Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 21(2009) 1415–1423
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Phosphorus utilization and microbial community in response to lead/iron addition to a waterlogged soil

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Received 28 December 2008; Revised 24 February 2009; accepted 03 April 2009

Abstract

Constructed wetlands have emerged as a viable option for helping to solve a wide range of water quality problems. However, heavy metals adsorbed by substrates would decrease the growth of plants, impair the functions of wetlands and eventually result in a failure of contaminant removal. *Typha latifolia* L., tolerant to heavy metals, has been widely used for phytoremediation of Pb/Zn mine tailings under waterlogged conditions. This study examined effects of iron as ferrous sulfate (100 and 500 mg/kg) and lead as lead nitrate (0, 100, 500 and 1000 mg/kg) on phosphorus utilization and microbial community structure in a constructed wetland. Wetland plants (*T. latifolia*) were grown for 8 weeks in rhizobags filled with a paddy soil under waterlogged conditions. The results showed that both the amount of iron plaque on the roots and phosphorus adsorbed on the plaque decreased with the amount of lead addition. When the ratio of added iron to lead was 1:1, phosphorus utilized by plants was the maximum. Total amount of phospholipids fatty acids (PLFAs) was 23%–59% higher in the rhizosphere soil than in bulk soil. The relative abundance of Gram-negative bacteria, aerobic bacteria, and methane oxidizing bacteria was also higher in the rhizosphere soil than in bulk soil, but opposite was observed for other bacteria and fungi. Based on cluster analysis, microbial communities were mostly controlled by the addition of ferrous sulfate and lead nitrate in rhizosphere and bulk soil, respectively.

Key words: phosphorus; lead; PLFA; iron plaque; microbial community; waterlogged soil
DOI: 10.1016/S1001-0742(08)62434-1

Introduction

Wetlands, commonly known as biological filters, have emerged as a viable option for helping to solve a wide range of water quality problems (Collins *et al*., 2005). Recently, constructed wetlands have been built as a replacement of natural wetlands to treat wastewater with many advantages (Sheoran and Sheoran, 2006). To optimize the potential and prolong the life of constructed wetlands, the design of substrates (Xu *et al*., 2006) and plants for constructed wetlands has become a research focus. Suitable plants such as *Typha latifolia* L., *Canna indica* L., *Coix lacryma-jobi* L., *Zizania latifolia* L., and *Phragmites australis* L. have been used for constructed wetlands (Xu *et al*., 2005; Hung *et al*., 2007). *T. latifolia* has been widely used for phytoremediation of Pb/Zn mine tailings under waterlogged conditions due to its higher accumulation of heavy metal in the root system (Manios *et al*., 2003; Ye *et al*., 1997). Heavy metals adsorbed by substrates have shown to decrease the growth of plants, impair the functions of wetlands and eventually result in a failure of contaminant removal (Yang *et al*., 2006).

Although plants play an important role in removing nitrogen, phosphorus and heavy metals in wetlands (Weis and Weis, 2004), the function of the microbial community cannot be neglected, especially for nitrogen, sulfur, iron, organic matter and phosphorus cycle (Steingruber *et al*., 2001). One of the most important characteristics for wetland plants is oxygen transport from stems to the roots and the subsequent release of O_2 into the rhizosphere (Armstrong, 1994). Iron (hydr) oxide precipitates, or plaque, occurring on the surface of roots, have been a focus on its special characteristic in recent years (Greipsson, 1995; Hansel *et al*., 2001). However, the mechanism of plaque formation is still uncertain, especially the role of microbes. Acdithiobacillus ferroxidans or *Leptospirllus* spp., strain BrT has been studied during the course of plaque formation (Weiss *et al*., 2004). Whereas the effect of heavy metal contamination on a microbial system in the rhizosphere is complex, the effect of plaque on microbial communities remains unclear (Collins *et al*., 2005). The effect of iron plaque on the uptake of nutrients and heavy metals has been studied, especially for rice (Batty *et al*., 2000; Zhang *et al*., 1999). However, little is known for wetland plants (Taylor *et al*., 1984; Hansel *et al*., 2001; Xu *et al*., 2009).

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Monitoring soil microbial community structures has been considered a very time-consuming task (Frostegård *et al*., 1993b). Phospholipid fatty acids (PLFAs) are specific components of cell membranes that are only found in living cells. The PLFA analysis has been shown as a useful tool for rapid characterization of broad taxonomic groups of microorganisms in the environment, although PLFA profiles cannot be linked to microorganisms at species level (Zelles *et al*., 1992; Frostegård *et al*., 1993a). However, there are very few studies using PLFA as indicators of microbial communities in saturated soils contaminated with heavy metals (Merila *et al*., 2002; Kaur *et al*., 2005; Zhukova, 2005).

Heavy metals have an obvious effect on or are potentially harmful to biota (Hu *et al*., 2007). The change of the microbial community may influence the element's biochemical reactions and plant utilization. The present study aimed to examine the effects of iron and lead addition on (1) the formation of iron plaque on the roots and phosphorus utilization by wetland plant species *T. latifolia*; and (2) changes of microbial community structure in rhizosphere and bulk soils under waterlogged conditions.

1 Materials and methods

1.1 Soil

The soil for experiment was collected from the depth between 0 and 30 cm in an abandoned paddy field in Jiaxing of Zhejiang Province, China. It is clayey illitic thermic typic epiaqualfs under long-term agricultural use. The soil was sieved through a 2-mm mesh after being air-dried. The pH was 5.61 (soil:water, 1:2.5, *W/W*) and organic matter content was 3.62%.

1.2 Greenhouse experiment

The study was conducted in a plastic greenhouse. The seedlings of *T. latifolia* purchased from Zhongzhu Horticulture Corporation were transplanted into plastic pots with each containing 4.0 L of Hoagland solution (Gamborg and Wetter, 1975). After two weeks, uniform seedlings were selected and transplanted into rhizobags (10 cm × 15 cm) filled with 0.5 kg of the same soil. The rhizobags were then put in plastic pots (upper diameter 20 cm, height 16 cm) filled with 1.5 kg soil, which were treated with four levels of lead (0, 100, 500, 1000 mg Pb/kg as $Pb(NO_3)_2$) and two levels of iron (100 and 500 mg Fe/kg as $FeSO_4$). The experiment was replicated three times. The pots were arranged randomly, and their position was rotated regularly to ensure uniform conditions, and kept about 0.5 cm thickness of water above soil surface for eight weeks.

1.3 Analytical methods

1.3.1 Phospholipid fatty acid (PLFA) analysis

PLFAs were extracted from freeze-dried soil with chloroform/methanol/citrate buffer (1:2:0.8, *V/V/V*) (0.15 mol/L, pH 4.0) (modified according to the method by Frostegård *et al*., (1993b)). Pooled supernatants (two repeated extractions) were split into two phases by the addition of chloroform and the above extracting buffer. The lipid-containing phase was transferred to sterilized glass tubes, dried under N_2, dissolved in 600 μL of chloroform, and transferred to a silica gelcartridge (500 mg, 3 mL) (Supelco, USA) and washed by 5 mL chloroform. Following the elution of neutral lipids and glycol-lipids with 10 mL chloroform and 10 mL acetone, respectively, phospholipids were eluted with 8 mL methanol and dried under N_2. Nonade canoate acid methyl ester (C19:0) was added as an internal standard. PLFAs were subsequently derivatized by mild-alkali methanolysis (He *et al*., 2007). The resulting fatty acid methyl esters were then separated and identified by Agilent 6890N gas chromatography (Agilent, USA) fitted with a MIDI Sherlocks microbial identification system (Version 4.5, MIDI, USA). Individual fatty acids were designated in terms of total number of carbon atoms: number of double bonds, followed by the position (ω) of the double bond from the methyl end of the molecule. The prefixes "a" and "i" indicate anteiso- and iso-branching, respectively, "10Me" describes a methyl group on the tenth carbon atom from the carboxyl end of the molecule, and "cy" represents a cyclopropane fatty acid. Main microbial taxa, including bacteria, gram-positive bacteria, gram-negative bacteria, anaerobe, aerobe, methane oxidizing bacteria, sulfate-reducing bacteria, fungi, and actinomycetes, were indicated by the referred individual PLFA biomarkers (Ekelund *et al*., 2003; Zelles, 1992; Findlay and Dobbs, 1993; Vestal and White, 1989). PLFAs contributed less than 0.1% of the total amount extracted from each sample or PLFAs observed in only one sample were eliminated from the data set, yielding series PLFAs for statistical analysis.

1.3.2 Iron plaque extraction and determination

Fresh roots were washed with tap water, then rinsed in deionized water, and dried by filter paper. The plaque was then extracted using the cold DCB (dithionite-citrate-bicarbonate) technique (Taylor and Crowder, 1983). Roots (about 1 to 3 g fresh weight) were mechanically agitated at room temperature (25°C) for 3 h in the mixture of 40 mL of sodium citrate (0.3 mol/L), 5 mL of sodium bicarbonate (1.0 mol/L), and 3 g of sodium dithionite. Roots removed from the resulting solution were rinsed with deionized water, and the rinse added to the DCB extract. Deionized water was then added to the solution to make total 100 mL with deionized water. The concentration of iron was determined by an atomic spectrophotometer (NovAA300, Germany).

Plant samples were divided into above-ground parts and roots. Sample were dried at 105°C for 30 min, followed by drying at 80°C for 24 h, and then were ground into powder and sieved through a 0.5-mm mesh. Samples (about 0.2–0.5 g dry weight) were digested by 10 mL concentrated nitric acid by microwave digestion. The plant digestion solution and the phosphorus in DCB extract were measured according to molybdenum antimony colorimetry method (Lu, 2000).

1.4 Calculation and statistical analysis

The PLFA data were subjected to principal component analysis (PCA) and Hierarchical Cluster method using software SPSS15.1 to examine patterns of species combinations between rhizosphere and bulk soil. The means were compared using analysis of variance and post hoc multiple comparisons. Some data processing was carried out by software Micro EXCEL 2003.

The comparison of the different treatments richness, evenness, and diversity was done using a two-way analysis of variance. Richness (S) refers to the number of PLFAs detected in the given sample. The PLFA evenness (E), a measure of how evenly PLFAs were distributed in a given sample, was calculated as $E = H/\ln S$. Diversity was calculated as the Shannon index where $H' = -\sum p_i \ln p_i$, and p_i is the proportional amount of each PLFA (Hinojosa *et al*., 2005).

2 Results and discussion

2.1 Phosphorus accumulation in plant and iron plaque on roots

The change in amount of iron plaque on the *T. latifolia* roots was like an inverted "V" with increasing amount of lead supplied (Fig. 1a). Also, the amount of plaque at 500 mg Fe/kg was more than that at 100 mg Fe/kg. The amount of phosphorus adsorbed to plaque showed a similar trend (Fig. 1b), and the correlation coefficient was 0.78 (n = 24, p < 0.01) between the phosphorus adsorbed and the amount of iron plaque on the root, indicating that plaque had a high capacity for adsorbing phosphorus. Phosphorus accumulation in plants of aboveground parts and roots had the similar trend (Figs. 1c and 1d). Phosphorus is an important limiting factor for plant growth, especially in heavy metals contaminated soil (Howells and Caporn, 1996). Heavy metals adsorbed by substrates would decrease the growth of plants, impair the functions of wetlands and eventually result in a failure of contaminant removal (Yang *et al*., 2006). Our results showed that the amount of iron plaque was tightly related to the phosphorus utilization by plants, which is consistent with the result reported in previous studies (Zhang *et al*., 1999; Liang *et al*., 2006). A possible explanation was that iron addition would promote the formation of iron plaque and the enrichment of phosphorus on the roots, which caused a higher phosphorus availability. With increasing levels of lead, phosphorus bioavailability decreased for the combination of lead and phosphorus treatment (Chen *et al*., 2006b). However, phosphorus on iron plaque is increasingly absorbed by plant under phosphorus deficiency (Greipsson, 1995). The beneficial effect of iron addition was attributed to the strong competition of Fe with heavy metals for sensitive metabolic sites within the leaves (Kuo, 1986) or in root tips (Chen *et al*., 2006a). The pH value was decreased as a result of the ferrous oxidized

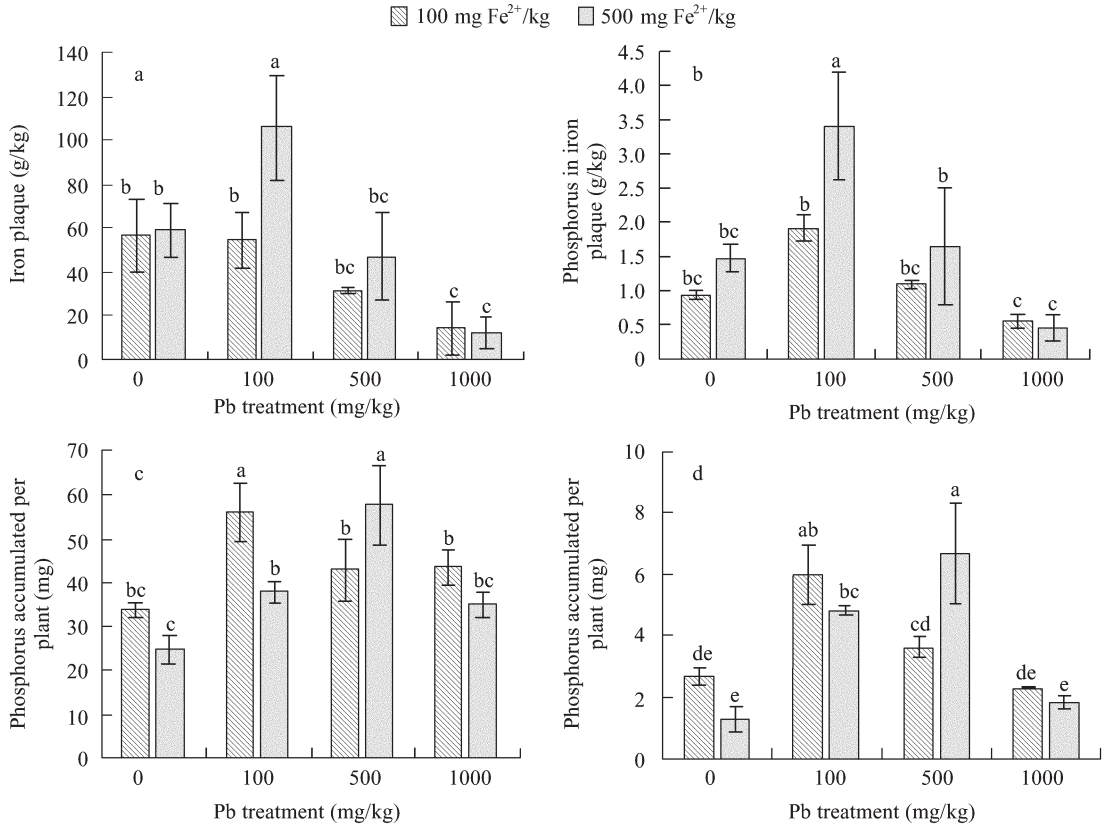

Fig. 1 Effects of Pb treatments on the amount of iron plaque on the roots (a), phosphorus in the iron plaque (b), phosphorus accumulated in aboveground plant (c), and roots (d) of *T. latifolia* grown in the waterlogged soil for 8 weeks. Error bars represent standard deviation. Significance of treatment effects is indicated by different letters (p < 0.05).

in the rhizosphere, which was beneficial to phosphorus availability in lead contaminated soil (Chen *et al*., 2006b). The phosphorus accumulation reached a maximum at the treatment of 100 mg Pb/kg at 100 mg Fe/kg level, and the treatment of 500 mg Pb/kg at 500 mg Fe/kg level. The phosphorus accumulation in both above-ground parts (p = 0.002) and root (p = 0.0001) was significantly affected by lead, and there was a statistically significant interaction between lead and iron in phosphorus accumulated in above-ground parts (p = 0.001) and root (p = 0.007). It suggests that phosphorus utilized was not only affected by iron plaque as the soil contaminated by lead, but the interaction of abiotic factors may also have unpredictable effects.

2.2 Biomass and diversity of microbial community in rhizosphere and bulk soil

The amount of fatty acid detected in the rhizosphere and bulk soil is shown in Table 1. The amount of PLFAs in the rhizosphere was 23%–59% higher than that in the corresponding bulk soil. In the rhizosphere, the control (no addition of lead) was richer in microbes than the other treatments at 100 mg Fe/kg level. The microbial mass characterized by PLFAs in the rhizosphere planted with *T. latifolia* was more than that of the bulk soil (Table 1).

The difference of microbial biomass between the rhizosphere and bulk soil may be related to the excretion of large amounts of organic matter by plant roots, which promotes the high density and diversity of microorganisms (Soderberg and Bååth, 1998). At the same time, *T. latifolia* is an emergent macrophyte with a high rate of radial oxygen loss (Jepersen *et al*., 1998). Oxygen diffused from root changed the rihzosphere condition, which formed an oxic and anoxic interface. The microbe mass in the rhizosphere treated with 100 mg Fe/kg and of the control was the greatest, up to (145.0 ± 8.9) nmol/g soil, but abundance of signature PLFA was not affected by lead addition. This was possibly due to the reduction of heavy metal toxicity with the increasing sulfate, because the iron was supplied as $FeSO_4{\cdot}7H_2O$, and sulfate reducing bacteria under anaerobic conditions were promoted, which likely accelerated sulfate reduction. Microbe-driven sulfate and iron reduction are processes occurring naturally in wetland sediments, which facilitate the removal of metals from acid mine water through increasing pH, and in turn result in the precipitation of metals either as hydroxides or as sulfides (Kalin, 2001; Russell *et al*., 2003). Although some oxygen could diffuse from root surface into the rhizsosphere, which could improve the microorganisms, mono-sulfide (S^{2-}) may be oxidized again, and lead combined with mono-sulfide (S^{2-}) released to the pore water. We found that at the 100 and 500 mg Fe/kg levels, the pH value in the rhizosphere was 1–1.5 units, lower than that in the corresponding bulk soil (data not shown). pH is a confounding factor in elucidating the effect of heavy metals (Frostegård *et al*., 1993a).

The richness, evenness, and diversity in response to different levels of lead in the rhizosphere and bulk soil are shown in Table 2. At the 100 mg Fe/kg level, the microbial community diversity characterized by Shannon indexes (H) reached the maximum at 100 mg Pb/kg both in bulk soil and rhizosphere, however, the evenness was the lowest. Shannon index (H) in the bulk soil increased with increasing lead level at 500 mg Fe/kg. The microbial community richness in the rhizosphere was higher than that in bulk soil except in the treatments of 0 and 100 mg Pb/kg at 100 mg Fe/kg, suggesting that root activity stimulated microbial growth. The evenness in bulk soil was higher than that of rhizosphere. In bulk soil, the microbial community diversity was influenced by the interaction between the addition of lead and iron (p = 0.013). Doleman and Haanstra (1984) reported that Fe content in soil was the main abiotic factor affecting negatively the toxicity of Pb on soil respiration.

Table 1 Total phospholipid fatty acids (PLFAs) in rhizosphere and bulk soil under different treatments

	Fe^{2+} treatment (mg/kg)	PLFAs (nmol/g soil)			
		0	100 mg Pb/mg	500 mg Pb/mg	1000 mg Pb/mg
Bulk soil	100	91.7 ± 1.7 b	96.3 ± 2.2 ab	97.7 ± 4.6 a	84.3 ± 4.3 c
	500	96.3 ± 1.0 ab	94.3 ± 1.4 ab	94.0 ± 1.1 ab	97.6 ± 3.3 ab
Rhizosphere	100	145.0 ± 8.9 a	118.2 ± 4.6 b	136.8 ± 7.0 ab	128.1 ± 4.6 ab
	500	129.8 ± 5.1 ab	130.6 ± 5.0 ab	127.0 ± 7.4 ab	135.6 ± 5.9 ab

Data are expressed as mean ± s.d., n = 3. Data within a row with different letters indicated significant difference at $p < 0.05$.

Table 2 Richness (R), evenness (E), and diversity (H) calculated from phospholipids-linked fatty acids in bulk soil and rhizosphere

Treatments (mg/kg)		Bulk soil			Rhizosphere		
Fe	Pb	R	E	H	R	E	H
100	0	39.7 bc	0.84 abc	3.08 ab	42.3 b	0.80 ab	2.97 c
100	100	53.3 a	0.80 c	3.16 a	52.0 a	0.78 b	3.10 abc
100	500	44.0 b	0.81 c	3.05 ab	51.7 a	0.78 b	3.08 abc
100	1000	32.7 c	0.86 ab	2.98 b	42.3 b	0.80 ab	2.99 abc
500	0	33.0 c	0.86 ab	2.98 b	35.0 c	0.85 a	3.01 abc
500	100	31.3 c	0.87 a	3.00 b	38.7 bc	0.82 ab	2.98 bc
500	500	43.3 b	0.82 bc	3.09 ab	53.7 a	0.79 ab	3.13 a
500	1000	52.7 a	0.80 c	3.17 a	53.3 a	0.78 b	3.12 ab

Data within a column with different letters indicated significant difference at $p < 0.05$.

2.3 Microbial types defined by biomarker PLFAs

PLFA analysis is better for studies of viable organisms and provides a more suitable than conventional cultivation techniques for inferences about community composition. Many lipids are associated with taxonomic or functional groups of microorganism.

I15:0 and a15:0 are indicators of Gram-positive bacteria (G+) (Frostegård *et al*., 1993a), varied from 14.5% to 16.0% of the PLFA in bulk soil (Table 3), and from 9.7% to 13.0% in the rhizosphere soil (Table 4). The relative abundance G+ increased slightly according to the lead amendment in bulk soil ($p = 0.0028$). However, the relative amount of G+ bacteria reached maximum at 100 mg Pb/kg, then decreased with the addition of lead at both iron levels ($p = 0.0126$) in rhizosphere soil. Soil with high lead loading tended to have a lower level of indicator fatty acids for G+ bacteria than the low lead treated soil in rhizosphere. There was no significant difference in relative abundance G+ between 100 and 500 mg Fe/kg levels.

Mono-unsaturated fatty acids such as 17:1ω8c, 16:1ω9c, 15:1 ISO G, 16:1 ISO H, 16:1 2OH, 15:1ω6c, 16:1ω5c, 16:1ω7c, 18:1ω7c and 18:1ω9c, were characterized as Gram-negative bacterial (G–) (Ratledge and Wilkinson, 1988). The relative abundance in the rhizosphere was 7.5%–18.3% higher than that in the bulk soil (Table 3). In bulk soil, relative amount of G– bacteria decreased with the addition of lead except the treatment at 1000 mg Pb/kg and 100 mg Fe/kg. However, in rhizosphere it increased to maximum at 100 mg Pb/kg then decreased at both 100 and 500 mg Fe/kg levels.

The signature fatty acids of anaerobic bacteria (AnB) are cyclo-17:0 and cyclo-19:0 (Findlay and Dobbs, 1993). There was a similar change in these fatty acids at both iron levels in rhizosphere and bulk soil (Table 3). PLFAs characterized anaerobic bacteria were the lowest for the treatment of 100 mg Pb/kg, and then increased with the addition of lead. The relative abundance of AnB in

Table 3 Relative abundance of eight types of microbe characterized by PLFAs in bulk soil

Microbia	Relative abundance (%)			
	0 mg Pb/kg	100 mg Pb/kg	500 mg Pb/kg	1000 mg Pb/kg
	100 mg Fe^{2+}/kg			
Gram positive bacteria	14.55 ± 0.48 a	15.64 ± 0.46 a	15.90 ± 0.75 a	16.03 ± 0.70 a
Gram negative bacteria	19.60 ± 0.48 a	18.05 ± 0.51 a	17.57 ± 2.21 a	19.16 ± 2.48 a
Anaerobic bacteria	8.19 ± 0.16 a	5.89 ± 0.06 c	7.17 ± 0.10 b	7.30 ± 0.27 b
Aerobic bacteria	8.56 ± 0.33 ab	8.72 ± 0.41 ab	8.60 ± 1.03 ab	9.57 ± 1.39 a
Sulfate reducing bacteria	7.33 ± 0.19 b	7.14 ± 0.47 bc	6.61 ± 0.19 c	7.15 ± 0.75 bc
Methane oxidizing bacteria	6.40 ± 0.25 a	6.25 ± 0.44 a	5.89 ± 0.92 a	6.49 ± 0.91 a
Actinomycetes	9.97 ± 0.33 bcd	10.36 ± 0.75 bc	9.18 ± 0.19 d	10.22 ± 0.78 bc
Fungi	1.67 ± 0.16 a	1.63 ± 0.04 a	1.47 ± 0.86 ab	1.36 ± 0.17 ab
	500 mg Fe^{2+}/kg			
Gram positive bacteria	14.56 ± 0.55 a	15.65 ± 1.68 a	15.67 ± 1.26 a	15.77 ± 0.65 a
Gram negative bacteria	18.78 ± 0.67 a	18.33 ± 1.21 a	18.27 ± 1.21 a	17.72 ± 0.40 a
Anaerobic bacteria	8.70 ± 0.23 a	6.91 ± 0.47 b	7.27 ± 0.21 b	7.30 ± 0.27 b
Aerobic bacteria	8.00 ± 0.62 b	9.03 ± 0.71 ab	8.81 ± 0.61 ab	8.23 ± 0.49 b
Sulfate reducing bacteria	8.19 ± 0.01 a	7.45 ± 0.63 b	6.83 ± 0.11 bc	7.03 ± 0.15 bc
Methane oxidizing bacteria	6.04 ± 0.46 a	6.34 ± 0.72 a	6.12 ± 0.22 a	5.66 ± 0.14 a
Actinomycetes	11.37 ± 0.09 a	10.60 ± 0.81 ab	9.57 ± 0.15 cd	9.68 ± 0.02 cd
Fungi	1.30 ± 0.04 b	1.55 ± 0.39 ab	1.47 ± 0.18 ab	1.37 ± 0.04 ab

Data are expressed as means ± s.d. (n = 3). Data within a row with different letters indicated significant difference at $p < 0.05$.

Table 4 Relative abundance of eight types of microbe characterized by PLFAs in rhizosphere soil

Microbia	Relative abundance			
	0 mg Pb/kg	100 mg Pb/kg	500 mg Pb/kg	1000 mg Pb/kg
	100 mg Fe^{2+}/kg			
Gram positive bacteria	11.40 ± 0.98 ab	13.05 ± 0.51 a	11.45 ± 1.24 ab	9.70 ± 1.13 c
Gram negative bacteria	19.65 ± 0.67 ab	20.79 ± 0.97 a	19.46 ± 0.34 ab	18.88 ± 1.39 b
Anaerobic bacteria	7.86 ± 0.54 abc	6.71 ± 0.59 d	6.78 ± 0.12 d	8.47 ± 0.84 ab
Aerobic bacteria	9.30 ±0.40 bcd	11.16 ± 0.43 a	9.61 ± 0.12 bc	8.29 ± 0.92 d
Sulfate reducing bacteria	5.98 ±0.48 b	5.97 ± 0.37 b	5.98 ± 0.27 b	6.45 ± 0.62 ab
Methane oxidizing bacteria	6.02 ±0.53 bc	7.46 ± 0.35 a	6.14 ± 0.14 b	5.13 ± 0.78 c
Actinomycetes	8.41 ± 0.77 a	8.47 ± 0.34 a	8.25 ± 0.43 a	8.98 ± 1.19 a
Fungi	10.37 ±2.35 a	6.18 ± 1.99 a	10.06 ± 0.37 a	10.11 ± 0.37 a
	500 mg Fe^{2+}/kg			
Gram positive bacteria	10.83 ± 0.48 bc	11.92 ± 0.25 ab	11.35 ± 1.69 bc	10.99 ± 0.59 bc
Gram negative bacteria	19.67 ± 0.81 ab	20.13 ± 0.50 ab	19.90 ± 0.56 ab	19.57 ± 0.34 ab
Anaerobic bacteria	8.86 ± 0.65 a	7.26 ± 0.53 cd	7.58 ± 0.22 bcd	8.48 ± 0.50 ab
Aerobic bacteria	9.38 ± 0.69 bc	10.38 ± 0.19 ab	9.75 ± 0.98 b	8.62 ± 0.19 cd
Sulfate reducing bacteria	6.39 ± 0.49 ab	6.51 ± 0.24 ab	6.14 ± 0.34 ab	6.73 ± 0.39 a
Methane oxidizing bacteria	5.76 ± 0.15 bc	6.45 ± 0.38 b	6.26 ± 0.90 b	5.12 ± 0.38 c
Actinomycetes	9.21 ± 0.62 a	9.25 ± 0.20 a	8.46 ± 0.55 a	9.30 ± 0.55 a
Fungi	8.01 ± 2.96 a	7.31 ± 1.85 a	7.50 ± 1.66 a	6.73 ± 1.87 a

Data are expressed as means ± s.d. ($n = 3$). Data within a row with different letters indicated significant difference at $p < 0.05$.

rhizosphere (p = 0.0314) and in bulk soil (p = 0.0311) initially decreased, and then increased with the addition of lead. There was a significant interaction between lead and iron addition on relative abundance of AnB in bulk soil (p = 0.0399).

The characteristic fatty acids of aerobic bacteria, 16:1ω7c and 18:1ω7c fatty acid, were 8.0%–9.6% in bulk soil, and 8.3%–11.2% in rhizosphere (Table 3). The trend was similar in bulk soil and in rhizosphere at the two iron levels, increasing first and reaching the maximum at 100 mg Pb/kg, and then decreasing except the treatment of 1000 mg Pb/kg at 100 mg Fe/kg. The relative abundance of aerobic bacteria in rhizosphere changed with the addition of lead (p = 0.0259).

The signature fatty acids of sulfate-reducing bacteria are i17:1ω7c, 11:1ω6c, 10Me16:0 (Robie and White, 1989). The biomarker fatty acid varied from 6.6% to 8.2% in bulk soil (Table 3) and 6.0% to 6.7% in rhizosphere (Table 4). Based on the analysis of variance of lead and iron, the relative abundance of SRB in rhizosphere increased with the addition of iron (p = 0.0228), however, it initially decreased, and then increased with the dosage of lead in bulk soil (p = 0.0031).

The characteristic fatty acid of methane-oxidizing bacteria was embodied by 16:1ω5c, 16:1ω7c (Bowman *et al*., 1991). The trend of relative abundance of fatty acid biomarkers of methane-oxidizing bacteria was similar in rhizosphere at two iron levels, and methane-oxidizing bacteria were higher in rhizosphere than in bulk soil in 100 and 500 mg Pb/kg treatments.

The 10Me16:0, 10Me17:0, and 10Me18:0 PLFAs are considered as actinomycetes biomarkers (Zelles *et al*., 1992). The biomaker fatty acid varied from 9.6% to 11.4% of the PLFA in bulk soil, and from 8.2% to 9.3% in rhizosphere soil. The relative abundance of actinomycetes in bulk soil changed with the addition of iron (p = 0.0407).

The signature fatty acid of fungi is 18:2ω6, 9c (Vestal and White, 1989). The relative abundance of fungi in rhizosphere was five to eight times higher than that of bulk soil. The fungi tended to decrease with the addition of lead at 100 mg Fe/kg level in bulk soil (Table 3). Generally it was found that the abundance of fungi at 100 mg Fe/kg level was higher than that at 500 mg Fe/kg level in both bulk and rhizosphere soil.

This study showed that microbial community structure can be characterized by signature PLFAs in bulk soil (Table 3) and in rhizosphere (Table 4) with the addition of different amount of ferrous sulfate and lead nitrate. The relative abundance of G+ bacteria and actinomycetes characterized by PLFAs were higher in bulk soil than that in rhizosphere soil. However, aerobic bacteria, fungi, methane oxidizing bacteria, and G– bacteria were opposite that of G+ and actinomycetes. The soil was waterlogged during the experimental period, which created an anaerobic environment. Iron and lead were added together at different ratios, with iron oxidized around the roots to form an iron plaque on the root or precipitated (GreiPsson, 1995; Hansel *et al*., 2001), which may reduce the iron toxicity. Iron oxide formed in rhizosphere reduced the toxicity of lead, which was supported by the report of Doelman and Haanstra (1984) that Fe content had been found to be the main abiotic factor affecting negatively the toxicity of Pb on soil respiration. Moreover, the sulfate increased with the amendment of iron had a large influence on the speciation of iron and lead. Therefore, the interaction of abiotic factors may have unpredictable effects. The PLFAs affected by these factors varied in a complicated manner with the addition of lead at the different iron levels. Gram-negative bacteria in bulk soil were not affected by lead addition (Doelman and Haanstra, 1979). The fungal marker PLFA decreased with the addition of lead at 100 mg Fe/kg level in bulk soil, however, there was no significant decrease with the addition of lead in rhizosphere soil. The results are consistent with a reported reduction in the fungal marker PLFA (18:2ω6,9) in coniferous forest soils near metal smelters (Pennanen *et al*., 1996). It has often been stated that fungi are generally more tolerant of heavy metals than bacteria (Hiroki, 1992). Aerobic bacteria in biomarkers of PLFA had a similar trend at two iron levels in rhizsosphere, the relative abundance reaching a maximum at 100 mg Pb/kg, and then decreased above 100 mg Pb/kg addition level. This suggested that aerobic bacteria activity was stimulated by a low dose of lead, but inhibited by high doses. Sulfate reducing bacteria at 500 mg Fe/kg level was higher than that at 100 mg Fe/kg in rhizosphere (Table 4). This may be caused by the addition of sulfate added with ferrous sulfate that gave the sulfate reducing bacteria resource.

2.4 Cluster analysis and principal component analysis of microbial community

The microbial community may be affected by many factors, such as nutrients, heavy metals, pH, and water potential. Based on the fatty acids detected in bulk soil and rhizosphere in each treatment, hierarchical cluster analysis was used. A dendrogram of cluster is shown in Fig. 2. In bulk soil, the control and the treatments of lead nitrate addition were divided into two groups, and then it was mainly clustered four groups with lead addition levels. However, it was clustered into two types according to the amount of added iron, only A1 (the treatment of 100 mg Fe/kg and 100 mg Pb/kg) deviates from this pattern in rhizosphere. The results indicated that microbes were more obviously affected by lead nitrate than iron in bulk soil. However, ferrous sulfate played a key role in the rhizosphere. This may be the result of iron plaque formation on the roots in rhizosphere.

Principal component analysis (PCA) data gave loading values for individual PLFAs in bulk soil and rhizosphere at the amendment of 0 to 1000 mg Pb/kg at two levels of iron (Fig. 3). The PC1 and PC2 could explain 35% and 25% of the variation, respectively, in bulk soil. Saturated PLFAs were separated from the others by PC2, and the saturated PLFAs were concentrated at the first quadrant. 16:1ω7c, 18:1ω7c were separated with cy17:0, 19:0 by PC1. However, 81.3% variation can be explained by PC1, but only 9.9% by PC2. It was found 18:2ω6,9c was separated from the others by PC2, and 16:1ω7c, 18:1ω7c

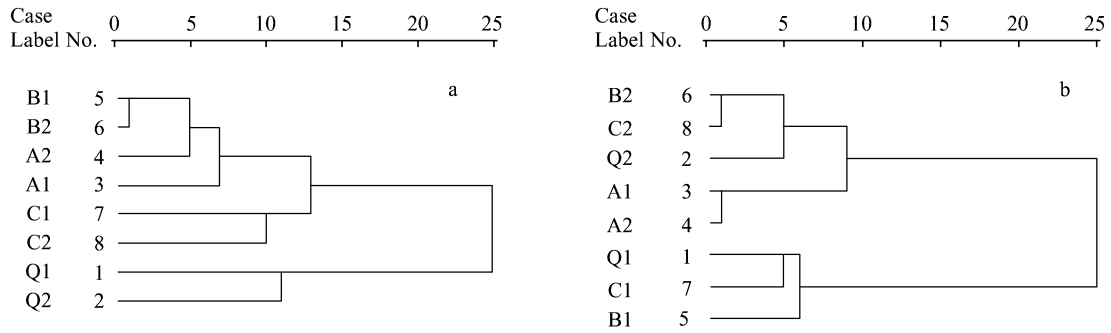

Fig. 2 Dendrogram of cluster of microbial biomarkers PLFAs at different Pb and Fe^{2+} treatments in bulk soil (a) and rhizosphere (b). Q: control (no lead), A: 100 mg Pb/kg, B: 500 mg Pb/ kg, C: 1000 mg Pb/kg, 1: 100 mg Fe^{2+}/kg, 2: 500 mg Fe^{2+}/kg.

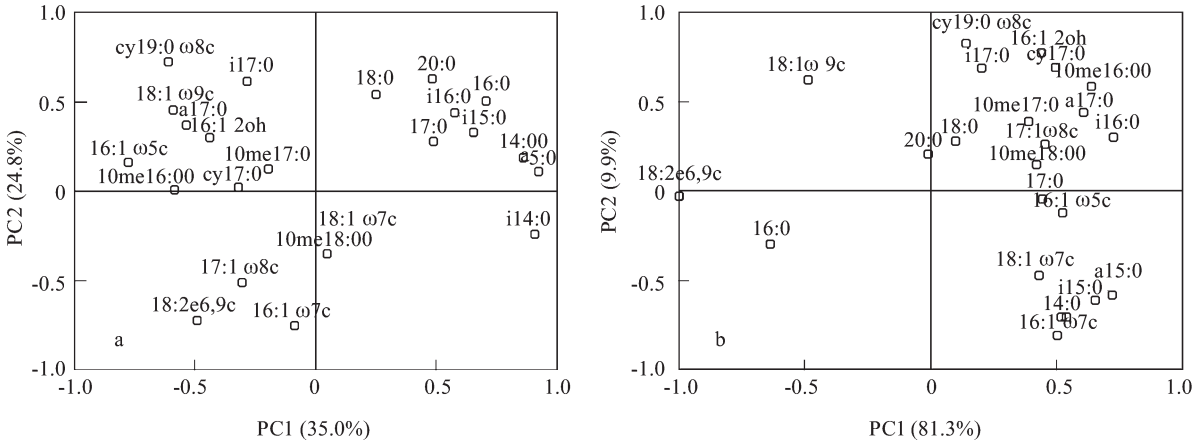

Fig. 3 Loadings for individual PLFAs in bulk soil (a) and rhizosphere (b) were plotted along the first two principal components.

were separated with cy17:0, cy19:0 by PC1.

3 Conclusions

Our study exemplifies that phosphorus utilization by plants was improved after the addition of ferrous sulfate in the lead contaminated soil under waterlogged conditions. When the concentration ratio of iron to lead was 1:1, the phosphorus utilized by plants reached the maximum. The difference of microbial community structure between rhizosphere and bulk soil by PLFA profiles was obvious under the addition of iron. Microbes were more obviously affected by lead nitrate than iron in bulk soil. However, ferrous sulfate played a key role in the rhizosphere. Excess addition of ferrous sulfate had not further effect on microbial community structure in non-polluted soil, and enhanced the Shannon index in the lead contaminated soil. Our results would provide a new way for effectively enhancing the removal of phosphorus in constructed wetland as substrate contaminated by heavy metals, especially for the treatment of mining wastewater.

Acknowledgments

This work was jointly supported by the National Nature Science Foundation for Distinguished Young Scholars of China (No. 40425007), and the Science and Technology Project of Zhejiang Province (No. 2005E10047, 2005C13003). We thank Prof. Dennis E. Rolston and Prof. C. X. Tang for helping to edit the manuscript.

References

Armstrong W, Strange M E, Cringle S, Beckett P M, 1994. Microelectrode and modeling study of oxygen distribution in roots. *Annals of Botany*, 74: 287–299.

Batty L C, Baker A J M, Wheeler B D, Curtis C D, 2000. The effect of pH and plaque on the uptake of Cu and Mn in *Phragmites australis* (Cav.) Trin ex. Steudel. *Annals of Botany*, 86: 647–653.

Bowman J P, Skerratt J H, Nichols P D, Sly L I, 1991. Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria. *FEMS Microbiology Ecology*, 85: 15–22.

Chen R F, Shen R F, Gu P, Dong X Y, Du C W, Ma J F, 2006a. Response of rice (*Oryza sativa*) with root surface iron plaque under aluminium stress. *Annals of Botany*, 98: 389–395.

Chen S B, Zhu Y G, Ma Y B, 2006b. Effects of phosphate amendments on Pb extractability and movement of phosphorus in contaminated soil. *Acta Scientiae Circumstantiae*, 26(7): 1140–1144.

Collins B S, Sharitz R R, Coughlin D P, 2005. Elemental composition of native wetland plants in constructed mesocosm treatment wetlands. *Bioresource Technology*, 96(8): 937–948.

Doelman P, Haanstra L, 1979. Effects of lead on the decomposition of organic matter. *Soil Biology and Biochemistry*, 11: 481–485.

Doelman P, Haanstra L, 1984. Short-term and long-term effects of cadmium, chromium, copper, nickel, lead and zinc on soil microbial respiration in relation to abiotic soil factors. *Plant and Soil*, 79: 317–327.

Ekelund F, Olsson S, Johansen A, 2003. Changes in the succession and diversity of protozoan and microbial populations in soil spiked with a range of copper concentrations. *Soil Biology and Biochemistry*, 35(11): 1507–1516.

Findlay R H, Dobbs F C, 1993. Quantitative description of microbial communities using lipid analysis. In: Handbook of Methods in Aquatic Microbial Ecology (Kemp P F, Sheer B F, Sheer E B, Cole J J, eds.). London: Taylor & Francis Press, 271–284.

Frostegård A, Bååth E, Tunlid A, 1993a. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. *Soil Biology and Biochemistry*, 25(6): 723–730.

Frostegård Å, Tunlid A, Bååth E, 1993b. Phospholipids fatty composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Applied and Environmental Microbiology*, 59(11): 3605–3617.

Gamborg O L, Wetter L R, 1975. Plant tissue culture methods. National Research Council of Canada, Saskatoon.

Giller K E, Witter E, Mcgrath S P, 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. *Soil Biology and Biochemistry*, 30(10-11): 1389–1414.

Greipsson S, 1995. Effect of iron plaque on roots of rice on growth of plants in excess zinc and accumulation of phosphorus in plant in excess copper or nickel. *Journal of Plant Nutrition*, 18(8): 1659–1665.

Hansel C M, Fendorf S, Sutton S, Newville M, 2001. Characterization of Fe plaque and associated metals on the roots of mine-waste impacted aquatic plants. *Environmental Science & Technology*, 35: 3863–3868.

He Y, Xu J M, Ma Z H, Wang H Z, Wu Y P, 2007. Profiling of PLFA: Implications for nonlinear spatial gradient of PCP degradation in the vicinity of *Lolium perenne* L. roots. *Soil Biology and Biochemistry*, 39(5): 1121–1129.

Hinojosa M B, Carreira J A, Roberto G R, Richard P D, 2005. Microbial response to heavy metal-polluted soils: Community analysis from phospholipid-linked fatty acids and ester-linked fatty acids extracts. *Journal of Environmental Quality*, 34: 1789–1800.

Hiroki M, 1992. Effects of heavy metal contamination on soil microbial populations. *Soil Science and Plant Nutrition*, 38(1): 141–147.

Howells J C, Caporn S, 1996. Remediation of contaminated land by formation of heavy metal phosphates. *Applied Geochemistry*, 11(1-2): 335–342.

Hu Q, Qi H Y, Zeng J H, Zhang H X, 2007. Bacterial diversity in soils around a lead and zinc mine. *Journal of Environmental Sciences*, 19(1): 74–79.

Hung L Q, Asaeda T, FujinoT, Mnaya B J, 2007. Inhibition of *Zizania latifolia* growth by *Phragmites australis*: an experimental study. *Wetland Ecology and Management*, 15(2): 105–111.

Jespersen D N, Sorrell B K, Brix H, 1998. Growth and root oxygen release by *Typha latifolia* and its effects on sediment methanogenesis. *Aquatic Botany*, 61(3): 165–180.

Kalin M, 2001. Biogeochemical and ecological consideration in designing wetland treatment systems in post-mining landscapes. *Waste Management*, 21(2): 191–196.

Kaur A, Chaudhary A, Kaur A, Choudhary R, Kaushik R, 2005. Phospholipid fatty acid – A bioindicator of environment monitoring and assessment in soil ecosystem. *Current Science*, 89(7): 1103–1112.

Kuo S, 1986. Concurrent sorption of phosphate and zinc, cadmium, or calcium by a hydrous ferric oxide. *Soil Science Society of America Journal*, 50: 1412–1419.

Liang Y, Zhu Y G, Xia Y, Li Z, Ma Y, 2006. Iron plaque enhances phosphorus uptake by rice (*Oryza sativa*) growing under varying phosphorus and iron concentrations. *Annals of Applied Biology*, 149: 305–312.

Lu R K, 2000. Physic-chemistry Analysis of Soils. Beijing: China Agricultural Scientific and Technological Press.

Manios T, Stentiford E I, Millner P, 2003. Removal of heavy metals from a metaliferous water solution by *Typha latifolia* plants and sewage sludge compost. *Chemosphere*, 53(5): 487–494.

Merila P, Stromner R, Fritze H, 2002. Soil microbial activity and community structure along a primary succession transect on the land uplift coast in western Finland. *Soil Biology and Biochemistry*, 34(11): 1647–1654.

Parke R J, Dowling N J E, White D C, Herbert R A, Gibson G R, 1993. Characterization of sulphate-reducing bacterial populations within marine and estuarine sediments with different rates of sulphate reduction. *FEMS Microbiology Letters*, 102(3-4): 235–250.

Pennanen T, Frostegård Å, Fritze H, Bååth E, 1996. Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. *Applied and Environmental Microbiology*, 62(2): 420–428.

Rajapaksha R M C P, Tobor-Kapłon M A, Bååth E, 2004. Metal toxicity affects fungal and bacterial activities in soil differently. *Applied and Environmental Microbiology*, 70(5): 2966–2973.

Ratledge C, Wilkinson S G, 1988. Fatty acids, related and derived lipids, In: Microbial Lipids, Vol. 1. London: Academic Press. 23–52.

Robie J V, White D C, 1989. Lipid analysis in microbial ecology: Quantitative approaches to the study of microbial communities. *Bioscience*, 39(8): 535–541.

Russell R A, Holden P J, Wilde K L, Neilan B A, 2003. Demonstration of the use of *Scenedesmus* and *Carteria* biomass to drive bacterial sulfate reduction by *Desulfovibrio alcoholovorans* isolated from an artificial wetland. *Hydrometallurgy*, 71(1-2): 227–234.

Sheoran A S, Sheoran V, 2006. Heavy metal removal mechanism of acid mine drainage in wetlands: A critical review. *Minerals Engineering*, 19(2): 105–116.

Soderberg K H, Bååth E, 1998. Bacterial activity along a young barley root measured by the thymidine and leucine incorporation techniques. *Soil Biology and Biochemistry*, 30(10-11): 1259–1268.

Steingruber S M, Friedrich J, Gächter R, Wehrli B, 2001. Measurement of denitrification in sediments with the ^{15}N isotope pairing technique. *Applied and Environmental Microbiology*, 67(9): 3771–3778.

Taylor G J, Crowder A A, 1983. Use of the DCB technique for extraction of hydrous iron oxides from roots of wetland plants. *American Journal of Botany*, 70(8): 1254–1257.

Taylor G J, Crowder A A, Rodden R, 1984. Formation and morphology an iron plaque on the roots of *Tapha Latifolia* L. growth in solution culture. *American Journal of Botany*, 71(5): 666–675.

Vestal J R, White D C, 1989. Lipid analysis in microbial ecology – Quantitative approaches to the study of microbial communities. *BioScience*, 39(8): 535–541.

Weis J S, Weis P, 2004. Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. *Environmental International*, 30(5): 685–700.

Weiss J V, Emerson D, Megonigal J P, 2004. Geochemical control of microbial Fe(III) reduction potential in wetlands: comparison of the rhizosphere to non-rhizosphere soil. *FEMS Microbiology Ecology*, 48(1): 89–100.

Xu D F, Xu J M, He Y, Huang P M, 2009. Effect of iron plaque formation on phosphorus accumulation and availability in the rhizosphere of wetland plants. *Water, Air, and Soil Pollution*. 200: 79–87

Xu D F, Xu J M, Wang H S, Luo A C, Xie D C, Ying Q S, 2005. Absorbability of wetland plants on N and P from eutrophic water. *Plant Nutrition and Fertilizer Science*, 11: 597–601.

Xu D F, Xu J M, Wu J J, Muhammad A, 2006. Studies on the phosphorus sorption capacity of substrates used in constructed wetland systems. *Chemosphere*, 63(2): 344–352.

Yang B, Lan C Y, Yang C S, Liao W B, Chang H, Shu W S, 2006. Long-term efficiency and stability of wetlands for treating wastewater of a lead/zinc mine and the concurrent ecosystem development. *Environmental Pollution*, 143(3): 499–512.

Ye Z H, Baker A J M, Wong M H, Willis A J, 1997. Copper and nickel uptake, accumulation and tolerance in populations of *Typha latifolia* L. *New Phytologist*, 136(3): 469–480.

Zelles I, Bai Q Y, Beck T, Beese F, 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agriculture soils. *Soil Biology and Biochemistry*, 24(4): 317–323.

Zhang X K, Zhang F S, Mao D R, 1999. Effect of iron plaque outside roots on nutrient uptake by rice (*Oryza sativa* L.): Phosphorus uptake. *Plant and Soil*, 209(2): 187–192.

Zhukova N V, 2005. Variation in microbial biomass and community structure in sediments of Peter the Great Bay (Sea of Japan/East sea), as Estimated from fatty acid biomarkers. *Ocean Science Journal*, 40(3): 145–153.