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Technical Note

Effects of Cd(II) on wastewater biological nitrogen and phosphorus removal

Hong-bo Chen, Dong-bo Wang *, Xiao-ming Li *, Qi Yang, Kun Luo, Guang-ming Zeng, Mao-lin Tang

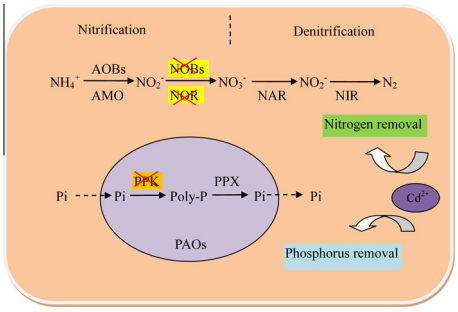
College of Environmental Science and Engineering, Hunan University, Changsha 410082, China
Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, China

HIGHLIGHTS

- Mechanisms for Cd(II) affecting the biological treatment processes were not well defined.
- Short-term exposure to Cd(II) did no affect biological nutrient removal.
- Long-term exposure to 10 mg L^{-1} Cd (II) decreased NOBs and PAOs abundance.
- NOBs were more sensitive to Cd(II) than AOBs.
- Long-term exposure to Cd(II) inhibited NOR and PPK activities.

GRAPHICAL ABSTRACT

This study showed the long-term exposure to 10 mg L^{-1} Cd(II) inhibited nitification and phosphorus uptake by decreasing the abundance of NOBs and PAOs and the activities of NOR and PPK.



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ABSTRACT

Short-term and long-term effects of Cd(II) on wastewater biological nitrogen and phosphorus removal were investigated with respect to microorganism abundances, enzyme activities, and polyhydroxyalkanoates (PHAs) and glycogen transformations. Though no obvious effects on wastewater biological nutrient removal were observed after short-term exposure, the long-term exposure of 10 mg L^{-1} Cd(II) inhibited nitrification and phosphorus uptake. Compared with the absence of Cd(II), the presence of 10 mg L^{-1} of Cd(II) decreased total nitrogen and phosphorus removal efficiencies from 97% and 98% to 88% and 18%, respectively. Mechanism studies revealed that Cd(II) affected the transformations of intracellular PHAs and glycogen, and the activities of oxidoreductase and polyphosphate kinase, resulted in the decrease of nitrite oxidizing bacteria and polyphosphate accumulating organisms abundance, which might be the major reason for the negative effects of long-term exposure to 10 mg L^{-1} Cd(II) on biological nitrogen and phosphorus removal.

1. Introduction

Wastewater biological nitrogen and phosphorus removal is a proven and effective method to avoid eutrophication problem. Nitrogen removal is generally achieved by alternately exposing bacteria including nitrifiers and denitrifiers to aerobic condition for nitrification and anoxic environment for densification (Metcalf and Eddy, 2003). While phosphorus removal is usually based on

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, China. Tel.: +86 731 88823967; fax: +86 731 88822829 (D. Wang).
E-mail addresses: w.dongbo@yahoo.com (D.-b. Wang), xmli@hnu.edu.cn (X.-m. Li).

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the enrichment of activated sludge with polyphosphate accumulating organisms (PAOs) which are able to store phosphorus under sequential anaerobic–aerobic conditions (Smolders et al., 1995). In recent decades, varieties of wastewater treatment processes with alternate anaerobic and oxic (and/or anoxic) procedures were developed to achieve wastewater biological nutrient removal.

Heavy metal pollution is a serious threat to ecosystem functioning because of the persistence and high toxicity to many aquatic organisms (Redeker and Blust, 2004). Therefore, the presence of heavy metal in wastewater may affect biological nitrogen and phosphorus removal in wastewater treatment plants (WWTPs).

Cd is a heavy metal of commercial importance, which is used widely in different industrial processes, such as batteries, pigments, chemical stabilizers and metal coatings (Olabarrieta et al., 2001). Meanwhile, Cd(II) is considered to be a highly toxic metal as it is toxic to organisms, including teratogenicity, carcinogenicity, endocrine and reproductive toxicities (Arao et al., 2009). The widespread industrial use of Cd(II) means it is ubiquity in aquatic environments and WWTPs. It has been reported that Cd(II) is highly soluble and can be transported into cells (Khaokaew et al., 2011). Therefore, once released into WWTPs, Cd(II) may exert toxic effects on activated sludge. As a potential threat, the toxic effect of Cd(II) on wastewater nutrient removal performance needs to be understood accurately and systematically. Unfortunately, up to now, the mechanisms by which heavy metals affect the biological treatment processes are still not well defined (Stasinakis et al., 2003). Moreover, though the effects of heavy metals such as chromium on activated sludge systems have been widely studied (Stasinakis et al., 2002; Chen and Gu, 2005; Vaiopoulou and Gikas, 2012), few studies have been conducted to evaluate the toxic effects of Cd(II) on wastewater biological nutrient removal.

It was reported that the combined presence of Cd(II) and Ni(II) could depress the degree of nitrification (Bagby and Sherrard, 1981). Besides, Cd(II) was found to reduce biological treatment efficiency in a fixed-film biological waste treatment system (Chang et al., 1986). However, the performance of activated sludge systems depends on the interaction of various species of bacteria. Those previous studies described the toxicity of Cd(II) in terms of plant performance without specifying the importance of Cd(II) effect on microbial abundance of microorganism related to biological nutrient removal, such as ammonia oxidizing bacteria (AOBs), nitrite oxidizing bacteria (NOBs), and PAOs as well as glycogen accumulating organisms (GAOs), thus the potential influence of Cd(II) on wastewater biological nitrogen and phosphorus removal is unknown and needed to be further explored. Moreover, effects of Cd(II) on the potential metabolic activities of microorganisms consisted of nitrification, denitrification and esterasic activities were assessed (Bonnet et al., 1999). Nevertheless, assay results showed the difficulty of linking the measured and potential enzyme activities of microorganism. And determination of enzyme activities such as ammonia monooxygenase (AMO), nitrite oxidoreductase (NOR), exopolyphosphatase (PPX) and polyphosphate kinase (PPK) and transformations of polyhydroxyalkanoates (PHAs) and glycogen will allow a more evaluation of Cd(II) toxicity to biological nutrient removal.

Therefore, the objective of this study was to investigate the acute and chronic effects of Cd(II) on biological nitrogen and phosphorus removal after short-term and long-term exposure. Scanning electron microscope (SEM) was used to determine the surface integrity of activated sludge. Nitrogen and phosphorus removal efficiencies were linked to the microbial community structures as assessed by fluorescent in situ hybridization (FISH) analysis. The transformations of intracellular PHAs and glycogen and the activities of some key enzymes related to biological nitrogen and phosphorus removal were further measured to explore the overall effect of Cd(II) on activated sludge.

2. Materials and methods

2.1. Synthetic wastewater

The synthetic feeding medium containing 15 mg L^{-1} PO_4^{3-}, 40 mg L^{-1} NH_4^+ and 300 mg L^{-1} of COD was used in this study and was prepared daily. Acetate was used as the sole carbon source because it was the most common volatile fatty acid (VFA) in domestic wastewaters (Chen et al., 2004). The concentrations of the other nutrients in the synthetic wastewater were presented as below: 0.01 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.005 g L^{-1} $CaCl_2$, and 0.5 mL L^{-1} trace element solution which contained (g L^{-1}): 0.03 $CuSO_4{\cdot}5H_2O$, 1.50 $FeCl_3{\cdot}6H_2O$, 0.12 $MnCl_2{\cdot}4H_2O$, 0.06 $Na_2MoO_4{\cdot}2H_2O$, 0.12 $ZnSO_4{\cdot}7H_2O$, 0.15 $CoCl_2{\cdot}6H_2O$, 0.18 KI, 0.15 H_3BO_3 and 10 EDTA.

2.2. Parent sequencing batch reactor (SBR) operation

Activated sludge was cultured in an anaerobic/oxic/anoxic parent SBR with a working volume of 12 L. The parent SBR was operated at 21 ± 2 °C with four 6-h cycles each day to achieve biological nitrogen and phosphorus removal. Each cycle consisted of 1.5 h anaerobic, 2 h oxic and 1.5 h anoxic periods, followed by 1 h for settling/decanting. Mixing was accomplished using magnetic a stirrer, and during the aerobic time, air was supplied into the SBR at a flow rate of 4 L min^{-1}. After settling period 8 L supernatant was discharged from the SBR and was replaced with 8 L of fresh wastewater before the anaerobic period started. Mean cell residence time (MCRT) was controlled at approximately 14 d by withdrawing the sludge from the SBR at the end of the anoxic period. The influent pH was adjusted to 7.2 by adding 1 M HCl or 1 M NaOH.

2.3. Short-term exposure experiments

The experiments were conducted on day 1 in three SBRs each with a working volume of 3 L to evaluate the short-term effects of Cd(II) on transformations of nitrogen and phosphorus. The SBRs were respectively exposed to no Cd(II) (the control), 1 and 10 mg L^{-1} Cd(II) after seeding with the concentrated sludge obtained from the parent SBR. The Cd(II) was used as chloride (C-2544, Sigma) dissolved in deionised water. The operated conditions were the same with the parent SBR. Because the Cd(II) concentrations of in the SBRs exposed to 1 and 10 mg L^{-1} Cd(II) might slowly decrease due to the discharge of effluent and sludge, a certain amount of Cd(II) were supplemented every other day to recover to the initial concentrations after determining the concentration of Cd(II) in the reactor.

2.4. Long-term exposure experiments

The three SBRs containing respectively 0, 1 and 10 mg L^{-1} Cd(II) in the short-term exposure experiments were continuously operated for 90 d to study the potential long-term effects of Cd(II) on biological nitrogen and phosphorus removal. The total concentrations of Cd(II) in each SBR were mentioned, and a certain amount of Cd(II) was supplemented every other day to recover to the initial concentrations of Cd(II) during the long-term exposure experiments.

2.5. Analytical methods

The Cd(II) concentrations were determined by atomic absorption spectroscopy using a spectrophotometer (6800, Shimadzu) with a GF-6501 graphite oven, an autosampler (ASC-6000, Hamamatsu) and Cd(II) cathode lamps. NH_4^+, NO_2^-, NO_3^-, soluble

orthophosphates (SOP), sludge volume index (SVI), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured using the standard methods (APHA, 1998). Analysis of AMO, NOR, PPX and PPK activities was performed according to the reference (Zheng et al., 2011). Sludge glycogen, poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV) and poly-3-hydroxy-2-methylvalerate (PH2MV) were measured according to the methodology described by the method reported previously (Chen et al., 2013a). The total PHAs was calculated as the sum of measured PHB, PHV, and PH2MV.

The cell viability of activated sludge was measured using the cell counting kit-8 (Sigma–Aldrich). SEM images were obtained by using a SEM (TM3000, Hitachi) to analyze the surface morphology of activated sludge after exposed to Cd(II). FISH technique was the same as described by our previous publication (Chen et al., 2013b). The following oligonucleotide probes used for hybridization are listed in Supplementary Material (SM) Table SM-1. EUB338-I, EUB338-II and EUB338-III were applied together (EUBmix, specific for most bacteria), NSO1225 (specific for *Betaproteobacterial* AOB), NIT3 (specific for *Nitrobacter sp.*), NTSPA662 (specific for *Nitrospira genera*), as well as PAO462, PAO651 and PAO846 (PAOmix, specific for *Accumulibacter*), GAOQ431, GAOQ989, GB_G2, TFO_DF218, TFO_DF618, DF988 and DF1020 (GAOmix, specific for *Candidatus Competibacter* phosphates and "*Defluviicoccus*"-related GAOs). 40 microscopic fields were analyzed for the hybridization of individual probes with a confocal scanning laser microscope (FV 500, Olympus). Furthermore, FISH quantification was performed with image database software (VideoTesT Album3.0).

3. Results and discussion

3.1. Effects of Cd(II) on the viability and settling property of activated sludge

It was well known that once enter cells, heavy metal ions can form stable combinations with thiol groups and destroy the structures and functions of protein, which commonly resulted in cell membrane damage and cell viability decrease (Vaiopoulou and Gikas, 2012). Nevertheless, no significant difference was observed on sludge surface in the SEM images of activated sludge exposed to no Cd(II), 1 mg L^{-1} and 10 mg L^{-1} Cd(II) except a slight decrease in size of flocs, revealing that the presence of Cd(II) had no observed damage on the surface and compaction properties of activated sludge (see Fig. 1). Furthermore, few negative influences on activated sludge viability were measured in this study after short-term and long-term exposure to either 1 or 10 mg L^{-1} Cd(II) (see Fig. 2).

So far negative effects of heavy metals on the structure of flocs have been reported for Cu, Hg, Cr and Zn (Lamb and Tollefson, 1973; Neufeld, 1976). In this study, the settling ability of activated sludge after exposure to Cd(II) was almost the same with that of the control. Relative SVI values were close to 100% during the long-term exposure to 1 and 10 mg L^{-1} Cd(II). This indicated that exposure to these concentrations of Cd(II) did not affect the settling ability of activated sludge.

3.2. Short-term effects of Cd(II) on biological nitrogen and phosphorus removal

Although few significant influences on the viability and settling ability of activated sludge were observed, it was still not clear whether the exposure to Cd(II) will affect on wastewater biological nutrient removal.

Fig. SM-1 showed that the cyclic profiles of NH_4^+, NO_2^-, NO_3^-, and SOP in the presence of 1 and 10 mg L^{-1} Cd(II) were the same as those in the absence of Cd(II), indicating that these concentrations of Cd(II) had no obvious effects on wastewater biological nitrogen and phosphorus removal after short-term exposure.

3.3. Long-term effects of Cd(II) on biological nitrogen and phosphorus removal

Despite no acute impacts were observed in short-term tests, the possible influence of Cd(II) on biological nutrient removal after long-term exposure remained unclear. Thus, long-term tests were carried out to investigate the chronic effects on wastewater biological nitrogen and phosphorus removal.

Fig. 3 summarized the effluent data of NH_4^+, NO_2^-, NO_3^-, and SOP during the 90 d long-term operation. As compared with the absence of Cd(II), 1 mg L^{-1} Cd(II) caused no significant effects on nitrogen and phosphorus effluent concentrations. In contrast, dramatic increases of NH_4^+ and SOP effluent concentrations were observed in the presence of 10 mg L^{-1} Cd(II). From Fig. 3, it can be seen that the presence of 10 mg L^{-1} Cd(II) decreased total nitrogen and phosphorus removal efficiencies from 97% and 98% to 88% and 18%, respectively. Those results revealed that long-term exposure to 10 mg L^{-1} Cd(II) had negative effects on wastewater biological nitrogen and phosphorus removal.

Fig. SM-2 displayed the cyclic variations of pH and DO in the SBRs after long-term exposure to no Cd(II), 1 and 10 mg L^{-1} Cd(II). It can be seen that cyclic pH and DO variations in all SBRs showed similar patterns, indicating that Cd(II) had no significant effect on pH and DO. The variations of NH_4^+, NO_2^-, NO_3^-, and SOP concentrations during one cycle after 90 d exposure were illustrated in Fig. 4. During the anaerobic stage, 1 and 10 mg L^{-1} Cd(II) caused no inhibition on SOP release as compared to the control. While in the subsequent oxic period, the uptake of SOP and the nitrification of NH_4^+ were decreased by 10 mg L^{-1} Cd(II) exposure. Although all the NO_2^- and NO_3^- had been reduced by the end of the anoxic period, high effluent concentrations of NH_4^+ and SOP were detected. Moreover, the transformation of NO_2^- was not affected by the exposure to Cd(II), suggesting that the decrease of NH_4^+ removal might be majorly due to the NH_4^+ oxidation to NO_3^-.

The long-term exposure to 10 mg L^{-1} Cd(II) did not affected denitrification, but inhibited nitrification and biological phosphorus uptake. Therefore, it is necessary to investigate the abundances of nitrifying bacteria, PAOs and GAOs in presence and absence of Cd(II) to understand the mechanism of Cd(II) affecting biological nutrient removal. Table SM-2 summarized the abundances of AOBs, NOBs, PAOs and GAOs in the activated sludge. It can be concluded that long-term exposure to 1 mg L^{-1} Cd(II) did not affect microorganism abundance. In contrast, long-term exposure to 10 mg L^{-1} Cd(II) seriously decreased the abundance of NOBs and PAOs, which might be the principal reason for the suppressed wastewater nitrogen and phosphorus removal efficiencies.

In addition, the abundance of AOBs was not affected but that of NOBs was severely decreased after long-term exposure to 10 mg L^{-1} Cd(II), which was consistent with the uninfluenced NO_2^- and decreased NO_3^- accumulation and confirmed that NOBs were more sensitive to Cd(II) as compared to AOBs.

The previous studies had reported that nitrifying organisms were much more sensitive to heavy metals than heterotrophic organisms (Bagby and Sherrard, 1981; Stasinakis et al., 2003). The addition of 0.5 mg L^{-1} Cr(VI) affected significantly the nitrification process, resulted in a reduction in effluent nitrate and increase in ammonium concentrations (Stasinakis et al., 2003). Harper et al. (1996) reported that continuous loading with Cr(III) did not reduce the ammonia removal until the concentration of Cr(III) up to 25 mg L^{-1}. In this study, the presence of 1 mg L^{-1} Cd(II) did not

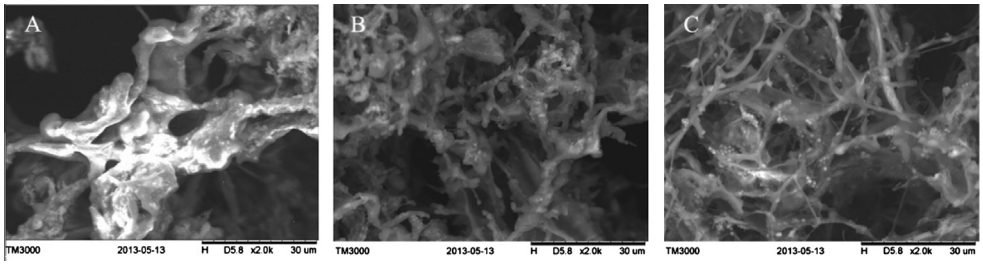

Fig. 1. SEM images of activated sludge exposed to no Cd(II) (control) (A), 1 mg L^{-1} (B), and 10 mg L^{-1} (C) Cd(II).

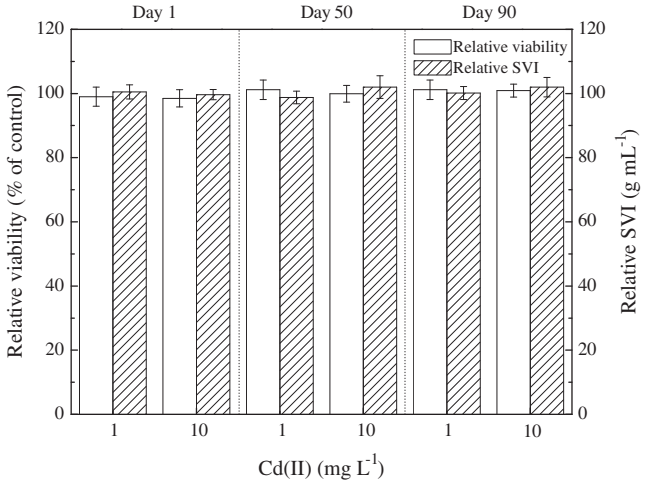

Fig. 2. Effects of 1 and 10 mg L^{-1} Cd(II) on relative viability and SVI at day 1, day 50 and day 90. Error bars represent standard deviations of triplicate measurements.

affect nitrogen and phosphorus removal efficiencies, while signs of process failure were observed after long-term exposure to 10 mg L^{-1} Cd(II). Those results suggested that the toxic effect of Cd(II) on nitrification was weaker than Cr(VI) but stronger than Cr(III).

Bagby and Sherrard (1981) reported that the simultaneous presence of 5.25 mg L^{-1} Cd(II) and 1.15 mg L^{-1} Ni(II) severely depressed the degree of nitrification, and nitrification process was almost totally inhibited when the loading increased to 10.17 mg L^{-1} Cd(II) and 5.35 mg L^{-1} Ni(II). In this study, NH_4^+ removal efficiency decreased from 100% to 90% after long-term exposure to 10 mg L^{-1} Cd(II). Those results revealed that the combined effects of Cd(II) and Ni(II) on activated sludge process were much stronger than the sole influence of Cd(II). In other words, the presence of Ni(II) enhanced the toxicity of Cd(II) to biological nutrient removal.

3.4. Effects of Cd(II) on the transformations of PHAs and glycogen and the activities of key enzymes

The transformations of intracellular PHAs and glycogen were found to be associated with PAOs and GAOs activities, and high glycogen accumulation commonly indicating the activated GAOs metabolism (Mino et al., 1998). Under anaerobic condition, PAOs consume glycogen and hydrolyze intracellular stored polyphosphate (poly-P) to provide energy for taking up VFA and storing them as PHAs (Mino et al., 1998). In the subsequent aerobic condition, PAOs replenish glycogen and take up excessive SOP to recover the intracellular poly-P levels by oxidizing the stored PHAs (Pijuan et al., 2004). Fig. 5 illustrated that after 90 d exposure to 10 mg L^{-1} Cd(II), the PHAs synthesis and glycogen consumption during the

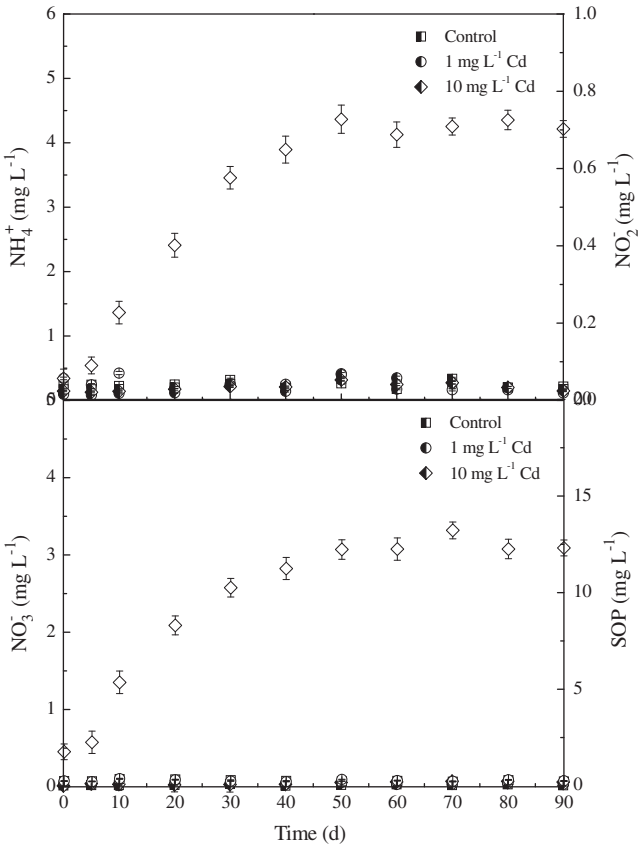

Fig. 3. Effluent concentrations of NH_4^+ (A, white), NO_2^- (A, half white), NO_3^- (B, half white) and SOP (B, white) during the long-term exposure to no Cd(II), 1 and 10 mg L^{-1} Cd(II). Error bars represent standard deviations of triplicate measurements.

anaerobic stage were 2.23 and 1.21 mM C g^{-1} VSS, respectively, compared with the 3.19 and 0.99 mM C g^{-1} VSS in control. Moreover, significant variations were also obtained for PHAs degradation (2.01 vs. 2.84 mM C g^{-1} VSS) and glycogen accumulation (1.54 vs. 1.19 mM C g^{-1} VSS) in the oxic period. These results suggested that long-term exposure to 10 mg L^{-1} Cd(II) affected the transformations of PHAs and glycogen. These results indicated that exposure to 10 mg L^{-1} Cd(II) had a long-term effect on biological phosphorus removal by decreasing PHAs synthesis and consumption and increasing glycogen degradation and replenishment, which generally caused inhibition to the activity of PAOs.

It was confirmed that under anaerobic conditions, external acetate was mainly used by PAOs for PHB synthesis, and to a small extent for PHV production. Nevertheless, when fed with acetate GAOs tended to produce more PHV than PAOs due to the partial conversion of pyruvate to propionyl-CoA through the succinate–propionate pathway (Zeng et al., 2002). After 90 d exposure to

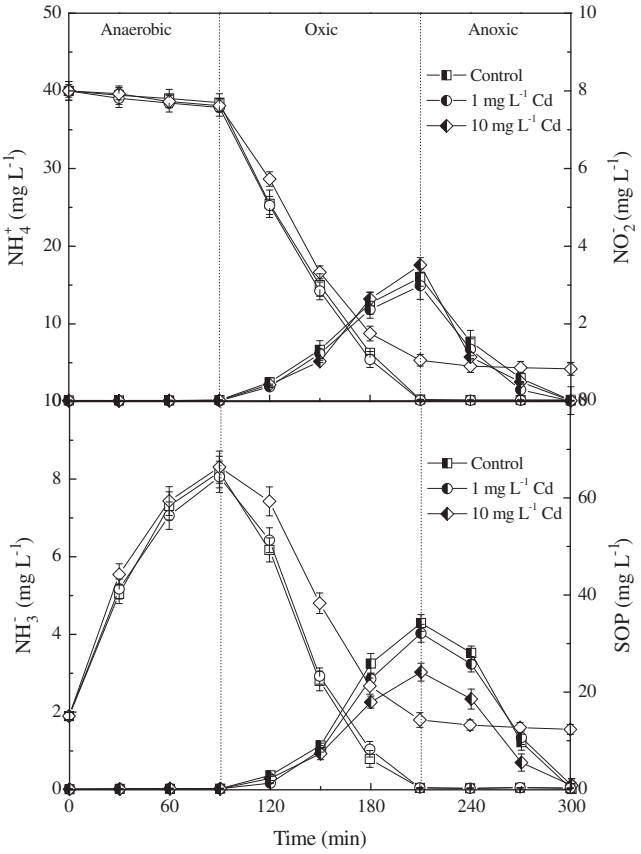

Fig. 4. Variations of NH_4^+ (A, white), NO_2^- (A, half white), NO_3^- (B, half white) and SOP (B, white) during one cycle after long-term exposure to no Cd(II), 1 and 10 mg L^{-1} Cd(II). Error bars represent standard deviations of triplicate measurements.

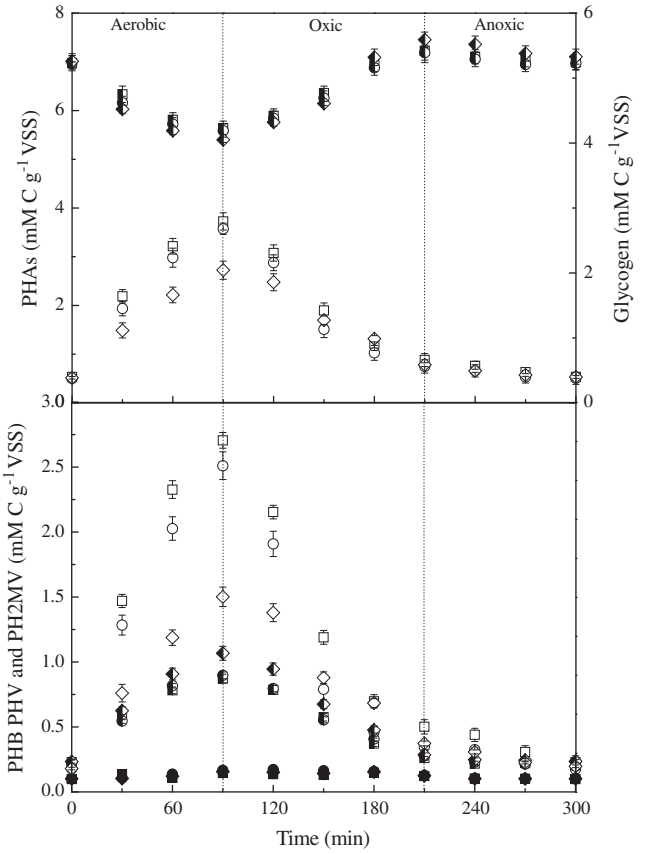

Fig. 5. Variations of PHAs (A, white), Glycogen (A, half white), PHB (B, white), PHV (B, half white) and PH2MV (B, black) during one cycle after long-term exposure to no Cd(II), 1 and 10 mg L^{-1} Cd(II). Error bars represent standard deviations of triplicate measurements.

10 mg L^{-1} Cd(II), the SBR had almost the same accumulation of PH2MV, but the PHV synthesis was higher and the PHB production lower as compared to the control. The decrease of PHAs synthesis after 90 d exposure to 10 mg L^{-1} Cd(II) was due to the decrease of PHB production. Those results were consistent with the results of population abundance analysis and verified the decrease of PAO abundance.

Further investigation showed that 10 mg L^{-1} Cd(II) affected the activities of enzymes relevant to biological nitrogen and phosphorus removal. AMO and NOR are two key enzymes in nitrification (Kristjansson and Hollocher, 1980), whereas phosphorus removal is directly related to the activities of PPX and PPK (Mino et al., 1998). In Table SM-2, as compared with the control, no obvious variations of AMO, NOR, PPX and PPK activities was measured in the presence of 1 mg L^{-1}. However, after 90 d exposure to 10 mg L^{-1} Cd(II), the activities of NOR and PPK decreased to nearly 70% and 49% of that of the control, respectively, kept consistent with the lower nitrification and phosphorus uptake. Moreover, the activities of AMO and PPX were not affected by the exposure to 10 mg L^{-1} Cd(II), in accordance with the uninfluenced NH_4^+ oxidation to NO_2^- and anaerobic phosphorus release. These results indicated that 10 mg L^{-1} Cd(II) inhibited biological nitrogen and phosphorus removal by decreasing the activities of NOR and PPK.

4. Conclusion

Neither 1 nor 10 mg L^{-1} of Cd(II) obviously affected wastewater biological nutrient removal after short-term exposure. Long-term exposure of 10 mg L^{-1} Cd(II) had negative effects on wastewater biological nitrogen and phosphorus removal due to the inhibition to nitrification and phosphorus uptake. 10 mg L^{-1} Cd(II) affected the transformations of PHAs and glycogen, as well as the activities of NOR and PPK, resulted in the decrease of the abundance of NOBs and PAOs, which might be the major reason for the negative effect of long-term exposure to 10 mg L^{-1} Cd(II) on biological nitrogen and phosphorus removal.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.05.057.

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