POLISHING DOMESTIC WASTEWATER ON A SUBSURFACE FLOW CONSTRUCTED WETLAND: ORGANIC MATTER REMOVAL AND MICROBIAL MONITORING

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Microbial monitoring of constructed wetlands (CWs) treating domestic wastewater is generally scarce, despite the need of more knowledge about its biocenosis. The sanitation quality of a wastewater treated in a CW is a crucial aspect, mainly when the receiving water body is used as a swimming and/or recreation area. The present study was carried out in a horizontal subsurface flow CW planted with* Phragmites australis *receiving pre-treated domestic wastewater (mean flow 50 m^3 day^{-1}), from a population of about 300 inhabitants. The monitoring programme undertaken during the first year operation, revealed removal efficiencies of 61% BOD_5, 44% COD, and 65% TSS for inlet water with ca. 90 mg L^{-1} BOD_5, 157 mg L^{-1} COD, and 17 mg L^{-1} TSS. Total Coliform (TC) and Faecal Coliform (FC) bacteria were removed from wastewater (mean inlet values of 5 × 10^6 CFU 100 mL^{-1} TC and of 9 × 10^5CFU 100 mL^{-1} FC), with efficiencies of 92 and 97%, respectively. The dynamics of microbial communities established in the system assessed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), had revealed a high bacterial diversity within the system, with no relevant differences in composition at the CW inlet and outlet but exhibiting temporal differences in bacterial communities.

KEY WORDS: microbial communities, constructed wetland, DGGE, *Phragmites australis*, domestic wastewater

INTRODUCTION

One of the goals of domestic wastewater treatment is to produce low organic load effluents in order to render its discharge harmless to receiving water bodies. According to EU Water Framework Directive (2000/60/EC) a "good ecological status" must be achieved for all European waters by 2015. Regarding this objective, it is crucial that systems treating wastewater fulfil entirely its function, removing organic load and disease-causing microorganisms.

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In the last years we have assisted in Portugal to an increasing use of constructed wetlands (CWs) to treat domestic wastewater in rural areas. Some of these areas are located on the mountains, namely near the "Gerês National Park," a watershed with bathing areas. However, functioning of these low technology systems is not yet fully understood and its microbial monitoring regarding its sanitation quality is generally insufficient. More recent reports deal with such aspects (Baptista et al. 2003; Ibekwe et al. 2003; Vacca et al. 2005; Faulwetter et al. 2009). Summarizing efficiency data, Vymazal (2009) reviewed 17 horizontal subsurface flow (HSSF) CW used primarily for treatment of municipal or domestic wastewater, with data on faecal coliform (FC) removal available for 6 of the systems reported. On 5 of these systems FC removal was between 3 and 4 log decrease and on the 1 system a 1 log decrease was achieved.

The role of the bacterial communities in wastewater treatment is indisputable. The diversity of microorganisms in the wetland environment may be critical for the proper functioning and maintenance of the system (Ibekwe et al. 2003). The start-up of some of these singular systems is the ideal occasion to follow the evolution of CWs biocenoses. Using culture-dependent and culture-independent microbial techniques we may assemble data for a better understanding of the processes that take place in planted beds (Vacca et al. 2005; Truu et al. 2009; Dong and Reddy 2010). Structure of bacterial communities has been estimated by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analyses on different kinds of CWs (Ahn et al. 2007; DeJournett et al. 2007; Jin and Kelley 2007; Baptista et al. 2008; Calheiros et al. 2009a, 2009c; Dong and Reddy 2010).

In the present study, we followed from start-up and for one year the operation of a HSSF CW. BOD_5 and COD reduction and removal of total coliforms (TC) and *Escherichia coli* (faecal coliforms (FC)) were assessed. The spatial and temporal dynamics of microbial communities established in the system was followed using PCR-DGGE.

METHODS

Constructed Wetland

The HSSF CW targeted in this study is located in northern Portugal, receiving a domestic wastewater with a mean flow of 50 m^3 day^{-1} for a population of about 300 inhabitants.

Operation started in September 2007. The treatment system comprises a pre-treatment unit, a septic tank (total volume 201.6 m^3) and two HSSF beds planted with *Phragmites australis* (462 m^2 each) operating in parallel. These reed beds are coated with geotextil membrane and filled with a substratum composed from bottom to top with: about 20 cm coarse gravel (stones sized roughly 2–4 mm), about 50 cm fine gravel (particles sized 2–4 mm) and about 10 cm local soil (a typical soil, mixture of mineral and organic constituents). The wastewater retention time on the reed beds was 4 days and the water in the beds was approximately 5 cm below the surface. The reeds were planted in the beds by hand, from unitary shoots purchased on flowerpots (11 plants m^{-2}). Generally, *Phragmites* was visually inspected for toxicity signs, such as chlorosis, necrosis and malformation and the development of the plants was followed on a monthly basis during the operation of the CW.

Sampling Campaigns

Monthly sampling campaigns were carried out between November 2007 and September 2008 for microbial and physico-chemical parameters. Wastewater samples (ww) were

collected from the inlet (IN) and outlet (OUT) of one of the macrophytes beds. Three substratum subsamples were pooled to form two composite samples (at a depth around 10 cm), from upstream (upS) and downstream (dwS) of the bed. The grab samples (INww, OUTww, upS, and dwS) were collected into sterile containers and were kept cold on a thermo-box until analysis performed within 4 hours.

Wastewater Analyses

The wastewater samples were analysed based on Standard Methods (APHA, AWWA, WEF 1998) for the following parameters: biochemical oxygen demand (BOD_5), determined by the manometric method using an OxiTop® self-check measuring system (WTW, Giessen, Germany), chemical oxygen demand (COD), determined by the dichromate digestion method (with potassium dichromate in sulphuric acid and silver sulphate as catalyst) and total suspended solids (TSS), determined after filtration under vacuum, through 47 mm diameter glass microfiber filters (934-AH Whatman) and after drying to constant weight at 105°C. *In situ* determination of pH, wastewater, and air temperature were performed with a HI 98128 (HANNA instruments). Culture dependent microbial analyses were used to enumerate microbial groups—heterotrophic bacteria, total coliforms (TC), and faecal coliforms (FC). Bacterial enumeration (CFU mL^{-1}) of heterotrophic bacteria was carried out on R2A medium (Merck) using the spread plate method, after threefold 100 μL inoculation of each selected decimal diluted samples and incubation at room temperature for five days. The coliform bacteria were enumerated on Chromocult® agar (CC) from Merck. As a differential medium CC allows to distinguish *E. coli* dark-blue to violet colonies (FC) from other coliform colonies, coloured salmon to red (TC). In CC medium the accompanying flora is represent by other Gram-negative *Enterobacteriaceae*, forming colourless colonies (Ent). The inoculated plates were incubated at 36 ± 1°C for 24 hours.

Substratum Analyses

Microbial analysis of the substratum (S-samples) was made using culture-dependent and culture-independent methods. Substratum samples up to 10 cm depth were collected, from December 2007 to September 2008, into 50 mL sterile polypropylene tubes and 10 g of each homogenized sample was suspended on 10 mL of NaCl 0.3 M sterile solution. For bacterial enumeration, serial decimal dilutions were performed from these suspensions using NaCl 0.3 M sterile solution. Three chosen dilutions of each S-sample were threefold inoculated (100 μL) on solid media (R2A and CC), such as with ww-samples.

Culture independent assessment of microbial diversity was made using PCR-DGGE. The 16S rRNA bacterial gene from S-samples (upS and dwS) was amplified by a polymerase chain reaction, and then a denaturing gradient gel electrophoresis (PCR-DGGE) was performed.

DNA extraction. DNA was extracted from upS and dwS samples using UltraClean Soil DNA kits (MOBIO, Laboratories) according to the manufacturer's protocol.

DGGE analysis of total bacterial community. PCR amplification of bacterial 16S rRNA gene fragments was performed using primers 338F_GC and 518R (Henriques et al. 2006a). Nested PCR amplifications were carried out as described in Calheiros et al. (2009a). DGGE analysis was performed on a DCodeTM 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 1xTAE buffer using a denaturing gradient ranging from 35% to 60% with 100% denaturant solution 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. 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^{TM} II software (Applied Maths). Dendrograms were generated by unweight pair group mean average (UPGMA) cluster analysis. DGGE banding data were used to estimate the *Shannon-Weaver index, H* (Shannon and Weaver 1963) and the *equitability index, E* (Pielou 1975).

RESULTS AND DISCUSSION

In this study the performance of a HSSF CW, in terms of BOD_5, COD, total coliforms (TC), and *Escherichia coli* (faecal coliforms—FC) removal, was assessed. The spatial and temporal dynamics of microbial communities established in the system was also followed through PCR-DGGE technique.

Plant Development

In general, plants developed and proliferated without showing signs of toxicity. At the beginning, a natural colonization by local plants (essentially Asteraceae, Borraginaceae, and Cyperaceae—*Scirpus*) occurred in the CW, although no intervention was necessary to control their growth, which was rapidly overlapped by *P. australis*. The CW was fully vegetated nearly exclusively by the reeds after one year operation. This plant has already proven to be resilient to high organic loads, up to 900 kg BOD_5 ha^{-1} d^{-1}, and to interruptions in feed, without jeopardizing the treatment success and its establishment (Calheiros et al. 2009b). Also, it has been used in CW for the treatment of various types of wastewater with subsurface flow (Vymazal 2009).

Organic Removal Efficiency

From November 2007 to September 2008, monthly samples of wastewater were collected from the HSSF CW (IN and OUT). The wastewater temperature determined *in situ* ranged between 11.4 and 22.1°C at the inlet and between 11.6 and 20.9°C at the outlet. Air temperature was always higher than wastewater temperature (Figure 1). Organic matter removal was assessed through determination of BOD_5, COD, and TSS. The COD and BOD_5 concentration (Figure 1) of incoming wastewater varied throughout the year.

Considering the mean flow reaching the CW studied, ca. 25 m^3 day^{-1}, the highest organic loading was about 87 kg BOD_5 ha^{-1} day^{-1} whereas the lowest was ca. 27 kg BOD_5 ha^{-1} day^{-1}. BOD_5 ranged between 50 and 160 mg L^{-1} at the inlet and 16 and 52 mg L^{-1} at the outlet and COD ranged between 104 and 229 mg L^{-1} and 33 and 141 mg L^{-1} at the inlet and outlet of the CW, respectively. Removal efficiencies up to 95% BOD_5 and 68% for COD were achieved. The highest removal efficiency attained for TSS was of 83%. During the experimental period the BOD_5, COD and TSS levels of outlet samples were

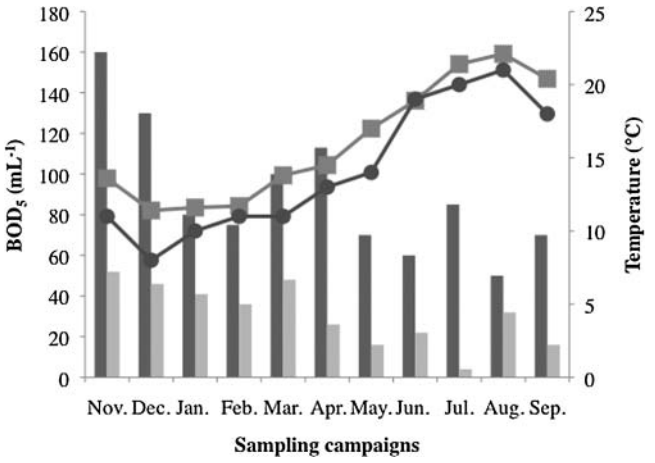

Figure 1 BOD_5 (mg L^{-1}) of wastewater samples (ww) collected at the inlet (INww ■) and outlet (OUTww ■) of the constructed wetland (bars); lines – inlet wastewater (●) and air (■) temperature (°C).

in compliance with the Portuguese legislation (DL n° 236/98 – 1 August), always below 40 mg L^{-1} BOD_5, 150 mg L^{-1} COD, and 60 mg L^{-1} TSS (Table 1).

The performance of the 17 horizontal flow CWs treating municipal and domestic wastewater from several countries summarized by Vymazal (2009) varied in area from 90 to 4495 m^2 with mean flows estimated between 0.9 and 180–400 m^3 day^{-1}. The lower BOD_5 removal efficiencies reported were generally associated to HSSF–CW with low BOD_5 at the influent (between 5.8 and 27.0 mg L^{-1}) whereas the systems with incoming BOD_5 ranging from 51.0 and 979.0 mg L^{-1} presented efficiencies between 85% and 98%. In the present study, during its first year functioning, influent BOD_5 varied between 50–160 mg L^{-1} and outflow BOD_5 varied between 4–52 mg L^{-1}. The 61% BOD_5 mean

Table 1 Mean values (± standard deviation) of temperature (°C), pH and BOD_5, COD and TSS (mg L^{-1}) determined on wastewater ww-samples collected at the inlet (IN) and outlet (OUT) of the macrophytes bed

	Samples			
	wwIN	wwOUT	Efficiency (%)	MDV
Temperature (°C)	16.0 ± 4.1 n = 11	15.4 ± 3.3 n = 11		
pH	6.64 ± 0.36 n = 11	6.65 ± 0.51 n = 11		6.0–9.0
BOD_5 (mg L^{-1})	90 ± 33 n = 11	35 ± 12 n = 10	61	40
COD (mg L^{-1})	157 ± 51 n = 8	88 ± 42 n = 8	44	150
TSS (mg L^{-1})	17 ± 10 n = 5	6 ± 4 n = 5	65	60

n = number of samples; MDV (*maximum discharge values,* according to Portuguese legislation—DL n° 236/98 1 August)

removal efficiency obtained is lower than the efficiencies of the systems reported by Vymazal (2009), however incoming BOD was closer to the lower range reported by that author, where lower efficiencies are expected. From the same geographic area, a similar HSSF CW monitored by Mina and Ferreira (2006) had shown higher BOD_5 removal efficiency (80–90% for an inlet of 113 ± 62 mg L^{-1}).

Concerning COD removal, Fountoulakis et al. (2009) reported for a pilot scale HSSF CW treating domestic wastewater, values higher than 75% for mean inlet COD of 99.6 ± 49.4 mg L^{-1}. Kouki et al. (2009) for HSSF CW system designed for rural domestic wastewater treatment presented even higher removal efficiencies, close to 90% for mean inlet COD of 1339 ± 352 mg L^{-1}.

Lower COD removal efficiencies were achieved on other HSSF CW with *P. australis* or other macrophytes: 73% for mean inlet COD of 228 ± 54 mg L^{-1} (Mina and Ferreira 2006), 70% for mean inlet COD of 226 ± 20 mg L^{-1} (Badkoubi et al. 1998) and 60% for mean inlet COD of 430 ± 86 mg L^{-1} (Krasnits et al. 2009). In this study, the lower BOD_5 and COD removal efficiencies recorded in the HSSF CW are probably due to the fact that the study was done during the first year functioning of the system and that low organic loadings were being applied. Calheiros et al. (2009a) have reported that higher pollutant removal in terms of BOD_5 and COD, were achieved in expanded clay units planted with *Thypha latifolia* after long-term operation.

Enumeration of Heterotrophic Bacteria and Coliforms

The heterotrophic bacterial counts from inlet and outlet wastewater ranged between 3.12×10^5 (December) and 4.50×10^6 CFU ml^{-1} (February) and 3.15×10^4 (December) and 4.80×10^5 CFU ml^{-1} (March), respectively. One log decrease was recorded on most sampling campaigns, however reduction of bacterial counts from inlet to outlet on June, July, and August samples were lower (Figure 2). For soil samples, upS, and dwS, heterothrophic bacterial counts ranged between 1.21×10^5 (August) and 5.40×10^6 CFU g^{-1} (June) and between 1.46×10^5 (March), and 3.50×10^6 CFU g^{-1} (May), respectively. On the majority of the samples, bacterial counts were of the same order of magnitude both upstream and downstream of the reed bed (Figure 2). The bacterial counts on the substratum were lower than that published in other studies (Calheiros et al. 2009c; Truu et al. 2005). Nevertheless, Truu et al. (2005) also found no significant differences between upstream and downstream on the substratum of a CW treating domestic wastewater.

The TC ranged between 2.70×10^3 (April) and 2.50×10^5 CFU mL^{-1} (September) on inlet wastewater and between 3.70×10^2 (April) and 1.92×10^4 CFU mL^{-1} (January) on outlet wastewater, whereas the FC counts varied between 2×10^2 (April) and 6×10^4 CFU mL^{-1} (September) and between 2×10^1 (September) and 1.60×10^3 CFU mL^{-1} (June) at the inlet and outlet, respectively (Figure 3). The removal efficiencies decreased from December 2007 to April 2008 (minimum efficiency value of 70%) increasing thereafter.

Despite the contradictory results on the removal efficiency of pathogenic bacteria by planted CW over non-planted ones (Wand et al. 2007), in our study the decreased of TC and FC from inlet to outlet of the system were lower than that reported by other authors when using planted systems (Vacca et al. 2005; Vymazal 2009). In a CW for dairy wastewater effluent, consisting of two HSSF reed beds operating in parallel preceded by a raw and a facultative pond for central collection of washwater, TC removal efficiencies of about 99% (approximately 10^6 per 100 ml at inlet), corresponding to a 2 log decrease, and FC removal efficiencies of about 99.9%, corresponding to a 3 log decrease, were observed between

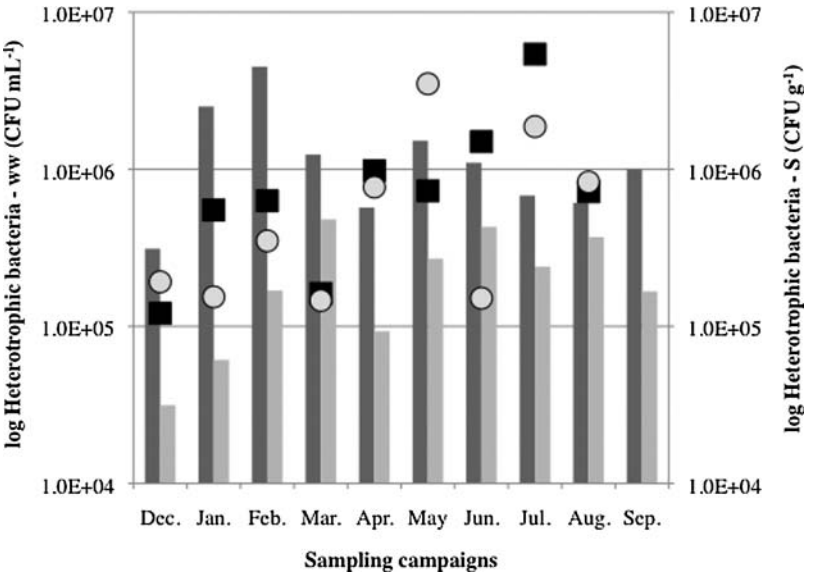

Figure 2 Heterotrophic bacterial counts from inlet (INww ■) and outlet (OUTww ■) CW wastewater samples (bars), and from upstream (upS ○) and downstream (dwS ■) macrophytes bed samples (symbols), during the experimental period.

the raw washwater pond and the average wetland effluent (Ibekwe et al. 2003). Similar results were recorded in a pilot scale HSSF CW (Fountoulakis et al. 2009), in a CW in Almendares river watershed (Cuba) where the average removal of faecal bacteria indicators (FBIs, i.e., total and faecal coliforms, *E. Coli,* and enterococci) was always higher than 2.4 log units (Garcia-Armisen et al. 2008), in pilot-scale CW systems treating domestic wastewater (Vacca et al. 2005) and on a combined subsurface vertical and horizontal flow

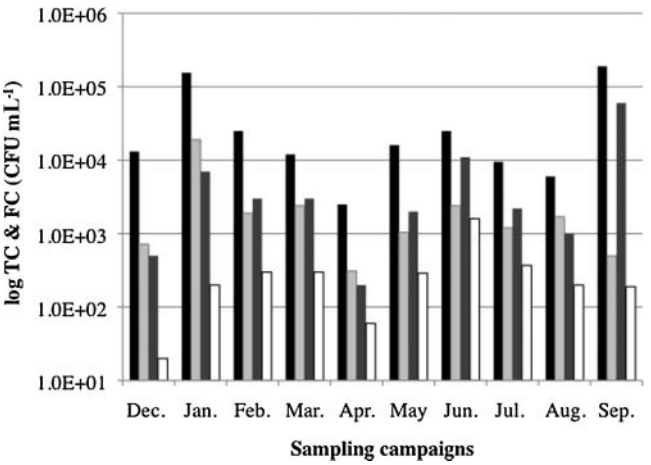

Figure 3 Counts of total coliformes — TC (wwIN ■ and wwOUT ■) and faecal coliforms — FC (wwIN ■ and wwOUT □), recovered on Chromocult® differential medium from inlet and outlet wastewater.

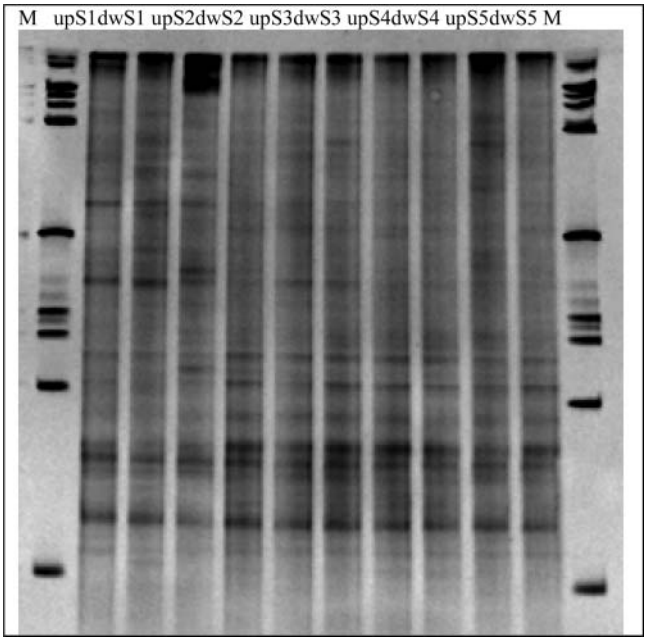

Figure 4 DGGE analysis of 16S rRNA gene fragments of total bacteria communities from substratum samples at upstream and downstream (upS and dwS) of the constructed wetland between December 2007 and April 2008; 1 – Dec.; 2 – Jan.; 3 – Feb.; 4 – Mar.; 5 -Apr. A DNA marker (M) was included in all the gel to serve as control.

CWs system designed for rural domestic wastewaters treatment operating with a theoretical hydraulic retention time of 3.6 days (Kouki et al. 2009).

According to Directive 96/7/EC, concerning the management of bathing water quality, a good to excellent quality of inland waters must not have more than 500–1000 FC CFU 100 ml^{-1}. Although in this study we have not determined the content in TC and FC on the receiving river water, the FC content on CW outlet reached densities comprised between 2×10^4 and 4×10^6 CFU 100 ml^{-1}. As this effluent discharge is made to an important water body used for swimming, attention must be drawn to microbial monitoring, particularly in summer period.

DGGE Analysis of Bacterial Communities

DGGE analysis of 16S rDNA fragment was used to investigate bacterial community dynamics in the substratum of the reed bed. DGGE profiles were obtained from the two sampling sites (upS and dwS) of reed bed, between December 2007 and July 2008. As an example, Figure 4 shows the DGGE profiles from upS and dwS samples collected between December 2007 and April 2008. Some of the bands were present in all S-samples throughout the experimental period.

Cluster analysis was performed to gain an overview on the relatedness of phylogenetic profiles representing the communities at each sampling point (Figure 5). Generally, the microbial community structure in this HSSF CW was found spatially homogeneous.

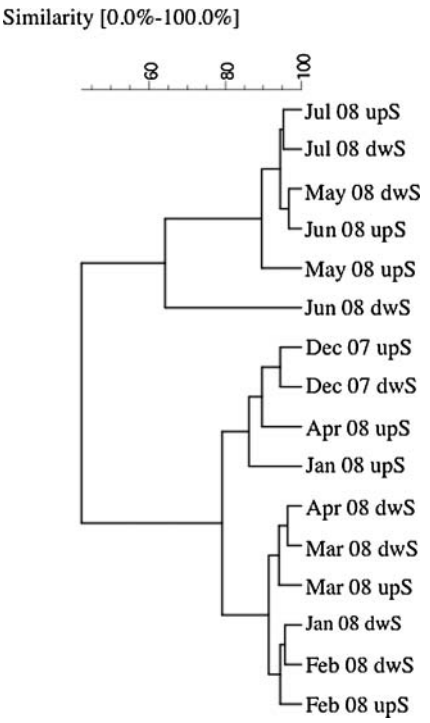

Figure 5 Cluster analysis of DGGE patterns of monthly upstream (upS) and downstream (dwS) samples from the constructed wetland, taken from December 2007 (Dec 07) to July 2008 (Jul 08). Similarities were calculated using the Bray–Curtis measure.

Most upstream and downstream samples, at each sampling campaign, exhibited a similar pattern—the greater phylogenetic distance between upS and dwS microbial communities was between January 08 and April 08 samples. Nevertheless, the phylogenetic distances of microbial communities exhibited between winter and early spring and late spring and early summer revealed a temporal heterogeneous pattern.

The spatial distribution of major microbial populations was studied by Krasnits et al. (2009) in a well established HSSF CW. Major microbial groups were characterized by fluorescent *in situ* hybridization (FISH) at the beginning, centre and end of the CW and at different depths, including the root zone and a relatively uniform microbial population distribution, was observed with respect to depth and length of the CW. However, Truu et al. (2005) described the microbial community structure in a HSSF CW treating wastewater from a hospital as spatially heterogeneous. The most important spatial pattern in microbial community structure within that CW was related to the depth gradient, followed by differences between inflow and outflow. PCR-DGGE fingerprints indicated a high diversity of bacterial community within the soil filter bed with significant differences in bacterial community structure between the upper and deeper layers; in general, the diversity of the bacterial community was higher in the upper layer than in the deeper horizon. The similar DGGE profiles obtain by Ibekwe et al. (2003) characterizing the microbial composition of two CWs designed to remove contaminants from dairy washwater showed no clear distinction between time and sampling locations. The differences in bacterial composition observed by Calheiros et al. (2009a) in well-established CWs planted with *T. latifolia* in

different substrates, were related to the type of substrate in the bed, with planted CWs units presenting a high similarity within the same year. In our system, similar bacterial communities were found upstream and downstream of the reed bed, nevertheless the temporal heterogeneous pattern exhibited may be associated with the development stage of the plants, considering that the study was carried out during first year operation. The greater differences observed on the reed bed bacterial community were recorded in January and April, corresponding respectively, to the dormant and sprout phases of the reeds.

The microbial communities diversity was assessed by the Shannon-Weaver diversity index, H (Shannon and Weaver 1963) and the equitability index, E (Pielou 1975) considering the number and relative intensity of the DNA bands of each sample. The upS samples presented H values between 1.33 and 1.45, while the H values of dwS samples ranged between 1.27 and 1.48. The higher H values were recorded on both samples of June, and the lower H values were recorded on dwS samples of January and March, which may be associated to the plants growth rate. The equitability index, E, was practically constant along experimental period (0.98–1). Microbial diversity of this CW reed bed was thus high. The macrophytes' importance on the bacterial community diversity had been emphasizing by several authors (Gersberg et al. 1986; Krasnits et al. 2009; Sleytr et al. 2009). Calheiros et al. (2009a) reported higher diversity in the near-root substratum, related to rhizosphere effects on bacterial communities, and our results seems to corroborate that the plants development stage influence the bacterial communities of the reed beds.

According to Garcia-Armisen et al. (2008) the type of processes occurring in a CW are difficult to compare as factors as climatic conditions, vegetal species, retention time and flow surface can severely affect the removal efficiency of the measured parameters, including the removal of faecal bacteria indicators (FBIs). In CW the removal of FBIs is mostly due to sorption, sedimentation and predation as light' effect is attenuated by plants (Garcia-Armisen et al. 2008). As so, when plants are in its growing phase the light effect may be more obvious, if light intensity do not decreased owing to year season.

The present system has shown to be adequate for BOD and COD polishing, however, coliform reduction was only achieved to small extents. Moreover, more research into the microbial population diversity, both spatially and temporally, is needed to help further understanding of horizontal subsurface systems functioning.

CONCLUSIONS

A horizontal subsurface flow CW, operating for one year, was evaluated in terms of performance, plant establishment and removal of total coliforms and faecal coliform.

The main conclusions of this investigation are summarized as follows:

- CW allowed incoming domestic wastewater to be polished to a level allowing discharge in compliance with the Portuguese legislation (DL n° 236/98, 1 August) considering the organic load.
- Microbial groups of sanitary importance (TC and FC) were reduced through the reed bed, during the first year functioning, allowing for one log reduction; attention must be paid to these values to achieved a "good ecological status" as require by EU Water Framework Directive (2000/60/EC).
- DGGE analysis revealed a high bacterial diversity within the system, with no relevant differences upstream and downstream of the reed bed, and temporal differences in bacterial communities may be associated to different life stages of the reeds.

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REFERENCES

Ahn C, Gilleveta PM, Sikaroodi M. 2007. Molecular characterization of microbial communities in treatment microcosm wetlands as influenced by macrophytes and phosphorus loading. Ecol Indic. 7(4)852–863.

APHA, AWWA, WEF. 1998. Standard methods for the examination of water and wastewater. 20th ed. Washington (DC): American Public Health Association.

Badkoubi A, Ganjidoust H, Ghaderi A, Rajabi A. 1998. Performance of a subsurface constructed wetland in Iran. Water Sci Technol. 38(1):345–350.

Baptista JDC, Donnelly T, Rayne D, Davenport RJ. 2003. Microbial mechanisms of carbon removal in subsurface flow wetlands. Water Sci Technol. 48(5):127–134.

Baptista JdeC, Davenport RJ, Donnelly T, Curtis TP. 2008. The microbial diversity of laboratory-scale wetlands appears to be randomly assembled. Water Res. 42(12):3182–3190.

Calheiros CSC, Duque AF, Moura A, Henriques IS, Correia A, Rangel AOSS, Castro PML. 2009a. Substrate effect on bacterial communities from constructed wetlands planted with *Typha latifolia* treating industrial wastewater. Ecol Eng. 35(5):744–753.

Calheiros CSC, Rangel AOSS, Castro PML. 2009b. Treatment of industrial wastewater with two-stage constructed wetlands planted with *Typha latifolia* and *Phragmites australis*. Bioresour Technol. 100:3205–3213.

Calheiros CSC, Duque AF, Moura A, Henriques IS, Correia A, Rangel AOSS, Castro PML. 2009c. Changes in the bacterial community structure in two-stage constructed wetlands with different plants for industrial wastewater treatment. Bioresour Technol. 100:(13):3228–3235.

DeJournett TD, Arnold WA, LaPara TM. 2007. The characterization and quantification of methanotrophic bacterial populations in constructed wetland sediments using PCR targeting 16S rRNA gene fragments. Appl Soil Ecol. 35(3):648–659.

Decreto Lei n° 238/98 de 1 de August (DL n° 238/98 1 August). Diário da República n° 176/98 — I Série A. Ministério do Ambiente. Lisboa.

Directive 96/7/EC of the European Parliament band of the Council. [cited 15 February 2006]. Available from: http://eur-lex.europa.eu.

Dong X, Reddy GB. 2010. Soil bacterial communities in constructed wetlands treated with swine wastewater using PCR-DGGE technique. Bioresour Technol. 101:1175–1182.

EU Water Framework Directive (2000/60/EC). Available from: http://eur-lex.europa.eu.

Faulwetter JL, Gagnon V, Sundberg C, Chazarenc F, Burr MD, Brisson J, Camper AC, Stein OR. 2009. Microbial processes influencing performance of treatment wetlands: A review. Ecol Eng. 35(6):987–1004.

Fountoulakis MS, Terzakis S, Chatzinotas A, Brix H, Kalogerakis N, Manios T. 2009. Pilot-scale comparison of constructed wetlands operated under high hydraulic loading rates and attached biofilm reactors for domestic wastewater treatment. Sci Total Environ. 407(8):2996–3003.

Garcia-Armisen T, Prats J, Marrero Y, Servais P. 2008. Faecal bacterial indicators removal in various wastewater treatment plants located in Almendares River watershed (Cuba). Water Sci Technol. 58(4):773–779.

Gersberg RM, Elkins BV, Lyon SR, Goldman CR. 1986. Role of aquatic plants in wastewater treatment by artificial wetlands. Water Res. 20(3):363–368.

Henriques IS, Alves A, Tacão M, Almeida A, Cunha A, Correia A. 2006a. Seasonal and spatial variability of free-living bacterial community composition along an estuarine gradient (Ria de Aveiro, Portugal). Estuar Coast Shelf S. 68:139–148.

Henriques IS, Moura A, Alves A, Saavedra MJ, Correia A. 2006b. Analysing diversity among b-lactamase encoding genes in aquatic environments. FEMS Microbiol Ecol. 56:418–429.

Ibekwe AM, Grieve CM, Lyon SR. 2003. Characterization of microbial communities and composition in constructed dairy wetland wastewater effluent. Appl Environ Microbiol. 69(9):5060–5069.

Jin G, Kelley TR. 2007. Characterization of microbial communities in a pilot-scale constructed wetland using PLFA and PCR-DGGE analyses. J Environ Sci Health A. 42(11):1639–1647.

Kouki S, M'hiri F, Saidi N, Belaïd S, Hassen A. 2009. Performances of a constructed wetland treating domestic wastewaters during a macrophytes life cycle. Desalination. 246(1–3):452–467.

Krasnits E, Friedler E, Sabbah I, Beliavski M, Tarre S, Green M. 2009. Spatial distribution of major microbial groups in a well established constructed wetland treating municipal wastewater. Ecol Eng. 35(7):1085–1089.

Mina IA-P, Ferreira MJ. 2006. Reviving Käthe Seidel's legacy to University of Minho. In: IWA, editor. 2006. Proceedings of the 10th International Conference on Wetland Systems for Water Pollution Control. Lisbon (Portugal): MAOTDR (Ministério do Ambiente, do Ordenamento do Território e do Desenvolvimento Regional). p. 641–649.

Muyzer G, De Wall EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analyses of polymerase chain reaction-aplified genes coding for 16S rRNA. Appl Environ Microbiol. 59(3):695–700.

Pielou EC. 1975. Ecological diversity. New York: John Wiley and Sons, Inc.

Shannon CE, Weaver W. 1963. The mathematical theory of communication. Urbana (IL): University of Illinois Press.

Sleytr K, Tietz A, Langergraber G, Haberl R, Sessitsch A. 2009. Diversity of abundant bacteria in subsurface vertical flow constructed wetlands. Ecol Eng. 35(6):1021–1025.

Truu J, Nurk K, Juhanson J, Mander U. 2005. Variation of microbiological parameters within planted soil filter for domestic wastewater treatment. J Environ Sci Health. 40:1191–1200.

Truu M, Juhanson J, Truu J. 2009. Microbial biomass, activity and community composition in constructed wetlands. Sci Total Environ. 407(13):3958–3971.

Vacca G, Wand H, Nikolausz M, Kuschk P, Kästnera M. 2005. Effect of plants and filter materials on bacteria removal in pilot-scale constructed wetlands. Water Res. 39(7):1361–1373.

Vymazal J. 2009. The use constructed wetlands with horizontal sub-surface flow for various types of wastewater. Ecol Eng. 35:L1–17.

Wand H, Vacca G, Kruschk P, Krüger M, Kästner M. 2007. Removal of bacteria by filtration in planted and non-planted sand columns. Water Res. 41:159–167.