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Key design factors affecting microbial community composition and pathogenic organism removal in horizontal subsurface flow constructed wetlands

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HIGHLIGHTS

- Key design factors affecting HSSF constructed wetlands performance were evaluated.
- Water depth of 0.27 m was more effective than 0.50 m for microbial removal.
- Fine granulometry was more effective than coarse gravel for microbial removal.
- Microbial removal in all HSSF wetlands analysed occurs mainly near the inlet.
- Microbial communities from constructed wetlands were affected by water depth.

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ABSTRACT

Constructed wetlands constitute an interesting option for wastewater reuse since high concentrations of contaminants and pathogenic microorganisms can be removed with these natural treatment systems. In this work, the role of key design factors which could affect microbial removal and wetland performance, such as granular media, water depth and season effect was evaluated in a pilot system consisting of eight parallel horizontal subsurface flow (HSSF) constructed wetlands treating urban wastewater from Les Franqueses del Vallès (Barcelona, Spain). Gravel biofilm as well as influent and effluent water samples of these systems were taken in order to detect the presence of bacterial indicators such as total coliforms (TC), *Escherichia coli*, fecal enterococci (FE), *Clostridium perfringens*, and other microbial groups such as *Pseudomonas* and *Aeromonas*. The overall microbial inactivation ratio ranged between 1.4 and 2.9 log-units for heterotrophic plate counts (HPC), from 1.2 to 2.2 log units for total coliforms (TC) and from 1.4 to 2.3 log units for *E. coli*. The presence of fine granulometry strongly influenced the removal of all the bacterial groups analyzed. This effect was significant for TC ($p = 0.009$), *E. coli* ($p = 0.004$), and FE ($p = 0.012$). Shallow HSSF constructed wetlands were more effective for removing *Clostridium* spores ($p = 0.039$), and were also more efficient for removing TC ($p = 0.011$) and *E. coli* ($p = 0.013$) when fine granulometry was used. On the other hand, changes in the total bacterial community from gravel biofilm were examined by using denaturing gradient gel electrophoresis (DGGE) and sequencing of polymerase chain reaction (PCR)-amplified fragments of the 16S rRNA gene recovered from DGGE bands. Cluster analysis of the DGGE banding pattern from the different wetlands showed that microbial assemblages separated according to water depth, and sequences of different phylogenetic groups, such as Alpha, Beta and Delta-Proteobacteria, Nitrospirae, Bacteroidetes, Acidobacteria, Firmicutes, Synergistetes and Deferribacteres could be retrieved from DGGE bands.

1. Introduction

Water shortages in arid and semi-arid areas such as the Mediterranean have prompted a need for wastewater treatment and subsequent reuse. Reclamation can be achieved through conventional intensive

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systems or natural, ecologically engineered treatments such as horizontal subsurface flow (HSSF) constructed wetlands. Depending on wastewater type, some pathogenic microorganisms may be present and, therefore, wastewater reclamation processes with disinfection could be required (Asano and Levine, 1998). Thus, research into sewage treatment is needed in order to reduce risks associated with improper sanitation, particularly in terms of wastewater reuse for crop irrigation.

Regardless of their location (water column, biofilms on surface material or sediment pore-water), pathogens must compete with the consortium of organisms surrounding them. As intestinal organisms, most may not survive and may also be destroyed by predation. Water temperature, organic matter concentration and hydraulic conditions such as flow, aspect ratio, and granular media type are some of the most important factors governing occurrence and growth of viable microbes in biofilms developed elsewhere (LeChevallier et al., 1988, 1996; Reasoner et al., 1989; Block, 1992; Block et al., 1993; Van der Kooij et al., 1995).

Molecular fingerprinting techniques (Friedrich et al., 2003; Ibekwe et al., 2003; Vacca et al., 2005) have been used to study the dynamics and structure of microbial communities in constructed wetlands but few studies have been performed on the effect of wetlands on the removal of specific pathogens. Although the work with powerful new techniques, such as quantitative real time polymerase chain reaction (qPCR), will undoubtedly change the scene of health and environmental microbiology during the following years, until now routine examination for pathogenic microorganisms is not recommended because of the high cost of the analysis and the generally low number of a specific pathogen that is present in an environmental sample. Therefore, indicator organisms are routinely used to study microbial removal in constructed wetlands.

In general, most studies on fecal microorganism removal in constructed wetlands only describe total and fecal coliform removal (Kadlec and Knight, 1996; Wang et al., 2005; Tanaka et al., 2006; Tunçsiper, 2007). Research using experimental, pilot and full-scale constructed wetlands has shown that fecal coliform bacteria inactivation usually ranges between 1.25 and 2.5 log units (Gersberg et al., 1989 a,b; Hiley, 1990; Rivera et al., 1995; Williams et al., 1995; Decamp et al., 1999; Arias et al., 2003; Vacca et al., 2005; Vymazal, 2005). However, fecal coliform inactivation rates of 3.0 log units and higher have been recorded in tertiary HSSF constructed wetlands treating slaughterhouse wastewater with extremely high influent concentrations of fecal bacteria (from 6.0 to 11.0 log units/100 mL). Furthermore, removals of 2.4 to 5.3 orders of magnitude for cultivable *Salmonella* cells were found (Pundsack et al., 2001). The high degree of inactivation observed in these wetlands was related to the high influent microbial concentration (Rivera et al., 1995).

It must also be taken into account that the concentration of coliform bacteria in wastewaters is subject to significant daily fluctuations, so the highest concentration in the influent of a given constructed wetland system will not necessarily coincide with the highest concentration in the effluent (Cooper et al., 1996).

In any case, subsurface flow constructed wetlands offer a suitable combination of physical, chemical and biological mechanisms required to remove pathogenic organisms. Physical factors include filtration and sedimentation (Gersberg et al., 1989a; Pundsack et al., 2001), while chemical mechanisms combine oxidation and adsorption to organic matter (Gersberg et al., 1989a). The biological removal features include oxygen release and bacterial activity in the rhizosphere, aggregation and retention in biofilms (Hiley, 1995; Brix, 1997), potential production of bactericidal compounds or antimicrobial activity of root exudates (Kickuth and Kaitzis, 1975; Seidel, 1976; Axelrood et al., 1996), as well as predation by nematodes and protists (Decamp and Warren, 1998; Decamp et al., 1999), attack by lytic bacteria and viruses (Axelrood et al., 1996), natural die-off (Gersberg et al., 1989a,b) and competition for limiting nutrients or trace elements (Gersberg et al., 1987a,b).

Fecal bacteria removal in constructed wetlands has been related to environmental factors such as granular medium or type of plant.

Some studies appear to show that granular media and the presence or absence of plants (macrophytes) in constructed wetlands are important for fecal bacteria inactivation, while in others' works there is no evidence of this fact. On the one hand, wetland plants play several roles in the HSSF constructed wetland. Their root systems provide surfaces for the attachment of microorganisms, enhance filtration effects, and stabilize the bed surface. The roots contribute to the development of microorganisms by the release of oxygen and nutrients. Moreover, the plants give the treatment site an attractive appearance. The effect of macrophytes on the system efficiency seems to vary depending on the season, wastewater type and plant species (Stein and Hook, 2003). On the other hand, the use of a small size granular medium instead of a large size one seems to improve the microbial inactivation ratio between 1.0 and 2.0 log units for both fecal coliforms and somatic coliphage removal (Ottová et al., 1997; Garcia et al., 2003). Nevertheless, several researchers have obtained contradictory results, and links between microbial removal and environmental factors have still not been definitively understood.

Another important design parameter for constructed wetlands is water depth. From practical experience, water depth in subsurface flow constructed wetlands has been normally set at 0.60 m because this is the maximum depth at which the roots and rhizomes of the macrophytes grow, and it is therefore the maximum depth at which the macrophytes can have effects on the process (Cooper et al., 1996). The mass transfer theory dictates that water depth influences the oxygen transfer coefficient from the atmosphere to the water. Water depth also determines the fraction of water volume in contact with the underground biomass of the macrophytes. Therefore, despite the fact that only the surface area is in contact with atmosphere, it can be reasonably supposed that water depth influences the efficiency of the subsurface flow constructed wetlands. However, the information available on the effect of water depth is scarce and contradictory (US EPA, 2000; Coleman et al., 2001).

Thus, the removal of pathogens in subsurface flow constructed wetlands used for wastewater treatment seems to be a very complex process and may vary in time and space, depending on many factors. Even though possible mechanisms of bacterial removal have been discussed in many papers (Burger and Weise, 1984; Armstrong et al., 1990; Morales et al., 1996; Decamp and Warren, 2000), no systematic analyses on the removal processes and the fate of potential pathogenic bacteria in constructed wetlands are yet known. In terms of bacterial removal, constructed wetlands are generally considered to feature a combination of chemical and physical factors, including mechanical filtration and sedimentation (Pundsack et al., 2001). Also, the relative importance of the different biochemical reactions that can take place in these natural treatment systems plays a determinant role on the efficiency of the system and the microbial removal.

In view of all the above, the objective of the present work was to evaluate and clarify the role of different design key factors, such as granular media, water depth and season effect, that could affect the removal of microbial indicators in order to improve our understanding of the microbial reduction in constructed wetlands. We have also performed a molecular analysis using denaturing gradient gel electrophoresis (DGGE) with the aim to determine the effect of wetland design on the microbial diversity of gravel biofilms.

2. Materials and methods

2.1. Pilot plant system

The pilot HSSF system used in this study treats part of the urban wastewater generated by the Can Suquet housing development in the municipality of Les Franqueses del Vallès (Barcelona, north-east Spain). A detailed description of the plant can be found in García et al.

(2004, 2005). The wastewater was previously screened and flowed to an Imhoff tank. After primary clarification, the effluent was equally divided and flowed to eight parallel HSSF constructed wetlands with a surface area of 54–56 m^2 each. The system had a valve and a flowmeter that allowed the adjustment and monitoring of the flow pumped to the wetlands. The plant began to operate in March 2001, when the beds were planted with *Phragmites australis*.

The aspect ratio of the HSSF constructed wetlands varied in pairs. Pair A had an aspect ratio of 1:1, B of 1.5:1, C of 2:1 and D of 2.5:1. Furthermore, the size of the granular medium within each pair also differed. Thus, type 1 contained a coarse granitic gravel (D60 = 10 mm, Cu = 1.6) while type 2 had fine granitic gravel (D60 = 3.5 mm, Cu = 1.7). For example, the HSSF constructed wetland A1 presented an aspect ratio of 1:1 and contained coarse gravel, while the HSSF constructed wetland A2 contained fine gravel and had the same aspect ratio. HSSF constructed wetland from types A, B, and C had an average water depth of 0.50 m, and D of 0.27 m.

All HSSF wetlands had 2 perforated tubes (0.1 m in diameter) inserted into the middle part of the gravel and uniformly distributed throughout the length of the bed, so that intermediate samples could be obtained. These tubes were perforated all along their depth, and they were installed at the bottom of the beds. They were referred to as P1 (near the inlet, at 1/4 of the length) and P2 (near the outlet, at 3/4 of the length). It should be taken into account that these perforated tubes had a minor impact on the flow field because of their small size in relation to the width of the HSSF wetlands.

2.2. Sampling

Influent and effluent water samples for evaluation of the removal efficiency of microbial indicators were taken once a month in types C and D HSSF constructed wetlands, from January 2003 to February 2005. Types A and B did not present significant differences in performance with type C, while type D differed in removal efficiency from the rest (Garcia et al., 2004, 2005). Thus, experiments were conducted in four HSSF wetlands: C1 and C2 were chosen as representatives of the deep beds, and D1 and D2 were chosen as shallow ones. The four wetland systems differ in terms of aspect ratio (length to width), granular medium size and water depth (Table 1). A 36 mm/day flow rate was used and all HSSF received the same flow and therefore they operated with the same hydraulic loading rate (HLR). The nominal hydraulic retention time (HRT) was 5.6 days for type C and 3 days for the type D.

In November 2004, samples from the perforated tubes (P1 and P2) and from deep (C1, C2) and shallow (D1, D2) wetland types were obtained for biofilm analysis. Gravel was quickly removed and placed in a container full of water in order to avoid drying of the biofilm and brought to the laboratory, where the contents were aseptically removed and analyzed. Prior to the analyses, gravel were rinsed with 200 mL of saline solution (NaCl 0.9%), to remove non-attached deposits and introduced into a plastic container with 15 mL of saline solution. Attached microorganisms were detached by sonication (3 min, 40 W), according to the procedures of the European Biofilm Workgroup, AGHTM Biofilm Group (1999). Details of the procedure of biofilm analyses can be found in Morató et al. (2005). For molecular analysis, aliquots of detached microorganisms were removed and centrifuged, and the pellets were then stored at −20 °C for DNA extraction. For epifluorescence microscopy (Nikon Optiphot, Barcelona), aliquots of samples were treated after sonication with a 2% v/v solution of formaldehyde, filtered through a Nuclepore filter (0.2 mm pore and 13 mm diameter) and examined to evaluate the number of total cells and viable microbial counts using Live/Dead (Molecular Probes, Inc.).

Heterotrophic plate counts (HPC) were carried out using the spread plate method with PCA agar (Merck). The plates were incubated for 72 h at 22 °C. The presence of fecal bacterial indicators such as total coliform bacteria (TC) and *Escherichia coli*, fecal enterococci (FE) and *Clostridium perfringens* and other microbial groups such as *Pseudomonas* and *Aeromonas* were assessed by membrane filtration according to standardized methods (APHA, 1995).

Table 1
Summary of the characteristics of the four SSF analyzed.

	SSF			
	C1	C2	D1	D2
Aspect ratio	2:1	2:1	2.5:1	2.5:1
Medium size (mm)	10	3.5	10	3.5
Porosity (%)	39	40	39	40
Water depth (m)	0.5	0.5	0.27	0.27

2.3. Statistical analysis

Statistical procedures for the evaluation of the effect of season, bed type (which included aspect ratio and water depth), and medium size on wetlands microbial removal performance were carried out using the SPSS statistical software package. One-way and three-way ANOVA methods were used to evaluate the influence of each factor considered for every microbial indicator parameter of the effluent and also to assess interactions between factors. For all ANOVA tests it was verified that the variables were distributed normally. Otherwise, the variables were log-transformed.

2.4. Molecular analysis of bacterial diversity

Bacterial DNA from gravel biofilm was extracted as described by Massana et al. (1997). Gravel samples were suspended in 2 mL of lysis buffer (50 mM Tris–HCl, pH 8.3; 40 mM EDTA, pH 8.0; 0.75 M sucrose). 0.5-mm-diameter sterile glass beads were added to the cultures, and vortexed three times during 30 s. DNA was extracted using the lysis/phenol extraction method as described below. Lysozyme (1 mg/mL final concentration) was added and samples were incubated at 37 °C for 45 min in slight movement. Then, sodium dodecyl sulfate (1% final concentration) and proteinase K (0.2 mg/mL final concentration) were added and samples were incubated at 55 °C for 60 min in slight movement. Nucleic acids were extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1, vol:vol:vol), and the residual phenol was removed once with chloroform–isoamyl alcohol (24:1, vol:vol). Nucleic acids were purified, desalted and concentrated with a Centricon-100 concentrator (Millipore). DNA integrity was checked by agarose gel electrophoresis, and quantified using a low DNA mass ladder as a standard (Invitrogen).

Fragments of the 16S rRNA gene suitable for denaturing gradient gel electrophoresis (DGGE) analysis were obtained by using the bacterial specific primer set 358f-907rM (Sánchez et al., 2007). Polymerase chain reaction (PCR) was carried out with a Biometra thermal cycler using the following program: initial denaturation at 94 °C for 5 min; 10 touchdown cycles of denaturation (at 94 °C for 1 min), annealing (at 63.5–53.5 °C for 1 min, decreasing 1 °C each cycle), and extension (at 72 °C for 3 min); 20 standard cycles (annealing at 53.5 °C, 1 min) and a final extension at 72 °C for 5 min.

PCR mixtures contained 1–10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 μM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) at a final concentration of 600 μg/mL was added to minimize the inhibitory effect of humic substances (Kreader, 1996). The volume of reactions was 50 μL. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen).

The DGGE was run in a DCode system (Bio-Rad) as described by Muyzer et al. (1988). A 6% polyacrylamide gel with a gradient of 40–80% DNA-denaturant agent was cast by mixing solutions of 0% and

80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). One thousand nanograms of PCR product was loaded for each sample and the gel was run at 100 V for 18 h at 60 °C in 1 × TAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SYBR Gold (Molecular Probes) for 45 min, rinsed with 1 × TAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Gel Doc EQ (Bio-Rad).

Prominent bands were excised from the gels, resuspended in milli-Q water overnight and reamplified for its sequencing. Purification of PCR products from DGGE bands and sequencing reactions were performed by Macrogen kit (South Korea) using primer 907rM and the Big Dye Terminator version 3.1 sequencing kit. Reactions were run in an automatic ABI 3730XL Analyzer-96 capillary type.

Sequences were subjected to a BLAST search (Altschul et al., 1997) to obtain an indication of the phylogenetic affiliation. Eighteen 16S rRNA gene sequences were sent to the EMBL database (http://www.Ebi.ac.uk/embl) and received the following accession numbers: from FN429742 to FN429759.

Digitized DGGE images were analyzed with Quantity One software (Bio-Rad). Bands occupying the same position in the different lanes of the gels were identified. A matrix was constructed for all lanes, taking into account the presence or absence of the individual bands. This matrix was used to calculate a distance matrix using hierarchical cluster analysis with the statistical software SPSS. Finally, a dendrogram comparing samples for each HSSF constructed wetland was obtained utilizing the unweighted-pair group method with average linkages (algorithm: Dice) and SPSS.

3. Results and discussion

3.1. Bacterial removal

All HSSF constructed wetlands received the same wastewater flow with the same quality. According to García et al. (2004, 2005), the shallow pair (D1, D2) had the lowest effluent concentrations of chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), ammonia and dissolved reactive phosphorus (DRP), and therefore it was more efficient in removing organic matter and nutrients. Thus, differences in performance between the shallow pair and the rest of the HSSF wetlands were not due to aspect ratio, but rather to water depth. The overall removal efficiency was approximately 60% (types A–C), and 75% (type D) for COD with an inlet value of 170 ± 55 mg/L; 60% and 80% for BOD_5 with an inlet value of 140 ± 54 mg/L; 30% and 50% for ammonia with an inlet value of 36.8 ± 11 mg/L; and 0% and 10% for DRP with an inlet value of 5.6 ± 1.9 mg/L, respectively. According to these conclusions, evaluation of the removal efficiency of microbial indicators, as well as molecular analyses, was performed in deep (C1, C2) and shallow (D1, D2) wetland types as representatives of different water depths.

Table 2 shows the overall averages and the standard error of the different microbial groups determined in the influent and the effluent water samples of deep and shallow wetlands (C and D types, respectively). The overall microbial inactivation ratio ranged between 1.4 and 2.9 log-units for heterotrophic plate counts (HPC), from 1.2 to 2.2 log units for total coliforms (TC) and from 1.4 to 2.3 log units for *E. coli*. The inactivation of *Pseudomonas* and *Aeromonas* ranged between 1.4 to 2.1 log units and 0.4 to 1.9 log units, respectively. For fecal enterococci (FE) and *Clostridium* it ranged between 1.4 to 2.2 log units and 1.2 to 1.6 log units respectively. The shallower HSSF wetlands, especially D1, showed a lower efficiency in the removal of the classical bacterial indicators such as TC, *E. coli* and FE. A similar trend was observed in the *Pseudomonas* group and with lesser differences, in the *Aeromonas* group. On the other hand, the shallower wetlands showed a higher efficiency in the removal of *Clostridium* spores.

Table 3 shows the ANOVA analysis of data from water from the outlet of the treatment plant, performed in order to assess the effect of the treatments (HSSF wetland type, granulometry and season) on microbial removal. The presence of fine granulometry strongly influenced the removal of each bacterial group. This effect was significant for TC ($p = 0.009$), *E. coli* ($p = 0.004$), FE ($p = 0.012$) and *Clostridium* spores ($p = 0.036$). Removal of the last bacterial groups including *Clostridium* spores was higher in summer season, and this effect was significant for TC ($p = 0.011$), *E. coli* ($p = 0.028$) and *Clostridium* spores ($p = 0.001$).

Although no clear relationship could be found between microbial removal and HSSF constructed wetland type, ANOVA interaction terms indicated that this fact was largely dependent on granulometry. In this sense, shallow HSSF wetlands were more efficient for removing total coliforms ($p = 0.011$) and *E. coli* ($p = 0.013$) when fine granulometry was used. Also, shallow HSSF wetlands were more effective for removing *Clostridium* spores ($p = 0.039$) but, in this case, this effect was independent of granulometry.

Granular media type and its granulometry can be identified as another key factor in the microbial removal process (Polprasert and Hoang, 1983). On average, smaller granular media improve the microbial inactivation ratio between 1 and 2 log units for both fecal coliforms and somatic coliphages (Ottová et al., 1997; García et al., 2003). Garcia et al. (2005) reported that the slightly higher removal efficiency observed in HSSF constructed wetlands with a fine medium occurs in conjunction with a clearly greater macrophytes development (1800 and 600 g/m^2 of dry weight for aerial and underground biomass, respectively) than that observed in HSSF constructed wetlands with a coarse gravel (540 and 270 g/m^2, respectively). As a consequence, in shallow HSSF wetlands, a larger fraction of the water volume will be in contact

Table 2
Overall averages[a] and standard error (n = 17) of the different microbial groups determined in the influent and the effluent of SSF types C (deep) and D (shallow).

	Influent	C1	C2	D1	D2
Heterotrophic plate count (HPC)	1.73E+07	5.73E+05	1.90E+04	4.84E+05	4.70E+05
	9.24E+06	*5.65E+05*	*5.54E+03*	*3.66E+05*	*4.35E+05*
Total coliforms (TC)	4.40E+07	2.52E+05	2.30E+05	2.27E+06	4.88E+05
	1.62E+07	*3.30E+04*	*5.87E+04*	*9.31E+05*	*1.66E+05*
Escherichia coli	6.32E+06	4.13E+04	4.26E+04	2.17E+05	3.17E+04
	3.43E+06	*9.35E+03*	*1.40E+04*	*8.63E+04*	*1.82E+04*
Fecal enterococci (FE)	3.16E+05	7.04E+03	3.70E+03	1.22E+04	1.97E+03
	6.95E+04	*3.02E+03*	*1.29E+03*	*3.35E+03*	*3.35E+03*
Clostridium perfringens spores	1.56E+05	9.72E+03	7.20E+03	9.50E+03	4.00E+03
	6.54E+04	*5.19E+03*	*2.09E+03*	*7.68E+03*	*1.80E+03*
Pseudomonas	1.61E+08	1.19E+06	3.31E+06	6.73E+06	1.18E+06
	4.96E+07	*2.38E+05*	*1.35E+06*	*2.70E+06*	*5.02E+05*
Aeromonas	1.75E+07	2.21E+05	3.02E+05	6.26E+06	3.07E+05
	6.57E+06	*6.86E+04*	*9.45E+04*	*5.55E+06*	*1.20E+05*

[a] CFU/mL for HPC and CFU/100 mL for all other microbial parameters.

Table 3
Probabilities of the ANOVA test on the effects of the factors (one-way) and their interactions (three-way) on the bacterial indicators from the outlet of the treatment plant (log CFU/mL or CFU/100 mL).

Factor	HPC 22 °C	Total coliforms	*Escherichia coli*	Fecal enterococci	*Clostridium* spores
Type of CWs	***Deep***	***Deep***	**Deep**	**Deep**	***Shallow***
	0.005	***0.041***	**0.047**	**0.049**	***0.039***
Granulometry	Fine	***Fine***	***Fine***	***Fine***	**Fine**
	0.16	***0.009***	***0.004***	***0.012***	**0.036**
Season	Winter	***Summer***	***Summer***	Summer	*Summer*
	0.98	***0.011***	***0.028***	0.61	*0.001*
CWsType * granulometry	***D-1/2***	***Sh-2***	***Sh-2***	Sh-2	Sh-2
	0.047	***0.011***	***0.013***	0.052	0.572
CWsType * season	–	***Sh-Summer***	–	–	
	0.79	***0.011***	0.17	0.22	0.126

Coarse gravel (1) and fine gravel (2) Significant values ($p < 0.05$) are in bold and italics.

with the root system of the macrophytes, maybe favoring the microbial removing process.

In order to assess the effect of the different treatments (HSSF wetland type, granulometry and season) on microbial removal, an ANOVA data analysis from the gravel biofilm of the two perforated tubes (P1, near the inlet; P2, near the outlet) was performed (Table 4). Similarly to the results obtained with water samples, the presence of fine granulometry strongly influenced microbial removal. This effect was significant for all bacterial groups analyzed, with the exception of *Clostridium* spores. The higher specific surface area available for microbial attachment in the fine medium, which is considered the main mechanism for phosphorus removal in constructed wetlands (Vymazal, 2003), could explain the better performance for those with fine gravel.

Samples obtained from the P2 sampler (near the outlet) showed less bacterial charge for all groups, indicating a significant effect of the HSSF constructed wetland length. Interestingly, it seems that shallow wetlands were more efficient to remove *E. coli* ($p = 0.009$) on the biofilm, and a similar trend was observed for FE and *Clostridium* spores. On the other hand, the removal of HPC (22 °C) and TC attached to the biofilm was higher in deep HSSF wetlands, especially those with fine granulometry. Concerning to the seasons effect, a higher removal of *E. coli* ($p = 0.048$) and FE ($p = 0.076$) was observed in summer, while HPC (22 °C) ($p = 0.003$) removal was higher in winter. A clear decrease of the HPC in winter as a consequence of the temperature effect could increase the number of the bacterial indicators – *E. coli* and FE – or can suppress its detection, due to the reduction of some coliform antagonists belonging to the HPC (LeChevallier and McFeters, 1985), that could cause injury to the coliform population.

Finally, with the aim to assess the effect of the treatments (HSSF wetland type, granulometry and season) on total and viable microbial counts (Live/Dead), another ANOVA data analysis from the gravel biofilm of the two perforated tubes (P1, near the inlet; P2, near the outlet) was carried out (Table 5). Samples obtained from the P2 sampler (near the outlet) showed lower total cell numbers ($p = 0.012$), indicating a significant effect of the HSSF constructed wetland length. Similar to plate cultures, shallow HSSF wetlands were more efficient for microbial removal, showing a significant decrease of the total cell numbers ($p = 0.001$) and, as a consequence, the live ($p = 0.002$) and dead ($p = 0.001$) cell counts.

The oxidation and reduction potential (E_H) profiles carried out in summer 2001 and winter 2003 in the perforated tubes of all the HSSF constructed wetlands used in the present study indicate that the water inside the wetlands was under reducing conditions. Nevertheless, the E_H values were higher in the shallower HSSF wetlands (on average, they ranged from −144 to −131 mV) compared to the deep ones (from −183 to −151 mV), and therefore shallow wetlands had more oxidized conditions. Rivera et al. (1995) speculate that the redox status of the constructed wetlands affects the microbial removal efficiency as it occurs in other wastewater treatment systems. Differences in redox status in HSSF constructed wetlands at different depths can be explained because the mass transfer coefficient of oxygen from the atmosphere to the bulk water is inversely proportional to water depth (Kadlec and Knight, 1996). Therefore, it can be concluded that a lower water depth promotes more energetically favorable biochemical reactions, with more oxidized conditions that give in turn more efficiency as it has been previously described for the same system (García et al., 2004, 2005).

3.2. Bacterial community composition

Analysis of the bacterial community composition by PCR-DGGE was performed on gravel biofilm samples from perforated tubes P1 and P2 collected in November 2004 for deep and shallow HSSF constructed wetlands (types C and D, respectively). Banding patterns for the 16S rRNA DGGE-PCR amplicons are presented in Fig. 1. The number of bands per lane varied from 13 to 25. Some differences could be observed in band position, intensity, and number of bands present in the different

Table 4
Probabilities of the ANOVA test on the effects of the factors (one-way) and their interactions (three-way) on the bacterial indicators from the gravel biofilm (log CFU/cm^2).

Factor	HPC 22 °C	Total coliforms	*Escherichia coli*	Fecal enterococci	*Clostridium* spores
Type of CWs	Deep	Deep	***Shallow***	Shallow	Shallow
	0.185	0.088	***0.009***	0.527	0.662
Granulometry	***Fine***	***Fine***	***Fine***	***Fine***	Fine
	0.001	***0.001***	***0.001***	***0.001***	0.278
Season	***Winter***	Winter	***Summer***	Summer	–
	0.003	0.258	***0.048***	0.076	
Inlet–outlet	***Outlet***	***Outlet***	***Outlet***	***Outlet***	***Outlet***
	0.035	***0.001***	***0.001***	***0.001***	***0.035***
CWsType*Granulometry	***Deep-2***	Deep-2	Shallow-2	Shallow-2	Shallow-2
	0.036	0.778	0.206	0.533	0.472
Granulometry*Inlet–outlet	2-Outlet	2-Outlet	2-Outlet	***2-Outlet***	2-Outlet
	0.07	0.283	0.365	***0.006***	0.301

Significant values ($p < 0.05$) are in bold and italics.

Table 5
Probabilities of the ANOVA test on the effects of the factors (one-way) and their interactions (three-way) on the microbial cell counts from the gravel biofilm ANOVA of data from the gravel biofilm (log CFU/cm^2).

Factor	TCN Total	TCN Live	TCN Dead
Type of CWs	***Shallow***	***Shallow***	***Shallow***
	0.001	***0.002***	***0.001***
Inlet–outlet	***Outlet***	Outlet	***Outlet***
	0.012	0.58	***0.001***
CWsType * inlet–outlet	Shallow-Outlet	Shallow-Outlet	***Shallow-Outlet***
	0.059	0.905	***0.001***

Significant values ($p < 0.05$) are in bold and italics.

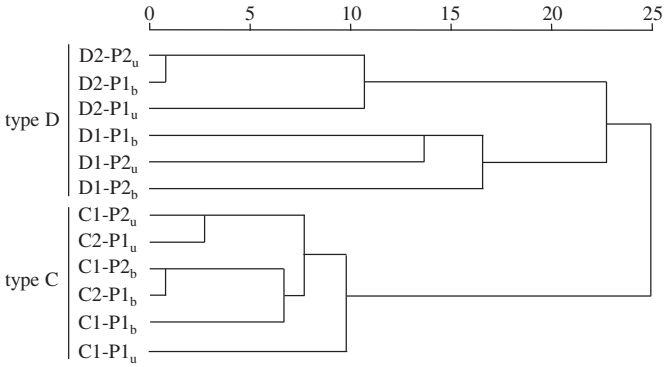

Fig. 2. Dendrograms generated from the DGGE profiles of the different samples analyzed for each wetland, determined by the unweighted-pair group method using average linkages. The scale bar is linkage distance (P1, near the inlet; P2, near the outlet; u: upper part; b: bottom part).

samples for each HSSF wetland analyzed, demonstrating different bacterial community developments.

The dendrogram based on the DGGE banding pattern (Fig. 2) separates the samples according to the different water depth designs. Thus, samples split in two main clusters corresponding to deep (type C) and shallow wetlands (type D), confirming that it was the main factor affecting microbial communities. For deep HSSF wetlands (type C), gravel size and sample position (P1 or P2 perforated tubes) in the two systems analyzed (C1 and C2) did not seem to exert a strong effect on bacterial community structure. On the contrary, samples from shallow wetlands (D1 and D2) were separated according to gravel size, although gravel position (P1 or P2) did not have any influence on the bacterial assemblage composition.

The number of DGGE bands is a representation of the diversity of phylotypes present in the different microbial assemblages. For type C and D2 wetlands, it was observed a higher band number (between 20 and 25) near the upper part of the inlet (samples P1u) compared to

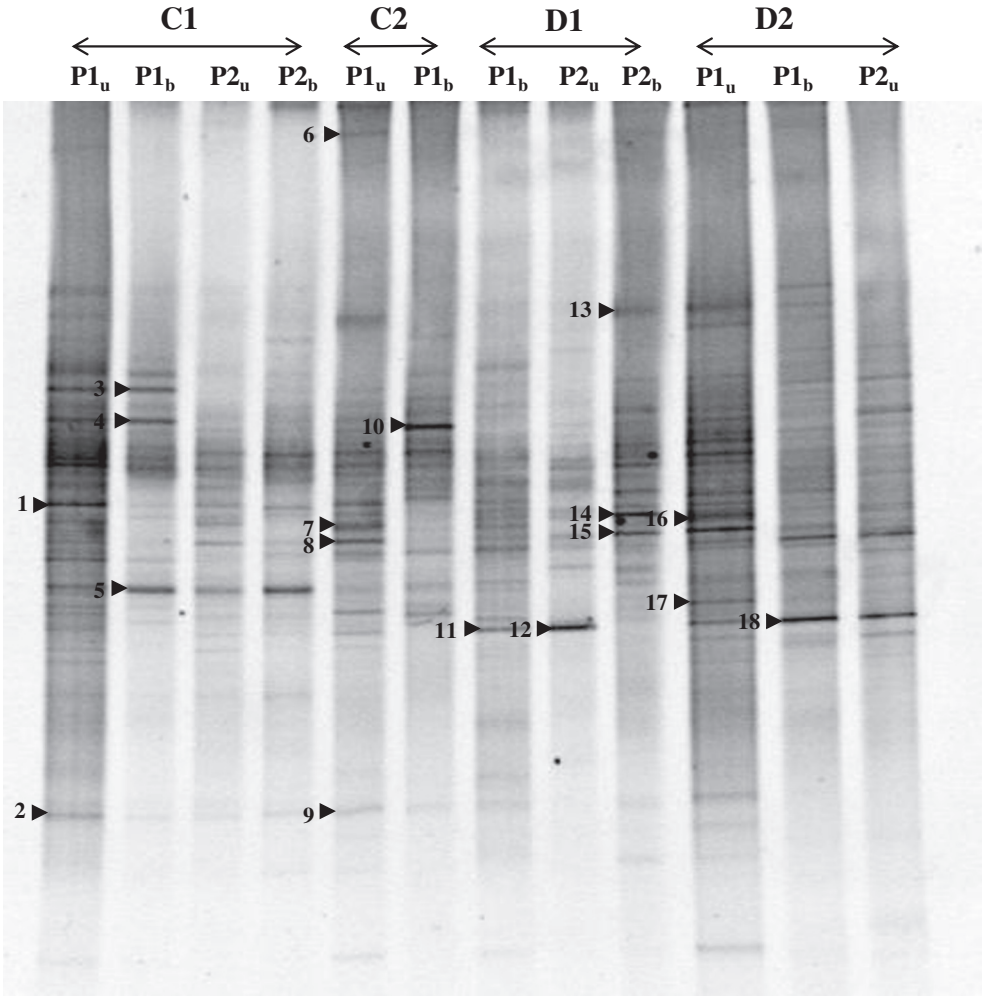

Fig. 1. DGGE fingerprints from samples of gravel biofilm obtained from different positions (u: upper part; b: bottom part) of P1 (near the inlet) and P2 (near the outlet) perforated tubes of constructed wetlands C1, C2, D1 and D2.

Table 6
Phylogenetic affiliation of sequences obtained from DGGE bands, with closest uncultured and cultured matches. Number of bases used to calculate the sequence similarity is shown in parentheses in the third column.

Band	Closest match	Similarity (%) (no bases)	Taxonomic group	Acc no. (GenBank)	Cultured closest match (% similarity)
FRV03-1	Uncultured *Catellibacterium* sp.	97.5 (505)	αproteobacteria	EU887785	*Rhodobacter* sp. (96.5)
FRV03-2	Uncultured bacterium	93.5 (488)	Acidobacteria	AB291529	*Pelobacter carbinolicus* (80.4)
FRV03-3	Uncultured bacterium	86.3 (441)	Synergistetes	EU864485	*Synergistes* sp. (81.8)
FRV03-4	Uncultured bacterium	89.3 (460)	Firmicutes	FJ390537	*Clostridium ruminantium* (88.3)
FRV03-5	Uncultured *Deferribacter* sp.	99.6 (518)	Deferribacteres	EU887800	*Aminobacterium colombiense* (88.5)
FRV03-6	Unidentified bacteria	96.5 (517)	Bacteroidetes	AJ224942	*Owenweeksia hongkongensis* (90.7)
FRV03-7	*Desulfococcus biacutus*	93.7 (463)	δproteobacteria	AJ277887	
FRV03-8	Uncultured bacterium	92.8 (505)	β-proteobacteria	DQ836763	*Denitratisoma oestradiolicum* (89.9)
FRV03-9	Uncultured Acidobacteria bacterium	99.6 (525)	Acidobacteria	DQ383312	*Desulfuromonas alkaliphilus* (85.9)
FRV03-10	Uncultured *Thiobacillus* sp.	98.9 (539)	β-proteobacteria	AY082471	*Thiobacillus sayanicus* (97.4)
FRV03-11	Candidatus *Nitrospira defluvii*	100 (532)	Nitrospirae	EU559167	
FRV03-12	Candidatus *Nitrospira defluvii*	99.8 (532)	Nitrospirae	EU559167	
FRV03-13	Uncultured bacterium	95.7 (517)	Bacteroidetes	EU234211	*Flexibacter* sp. (85.2)
FRV03-14	Uncultured ß-proteobacterium	96.1 (519)	β-proteobacteria	AB113610	*Siderooxidans ghiorsii* (90.4)
FRV03-15	Uncultured bacterium	95.4 (520)	β-proteobacteria	DQ836763	β-proteobacterium G5G6 (93.6)
FRV03-16	Uncultured bacterium	96.5 (526)	β-proteobacteria	DQ836763	β-proteobacterium G5G6 (93.6)
FRV03-17	Uncultured bacterium	93.0 (412)	β-proteobacteria	EU925882	*Dechloromonas* sp. (80.8)
FRV03-18	Candidatus *Nitrospira defluvii*	100 (533)	Nitrospirae	EU559167	

the outlet (samples P2) and the bottom part of the inlet (samples P1b), indicating that sample position had some effect on diversity. Unfortunately, we could not obtain enough DNA for sample D1P1u.

A total of 38 band positions were excised and sequenced in order to determine their phylogenetic affiliation, although only 18 bands produced DNA sequences with enough quality. The closest matches (and percentages of similarity) for the sequences retrieved were determined by a BLAST search (Table 6). The number of bases used to calculate each similarity value is also shown in Table 6 as an indication of the quality of the sequence.

Different phylogenetic group sequences could be retrieved from deep and shallow wetlands (types C and D, respectively), which differed in water depth. Table 6 shows that sequence identity of most of the bands is closely associated with different species of bacteria from sludge or wastewater environments.

Some of them belonged to Beta-proteobacteria, and had uncultured closest matches; one of these sequences, although with a low similarity, was associated to *Denitratisoma oestradiolicum*, a denitrifying bacterium isolated from activated sludge of a wastewater treatment plant able to use 17-beta-oestradiol as the sole carbon and energy source (Fahrbach et al., 2006). Another sequence corresponded to an uncultured *Thiobacillus* sp. related to *Thiobacillus sayanicus*, an autotrophic sulfur-oxidizing bacterium able to utilize thiosulfate and hydrogen sulfide. In general, sequences from Beta-proteobacteria are recurrent in this kind of environments (Sánchez et al., unpublished).

Other sequences, particularly abundant in the shallower HSSF wetlands (type D), were related with a high similarity (near 100%) to a microorganism from the nitrogen cycle, Candidatus *Nitrospira defluvii*. This nitrite-oxidizing bacterium was described as selectively enriched from a nitrifying activated sludge from a wastewater treatment plant, and is capable to further oxidize nitrite to nitrate (Spieck et al., 2006). The abundance of this kind of organisms involved in nitrogen removal in wastewater treatment plants usually accounts for 1–10% of the total bacterial population (Daims et al., 2001; Juretschko et al., 2002).

Acidobacteria was another taxonomic group observed in our study. Sequences belonging to this widespread distributed phylum have been found traditionally in soil, aquatic environments and wastewater treatment plants (Ludwig et al., 1997; Juretschko et al., 2002). On the other hand, the cluster Alpha-proteobacteria was represented by *Catellibacterium* sp., a strictly aerobic microorganism originally isolated from activated sludge belonging to the 'Rhodobacter group' characterized by the absence of photosynthetic activity (Tanaka et al., 2004).

Uncultured members of other groups, such as Bacteroidetes, Synergistetes, Firmicutes and Deferribacteres were also retrieved. One of these sequences had a high similarity (99.6%) with *Deferribacter* sp., a genus usually found in hydrothermal vents, petroleum reservoirs and gas fields (Takai et al., 2003; Mochimaru et al., 2007). A sequence belonging to the Delta-proteobacteria and related to *Desulfococcus biacutus* was also detected, although similarity was low (93.7%). This strictly anaerobic sulfate-reducing bacterium, able of growing with acetone, was originally isolated from anaerobic digester sludge of a wastewater treatment plant (Platen et al., 1990).

In general, most of the sequences retrieved from deep HSSF wetlands (type C) gravel biofilm (bands 2, 3, 4, 5, 7, 8 and 9) corresponded to cultured closest matches represented by anaerobic microorganisms, in accordance with lower redox potential (E_H) measurements found in this type of systems. In contrast, closest matches of shallower HSSF wetlands (type D) were mainly related to aerobic bacteria.

4. Conclusion

Water depth and gravel granulometry are the two most important key design factors controlling the efficiency of subsurface flow constructed wetlands for wastewater treatment. During the period of activity evaluated, the HSSF wetland analyzed in this study with a water depth of 0.27 m and a granular medium with a size of 3.5 mm proved to be more effective than those designed with a water depth of 0.50 m and a granular medium size of 10 mm for the removal of TC ($p = 0.011$), *E. coli* ($p = 0.013$), FE ($p = 0.052$) and total cell numbers ($p = 0.001$). Microbial removal in all HSSF wetlands analyzed occurs mainly near the inlet by a combination of biological and physical mechanisms (i.e., sedimentation and filtration). Microbial removal effectiveness in HSSF wetlands with a fine medium can be partially explained by the fact that a larger fraction of the water volume is in contact with the root system of the macrophytes.

Water depth is an important parameter that should be taken into account for HSSF wetland design and for predictive performance models, although models currently available in the scientific and technical literature still do not include water depth as a variable. Measurements of E_H in different periods have shown that shallower HSSF wetlands have more oxidized conditions. Furthermore, microbial communities from constructed wetlands appear to be affected by water depth, being the communities from shallow HSSF wetlands more related to aerobic microorganisms, in accordance with the higher values of E_H found in this type of natural treatment systems.

Concerning to the season effect, a significant higher removal for bacterial indicators was observed in summer, while the HPC (22 °C)

removal was higher in winter as a clear effect of the temperature decreasing.

On the other hand, DNA-based molecular tools showed that the HSSF constructed wetlands analyzed in our study contain significant hidden diversity of unknown and uncultured microorganisms that have the potential to act as degraders of environmental pollutants. Therefore, further attempts to isolate the key microorganisms involved in these processes will be essential in order to explore the degradation capacity of the microbial communities developed in the different designed wetlands.

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