Substrate effect on bacterial communities from constructed wetlands planted with Typha latifolia treating industrial wastewater


Keywords: Bacterial communities, Constructed wetland, DGGE, Typha latifolia, Industrial wastewater

Abstract

Constructed wetlands (CWs) have been recognized as being able to effectively treat wastewater from municipal and industrial sources. This study focused on the effect of different substrates and long-term operation of horizontal subsurface flow CWs treating tannery wastewater on the bacterial communities. The CWs were planted with Typha latifolia in three types of substrate: two units with different types of expanded clay aggregates and one unit with fine gravel. Another unit with expanded clay was left unvegetated. Changes in the bacterial community related to the type of substrate, different hydraulic loading rates and along CW operation were examined using denaturating gradient gel electrophoresis (DGGE). Bacterial enumeration was also performed and several bacterial isolates were retrieved from the CWs. Phylogenetic affiliations of those isolates were obtained on the basis of 16S rRNA gene sequences and revealed that they were closely related to the genera Bacillus (TM1S1, TM1R3, TNR1 and TAR1), Paracoccus (TM1R2), Pseudomonas (TM1R1) and Halomonas (TM1S2).

The type of substrate and the presence of T. latifolia had a major effect on the species richness and the structure of bacterial communities as inferred by numerical analysis of DGGE profiles.

Introduction

The tannery industry converts rawhide or skin, a putrescible material, into leather, a stable material, so that it can be used in the manufacture of a wide range of consumer products. Most of the steps of the tannery operations are carried out in water. Consequently, the wastewater treatment is of major concern. Tannery wastewater composition varies considerably with the production process that is engaged by the specification of the final product. Problems related to high organic content, and the presence of sulphides and chromium are often encountered imposing treatment needs in order to avoid negative impacts on the environment (COTANCE, 2002).

Constructed wetlands (CWs) are an effective method for wastewater treatment (Vymazal, 2005), inclusively for several types of industrial wastewater (Kadlec et al., 2000). In the case of the tannery industry these systems have already shown favorable performance (Calheiros et al., 2007, 2008a). The treatment mechanisms in CWs encompass a mix of physical, chemical, and biological processes. The vegetation is essential in wetland treatment systems (Kadlec et al., 2000); however, the main role in the transformation and mineralization of
of the systems was carried out according to Calheiros et al. (2008a).

Wastewater samples were collected periodically from the inlet and outlet of the CW units and physico-chemical parameters were determined based on Standard Methods (APHA, 1998): chemical oxygen demand (COD; Closed Reflux, Titrimetric Method), biochemical oxygen demand (BOD5; 5-day BOD Test), total suspended solids (total suspended solids, TSS; dried at 103–105 °C method), Kjeldahl nitrogen (TKN; Kjeldahl method), nitrate nitrogen (NO3−–N; nitrate electrode method), ammonia nitrogen (NH3–N; phenate method), total phosphorus (total P; manual digestion and flow injection analysis for total phosphorus) and pH. The sulphate determination (SO42−; 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 samples were properly stored. Dissolved oxygen (DO) and conductivity were registered with a WTW handheld multi-parameter instrument 340i at the inlet and outlet of the units. The substrates used in the units were analyzed for organic matter content, based on Houba et al. (1995).

**Bacterial enumeration**

Microbiological analyses were performed simultaneously with physico-chemical analysis. Colony forming units (CFUs) were determined based on the surface-plate counting procedure. Briefly, three subsamples were pooled to form two composite samples (10 g) of plant roots and substrate (from a depth between 10 and 15 cm) of each CW, being placed separately in sterile tubes with 10 mL of saline solution (0.15 mol L−1 NaCl) and shaken on a vortex mixer 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 CFU were counted. The same procedure was used for bacterial enumeration of the wastewater at the inlet and outlet of the CWs.

**Methods**

**CWs set-up and physico-chemical analysis**

The setup conditions of the CWs, located at a tannery wastewater treatment plant in Portugal, are described in Calheiros et al. (2008a). Briefly, the CWs consisted of three units planted with Typha latifolia in different substrates: unit one (U1) and unit three (U3) had expanded clay aggregates being respectively Filtralite®MR 3–8 (FMR) and Filtralite®NR 3–8 (FNR) (from maxit, Argilas Expandidas, SA, Portugal), and unit two (U2) had fine gravel: AGH 4–8 (FG) (from Areipor-Areias Portuguesas, Lda, Portugal). An unvegetated unit (Uc), with the expanded clay aggregate FMR as substrate, was also included (Fig. 1). The CWs operated with horizontal subsurface flow and had a surface area of 1.2 m2. They received tannery wastewater after primary treatment under different hydraulic loading rates. The two units with FMR, U1 and Uc, had been in operation for 17 months during which two hydraulic regimes, 3 and 6 cm d−1, were tested (Calheiros et al., 2007). The units U2 and U3 had an acclimation period of 3 weeks before the tannery wastewater was applied. After that, all the systems were operated for 31 months under different hydraulic loadings and interruptions in feed (18, 6 and 8 cm d−1). Maintenance
Bacteria isolation, DNA extraction and RAPD typing

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). Molecular typing of bacterial isolates was performed by random amplified polymorphic DNA (RAPD) analysis. Amplification was performed in 25 μL reaction mixtures containing: 0.75 U Taq polymerase, 1.5 mM MgCl₂, 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, CA, 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, CA, USA).

DNA sequence and phylogenetic 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 by Lane (1991). PCR products were purified with Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany). DNA sequencing was conducted under BigDye™ terminator cycling conditions, and analyzed 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).

Analysis of bacterial communities of substrate and roots from CWs

Genomic DNA from substrate and root samples (six subsamples were pooled to form one composite sample of plant roots and substrate for each CW) was extracted using the Ultra Clean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA), according to the manufacturer’s protocol. PCR amplification of bacterial 16S rRNA gene fragments was performed using primers 338F,GC and 518R, as described previously (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. DGGE analysis was performed on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, 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 to cover an adequate range of bands. Electrophoresis conditions and image acquisition were as described previously (Henriques et al., 2006b).

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 unweighted pair group mean average (UPGMA) cluster analysis. Cluster analysis and multidimensional scaling (MDS) 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 to describe possible changes in the dominance among phylotypes (Fromin et al., 2002).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of bacterial isolates obtained in this study were deposited in GenBank under the accession numbers TM1R1: EU430693, TM1R2: EU430694, TM1R3: EU430695, TM1S1: EU430696, TM1S2: EU430697, TNR1: EU430700, TAR1: EU430701.

Data analysis

Statistical analysis was 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 characterization

In Fig. 2 the removal of several wastewater components by the CWs is illustrated. COD, BOD₅ and TSS inlet concentration varied between 835–2261, 430–850 and 43–110 mg L⁻¹, respectively. In general, the FMR unit U1 presented higher removal levels of organic matter, followed by the FNR unit U3, the fine gravel unit U2 and the FMR unvegetated unit Uc.

The inlet concentrations of TKN, NH₃⁻N and SO₄²⁻ ranged between 102–160, 60–98 and 78–1070 mg L⁻¹, respectively. For NO₃⁻, the inlet varied between 17–59 mg L⁻¹ and the outlet between 9–47 mg L⁻¹. Total phosphorus ranged between

null
U3, although for U1 no significant differences were found. When Student’s t-test was applied to compare the CFUs between the planted units and no significant differences were found. One-way ANOVA was applied to compare bacterial counts from roots and substrate, is shown in Fig. 3. One-way ANOVA was applied in order to compare the diversity of bacterial communities within each unit. The microbial community structure of the planted units was significantly different from that of the control. The bacterial community composition shifted depending on the sampling period and sampling position. The number of aerobic bacteria present in the wastewater at the inlet and outlet of the CWs was also determined. Samples collected periodically (February, July, September, December 2005 and February, June, October 2006) showed a variation between $3.9 \times 10^3$ and $4.2 \times 10^5$ for the inlet, between $3.2 \times 10^3$ and $2.2 \times 10^6$ for the outlet of the vegetated units (U1, U2 and U3) and between $2.6 \times 10^4$ and $1.2 \times 10^6$ for the outlet of the unvegetated unit (Uc).

Dominant bacterial isolates obtained from the plates were further molecular typed by RAPD-PCR. Seven different RAPD types were then characterized through sequencing of the 16S rRNA encoding gene. According to BLAST results, 2 strains were affiliated with γ-Proteobacteria, 4 with Firmicutes and 1 with α-Proteobacteria. These strains were isolated from plant roots (TM1R1, TM1R2, and TM1R3) and substrate (TM1S1 and TM1S2) of unit U1 and from plant roots of unit U2 (TAR1) and unit U3 (TNR1) (Table 1).

**Fig. 4** shows clustering analysis of DGGE banding patterns. In general, samples clustered mostly according to the place of collection (substrate or root) and the type of substrate, being the temporal factor less relevant in bacterial assemblage composition. Multidimensional scaling diagrams of similarity matrices (Fig. 6), calculated from the DGGE patterns of samples, showed that the bacterial community changed according to the different substrates. Concerning the analysis between the FMR units U1 and the unvegetated control (Uc), differences between community profiles within each unit are clear, with a higher dispersion for the vegetated unit U1.

**Discussion**

This study intended to investigate the bacterial communities in CWs established with different substrates, namely expanded clay aggregates and fine gravel. Close attention was given to bacteria, although it is known that fungi may also play an important role in the function of wetlands (Baptista, 2003).
The complex physico-chemical composition of the tannery wastewater passing through the wetland is of major importance since it can have a relevant effect on the vegetation (Calheiros et al., 2007, 2008a,b) and in the bacterial communities that inhabit these ecosystems. The wastewater content varies in terms of nutrients and toxic substances and can limit or promote bacterial activity and growth. The main role concerning the direct degradation of organic chemicals in wastewater treatment is played by microorganisms despite the capacity of plants to detoxify xenobiotics (Stottmeister et al., 2003). The organic compounds degradation in the horizontal subsurface flow wetlands is carried out aerobically and anaerobically at different extents by bacteria attached to plant roots and substrate surfaces (Kadlec et al., 2000).

The three tested substrates have proven to be adequate for T. latifolia development although there was higher plant propagation for the expanded clay aggregates (Calheiros et al., 2008a). The macrophytes are important in the wetlands since they provide structure and a source of reduced carbon for the microbes that mediate most of the pollutant transformations occurring in the wetlands (Kadlec et al., 2000). Collins et al. (2004) concluded that plants do have an effect on water quality, in part because they affect bacterial assemblages. Higher pollutant removals, in terms of COD and BOD$_5$, were achieved in expanded clay planted units after long-term operation. The similar behavior of the expanded clay systems (U1 and U3) concerning the pollutant removal may be attributed to the fact that they may have similar functional group of microorganisms.

The substrate is an important wetland component since it supports plant growth, establishment of microbial biofilms and influences the hydraulic processes (Stottmeister et al., 2003).
Fig. 4 – DGGE analysis of 16 rRNA gene fragments of total bacterial population from samples of the root and substrate of constructed wetlands planted with *T. latifolia* in different matrixes. (A) Gel image of root and substrate samples collected in U1 in September 2004 (lanes 1 and 2), February 2005 (substrate, lane 4), June 2005 (lanes 5 and 6), July 2005 (lanes 7 and 8), July 2006 (lanes 9 and 10), October 2006 (lanes 11 and 12) and (B) gel image of root and substrate samples collected in U2 in February 2005 (lanes 19 and 20), June 2005 (lanes 21 and 22), July 2005 (lanes 23 and 24), July 2006 (lanes 25 and 26), October 2006 (lanes 27 and 28). A DNA marker (M) was included in all the gels to serve as control.

2003). A porous matrix, such as expanded clay, provides a greater surface area for treatment contact and biofilm development. Each substrate used here has different characteristics in terms of pH, electrical conductivity, porosity, and organic matter content. The higher organic matter content verified at the inlet substrate of all units was attributed to the fact that at this point the wastewater organic loading was higher than at the outlet.

The number of CFU found in the vegetated units is within the range of what has been published by Truu et al. (2005) for aerobic heterotrophic bacteria in horizontal subsurface flow CW for domestic wastewater treatment, filled with coarse

**Table 2** – Number of bands and Shannon diversity (H) and equitability (E) indexes, calculated for the constructed wetlands (CWs). U1: CW with *Typha latifolia* planted in Filtralite® MR 3–8; U2: CW with *T. latifolia* planted in fine gravel, AGH 4–8; U3: CW with *T. latifolia* planted in Filtralite® NR 3–8; Uc: unvegetated unit with Filtralite® MR 3–8.

<table>
<thead>
<tr>
<th>Samples</th>
<th>U1</th>
<th>U2</th>
<th>U3</th>
<th>Uc</th>
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<td>E</td>
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<td>E</td>
</tr>
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<td>0.76</td>
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<td>n.d.</td>
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<td>0.94</td>
</tr>
<tr>
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<td>26</td>
<td>0.88</td>
<td>0.62</td>
<td>14</td>
</tr>
<tr>
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<td>29</td>
<td>1.02</td>
<td>0.70</td>
<td>22</td>
</tr>
<tr>
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<td>30</td>
<td>1.01</td>
<td>0.68</td>
<td>22</td>
</tr>
<tr>
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<td>1.64</td>
<td>1.16</td>
<td>18</td>
</tr>
<tr>
<td>Jul_S_05</td>
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<td>0.76</td>
<td>0.55</td>
<td>19</td>
</tr>
<tr>
<td>Sep_S_05</td>
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<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
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<td>22</td>
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<td>25</td>
</tr>
<tr>
<td>Oct_S_06</td>
<td>22</td>
<td>1.02</td>
<td>0.76</td>
<td>22</td>
</tr>
</tbody>
</table>

n.a., not applicable; n.d., not determined.

* In samples column is presented the month, location (R: root or S: substrate) and year, that each sample was collected.
sand and dominated by *Scirpus sylvaticus*, *Urtica dioica* and *Epilobium hirsutum*. The fact that there were no significant differences in bacterial numbers between the substrate and the roots in unit U1 was attributed to the diffuse propagation of *T. latifolia*, since this unit was established for units longer than U2 and U3. Significantly higher numbers of CFUs were frequently found in the wastewater samples collected from the CWs outlet when compared to the inlet, which can be due to the fact that the water passing through the wetland washes out bacteria from the substrate. Although in this study an aerobic plate count method was followed it would be interesting, in the future, to undertake anaerobic enumeration considering the fact that the organic degradation can occur both aerobically and anaerobically in the CW systems. Attempts to evaluate the relative importance of different microbial reactions on organic matter removal (in terms of COD), in horizontal subsurface flow CWs treating urban wastewater, have been carried out using a 2D simulation model (Ojeda et al., 2008). They indicated that the microbial anaerobic reactions involved in organic matter removal (methanogenesis and sulphate reduction) occurred over larger areas of the wetlands than anoxic (denitrification) and aerobic reactions.
The microbial diversity in the substrate and rhizosphere is of great importance since it may be influenced by the type and amount of plants. Most of the bacterial isolates retrieved from the roots and substrate of the CWs presented here were similar to environmental isolates reported from sources such as river and sea waters, sediments and soil. Two isolates affiliated with \( \gamma \)-Proteobacteria were recovered from substrate and roots from the CWs. Franco et al. (2005), based on enrichment cultures with soil collected nearby the same CWs, have isolated bacteria able to degrade polyphenols used in the tannery process being all of them members of \( \gamma \)-Proteobacteria.

The dynamics of the bacterial community in the CWs over 3 years of operation were analyzed by comparison of 16S rDNA PCR-DGGE profiles. Data from this study showed that there was a diverse community of bacteria in the CWs, and that might influence to different extents the final effluent quality. Although the FMR units U1 and Uc had been in operation for a bit longer than U3 and U2, which could indicate that these systems would be more stable and resistant to the stress caused by the hydraulic alterations and wastewater contaminations, no direct relationship could be found.

In this study the community composition was not deepened in relation to the identification of bacterial groups. Despite that, species richness and the structure of bacterial communities have been inferred by numerical analysis of DGGE profiles (Moura et al., 2007). DGGE profiles showed a high variability among bacterial assemblages. The presence of vegetation seemed to have a major effect on the community diversity, as lower bacterial diversity (lower \( H \) values) was observed in the unvegetated unit (Uc). Similarly, Baptista et al. (2003) reported that microbial communities from inlet and outlet of horizontal subsurface flow CW with plants are different when compared to an unplanted wetland, treating a solution of filtered beer. In this study, species richness was higher in the unit with the expanded clay FNR (U3), followed by FMR (U1) and FG (U2). The differences observed might be explained in part by the substrates that differ in characteristics such as pH, conductivity and porosity (Calheiros et al., 2005; Ibekwe et al., 2003). In general, all units showed higher diversity near root comparing to substrate, which can be related to rhizosphere effects on bacterial communities. Previous studies have reported the importance of plant metabolites excreted to the rhizosphere, such as vitamins, that may stimulate microbial growth (Stotmeister et al., 2003). Additionally, Marschner et al. (2002) have studied the microbial community structure in the rhizosphere of cluster roots of Lupinus albus and reported that changes in the community structure may be related with organic acid plant exudates.

Differences in microbial community structure were detected by using the equitability index (Pielou, 1975). The equitability was higher in the unit with the sand FG (U2) followed by the units with expanded clay FNR (U3) and then FMR (U1), and finally the unvegetated unit (Uc). Vacca et al. (2005) have reported that when using CWs for domestic wastewater treatment, the rhizosphere of \( P. \) australis is colonized by distinct communities depending on the filter material (sand and expanded clay) and have concluded that the technology, filter material and plant have a selective influence on the microbial community within the CW. The functional diversity may be linked to certain extents to the ecophysiological roles played by functional groups although the present study did not allow such conclusions. In general, equitability was higher in root samples comparing to substrate samples. Again, this may be related to the rhizosphere effect on microbial communities that may promote the growth of certain bacterial groups.

The assessment of microbial communities in CWs has been addressed by several authors (Baptista et al., 2003; Ibekwe et al., 2003; Truu et al., 2005; Nicomrat et al., 2006). Ibekwe et al. (2003) have characterized the microbial communities and composition in CW for dairy wastewater and reported that these systems are dependent on microbial communities for optimal wastewater treatment. Since little information is available regarding the bacterial communities that inhabit these ecosystems, the PCR-DGGE technique employed here was of great importance in obtaining new data concerning the microbial structure and dynamics of CWs for tannery wastewater treatment. The study of dynamics of microbial communities from CWs is thus essential in understanding these treatment systems, being a valuable tool for improv-

![Multidimensional scaling diagram of DGGE patterns](image-url)
ing its design and operation. A more extensive investigation may also be undertaken at other levels, mainly those related to the detection of microbial genes encoding enzymes linked to degradation pathways, and analysis of their quantitative expression within each CW.

Conclusions

(1) The type of substrate and the presence of T. latifolia seemed to have a major effect on the dynamics and diversity of the bacterial community.

(2) The variations introduced in the systems in terms of hydraulic regimes and wastewater contamination did not result in substantial changes in the diversity of the microbial communities along the systems operation.

(3) A high diversity of bacterial populations was found in the CW units (according to DGGE profiles) and that could contribute to the resilience and resistance of the CWs to stress created by the wastewater loads applied.

(4) The type of substrate was more relevant in determining the bacterial composition than the temporal variability, with the planted CWs units presenting a high similarity within the same year for root and substrate.

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POCI/AMB/60126/2004. The work was supported by the project POCI/AMB/60126/2004.


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