Analysis of the Metabolic Utilization of Carbon Sources and Potential Functional Diversity of the Bacterial Community in Lab-Scale Horizontal Subsurface-Flow Constructed Wetlands

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Microorganisms are an integral part of the biogeochemical processes in wetlands. To improve the performance of constructed wetlands, it is very important to know the metabolic properties and functional diversity of the microbial communities. The purpose of this study is to analyze the metabolic properties and functional diversity of the microbial community in a horizontal subsurface-flow constructed wetland (CW) in a laboratory study through the sole-carbon-source utilization profiles using Biolog-ECO microplates. The technique has advantages over traditional cell culture techniques, such as molecular-level techniques–RNA amplification, which are time-consuming, expensive, and only applicable to the small number of species that may be cultured. This CW was designed to treat rural eutrophic water in China, using the plant *Cyperus alternifolius* L. This study showed that the metabolic activities of upper front substrate microorganisms (UF) were greater than those of the lower back substrate microorganisms (LB) in the CW. Integrated areas under average well color development (AWCD) curves of substrate microorganisms in the UF were 131.9, 4.8, and 99.3% higher than in the lower front part (LF), the upper back part (UB), and the LB part of the CW, respectively. Principal components analysis showed significant differences in both community structure and metabolic utilization of carbon sources between substrate microorganisms from different sampling sites. Carbon source utilization of polymers, carbohydrates, carboxylic acids, and amino acids was higher in UF than in LF, but that of amines and phenolic compounds was very similar in UF and LF. The richness, evenness, and diversity of upper substrate microbial communities were significantly higher than those of lower substrate. The LF substrate microbial communities had lower evenness than the other sampling plots, and the lowest richness of substrate microbial community was found in the LB part of the CW.

Activated sludge and bacterial bed processes have been used to treat the wastewater from large and small cities to protect the water environment. However, these processes are not economically adapted for dispersed population in rural areas, mainly due to the construction cost of sewage collectors. In rural areas, constructed wetlands (CWs) may be a promising tool for the treatment of agricultural runoff, which is difficult and expensive to treat with conventional wastewater purification techniques.

Horizontal subsurface-flow constructed wetlands (HSSFs) have proven to be effective in wastewater treatment, particularly for total suspended solids, organic matter, and N (Green and Upton, 1995; Merlin et al., 2002; Garcia et al., 2004; Healy and Cawley, 2002; Gale et al., 1994). In the construction of wetlands, it is important to create optimal physical, chemical, and biological environments for nutrient removal. If a CW provides a diversity of environmental conditions and multiple plant species, the treatment efficiency can be improved.

Microorganisms have central crucial roles in CWs. The removal of organic pollutants and the operating performance of CWs are mainly influenced by the structure and metabolic function of the microbial community (Liang et al., 2003). Few evaluations of microbial biomass in HSSF exist in China. At present, a majority of CW studies focused on litter decomposition rates in China (Gao et al., 2006). The differences in microbial composition and catabolic capabilities were indicative of the ability of distinct microbial communities to decompose different types of litter. Microbial diversity and the utilization of a wide range of carbon sources are essential in determining the functionality of wetlands, especially constructed wetlands.

Changes in the functionality of HSSFs may be caused by shifts in either community structure or physiological composition (Garland and Mills, 1994). To understand the role of bacterial communities in the CWs, it is essential to take into account the functional diversity of the microorganism community and also the influence of medium disturbances on the change of this diversity. Traditional methods for studying microbial community

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Abbreviations: AWCD, average well color development; COD, chemical oxygen demand; CW, constructed wetland; HSSF, horizontal subsurface-flow constructed wetland; LB, lower back substrates; LF, lower front substrates; OD, optical density; PC, Principal Component; TN, total nitrogen; TP, total phosphorus; UB, upper back substrates; UF, upper front substrates.

diversity were culture-based techniques that often do not reflect the total diversity because many microorganisms are viable but nonculturable (Kunc, 1994). Alternative methods involve fluorescence in situ hybridization, epifluorescence microscopy, and 16S ribosomal DNA polymerase chain reaction amplification–denaturing gradient gel electrophoresis (DeJournett et al., 2007; Jin and Kelley, 2007; Dong and Reddy, 2010). Such analyses, however, are relatively expensive, labor intensive, and require some expertise (Garland, 1997), and therefore restrict the number of samples that may be analyzed over a short period of time. Biolog-ECO is a rapid method to assess the immediate carbon source utilization of samples by avoiding the isolation and culturing of microbes (Garland and Mills, 1994).

Substrate utilization patterns are one widely used method to obtain metabolic "patterns" of microbial community structure and functional diversity (Garland and Mills, 1994; Staddon et al., 1998). This rapid method employs the commercial Biolog microtiter plate assay (Biolog Inc., Hayward, CA) for monitoring shifts of the metabolic patterns of bacterial communities. The Biolog-ECO plates system used in this study includes 31 carbon substrates, predominantly amino acids, carbohydrates, and carboxylic acids. Each well also contains a redox indicator dye (tetrazolium violet), which changes color in response to microbial growth. The amount of color development in each well is assessed spectrophotometrically and indicates the utilization of a particular carbon source. This technique is simple and uses an automated measuring apparatus. Because it measures utilization of carbon, an important major factor that regulates microbial growth and community structure in substrate, this method provides a more meaningful assay of community structure than isolation-based methods (Knight et al., 1997; Baath et al., 1998). Biolog-ECO plates may also be used to characterize the functional diversity of microbial communities by multivariate statistical analysis (Preston-Mafham et al., 2002).

The purpose of this study was to demonstrate that this technique is applicable to indicate the carbon metabolic properties and discrimination of functional diversity of the bacterial communities in lab-scale HSSF, and to suggest that its use may have applications both in the laboratory and full-scale CW systems. The results obtained in this study could provide a basis for future studies in improving the performance of HSSF.

Materials and Methods

Lab-scale HSSF Setup and Operation

Figure 1 illustrates the structure of the HSSF reactor. The length, breadth, and height of the reactor were 150 cm, 40 cm, and 80 cm, respectively. This HSSF reactor was constructed from 8-mm polyvinyl chloride boards and packed with washed pea-sized gravel (diam. 4–6 mm), which are conventionally used in subsurface-flow CWs. The packed bed height was 60 cm. The porosity of the media was 0.41. Total working volume was 0.36 m^3.

Neighboring sampling ports were 15 cm apart along the height and 30 cm apart along the length of the reactor. A 10-cm-diam. pipe was screwed into each sampling port and fitted with a stopper. There were four water sampling ports in the same sites on the opposite board. A 2-cm-diam. pipe was screwed into each sampling port and controlled with a tap.

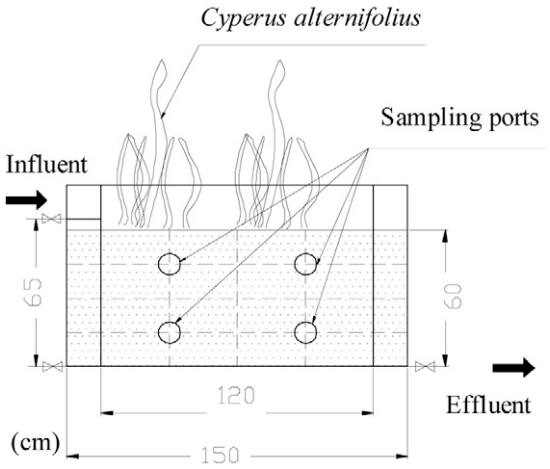

Fig. 1. Structure of pilot-scale horizontal subsurface-flow reactor constructed wetland in a laboratory study; sampling ports are UF, LF, UB, LB (UF = upper front substrates, LF = lower front substrates, UB = upper back substrates, LB = lower back substrates).

Cyperus alternifolius L. plants were obtained from a field wetland and were planted in the HSSF reactor during March 2006. The reactor was fed with eutrophic water (Table 1) from a Chinese rural pond with a hydraulic retention time of 3 d (flow rate = 48 L d^{-1}) beginning in March 2006. The water level was maintained 55 cm below the upper surface of the reactor (Fig. 1).

Sampling and Microbial Extraction

To reach steady-state conditions and to obtain a more reliable estimate of the removal efficiency of the system, sampling started once the concentration of chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) had stabilized at the effluent of the system. Samples (gravel with biomembrane) were collected in October 2006, 7 mo after planting. Biomembrane growing in the surface of rocks was the research aim here. Samples of substrate were obtained from the four sampling ports on the side (Fig. 1). All samples were collected in 100-mL clean glass bottles, which were transported refrigerated to the laboratory where they were stored at 4°C until analysis. The sample holding time was <3 h.

Forty grams of each wet sample was added to 100-mL sterile NaCl solution (0.85%) and shaken in an incubator shaker for 30 min at 200 cycles min^{-1} at 25°C. These solutions were then centrifuged at 1800 × *g* for 5 min. The suspensions were further analyzed.

Table 1. Hydrochemistry of the water from inflow of the constructed wetlands.

Index	Range
COD† (mg L^{-1})	62.7–177.8
TN (mg L^{-1})	2.87–6.64
NH_3–N (mg L^{-1})	0.15–0.69
TP (mg L^{-1})	0.76–1.64
PO_4^3–P (mg L^{-1})	0.13–0.72
Chlorophyll *a* (mg m^{-3})	33.2–256.4
pH	7.3–7.8

† COD = chemical oxygen demand; TN = total nitrogen; TP = total phosphorus.

Inoculation of Biolog Microplates

Aliquots of the microbial suspensions described above were transferred to each well in the Biolog-ECO plates. All samples were inoculated in triplicate on ECO plates. Each well in the Biolog-ECO plates was inoculated with 0.15 mL of microbial suspensions. The ECO plates were incubated at 25°C for 168 h, and the optical density (OD) at 590 nm was read for each well at 8-h intervals over a period of 168 h.

Statistical Methods

According to the procedure described by Glimm et al. (1997), principal components analysis was applied to statistically analyze the Biolog-ECO microplate data. Each 96-well plate consists of three replicates, each comprising 31 sole carbon sources and a blank. All ODs_{590nm} were read with a Multiskan-Microplate Photometer (Thermo Fisher Scientific Inc., Hudson, NH). The OD_{590nm} of the control well for each replicate (containing no carbon source) was subtracted from the OD_{590nm} of each of the other wells to adjust for coloration inherent in the samples or due to autophagia. These adjusted OD_{590nm} values were standardized by dividing by the average well color development (AWCD) for the replicate, in line with the recommendations of Garland (1996). The AWCD for replicate j at time t is given as

$$\mathrm{AWCD}_{jt} = \frac{1}{31}\sum_{i=1}^{31}\mathrm{OD}_{ijt} \qquad [1]$$

where $OD_{i,j,t}$ denotes the corrected OD for well i of replicate j measured at time t, and hence the adjusted OD is:

$$\overline{\mathrm{OD}} = \frac{\mathrm{OD}_{ijt}}{\mathrm{AWCD}_{jt}} \qquad [2]$$

These adjusted ODs are the basis for the subsequent analysis.

Principal components analysis using SPSS 13.0 (SPSS Inc., Chicago, IL) on a personal computer was then performed on the data, at the selected time point using the covariance matrix so that scale is maintained. The number of components was determined using a screen plot.

Normalized absorbance readings were analyzed to give substrate richness (the number of substrates used), substrate evenness (the distribution of color development between substrates), and diversity indexes (a composite measure of richness and evenness) (Magurran, 1988). These indexes were calculated and are presented in Table 2.

Analytical Methods

The influent and effluent samples were analyzed for physical, chemical, and microbiological variables (COD, TP, TN, chlorophyll *a*, ammonia [NH_3–N], and pH) using Chinese Standard Methods (SEPA, 2002).

Results and Discussion

Variation of Average Well Color Development and Analysis of Catabolism of Carbon Sources

Average well color development values represented carbon source utilization by the sampled microbial communities. Differences in either maximum color development or rates of color development result in different areas under the time-course profile. Thus, these areas are the most useful summary statistics for detecting differences among the samples. Figure 2 shows that the AWCD for metabolism of substrate microbial communities has a strong nonlinear correlation with incubation time, and that the time-course of AWCD variation is similar to kinematic models of microbial population growth (S-curve). Therefore, a modified logistic equation ($y = y_0 + a/\{1 + \exp[-(x - x_c)/k]\}$) was used to realize the fitting of varia-

Table 2. Expressions for richness, evenness, and diversity of microbial communities.

Index	Definition	Formula
RI	Richness	$RI = (S - 1)/\ln R$†
H′	Diversity, measure of richness, and evenness, calculated from Shannon–Weaver Index	$H' = -\Sigma[(R_i/R)]\ln(R_i/R)$
EI	Evenness	$EI = \Sigma[R(R-1)/R_i(R_i-1)]/e^{H'}$

† S = the number of cells which the substrates were utilized.

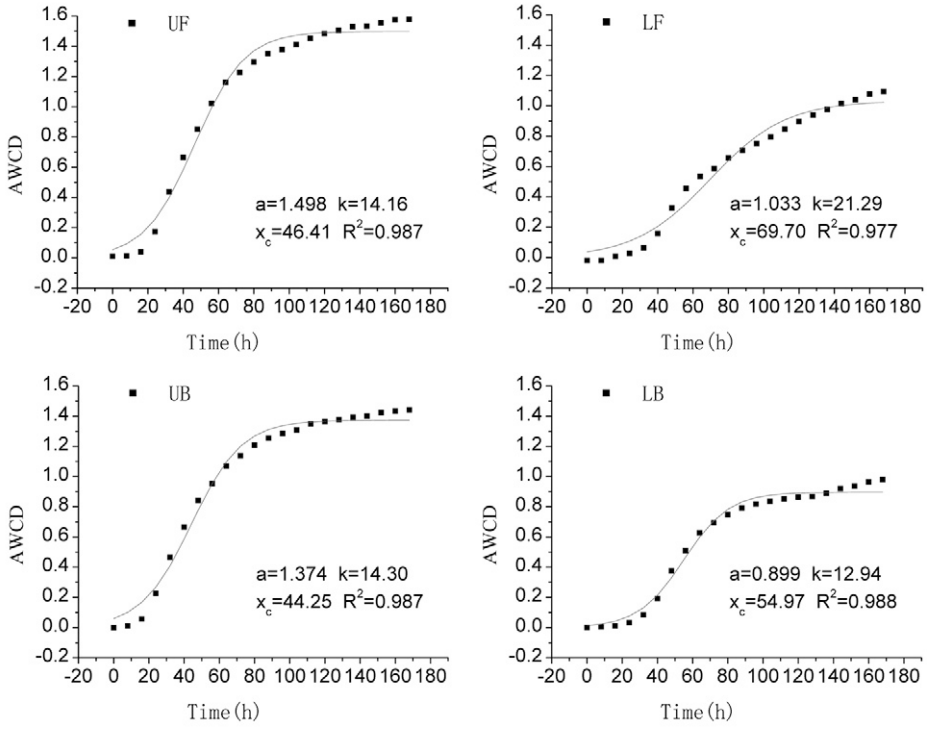

Fig. 2. Variation in average well color development (AWCD; see Eq. [1]) over time in Biolog-ECO plates (UF = upper front substrates, LF = lower front substrates, UB = upper back substrates, LB = lower back substrates; see Fig. 1). Variables: *a* is the maximum value of absorbance in Biolog test, *k* represents variability index of the mean absorbance values, and x_c is incubation time at half of maximal optical density.

tion of AWCD with Origin 6.0 (OriginLab Corporation, Northampton, MA) in this paper, where a is the maximum value of absorbance in Biolog test, $1/k$ represents variability index of the mean absorbance values, and x_c is incubation time at half of maximal OD. The detailed values of curve integration were obtained and are shown in Table 3.

Approaches to the analysis of Biolog data include the AWCD method, a curve-integration method, a value, and x_c value (Guckert et al., 1996). In the present study, a curve-integration approach was found to best represent the capacity of communities in utilization of C sources. Therefore, the integrated areas under AWCD curves were used to assess the capacity of C-source consumption, with the incubation time ranging from 0 to 96 h.

There were 31 different carbon sources in the Biolog-ECO plates. For each plate, the microbes were found to utilize >27 kinds of carbon sources. The different microbial communities had significantly different respiration responses to carbon compounds. Microbial community, as well as microbial biomass, appeared to play a role in litter decomposition rates (Blume et al., 2002).

As shown in Fig. 2, AWCD values in the first 16 h showed little variation, and over 168 h, samples developed sigmoid AWCD curves. In this lab-scale HSSF, hydraulic retention time was 72 h and AWCD was analyzed within 96 h.

Ninety-six-hour AWCD data successfully distinguished among the four substrate samples' expression of heterotrophic bacterial activity in ECO plates (Fig. 2). The result of comparison of integrated areas under AWCD curves of the four substrate samples was UF > UB > LB > LF and the proportion of areas were 2.32:2.21:1.16:1, respectively.

The variation of AWCD indicated that the carbon metabolizing activity of upper-substrate microorganisms was greater than that of lower-substrate microorganisms. The carbon metabolic activity of LF substrate microbial communities is the lowest. The substrate microorganisms in UF showed 131.9, 4.8, and 99.3% higher integrated areas under AWCD curves than in LF, UB, and LB of the CW, respectively. The values of upper substrates tended to be higher than those of lower substrates in the vertical direction, whereas in the horizontal direction, the values of front substrates were higher than that of back substrates in the upper layer, while the opposite trend was observed in the lower layer (see Table 3).

Plant material is known to be the dominant factor determining microbial biodiversity for most rhizosphere systems (Denton et al., 1998). The observed differences in microbial diversity between upper and lower layers may reflect variation in the presence of carbon substrates as well as differential use of the substrates by microorganisms. Plant root systems developed much better in the upper layer than the lower one. The secretion of the rhizosphere can result in higher growth rates of microorganisms. Furthermore, metabolic activity is also influenced by a multitude of factors such as the concentration of dissolved oxygen and dissolved organic matter, the intensity of root penetration, and oxidation redox potential (Armstrong et al., 1990). The release of oxygen through roots and rhizomes (transported by the aerenchyma helophytes into the rhizosphere) contributes to a complex environment of aerobic, anoxic, and anaerobic microzones in the subsurface of the HSSF (Randerson, 2006). The amount of aerobic microorganisms with strong metabolic capacity was higher in upper samples than in the lower sites, which implies that upper microorganisms in HSSF show stronger metabolic capability of the microbial communities.

Table 3. Integrated parameters of average well color development (AWCD) curves.

Sampling port†	Integrated areas under AWCD curves (0–96 h)
UF	74.13
LF	31.96
UB	70.75
LB	37.2

† UF = upper front substrates, LF = lower front substrates, UB = upper back substrates, LB = lower back substrates.

The differences in metabolic capability of the microbial communities in the horizontal layer may be caused by the variation of organic substrates and nutrients along the length of CW. As described in Fig. 3, the removal of contaminants including COD, TP, TN, PO_4^{3-}–P (orthophosphate) and NH_3–N (ammonia) mainly decreased in the front part of the CW, that is, in the first 25% of the length. Thus, metabolic activity of bacteria in the upper layer was UF > UB described with AWCD after curve integration because microorganisms in UF had higher levels of nutrients to utilize.

In the lower layer, however, microbial function was reversed, LB > LF, which may be induced by changing of microbial communities. Contaminants in rural eutrophic water were mainly from plankton (the amount of chlorophyll a in water was >200 mg m^{-3} in autumn in this study) and suspended solids. Subsurface-flow CWs have proven to be effective in wastewater treatment, particularly for total suspended solids (Green and Upton, 1995). The removal of plankton and suspended solids in the interval 0 to 25% length in our HSSF was mainly due to the media filtration, and then the greater removal of COD and TN appeared further downstream. As measured in this study (Fig. 3), the concentrations of COD, TP, TN, PO_4^{3-}–P, and NH_3–N in LF were markedly higher than those in LB. This

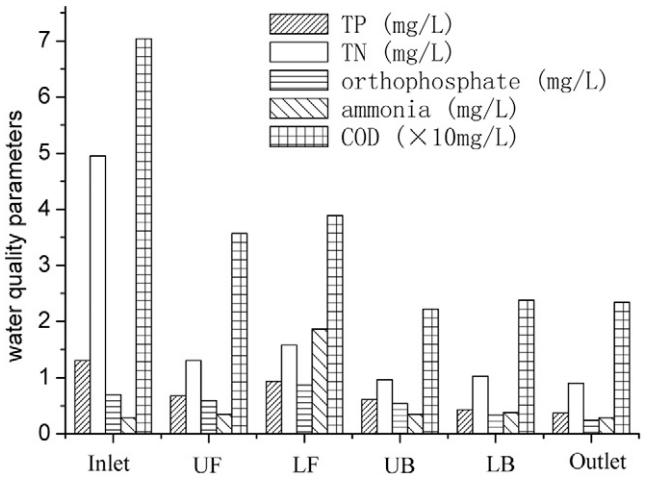

Fig. 3. Variation of chemical water parameters concentration at different sampling ports of horizontal subsurface-flow reactor constructed wetland in a laboratory study. UF = upper front substrates, LF = lower front substrates, UB = upper back substrates, LB = lower back substrates, TP = total phosphorus, TN = total nitrogen, COD = chemical oxygen demand.

may result in the accumulation of inactive microorganisms without strong metabolic capacity in LF in long-term anaerobic conditions. Thus, the metabolic capability of the microbial communities in LF was lower than in LB, a reversal of the situation in the upper layer.

The variation of metabolic capability of the microbial communities with experimental time may be related to the accumulation of biomass and substances in HSSF. The biomass accumulated in entry site due to higher concentration of nutrients, which may result in the change of microbial communities, especially in LF. At the extended performance period, the metabolic capability of the microbial communities in HSSF substrate also changed. In general, the mechanism of the variation of metabolic capability of the microbial communities is still being researched. Therefore, more studies on the structure of microbial communities may be performed in the future, associated with the molecular biology.

It is known that higher levels of soil nutrients can result in higher growth rates of plants, which leads to enhanced rates of nutrient cycling. The difference of metabolic capability of the microbial communities in the same layers responded to impacts of changing nutrient levels. The removed N is either accumulated in the sediments or eliminated by denitrification, whereas P removal is only due to sediment accumulation.

The AWCD mainly reflected the species metabolic activity and the ability of the bacterial community to respond to the substrates in HSSF. The reduction of the microbial activity as shown by AWCD was possibly due to the reduction in the numbers and species diversity of the biota.

Principal Components Analysis of Carbon Source Utilization Patterns

Principal components analysis using all 31 carbon sources revealed a separation of substrate samples, indicating the different patterns of potential C utilization and different microbial communities. The time selected for analysis using the method described above was 96 h. Five principal components (PCs) were selected to be retained from a screen plot. The first two principal components (PC1 and PC2) explained 73.03% of the total variance in the data and are plotted against each other in Fig. 4 for illustration. The microbial community composition of each ecosystem was clearly separated by PC1 and PC2. Analysis of the data with the data point included gives very similar results. As shown in Fig. 4, samples isolated from upper layer were in the positive side of PC2, and near to the zero of PC1, while samples isolated from lower layer were both in the negative side of PC2, and LF and LB samples were in the different sides of PC1. Then the four samples were differentiated from each other, which reflected different structures and characteristics of microbial communities in different places in

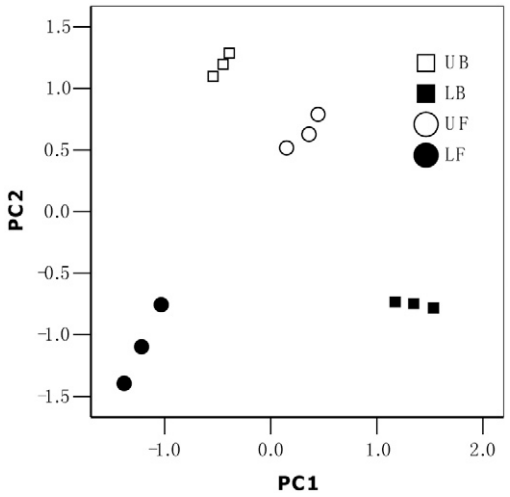

Fig. 4. Principal components analysis (PCA) of Biolog results for substrate samples in constructed wetlands after 96 h of incubation at 25°C on Biolog-ECO plates for all four sites (each data point is average of three replicates); Principal Component 1 (PC1) plotted against Principal Component 2 (PC2) generated by PCA, showing the different patterns of substrate utilization by substrate microbial communities. UF = upper front substrates, LF = lower front substrates, UB = upper back substrates, LB = lower back substrates.

CWs. Samples from the four ports appeared to have markedly different metabolic activities (Fig. 4). Therefore, this technique appears to show promise as a monitoring tool for HSSF at the community carbon metabolic level.

The data here showed that the composition and structure of microbial communities changed to adapt to the special environment. It is in agreement with others that subsurface microbial communities are distinct in composition from surface communities (Blume et al., 2002). All studies imply microbial communities contained in deeper layers are specialized for their environment and are fundamentally distinct from surface communities.

Relative Utilization of Carbon Sources

To gain a better understanding of microorganism metabolism in the HSSF, Biolog-ECO was used to characterize microbial community function based on sole carbon source utilization patterns. Biolog-ECO plates consist of 31 different carbon sources: 4 polymers, 10 carbohydrates, 7 carboxylic acids, 6 amino acids, 2 amines, and 2 phenolic compounds. Table 4 shows the utilization of compounds in each of these chemical classes in the Biolog-ECO plates.

Interestingly, the analyses of the response patterns revealed carbon source utilization of polymers, carbohydrates, carboxylic acids, and amino acids were greater in UF than in LF, but that of amines and phenolic compounds were very similar in UF and LF. Carbon source utilization of carbohydrates,

Table 4. Relative utilization of carbon sources in horizontal subsurface-flow reactor stated by optical densities after 96 h of plate incubation, R_{96}.

Sampling port†	Polymers	Carbohydrates	Carboxylic acids	Amino acids	Amines	Phenolic compounds
UF	1.836	1.509	1.110	1.579	0.741	0.790
LF	1.221	0.628	0.827	0.594	0.603	0.792
UB	1.801	1.533	1.029	1.087	1.428	0.380
LB	0.988	0.823	0.421	0.991	1.564	0.577

† UF = upper front substrates, LF = lower front substrates, UB = upper back substrates, LB = lower back substrates.

amino acids, and amines were lowest in LF, whereas that of polymers and carboxylic acids were lowest in LB (see Table 4). The utilization of carbon sources in sampling sites was different, likely due to microbial types and substrate–root–microbes interactions (Liang et al., 2003).

Principal components analysis of carbon source utilization patterns implied that the four samples appeared to have obviously different metabolic activities (see Fig. 4), which is also reflected in Table 4. The upper layer removed more polymers, carbohydrates, carboxylic acids, and amino acids, likely related to abundant organic substances in the bulk water. The decrease in the removal rates of the four carbon sources could be due to changes in microbial population in the lower sites. The decreased utilization of the first four organic substance groups and anaerobic conditions were observed in LF where the metabolic levels of carboxylic acids and phenolic compounds were increased.

Diversity Indices Analysis of Community Carbon Metabolic Profiles

Table 5 shows that the richness, diversity, and evenness indices for microbial communities in HSSF changed with sampling sites. Richness is generally defined as the number of different groups of microorganisms found occurring together. Like metabolic rate, the richness of upper layer was higher than the lower layer, and the trend followed was UB > UF > LF > LB. The richness of lower back-end substrates microorganisms was markedly lower than others. Shannon indices are the indicators of metapopulation species diversity. The upper sampling ports had higher Shannon diversity indices of carbon substrate utilization compared with the lower layer. The results showed that Shannon indices decreased as follows: UF > UB > LF > LB, which has the similar pattern described above. Evenness is the expected distribution of microbial groups represented by the distribution of color development between substrates in Biolog-ECO plates. Significantly lower evenness was observed in LF, and no significant differences were found among measures of carbon source utilization evenness in the three other sampling ports (see Table 5). This may be induced by accumulation of a great amount of organic pollutants in LF. Thus, catabolic evenness was highly related to microbial communities and they show strong utilization of particular carbon sources.

The application of Biolog to describe microbial community structure may have some limitations. For example, organisms that can use the carbon sources in Biolog wells may not represent all microbial communities in substrates, just as culture-based techniques may lead to selective overrepresentation of the contributions of certain organisms. Recent studies often use molecular biotechnology or advanced biochemical techniques to analyze the structure and diversity of microorganisms (Garland, 1997). Fluorescent in situ hybridization has been used to characterize major microbial groups at the beginning, center, and end of a well-established HSSF and at different depths including the root zone (Krasnits et al., 2009). However, Biolog is more suitable for inexpensive, rapid analysis of metabolic capacity of C resource of microorganisms and comparison of community diversity.

Conclusions

In general, HSSFs have proven to be useful for the degradation of organic compounds from urban wastewater.

This study applied Biolog-ECO plates to investigate microbial metabolic activities at the community level in lab-scale CWs. Biolog-ECO plates differ from the Biolog-GN plates (Biolog Inc., Hayward, CA) used in earlier studies, where the carbon substrates in the GN plates are designed to differentiate and identify gram-negative isolates (Guckert et al., 1996). Rather than 95 different carbon substrates on each plate, one Biolog-ECO plate contains three replications of 31 different substrates that are most useful for environmental community analysis because there is evidence that the selection of the carbon substrates allows greater discrimination between communities (Preston-Mafham et al., 2002); they are also more convenient because replication of subsamples is achieved without the need for multiple plates. Biolog-ECO was an effective technique for finding similarities and differences in the functional diversity of microbial communities in our constructed wetlands (HSSF). And the media depth and the operation of this HSSF were similar to the large-scale subsurface-flow constructed wetlands. The results from this small-scale study can be used for large-scale wetlands in the field:

1. Biolog test suggested carbon sources metabolism declined from the front to the back and from the upper to the lower portions of the HSSF. The result of comparison of integrated areas under AWCD curves of the four substrate samples was UF > UB > LB > LF. Carbon source utilization of polymers, carbohydrates, carboxylic acids, and amino acids were higher in UF than in LF, but that of amines and phenolic compounds were very similar in UF and LF, likely due to different microbial communities present in the four sampling ports.
2. Principal components analysis of metabolic data shows that a standard statistical program, such as SAS or SPSS, can differentiate community structure and characteristic carbon sources metabolism of every sampling port in HSSF effectively. These differences are likely related to variations of bed environment and distribution of nutrient substances.
3. Biolog results showed functional microbial diversity among microorganisms in different places in HSSF. The richness, evenness, and diversity of upper-substrate microbial communities were significantly higher than

Table 5. Means for Shannon indices of functional diversity and measures of richness and evenness of carbon source utilization in four sampling ports in horizontal subsurface-flow reactor after 96 h of incubation at 25°C using a set of 31 Biolog microplate wells that correspond to substrate on Biolog-ECO plates.

Sampling port†	Richness, RI	Diversity, *H′*	Evenness, EI
UF	2.72	3.124	0.945
LF	2.486	2.532	0.883
UB	2.738	2.987	0.926
LB	1.578	2.256	0.948

† UF = upper front substrates, LF = lower front substrates, UB = upper back substrates, LB = lower back substrates.

those of the lower substrate, which suggested possible differences in functional diversity. The LF substrate microbial communities had lower evenness than the other sampling plots, and the lowest richness of substrate microbial community was found in the LB part of the constructed wetland.

4. Nutrients, oxygen, and roots are widely believed to influence wetland microorganisms. In this research, nutrients were similar in UB and LB. Thus, anaerobic environment and roots may contribute to the difference in metabolic utilization of carbon sources between UB and LB. In future application studies, aerating in the lower substrate is suggested to optimize the structure or function of microbial communities, which will enhance the nutrient removal yield.

   The technique might find application in monitoring the health of the microbial population in the CW and it might be expected that significant changes in the community structure precede a "crash" of an operating unit. The technique might also find application in searching for similarities and differences in the functional diversity of microbial communities in different HSSF operating under similar conditions.

5. Although further studies are needed, Biolog application provides numerous data, whereas limited information about community structure can be gained from the analysis of microbial metabolization function. In recent years, an increasing number of studies using molecular strategies to research the soil microbial structural diversity in genetic profiling have been published. Therefore, in the future work, the combination of polyphasic methods is required to obtain accurate information on microbial community characteristics.

Acknowledgments

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