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

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ABSTRACT

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.

1. 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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2. Methods

2.1. 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.

2.2. 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.

2.3. 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.

2.4. 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.

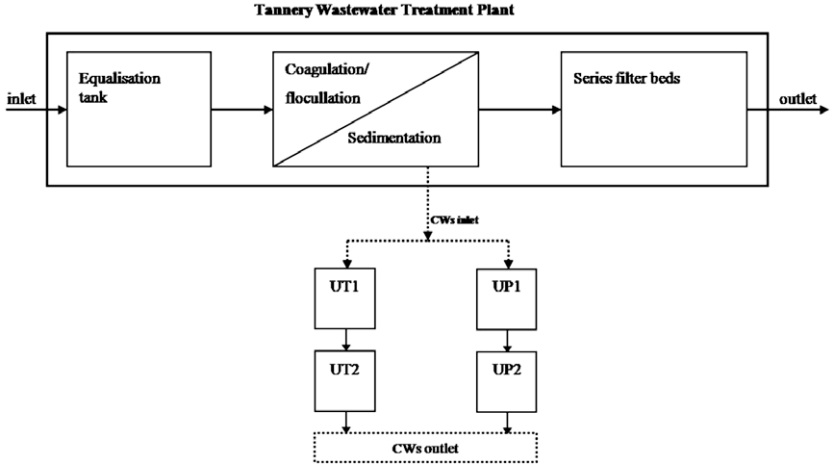

Fig. 1. Schematic representation of the constructed wetlands (CWs). UT1 and UT2: CW with *Typha latifolia* planted in Filtralite®MR 3-8, UP1 and UP2: CW with *Phragmites australis* planted in Filtralite®MR 3-8.