Mitigation of Wastewater-Borne Chlorpyrifos in Constructed Wetlands: an Ecological Suitability Assessment by Macrophyte and Microbial Responses

Chuan Wang1,2, Biyun Liu1, Dong Xu1, Liping Zhang1, Feng He1, Qiaohong Zhou1*, Zhenbin Wu1

1State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
2University of Chinese Academy of Sciences, Beijing 100049, China

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Abstract

Plants and microorganisms are the main biotic compartments for phytoaccumulation and metabolic transformation of organic contaminants in constructed wetlands (CWs). However, how they cope with special pollutants during the treatment process has not been well characterized. In this study, responses of Iris pseudoacorus (I. pseudoacorus) and microbial communities were intensively investigated in pilot-scale CWs treating wastewater-borne chlorpyrifos, an organic phosphorus pesticide. Chlorpyrifos was associated with inhibited plant growth, decreased photosynthetic activity, and a significant increase in oxidative products. Superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities were affected by chlorpyrifos, whereas catalase (CAT) activity was almost unaffected. Although chlorpyrifos stimulated the antioxidant system, there was little indication of oxidative damage in I. pseudoacorus. Urease, β-glucosidase, and phosphatase activities in substrate were elevated by 73.73%, 17.20%, and 16.23%, respectively, which may indicate enhancement of nitrogen, carbon, and phosphorus cycling. Fatty acid methyl ester (FAME) profiles showed that aerobic prokaryotes, which are likely the functional group responsible for chlorpyrifos degradation, increased from 16.37% to 31.32% after chlorpyrifos addition. Biomarkers for fungal/bact and trans/cis remained unchanged after the chlorpyrifos addition, suggesting that chlorpyrifos did not negatively influence the substrate microbial communities in CWs.

Keywords: constructed wetlands (CWs), chlorpyrifos, oxidative product, antioxidant enzyme, fatty acid methyl ester (FAME)
Introduction

Chlorpyrifos, a broad spectrum organophosphate insecticide, is applied on a large scale in croplands for the control of foliar insects [1-3]. Approximately 140,000 t of chlorpyrifos are applied annually on a global scale, accounting for 1/3 of total organophosphorus pesticide use. Annually, 5,000 t (of the active ingredient) are used in the United States and 18,000 t are used in China [4-5]. Chlorpyrifos and other pesticides may have negative effects on non-target aquatic invertebrates and fish through irrigation runoff, in which pesticides are either dissolved or particle-associated [6].

Constructed wetlands (CWs) are ecosystems containing substrate, microorganisms, and plants that have been proposed as structural best management practices for the mitigation of chlorpyrifos contamination in agriculturally dominated watersheds [7]. Results from field studies indicate that CWs can efficiently remove pesticides from agricultural runoff. Stearman et al. evaluated pesticide removal rates with different wetland areas and flow rates, and found that the optimal combination eliminated 90.2% of added metolachlor and 83% of added simazine [8]. A field experiment in Norway showed that CWs reduced mean pesticide peak concentrations up to 91% in vegetated wetland cells [9].

However, stress caused by exogenous chlorpyrifos also needs to be considered. Antioxidative enzymes and oxidative products are often used as indicators of plant physiological stress caused by pesticides or herbicides [10-11]. Antioxidative enzymes, which act as scavengers of reactive oxygen intermediates (ROIs) in plants, can be elevated under stressful conditions [12]. Superoxide dismutase (SOD) acts as the first line of antioxidative defense and converts superoxide anion \( \text{O}_2^- \) into hydrogen peroxide \( (\text{H}_2\text{O}_2) \), after which ascorbate peroxidase (APX) and catalase (CAT) detoxify \( \text{H}_2\text{O}_2 \) into \( \text{O}_2 \) and malonaldehyde (MDA) [12]. Water-soluble sugar (WSS) functions as osmotic adjustment to relieve injuries to the plasmalemma [13].

Enzymatic activities in soil microorganisms are sensitive indicators that can be used to monitor changes in soils [14]. Urease, \( \beta \)-Glucosidase, and phosphatase are used as bioactivity indices in soils, sediments, and other substrates. Urease, \( \beta \)-glucosidase, and phosphatase are responsible for hydrolysis of urea, degradation of cellulose, and cleavage of a variety of ester-phosphate bonds, respectively, forming inorganic nutrients and maintaining biological soil quality [15-18]. Ester-linked fatty acid methyl ester (EL-FAME) profiles have been widely used to “fingerprint” microbial communities in various environments [19-20], which is a useful tool for the study of microbial communities in contaminated soils and aquifers, and for the evaluation of recovery following reclamation [21-23].

Most studies related to pesticides and CWs have focused on the effectiveness of remediation technologies, and few have included ecological suitability assessment of the technology itself. In the present study, the responses of biosystems in CWs, including macrophytes and microorganisms, were evaluated during the process of chlorpyrifos removal from contaminated water.

Material and Methods

Pilot-Scale CWs

Experimental systems were placed in the open air in Wuhan, China (30.55 E and 114.36 N). Six pilot-scale vertical flow CWs were made from polyethylene (PE) columns with a height of 600 mm and a diameter of 500 mm. Each column was filled to a depth of 300 mm with washed river sand (mainly \( \text{Si}_2\text{O}_3, \text{Al}_2\text{O}_3, \text{Fe}_2\text{O}_3 \), 1-5 mm in diameter) as substrate and planted with three uniform \( \text{Iris pseudoacorus} \) (I. pseudoacorus).

Experimental Design and Sampling

During a 15-day acclimatization stage, each column received synthetic wastewater containing 7-8 mg L\(^{-1}\) of total nitrogen (TN), 0.2-0.4 mg L\(^{-1}\) of total phosphorus (TP), and 60-70 mg L\(^{-1}\) of chemical oxygen demand (COD\(_{\text{cr}}\)) as inflow water. The average removal rates of three batches for TN, TP, and COD\(_{\text{cr}}\) were 63.37%, 80.96%, and 90.31%, respectively (Table 1). A 30-day experimental stage followed immediately after acclimation. In the experimental stage, three of the CWs were assigned to a control group and three CWs were assigned to a chlorpyrifos treatment group. Chlorpyrifos was added into the inflow water of the latter group with a final

<table>
<thead>
<tr>
<th></th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
<th>Average removal rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influent</td>
<td>Effluent</td>
<td>Influent</td>
<td>Effluent</td>
</tr>
<tr>
<td>TN (mg L(^{-1}))</td>
<td>7.22±0.13</td>
<td>1.96±0.37</td>
<td>6.99±0.08</td>
<td>3.01±0.34</td>
</tr>
<tr>
<td>TP (mg L(^{-1}))</td>
<td>0.24±0.00</td>
<td>0.03±0.01</td>
<td>0.36±0.00</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>COD (mg L(^{-1}))</td>
<td>64.00±5.66</td>
<td>11.67±3.50</td>
<td>62.50±0.71</td>
<td>3.50±1.47</td>
</tr>
</tbody>
</table>

Table 1. Purification effects for water quality indices TN, TP, and COD\(_{\text{cr}}\) from constructed wetlands.
concentration of 1 mg L⁻¹. This concentration is between the solubility (1.39 mg L⁻¹ at 25°C) of chlorpyrifos and the chlorpyrifos concentration of contaminated water in the field [24-25]. The hydraulic load was 25.48 mm d⁻¹ and the hydraulic retention time (HRT) was two days.

Macrophyte Growth and Chlorophyll Measurements

Young leaves grew out after the acclimatization stage, and the lengths of these leaves were measured at the start and end of the experimental stage. The number and length of withered leaves were also recorded. Chlorophyll was extracted by ethanol (96%, v/v) and detected by spectrophotometry. The formulas used to calculate the fresh concentrations of chlorophyll a, chlorophyll b, and carotenoid are as follows:

- chlorophyll a (Chla) = \( (13.95A_{663} - 6.88A_{649}) \times \frac{total \ volume}{fresh \ weight} \)
- chlorophyll b (Chlb) = \( (24.96A_{663} - 7.32A_{649}) \times \frac{total \ volume}{fresh \ weight} \)
- carotenoid (Car) = \( \frac{(1000A_{470} - 2.05Ca - 114.8Cb)}{245 \times \frac{total \ volume}{fresh \ weight}} \)

Oxidative Products and Antioxidant Enzymes

WSS and H₂O₂ levels were determined colorimetrically and MDA was measured using the thiobarbituric acid method as described by Kong and Lin [26-27]. Fresh tissue (0.5 g) was homogenized in 5 mL cold K₂HPO₄/KH₂PO₄ buffer (50 mM, pH 7.0), which included 1% (w/v) polyvinylpyrrolidone. The homogenates were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used for activities of SOD, APX, and CAT assays.

The activity of SOD was determined by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture (9 mL) consisted of 50 mM potassium phosphate buffer (pH 7.8, 6 mL), distilled water (4 mL), 100 mM EDTA, and enzyme extract (0.15 mL). The reaction was initiated by illuminating the reaction mixture of 1.0 mL min⁻¹.

The activity of APX was determined at 290 nm by the rate of generation of ascorbic acid. The reaction mixture (9 mL) contained 0.1 mM EDTA, 0.5 mM ascorbate, and 0.1 mM H₂O₂. The reaction was initiated by adding 0.1 mL enzyme extract to the reaction mixture. One unit of enzyme activity was calculated as the amount of enzyme extract required to oxidize 1 mM of ascorbate (mg min⁻¹).

The specific activity of CAT was measured according to the decomposition rate of H₂O₂. The reaction mixture (12 mL) contained 0.8 mL of enzyme extract, 0.2 M phosphate buffer (pH 7.8, 6 mL), distilled water (4 mL), and 10 mM H₂O₂ (1.2 mL). CAT activity was estimated at 25°C by monitoring the decrease in absorbance of H₂O₂ at 240 nm and was expressed as mM of H₂O₂ decomposed (mg min⁻¹).

Enzyme Activity and EL-FAME Analysis of Substrate

The activities of urease, β-glucosidase, and phosphatase were measured as previously described [28]. Mild alkaline methanolyis was used for the extraction of EL-FAMEs as described by [29] with some modifications. Six grams of soil was placed in a 50 mL teflon-lined screw-cap test tube and was mixed with 15 mL 0.2 M KOH in MeOH. Cells were lysed and saponified by incubating the test tubes at 37°C for 1 h and gently agitating them every 10 min. After incubation, 3 mL 1 M acetic acid was added to adjust the pH of the mixture. Then 10 mL hexane was added to extract the FAMES from the aqueous phase into the organic phase. Soil samples were centrifuged at 4,800 rpm for 10 min to separate soil organic matter from the organic phase. The organic phase was then transferred to a gas chromatograph (GC) vial, evaporated under a stream of N₂, and finally redissolved into 0.5 mL hexane.

GC analysis was performed using an Agilent 6890N gas chromatograph (Agilent Technologies, USA), equipped with an Agilent 5793 inertMSD. A fused silica capillary column (DB 5 ms, 0.25 mm × 30 mm × 0.25 µm) was employed. The GC oven temperature program consisted of an initial temperature of 80°C for 2 min, with a ramp of 50°C min⁻¹ to 150°C and holding for 2 min, a ramp of 2.5°C min⁻¹ to 195°C and holding for 2 min, and finally a ramp of 2.5°C min⁻¹ to 215°C and holding for 5 min. The injector temperature was 250°C and the injected volume was 1µL. Helium (purity > 99.999%) was used as carrier gas at a constant flow rate of 1.0 mL min⁻¹.

Data Analysis

Differences between control and treatment groups, and between young and adult leaves were analyzed by one-way ANOVA with least significant difference (LSD) for multiple comparisons. Calculations were made with support from the statistical program SPSS, version 16.0.

To ascertain the soil microbial community composition, individual percentage of FAMES of the substrate samples were first subjected to detrended correspondence analysis (DCA). According to the length of the first gradient (0.797), principal component analysis (PCA) of unstrained ordination based on a correlation matrix was used (CANOCO software 4.5). The data were log(x + 1), transformed to allow analysis of patterns
related to the whole community by weighing rare fatty acids (FAs) similarly to common FAs.

Results and Discussion

Macrophyte Growth and Chlorophyll

Throughout the experiment, new leaves formed and young leaves elongated. However, terminal wilting was also observed in adult leaves. Chlorpyrifos significantly inhibited the growth of I. pseudoacorus (Fig. 1a). Compared with the start of the experimental stage, the leaf number and length at the end of experiment increased by 16.28% and 14.29%, respectively, in the treatment group, and by 32.44% and 33.85%, respectively, in the control group. The withering rate was not significant between groups.

There was no difference in Chla, Chlb, and Car between young and adult leaves, so data were combined before evaluating differences between the treatment and control groups. The concentrations of Chla, Chlb, and Car were all significantly lower in the treatment group than in the control group (25.04%, 20.94%, and 19.02% lower, respectively; Fig. 1b). Total chlorophyll and carotenoid are reported to decrease with increasing concentrations of a pesticide mixture that includes chlorpyrifos, atrazine, metalaxyl, disulfoton, and ethion [30]. These decreases in pigment content can be partially explained by herbicidal effects on photosystem II (PSII), but can also be attributed to the adverse effects of pesticides on photosynthetic electron transport capacity. Carotenoids function as light-harvesting pigments and prevent photochemical formation of singlet oxygen via chlorophyll [31]. Consequently, macrophytes with decreased chlorophyll and carotenoid content may be vulnerable to oxidative intimidation that results in a decrease of photosynthetic efficiency.

Ratios of Chla/Chlb and Car/Chla were larger in young control leaves than in mature control leaves, whereas there were no differences in these ratios within the treatment group (Fig. 1c). The two ratios were more sensitive to chlorpyrifos in young leaves than that in mature leaves. There was no difference between control and treated mature leaves in the Chla/Chlb or Car/Chla ratio. However, in young leaves, Chla/Chlb was 3.27 for controls and 3.02 for treated plants, and Car/Chla was 0.19 for controls and 0.21 for treated plants. Although the difference in Car/Chla between treated and control young leaves was statistically significant, it was quite small. Reductions in Chla/Chlb after pesticide exposure have been described as an indicator of increased grana stacking, reduced intergrana lamellae, and elevated PSII/PSI ratio, which results in an increased capacity for post-illumination ATP production [32]. Car/Chla generally increases after being treated with herbicides [33]. He et al. reported that Cars/Chla ratios in various species increased after treatment with herbicides, such as bentazon and molinate treatment of Anabaena cylindrical, oxyfluorfen treatment of Nostoc muscorum and Phormidium foveolarum, and 2,4-D treatment of Anabaena fertilissima [34].

Fig. 1 Macrophyte growth and chlorophyll levels after chlorpyrifos treatment. Data are presented as means ± SE (n = 9). Different letters (a, b or p, q) indicate significant differences between young and mature leaves. Asterisks (*) indicate significant differences between control and treated groups (one-way ANOVA, p<0.05).
Oxidative Products and Antioxidant Enzyme Activities

Concentrations of WSS and MDA in young leaves were lower than those in mature leaves within the control group; however, no such differences were found within the treatment group. H₂O₂ concentration was higher in young leaves than in mature leaves in both the control and treatment groups (Fig. 2a). In both young and mature leaves, WSS, H₂O₂, and MDA concentrations were greater in the chlorpyrifos treatment group than in the control group. Relative to the concentrations of these substances in leaves from treated plants, concentrations in young and mature leaves from control plants were 72.99% and 26.55% greater, respectively (WSS); 63.67% and 176.62% greater, respectively (H₂O₂); and 35.69% and 14.57% greater, respectively (MDA).

There was no difference in SOD activity in young and mature leaves. CAT activity was greater in young control leaves than in mature control leaves, but there was no difference between young and mature treated leaves. AOX activity was lower in young control leaves than in mature control leaves, whereas it was higher in young treated leaves than in mature treated leaves (Fig. 2b). Chlorpyrifos stimulated SOD activity in young leaves, but had no effect on CAT activity. AOX was stimulated by chlorpyrifos in young leaves, but was inhibited by chlorpyrifos in mature leaves.

The balance between SOD and AOX or CAT activities in cells is crucial for determining the steady-state level of H₂O₂ and superoxide radicals [35]. SOD acts as the first line of defense converting O₂⁻ into H₂O₂, AOX, and CAT, and then detoxifying H₂O₂ into O₂ and MDA [12]. Consequently, an increase in SOD activity would lead to a corresponding increase in H₂O₂ level, which was consistent with the results presented in section 3.2. It has been proposed that AOX may be responsible for the fine modulation of reactive oxygen intermediates (ROIs) for signaling, whereas CAT is responsible for the removal of excess ROIs during stress [12]. Given that we found no change in CAT activity with exposure to chlorpyrifos, it is unlikely that the H₂O₂ levels we observed didn’t cause oxidative damage. As indicated by the results presented in Fig. 1, chlorpyrifos didn’t cause any physiological damage except for the inhibition of *I. pseudoacorus* growth.

Substrate Enzyme Activities

Urease, β-glucosidase, and phosphatase activities in treated substrate were 73.73%, 17.20%, and 16.23% greater, respectively, than in the control substrate (Fig. 3). Urease activity is reportedly enhanced by chlorpyrifos, acetochlor, or both together [36]. Urease is more responsive than phosphatase to pesticides in soils [37]. β-glucosidase may regulate the supply carbon for...
microorganisms that are unable to directly utilize cellobiose [16]. Because of its role in the C cycle, β-glucosidase has microbial ecological significance and has been suggested as an assay for the 40 decomposition rate of organic matter [38]. β-glucosidase, acid phosphomonoesterase, alkaline phosphomonoesterase, and phosphotriesterase activities are reportedly stimulated by 10 pesticides [39]. Significant correlation has been observed between activity of the phosphatase, phosphotriesterase, and chlorpyrifos biotransformation rates [40]. The activities of all three soil enzymes in the present study increased with chlorpyrifos treatment, indicating stimulation of elements N, C, and P cycles.

Table 2. Ratios of characteristic fatty acids and diversity indices in substrate samples collected from constructed wetlands before and after a 30-d experimental period.

<table>
<thead>
<tr>
<th>Ratios</th>
<th>Before</th>
<th>After</th>
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<tbody>
<tr>
<td></td>
<td>treated</td>
<td>control</td>
<td></td>
</tr>
<tr>
<td>Fungal/bact</td>
<td>0.33±0.02a</td>
<td>0.21±0.01a</td>
<td>0.23±0.02a</td>
</tr>
<tr>
<td>(i15:0+a15:0)/16:0</td>
<td>0.15±0.04b</td>
<td>0.08±0.02c</td>
<td>0.16±0.05a</td>
</tr>
<tr>
<td>trans/cis</td>
<td>1.23±0.42b</td>
<td>1.63±0.48a</td>
<td>2.02±0.24a</td>
</tr>
<tr>
<td>MUFA/Branched</td>
<td>2.24±0.28c</td>
<td>8.69±0.82a</td>
<td>3.02±0.70a</td>
</tr>
<tr>
<td>Diversity indices</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shannon-Wiener</td>
<td>2.00±0.21a</td>
<td>1.95±0.11a</td>
<td>2.02±0.25a</td>
</tr>
<tr>
<td>Simpson</td>
<td>0.19±0.04c</td>
<td>0.20±0.04c</td>
<td>0.20±0.06c</td>
</tr>
<tr>
<td>Group (% of total FAs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>6.89±1.32a</td>
<td>4.73±0.52a</td>
<td>5.84±1.13a</td>
</tr>
<tr>
<td>Group II</td>
<td>16.83±3.24c</td>
<td>34.25±8.03a</td>
<td>18.47±7.53a</td>
</tr>
<tr>
<td>Group III</td>
<td>6.68±1.44c</td>
<td>4.07±0.78a</td>
<td>6.79±1.32a</td>
</tr>
<tr>
<td>Group IV</td>
<td>4.69±1.36c</td>
<td>2.25±0.54b</td>
<td>4.53±0.95a</td>
</tr>
</tbody>
</table>

Group I: Polyunsaturated FA, indicators of eukaryotic microbes; Group II: monounsaturated FA, indicators of aerobic prokaryotic microbes; Group III: branched-chain FA(C14-C16), indicators of anaerobic and Gram-positive bacteria; Group IV: branched-chain FA(C17-C19), including methyl and cyclopropyl FA, indicators of sulfate-reducing bacteria and other anaerobic bacteria.
Mitigation of Wastewater-Borne Chlorpyrifos: Microbial Community Structure and Changes in Soil Health

Variations in microbial community structure were analyzed by PCA. As shown in Fig. 5, the first principle component (PC1) explained 50.4% of the total variance and PC2 explained 14.7% of the total variance. The microbial community structure in the control group was almost unchanged throughout the course of the experiment, but it was separated along PC2 from the community structure of the treated group (Fig. 5a).

Monounsaturated FAs (16:1ω9, 18:1ω9t, and 18:1ω9c) and two saturated FAs (12:0 and 14:0) were most positively correlated with PC2, while cyclopropyl FAs (cy17:0 and cy19:0), two branched-chain FAs (a15:0 and n16:0), and saturated FA 17:0 were most negatively correlated with PC2 (Fig. 5b). These FAs could explain the changes in substrate microbial community structure in CWs after treatment with wastewater-borne chlorpyrifos.

The ratio of FAME fungal-to-bacterial biomarkers reflected changes in soil health, mainly soil organic matter and inorganic N concentrations [22]. The ratio of trans/cis FAMEs indicates the degree of stress in the microbial community [42]. The (i15:0+a15:0)/16:0 ratio reflects the proportion of microbes that are bacteria [43], and the ratio of MUFA/Branch indicates the comparative advantage of aerobic to anaerobic bacteria. The observed changes in (i15:0+a15:0)/16:0 and MUFA/Branch indicate that the overall proportion of the microbial community that was made up of bacteria decreased after treatment with chlorpyrifos, but the proportion of aerobic bacteria increased. This suggests that aerobic bacteria may be responsible for chlorpyrifos degradation by using it as a carbon source. Three aerobic bacterial consortia developed from pesticide-contaminated soils are reported to be able to degrade chlorpyrifos efficiently [44]. A major chlorpyrifos metabolite, 3,5,6-trichloro-2-pyridinol (TCP), has been identified in the degradation process by cyanobacterium Synechocystis sp. strain PUPCCC 64 [2]. Another strain of Alcaligenes sp., capable of degrading chlorpyrifos and its major metabolite TCP, has been isolated from paddy field soil [45]. Fungi are also capable of degrading chlorpyrifos, but there are fewer fungal species than bacterial species degrading this pesticide [46]. Fungal/bacterial ratios are indicators for both soil health and the sustainability of agro ecosystems [22, 47]. We found no negative influence of removing chlorpyrifos from wastewater by CWs on microbial communities in the CW substrate.

Conclusions

Chlorpyrifos at a concentration of 1 mg L\(^{-1}\) caused inhibition of growth and photosynthetic efficiency of I. Pseudoacorus in CWs. Although the antioxidant system was apparently induced by chlorpyrifos treatment, I. Pseudoacorus was able to cope with these changes and protect itself against oxidative damage. The activities of urease, β-glucosidase, and phosphatase in substrate samples were increased by chlorpyrifos treatment. However, the degree of stress in the microbial community and the health of the substrate were not greatly affected by chlorpyrifos. The biotic system in CWs is capable of resisting pesticide chlorpyrifos. Therefore, CWs may be an appropriate choice for disposal of wastewater containing chlorpyrifos or other organophosphorus pesticides that have a similar degradation pathway.

Acknowledgements

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