Changes in the bacterial community structure in two-stage constructed wetlands with different plants for industrial wastewater treatment

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A B S T R A C T

This study focused on the diversity of bacterial communities from two series of two-stage constructed wetlands (CWs) treating tannery wastewater, under different hydraulic conditions. Series were separately planted with *Typha latifolia* and *Phragmites australis* in expanded clay aggregates and operated for 31 months. The effect of plant species, hydraulic loading and unit stage on bacterial communities was addressed through bacterial enumeration and denaturating gradient gel electrophoresis (DGGE). Diverse and distinct bacterial communities were found in each system unit, which was related in part to the type of plant and stage position (first or second unit in the series). Numerical analysis of DGGE profiles showed high diversity in each unit with an even distribution of species. No clear relation was established between the sample collection time, hydraulic loading applied and the bacterial diversity.

Isolates retrieved from plant roots and substrates of CWs were affiliated with γ-*Proteobacteria*, *Firmicutes*, α-*Proteobacteria*, *Sphingobacteria, Actinobacteria* and *Bacteroidetes*.

Both series were effective in removing organic matter from the inlet wastewater, however, based on batch degradation experiments it seems that biodegradation was limited by the recalcitrant properties of the wastewater.

Introduction

Tannery wastewater composition is complex due to the variety of chemicals used during leather production. Several studies regarding the composition and toxicity of tannery wastewater (Cotman et al., 2004), its effects on the development of different plant species (Calheiros et al., 2007, 2008a) and its effects on chemical and biological soil characteristics (Alvarez-Bernal et al., 2006), have provided an overview on the impacts of this type of wastewater on the environment and in treatment systems.

Industrial wastewaters that are able to be treated by biological means are good candidates to be addressed by constructed wetlands (CWs). The proper functioning of the wetland system is dependent on the interactions between plants, soil, wastewater characteristics, microorganisms and operational conditions (Aguilar et al., 2008). Several design features may affect the processes occurring in a constructed wetland (Kadlec et al., 2000); for instance, changes in design and loading regime may improve the oxidation in CWs (Brix and Schierup, 1990). It has been suggested that macrophyte species also affect the pollutant removal efficiency in CWs, although differences in performance associated with different plant species are difficult to demonstrate due to inherent variation between studies and monitoring practices (Brisson and Chazarenc, 2008). Plant roots and rhizomes are important for the microbial transformation processes and subsequently to wastewater purification process (Münch et al., 2007; Stottmeister et al., 2003). Microbial assemblages can be found as a biofilm on substrate and root surfaces (Gagnon et al., 2007). Many parameters affect biofilm structure, especially nutrient availability or other environmental conditions (Kierek-Pearson and Karatan, 2005).

Detailed knowledge about the microbial assemblages is needed to understand and explain the CWs functioning and thus the phytoremediation processes. Molecular tools, such as denaturating gradient gel electrophoresis (DGGE), may be applied in order to study the microbial community structure, composition and diversity on CWs systems (Ibekwe et al., 2003; Truu et al., 2005).

In this study, the effect of plant species, hydraulic loading and unit stage in the dynamics of microbial communities occurring in horizontal subsurface flow CWs was assessed by bacterial enumeration and DGGE of the 16S rRNA gene.

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Methods

Systems design

The experimental systems consisted of two series of two-stage CWs operating in horizontal subsurface flow mode, for tannery wastewater treatment. They were located after the primary treatment of a wastewater treatment plant, from a leather company in the North of Portugal (Fig. 1). The series were planted with *Typha latifolia* (UT series) and *Phragmites australis* (UP series) in expanded clay aggregates named Filtralite®MR3-8 (FMR) (from maxit – Argilas Expandidas, SA – Portugal). The surface area of each bed was 1.2 m^2 (length: 1.2 m and width: 1 m), the effective depth of the substrate was 0.60 m and the average depth of liquid in the bed was 0.55 m.

The systems were aligned to work in series and operated for 31 months under different hydraulic conditions and interruptions in feed. The overall hydraulic retention time in the two-stage system series, for each HLR was 7, 2 and 5 days (in order of application). The first units of *T. latifolia* (UT1) and *P. australis* (UP1) series had already been in operation for 17 months receiving tannery wastewater (Calheiros et al., 2007).

Briefly, for 2 months the first unit of each system was subject to a hydraulic loading rate (HLR) of 18 cm d^{-1}. By the third month, the systems were not fed during 24 days due to the shutdown of the production plant and a second period of operation occurred subsequently during 23 months under a HLR of 6 cm d^{-1}. During this time, the wastewater supply was stopped twice. By day 479, a mowing was made leaving around 10 cm of aboveground plant material. A third period of operation occurred during 6 months under a HLR of 8 cm d^{-1}, and the systems were not fed due to the shutdown of the production plant by the second month within that period.

Analytical methodology

Wastewater samples were collected from the inlet and outlet of the CW units (UT1, UT2, UP1 and UP2) simultaneously with the microbiological samples, and physico-chemical parameters were determined based on Standard Methods (APHA, 1998): chemical oxygen demand (COD; closed reflux, titrimetric method), biochemical oxygen demand (BOD_5; 5-day BOD test), total suspended solids (TSS; total solids dried at 103–105 °C method), Kjeldahl nitrogen (TKN; Kjeldahl method), nitrate nitrogen (NO_3^-–N; nitrate electrode method), ammonia nitrogen (NH_3–N; phenate method), total phosphorus (total P; manual digestion and flow injection analysis for total phosphorus) and pH. The sulphate determination (SO_4^{2-}; turbidimetric method) was done based on the method of the Association of Official Analytical Chemists (AOAC, 1995). The analyses were done immediately after sample collection, otherwise were properly stored according to APHA (1998). Dissolved oxygen (DO) and conductivity were registered with a WTW handheld multi-parameter instrument 340i at the inlet and outlet of the units.

Removal of COD in batch enrichment cultures

Enrichment cultures were carried out in 250 ml flasks containing: 60 ml of sterile minimal salts medium (Caldeira et al., 1999), effluent (75 ml) coming from the outlet of UT1 and UP1 units, and suspensions resulting from washing the substrate (15 ml) (used as inocula). For the inocula, substrate samples (15 g) were collected from the CWs and were added to sterile saline solution (0.85% w/v NaCl), and vortexed for 10 min. In assay A, inocula was obtained from the CW planted with *T. latifolia* (UT1), in assay B the inocula was originated from the CW planted with *P. australis* (UP1), while assay C was run with sterile saline solution instead of the inocula, under the same conditions. Cultures were incubated for 10 days on a rotary shaker (100 rpm) at 25 °C. Each assay was carried out in triplicate. Organic matter degradation was monitored by analyzing the COD. At the beginning and at the end of the experiment CFUs were determined based on the surface-plate counting procedure as described below.

Microbial counts

Colony forming units (CFUs) were determined based on the surface-plate counting procedure. Briefly, two sets of three subsamples were pooled to form one composite sample (10 g) of plant roots and substrate (from a depth between 10 and 15 cm) of each CW (UT1, UT2, UP1 and UP2), placed separately in sterile tubes with 10 ml of saline solution (0.85% w/v NaCl) and vortexed for 1 min at room temperature. Serial dilutions were made in duplicate and 0.1 ml of each dilution was spread onto nutrient agar (LABM, UK). Plates were incubated at 25 °C for 4 days after which CFUs were counted. The same procedure was used for bacterial enumeration of the wastewater at the inlet and outlet of the CWs.

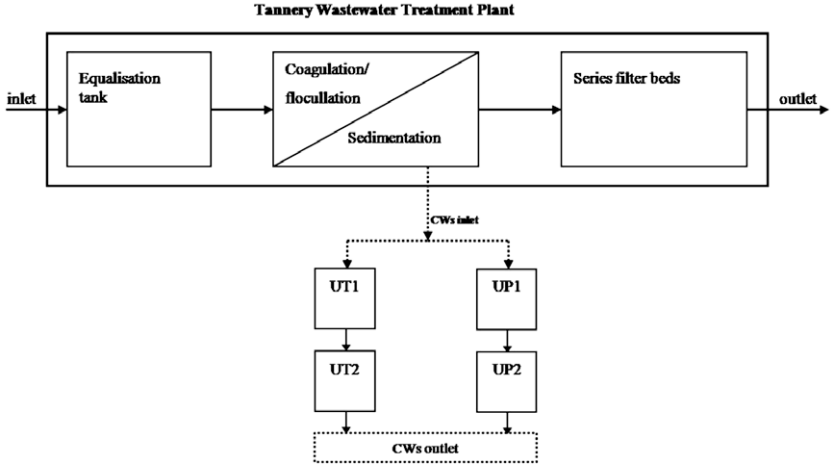

Fig. 1. Schematic representation of the constructed wetlands (CWs). UT1 and UT2: CW with *Typha latifolia* planted in Filtralite®MR 3-8, UP1 and UP2: CW with *Phragmites australis* planted in Filtralite®MR 3-8.

Isolation and identification of bacteria

Bacteria isolation and DNA extraction

Different bacterial colonies were isolated based on size, morphology and pigmentation, from nutrient agar plates using a streak-plate procedure. DNA of each isolate was obtained by picking a colony with a sterile toothpick, suspending the cells in 20 μL sterile water and incubating for 10 min at 100 °C (Henriques et al., 2006b).

Random amplified polymorphic DNA (RAPD)

Molecular typing of bacterial isolates was performed by RAPD analysis. Amplification was carried out in 25 μL reaction mixtures containing: 0.75 U *Taq* polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.0 μM primer M13 (MWG-Biotech AG) and 0.5 μL of crude cell lysates. The thermal cycling profile was as follows: initial denaturation (94 °C for 5 min); 45 cycles of denaturation (94 °C for 1 min), annealing (34 °C for 2 min), and extension (72 °C for 2 min); and a final extension (72 °C for 10 min) (Silva et al., 2006). The reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, California, USA) using *Taq* polymerase and nucleotides purchased from MBI Fermentas (Vilnius, Lithuania). Polymorphic DNA fragments were analyzed by electrophoresis in a 1% agarose gel in Tris–acetate–EDTA (TAE) buffer, after staining with ethidium bromide. Gel image was acquired using a molecular image FX apparatus (Bio-Rad Laboratories, Hercules, California, USA).

DNA sequencing analysis

Isolates displaying unique RAPD profiles were subsequently identified by 16S rRNA gene sequencing analysis. Amplification was performed with universal bacterial primers 27F and 1492R, as described (Lane, 1991). PCR products were purified with a Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany). DNA sequencing was conducted under BigDye™ terminator cycling conditions, using an automatic sequencer 3730xl (Macrogen Inc., Seoul, Korea). To determine the phylogenetic affiliation, similarity searches were performed using the BLAST program (Altschul et al., 1997).

Nucleotide sequence acession numbers

The 16S rRNA gene sequences of bacterial isolates used in this study are deposited in GenBank under the accession numbers PM1R1: EU430690, PM1R2: EU430691, PM1R3: EU430692, TM2R1: EU430698, TM2R3: EU430699. Additionally isolates TM1R1: EU430693, TM1R2: EU430694, TM1R3: EU430695, TM1S1: EU430696, TM1S2: EU430697 were also obtained from the same samples during a previously conducted study (Calheiros et al., 2008c) and included in the analysis.

Denaturating gradient gel electrophoresis of total community DNA

Total community DNA extraction from CWs

Genomic DNA from substrate and root samples of each unit (six subsamples were pooled to form composite samples of plant roots and of substrate for UT1, UT2, UP1 and UP2) and extracted using the Ultra Clean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA), according to the manufacturer's protocol.

Polymerase chain reaction

PCR amplification of bacterial 16S rRNA gene fragments was performed using primers 338F_GC and 518R (Henriques et al., 2006a). Nested PCR amplifications were performed using as template 1 μL of the DNA amplicon obtained after the first amplification round and using the same primers and conditions applied in the first PCR amplification.

Denaturating gradient gel electrophoresis

DGGE analysis was performed on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA). Samples containing approximately equal amounts of nested-PCR amplicons were loaded onto 8% (w/v) polyacrylamide gels (37.5:1, acrylamide/bis-acrylamide) in 1× TAE buffer using a denaturing gradient ranging from 35% to 60% (100% denaturant solution is defined as 7 M urea and 40% (v/v) formamide (Muyzer et al., 1993)). A standard marker was also included in all gels, to serve as an indicator of the analysis quality. The standard marker was constructed using bacterial isolates (obtained as described above) selected in order to cover an adequate range of bands. Electrophoresis conditions and image acquisition were as described previously (Henriques et al., 2006b).

DGGE data analysis

DGGE profiles, concerning the presence and intensity of the bands, were analyzed using GelCompar® II software (VERSION 4.6; Applied Maths, Sint-Martens-Latem, Belgium). Detected band patterns were transferred to an absence/presence matrix. The binary matrix was transformed into a similarity matrix using the Bray–Curtis measure. Dendrograms were generated by unweight pair group mean average (UPGMA) cluster analysis. Cluster analysis and non-metric multidimensional scaling (MDS) diagram construction were performed using PRIMER 5 for Windows (Version 5.2., 2001, PRIMER-E Ltd.) (Clarke and Gorley, 2001).

DGGE banding data were used to estimate diversity, H (Shannon and Weaver, 1963) and equitability, E (Pielou, 1975) indexes.

Data analysis

Statistical analysis were performed using the software SPSS (SPSS Inc., Chicago, IL, USA; Version 12.0). When applicable, the data were analyzed through one-way analysis of variance (ANOVA) and Student's t-test. To detect the statistical significance of differences ($p < 0.05$) between means of observation, the Duncan test was performed. When applicable, values were presented as the mean ± standard error.

Results

Physico-chemical data

In Table 1, the mean composition of the wastewater at the inlet and outlet of the CWs is presented. Data presented here correspond to the sampling points used for microbiological analysis. The conductivity and pH were similar for the outlet of all units. DO at the inlet and outlet of the units was low, varying between 0.2–1.9 mg L^{-1} and 0.1–0.9 mg L^{-1}, respectively. Total phosphorus concentration was similar for the inlet and outlet of the units. The COD and BOD_5 removal efficiency of the first units (UT1 and UP1) varied between 41–80% and 29–65%, respectively. For the second units (UT2 and UP2) COD and BOD_5 removal varied between 28–67% and 22–62%, respectively. Removal of TSS varied between 50% and 77% for the first units and between 52% and 78% for the second units. The TKN and NH_3 removal efficiency of the first units varied between 25–44% and 21–51% respectively. For the same parameters, removal for the second units varied between 30–42% and 15–32%, respectively. SO_4^{2-} removal varied between 12% and 56% for the first units and between 7% and 54% for the second units.

Based on these data, no significant differences were found regarding the removal of COD, CBO_5, TSS, TKN, NH_3 and SO_4^{2-} between UT1 and UP1 (first units) and between UT2 and UP2 (second units). The organic loading at the first units (UT1 and UP1) ranged

Table 1
Mean composition of the water at the inlet and outlet of the constructed wetlands (CWs). UT1 and UT2: CW with *Typha latifolia* planted in Filtralite®MR 3-8, UP1 and UP2: CW with *Phragmites australis* planted in Filtralite®MR 3-8.

Parameters	Inlet	UT1	UT2	UP1	UP2
pH	6.24 ± 0.37	8.08 ± 0.05	8.12 ± 0.07	7.79 ± 0.25	7.95 ± 0.20
Conductivity (mS cm^{-1})	7.47 ± 0.64	7.81 ± 0.47	6.85 ± 0.53	7.34 ± 0.49	6.97 ± 0.50
DO (mg O_2 L^{-1})	0.8 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
COD (mg O_2 L^{-1})	1758 ± 138	672 ± 69	315 ± 39	712 ± 83	339 ± 36
BOD_5 (mg L^{-1})	712 ± 38	346 ± 25	208 ± 26	361 ± 28	207 ± 25
TSS (mg L^{-1})	88 ± 7	29 ± 3	9 ± 1	30 ± 3	10 ± 1
TKN (mg TKN-N L^{-1})	136 ± 6	89 ± 5	58 ± 4	88 ± 4	55 ± 3
NH_3 (mg NH_3–N L^{-1})	79 ± 4	50 ± 3	39 ± 2	52 ± 4	40 ± 3
NO_3^- (mg NO_3^-–N L^{-1})	43 ± 5	30 ± 3	21 ± 2	30 ± 3	23 ± 3
Total P (mg L^{-1})	0.30 ± 0.08	0.24 ± 0.07	0.20 ± 0.06	0.23 ± 0.07	0.20 ± 0.07
SO_4^{2-} (mg SO_4^{2-} L^{-1}	265 ± 103	187 ± 91	151 ± 86	190 ± 96	154 ± 90

The values are means ± SE.

between 769 and 2500 kg COD ha^{-1} d^{-1} and between 324 and 1296 kg BOD_5 ha^{-1} d^{-1}. For the first units organics removal up to 1213 kg COD ha^{-1} d^{-1} and 419 kg CBO_5 ha^{-1} d^{-1} were achieved. For the second units organics removal up to 562 kg COD ha^{-1} d^{-1} and 283 kg CBO_5 ha^{-1} d^{-1} were achieved, however organic loadings were lower, ranging between 173 and 1332 kg ha^{-1} d^{-1} for COD and between 127 and 918 kg ha^{-1} d^{-1} for CBO_5.

Enrichment cultures

Organic matter degradation in batch cultures with inocula obtained from the two CWs (UT1 and UP1) substrate is shown in Fig. 2. COD decreased from around 500 to nearly 200 mg L^{-1} in 2 days. In the experiment using as inoculum just the wastewater from CW outlet, the COD reduction was slightly lower. The average CFU ml^{-1}, at the beginning of the experiment, for each assay was: $4.4 \times 10^4 \pm 3.2 \times 10^3$ for A (with UT1 inocula), $3.4 \times 10^4 \pm 5.0 \times 10^3$ for B (with UP1 inocula) and $1.7 \times 10^4 \pm 2.3 \times 10^3$ for C (without inoculum). At the end of the experiment the CFU ml^{-1}, for each assay, was: $2.8 \times 10^7 \pm 4.2 \times 10^6$ for A, $5.1 \times 10^7 \pm 3.8 \times 10^6$ for B and $2.4 \times 10^6 \pm 5.2 \times 10^5$ for C.

Bacterial counts and isolation

Monitoring of bacterial counts in the wetlands was undertaken from July 2004 to October 2006. At the inlet of units UT1 and UP1, the average CFU was $7.2 \times 10^4 \pm 4.8 \times 10^4$ CFU ml^{-1}. For the outlet of the units counts were, in average, $4.2 \times 10^5 \pm 2.2 \times 10^5$ CFU ml^{-1} for UT1, $1.3 \times 10^6 \pm 6.3 \times 10^5$ CFU ml^{-1} for UT2, $5.1 \times 10^5 \pm 2.6 \times 10^5$ CFU ml^{-1} for UP1 and $5.1 \times 10^5 \pm 2.5 \times 10^5$ CFU ml^{-1} for UP2. ANOVA one-way was performed to compare the counts in terms of CFU ml^{-1} and significant differences between the wastewater inlet and the outlet of all CWs were found.

Bacterial counts from the root and substrate samples of each unit are shown in Fig. 3. The CFU varied between 2.0×10^6 and 2.7×10^8 CFU g^{-1} in UT1 and between 1.2×10^6 and 6.0×10^7 CFU g^{-1} in UT2. Counts in UP1 varied between 1.3×10^7 and 4.4×10^8 CFU g^{-1} and in UP2 varied between 2.0×10^6 and 3.2×10^8 CFU g^{-1}. When Student *t*-test was applied to compare the CFUs from the root and substrate of each unit independently, no significant differences were seen in UT1 and UP1, although for UT2 and UP2 counts in the root were significantly higher than in

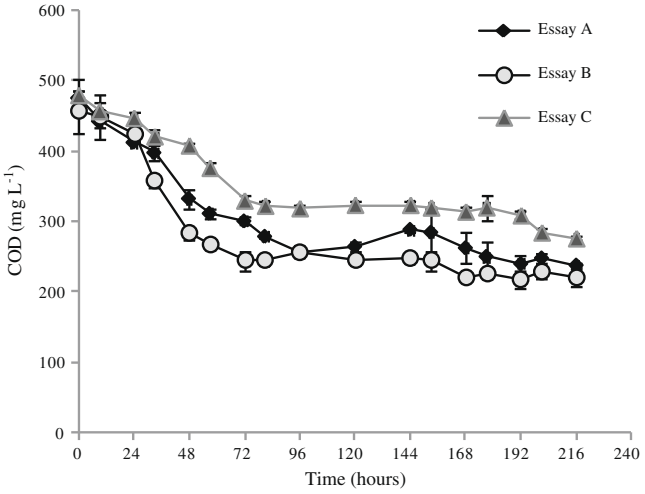

Fig. 2. Biodegradation of organic matter from tannery wastewater during a batch culture. (A) with inocula from a constructed wetland with *Typha latifolia* plus outflow wastewater; (B) with inocula from a constructed wetland with *Phragmites australis* plus outflow wastewater; (C) with outflow wastewater as inocula. Dispersion bars represent standard error of the mean.

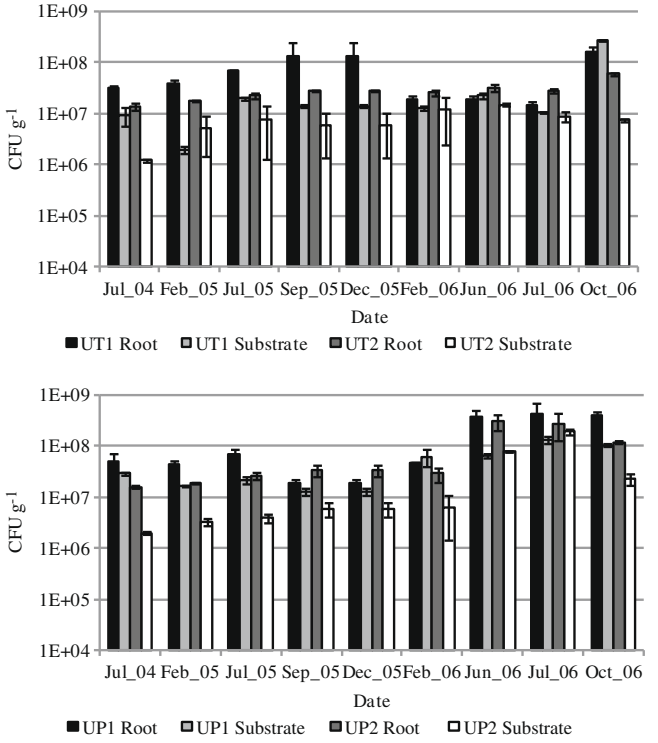

Fig. 3. Bacterial enumeration analyzed by plate counts and expressed in CFUs g^{-1} for substrate and plant roots of constructed wetlands (CWs). Dispersion bars represent standard error of the mean. UT1 and UT2: CW with *Typha latifolia* planted in Filtralite®MR 3-8, UP1 and UP2: CW with *Phragmites australis* planted in Filtralite®MR 3-8.

Table 2
Phylogenetic affiliation of bacterial strains isolated from the constructed wetlands.

Isolate	NCBI accession no.	Phylogenetic affiliation	Closest relative (accession no.)	Similarity (%)	Isolation source
PM1R1	EU430690	γ-*Proteobacteria*	*Halomonas venusta* (AY870665)	99	Galician oyster
			Halomonas sp. *Sa1 3-1I* (AB305227)	99	Saline sand from Cachuma lake
PM1R2	EU430691	*Sphingobacteria*	*Cytophaga* sp. MBIC01539 (AB086623)	97	Green algae
			Cyclobacterium sp. V4.MS.32 (AJ244689)	97	Meso-eutrophic environment
PM1R3	EU430692	γ-*Proteobacteria*	*Providencia alcalifaciens* NCTC 10286 (DQ885261)	100	nd
			Providencia sp. H2-3 (EF061136)	99	nd
TM1R1	EU430693	γ-*Proteobacteria*	*Pseudomonas stutzeri* ST27MN3 (U26419)	99	Sea
			Pseudomonas stutzeri 19smn4 (U22426)	99	Sea
TM1R2	EU430694	α-*Proteobacteria*	*Paracoccus* sp. G30 (DQ667122)	98	Antarctic seawater
			Paracoccus sp. G29 (DQ667121)	98	Antarctic seawater
TM1R3	EU430695	*Firmicutes*	*Bacillus pumilus* MLA14 (EF462914)	99	nd
			Bacillus sp. NS88 (EF633174)	99	*Phyllostachys edulis*
TM1S1	EU430696	*Firmicutes*	*Bacillus* sp. BCw063 (DQ492812)	99	Arctic sea water
			Bacillus sp. GB02-29 (DQ079002)	99	Hydrothermal sediments and plumes
TM1S2	EU430697	γ-*Proteobacteria*	*Halomonas* sp. 3014 (AM1 10971)	99	Deep sea sediment
			Halomonas sp. A3-3 (AB219125)	99	Fermented foods
TM2R1	EU430698	*Actinobacteria*	*Microbacterium* sp. 4_C16_10 (EF540500)	99	Waste from oil-shale chemical industry
			Microbacterium sp. SP-1 (DQ898301)	99	Plant
TM2R3	EU430699	*Bacteroidetes*	Flavobacteriaceae bacterium D1 1-24b1 (AM403225)	96	Marine aquaculture biofilter
			Bacterium RC-III-71 (AJ252729)	95	Potato plant root

the substrate. When comparing root and substrate's CFU from UT and UP series through Student *t*-test application, no significant differences were seen.

Genotyping of the dominant bacterial was done by RAPD-PCR, and isolates displaying distinct RAPD profiles were characterized through the determination of the nucleotide sequence of the 16S rRNA encoding gene. According to BLAST results (Table 2), four strains were affiliated with γ-*Proteobacteria*, two with *Firmicutes*, one with α-*Proteobacteria*, one with *Sphingobacteria*, one with *Actinobacteria* and one with *Bacteroidetes*. These strains were isolated from plant roots (TM1R1, TM1R2 and TM1R3) and substrate (TM1S1 and TM1S2) of UT1 and from roots of UT2 (TM2R1 and TM2R3) and UP1 (PM1R1, PM1R2 and PM1R3). Most of them shared 99% similarity with sequences deposited in GenBank.

Molecular analysis of bacterial communities within the systems

The molecular analysis was conducted on samples from root and substrate of the two-stage series of wetlands, from July 2005 to October 2006. During this time the CWs operated under different hydraulic conditions.

Comparisons between the different profiles revealed several differences in position, number and intensity of the bands along the sampling for each unit (Table 3). The total number of different band positions in the gel was 47 (UT1), 48 (UT2), 64 (UP1) and 34 (UP2). The number of DGGE bands per lane varied between 21 (substrate sample from October 2006) and 31 (root sample of July 2006) for UT1, and between 18 (root sample from September 2005) and 26 (substrate sample from July and root sample from October 2006) for UT2. The number of DGGE bands per lane varied between 21 (substrate sample from October 2006) and 41 (root sample from September 2005) for UP1 and for UP2 varied between 16 (root sample from September 2005 and February 2006) and 23 (substrate sample from June 2006). On average, the Shannon's diversity index (*H*) was in average of 1.20 ± 0.03 for UT1, 1.15 ± 0.04 for UT2, 1.31 ± 0.03 for UP1 and 1.10 ± 0.02 for UP2. The equitability index (*E*) was 0.84 ± 0.02 for UT1, 0.85 ± 0.02 for UT2, 0.87 ± 0.01 for UP1 and 0.86 ± 0.01 for UP2.

In order to assess the relatedness of phylogenetic profiles corresponding to the communities of each CW unit stage, at each sampling time and location (substrate and root), a cluster analysis was performed. The samples from each unit clustered together (Fig. 4) but the similarity of the communities between units was less than 60%. The type of plant within each unit and the stage of the units (first or second) had an evident effect on the bacterial communities while the sampling time did not. Some samples from UT1 (July and September 2005, February 2006), UT2 (September 2005, June and July 2006), UP1 (July 2005) and UP2 (September 2005, February and June 2006) clustered according to the location of collection (substrate and root), having, in general, similarity higher than 80% (except UP2 February 2006).

The MDS presented in Fig. 5 allows visualizing how the samples are plotted together providing an insight in the level of dispersion between them, facilitating the interpretation of the results previously addressed in the dendrograms. The difference between the bacterial assemblages of each unit is evident, reinforcing the effect of type of plant and stage of the units (first or second).

Table 3
Shannon diversity (*H*) and equitability (*E*) indexes, calculated for the two-stage constructed wetlands (CWs). UT1 and UT2: CW with *Typha latifolia* planted in Filtralite®MR 3-8, UP 1 and UP2: CW with *Phragmites australis* planted in Filtralite®MR 3-8.

Samples[a]	UT1		UT2		UP1		UP2	
	H	*E*	*H*	*E*	*H*	*E*	*H*	*E*
Jul_R_05	1.10	0.75	nd	nd	1.37	0.86	nd	nd
Jul_S_05	1.26	0.88	nd	nd	1.37	0.89	nd	nd
Sep_R_05	1.12	0.76	1.03	0.82	1.38	0.85	0.94	0.78
Sep_S_05	1.01	0.72	0.95	0.74	1.42	0.95	1.07	0.85
Feb_R_06	1.26	0.86	nd	nd	1.46	0.92	1.00	0.83
Feb_S_06	1.23	0.88	nd	nd	1.30	0.84	1.09	0.87
Jun_R_06	1.30	0.90	1.10	0.84	1.35	0.87	1.16	0.88
Jun_S_06	1.21	0.88	1.19	0.88	1.30	0.90	1.11	0.82
Jul_R_06	1.35	0.91	1.30	0.93	1.31	0.85	1.13	0.90
Jul_S_06	1.07	0.76	1.21	0.85	1.17	0.83	1.17	0.90
Oct_R_06	1.34	0.91	1.20	0.85	1.16	0.86	1.15	0.87
Oct_S_06	1.16	0.87	1.25	0.90	1.14	0.86	1.13	0.87

nd: not determined.
[a] In samples, column is presented the month, location (R: root or S: substrate) and year, that each sample was collected.

Discussion

Wastewater characterization and organic matter removal

Tannery wastewater characterization is of major importance since raw and treated wastewaters have different toxic impacts

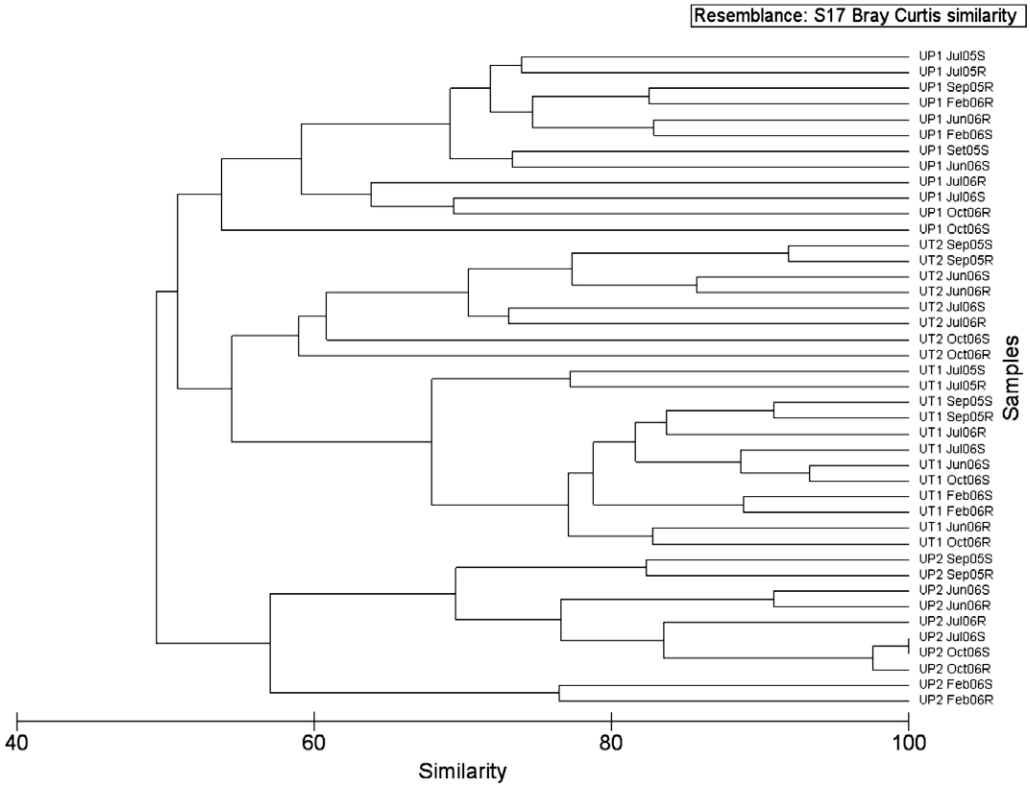

Fig. 4. Cluster analysis of DGGE patterns of root (R) and substrate (S) samples taken in different months, from UT1, UT2 (units with *Typha latifolia* planted in Filtralite®MR 3-8), UP1 and UP2 (units with *Phragmites australis* planted in Filtralite®MR 3-8). Similarities were calculated using the Bray–Curtis measure.

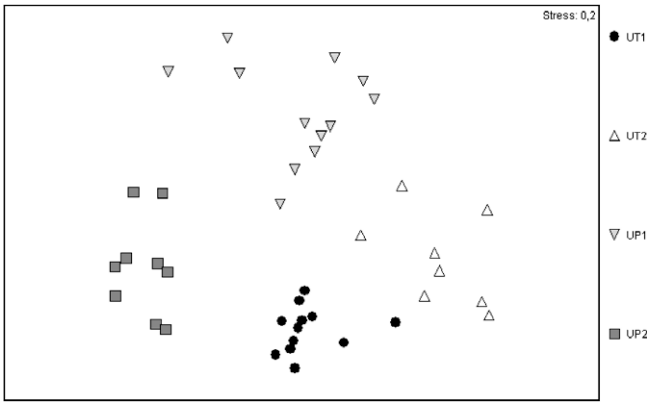

Fig. 5. Multidimensional scaling diagram of DGGE patterns of samples collected in the constructed wetland units (CWs) of UT1, UT2, UP1 and UP2. UT1 and UT2: CWs with *Typha latifolia* planted in Filtralite®MR 3-8, UP1 and UP2: CWs with *Phragmites australis* planted in Filtralite®MR 3-8.

on vegetation and microorganisms (Cotman et al., 2004; Alvarez-Bernal et al., 2006; Calheiros et al., 2008a). Considering wastewater treatment with CWs, Collins et al. (2004) have concluded that water quality is affected by plants and this effect resulted in part from their influence on bacterial assemblages. Plants may also stabilize microbial communities within these systems (Weber et al., 2008). Ibekwe et al. (2003) have suggested that processes such as nitrification and denitrification may be more efficient if a diverse microbial community is present.

Studies with single stage CWs for tannery wastewater treatment had already been undertaken (Calheiros et al., 2007, 2008b) although the implementation of two-stage systems, as was used in this study, promotes higher pollutant removal rates (Calheiros et al., unpublished).

The low DO concentration (<0.85 mg L^{-1}) at the systems outlet suggest that there were reducing conditions in the rhizosphere, although this fact provides little information about the processes occurring in the bed (Vymazal and Kröpfelová, 2008). Vymazal and Kröpfelová (2008) have reported that the DO at the inflow of horizontal subsurface flow is a negligible portion of the amount used in the bed, but the release of oxygen from the plant internal root zones in the rhizosphere is important (Stottmeister et al., 2003). Thus, several wetland plants have shown to enhance root zone aeration (Brix and Schierup, 1990; Sorrell et al., 2000). In this study, the wastewater entering in the CWs units contained high amounts of organic matter that varied according to the changes in the hydraulic loadings. The organic loadings were much higher than those suggested in the literature for subsurface flow systems (USEPA, 2000) and this fact may probably have had consequences in the bacterial communities. The observed reduction in BOD_5, COD, TSS, total phosphorus, TKN, NH_3 and SO_4^{2-} is related to the treatment mechanisms occurring in CWs that comprise several physical, chemical, and biological processes.

Biodegradation of the tannery wastewater was tested in batch assays using as inocula material obtained from the CWs. Organic matter removal in assays using as inocula bacterial suspensions detached from the substrates was higher (30–38%) in the first 48 h when compared to removal in assays supplied with only wastewater from the outlet of the units, which contained microbial populations naturally detaching from the beds (15%). This result indicates that microbial communities within the systems (present in the substrate) should play an active role in the organic matter degradation. After 48 h, degradation rate was reduced probably due to nutrient scarcity or to the fact that the remaining organic matter was recalcitrant.

Bacteria quantification and isolation

The CFU calculation provided quantitative data in relation to the units in series and along systems operation. The variation of CFUs for root and substrate samples was in the range of values found in other reports (Truu et al., 2005) concerning subsurface flow wetlands. Truu et al. (2005) have compared the number of cultivable heterotrophic bacteria between two sampling depths of a horizontal subsurface flow CW and no significant differences have been found. The fact that there were no significant differences in bacterial numbers between the substrate and the roots in units UT1 and UP1 might be attributed to the diffuse propagation of *T. latifolia* and *P. australis*, respectively, since these units were established for longer than units UT2 and UP2. Gagnon et al. (2007) have reported higher microbial aerobic respiration on root surface that on the substrate, suggesting an influence of root oxygen release on the microbial population. The authors have shown that microbial density and activity are enhanced by the presence of plants and may differ according to plant species. Hatano et al. (1993) have reported that plants have a significant effect on the microbial populations when studying decomposition activity of gravel filled wetlands. Average CFUs values of $8.6 \times 10^5\ g^{-1}$ for a *Typha* unit and $4.6 \times 10^6\ g^{-1}$ for a *Phragmites* unit were registered and, regarding the time of sampling, little effect on the bacteria populations were seen for the *Typha* unit in contrast with the *Phragmites* unit that exhibited a significant increase between June and October samplings. Vymazal et al. (2001) have reported that there are significantly more bacteria on *P. australis* roots when compared to the roots of *Phalaries arundinacea*. In this study, no significant differences were seen when comparing CFUs of *Typha* and *Phragmites* roots and the substrate.

The bacterial isolates retrieved from the roots and substrate samples of the CWs were closely related to environmental isolates reported from sources such as sediments, sand, plant, roots and biofilters. They were affiliated with γ-*Proteobacteria*, *Firmicutes*, α-*Proteobacteria*, *Sphingobacteria*, *Actinobacteria* and *Bacteroidetes*. Lefebvre et al. (2006) have studied the microbial diversity in hypersaline tannery wastewater and reporting a high diversity in this type of environment being several domains present such as *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. Aguilar et al. (2008) have reported bacteria isolates showing similarities to α, γ and β-*Proteobacteria* subgroups, some particularly closely related with *Pseudomonas stutzeri*, and affiliated with the *Actinobacter* sp., when doing a study on identification and characterization on sulphur-oxidizing bacteria in a wetland treating tannery wastewater.

Dynamics of the microbial community

Mathematical indexes were used to follow changes in the bacterial communities. The diversity and equitability indexes calculated based on DGGE profiles were similar for all the wetland units, suggesting that despite the high diversity, the distribution of species is even. Li et al. (2008) have used the Shannon index in order to indicate the ecological diversity and pollution level in horizontal subsurface flow CWs, and have concluded that in most cases the *H* value was above 1.0. In this study, the *H* values were in general above 1.0 and results from CWs outlet showed that the wastewater depuration is relevant and notorious. The diversity of microorganisms may be critical for optimal wastewater treatment (Ibekwe et al., 2003).

The distinctive assemblages corresponding to each unit might be influenced by the morphology and development of the plant species and by the different loadings applied to the first (UT1 and UP1) and second units (UT2 and UP2). Gagnon et al. (2007) have suggested that the first two characteristics are key factors influencing the microcosm. Münch et al. (2007) have also related the root surface microbial colonization of *Gliceria maxima* with the age of plant roots. On the other hand, Truu et al. (2005) have compared the structure of the bacterial community of CWs using PCR-DGGE and have reported that there is a higher diversity in the upper than in the deeper layer of the CW.

Several studies suggest that plants have a significant effect on the microbial populations (Li et al., 2008; Gagnon et al., 2007; Truu et al., 2005; Collins et al., 2004; Hatano et al., 1993). Weber et al. (2008) have assessed changes in the microbial community in CWs exposed to acid mine drainage and have suggested that microbial ecology in planted wetlands may be more robust and buffered against significant shifts in microbial composition in response to various disturbances. The studies of Baptista et al. (2008) on the microbial diversity on laboratory-scale wetlands have indicated, however, that plants do not appear to have a strong effect on the abundance or diversity of the heterotrophs, sulphate-reducing bacteria and Archaea, since neither the quantity nor the community structure of these groups appears to be statistically distinguishable. Hatano et al. (1993) have reported, in the study of three subsurface flow CWs, that besides the presence of bacteria in the systems, relatively small fungal populations are also present.

Cluster analysis showed that samples from the same unit clustered together and the similarity between those samples was higher than with samples from the other units, despite the fact that within each cluster the sample dispersion was notorious. Also, there was no clear relation between the sample collection time and the hydraulic loading applied but the spatial factor (unit type and stage) was crucial.

Conclusions

In this study, the microbial dynamics of root and substrate from two-stage series of CWs with horizontal subsurface flow, treating tannery wastewater, was investigated. The application of molecular tools, namely DGGE allowed deepening the research in this field since very few studies were undertaken with the specificity of this type of industrial application. The main conclusions of this investigation are summarized as follows.

- The clustering analysis suggested that a diverse and distinct bacterial community inhabited each constructed wetland, although an even distribution of species was observed within each unit.
- The type of plant and the stage position (first or second unit in the series) seemed to have a major effect on the dynamics of bacterial communities.
- The different hydraulic loadings corresponding to organic matter increases in the systems did not result in evident changes in the microbial communities.
- The interaction between plant roots, microorganisms and substrate, along operation time, might have contributed to the establishment of diverse assemblages.
- Organic matter biodegradation might be limited by the recalcitrant properties of the wastewater.

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