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Effects of Cd^{2+} and Pb^{2+} on the substrate biofilms in the integrated vertical-flow constructed wetland

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Abstract

The effects of single Cd^{2+} and Pb^{2+}, and combined Cd^{2+} and Pb^{2+} on dehydrogenase activity and polysaccharide content of the substrate biofilms in the integrated vertical-flow constructed wetland (IVCW) were studied. Dehydrogenase activities decreased linearly with the increasing concentrations of Cd^{2+} and Pb^{2+} at different times (6, 24, 72, and 120 h). The activities at both 6 and 24 h were significantly higher than that at 72 and 120 h in the case of single and combined treatments. The single Cd^{2+} and Pb^{2+} treatments significantly inhibited dehydrogenase activities at concentrations in excess of 20 μmol/L Cd^{2+} and 80 μmol/L Pb^{2+}, respectively. The inhibitory effect of Cd^{2+} was much greater than that of Pb^{2+}. At the same time, the combined treatment of Cd^{2+} and Pb^{2+} significantly inhibited dehydrogenase activities at all five concentrations studied and the lowest combined concentration was 1.25 μmol/L Cd^{2+} and 5 μmol/L Pb^{2+}. A synergistic effect of Cd^{2+} and Pb^{2+} was observed. On the other hand, polysaccharide contents varied unpredictably with the increasing concentrations of Cd^{2+} and Pb^{2+} and extended experimental time. There were no significant statistical differences within the range of concentration and time studied, whether singly or in combination. These results implied that the effects of heavy metals on biofilms should be a concern for the operation and maintenance of constructed wetlands.

Key words: Cd^{2+}; Pb^{2+}; biofilms; dehydrogenase activity; polysaccharide content; integrated vertical-flow constructed wetland (IVCW)

Introduction

Constructed wetlands have proved to be an effective low cost system for wastewater treatment, which are able to remove significant amounts of suspended solids, organic matter, nutrients, heavy metals, trace elements, pesticides, and pathogens through chemical, physical, and biological processes (Kohler *et al*., 2004). The interactions of substrates, wetland plants, and microorganisms in the wetland system probably are the main mechanisms of wastewater purification (Liang *et al*., 2003). Biofilms formed as the microorganisms attached on different parts of plants and substrates, and were chiefly composed of bacterial cells and extracellular polymeric substances (Nielsen *et al*., 1997). Jahn and Nielsen (1998) reported that cell biomass of biofilms from three different gravity sewers was only a minor fraction of the organic matter in biofilms, and 70%–98% of total organic carbon was found to be extracellular. Polysaccharide accounted for 40%–95% of extracellular polymeric substances (Flemming and Wingender, 2001). Therefore, it contributed much to the biomass of biofilms and might impact the efficiency of wastewater treatment because the efficiency relied heavily on appropriate accumulation of biofilm biomass. On the other hand, dehydrogenase activity was correlated significantly with the removal of substrate loading, and the removal efficiency of organic matter in wastewater could be forecasted by means of determining the dehydrogenase activity of biofilms (Yin *et al*., 1995).

Some publications report that biofilms play an important role in removing various pollutants (Costley and Wallis, 2001; Larsen and Greenway, 2004; Bigambo and Mayo, 2005). However, few researchers have focused on the responses of biofilms to pollutants, namely, the effects of pollutants (e.g., heavy metals) on biofilms. The accelerating industrialization in developing countries with an enormous and increasing demand for heavy metals leads to anomalously high concentrations of metals in the environment. The potential of applying constructed wetland technology to treat industrial wastewaters, particularly metal-containing wastewater, has received increasing attention (Zhao *et al*., 2001; Mungur *et al*., 1997; Song *et al*., 2001). Cheng *et al*. (2002) found that heavy metals such as Cu, Cd, Zn, Pb, Al, and Mn could be readily removed by constructed wetland, and most of the metals accumulated mainly in the top layer of the inflow chamber except that Al and Pb were evenly distributed through the whole substrate column. Noticeably, these metals remained in the wetland substrates would have effect on

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biofilms. Pb and Cd, as two common heavy metals, are also the representative contaminants in aquatic systems, with a high risk for aquatic ecosystems and a severe threat to production of safe drinking water and to human health. Consequently, the objective of this study was to assess the effects of single Cd^{2+} and Pb^{2+} and the combined effect of these two metal ions on dehydrogenase activity and polysaccharide content of the substrate biofilms in the integrated vertical-flow constructed wetland (IVCW), and then provide a scientific basis to improve the performance of constructed wetlands.

1 Materials and methods

1.1 Integrated vertical-flow constructed wetland

A twin-chamber (a down-flow chamber and an up-flow chamber, indicating the direction of the passing water) middle-scale plot system of IVCW, based on the design given by Perfler *et al*. (1999), was constructed beside the East Lake, one of the eutrophic urban lakes in Wuhan City, China. In the down-flow chamber and up-flow chamber, the depths of the substrates were 65 and 55 cm, and *Canna indica* and *Acorus calamus* were planted, respectively. The influent of the IVCW system came from East Lake, passing a tank for primary precipitation. The hydraulic loading was 800 mm/d, and the hydraulic retention time was 5.3 h, intermittently loaded.

1.2 Sampling

The substrate biofilm samples were collected from five representative sites of the IVCW system and combined in a composite sample. All litter was removed and the samples were taken to the laboratory in sealed polypropylene bags and stored at 4°C.

1.3 Spatial distributions of biofilms

The spatial distributions of dehydrogenase activity, polysaccharide content, and volatile weight of biofilms in the IVCW system were examined to obtain biofilm samples that contained high amount of microbial activity and biomass at beginning. The down-flow and up-flow chambers were divided into three layers according to their respective depths, namely, surface 0–10 cm, subsurface 10–30 cm, and below 30 cm (30–55 cm for the up-flow chamber and 30–65 cm for the down-flow chamber).

1.4 Correlation between sample volume and net weight of biofilms

Fifty grams of substrates in triplicates were weighed and put in 250 ml conical flasks with a 2:1 (water:substrate) ratio. The flasks were agitated on a shaker (25°C, 120 r/min) for 1 h. The suspended solutions of biofilms were filtered through 0.45 μm pre-dried membranes. The net weight of biofilms was estimated by determining the difference between the dry weight of the membrane after filtration (membrane plus biofilms) and before filtration, with the dry weight quantity obtained by heating the membranes to constant weight in oven at 105°C. Finally, the correlation between different sample volumes and corresponding net weights of biofilms was analyzed.

1.5 Preparation of artificial wastewater

Artificial wastewater consisted of glucose and salts in the following concentrations (mg/L): glucose 100, $(NH_4)_2SO_4$ 20, $K_2HPO_4{\cdot}3H_2O$ 10, $MgSO_4$ 10, $CaCl_2{\cdot}2H_2O$ 10, NaCl 10, $FeCl_3{\cdot}6H_2O$ 0.4, as described by Cao *et al*. (2002).

1.6 Effects of Cd^{2+} and Pb^{2+}

Heavy metal stock solutions were initially prepared with $CdCl_2{\cdot}2.5H_2O$ and $Pb(CH_3COO)_2{\cdot}3H_2O$ (AR). The heavy metal solutions were obtained using artificial wastewater, and the final concentrations of Cd^{2+} and Pb^{2+} were as follows: 2.5, 5, 10, 20, 40 μmol/L and 10, 20, 40, 80, 160 μmol/L, respectively. The combined concentration of Cd^{2+} and Pb^{2+} was also explored by the addition of half of the concentrations of single Cd^{2+} and Pb^{2+}, namely, Cd^{2+} 1.25 + Pb^{2+} 5, Cd^{2+} 2.5 + Pb^{2+} 10, Cd^{2+} 5 + Pb^{2+} 20, Cd^{2+} 10 + Pb^{2+} 40, Cd^{2+} 20 + Pb^{2+} 80 in μmol/L.

Two hundred and fifty milliliters conical flasks with a 2:1 (solution:substrate) ratio (100 ml heavy metal solution of different concentrations to 50 g of substrates) were cultured at 25°C on a shaker agitating at 120 r/min. The flasks were divided into three groups (single Cd^{2+} and Pb^{2+} treatments and their combined treatment) of three replicates. Other three flasks fed with artificial wastewater without heavy metals were used for controls. Dehydrogenase activity and polysaccharide content of biofilms were measured at 6, 24, 72, and 120 h, respectively.

1.7 Analytical methods

The phenol sulphuric acid assay was used to quantify polysaccharide of biofilms (Liu *et al*., 2000). One milliliter diluted suspended solution (1 in 10 dilution) and 1 ml 50 g/L phenol were added in a glass tube. The tubes were then mixed on a vortex mixer for 30 s. Five milliliters concentrated H_2SO_4 was added to each tube, and again the tubes were mixed on a vortex mixer for 10 s. The absorbance of the solution was measured at 490 nm when the tubes were cool. Polysaccharide content was related to gram of fresh weight (fw) substrate, and glucose was used to construct the standard curve for polysaccharide content.

Dehydrogenase activity was determined by reduction of triphenyl tetrazolium chloride (TTC) to triphenyl formazan (TF) (Zhu *et al*., 1996; Liu *et al*., 2000). Briefly, 2 ml suspended solutions, 2 ml Tris-HCl buffer solutions (pH 7.6), 2 ml 0.1 mol/L glucose, and 2 ml 0.5% (*W/V*) TTC were incubated for 12 h at 37°C and stopped by adding concentrated H_2SO_4. After 5 ml acetone was added, the suspensions were centrifuged at 5,000 r/min for 5 min, and the supernatant was assayed at 492 nm. Dehydrogenase activity was calculated as microgram TF produced per gram of fw substrate per 12 h. A standard curve was prepared by using Na_2S (10%) as reducing agent to reduce TTC to TF (0–60 μg) and reading the absorbance at 492 nm.

The volatile weight of biofilms was determined by

measuring the difference between the dry weight (quantified by heating the substrates to constant weight in oven at 105°C) and the ash weight (quantified by heating the substrates to constant weight in furnace at 550°C) (Liu *et al*., 2000), and g/g fw substrate gave the unit of the volatile weight.

1.8 Data analysis

Statistical analysis was made with the software package SPSS 13.0 for Windows (SPSS Inc., USA). Multivariate analysis of variance was used to analyze the data for dehydrogenase activities and polysaccharide contents of biofilms. To further visualize the effects of concentrations of Cd^{2+} and Pb^{2+} on dehydrogenase activities, simple linear regression was used. Statistical significance was tested at 0.05 level.

2 Results

2.1 Spatial distribution of biofilms

As shown in Table 1, on the basis of the investigations carried out in the IVCW system, it was established that microbial activity and biomass were the greatest within the top 10 cm in the down-flow chamber. The wetland system had been operated for several years, and the surface 0–5 cm substrates contained a lot of litter, therefore in this study, the biofilm samples in the depth of 8–10 cm in the down-flow chamber were collected to study the effects of Cd^{2+} and Pb^{2+} on dehydrogenase activity and polysaccharide content of biofilms.

2.2 Correlation between sample volume and net weight of biofilms

The volume of well-proportioned suspended solution of the substrate biofilms was correlated significantly with the corresponding net weight of biofilms (R^2 = 0.993, P < 0.05, Fig.1). It was inferred that the volume of suspended solution could substitute for the weight or concentration of biofilms and could be used for calculating quantificationally dehydrogenase activity and polysaccharide of biofilms.

2.3 Effects of Cd^{2+} and Pb^{2+} on dehydrogenase activity and polysaccharide content of biofilms

2.3.1 Single and combined effects of Cd^{2+} and Pb^{2+} on dehydrogenase activity

Dehydrogenase activities decreased with the increasing concentrations of Cd^{2+} and Pb^{2+} and extended experimental time, whether singly or in combination (Fig.2). It was worth noting that dehydrogenase activities in the controls also decreased with time, and the activities reduced about 50% after 72 h. The dehydrogenase activities at 20, 40 μmol/L (for Cd^{2+}) and 80, 160 μmol/L (for Pb^{2+}) were significantly lower than the control under the single treatments of Cd^{2+} and Pb^{2+} at 6 and 24 h. The activities at all five concentrations studied were significantly lower than the control under the combined treatment of Cd^{2+} and Pb^{2+}. In other words, dehydrogenase activities were significantly inhibited at higher concentrations of single Cd^{2+} (20, 40 μmol/L) and Pb^{2+} (80, 160 μmol/L), and at the same time were significantly inhibited at all five combined concentrations of Cd^{2+} and Pb^{2+}. Moreover, under the single treatments of Cd^{2+} and Pb^{2+}, dehydrogenase activities at different times in descending order were: 6 h > 24 h > 72 h > 120 h, but under the combined treatment of Cd^{2+} and Pb^{2+}, the activities were found to descend in the order: 24 h > 6 h > 72 h > 120 h. Dehydrogenase activities at both 6 and 24 h were significantly higher than that at 72 and 120 h in single and combined treatments.

When the concentration of Cd^{2+} exceeded 10 μmol/L, dehydrogenase activities decreased rapidly at 6 and 24 h (Fig.2), and both reached their lowest values at 40 μmol/L Cd^{2+} with only 57.0% (at 6 h) and 48.9% (at 24 h) of the activity at 10 μmol/L Cd^{2+}. Comparing the activity at 40 μmol/L Cd^{2+} with the control, the largest inhibitory efficiencies at 6 and 24 h were 43.0% and 54.2%, respectively. Similarly, dehydrogenase activities decreased rapidly when the concentrations of Pb^{2+} were in excess of 40 μmol/L (at 6 h) and 20 μmol/L (at 24 h) (Fig.2). The activities at both 6 and 24 h reached their lowest values at 160 μmol/L Pb^{2+}, with 55.1% of the activity at

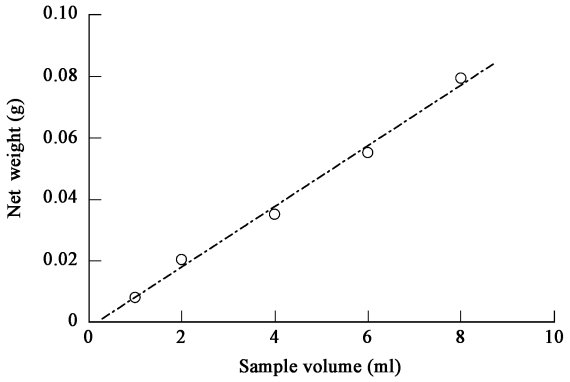

Fig. 1 Correlation between sample volume and net weight of biofilms used in the form of suspended solution.

Table 1 Spatial distribution of dehydrogenase activity, polysaccharide content, and volatile weight of biofilms

Sampling location	Depth (cm)	Dehydrogenase activity (μg TF/(g fw substrate·12 h))	Polysaccharide content (μg/g fw substrate)	Volatile weight (g/g fw substrate)
Up-flow chamber	0–10	11.29 ± 1.54	2,660.4 ± 55.9	0.0089 ± 0.0001
	10–30	1.68 ± 0.23	816.9 ± 13.0	0.0032 ± 0.0003
	30–55	4.41 ± 0.33	1,275.0 ± 115.4	0.0037 ± 0.0001
Down-flow chamber	0–10	25.75 ±1.35	4,652.5 ± 165.6	0.0103 ± 0.0005
	10–30	15.14 ±2.69	1,348.1 ± 17.6	0.0048 ± 0.0004
	30–65	8.04 ±1.03	1,203.2 ± 40.7	0.0041 ± 0.0002

TF: triphenyl formazan; fw: fresh weight. The value is expressed as mean ± standard deviation.

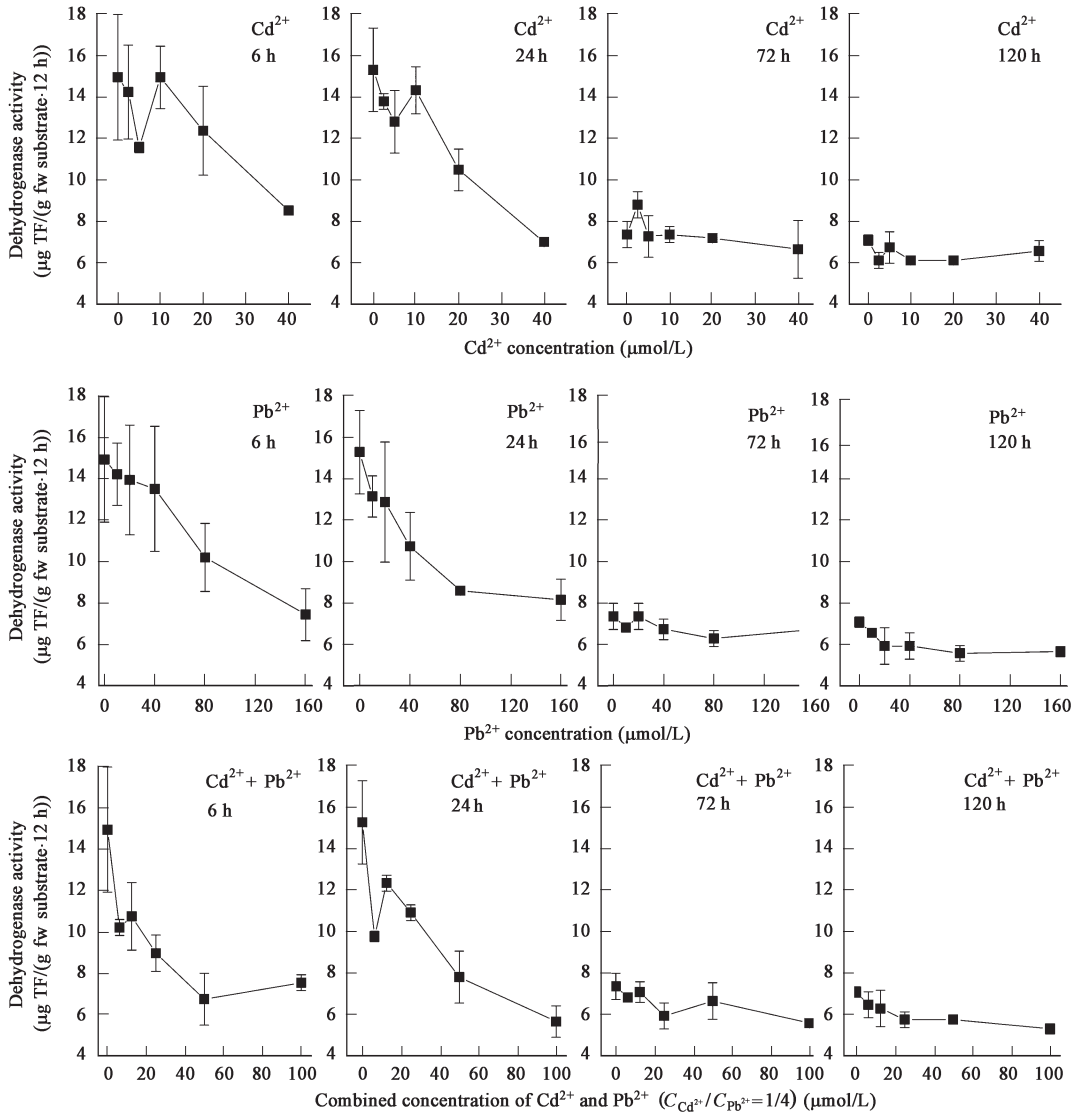

Fig. 2 Effects of Cd^{2+}, Pb^{2+}, and the combination of Cd^{2+} and Pb^{2+} on dehydrogenase activity of biofilms.

40 μmol/L Pb^{2+} (at 6 h) and 63.3% of the activity at 20 μmol/L Pb^{2+} (at 24 h). The largest inhibitory efficiencies at 6 and 24 h were 50.1% and 46.7%, respectively. As for the combined treatment of Cd^{2+} and Pb^{2+}, dehydrogenase activities decreased sharply with the increasing metal ion concentrations at both 6 and 24 h (Fig.2). The inhibitory efficiency ranged between 28.0% and 54.9% at 6 h and ranged between 19.2% and 63.0% at 24 h. It was interesting that dehydrogenase activity suddenly increased at the combined concentration of 2.5 μmol/L Cd^{2+} and 10 μmol/L Pb^{2+} at 24 h and higher than that at 6 h, thereafter, the activity decreased but still was higher than that at the same concentration at 6 h.

Under the single and combined treatments of Cd^{2+} and Pb^{2+}, the relationships between dehydrogenase activities and concentrations of metal ions at the different designated times were estimated by a linear regression analysis. The results indicated that dehydrogenase activities showed linear relationships to the concentrations of Cd^{2+} at 6 h (R^2 = 0.706), 24 h (R^2 = 0.916) and 72 h (R^2 = 0.417) except that at 120 h. In particular, the activities were significantly linear with the concentrations of Cd^{2+} at 6 and 24 h ($P < 0.05$ and $P < 0.01$, respectively). Similarly, for the single treatment of Pb^{2+}, the activities decreased linearly with the concentrations of Pb^{2+} at all designated times. The relationship between the dehydrogenase activity and the concentration of Pb^{2+} at 6 h was of overriding importance (R^2 = 0.974, $P < 0.001$). At the same time, it indicated a significant linear relationship at 24 h (R^2 = 0.782, $P < 0.05$) and only a weak relationship at 72 h (R^2 = 0.298). Likewise, for the combined treatment of Pb^{2+} and Cd^{2+}, changes of affected activities with the combined concentrations of Cd^{2+} and Pb^{2+} showed definite linear relationships at all the times, and the relationships were significant at 24 h ($P < 0.05$) and 120 h ($P < 0.05$), respectively.

Compared with the control, dehydrogenase activities at 6, 24, 72, and 120 h respectively decreased 43.0%, 54.2%, 9.6%, 7.5% at 40 μmol/L Cd^{2+}; decreased 50.1%, 46.7%, 8.4%, 20.2% at 160 μmol/L Pb^{2+} and decreased 54.9%, 49.0%, 9.6%, 18.9% at the combined concentration of 10 μmol/L Cd^{2+} and 40 μmol/L Pb^{2+}. Noticeably at the same

experimental time, these values under single and combined treatments of Cd^{2+} and Pb^{2+} were respectively close to each other. Based on the magnitude of the respective metal concentration resulting in the same quantum of decrease in the dehydrogenase activities obtained under the single treatments of Cd^{2+} and Pb^{2+}, the inhibitory effect of Cd^{2+} was about four times that of Pb^{2+}. Comparison of the single and combined treatments of Cd^{2+} and Pb^{2+} indicated that the combined effect on dehydrogenase activity was much more severe than the sum of the effect of each individual metal ion at the same concentration. Synergistic effect of Cd^{2+} and Pb^{2+} was observed.

2.3.2 Single and combined effects of Cd^{2+} and Pb^{2+} on polysaccharide content

Unlike the response of dehydrogenase activity, polysaccharide content of substrate biofilms was not affected by single and combined exposures to Cd^{2+} and Pb^{2+} or experimental time (Fig.3). No significant statistical differences were observed within the range of concentration and time studied.

3 Discussion

Changes of dehydrogenase activity have been used as a measure of cell inhibition by toxicants in water or activated sludge (Chang-Won *et al.*, 1994). The dehydrogenase activities in this study demonstrated marked inhibitory responses to Cd^{2+} and Pb^{2+}, especially in the first 24 h, whether individually or in combination. When the metal was introduced into the conical flasks, the bulk of the metal was gradually immobilized by the substrates through complexation or chelation. The small percentage of the metal leaving in the aqueous phase could influence availably the metabolic activities of microorganisms, and was called available metal. The amount of available metal ion much more than that of the total metal ion could reflect the effect of metal ion on the substrate microorganisms and plants (Huang *et al.*, 2000). In this work, only a small quantity of Cd^{2+} and Pb^{2+} remained in the suspended solution of substrate biofilms over a period of time (72 h), which could significantly reduced their toxicity to the microorganisms

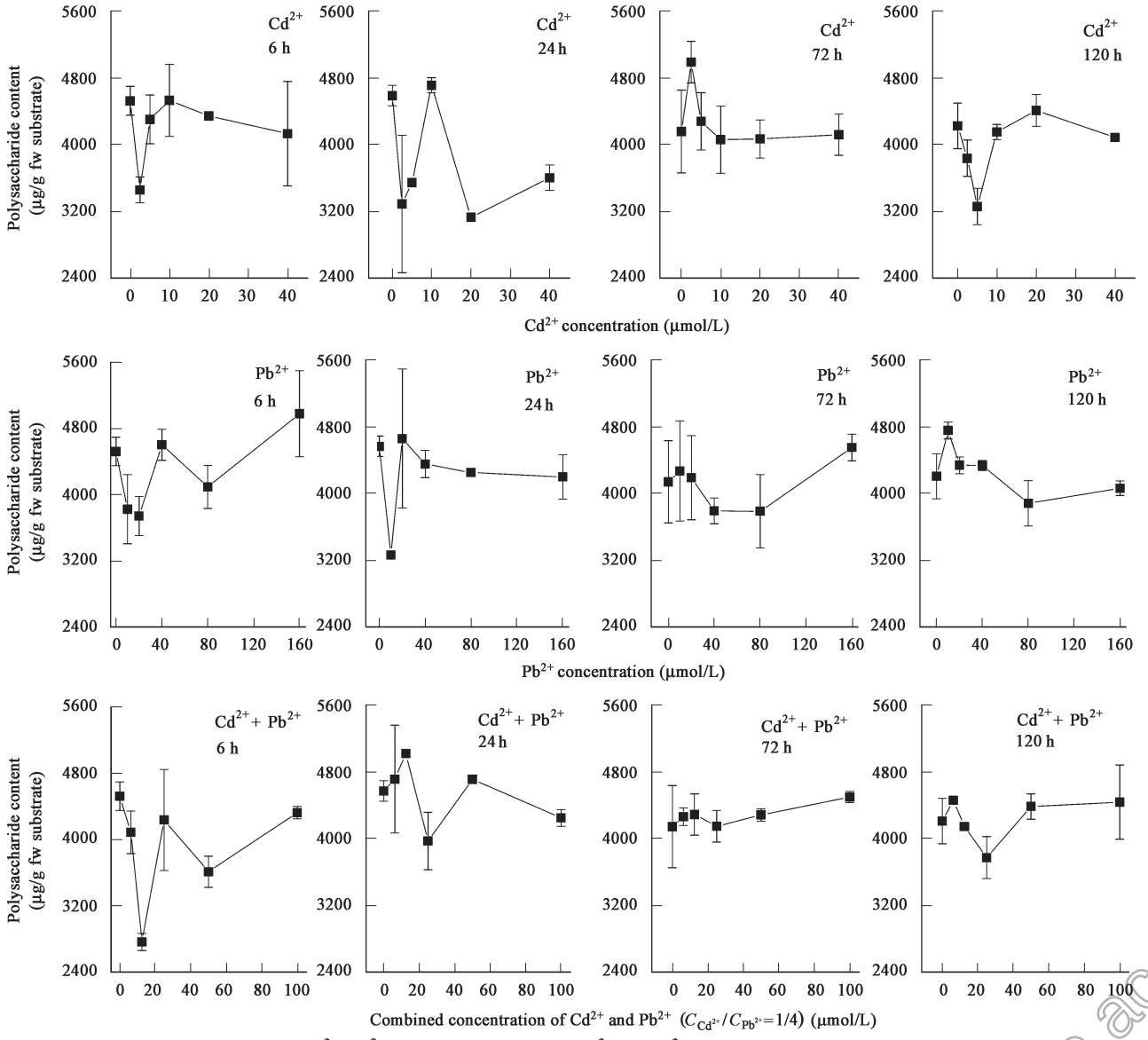

Fig. 3 Effects of Cd^{2+}, Pb^{2+}, and the combination of Cd^{2+} and Pb^{2+} on polysaccharide content of biofilms.

in biofilms. Therefore, the inhibitory effects of Cd^{2+} and Pb^{2+} deteriorated progressively, and the activities at the single and combined concentration gradients of Cd^{2+} and Pb^{2+} exhibited smooth changes at 72 and 120 h. The significant decreases of dehydrogenase activities at 72 and 120 h could be explained by the decreasing controls with time. A general growth process of biofilms comprised adaptive phase, increased phase, stationary phase, and declining phase (Liu *et al*., 2000). Dehydrogenase activities in the controls decreased over time, it might be due to the fact that biofilms in the controls at 72 and 120 h were at different growth phases in comparison to that at 6 and 24 h. Tetrazolium salts (e.g., INT, TTC) reduction had been used as an indicator of viable respiring bacteria (Roslev and King, 1993; Rodriguez *et al*., 1992). And since respiration was closely associated with cellular metabolism, detection of dehydrogenase activity by reduction of tetrazolium salts such as TTC to TTC-Formazan had been used to detect respiring cells and to measure their activity. In this study, the dehydrogenase activity at 24 h increased compared with that at 6 h under the combined treatment of Cd^{2+} and Pb^{2+}, it was probably due to the fact that the activity under the combined treatment was affected in different ways compared to single treatment, and the combined toxicity of Cd^{2+} and Pb^{2+} was likely to stimulate the increase or emergence of certain viable bacteria.

Single Cd^{2+} and Pb^{2+} significantly inhibited dehydrogenase activities at concentrations in excess of 20 μmol/L Cd^{2+} (equal to 2.24 mg/L Cd^{2+}) and 80 μmol/L Pb^{2+}. This result was in agreement with Nie (2003) who found that Cd^{2+} (2 mg/L) inhibited the activity and growth of algae-bacteria biofilms. The inhibitory effect of Cd^{2+} on the activity was much stronger than that of Pb^{2+}. The difference in characters of Cd^{2+} and Pb^{2+} such as standard electrode potential, covalent radius, and atomic radius was a probable explanation for the difference in toxicity. Furthermore, the combination of Cd^{2+} and Pb^{2+} could significantly inhibit the activities at all the five concentrations, and the lowest concentration was 1.25 μmol/L Cd^{2+} and 5 μmol/L Pb^{2+}. The synergistic effect of Cd^{2+} and Pb^{2+} increased the toxicity to biofilms. These results showed that if the Cd^{2+} and Pb^{2+} introduced into constructed wetlands went beyond certain limits, the activity of biofilms would be significantly affected, and the capacity of removing other pollutants (e.g., N, COD) by biofilms would further be impacted.

The measurement of dehydrogenase activity gave indication of microbial activity at the time of sampling, and analysis of polysaccharide content measured biofilm accumulation over time. Polysaccharide played an important role in the immobility of cells, formation of biofilms, and adsorption of dissolved ions, and its secretion and accumulation were related to cellular activity and amount (Liu *et al.*, 2000). Ragusa *et al*. (2004) reported that bacteria growing in the biofilms produced extracellular polymeric substances that help them attach to surface, bind together, and protect them from the surrounding environment, and polysaccharide was the major component. The fact that the single and combined treatments of Cd^{2+} and Pb^{2+} or experimental time had no significant effects on polysaccharide content of biofilms indicated that the biofilms did not adapt to the altered environment in the short term.

4 Conclusions

Cd^{2+} and Pb^{2+} showed significant inhibitory effects on the activity of substrate biofilms in the IVCW system in short order. Unlike organic pollutants, heavy metals could not be degraded through biological processes and thus would pose threats to the plants and microorganisms in constructed wetlands. The present study provided evidences for the operation and maintenance of constructed wetlands, and Cd^{2+} and Pb^{2+} at high concentrations should be controlled in the influent. The most fundamental characteristic of wastewater treatment facilities (including wetlands) was that their functions relied heavily on the metabolism of microorganisms contained within sludge or biofilms. Therefore, further study is needed to demonstrate the changes of not only microbial diversity but also the viable bacteria cell number and their function in the biofilms affected by heavy metals.

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