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Effect of copper ion on the anaerobic and aerobic metabolism of phosphorus-accumulating organisms linked to intracellular storage compounds

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ABSTRACT

The shock load effect of heavy metals (Cu (II)) on the behavior of poly-phosphate-accumulating organisms (PAOs) was investigated with respect to the transformations of poly-P, intracellular polyhydroxyalkanoates (PHAs) and glycogen. The PAOs biomass was exposed to different concentrations of Cu (II) at various pH and biomass levels. The results showed that when the mixed liquor suspended solid (MLSS) concentration was 2500–4000 mg/L, the P removal was not adversely affected by spiking with 2 mg Cu^{2+}/L; however, it deteriorated completely after a Cu (II) shock concentration of 4 mg/L. Nevertheless, the tolerance of PAOs biomass to Cu (II) shock could be enhanced by increasing the MLSS. Moreover, in the presence of 2 mg Cu^{2+}/L, the P removal efficiency was highest at an initial pH of 6.2 and lowest at an initial pH of 6.9, indicating that the Cu inhibitory effect was reduced by increasing the pH to 7.6. The inhibition by Cu (II) was related to the transformation of intracellular storage compounds of PAOs. Specifically, poly-P degradation might be inhibited, which reduced the energy available for PHA production and eventually led to poor P removal.

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1. Introduction

Enhanced biological phosphorus removal (EBPR) characterized by cycling anaerobic and aerobic reactions is a widely applied technology in wastewater treatment facilities to remove phosphorus from wastewater. The EBPR process is based on the enrichment of activated sludge with poly-phosphate-accumulating organisms (PAOs), which are able to take up volatile fatty acids (VFAs) anaerobically and convert them to intracellular polyhydroxyalkanoates (PHAs). The energy for these biotransformations is primarily generated by degradation of poly-phosphate (poly-P) and the release of phosphate from the cells [1]. Aerobically, PAOs are able to use their stored PHA as an energy source for biomass growth, glycogen replenishment, P uptake and poly-P storage [2]. Since EBPR was first discovered in the 1960s, there have been numerous studies conducted to investigate the mechanisms and to optimize this process [3].

Successful operation of the EBPR process depends on numerous process operational factors, particularly the variation in wastewater quality. Metals in both inorganic and organic form are known to affect the quality of wastewater treatment [3]. This is because metals are toxic to most microorganisms at specific concentrations [4] and have been detected in significant concentrations in various wastewater streams [5,6]. Specifically, shock loads of metals in influent can lead to a loss in sludge viability, changes in the sludge community structure, loss of floc structure, and even complete failure of biological processes [7–9].

Heavy metals have been reported to inhibit nitrification and denitrification processes [6,10,11], as well as to reduce the microbial oxidation of organic compounds [12,13]. Moreover, monitoring of a full-scale advanced municipal wastewater treatment plant (WWTP) showed that the P removal efficiency decreased dramatically after tin (Sn) levels in the solids fraction of the MLSS exceeded 4 μg Sn/L [14]. Their study was the first to provide information regarding the effects of metal on the EBPR process; however, a possible mechanism for the Sn inhibition of the PAOs was not provided.

Previous studies have also demonstrated that toxic effects of heavy metals correlate well with pH [13,14]. The pH of the system has long been considered the most important operating parameter influencing metal solubilization and toxicity [15]. In an activated sludge system, an increase in pH can reduce the availability of heavy metal fractions, which in turn reduces heavy metal toxicity to microorganisms [5]. However, no detailed description of heavy metal toxicity to PAOs at various pHs has been reported to date.

Most previous studies of metal toxicities toward wastewater microorganisms have been directed toward the COD removal or nitrification efficiencies, and few have been devoted to the EBPR. Accordingly, extremely little is known about the metal interaction with PAOs biomass, especially from the intracellular storage compounds transformation point of view. Therefore, this study was conducted to investigate the shock load effect of heavy metals (Cu (II)) on the release and uptake of phosphorus from the biomass of

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PAOs and to correlate these effects with the anaerobic and aerobic metabolism of PAOs. The PAOs sludge was exposed to different concentrations of Cu (II) at various pH levels and biomass concentrations. The heavy metal investigated in this study was copper because of its widespread industrial use and documented toxicity toward microorganisms.

2. Materials and methods

2.1. Sludge

The experimental sludge used in this study was obtained from a laboratory scale plug-flow anaerobic–aerobic (A/O) reactor with a working volume of 32 L. A more detailed description of this system can be found in Wang et al. [16]. The EBPR biomass was enriched using acetate as the sole carbon source. The experiments reported below were conducted when the A/O process reached steady state.

2.2. Synthetic wastewater

The influent solution consisted of NaAc (400 mg COD/L), KH_2PO_4 (15 mg P/L), NH_4Cl (25 mg/L), $MgSO_4{\cdot}7H_2O$ (150 mg/L), $CaCl_2$ (18 mg/L), and trace elements 0.4 mL/L. The mineral salt solution was reported in Wang et al. [16]. The temperature was maintained at 18–22 °C.

2.3. Batch experiments with PAOs sludge

Batch tests were conducted in 2 L jacketed magnetically stirred glass reaction vessels. The sludge (15 L) for the batch tests was taken from the final aerobic compartment of the A/O system on days 133, 156 and 200. The sludge was then washed three times with tap water, after which it was transferred into the reaction vessels.

2.3.1. Batch Cu (II) toxicity experiments (Expt. I)

The washed sludge was resuspended with the synthesis water containing the trace elements to a volume of 18 L and then evenly divided into nine batch reactors. The experiment was divided into three runs according to the different Cu (II) concentrations, and each run was operated three times. $CuCl_2$ was added with different initial Cu (II) concentrations of 2 and 4 mg/L, respectively, and a blank test in which no Cu (II) was added. At the beginning of the anaerobic period, acetate and KH_2PO_4 were added into each batch reactor rapidly to reach initial concentrations of 300 mg COD/L and 10 mg PO_4^{3-}-P/L, respectively. The mixed liquor was then incubated for 2 h under anaerobic conditions by injecting nitrogen gas above the water surface, after which it was exposed to aerobic conditions for 3–4 h. The initial pH of the mixed sludge in the reactors was controlled below 8.0 during both the anaerobic and aerobic periods by a one-way controller through dosing with 1.0 M HCl.

2.3.2. Batches at different initial pH values in the presence of 2 mg Cu^{2+}/L (Expt. II)

The washed biomass was resuspended to a volume of 2 L using the procedures described for Expt. I. The experiment was divided into three runs according to the different pH levels, and each run was conducted three times. At the beginning of each cycle, acetate, KH_2PO_4 and $CuCl_2$ were added into the reactors to reach the initial concentrations of 300 mg COD/L, 10 mg PO_4^{3-}-P/L and 2 mg Cu^{2+}/L, respectively. The initial solution pH was adjusted to the desired values of 6.2, 6.9 and 7.6, respectively, by adding either 1.0 M HCl or 1.0 M NaOH.

2.3.3. Batch different biomass in the presence of Cu^{2+} experiments (Expt. III)

The washed sludge was evenly divided into three parts, and each part was further divided into three subparts according to a volume proportion of 1:2:3 to give different biomass levels. After being transferred to 2 L batch reactors, the sludge was resuspended with the same effluent to a volume of 2 L. Acetate, KH_2PO_4 and $CuCl_2$ were added into each batch reactor to give initial concentrations of 270 mg COD/L, 10 mg PO_4^{3-}-P/L and 2 mg Cu^{2+}/L, respectively.

2.4. Analytical methods

The liquid samples were immediately filtered through Millipore filter units (0.45 μm pore size) for analysis of phosphate (PO_4^{3-}-P). The PO_4^{3-}-P, NH_4^+-N, NO_3^--N, NO_2^--N, MLSS, mixed liquor volatile suspended solid (MLVSS) and biomass-P content were analyzed in accordance with the standard methods [17]. VFAs (acetate, HAc) were measured using an Agilent 6890N gas chromatograph (GC) equipped with a 30 m × 0.53 mm × 1 μm (length × ID × film) DB-WAXetr column and a flame ionization detector (FID) at 220 °C. Intracellular glycogen was analyzed using the method described Jenkins et al. [18].

Poly-β-hydroxybutyrate (PHB) and poly-hydroxyvalerate (PHV) were measured according to a modification of the method described by Oehmen et al. [19]. Briefly, weighed freeze-dried samples of biomass were put into screw-topped glass tubes and 2 mL of chloroform, 2 mL of an acidified methanol solution (3% H_2SO_4) and 0.1 mL benzoic acid methanol solution were added (2 g of benzoic acid dissolved in 100 mL methanol was used as an internal standard). The samples were then digested for 4 h at 100 °C. After cooling, 1 mL of distilled water was added and mixed vigorously with each sample. When the phases were separated, approximately 1 mL of the bottom organic layer was transferred to the GC vials for analysis. It should be noted that PH2MV was not analyzed because acetate was added as the sole carbon source, which resulted in the synthesis of very little PH2MV. The total PHA in the samples was calculated as the sum of the measured PHB and PHV.

On-line monitoring was installed in the batch reactor with DO (WTW inoLab Oxi level 2 oxygen meters), pH and ORP (WTW pH/Oxi 340i) sensors. The temperature was measured by a probe incorporated into the DO probe.

3. Results and discussion

3.1. Effect of Cu (II) concentrations on P removal

Lower and higher levels of the metals were selected based on the tolerance of the PAOs biomass to Cu (II) when compared with the blank test. The MLSS and MLVSS concentrations were maintained at around 3400 and 2300 mg/L, respectively.

3.1.1. Phosphorous release and uptake linked to HAc uptake by PAOs

Typical phosphorus release and uptake patterns of an EBPR were observed in the blank test and in response to 2 mg Cu^{2+}/L shock, but these levels began diminishing as the Cu (II) concentration increased to 4 mg/L (Fig. 1a). For the case of 2 mg Cu^{2+}/L shock, the P release and uptake rates decreased slightly when compared with those of the blank test, indicating that Cu (II) did not produce a significant toxic effect on the EBPR process at this concentration. However, as the Cu (II) dose increased to 4 mg/L, the net released-P was reduced by approximately 25% when compared with that measured in the blank test.

In the subsequent aerobic phase, for the 4 mg/L Cu (II) test, there was less P release, followed by a plateau, during which HAc uptake

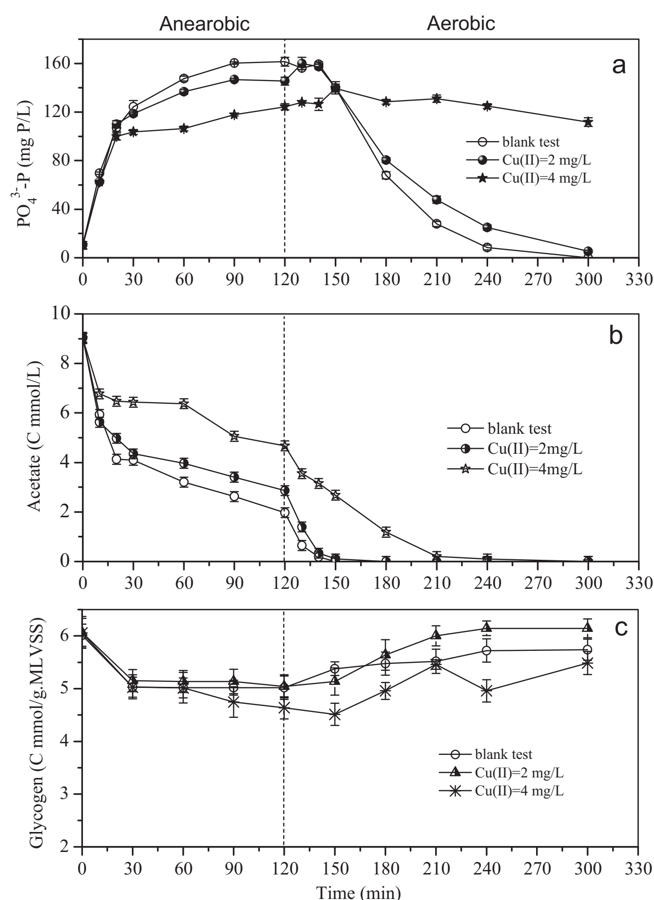

Fig. 1. Variations in phosphate, acetate and glycogen in batch reactors with different Cu (II) shock loads.

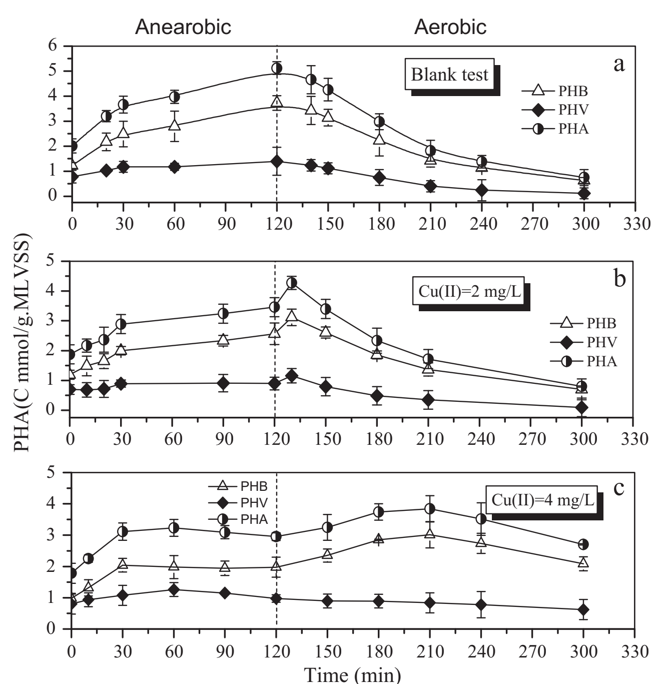

Fig. 2. PHA variations in batch reactors with different Cu (II) shock loads.

and PHA synthesis occurred (Figs. 1 and 2); after the HAc uptake ceased at approximately 210 min, a slow P uptake was observed, with an uptake rate of 0.19 mmol P/g MLVSS h (Fig. 1). Conversely, in the control and the 2 mg/L Cu (II) reactors, the aerobic P uptake rates were 0.27 mmol P/g MLVSS h and 0.41 mmol P/g MLVSS h, respectively. Obviously, the P uptake was severely inhibited in response to 4 mg Cu^{2+}/L shock, even though the corresponding PHA was sufficient (Fig. 2c). As a result, the mean effluent P concentration in the 4 mg/L Cu (II) test was much higher (112 mg/L) than the P concentration in the feed (10 mg P/L) (Fig. 1a). Indeed, this was typical behavior of a toxic effect of elevated levels of Cu (II) on the activity of PAOs.

A gradual decrease in the HAc uptake rate was observed in response to the 4 mg Cu^{2+}/L shock during the anaerobic phase, eventually resulting in incomplete HAc consumption (Fig. 1b and Table 1). Overall, approximately 48% of the influent HAc was residual to the subsequent aerobic phase. Nevertheless, the HAc uptake still occurred during the aerobic phase (Fig. 1b). When comparing the conversion rate of acetate to PHA, it was 0.27 and 0.20, respectively, in the anaerobic and aerobic phases (Table 2). These findings suggest that not all of the consumed acetate during the aerobic phase is linked to phosphorus release and glycogen degradation, and that a fraction is oxidized for energy requirements.

3.1.2. Effect of Cu (II) concentrations on the synthesis and consumption of PHA and glycogen by PAOs

The greatest anaerobic PHA storage was observed in the control, while the lowest value was observed in the case of 4 mg Cu^{2+}/L shock (Fig. 2), indicating that 4 mg Cu^{2+}/L induced the suppression of the PHA synthesis (Fig. 2c). This was consistent with the corresponding P release in that the lowest P release also occurred in the 4 mg Cu^{2+}/L test (Fig. 2). Similar phenomena have been described previously, with a lower P release being found to lead to lower PHA production because the energy for PHA formation is produced by poly-P hydrolysis and P release [1].

It should be noted that the level of glycogen hydrolysis was clearly reduced from the 4 mg Cu^{2+}/L shock test to the blank test, which was not similar to the variations of the PHA synthesis.

Table 1
Phosphorus release and uptake rate, HAc uptake rate, PHA and glycogen consumption and synthesis rates calculated using the experimental data from batch reactors (mean values).

	Experiment I (Cu (II) (mg/L))			Experiment II (pH)			Experiment III (MLSS (mg/L))		
	Blank (0)	2	4	6.2	6.9	7.6	4080 (0.49)[a]	2600 (0.77)[a]	1360 (1.47)[a]
P-release rate (mmol P/g VSS h)	0.87	0.78	0.65	0.46	0.41	0.50	0.45	0.47	0.44
C-uptake rate (mmol C/g VSS h)	1.59	1.39	0.98	1.02	1.29	1.18	1.31	1.91	2.39
PHA-produced rate (mmol C/g VSS h)	0.70	0.36	0.26	0.35	0.38	0.47	1.48	1.18	1.17
Glyc-degraded (mmol C/g VSS h)	0.50	0.51	0.71	0.44	0.54	0.71	0.41	0.59	0.86
P-uptake rate (mmol P/g VSS h)	0.62	0.54	−0.05	0.30	0.20	0.27	0.41	0.46	0.37
PHA degradation rate (mmol C/g VSS h)	0.65	0.40	0.04	0.29	0.25	0.33	1.31	1.08	0.65
Glycogen synthesis rate (mmol C/g VSS h)	0.24	0.37	0.28	0.80	0.66	0.92	0.11	0.361	0.50

Note: All rates were calculated based on 2 h of anaerobic reaction and 3 h of aerobic reaction.

[a] Cu (II) load: mg Cu^{2+}/g MLSS.

Table 2
Ratios and rates obtained in the batch experiments conducted in this study (mean values).

	Experiment I (Cu (II) (mg/L))			Experiment II (pH)			Experiment III (MLSS (mg/L))		
	Blank (0)	2	4	6.2	6.9	7.6	4080 (0.49)[g]	2600 (0.77)[g]	1360 (1.47)[g]
PHA-produced/C-uptake[a]	0.44	0.27	0.27	0.34	0.29	0.39	1.13	0.61	0.49
aerobic C-uptake rate[b] (mmol C/g VSS h)	1.77	1.28	1.02	2.48	2.05	2.56	0.36	1.20	1.43
PHA-produced/C-uptake[b]	–	0.55[c]	0.20[d]	0.04[c]	0.15[e]	0.10[c]	–	–	0.42[f]

[a] Anaerobic period.
[b] Aerobic period.
[c] Ratio calculated based the reaction of 120–130 min.
[d] Ratio calculated based the reaction of 120–210 min.
[e] Ratio calculated based the reaction of 120–150 min.
[f] Ratio calculated based the reaction of 120–240 min.
[g] Cu (II) load: mg Cu^{2+}/g MLSS.

For the 4 mg Cu^{2+}/L shock test, the greatest glycogen hydrolysis of 1.42 mmol C/g MLVSS was accompanied by the smallest PHA production. However, for the control, the degraded-glycogen was 0.99 mmol C/g MLVSS, but accompanied by higher PHA production (Fig. 1c). The higher conversion of glycogen to PHA in the 4 mg Cu^{2+}/L shock test during the anaerobic period may have been due to the maintenance adenosine triphosphate (ATP) required by the cell.

With respect to the aerobic phase, the PHA degradation and glycogen production displayed very similar transformation trends in the control and 2 mg Cu^{2+}/L shock test (Figs. 1 and 2). However, the level of the PHA degradation decreased in both the 2 mg Cu^{2+}/L and 4 mg Cu^{2+}/L treatments when compared with the control. It should be noted that, for the 4 mg Cu^{2+}/L shock, PHA accumulation instead of degradation occurred along with HAc uptake and a slight P release (Figs. 1 and 2), although the conversion of HAc to PHA decreased when compared with that observed in the anaerobic phase (Table 2). After the HAc was depleted, a slight phosphorus uptake that was linked with PHA degradation and glycogen synthesis occurred (Figs. 1 and 2). Similar findings have been reported in several previous studies [20,21], and these results have been explained by PAOs having the ability to take up acetate under aerobic conditions, linking this consumption to phosphorus release, PHA formation and the growth of PAOs.

Nevertheless, for the 4 mg Cu^{2+}/L shock test, although PHA synthesis occurred in the aerobic phase and achieved a maximum value of 3.84 mmol C/g MLVSS at approximately 210 min, it was still approximately 25% lower than that of the control (Fig. 2a and c). This corresponds well with the lower P uptake that was observed in the 4 mg Cu^{2+}/L shock case (Fig. 1a), as an energy source for the phosphorus uptake is provided by the anaerobically formed PHA [22].

Additionally, as shown in Fig. 2, the anaerobic PHA production occurred and displayed similar trends in all cases; however, the PHA degradation had completely different characteristics. These findings suggest that the aerobic PHA degradation was more affected by the Cu (II) inhibition than the anaerobic PHA synthesis.

3.2. Anaerobic and aerobic metabolism of PAOs at different initial pH values in the presence of 2 mg/L Cu (II)

3.2.1. pH variations in batch reactors

In the anaerobic phase, different trends in the pH variation were observed among the three treatments (Fig. 3). In systems having with an initial pH of 6.2 and 6.9, an increasing trend was evident during the anaerobic phase. However, systems with an initial pH of 7.6 did not exhibit a similar trend; rather, the pH decreased from 7.6 to 7.3. Clearly, all pH values were near neutral at the end of the anaerobic phase, regardless of whether the initial pH was alkaline or acidic. Our findings agree well with those of Liu et al. [23], who reported that the EBPR system has the buffering capacity to automatically adjust its pH to around 7.0. From the microbiological point of view, the pH in the cell for most bacteria is maintained relatively constant in a rather narrow neutral range, and can decrease or increase when the bulk pH is below or above their cell pH [24,25].

Additionally, a rapid increase in pH occurred in all tests at the beginning of the aerobic period, which may have been due to the stripping of CO_2 after intensified aeration.

3.2.2. Effect of 2 mg/L Cu (II) shock on the P transformation at various initial pH values

The addition of 2 mg/L Cu (II) did not significantly inhibit the release of P (Fig. 4). The highest phosphate release occurred at an initial pH of 7.6, followed by pH values of 6.2 and 6.9 (Fig. 4 and Table 1). According to equation $\alpha_{PAO} = 0.16 \times pH_{out} - 0.7985$ [26], the higher P release was due to the higher energy demand for active uptake of VFA (α_{PAO}) at the higher pH, which caused more poly-P degradation for the production of more ATP [26]. Conversely, a minor released occurred at an initial pH of 6.9, but not 6.2, primarily because cell acidification led to leakage of phosphate from the cell or extracellular polymeric substances (EPS) [23,25].

During the aerobic period, the influence of 2 mg Cu^{2+}/L shock on P uptake at various pH values behaved differently from the P release, indicating that pH impacted the Cu inhibitory effect on the P uptake. As shown in Figs. 4a and 5a, the mean P removal efficiency decreased from 83% to −60% as the initial pH increased from 6.2 to 7.6, and the poorest P removal of −124% was achieved in the case of an initial pH of 6.9. Our findings are partially inconsistent with those of Liu et al. [23], who found that a higher initial pH of wastewater was associated with a lower P removal efficiency. It is possible that the increase in pH may decrease the inhibitory effect of the added

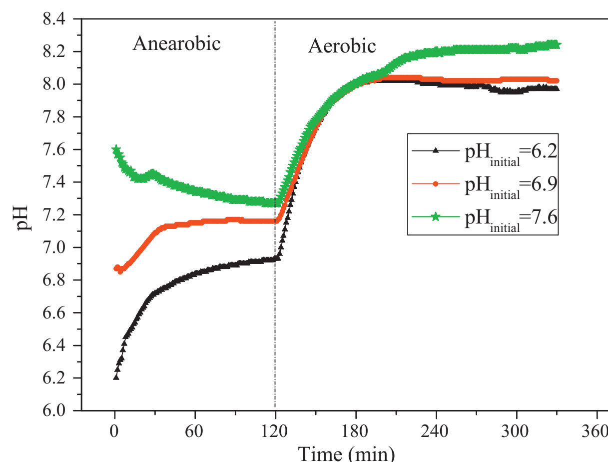

Fig. 3. pH variations in batch reactors at different initial pH values in the presence of 2 mg/L Cu (II).

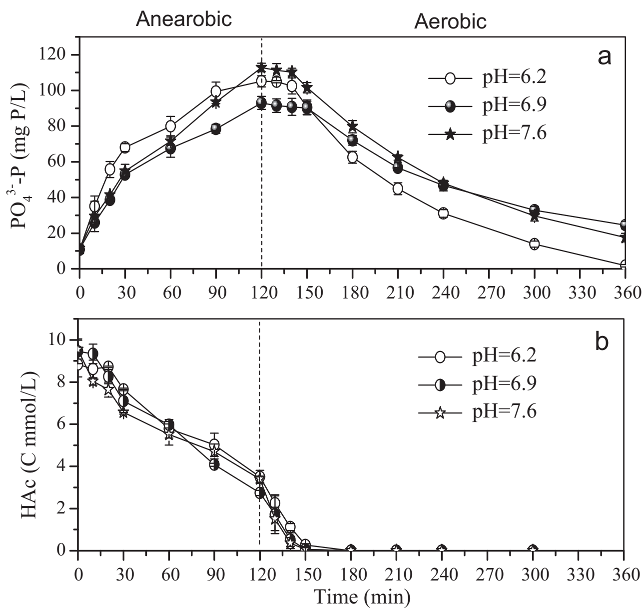

Fig. 4. Variations in phosphate and acetate in batch reactors at different initial pH values in the presence of 2 mg/L Cu (II).

Cu (II), for which the P removal efficiency was comparatively higher at pH 7.6 than at pH 6.9. Indeed, the increase in pH can reduce the availability of heavy metal fractions by precipitating metals as hydroxide, which can in turn reduce heavy metal toxicity toward microorganisms [14].

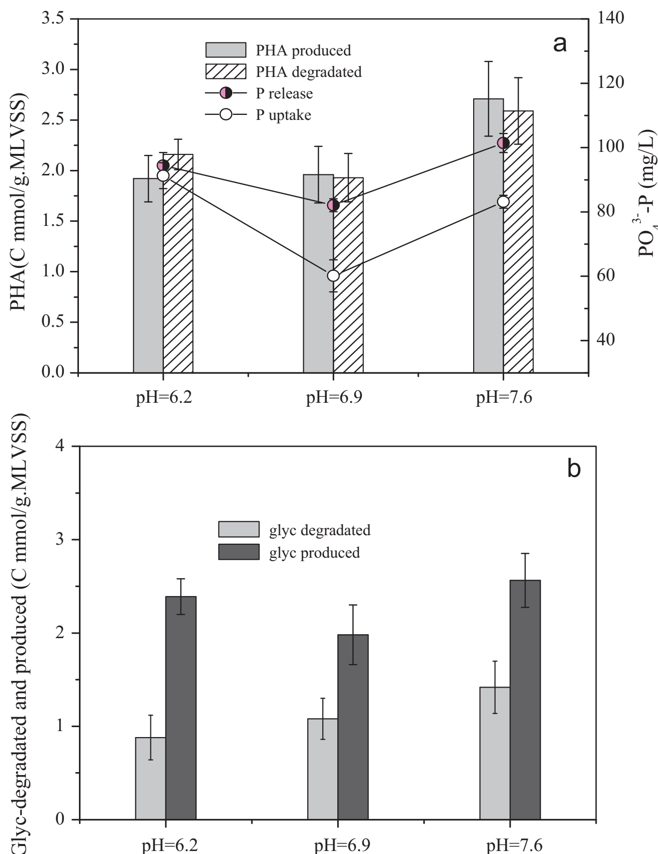

Fig. 5. Effect of 2 mg/L Cu (II) shock on the transformations of P, PHA and glycogen at various initial pH values.

3.2.3. Effect of 2 mg/L Cu (II) shock on acetate anaerobic transformation at various initial pH values

As Figs. 3 and 4b showed, there was no obvious relationship between the HAc uptake rate and pH in the presence of 2 mg Cu^{2+}/L. Similar observations were also found by Filipe et al. [2,26], who examined VFA uptake rates using the entire stage pH controlling strategy, and concluded that the acetate uptake rate was independent of pH. Conversely, Liu et al. [23] employed an initial pH controlling strategy similar to that used in the present experiment and obtained that the acids uptake increased as the initial pH increased from 6.4 to 8.0. The discrepancy between our findings and those of Liu et al. [23] may have been due in part to the different acclimated sludges used in the experiments as well as the addition of Cu (II) in the present experiment.

3.2.4. Effect of 2 mg/L Cu (II) shock on the PHA and glycogen transformations at various pH values

The highest PHA accumulation was observed when the initial pH was 7.6 and the lowest occurred when the initial pH was 6.2, with mean PHA storage values of 1.92, 1.96 and 2.71 mmol C/g MLVSS being observed at the end of the anaerobic phase when the initial pH values were 6.2, 6.9 and 7.6, respectively. These findings correspond well with the variations in the P release shown in Fig. 5a, which was likely because the anaerobic phosphate release and PHA accumulation are metabolically linked, and a higher P release generally leads to higher PHA synthesis if the substrate is sufficient [25]. Additionally, the lowest PHA synthesis was observed when the initial pH was 6.2, which may have been due to the fact that intracellular acidification inhibits PHA formation as proposed in a previous study [25]. The amount of glycogen degradation increased from 0.88 ± 0.24 to 1.42 ± 0.28 mmol C/g MLVSS as the pH increased from 6.2 to 7.6 (Figs. 3 and 4).

During the aerobic phase, the transformation of the intracellular storage compounds displayed a pattern different from that obtained in the anaerobic phase (Table 1). The degradation of PHA decreased from 2.16 to 1.93 mmol C/g MLVSS as the pH increased from 6.2 to 6.9, which was followed by an increase to 2.59 mmol C/g MLVSS with pH up to 7.6. The glycogen production showed a trend similar to that of the PHA transformation with the smallest glycogen production of 1.98 mmol C/g MLVSS being observed when the initial pH was 6.9.

3.3. Endurance of PAOs biomass to Cu (II)

The endurance of the PAOs biomass to Cu (II) was examined using sludge samples with MLSS concentrations of 4080 ± 86 mg/L, 2600 ± 334 mg/L and 1360 ± 47 mg/L, respectively.

As shown in Fig. 6, the inhibition level of Cu (II) was strongly dependent on the MLSS concentrations. The anaerobic released-P decreased with increasing Cu (II) loading from 0.49 mg Cu^{2+}/g MLSS to 1.47 mg Cu^{2+}/g MLSS (Fig. 5). At Cu (II) loading levels lower than 0.49 mg Cu^{2+}/g MLSS, the inhibitory effect of Cu (II) on the release of P was not significant and the specific P release rate was maintained at approximately 0.45 mmol P/g VSS h (Table 1), indicating that PAOs can tolerate Cu (II) loadings as high as 0.49 mg Cu^{2+}/g MLSS. However, after Cu (II) loading up to 1.47 mg Cu^{2+}/g MLSS, both the anaerobic P release and the aerobic P uptake were inhibited, resulting in the effluent P concentration being greater than that of the influent. This situation could potentially lead to failures of EBPR systems (Fig. 6a). Moreover, these results showed that the toxicity of a heavy metal could be minimized by increasing the MLSS in activated sludge wastewater treatment systems.

The specific PHA synthesis and degradation rates increased as the MLSS concentrations increased (Table 1 and Fig. 6). Similar to the results of Expt. I, the aerobic PHA synthesis was also observed in the case of lower MLSS value of 1360 ± 47 mg/L, concurrent with a

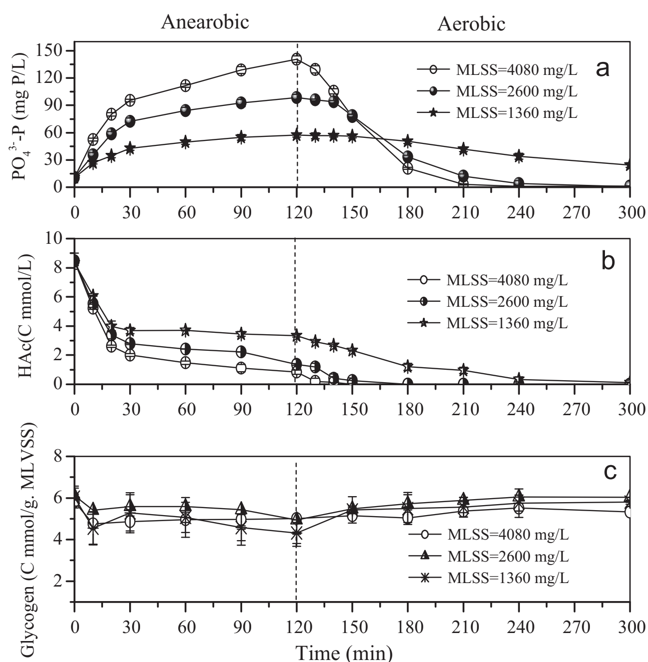

Fig. 6. Variations in phosphate, acetate and glycogen in batch reactors with different MLSS concentrations in the presence of 2 mg/L Cu (II).

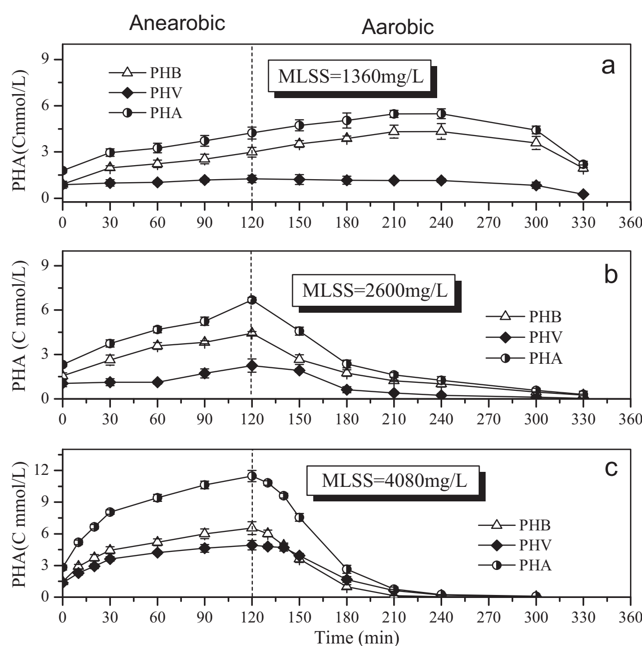

Fig. 7. PHA variations in batch reactors with different MLSS concentrations in the presence of 2 mg/L Cu (II).

higher uptake of acetate. These findings indicate that the inhibition of 2 mg/L Cu (II) at a lower MLSS resulted in incomplete HAc consumption, which indirectly affected the subsequent aerobic PHA degradation and P uptake. Indeed, the lower MLSS concentration resulted in a higher metal uptake density, which led to poorer P removal efficiencies and lower PHA transformation rates (Table 1).

3.4. Mechanisms for Cu (II) (heavy metal) inhibition of the metabolism of PAOs

It has been reported that the toxicity of heavy metal in activated sludge depends primarily on metal species and concentration [13]. For Expts. I and III in our study, HAc was not depleted during the anaerobic phase when suffering elevated Cu (II) shock addition; therefore, the PHA synthesis occurred aerobically. These different metabolic patterns may in part explain the higher sensitivity shown by PAOs exposed to toxic substances.

It is generally believed that the toxic effects of metals primarily occur through interaction with intracellular functional groups, which destroys protein structure and function [27]. However, Hu et al. [11] reported that Cu has a unique mode of action that involves rapid loss of membrane integrity. Their findings were also confirmed by other researchers, who found that disruption of the cytoplasmic membrane was the major mechanism of Cu microbial toxicity [28,29]. Moreover, some researchers have suggested that heavy metals could cross the cell membrane to interact with the intracellular enzymes; thus, interfering with some essential cellular metabolism of bacteria [30]. For EBPR processes, the synthesis and degradation of poly-P, PHA and glycogen in PAO are known to be involved in a number of enzymes. For example, poly-P kinase (PPK) and exopolyPase (PPX) have been found to play a role in poly-P metabolism. PPK induces the synthesis of an exopoly-phosphatase and is associated with the bacterial membrane in some organisms [31]. PPX is an extracellular enzyme and is involved in poly-P degradation [32]. As Cu may cause serious damage to the cell membrane, PPK and PPX could both be destroyed by Cu.

Conversely, little is known about how PHA/glycogen synthesis and degradation are regulated and which enzymes are involved in their regulation. If the active sites of enzymes involved in PHA and glycogen transformations are also located near the outside of the cytoplasmic membrane similar to PPK, the addition of Cu (II) may not only inhibit the activity of enzymes, but also destroy the location of these enzymes.

There are three possible mechanisms of the Cu inhibiton of P removal based on the results of this study. First, the anaerobic degradations of poly-P and glycogen are inhibited by Cu (II). As discussed above, a higher concentration of Cu may cause severe damage to the cell membrane; thus, destroying enzymes such as PPX. Consequently, the poly-P degradation is reduced, leading to insufficient energy available for PHA synthesis. This would directly lead to a subsequent reduction in PHA utilization, and could also indirectly cause reduced P removal efficiency due to energy limitation. In contrast, the hydrolysis of glycogen, which is a source of reducing power for the PHA synthesis, did not change greatly in response to spiking with Cu (II) (Figs. 1b, 2b and 3b), suggesting that the inhibition of glycogen degradation may not be the main cause of the Cu inhibition to PAOs.

Second, the present experimental results show that Cu (II) has a more severe inhibitory effect on aerobic PHA degradation than on anaerobic PHA synthesis. Therefore, it is likely that the PHA degradation enzyme is directly inhibited by Cu (II), which leads to reduced PHA available for subsequent energy sources for growth, and for the need to assimilate phosphate to synthesize poly-P.

Finally, the enzymes involved in P-uptake, such as PPK, may have been inhibited by Cu (II). Previous studies mentioned that PPK may be destroyed by Cu (II), because Cu (II) can destroy the cell membrane. As shown in Figs. 1a, 2c, 6a and 7a, during the aerobic phase, the P uptake greatly declined in response to the elevated Cu (II) addition, although the corresponding PHA was sufficient for PAOs to degrade and produce energy available for P uptake. This could mean that the PAOs capacity for P-uptake and poly-P formation was lost, resulting in inhibition in response to the addition of Cu (II).

4. Conclusions

The presence of Cu (II) changed the anaerobic and aerobic metabolism of PAOs by modifying the transformation patterns of

PHA, poly-P and glycogen. The inhibition of aerobic PHA degradation in response to Cu (II) was greater than the inhibition of anaerobic PHA synthesis. Moreover, the higher MLSS levels were associated with the greater tolerance to Cu (II) toxicity. Therefore, the toxicity of the heavy metal could be minimized by increasing the MLSS in activated sludge reactors. Additionally, at higher pH values, the heavy metal toxicity to PAO microorganisms was reduced via a reduction in the availability of heavy metal fractions. It was speculated that the presence of Cu (II) could have inhibited both the synthesis and activity of the enzymes involved in the synthesis and degradation of poly-P, PHA, and glycogen in PAOs.

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References

[1] T. Mino, V. Arun, T. Yoshiaki, T. Matsuo, Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal process, in: R. Ramadori (Ed.), Advances in Water Pollution Control – Biological Phosphate Removal from Wastewaters, Pergamon Press, Oxford, UK, 1987.

[2] C.D.M. Filipe, G.T. Daigger, C.P.L. Grady Jr., Effects of pH on the aerobic metabolism of phosphate-accumulating organisms and glycogen-accumulating organisms, Water Environ. Res. 73 (2001) 213–222.

[3] S. Rayne, S. Carey, K. Forest, Evidence for tin inhibition of enhanced biological phosphorus removal at a municipal wastewater treatment plant, J. Environ. Sci. Health A: Tox. Hazard. Subst. Environ. Eng. 40 (2005) 535–551.

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

[5] K.B. Chipasa, Accumulation and fate of selected heavy metals in a biological wastewater treatment system, Waste Manage. 23 (2003) 135–143.

[6] Z. Hu, K. Chandran, D. Grasso, B.F. Smets, Comparison of nitrification inhibition by metals in batch and continuous flow reactors, Water Res. 38 (2004) 3949–3959.

[7] J.J. Kelly, M.M. Häggblom, R.L. Tate, Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter as indicated by analysis of microbial community phospholipid fatty acid profiles, Biol. Fertil. Soils 38 (2003) 65–67.

[8] G. Cabrera, R. Pérez, J.M. Gómez, A. Ábalos, D. Cantero, Toxic effects of dissolved heavy metals on *Desulfovibrio vulgaris* and *Desulfovibrio* sp. strains, J. Hazard. Mater. 135 (2006) 40–46.

[9] L. Altaş, Inhibitory effect of heavy metals on methane-producing anaerobic granular sludge, J. Hazard. Mater. 162 (2009) 1551–1556.

[10] K.O. Waara, Effects of copper, cadmium lead and zinc on nitrate reduction in a synthetic water medium and lake water from northern Sweden, Water Res. 26 (1992) 355–364.

[11] Z Hu, K. Chandran, D. Grasso, B.F. Smets, Impact of metal sorption and internalization on nitrification inhibition, Environ. Sci. Technol. 37 (2003) 728–734.

[12] S.Y. Chang, J.C. Huang, Y.C. Liu, Effects of Cd (II) and Cu (II) on a biofilm system, J. Environ. Eng. 112 (1986) 94–104.

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

[14] F.B. Dilek, C.F. Gokcay, U. Yetis, Combined effects of Ni (II) and Cr (VI) on activated sludge, Water Res. 32 (1998) 303–312.

[15] A.P. De Mora, J.J. Ortega-Calvo, F. Cabrera, E. Madejón, Changes in enzyme activities and microbial biomass after "in situ" remediation of a heavy metal-contaminated soil, Appl. Soil Ecol. 28 (2005) 125–137.

[16] Y.Y. Wang, F. Jiang, Z.X. Zhang, M.Y. Xing, Z.B. Lu, M. Wu, J. Yang, Y.Z. Peng, Impact of carbon source on the enhanced biological phosphorus removal and microbial community dynamics in a continuous flow anaerobic/aerobic (A/O) process, Bioresour. Technol. 101 (2010) 98–104.

[17] APHA, Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association, American Water Works Association, Water Pollution Control Federation, Washington, DC, 1998.

[18] K. Jenkins, M.G. Richard, G.T. Daigger, Manual on the Causes and Control of Activated Sludge Bulking and Foaming, 2nd ed., Lewis Publishers, Boca Raton, FL, 1993.

[19] A. Oehmen, R.J. Zeng, Z. Yuan, J. Keller, Anaerobic metabolism of propionate by polyphosphate accumulating organisms in enhanced biological phosphorus removal systems, Biotechnol. Bioeng. 91 (2005) 43–53.

[20] J. Ahn, S. Schroeder, M. Beer, S. McIlroy, R.C. Bayly, J.W. May, G. Vasiliadis, R.J. Seviour, Ecology of the microbial community removing phosphate from wastewater under continuously aerobic conditions in a sequencing batch reactor, Appl. Environ. Microbiol. 73 (2007) 2257–2270.

[21] M. Pijuan, A. Guisasola, J.A. Baeza, J. Carrera, C. Casas, J. Lafuente, Aerobic phosphorus release linked to acetate uptake: influence of PAO intracellular storage compounds, Biochem. Eng. J. 26 (2005) 184–190.

[22] R.J. Seviour, T. Mino, M. Onuki, The microbiology of biological phosphorus removal in activated sludge systems, FEMS Microbiol. Rev. 27 (2003) 99–127.

[23] Y. Liu, Y. Chen, Q. Zhou, Effect of initial pH control on enhanced biological phosphorus removal from wastewater containing acetic and propionic acids, Chemosphere 66 (2007) 123–129.

[24] E. Padan, K. Zilberstein, S. Schuldiner, pH homeostasis in bacteria, Biochem. Biophys. 650 (1981) 151–166.

[25] P.L. Bond, L.L. Blackall, J. Keller, Anaerobic phosphate release from activated sludge with enhanced biological phosphorus removal, a possible mechanism of intracellular pH control, Biotechnol. Bioeng. 63 (1999) 507–515.

[26] C.D.M. Filipe, G.T. Daigger, C.P.L. Grady Jr., A metabolic model for acetate uptake under anaerobic conditions by glycogen-accumulating organisms: stoichiometry, kinetics and effect of pH, Biotechnol. Bioeng. 76 (2001) 17–31.

[27] D.H. Nies, Microbial heavy metal resistance, Appl. Microbiol. Biotechnol. 51 (1999) 730–750.

[28] S.V. Avery, N.G. Howlett, S. Radice, Copper toxicity towards *Saccharomyces cerevisiae*: dependence on plasma membrane fatty acid composition, Appl. Environ. Microbiol. 62 (1996) 3960–3966.

[29] R.K. Sani, B.M. Peyton, L.T. Brown, Copper-induced inhibition of growth of *Desulfovibrio desulfuricans* G20: assessment of its toxicity and correlation with those of zinc and lead, Appl. Environ. Microbiol. 67 (2001) 4765–4772.

[30] V. O'Leary, M. Solberg, Effect of sodium nitrite inhibition on intracellular thiol groups and on activity of certain glycolytic enzymes in *Clostridium perfringens*, Appl. Environ. Microbiol. 31 (1976) 208–212.

[31] K.H. Ahn, A. Kornberg, Polyphosphate kinase from *Escherichia colipurification* and demonstration of a phosphoenzyme intermediate, J. Biol. Chem. 256 (1990) 11734–11739.

[32] R. Goel, T. Mino, H. Satoh, T. Matsuo, Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor, Water Res. 32 (1998) 2081–2088.