Structural and metabolic responses of microbial community to sewage-borne chlorpyrifos in constructed wetlands

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ABSTRACT

Long-term use of chlorpyrifos poses a potential threat to the environment that cannot be ignored, yet little is known about the succession of substrate microbial communities in constructed wetlands (CWs) under chlorpyrifos stress. Six pilot-scale CW systems receiving artificial wastewater containing 1 mg/L chlorpyrifos were established to investigate the effects of chlorpyrifos and wetland vegetation on the microbial metabolism pattern of carbon sources and community structure, using BIOLOG and denaturing gradient gel electrophoresis (DGGE) approaches. Based on our samples, BIOLOG showed that Shannon diversity (H') and richness (S) values distinctly increased after 30 days when chlorpyrifos was added. At the same time, differences between the vegetated and the non-vegetated systems disappeared. DGGE profiles indicated that H' and S had no significant differences among four different treatments. The effect of chlorpyrifos on the microbial community was mainly reflected at the physiological level. Principal component analysis (PCA) of both BIOLOG and DGGE showed that added chlorpyrifos made a difference on test results. Meanwhile, there was no difference between the vegetation and no-vegetation treatments after addition of chlorpyrifos at the physiological level. Moreover, the vegetation had no significant effect on the microbial community at the genetic level. Comparisons were made between bacteria in this experiment and other known chlorpyrifos-degrading bacteria. The potential chlorpyrifos-degrading ability of bacteria in situ may be considerable.

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Introduction

Chlorpyrifos is one of the chlorinated organophosphate (OP) pesticides that have been used for pest control in agriculture since the 1960s (Maya et al., 2011). The half-life of chlorpyrifos is usually 60 to 120 days in soil, but it can range from 2 weeks to over 1 year depending on the illumination intensity, soil type, temperature and other factors (Anwar et al., 2009). Long-term usage of chlorpyrifos has caused agricultural non-point source pollution, and increased risk to the quality of aquatic environments (van Dijk and Guicherit, 1999; Spalding et al., 2003; Leu et al., 2005). Accumulation of chlorpyrifos in water bodies could cause potential damage (such as carcinogenicity, neurotoxicity, and reproductive and developmental toxicity) to both aquatic organisms and humans (Jorgenson, 2001; Robles-Mendoza et al., 2009). Thus, the demand for cost-effective methods to remove chlorpyrifos in pesticide wastewater is increasing.

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Constructed wetlands (CWs) have been proven to be an effective management practice to reduce aqueous concentrations of pesticides (Moore et al., 2009) and to control pesticide mitigation (Schulz and Peall, 2001; Moore et al., 2007; Budd et al., 2011). The degradation of pesticides in CWs mainly takes place by photolytic degradation, substrate sorption, plant uptake, and microbial degradation processes (Zhang et al., 2014). The efficiency of different microbial degradation pathways has also been studied. Numerous studies have concentrated on the selection of specific pesticide-degrading bacteria in different pesticide-polluted samples (Lakshmi et al., 2009; Sasikala et al., 2012). Nevertheless, research on the effect of pesticides on microbial community dynamics should not be neglected. This can provide background information for pesticide testing (Engelen et al., 2003).

In addition to microbial degradation, plant uptake is another major method that can decrease chlorpyrifos. Also, there have been a large number of studies focused on the interaction of plants and microbes being treated with chlorpyrifos (Fang et al., 2009; Xie et al., 2010). The roots of plants could provide a stable environment and suitable attachment points for rhizosphere microorganisms, and root exudates could also provide energy and carbon sources for the growth of microbes (Faulwetter et al., 2009).

In this article, the short-term response (30 days) of microbial community structure was examined before and after the addition of chlorpyrifos in both vegetated and non-vegetated CWs. Both BIOLOG (Garland and Mills, 1991) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) techniques (Muyzer, 1999) were used to reveal the basic features of the microbial community, with respect to metabolism and genotypic structure, respectively (Bushaw-Newton et al., 2012). The aim of this research was to explore the response of microbes in rhizosphere substrates of constructed wetlands being treated with chlorpyrifos at the physiological and genetic levels.

1. Materials and methods

1.1. Experimental systems and operating conditions

Six sets of vertical flow CWs were made from polyethylene (PE) buckets with a height of 600 mm and a diameter of 250 mm, which were equally separated into two groups on vegetated and non-vegetated CW systems. Each system was filled with river sand (1–5 mm) with a height of 300 mm, and only the vegetated group was planted with three uniform *Iris pseudacorus*. The river sand was passed through a sieve to remove the large particles, and then washed with tap water in order to remove the small particles. Therefore, the remaining river sand was 1–5 mm in diameter. The *I. pseudacorus* used in this experiment was obtained from the cultivation base of vegetation of the institute of hydrobiology. Fig. 1 shows the ichnography of the simulative vertical flow CWs. After an acclimatization stage of 15 days, the experimental stage lasted for 30 days. In the acclimatization stage, the artificial sewage, which was composed by 7–8 mg/L of total nitrogen (TN), 0.2–0.4 mg/L of total phosphorus (TP), and 60–70 mg/L of chemical oxygen demand (COD), was discharged into the
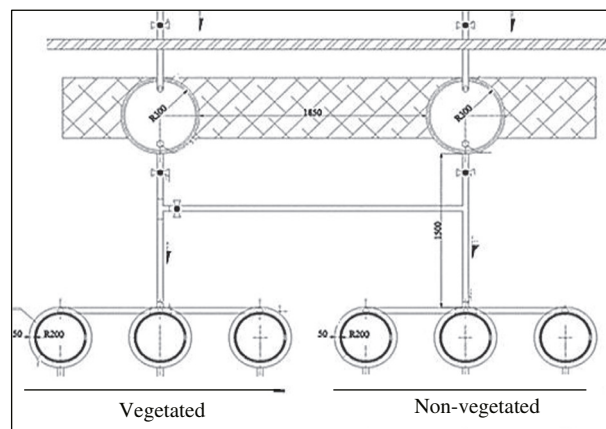

Fig. 1 – Ichnography of simulative vertical flow CWs.

CWs. In the experimental stage, chlorpyrifos was added in the artificial sewage until its concentration reached 1 mg/L. Chlorpyrifos (purity 99.9%) was purchased from Sigma-Aldrich Company. The hydraulic loading was 25.48 mm d^{-1}. Water samples were collected on days 4, 7, 17 and 29 after the chlorpyrifos was added. The chlorpyrifos removal rates on days 4, 7, 17 and 29 in the vegetated systems amounted to (93.97 ± 1.62)%, (96.57 ± 1.46)%, (96.14 ± 1.71)% and (95.72 ± 0.64)%, respectively, and in the non-vegetated systems amounted to (87.65 ± 3.77)%, (96.12 ± 1.28)%, (94.33 ± 1.03)% and (91.67 ± 2.32)%, respectively.

1.2. Substrate sample collection

Samples were collected from the rhizosphere substrate, and each substrate sample was collected from 5 uniformly distributed spots of the surface layer (0–10 cm) from one randomly selected bucket, and then mixed evenly. Substrate samples were taken at the beginning and the end of the experimental stage, and stored at −80°C prior to analysis. The samples collected at the beginning and end of the experimental stage in vegetated systems were designated Pbs and Pas respectively, while those in non-vegetated systems were designated non-Pbs and non-Pas respectively.

1.3. BIOLOG ECO microplate experiment

The BIOLOG ECO microplates contained 31 carbon sources of different groups: 12 kinds of carbohydrates (CHs), 6 kinds of amino acids (AAs), 5 kinds of carboxylic acids (CAs), 4 kinds of polymers (PMs), 2 kinds of amines (AMs), and 2 kinds of phenolic compounds (PCs). A 250 mL conical flask containing 10 g substrate and 100 mL of sterile saline was shaken for 30 min (200 r/min) at room temperature. The supernatant was diluted 25 times, and 150 μL of the diluted bacterial suspension was added to each well of a BIOLOG ECO microplate. The microplate was incubated at 30°C in darkness. Optical density (OD) was measured with SpectraMax M5 at 590 nm (color and turbidity) and 750 nm (turbidity) wavelengths every 12 hr until no more growth in OD value could be observed.

1.4. Extraction of genomic DNA

The SDS based method of DNA extraction (Zhou et al., 1996) and E.Z.N.A Gel Extraction Kit (OMEGA, USA) were used to obtain the genomic DNA of all the samples. The prepared DNA was stored at −20°C before the next step.

1.5. PCR amplification

The extracted DNA was subjected to PCR to amplify the bacterial V3 region of the 16S rDNA gene (from 1055 to 1406), with PCR primer pair GC-338f and 518r. GC-338f had a 40-bp GC clamp CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGG attached at the 5′-end. PCR reaction system was composed of 15 μL 2× Taq PCR MasterMix (ZT201, Zomanbio), 0.2 μL of each primer (100 μmol/L) and 1 μL of the extracted DNA (10–20 ng/μL), and then adjusted the volume to 30 μL using sterile deionized water. PCR amplification was performed using a Thermal Cycler (Bio-Rad T100, USA) under the following conditions: 95°C for 5 min, followed by 35 cycles consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension period at 72°C for 10 min. The PCR products staining with GelRed were verified by running a 1% (w/v) agarose gel electrophoresis in 0.5× TBE buffer.

1.6. DGGE experiment

The DGGE experiment was carried out using a DCode™ Universal Mutation Detection System (Bio-Rad, USA). Using a 40% polyacrylamide gel at 60°C, PCR-amplified samples (200–300 ng) were subjected to electrophoresis for 7 hr at 150 V. A 40% to 60% denaturing gradient was performed. After electrophoresis, the gel was stained with SYBR Green I (Molecular Probes, USA) for 30 min with 1× TAE buffer. Then the gel was examined under a Gel Doc™ XR⁺ Imaging System (Bio-Rad, USA).

1.7. Cloning and DNA sequencing analysis

The excision and reamplification of the desired bands were carried out. The desired bands were excised, resuspended in 30 μL sterile water and stored at 4°C overnight. The PCR amplification was performed as above, except using 338f as the forward primer. PCR products were purified using the Agarose Gel DNA Extraction Kit (TaKaRa) and cloned into pMD-18T vector (TaKaRa). Five positive clones of each band were sequenced by WuHan tsingke BioTech Company. The sequences obtained from the company were analyzed at the NCBI with the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Cluster analysis was performed by MEGA 5.1 with the neighbor-joining method.

1.8. Statistical analysis of BIOLOG and DGGE

Average well color development (AWCD), Shannon–Wiener diversity index (H') and richness (S) were calculated to show the properties of carbon sources and microbial community (da Mota et al., 2005; Rogers and Tate, 2001; Classen et al., 2003). The computation formulas of the three indexes are as follows.

$$AWCD = \sum(C–R)/n$$

where C is the difference in OD value of each carbon source, R is the OD value of the control hole, and n is the total number of carbon source classes ($n = 31$ in this research).

$$H' = -\sum(P_i \times \log P_i)$$

where P_i (in the BIOLOG experiment) is a probability that represents the difference of OD value between a medium pore and control, namely $P_i = (C–R)/\sum(C–R)$ (Dobranic and Zak, 1999). The natural logarithm was used in this paper where P_i (in the DGGE experiment) stands for the observed number of clones of a given species divided by the total number of organisms.

S = the number of reaction (in the BIOLOG experiment); the observed number of bands (in the DGGE experiment).

One-way analysis of variance (one-way ANOVA) was used to analyze the data of AWCD, H' and S. The standardized data of band intensity was processed in PAST3 for principal component analysis (PCA). Principal component analysis was processed on BIOLOG data as well. The banding patterns of DGGE profiles were analyzed using the Quantity One V4.62 Software (Bio-Rad).

2. Results

2.1. BIOLOG analysis of cultivable bacteria

2.1.1. Metabolic diversity and richness of microbial community
The following BIOLOG indices were calculated: AWCD and richness (S), which are indicators reflecting carbon source utilization ability (Choi and Dobbs, 1999; Zhang et al., 2013). In addition, Shannon's diversity (H'), which stands for the diverse utilization of carbon sources by soil microorganisms (Shannon, 2001), was calculated. Moreover, S and H' were calculated using the data from the 180 hr incubation readings (Table 1). Before the addition of chlorpyrifos, S (BIOLOG) ranged from 5.25–13.00, and H' (BIOLOG) ranged from 1.43–2.20. Before adding chlorpyrifos, H' (BIOLOG) and S (BIOLOG) were both higher in non-vegetated CWs than in vegetated CWs ($p < 0.05$). When comparing samples after the addition of chlorpyrifos to those before chlorpyrifos addition, S (BIOLOG) and H' (BIOLOG) in CWs were increased to 18.75–19.83 and 2.87, respectively. However, H' (BIOLOG) and S (BIOLOG) had no significant differences between non-Pas and Pas.

2.1.2. AWCD and its PCA analysis of microbial BIOLOG-ECO profiles
The utilization abilities of the six groups of carbon sources by microbes are shown in Fig. 2. CHs, AAs and CAs were the main carbon sources used by microbes in all treatments. CHs, AAs, CAs and AMs were increased significantly after adding chlorpyrifos into artificial sewage. However, PCs and AMs had significant difference between non-Pbs and Pbs. The AWCD values of these two groups could be detected in the non-Pbs treatment, but decreased to zero in the Pbs treatment. By contrast, there was no significant difference between non-Pas and Pas among the six groups of carbon sources.

The first two principal components were sufficient to explain about 86.99% of the total variance in AWCD data

Table 1 – The diversity and number of band intensity by DGGE profile. The metabolic diversity and reaction number as evaluated by BIOLOG ECO microplate incubated for 180 hr.

Treatment	H′ (BIOLOG)	S (BIOLOG)	H′ (DGGE)	S (DGGE)
Pbs	1.43 ± 0.37a [*]	5.25 ± 1.77a	2.91 ± 0.08a	19.00 ± 1.00a
non-Pbs	2.20 ± 0.25b	13.00 ± 4.95b	2.92 ± 0.11a	19.00 ± 1.73a
Pas	2.87 ± 0.06c	19.83 ± 0.29c	3.00 ± 0.15a	20.67 ± 3.06a
non-Pas	2.87 ± 0.65c	18.75 ± 0.35c	2.94 ± 0.10a	19.67 ± 1.53a

Pbs and Pas represent samples before and after addition of chlorpyrifos in the vegetation systems; non-Pbs, non-Pas represent samples before and after addition of chlorpyrifos in the non-vegetation systems.

[*] Data in the table are Mean ± SE. Different letters represent the significant difference at $p < 0.05$.

(Fig. 3). The difference between vegetated and non-vegetated samples disappeared after the addition of chlorpyrifos. Loading values of each carbon source for the first two principal components are shown in Fig. 4. Non-Pbs, Pas and non-Pas were mainly affected by CHs, PCs and AMs along principal component 1. Pbs was mainly affected by PMs along principal component 1. Non-Pas and Pas were mainly influenced by PCs along principal component 2. Non-Pbs and Pbs were mainly influenced by CAs and AMs.

2.2. PCR-DGGE profile

2.2.1. DGGE profiles and PCA analysis
A 180 bp of the V3 region of the 16S rDNA gene was amplified by PCR from the genomic DNA, and DGGE profiles were performed in different treatments (Fig. 5). There were no significant differences among the four treatments, as was also proved by the indices (H′ and S as calculated in Table 1) of the band intensity. Bands a, n, o, u, and x were the dominant bacteria in all samples. Bands d, e and j were mainly harbored in Pbs and non-Pbs. Meanwhile, the bacteria of bands h, k and t mainly existed in Pas and non-Pas.

The first two PCs explained 51.56% of the total variance (Fig. 6). The main differences among the four treatments occurred between before and after the addition of chlorpyrifos. The loading values of each band for the first two PCs are shown in Fig. 7. Pas and non-Pas were mainly affected by bands f, k, p, s, and t using PC1. Pbs and non-Pbs were mainly influenced by bands e, l, and r by using PC1. The four treatments were not separated by using PC2.

2.2.2. Phylogenetic analysis based on DGGE clones
There were 13 clones sequenced and blast-searched in NCBI (Table 2). Nine out of these were similar to alpha proteobacteria. One was similar to gamma proteobacteria. The others were similar to beta proteobacteria. Band d showed approximately 88% similarity with *Flavobacterium* sp. UKS19, and mainly existed in non-Pbs and non-Pas. Bands e (showing 98% identity to Uncultured *Shewanella* sp.) and j (showing 100% identity to Uncultured *Thauera* sp. clone Z142) were mainly observed in Pbs and non-Pbs. Band k mainly occurred in Pas. Band t (showing 98% identity to *Sphingomonas* sp. 9PNM-6) mainly appeared in Pas and non-Pas. The others were present in all treatments. A phylogenetic tree was constructed based on the 13 clones and 10 known chlorpyrifos-degrading bacteria (Fig. 8). Bands j, o (showing 100% identity to *Thauera* sp. R-26885), n (showing 99% identity to *Rubrivivax gelatinosus* OTSz_B_215), e, and d had close relationships with 4 known chlorpyrifos-degrading bacteria.
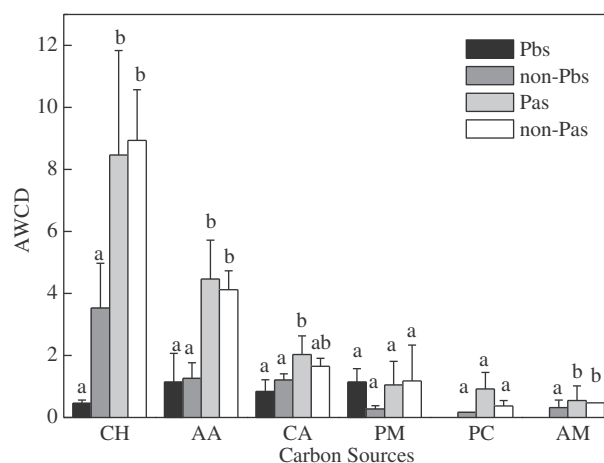

Fig. 2 – AWCD value of utilization of the six groups of carbon sources located in BIOLOG ECO plate by wetland substrate bacterial community of different samples. Significant comparison was made between different treatments in each carbon source. Different letters represent the significant difference at $p < 0.05$. Pbs, Pas, non-Pbs and non-Pas refer to the samples as explained in Table 1. CH: carbohydrates; AA: amino acids; CA: carboxylic acids; PM: polymers; PC: phenolic; AM: amines; AWCD: average well color development.
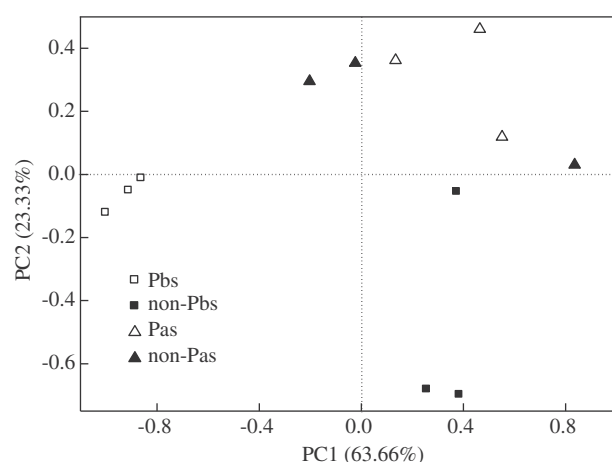
Fig. 3 – PCA analysis of microbial BIOLOG-ECO profiles based on PC1 by PC2 axes, with all the sample replicates projected. Pbs, Pas, non-Pbs and non-Pas refer to the samples as explained in Table 1. PCA: principal component analysis.
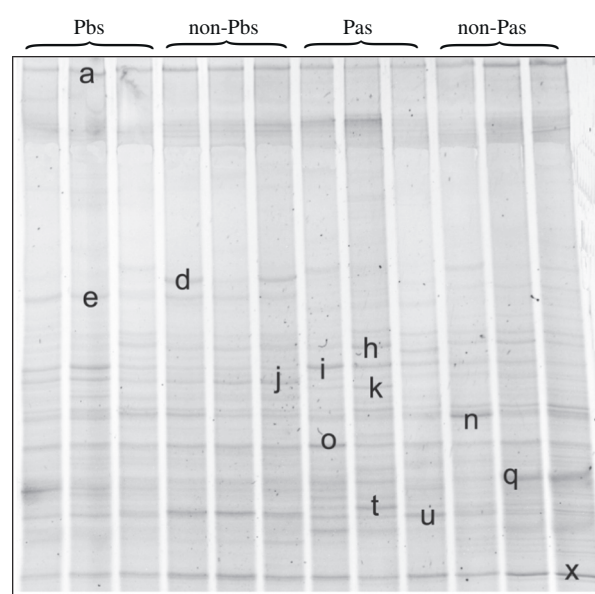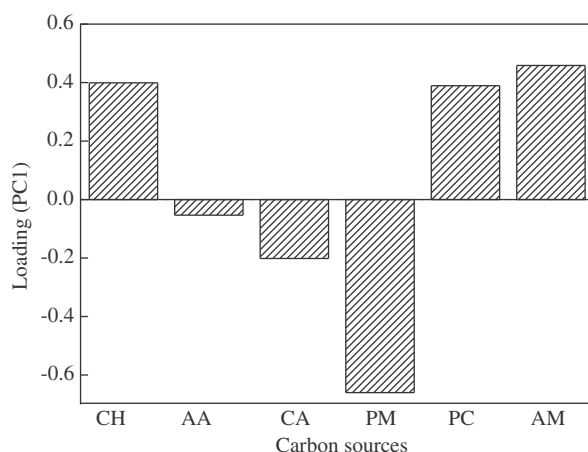

Fig. 5 – DGGE patterns of the PCR-amplified 16S rRNA gene of different wetland substrate treatments. Pbs, Pas, non-Pbs and non-Pas refer to the samples as explained in Table 1. a–x are explained in Table 2.

3. Discussion

BIOLOG plates show the patterns of metabolic response, which is useful for the classification and characterization of microbial communities (Garland and Mills, 1991). H' (BIOLOG) and S (BIOLOG) in non-Pbs were much higher than in Pbs (Table 1, $p < 0.05$). Namely, the utilization ability of microbes in non-Pbs was much higher than that of Pbs. The same result is shown in Fig. 4, where the AWCD of CHs, PCs and AMs were higher in non-Pbs than Pbs, but another study concluded that the AWCD of carbon source utilization was lower in non-vegetated than in vegetated petroleum-contaminated soil (Banks et al., 2003). As vegetation could absorb part of the carbon sources in the sewage, the concentration of carbon sources in non-Pbs was higher than that of Pbs. The microbes in non-Pbs should be more capable of dealing with sewage. Thus the microbes in non-Pbs had higher utilization ability of carbon sources than Pbs. After the addition of chlorpyrifos,

H' (BIOLOG) and S (BIOLOG) of vegetated and non-vegetated CWs were raised, and the difference of the two indices between Pas and non-Pas disappeared (Table 1 and Fig. 3). This indicated that chlorpyrifos could promote the carbon source utilization of soil microbes, as research has shown previously (Fang et al., 2009).

Six groups of carbon sources were selected to compare the variation of the four treatments (Fig. 2). Of these carbon sources, CHs, CAs and AAs contain D-Mannitol, i-Erythritol, Pyruvic Acid Methyl Ester, and D-Malic Acid, with relatively lower molecular weight, so that they could be more easily used by microbes (Farenhorst et al., 2008; Gilbert, 2011). Chlorpyrifos served as an enhancer of carbon source utilization in the substrate. This
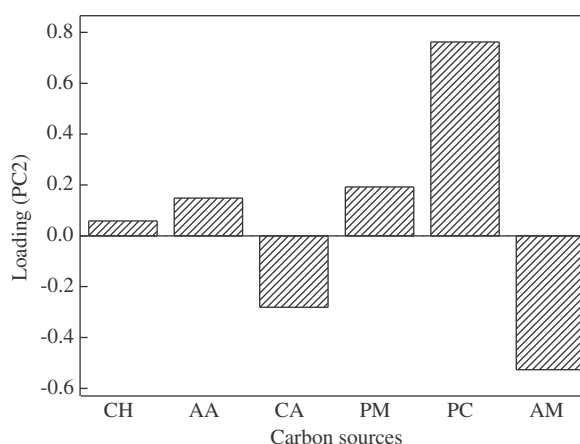

Fig. 4 – Loading values of each carbon source for the first two PCs based on the PCA analysis of BIOLOG-ECO profiles in the 12 sediment samples from the CWs. PCs: phenolic compounds; PCA: principal component analysis; CWs: constructed wetlands.
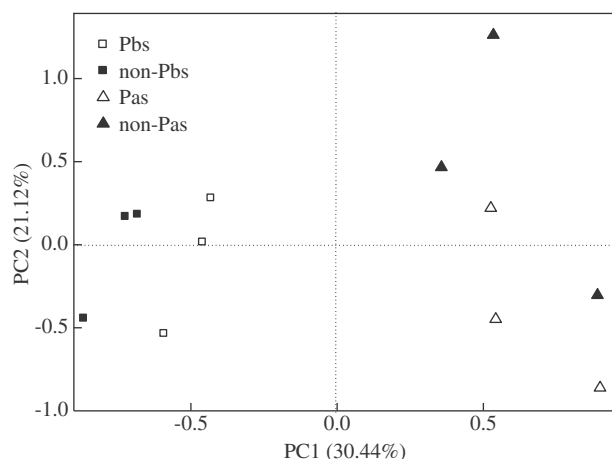
Fig. 6 – PCA analysis of microbial DGGE profiles based on PC1 by PC2 axes, with all the sample replicates projected. Pbs, Pas, non-Pbs and non-Pas refer to the samples as explained in Table 1.

phenomenon (Figs. 2 and 4) was observed for each of the six groups specifically, except for PMs. Before adding chlorpyrifos, PCs and AMs could only be detected in non-Pbs. Nevertheless, CHs, AAs, CAs and PMs were not affected by plants. This indicated that PCs and AMs may be inhibited by plants.

The BIOLOG data showed the variation of physiological level among the four treatments, while the DGGE analysis revealed the changes at the genetic level. Previous studies had shown that chlorpyrifos-degrading bacteria accelerated chlorpyrifos degradation, and induced the utilization of chlorpyrifos for long-term use (Head and Cain, 1991; Singh et al., 2000). In this article, H' (DGGE) and S (DGGE) had no significant difference among the four treatments. This meant that the indigenous species mainly changed at the physiological level, not the genetic level. There should be sufficient chlorpyrifos-degrading bacteria for coping with sewage-borne chlorpyrifos. Studies

have shown that pesticides may have no significant effect on microbial community and structure between vegetation and non-vegetation cases (O'Flaherty et al., 2003). The same result could be concluded in this paper. Other studies have shown that this may be due to the high tolerance and adaption of some microbes in situ to chlorpyrifos (Fang et al., 2009; Chen et al., 2014). There were 5 dominant bands (a, n, o, u, x) sequenced. Three out of the five bands were similar to chlorpyrifos-degrading bacteria in CWs (Pinjari et al., 2012; Sasikala et al., 2012; Feng et al., 2014). Hence, there might be considerable potential degrading ability of chlorpyrifos in each treatment. Three bands (h, k, t) were induced by the addition of chlorpyrifos and may be chlorpyrifos-degrading bacteria (Li et al., 2007). Above all, there was plenty of potential in the in situ microbial community for degrading chlorpyrifos. Another three bands (d, e, j) were suppressed by chlorpyrifos.

Chlorpyrifos had different effects on chlorpyrifos-degrading bacteria at different concentrations. Band j might be a chlorpyrifos-degrading bacterium, and band e had a close relationship with several chlorpyrifos-degrading bacteria, as presented in Fig. 8. Although bands e and j may have the characteristics of chlorpyrifos-degrading bacteria, their abundance could also be decreased at the concentration of 1 mg L^{-1} of chlorpyrifos. As shown in Fig. 6, the main difference in the four treatments was between before and after the addition of chlorpyrifos, based on DGGE profiles. This was different from the results of the PCA analysis on the physiological level. It showed that microbial community structures were more sensitive to chlorpyrifos than vegetation.

4. Conclusions

The results of BIOLOG analysis suggested that the differences in values of H' (BIOLOG) and S (BIOLOG) between vegetated and non-vegetated systems disappeared after adding chlorpyrifos. CHs, AAs and CAs were mainly affected by chlorpyrifos, while PCs, PMs and AMs were influenced by both vegetation and chlorpyrifos. DGGE results showed that the vegetation had little impact on the microbial community and
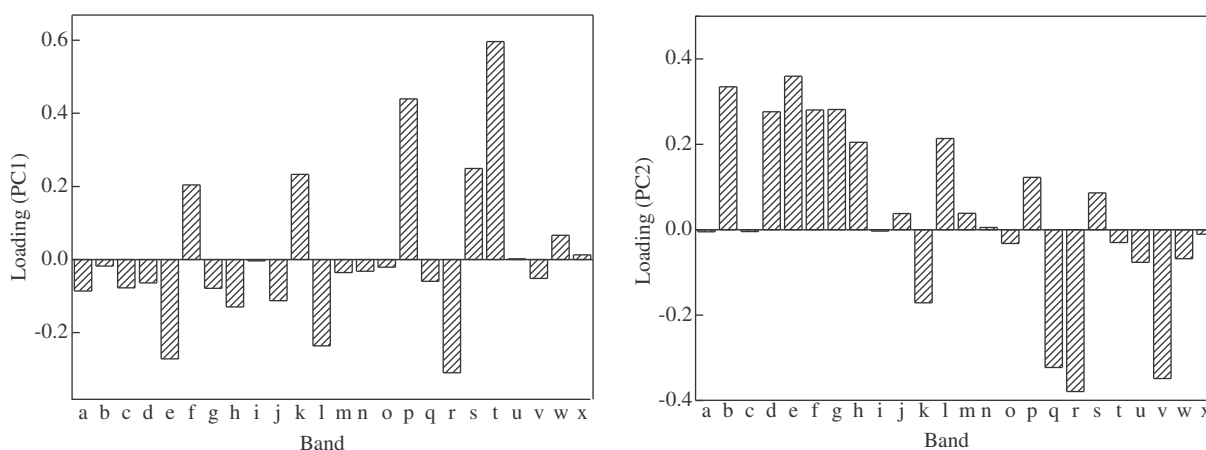

Fig. 7 – Loading values of each band for the first two PCs based on the PCA analysis of DGGE profiles in the 12 sediment samples from the CWs.

Table 2 – Phylogenetic affiliation of the 16S rDNA sequences from DGGE bands.

Bands	Best match in database	Class (proteobacterium)	Treatment	GenBank accession no.	Similarity (%)
1(a)	*Rhizobium* sp. MF10	*Alpha*	All	HF675175.1	98%
2 (d)	*Flavobacterium* sp. UKS19	*Alpha*	Non-Pbs, non-Pas	JQ687101.1	97%
3 (e)	Uncultured *Shewanella* sp.	*Gamma*	Pbs, non-Pbs	KC166793.1	98%
4(h)	*Sphingomonas* sp. MRHull-FeSM-02E	*Alpha*	All	KC404022.1	100%
5 (i)	*Sphingomonas sp.* LH128	*Alpha*	All	KC599553.1	100%
6 (j)	Uncultured *Thauera* sp. clone Z142	*Beta*	Pbs, non-Pbs	EU029354.1	100%
7 (k)	*Porphyrobacter* sp. W4	*Alpha*	Pas	KC248039.1	100%
8 (n)	*Rubrivivax gelatinosus* OTSz_B_215	*Beta*	All	FM886868.1	99%
9 (o)	*Thauera* sp. R-26,885	*Beta*	All	AM084104.1	100%
10 (q)	*Rhodoplanes* sp. JA527	*Alpha*	All	HE962154.1	97%
11 (t)	*Sphingomonas* sp. 9PNM-6	*Alpha*	Pas, non-Pas	JQ608327.2	98%
12 (u)	*Alpha proteobacterium* S-18	*Alpha*	All	AB578881.1	99%
13 (x)	*Hyphomicrobium* sp. AT4	*Alpha*	All	FN667866.1	96%

rDNA: ribosomal deoxyribonucleic acid; DGGE: denaturing gradient gel electrophoresis.

its structure. Based on the samples, the analysis result showed that the main difference in the microbial community structure took place after adding chlorpyrifos, compared to the original artificial sewage.

Microbial community structure and carbon source metabolism in the CWs changed in order to adapt to sewage containing chlorpyrifos. More research can contribute information on the relationship between the physiological and genetic level, and even at the functional level.
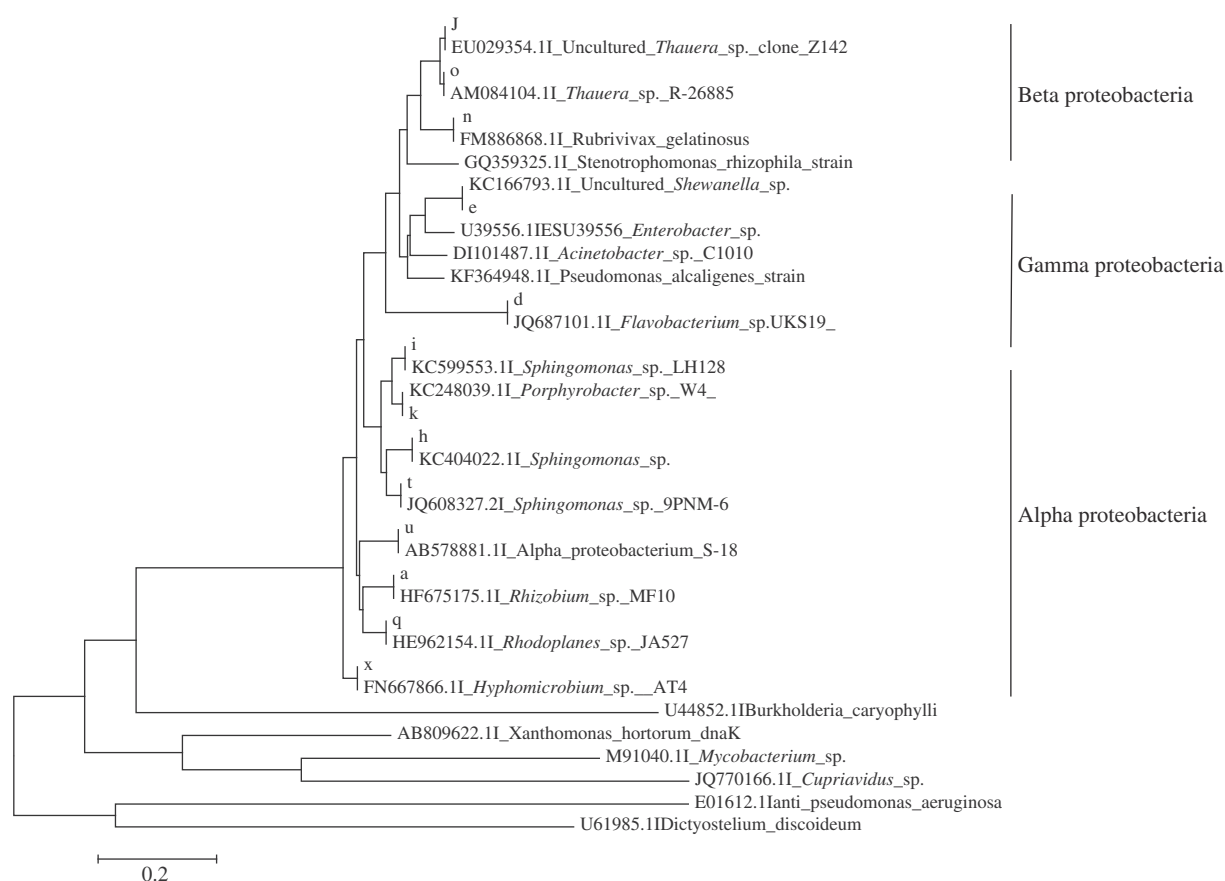
Acknowledgments

Fig. 8 – Phylogenetic tree of the 13 DGGE clones isolated from constructed wetland systems and some known chlorpyrifos-degrading bacteria based on their partial 180-bp 16S rRNA sequences. The scale represents 0.2 substitutions per base position.

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