Characteristics of the microbial communities in the integrated vertical-flow constructed wetlands

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Abstract

Microorganisms play an important role in removing pollutants from constructed wetlands. We investigated the microbial characteristics in a novel integrated vertical-flow constructed wetland (IVCW), which has been in operation in Wuhan, China since 1998. We used phospholipid fatty acid (PLFA) and *amo*A gene to analyze the structure and diversity of the microbial community within the IVCW. PLFA results suggested that the amount of bacterial PLFA was significantly higher than that of fungal PLFA, but the total microbial biomass represented by PLFA index was low in the system. Microbial spatial distribution showed significantly higher bacterial (both G$^+$ and G$^-$) and fungal biomass in the surface than in the subsurface layers. The ratios of monounsaturated to branched PLFA demonstrated that an anaerobic layer sandwiched by two aerobic layers existed in the IVCW, consistent with the redox potential results. Analysis of the *amo*A revealed the presence of *Nitrosomonas*-like sequences in the surface substrate of the downflow chamber and apparent diversities of ammonia-oxidizing bacteria in the system. These results suggest that microorganisms, despite their relatively low biomass, have inhabited the IVCW, and the results will offer some valuable information on microbe to system designers and managers.

Key words: ammonia-oxidizing bacteria; constructed wetland; microbial community; PLFA

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Introduction

Wetlands locate between terrestrial and aquatic systems and often possess the characteristics of both systems. They perform many important biogeochemical functions in watersheds due to their unique location (Wang *et al*., 2006). They have been found to be useful in remediating environment contaminated with biochemical oxygen demand (BOD), total suspended solid (TSS), nitrogen and phosphorus as well as various organic pollutants or metal oxides, or even pathogens (Thurston *et al*., 2001). However, as uncontrolled eco-systems, natural wetlands suffer from hydrological short-circuiting, poor control over chemical and biological processes (Mitsch, 1994), and are vulnerable to environmental changes. To better understand their functions and explore their applications, constructed wetland was introduced as a model system and has been used world-wide. These constructed wetlands have been proved valuable in not only removing environmental contaminants, but also providing habitat for wildlife and attractive destinations for tourists. The wetland remediation processes include biological processes

such as microbial metabolic activities, and plant uptake as well as physicochemical processes such as sedimentation, adsorption and precipitation (Kadlec and Knight, 1996) at the water-sediment, root-sediment and plant-water interfaces. For example, nitrification and denitrification reactions are the dominant nitrogen removal mechanisms in constructed wetlands, and the chemolitho-autotrophic ammonia-oxidizing bacteria (AOB) are responsible for the rate-limiting step of the nitrification reaction, and therefore contribute substantially to the global cycling of nitrogen.

Due to its importance in removing contaminants from the constructed wetlands, the microbial community within these wetlands has received great attention recently, especially with respect to pathogen removal (Perkins and Hunter, 2000; Thurston *et al*., 2001), metabolism process of pollutants (Giraud *et al*., 2001; Weaver *et al*., 2004) and impact on the microbial communities (Ibekwe *et al*., 2003; Hallberg and Johnson, 2005). Of all these studies, the investigation of microbial communities has become a focus in studying decontamination in the ecological system of constructed wetlands.

The integrated vertical-flow constructed wetlands (IVCW) are a new type of constructed wetland with subsurface flow. The one located in Wuhan, China, is one example and it has been in operation for 7 years. As a

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subproject of the international scientific fruit of INCO-DC project (No. ERBIC18CT960059) supported by the 4th Framework Programme of the European Commission, the IVCW was constructed to enhance the potential removal ability of a single vertical constructed wetland. The system integrated two kinds of water current patterns, including of vertical downflow and vertical upflow. The unique system design would result in different substrate physicochemical and biological characteristics within the wetland. In this article, the microbes in this IVCW were characterised to offer some valuable information to system designers and managers. Specifically, *amo*A gene sequence and phospholipids fatty acid (PLFA) profiles were analyzed to study the genetic diversities and spatial distribution of the microbes in the IVCW system.

1 Materials and methods

1.1 Experimental wetland system

The IVCW was installed near the East Lake in Wuhan, China. The vertical cross-section view of the wetland is shown in Fig. 1. Briefly, the system has a surface area of 162 m^2, which was divided equally into two chambers, with the first being a downflow chamber and the second upflow chamber. Each chamber composed of two layers with different particle sizes: 200 mm depth of coarse gravel (8–16 mm in diameter) on the bottom in both chambers, 550 mm (in the down-flow chamber), and 450 mm (in the up-flow chamber) depth of sand (0–4 mm in diameter) on the top. All water pipes were made from polyvinyl chloride (100 mm diameter). A 5‰ gradient vent-pipe was placed on the bottom to drain easily. For even distribution of water, two influent pipes with holes (5 mm diameter) on the underside were placed across the surface of the sand layer. On the bottom of both chambers, collecting pipes sent water coming from the down-flow chamber to the up-flow chamber. The effluent pipes were situated on the surface of the up-flow chamber. The system has been operating since it was built in April, 1998. *Zizania caduciflora* and *Acorus calamus* were originally planted in the down-flow and up-flow chambers, respectively, but *Z. caduciflora* was replaced by *Canna indica* in March of 2001, because *Z. caduciflora* withered in the winter and did not bourgeon well in the next spring. The source water was from East Lake, Wuhan and the hydraulic loading was 420 mm/d, with discontinuous filling. During sampling periods, the influent and effluent water quality is listed in Table 1. Conductivity, pH, and TDS were measured using a Thermo Orion 5 star portable meter *in situ*, while chemical oxygen demand (COD$_{Cr}$), total nitrogen (TN), and total phosphorus (TP) were measured within 24 h in laboratory according to Editorial board of environment protection bureau of China (1997).

Table 1 Influent and effluent water quality in the IVCW

Parameters (unit)	Influent	Effluent
Conductivity (μS/cm)	398.67 (1.53)*	273.67 (3.79)
pH	7.27 (0.08)	6.66 (0.11)
TDS (mg/L)	190.00 (1.00)	129.33 (1.53)
COD$_{Cr}$ (mg/L)	33.66 (1.05)	14.50 (2.58)
TN (mg/L)	5.35 (0.12)	1.89 (0.08)
TP (mg/L)	0.592 (0.04)	0.31 (0.02)

* Data in bracket represent standard deviation of the mean.

1.2 Substrate samples

The substrate samples were collected using cylindrical corer between September and October in 2004. Each core sample was divided into four subsamples according to the depth. The subsamples at 0–5, 15–20, 30–35 and 45–50 cm depths were designated S1 to S4 for the down-flow chamber and S5 to S8 for the up-flow chamber. And each sample was equally collected from five symmetrical sites. The sulfuric acid-potassium dichromate method was used to measure the organic matter content.

1.3 Phospholipid extraction and analysis

Phospholipids were extracted from each subsample and analyzed using GC/MS. Briefly, 5 g of fresh substrate without any root, withered branches or leaves was extracted with a chloroform: methanol: pH 7.4 phosphate buffer (1:2:0.8, *V/V/V*), and then the total lipids extracted were further fractionated into neutral lipids, glycol-lipids and phosphor-lipids on a silica-bonded phase column (SPE-Si, 500 mg/6 mL, Agilent). The polar lipids were transesterified with mild alkali to recover the PLFA as methyl esters in hexane. The PLFAs were separated, identified and quantified by a Hewlett-Packard 6890N GC/5873I MS with a nonpolar capillary column (HP-5 MS, 30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas. The temperature of the injector was set at 250°C. Samples (1 μL) were injected in the splitless mode. The initial column temperature was maintained at 80°C for 2 min,
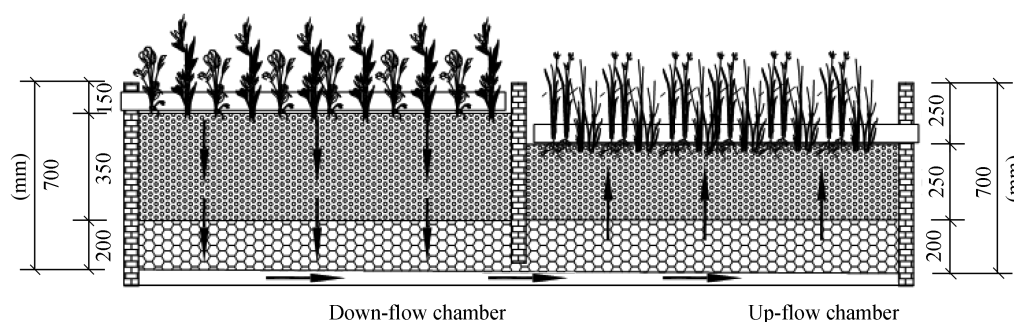

Fig. 1 Vertical cross-section view of the IVCW system.

then ramped to 150°C at 50°C/min, raised to 195°C at 2.5°C/min, which remained constant for 3 min before being increased to 240°C. Mass spectra were determined by electron impact at 70 eV. PLFAs were qualified and quantified according to the method described by Macnaughton *et al*. (1999). Fatty acid nomenclature used in this study comply with that described by Macnaughton *et al*. (1999) and Ponder and Tadros (2002).

1.4 Analysis of ammonia oxidizing bacteria using *amo*A gene

The primer pairs specific for *amo*A gene, *amo*A1F (5'-GGGGTTTCTACTCCTGGT-3') and *amo*A2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') (Hoshino *et al*., 2001) were used to amplify the 16S rDNA fragment from sample S1. Amplifications were performed in 25 μL reaction mixture, which contained 0.5 μL of 10 mmol/L dNTPs, 0.5 μL of 10 μmol/L primers, 1 U Taq polymerase and 1 μL template DNA. PCR profile consisted of initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 60 s. The final extension at 72°C lasted for 10 min. The PCR products were assessed using agarose gel (1.0%) electrophoresis.

After electrophoresis, the PCR products were excised from the gel, purified and concentrated using the Glass-milk DNA purification kit (Biostar International, Canada). The purified DNA was ligated to PMD18-T vector and the plasmids containing the PCR fragments were then used to transform *Escherichia coli* DH5α competent cells. Screened positive clones were cultured at 37°C for 2 h or more and then were sent to be sequenced.

1.5 Data analysis

The PLFA analysis was performed in triplicates and the data were analyzed by analysis of variance (ANOVA) and principal component analysis (PCA). All nucleotide sequences were submitted to Genbank (No. FJ603083-FJ603096) and aligned by the computer program Clustal X. The homologous sequences *Nitrosomonas oligotropha* (AF272406), *Nitrosomonas aestuarii* (AF272400), *Nitrosomonas marina* (AF272405), *Nitrosomonas cryotolerans* (AF272402), *Nitrosomonas communis* (AF272399), *Nitrosomonas europaea* (AF037107), *Nitrosospira briensis* (AY123821) were obtained from NCBI and a neithbor-joining phylogenetic UPGMA tree was constructed using Mega 2.1.

2 Results

2.1 PLFA types

The PLFA profiles from the IVCW are presented in Fig. 2. The PLFAs extracted from all the substrate samples at 0–50 cm depth contained saturated, monounsaturated (MUFA), branched, cyclopropyl, polyunsaturated (PUFA) and hydroxyl fatty acids. Straight-chain PLFAs were relative abundant within all the samples collected, with 16:0 having the highest concentration (540.0 pmol/g soil), followed by branched PLFAs (319.0 pmol/g soil). And cyclopropane PLFAs (cy17:0 and cy19:0), and finally hydroxyl PLFAs (2-OH 10:0, 2-OH 12:0, 3-OH 12:0, 2-OH 14:0, 3-OH 14:0 and 2-OH 16:0) have the lower concentrations, 76.1 and 57.1 pmol/g soil, respectively. Figure 3 shows the results of the ratios of MUFA to
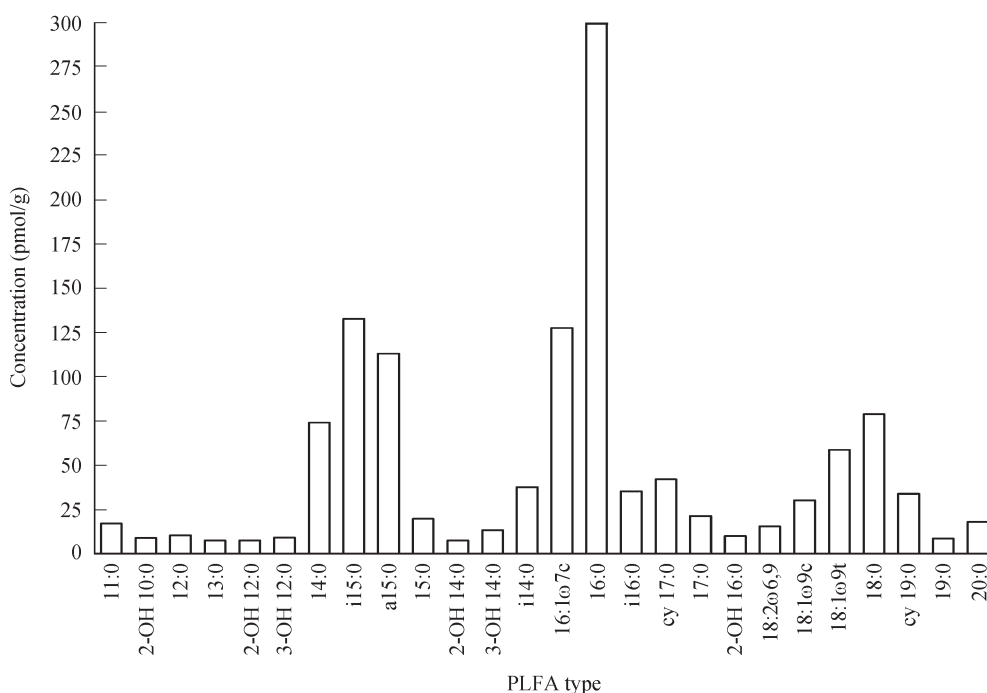

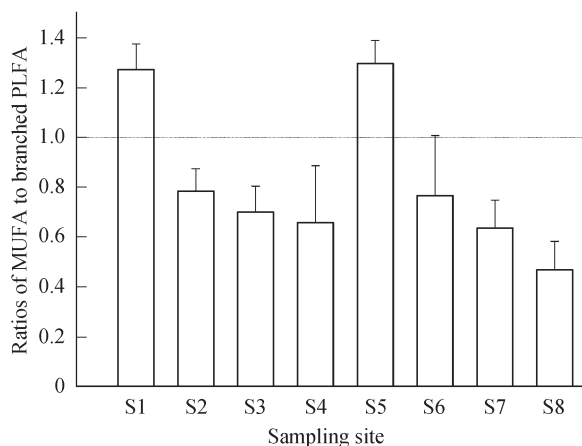**Fig. 2**   Concentrations of different types of phospholipids fatty acid (PLFA) in the IVCW.

Fig. 3 Ratios of MUFA to branched PLFA in the IVCW. S1 to S4 represent the samples at 0–5, 15–20, 30–35 and 45–50 cm depth in the downflow chamber, and S5 to S8 represent the samples at 0–5, 15–20, 30–35 and 45–50 cm depths in the upflow chamber, respectively.

branched fatty acids. And the results indicated that the ratios of all samples in the subsurface sites were less than 1.

2.2 Microbial PLFA contents

The microbial communities can be classified into different groups according to their PLFA composition. In this study, the most bacterial PLFAs were represented as the sum of the PLFAs from Gram-positive bacteria (G^+) (i15:0, a15:0, i16:0, 16:1ω7c, i17:0) and Gram-negative bacteria (G^-) (cy17:0, 18:1ω9c and cy19:0) (Tscherko *et al.*, 2004). Figure 4 shows the PLFA concentrations of total bacteria, G^+ and G^- bacteria at different locations in the IVCW. The contents in S1 and S5 were more abundant, and the concentrations were above 1363.4, 680.5 and 276.1 pmol/g soil, respectively. The biomass of fungal PLFA was measured as the amounts of 18:2ω6,9 (Fig. 5). And the contents in sites S1 and S5 were relative abundant, with 26.1 and 42.9 pmol/g soil, respectively.1
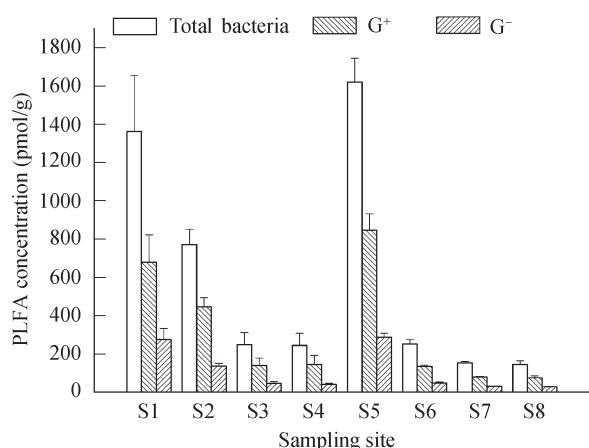

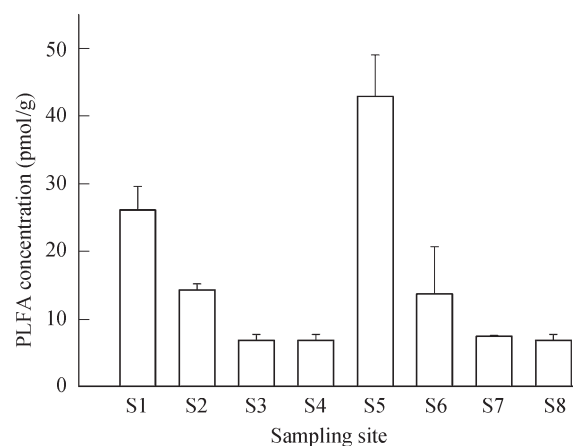**Fig. 4** Bacterial PLFA contents in the IVCW. S1 to S8 are the same as that in Fig. 3.

Fig. 5 Fungal PLFA contents in the IVCW. S1 to S8 are the same as that in Fig. 3.

2.3 Ammonia-oxidizing bacteria species

In this study, 14 full-length *amo*A genes of sample S1 were sequenced. The partial fragment of *amo*A gene is 491 bp and there were great diversities among the examined clones. The phylogenetic tree was constructed by neighbor-joining method based on genetic distance. All the 6 species of *Nitrosomonas* may exist in the substrate of constructed wetland. In the phylogenetic tree there were three different groups (Fig. 6) that are associated with the *amo*A gene of *Nitromonas* spp. In group I, there were 9 gene sequences associated with the *amo*A gene of *Nitrosomonas oligotropha*, and another two clones (8 and 9) related to *Nitrosomonas aestuarii* and *Nitrosomonas marina*. Clone 2 and *Nitrosomonas europaea* formed group II, while clone 1 and 4 formed group III that is rather distant to any known species. The latter two clones might be derived from a new *amo*A gene.

3 Discussion

3.1 Microbial biomass and communities in the IVCW

The microbial biomass represented by the PLFA index in the IVCW was relatively small. The average total PLFA concentration was about 1.48 nmol/g soil, which was far lower than that reported in other samples of soil (Suhadolc *et al.*, 2005) or sediment (Li *et al.*, 2007). The organic matter contents of samples S1–S8 were 3.7%, 0.7%, 0.3%, 0.2%, 4.6%, 0.5%, 0.3% and 0.3%, respectively. The organic matter, another indicator of biomass, was also lower in our system. Generally, substrate texture and nutrient source input could directly influence microbial biomass in the system. Indeed, Bauhus *et al.* (1998) suggested that microbial biomass was controlled by soil texture. Landgraf and Klose (2002) reasoned that systems with high organic matter inputs and available soil organic matters typically have higher microbial biomass contents. In our study, the system had been in operation since 1998, surprisingly the microbial biomass was rather small. The low microbial biomass in the IVCW could be attributed to the following reasons. First, sand and gravel were used to construct
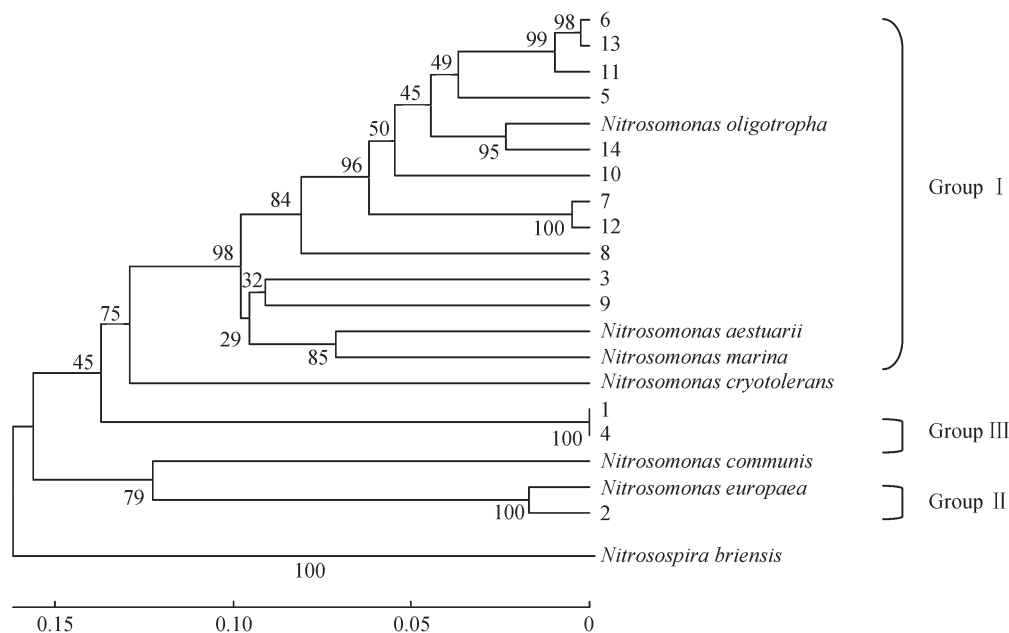
Fig. 6   A neighbor-joining phylogenetic tree based on *amo*A gene fragments of ammonia oxidizing bacteria in site S1.

the IVCW because of their low cost and relative high performance, but they may not be the appropriate substrate for microorganisms to grow on. Second, the nutritional substances introduced to the IVCW had decreased because the source water from the East Lake had improved quality since 2004 when sewerage discharge to the lake was stopped. Since the water quality improvement in natural and constructed wetlands is primarily attributable to bacteria (Ibekwe *et al*., 2003), appropriate substrates that support large amounts of bacterial growth are required to construct efficient wetlands.

Of all the samples analyzed, the ratio of G^+ PLFA over G^- PLFA was relatively constant, ranging from 2.46 to 3.45, and it did not change with water purification process, contradicting to the previous findings that G^- PLFA was increasing in this process (Kamaludeen *et al*., 2003). This difference might be due to the fact that the source of wastewater flowing through our wetlands was taken from a urban lake, which was much less contaminated than the industrial wastewater (tannery wastewater) used in their studies. The amount of fungal PLFA was significantly lower than that of bacterial PLFA ($P < 0.05$). The ratio of fungal PLFA to bacterial PLFA varied from 0.035 to 0.07, which is consistent with previous findings (Kamaludeen *et al*., 2003; Grayston *et al*., 2004).

3.2 Microbial spatial distributing characteristics

The ANOVA results indicated that the total bacterial PLFA concentrations of S1 and S5 (the 0–5 cm surface layer) were significantly higher than that of the other locations ($P < 0.05$), and followed by that of subsample S2, while the differences between the rest of the subsamples were not significant ($P > 0.05$). G^+ bacterial PLFA and G^- bacterial PLFA followed the same pattern as total bacterial PLFA, but the G^+ bacterial PLFA concentrations were higher than that of G^- bacterial PLFA in all samples. The single fungal PLFA content decreased with increasing

depth. The PLFA profiles, determined by principle component analysis (PCA), showed that the PCA1 accounted for 86.90% of the variance, while the PCA2 accounted for 8.29%. Together they explained 95.19% of the total variance in the first two dimensions of the plot, suggesting that they could be used as an integrated index of the PLFAs to determine the relationship among different locations in the IVCW. Compared to the distance of the locations along the abscissa (Fig. 7), the distance between S1 and S5 was the smallest, then S2, and then S3, S4, S6–S8.
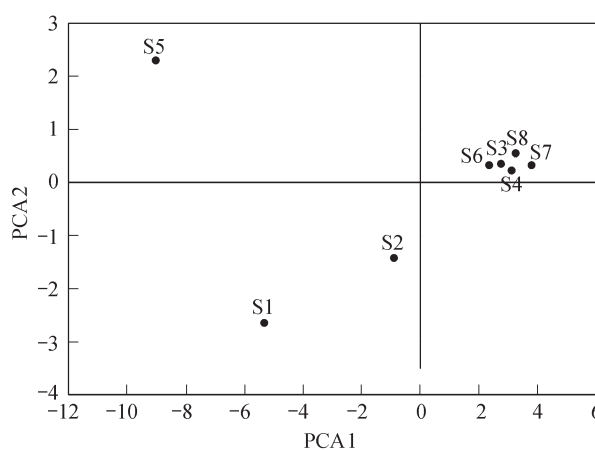

Fig. 7   Principal component scattering plot of substrate samples in the IVCW. S1 to S8 are the same as that in Fig. 3.

The differences in PLFAs between layers may be due to the vertical feeding which lead to the difference in available nutrients state and the physical-chemical conditions in these layers. In the surface layer, both organic matter and oxygen content should be the highest and the rhizosphere was also present. These conditions could stimulate the growth of microorganisms. Studies on the redox potential in the IVCW had shown that the wetland system could be divided into three functional zones along the water

flow direction, oxic (O) /anaerobic (A)/ oxic (O), with the redox potential of down-flow surface, bottom, and up-flow surface being 402 ~ 585 mV, –87 ~ –130 mV and 308 ~ 432 mV, respectively (Tao *et al.*, 2008).

Generally, MUFAs represent aerobic prokaryotes and eukaryotes, while most branched fatty acids represent anaerobic bacteria including sulphate reducing bacteria (SRB). Therefore, the ratio of MUFA to branched PLFA could be used to determine the relative dominance of aerobic and anaerobic bacterial groups in different locations (Rajendran *et al.*, 1997). A ratio less than 1 would indicate the dominance of anaerobic bacteria, and a ratio above 1 would indicate the predominance of aerobic bacteria. The ratios of all samples in the subsurface sites were less than 1, suggesting predominance of anaerobic organisms, and that the ratios in the surface layers were above 1, meaning there are likely more aerobic organisms living in the surface layers. The results also illustrated that the O/A/O layers existed in the IVCW. The O/A/O zones in the IVCW were achieved by the configuration design. The advantages of such design were obvious: complex organic substances could be degraded extensively and easily, and then the quality of effluent water would be better with higher dissolved oxygen content.

3.3 Diversity of ammonia-oxidizing bacteria in surface substrate of down-flow chamber in the IVCW

The use of the functional gene target of *amo*A sequences provided an example of diversity of ammonia oxidizers. PCR amplification of the surface substrate in down-flowing chamber produced DNA fragments of the expected size. The results suggested that *Nitrosomonas*-like sequence, especially *Nitrosomonas oligotropha*-like sequences was dominant in the surface substrate of down-flow chamber in the IVCW. Many studies showed that the AOB community structure varied with different treatment systems. Ibekwe *et al.* (2003) concluded that the population of AOB showed a higher percentage of *Nitrosospira*-like sequences from the wetland sample receiving dairy wastewater effluent. Tietz *et al.* (2007) studied the diversity of AOB in the vertical flow constructed wetland and revealed two dominant AOB lineages: *Nitrosomonas europaea*/"*Nitrosococcus mobilis*" and *Nitrosospira*. Dionisi *et al.* (2002) concluded that in the activated sludge system *Nitrosomonas oligotropha* was the dominant AOB. Ibekwe *et al.* (2002) studied AOB diversity along a transect of agronomic zones, suggesting that *Nitrosospira* was more dominant than *Nitrosomonas* in these agricultural soils.

The level of AOB diversity within a wastewater treatment system has a major influence on process stability. A treatment plant with greater diversity can cope better with changing conditions, and the greater the diversities, the more stable the process (Ibekwe *et al.*, 2003). The IVCW system had greater ammonia-oxidizing bacteria diversity, especially *Nitrosomonas* sp. Diversity does play a major role in wastewater treatment in the constructed wetlands, thereby, system designers and managers should make processes such as nitrification and denitrification more efficient in order to expand treatment type and capability.

4 Conclusions

In this study, we have provided information on microbial community structure from different locations in integrated vertical down-flow and up-flow constructed wetland. In order to offer some valuable information to system designers and managers, the biochemical and molecular biology tools were used to assess the inherent characteristics of this special system. The conclusions are: (1) the microbial biomass as determined by PLFA analysis was relatively low; (2) the IVCW consisted of aerobic, anaerobic and aerobic spatial layers; and (3) diversity of *amo*A sequences, especially that of *Nitrosomonas* sp., was relative great in the surface layer in down-flow chamber.

Acknowledgments

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