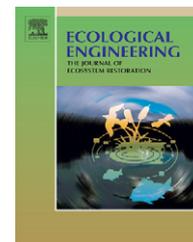


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## Short Communication

# Diversity of abundant bacteria in subsurface vertical flow constructed wetlands

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### ABSTRACT

Microorganisms are mainly responsible for the transformation and mineralization of degradable organic pollutants within constructed wetlands (CWs). There is still a lack of knowledge concerning microbial community composition within CWs. In order to elucidate the diversity of bacteria inhabiting subsurface vertical flow CWs, the molecular fingerprint technique “terminal restriction fragment length polymorphism” (T-RFLP) derived from total community DNA, was applied.

A comparison of the bacterial communities from a full-scale outdoor vertical flow CW with planted and unplanted indoor pilot-scale vertical flow CWs, operated under similar conditions, revealed that both systems are colonized by similar populations showing only little variation in their composition over filter depth. A comparison of bulk soil from an unplanted CW with the rhizosphere soil from the outdoor and indoor CWs showed differences in the bacterial composition, demonstrating the influence of the plants on the rhizosphere community. A comparison of the wastewater before and after the CW passage demonstrated that the bacterial diversity was clearly reduced within the planted outdoor system only.

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## 1. Introduction

Constructed wetlands (CWs) have received an increased attention in the recent years. As a result of the wide range of benefits in creating and maintaining wetlands they are implemented in a variety of geographic regions. When designing a CW it is important to create an environment which is optimal for reactions responsible for the wastewater purification. The complex microbial community mainly associated with the filter material or roots, created by interaction with the wastewater, is mainly responsible for the degradation performance of the system. Investigations of the microbial community compo-

sition and diversity in natural or constructed habitats are important for their characterization of such habitats, since microbes are the key factors in many environmental processes (Nogales et al., 2001).

This study focuses on a comparative overview of diversity patterns in such systems by a first experimental setup. The aim was to investigate the usefulness of a molecular method to characterize potential differences in microbial diversity within and between pilot-scale CWs (PSCWs) and a full-scale CWs (FSCWs) as done by Ansola et al. (2003). Possible differences between planted and unplanted CWs; within the filter bed depth profile and between the rhizosphere

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soils of *Miscanthus sinensis giganteus* and *Phragmites australis*, were investigated. Furthermore, changes in diversity pattern between the inflow and outflow effluent were surveyed to get an overview of the distribution of microorganisms within vertical flow constructed wetlands.

Only a few publications are available where the authors have tried to elucidate the complex microbial diversity within the filter body of CWs created to treat municipal wastewater. [Truu et al. \(2005\)](#) analysed the microbial community structure within a horizontal flow CW and found a decrease in bacterial diversity with increasing filter bed depth. Due to the great bacterial diversity within these systems, other studies focused on distinct functional groups relevant for the degradation process in wastewater like ammonia oxidizing bacteria ([Tietz et al., 2007a](#); [Gorra et al., 2007](#)), or methanotrophic bacteria ([DeJournett et al., 2007](#)). However, no study dealing with the total bacterial community composition within a subsurface vertical flow CW with intermittent loading with municipal wastewater has been published yet.

The efficiency of CWs concerning the removal of microorganisms, especially faecal indicator bacteria, is a topic that has been thoroughly investigated by conventional culture-based plate count techniques ([Sleytr et al., 2007](#)). Until now it has not been investigated whether bacteria found in the effluent of CWs are typical wastewater bacteria that pass the system or bacteria that are washed out from the soil particles in the CW. Therefore community fingerprints of the in- and outflows of the CW have been investigated in the present study.

This study was created to make a brief inventory to assess various patterns of bacterial diversity within the filter bed of indoor vertical flow PSCWs, an outdoor FSCW, and the inflow and outflow effluent. For this purpose, the common molecular fingerprinting technique “terminal restriction fragment length polymorphism” (T-RFLP, or TRF) ([Liu et al., 1997](#); [Ishida et al., 2006](#)) was used to characterize the microbial communities within the CWs. This culture-independent tool has been applied to analyse the bacterial diversity in a wide range of environmental habitats and is one of the easiest and cheapest molecular analyses available. T-RFLP profiles, based on the amplification of the phylogenetic marker gene of the 16S rRNA, display the complexity of the investigated bacterial communities.

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## 2. Materials and methods

### 2.1. Sampling

Soil- and wastewater samples were taken from a 2-year-old experimental full-scale subsurface vertical flow CW (FSCW) with a surface area of 20 m<sup>2</sup>, located outdoor in Ernstshofen (Lower Austria; coordinates: longitude: 14.482693; latitude: 48.127522) planted with *P. australis*. Bacterial communities in the rhizosphere of this plant are of great interest because of their potential for bioremediation of industrial effluent ([Chaturvedi et al., 2006](#)). Additionally, samples were taken from a 2-year-old indoor pilot-scale vertical flow CW (PSCW; coordinates: longitude: 16.223213; latitude: 48.150339). Six of the eight PSCWs units, with a surface area of 1 m<sup>2</sup> each, were planted with *M. sinensis giganteus*, whereas two beds were

unplanted. All systems were loaded intermittently four times a day with municipal wastewater with an organic load of 20 g COD/(m<sup>2</sup> day) for the indoor system and 27 g COD/(m<sup>2</sup> day) for the outdoor CW. A detailed description of the two sampling sites is given in [Langergraber et al. \(2007\)](#).

Samples for microbial community analyses were collected four times from different depths of the filter bed of the FSCW and from different PSCWs. Samples taken at any place from the filter bed with no reference to plants are referred to as “bulk soil” in contrast to samples, which were directly removed with a sterile spoon from roots and rhizomes (“rhizosphere soil”) of *P. australis* and *M. sinensis giganteus*. Rhizosphere samples were sampled at the same time and the same depths from 10 to 20 cm. The bulk soil samples were used to investigate potential differences in bacterial diversity from seven different depths (0–1, 1–5, 5–10, 10–20, 20–30, 30–40 and 40–50 cm), between the PSCW and the FSCW, and between rhizosphere soils compared to unplanted PSCW filter bed samples. Wastewater samples were collected two times from the inflows to the FSCW and the PSCWs, respectively, once from the outflow from the FSCW and once from three different PSCWs outflows.

### 2.2. Terminal restriction fragment length polymorphism (T-RFLP or TRF) of the community DNA

DNA extractions of the soil samples were carried out with the PowerSoil DNA isolation kit; for the wastewater samples the UltraClean™ Water DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) was used, following the manufacturer's recommendations.

T-RFLP analysis was done according to a modified protocol after [Hackl et al. \(2004\)](#) and [Sessitsch et al. \(2002\)](#).

Twenty-four profiles were compared and standardized to the lowest quantity, according to the method of [Dunbar et al. \(2001\)](#). TRFs of 50–500 base pairs (bp) in length and with heights of  $\geq 50$  fluorescence units (FU) were included in the analysis. TRFs that differed by less than 0.5 bp in different profiles were considered identical and were clustered. Fragment length and peak height were used as parameters for profile comparison.

The phylotype richness (S) was calculated from standardized profiles of individual samples as the total number of distinct TRF sizes from 50 to 500 bp according to [Dunbar et al. \(2001\)](#). Numbers of TRFs with intensities higher than  $\geq 500$  FU were designated as highly abundant TRFs.

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## 3. Results

### 3.1. TRF community profiles from the filter body samples

Twenty-four TRF community profiles derived from twelve bulk soil samples (ten planted and two unplanted), four rhizosphere soil samples and eight wastewater samples were analysed. For the wastewater samples the impact of the CW passage on changes in bacterial diversity of the outflow was analysed.

Phylotype richness (S) as shown in [Table 1](#) was calculated for all community profiles. The phylotype richness (num-

**Table 1 – Phylotype richness (S) of the filter body and wastewater samples from twenty-four profiles of the full-scale constructed wetland (FSCW) and the pilot-scale constructed wetlands (PSCWs); calculated from standardized fluorescence intensities.**

Sample [#]	Sample type	Depth (cm)	$S = \sum P \geq 50 \text{ (FU)}^a$	$\sum P \geq 500 \text{ (FU)}^a$
FSCW [1]	Bulk soil, <i>Phragmites a.</i>	0–1	36	5
FSCW [2]	Bulk soil, <i>Phragmites a.</i>	1–5	50; 47	0; 4
FSCW [2]	Bulk soil, <i>Phragmites a.</i>	5–10	29; 54	4; 3
FSCW [1]	Bulk soil, <i>Phragmites a.</i>	10–20	47	4
FSCW [1]	Bulk soil, <i>Phragmites a.</i>	20–30	51	3
FSCW [1]	Bulk soil, <i>Phragmites a.</i>	30–40	46	5
FSCW [1]	Bulk soil, <i>Phragmites a.</i>	40–50	49	2
PSCW [1]	Bulk soil, <i>Miscanthus s. g.</i>	5–10	49	2
PSCW [2]	Bulk soil, unplanted	1–10	56; 56	1; 4
FSCW [2]	Rhizosphere soil, <i>Phragmites a.</i>	10–20	37; 40	6; 2
PSCW [2]	Rhizosphere soil, <i>Miscanthus s. g.</i>	10–20	63; 49	1; 4
Inflow [2]	Wastewater indoor		35; 40	4; 5
Inflow [2]	Wastewater outdoor		78; 44	0; 4
Outflow [2]	Wastewater indoor, <i>Miscanthus s. g.</i>		39; 33	3; 6
Outflow [1]	Wastewater indoor, unplanted		58	2
Outflow [1]	Wastewater outdoor, <i>Phragmites a.</i>		39	2

<sup>a</sup>Fluorescence units.

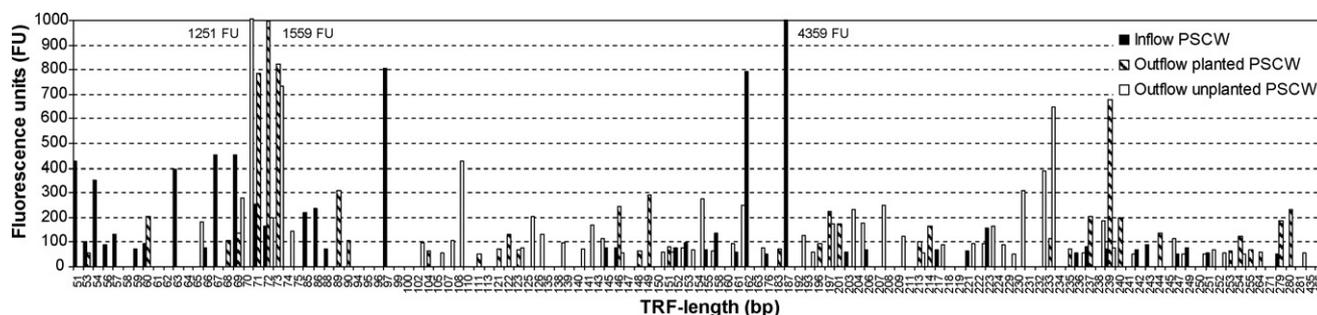
bers of TRFs with intensities  $\geq 50$  fluorescence units) ranged between twenty-nine and seventy-eight and peaks  $\geq 500$  FU varied from zero to six (data shown in Table 1). No differences between the individual layers and between the phylotype richness of rhizosphere soil and non-rhizosphere soil of the outdoor FSCW were detected, with exception of a lower diversity in the uppermost layer (FSCW 5; 0–1 cm). Between the outflows and the corresponding inflows no clear differences regarding the bacterial diversity were observed. In the unplanted PSCW, the diversity was higher in the outflow than the inflow, whereas in the FSCW the opposite was observed.

The TRF-profiles from outdoor bulk soil samples (FSCW) and indoor bulk soil samples (PSCW) implicate a similar community in both CWs (no data shown). The intensity of some peaks differed strongly between the out- and the indoor system. However samples from the seven different depths showed similar results, except the uppermost layer of the filter bed (like for S), which showed higher TRFs.

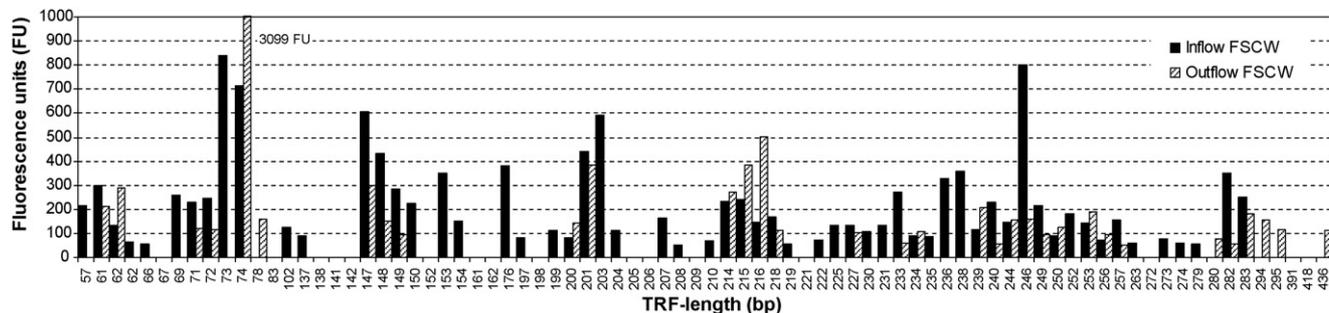
### 3.2. TRF community profiles from the wastewater samples

Figs. 1 and 2 show the TRF-profiles from the inflows and outflows of the PSCW and FSCW, respectively. From the forty

peaks occurring in the PSCW inflow only ten peaks were found in the planted PSCW outflow, whereas fifteen of the forty peaks were detected in the unplanted PSCW outflow (Fig. 1). The bacterial diversity in the outflow of the planted PSCW was not clearly reduced, and for the unplanted system even an increase of the diversity in the outflow was observed. On the other hand Fig. 2 shows the TRF-profiles from the FSCW in- and outflow. The FSCW shows a clear reduction of the bacterial diversity after the filter bed passage; from fifty-nine peaks in the inflow to thirty-three peaks in the outflow, whereas only twenty-eight peaks were identical within the FSCW in- and outflow. The intensive peak from 70 to 73 bp was detected in all outflow profiles (in highest intensities in the FSCW outflow) and also in the FSCW profiles derived from the outdoor filter bed samples (most abundant in the uppermost layer), but missing in the inflow samples. This fact suggests that this peak is originated from a soil-borne bacterium, which was washed out from the filter bed. Another reason could be that they may be insignificant members of the inflow but then find the CW an ideal habitat where they proliferate. Similarly, the peaks at 233 and 239 bp were more intensive in the outflow samples of both the planted and unplanted PSCWs but almost not detectable in the inflow (Fig. 1). This suggests that these peaks were also derived from soil bacteria rather



**Fig. 1 – Comparison of the inflow (T-RFLP profile pooled from two samples), the planted outflow (two samples) and unplanted outflow (one sample) TRF profiles from the indoor PSCW.**



**Fig. 2 – Comparison of the inflow (TRF profile pooled from two samples) and the outflow (one sample) TRF profiles from the outdoor FSCW.**

than from wastewater ones. In contrast, the most intensive peaks at 187 bp (<4000 FU) from the indoor inflow was not detected in neither the PSCW soil samples (data not shown) nor the outflow effluent samples (Fig. 1), which suggests that this peak represented a wastewater bacterium that was completely killed off in the CWs.

#### 4. Discussion

Community fingerprinting offers a useful tool to investigate functionally important microorganisms of an environmental habitat. Fingerprint techniques provide information on the diversity but with a resolution, which is surely not satisfactory to describe the full microbial diversity in complex habitats (Smalla et al., 2007). However the rather high detection sensitivity of the T-RFLP method has been demonstrated previously by Dunbar et al. (2000). T-RFLP analysis has the advantage of analytic consistency and a high throughput capability (Hartmann and Widmer, 2006).

Community profiles of the soil and wastewater samples obtained within this study, demonstrated a rather high bacterial diversity, which is typical for complex environmental habitats. A comparison between the bacterial diversity in the filter bed of the outdoor and the indoor systems revealed no clear differences, although the two systems are exposed to different temperatures, are planted with different helophytes, and treat municipal wastewater with different bacterial compositions (Figs. 1 and 2, inflow). Both systems are, however, very similar with respect to physiochemical factors such as pH, grain size distribution, as well as nutrient-, oxygen-, and water-content (unpublished data), which may have promoted the development of similar microbial communities. However, differences between the two rhizosphere soils (*P. australis* and *M. sinensis giganteus*) were found, and those were more clearly distinguishable than the differences between the rhizosphere and the bulk soil samples of the respective systems. This indicates an influence of the plant species on the rhizosphere bacteria, which has been frequently reported for soil systems (e.g. Smalla et al., 2001; Kowalchuk et al., 2002). Nevertheless, Zul et al. (2007) reported clear differences in the community composition in soils from lysimeters without plants, compared to populations in planted lysimeter soils, whereas no influence of plant species composition on bacterial diversity could be discerned.

For the bacterial diversity no clear correlation between the depth of the filter bed and the existence of distinct bacterial groups could be observed. Similarly, phylotype richness did not change with depth, with the exception of the 0–1 cm layer, which showed a reduced bacterial diversity. In a recent study, more than 50% of the microbial biomass and bacterial activity could be found in the first cm of the filter bed of the PSCWs and about 95% within the first 10 cm (Tietz et al., 2007b). This indicates that although the lower layers contain a lower biomass, they are probably composed of similar populations as the biomass in upper layers. In contrast to these results, Truu et al. (2005) found a higher bacterial diversity in the upper layers (0–10 cm) of a horizontal subsurface flow CW in comparison to the deeper layer of the filter bed (50–60 cm).

The filter bed of the CWs can be imagined as a sink for bacterial species, but additionally it can also be a source of bacteria. The number of bacteria in the wastewater is substantially reduced by the CW; bacterial removal rates range from 2.0 log units determined by total microscopic direct counts, up to 4.8 log units for *Enterococcus* (Sleytr et al., 2007). Nevertheless it seems that the diversity was not so strongly reduced; but this does not seem to be accompanied by a corresponding reduction in the bacterial community diversity. A clear reduction of the bacterial diversity between the in- and outflow was evident only in the FSCW (a planted outdoor system). Generally, the removal efficiency is considered to be a result of both chemical (e.g. adsorption), physical (e.g. filtration and sedimentation) and biological mechanisms. Examples of the latter are possible antimicrobial effect of root exudates, predation by nematodes and protists, lytic bacteria and viruses, retention in biofilms, and natural die-off (Vacca et al., 2005).

The study showed that CWs operated under similar conditions had communities with similar diversities. However, the diversity and composition of the rhizosphere communities seemed to be influenced by the plant species. While the microbial biomass generally decreases with depth of the bed, the results suggest that the microbial community composition show little variation with depth. Further research will give more precise information on the time dynamics of the microbial populations and the effect of different wastewater qualities. By analysing the sequence of the 16S rRNA genes, the most dominant species inhabiting the system can be identified, resulting in a more detailed description of the community structure. Modern techniques such as stable isotope probing could link phylogenetic assignment with metabolic activity

and give more information on the various microorganisms involved in wastewater purification.

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