

Hindawi Publishing Corporation
International Journal of Chemical Engineering
Volume 2010, Article ID 490240, 6 pages
doi:10.1155/2010/490240

Research Article

Seasonal and Spatial Changes of Microorganism Communities in Constructed Wetlands: A Community Level Physiological Profiling Analysis

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Received 15 January 2010; Accepted 22 March 2010

Academic Editor: Yves Andrès

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In constructed wetlands, microorganisms associated with plants are assumed to play a major role. A one-year survey was conducted in five vertical flow constructed wetland systems that had been operating from 2 months to 8 years in small French villages (100–500 People Equivalent) to provide a better understanding of microbiological activity. The objective of our study was to highlight the most important factor generating variability between microorganisms communities compared to treatment performances. Results of community level physiological profiling using Biolog Ecoplates were analyzed using principal component analysis. The greatest microbial activity was observed in the oldest wetland during summer. Profiles of fed and rest bed were differentiated by the nature of the main carbon source metabolized. Whereas carbohydrates and carboxylic acids appeared to be better assimilated with fed beds, it seemed that phosphate compounds as well as amines allowed better growth in the plates inoculated with samples of rest beds. In all fed beds, the most important parameters affecting the diversity were the season and the age of the wetlands. There were only slight profile differences between surface and subsurface samples and between the first and second stage samples.

1. Introduction

In constructed wetlands (CWs), it is widely recognized that microorganisms associated with macrophytes play a major role in pollutant removal. Over the last ten years a greater focus has been put on understanding microorganisms diversity and spatial distribution, on characterizing bacterial communities, or on monitoring microbial biomass. A large number of biochemical tools were successfully adapted to monitor microorganisms in CWs, such as epifluorescence, enzymatic activities, protein concentration, flow cytometry, fish probes, PCR, and DGGE [1–6].

In vertical flow constructed wetlands (VFCWs), pollutant removal efficiency (especially TKN removal) can be affected by several factors including season, CW age, and feeding mode (batch loads, periods of rest, etc.). Our

study aimed at verifying whether treatment performance was linked or not to microbial diversity by applying the community-level physiological profiles (CLPPs) method using Biolog EcoPlate for constructed wetlands microorganism communities. The CLPP method using microtiterplates with multiple sole-carbon sources has become a popular tool for the comparison of microbial communities with respect to their functional potential. CLPP was successfully adapted to the study of complex communities [7]. This method was shown to enable rapid assessment of variation in community structure in biological water treatment systems such as mixed liquor [8, 9] or biofilters [10, 11].

We analysed microbial diversity in five VFCWs designed to treat raw wastewater. We inoculated a total of 46 plates during two sampling campaigns. The first campaign (25 plates) was realized in February, April, June, and October

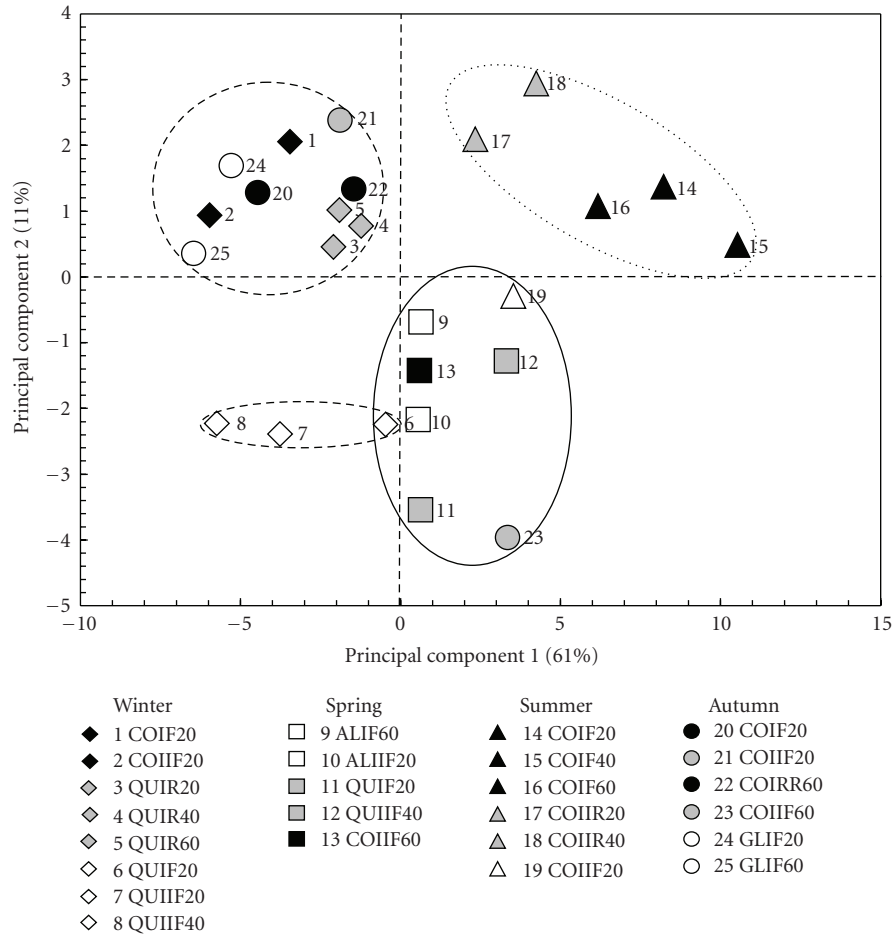


FIGURE 1: PCA of Biolog data of the first sampling campaign. A code identifies each sample: the first two letters refer to the CW name, followed by I for first stage and II for the second; we referred to the depth as 20 for 0–20 cm, 40 for 20–40, cm and 60 for 40–60; the feeding mode was expressed as F for bed fed for 3 days, R for bed in rest for 3 days, and RR for bed in rest for 8 to 10 days. For example, COIF20RR refers to Colomieu, stage I, 0–20 cm layer, in rest for 10 days.

2002 in 5 different sites and enabled to highlight season influence on bacteria community profile. The second campaign (21 plates) was performed in November 2003 in two sites to determine the vertical distribution of microbial activity in relation to CW age and feeding mode (fed and rest rotation).

2. Material and Methods

2.1. Wetlands Systems. The studied two-stage VFCWs, constructed by the Société d'Ingénieries Nature et Techniques, were designed to treat raw wastewater of small communities in the Rhone-Alpes region of France (Table 1).

Each stage was made of two to four beds fed according to 5–7 days rotation. Beds were 0.7 m. deep and planted with *Phragmites australis*. Average treatment performances observed during the two campaigns for the first stage of all VFCWs except Evieue are available in Table 2.

2.2. Sampling, Biofilm Solution Extraction, and Biolog Methods. For the first campaign, samples were taken at Colomieu,

Glandieu, Allèves, and Queige at four different dates, 2002 (March, April, June, October). For the second campaign, samples were taken at Colomieu and Evieue, in November 2003. A 60 cm long core, divided in three 20 cm sections, was taken at 50 cm of an inlet pipe. Between 2 and 15 grams of cores were first washed with a phosphate buffer solution (K_2HPO_4 , 9.3 g L^{-1} ; KH_2PO_4 1.8 g L^{-1} ; $\text{pH} = 7.2$) to separate gravel and obtain a final solution concentration having 2% w/w of extracted matter and was screened on a 5 mm filter. This method was effective in normalising the cell number density in the final inoculum for the Biolog EcoPlate. Carbon sources utilization pattern was realized using Biolog EcoPlate (Hayward, CA, USA). Each plate contains 31 of the most useful carbon sources for soil community analysis [12, 13]. Carbon sources are preloaded and dried into 96 wells (Figure 4). Tetrazolium Violet is used as a redox dye to indicate carbon sources use.

The extraction solution was diluted 20 times using phosphate buffer and vortexed for 10 minutes. Finally $150 \mu\text{L}$ aliquots of supernatant were added in each well. After injection, EcoPlate was incubated at 20°C in obscurity for

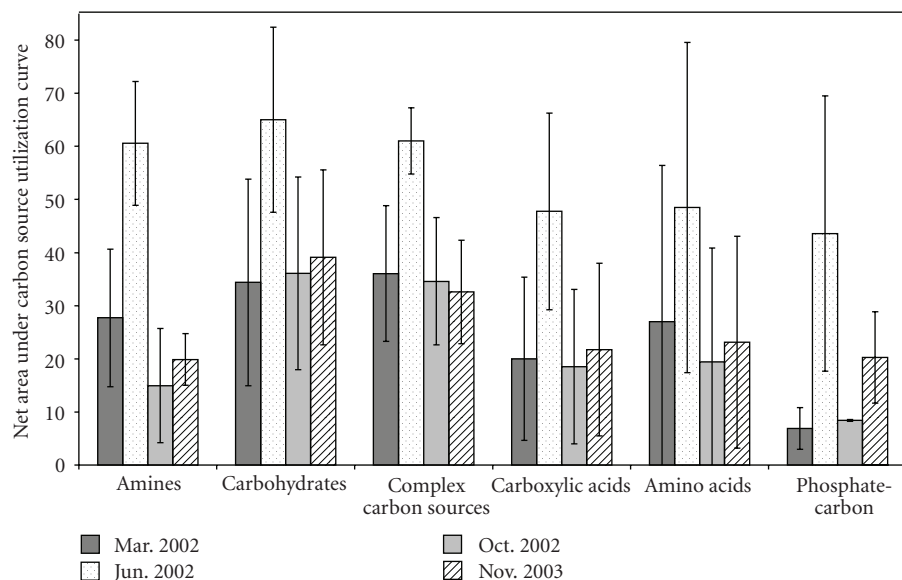


FIGURE 2: Seasonal metabolic response of samples taken in surface of the fed bed, first stage of Colomieu.

TABLE 1: VFCWs characteristics.

	Units	Glandieu	Queige	Allèves	Evieu	Colomieu
People equivalent	Pe	250	500	250	100*	200
First stage area	m ²	233	850	360	—	300
Second stage area	m ²	164	750	230	—	225
Initial organic load	g BOD ₅ m ⁻² d ⁻¹	64	32	—	—	48
Starting date	year	1999	1998	1999	2003	1993

* Evieu was only connected to 30 Pe for almost 3 months during the sampling campaign (November 2003).

at least 72 hours, during which coloration development was measured every 12 hours using a Vmax microplate reader (Molecular Device, Sunnyvale, CA, USA). Results (31 curves/sample) were analysed by curve integration and principal component analysis (PCA) according to Guckert et al. [14] and Kaiser et al. [9]. Since the inoculum density was controlled, data were not normalized by average area on each plate thus giving an indication of the global activity. Data were also compared using grouping of carbon sources. To simplify direct comparison, 6 main categories were chosen: amines, carbohydrates, complex carbon sources, carboxylic acids, amino acids, and phosphate-carbon (Figure 4).

3. Results and Discussion

3.1. Carbon Source Utilization. Microbial communities sampled in CWs were generally able to use all of the 31 carbon sources. The most important growths were observed on L-asparagine, L-serine, lactose, mannitol, acetyl glucosamine, glycogen, and cellobiose sources. As expected, elementary sugars or polymers such as glycogen were greatly metabolised, most probably in Krebs's cycle by oxidative phosphorylation. Complex carbon source and carbohydrates provided the best growth, most probably because they are the

more energetic compounds. The smallest microbial growths were reported on carbon sources containing aromatic cycle.

3.2. Sampling Campaign 1. Principal component 1 accounted for 61% of total variability and was mainly associated to carbon source B1, D2, and E2 (Figure 1). Principal component 2 accounted for 11% of total variability and was mainly associated with B4, C4, F4, and H4 (amino acids). There was little influence of sampling depth and CW age, with Biolog ecoplate profiles being mainly differentiated according to season. Samples taken in warm periods (with bed having full active macrophytes) had more positive score value for the first principal component than samples taken in winter or autumn (Figure 1). Less of the variability was explained by the second principal component differentiating between age of CW (all profiles of Queige and Allèves) and most probably spring profiles compared to the others. Similarities between three distinct groups were drawn with summer profiles in dotted line, spring profiles in indented line, and winter and autumn profiles in full line (Figure 1). This pattern could be linked to a decrease in activity of several bacteria species under cold conditions. Sludge accumulation in the first stages in winter [15], by limiting infiltration rate and oxygen renewal, may also affect microbial communities environment and redox. TKN

TABLE 2: Average performances (mass removal in %) observed in the first stage of the different VFCWs (adapted from Chazarenc and Merlin, [4]).

Name	Spring-Summer (average)			Autumn-winter (average)		
	TSS	COD	N-TKN	TSS	COD	N-TKN
Allèves	95.2 ± 5.5	92.3 ± 4.5	84.2 ± 12.4	95*	83.9*	85.6*
Colomieu	96.6 ± 4.5	93,4 ± 6,4	98.4 ± 0.6	96.1 ± 2.2	92.3 ± 4.2	76.7 ± 18.5
Glandieu	99.6*	98.7*	93.3*	98.5*	95.7*	—
Queige	92.5 ± 6.9	91.0 ± 9.4	77.7 ± 26.0	90.1 ± 8.1	83.0 ± 7.9	38.3 ± 14.3

* only one measurement available.

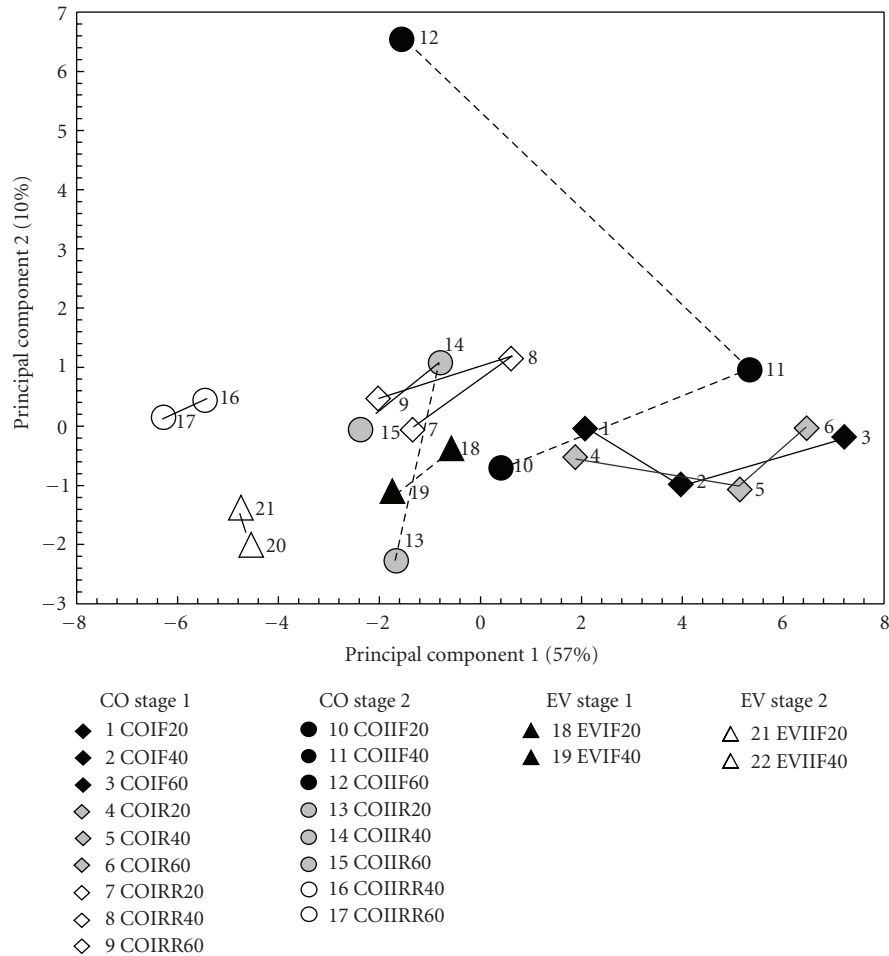


FIGURE 3: Ordination produced from PCA analysis of Biolog profile obtained during the second campaign. Samples connected with lines represent sampling from the same bed at different depths; full line: Colomieu stage 1; indented line: Colomieu stage 2; dotted line: Evieue. See legend in Figure 2 for sample codes.

removal efficiency decreased from 78% in summer to 38% in winter 2002 in Queige and from 98% to 77% in Colomieu (Table 2). To some extent, the spring group (full line in Figure 2) represented a transition between little diversified profiles (winter) and summer profiles.

Seasonal variations of bacterial community response in the upper layer of the fed bed in Colomieu illustrate well the seasonal changes observed in all samples (Figure 2). Net activities follow season temperature, with a maximum in June and lower values in late winter and autumn.

3.3. *Sampling Campaign 2.* Figure 3 shows PCA results obtained when comparing all profiles from the second sampling campaign. Principal component 1 accounted for 57% of total variability and was mainly associated to carbon source E1, F1. Principal component 2 accounted for only 10% of total variability and was mainly associated with E4, D1, and G1.

Profiles distribution along principal component 1 was mostly related to community diversity and activity. Slight differences were observed when comparing profiles for bed

Biolog EcoPlate	1	2	3	4
A	Water	β -methyl-D-glucoside	D-galactonic acid γ -lactone	L-arginine
B	Pyruvic acid méthyl ester	D-xylose	D-galacturonic acid	L-asparagine
C	Tween 40	i-erythritol	2-hydroxy benzoic acid	l-phenylalanine
D	Tween 80	D-mannitol	4-hydroxy benzoic acid	L-serine
E	α -cyclodextrin	N-acetyl-D-glucosamine	γ -hydroxy butyric acid	L-threonine
F	Glycogen	D-glucosamic acid	Itaconic acid	Glycyl-L-glutamic acid
G	D-cellobiose	Glucose-1-phosphate	α -ketobutyric acid	Phenylethyl-amine
H	α -D-lactose	D,l- α -glycerol phosphate	D-malic Acid	Putrescine







	Amines		Carboxylic acids
	Carbohydrates		Amino acids
	Complex carbon sources		Phosphate-carbon

FIGURE 4: Carbon sources in Biolog EcoPlate wells. Codification matrix and groups of carbon source isolated (real configuration is in triplicate).

fed and in rest for 3 days in the first stage of Colomieu (1–3 compared to 4–6) (Figure 3). This suggests that the microbial metabolic response is very similar between the two beds and that the growing conditions (amount of nutrients, humidity, etc.) were the same after few days of rest. On the other hand, a net difference appears after 10 days of rest (7–9) (Figure 3), suggesting that microbes were less active and probably less diversified. Whereas carbohydrates and carboxylic acids appeared to be better assimilated with fed beds, it seemed that phosphate compounds as well as amines allowed better growth in the plates inoculated with samples of rest beds [16]. Thus, a change in microbial communities should also appear between fed and long-period rest beds, predicting development of different purifying mechanisms (e.g. endogenous respiration).

Because the second stages were shown to be mostly efficient for TKN removal [15], we expected more differences in microbial communities between stages. A strong difference is only apparent in the deeper profile of the second

stage in Colomieu (12) (Figure 3). This difference was also apparent in the ACP of the first campaign (23) (Figure 1). Because Colomieu is the oldest CW, accumulation of organic matter in the deeper zone of the filtration matrix, by generating a reduced environment, could explain its distinct microbial composition. Except for this difference, profiles from Evieu were comparable to those of Colomieu. However, considering principal component 1, Evieu profiles of fed beds were similar to those observed in rest for almost 10 days in Colomieu. This could be a consequence of both the very low organic load in Evieu during the sampling period (Table 1) and the absence of a well-established surface layer. This was shown to enhance biological and hydraulic activities in the oldest CW such as Colomieu [4, 15].

One can suppose that wastewater greatly contribute to microbial establishment in CW; it could have been very interesting to compare profiles of influent versus CW effluent to see and study the wetland contribution as showed by Hench et al. [17].

4. Conclusion

Season was the most important parameter generating variability between bacterial community profiles. Small differences between same samples taken at different depth were observed except for the one of Colomieu 40–60 cm. Profile differences between fed beds and bed in rest for almost 10 days suggest that other degradation mechanisms such as endogenous respiration appeared over time (this could explain low sludge production in French VFCWs). Biolog Ecoplates are a quantitative tool for a simple comparison of microbial metabolic pattern in VFCWs. However care must be taken in interpreting results as the statistical analysis highlights mostly relative differences from a sample profile to another. Furthermore a wide range of bacteria such as strict anaerobic ones are not detected using microplates. Finally, this tool enabled us to see clearly differences between samples taken in the same CW and could be useful to optimize batch loading, to identify dead volumes or bypass, or to estimate dysfunction. In the future, wastewater contribution to bacteria communities should be studied.

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