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Efficiency of natural systems for removal of bacteria and pathogenic parasites from wastewater

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ABSTRACT

A combined constructed wetland formed by a facultative pond (FP), a surface flow wetland (SF) and a subsurface flow wetland (SSF) was studied from December 2004 until September 2005 in north-western Spain in order to evaluate their efficiency in the removal of pathogenic and indicator microorganisms and to determine their relationships. Microbial removal ranged from 78% for coliphages to over 99% for helminth eggs, depending on the treatment system. The highest removal of indicator bacteria (total coliforms, *E. coli*, faecal streptococci and *Clostridium perfringens*) occurred in the stabilization pond, reaching 84%, 96%, 89% and 78%, respectively. However, the greatest removal of protozoan pathogens (*Cryptosporidium* and *Giardia*) and coliphages was found in the SSF wetland, 98%, 97% and 94%, respectively. In contrast, the SF wetland was most efficient in the removal of pathogenic parasites when considering superficial removal rates. Seasonal differences in organism removal were not statistically significant during the study period. First-order removal rate constants ranged from 0.0027 to 0.71 m/d depending on the microorganism and type of wetland. Significant correlations were found between pathogenic parasites and faecal indicators in the influent of the treatment system but not in the other sampling points suggesting that such relations varied along the system due to the different survival rates of the microorganisms.

1. Introduction

Natural wastewater treatment systems have been widely used over the last few years as an alternative to conventional systems for the sanitation of small communities due to their minimum electric requirements and low maintenance costs (Mara et al., 1992; Brix, 1994; Vymazal, 2002; Bécares, 2006; Puigagut et al., 2007). Previous studies have focused attention on the ability of these systems to reduce microorganisms from wastewater, especially indicator microorganisms like the coliform group of bacteria (total and faecal coliforms) and the faecal streptococci group of bacteria (e.g. García and Bécares, 1997; Perkins and Hunter, 2000; Hench et al., 2003). Alternative faecal indicators (coliphages, spore-forming anaerobic bacterium *Clostridium perfringens*) and pathogenic microorganisms, i.e., protozoan parasites and helminths, have also been incorporated in order to provide better information on disinfection capacities in natural systems for wastewater treatment (Grimason et al., 1996; Karpiscak et al., 2001; Stott et al., 2003). Encysted organisms are extremely resistant to the environmental stress and persist for longer time than conventional indicator bacteria being able to survive in water for several weeks depending on the temperature, physico-chemical characteristics, sunlight, etc., (Fayer et al.,

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1998; Araki et al., 2001; Karim et al., 2004). These observations are consistent with those made previously in surface waters and freshwaters sediments (Davies et al., 1995; Medema et al., 1997). However, one of the major limitations in this field of investigation is the lack of studies comparing removal rates of indicator bacteria and pathogenic parasites in natural wastewater treatment systems.

The aims of this study were (i) to evaluate the efficiency of a combined constructed wetland (facultative pond, surface flow wetland and subsurface flow wetland) in the removal of total coliforms, *E. coli*, faecal streptococci, *C. perfringens*, coliphages, helminth eggs and protozoan parasites (*Cryptosporidium* and *Giardia*) (ii) to study the relationships between faecal indicators and pathogenic parasites in the different aquatic environments of the system and (iii) to determine the mechanisms responsible for their removal.

2. Materials and methods

2.1. Site description

The study was performed in a full wastewater treatment plant in Cubillas de los Oteros, a small village (150 habitants) in the province of León (north-western Spain). This system was continuously fed with domestic raw wastewater at a flow rate of 20.04 m^3 day^{-1} with an organic load of 1.17 g $BODm^{-2}$ day^{-1}. The system consists of a facultative pond of 1073 m^2 surface, 1.6 m depth, 75.9 days hydraulic retention time (HRT) and an inlet organic load of 1.86 g $BODm^{-2}$ day^{-1} followed by a surface flow wetland planted with *Typha latifolia* (44 m^2, 30 cm layer of 6–8 mm gravel, 40 cm depth of water, 1.2 days HRT and an inlet organic load of 19.1 g $BODm^{-2}$ day^{-1}) and by a subsurface flow wetland planted with *Salix atrocinerea* (585 m^2, 55 cm layer of 6–8 mm gravel, 5.7 days HRT and an inlet organic load of 2.14 g $BODm^{-2}$ day^{-1}).

2.2. Sample collection

Wastewater samples were monthly collected from December 2004 until September 2005 from the different stages of the treatment system in sterile plastic containers (10 L for pathogenic parasites and 1 L for bacteria and coliphages) and transported to the laboratory for analysis. All samples were kept refrigerated until the microbiological analyses, which were done a few hours after sampling.

2.3. Microbiological analyses

Total coliforms, *E. coli* and faecal streptococci were detected by the membrane filtration method according to Standard Methods for the Examination of Water and Wastewater (APHA, 1998), using 0.45 μm pore-size membrane filters (Millipore Corp., Berdford, MA) and selective agars, Chromocult® (*E. coli* and total coliforms) and KF-streptococcus (faecal streptococci). *C. perfringens* was determined by tube dilution using SPS agar (Angelotti et al., 1962). Coliphages were detected by the double agar layer method using *Escherichia coli*, strain ATCC 13706, as described by Adams (1959). All bacteriological media were obtained from Merck (Darmstadt, Germany). *Cryptosporidium* oocysts and *Giardia* cysts were concentrated by calcium carbonate flocculation procedure (Vesey et al., 1993). Staining for visualization of oocysts/cysts was performed using specific fluorescent monoclonal antibodies (Aqua-Glo G/C Direct, Waterborne, Inc., New Orleans, La.) and epifluorescence microscope at 200 x and 400 x magnifications (Olympus BX 60 F5 equipped with Nomarski DIC optics, a blue filter (480 nm-excitation, 520 nm-emission) for the detection of FITC-mAb labelled oocysts and a UV filter block (350 nm-excitation, 450 nm-emission) for DAPI. The recovery efficiency of this method ranged from 30% to 60% (mean 44.1±10.8%, $n=5$). For these recovery studies, wastewater samples (10 L) previously determined to be negative for *Cryptopsoridium* were seeded with

Table 1 – Influent and final effluent average microbial densities (in $\log_{10}$ units for bacteria and coliphages) and removal efficiency (in %) at each stage of the combined constructed wetland

	Influent[a]	Removal (%)				Final effluent[a]
		Facultative pond (FP)	Surface flow wetland (SF)	Subsurface flow wetland (SSF)	Cumulative treatment system[b]	
Cryptosporidium (oocysts/L)	45.7* (7.55)	75.78	47.8	98.89	99.87	<1
Giardia (cysts/L)	280.94* (99.14)	87.49	63.08	97.69	99.91	<1
Helminths (eggs/L)	9.56* (10.05)	92.46	>99.99	–	>99.99	<1
Total coliforms (CFU/100 mL)	6.75* (0.66)	84.82	36.07	69.27	97.12	4.40 (1.53)
E. coli (CFU/100 mL)	6.35* (0.55)	96.81	37.56	72.02	99.33	3.23 (1.6)
Faecal streptococci (CFU/100 mL)	5.31* (0.78)	89.61	61.97	54.67	99.29	2.62 (0.96)
Clostridium perfringens (CFU/mL)	2.61* (0.87)	78.44	33.93	47.68	94.63	1.08 (0.62)
Coliphages (PFU/100 mL)	4.86 (1.45)	53.92	14.58	94.88	78.39	2.62 (3.11)

[a] Arithmetic mean (standard error in parentheses) of 10 samples (3 replicate/sample) collected monthly over 1 year sampling period. Values followed by an asterisk are significantly different (Tukey HSD test, $p<0.05$) when compared between influent and effluent concentrations.
[b] The cumulative treatment system removal was assessed by the difference between the influent and final effluent (SSF effluent), expressed as a percent (%).

Table 2 – Significant differences (ANOVA, Tukey HSD post-hoc test, $p<0.05$) in the removal efficiency of the microorganisms between the different wetlands

	Cryptosporidium	*Giardia*	Helminths	TC	*E. coli*	FS	*Clostridium perfringens*	Coliphages
FP-SF	0.00013	0.00013	–	0.00572	0.00332	–	0.04585	–
FP-SSF	0.00012	0.00078	–	–	–	–	–	–
SF-SSF	0.00013	0.00013	–	–	–	–	–	0.00866

Facultative pond (FP), surface flow wetland (SF) and sub-surface flow wetland (SSF).

1×10^4 purified *Cryptosporidium parvum* oocysts (Iowa Strain Lot# 60710, Sterling Parasitol. Lab., Tucson, Arizona). The concentration and further identification of intestinal helminth eggs in wastewater was performed using the modified Bailenger method applied to wastewater (Bouhoum and Schwartzbrod, 1989). The number of eggs L^{-1} in wastewater was subsequently determined by the McMaster technique. All microbiological analyses were conducted by triplicate.

2.4. Data analyses

Statistical analyses were carried out using the STATISTICA 6.0 software package (StatSoft, Inc., 2001). One way analysis of variance (ANOVA) was used to check the influence of each factor considered (type of wetland and seasonal variation) in the removal of microorganisms. Assumptions for ANOVA test were previously checked and data were logarithmically transformed ($\log_{10} X+1$) when necessary. Subsequent pair-wise comparisons were performed using Tukey HSD post hoc tests (HSD, honestly significant difference). Organisms removal kinetic constants were estimated following the equation: $\ln C_0/C_1=k_1/\mathrm{HLR}$ (García et al., 2004), where C_0 and C_1 are the influent and effluent organism concentration, k_1 is the first-order kinetic constant and HLR is the hydraulic loading rate.

3. Results

3.1. Microbial removal by constructed wetlands system

A summary of the average microbial concentration both in the influent and final effluent and average percent removal in the wetland is presented in Table 1. Other than coliphages, all microorganisms showed mean concentrations significantly higher (1–3 orders of magnitude) in the influent than in the final effluent. The overall removal in the system ranged from 78.39% for coliphages to >99.99% for helminths. The SSF wetland achieved the highest removal of protozoan pathogens (*Cryptosporidium* and *Giardia*) and coliphages, 98.8%, 97.6% and 94.8%, respectively. In contrast, the highest removal efficiencies for indicator bacteria were reported in the facultative pond (78–96%). Heminths, mainly *Ascaris lumbricoides* and *Hymenolepis* species (*H. nana* and *H. diminuta*), were only detected in raw wastewater and pond effluent. Eggs of *Trichuris trichiura*, *Toxocara* spp. and *Strongyles* were occasionally found.

Statistical differences ($p<0.05$) in the removal of the microorganisms were highly dependent on the type of wetland (Table 2), with higher differences for (oo)cysts than faecal indicators. Table 3 shows the microbial removal rates (cfu/cyst removed m^{-2} day^{-1}) in the different wetlands of the system. The SF wetland was most effective in the removal of (oo)cysts and eggs, both in winter and summer. In contrast, the facultative pond obtained the higher removals for all bacteria in winter but not in summer. The evaluation of the pair-wise post-hoc comparisons showed that there were almost no seasonal variation in microbial removal during the study period. Significant differences ($p<0.05$) were only observed in SF wetland between both periods (winter and summer) for *Giardia* and faecal streptococci.

In order to determine the effect of HLR in organism removal, kinetic constants (k_d) were assessed for each microorganism in the different wetlands of the system, both in winter and summer (Table 4). Due to the low differences in the flow during the study period, the linear regressions were not

Table 3 – Removal loading rates [a] in the different wetlands in winter ($n=4$) and summer period ($n=6$). Facultative pond (FP), surface flow wetland (SF) and sub-surface flow wetland (SSF)

	Winter				Summer			
	FP	SF	SSF	Total treatment system	FP	SF	SSF	Total treatment system
Cryptosporidium	2.81	3.21	2.13	2.7	2.79	3.38	2.28	2.71
Giardia	3.66	3.69	2.42	3.49	3.6	4.03*	2.58	3.47
Helminths	2.06	2.51	–	1.89	2.08	2.64	–	1.92
Total coliforms	9.02	6.88	5.76	8.87	8.83	7.01	7.74	8.7
E. coli	8.81	2.01	4.74	8.61	8.38	6.38	5.28	8.2
Faecal streptococci	7.45	1.79	3.94	7.25	7.55	7.93*	4.9	7.43
Clostridium perfringens	7.28	3.92	4.32	7.18	6.29	4.1	3.56	6.17
Coliphages	6.04	–	–	–	3.37	2.93	8.12	7.15

[a] The removal rates were expressed as log cfu or cysts removed m^{-2} day^{-1}. Values followed by an asterisk are significantly different (Tukey HSD test, $p<0.05$) when compared between winter and summer within the same wetland.

Table 4 – Organisms decay constants (k_d; m/d) in the different wetlands of the system for both winter ($k_{d,w}$) and summer ($k_{d,s}$)

Microorganisms	FP		SF		SSF	
	$k_{d,w}$	$k_{d,s}$	$k_{d,w}$	$k_{d,s}$	$k_{d,w}$	$k_{d,s}$
Cryptosporidium	–	–	0.0096	0.0842	0.0119	0.0359
Giardia	–	0.0027	–	–	0.0284	0.0721
Helminths	–	0.0312	–	–	–	–
Total coliforms	0.0494	0.0102	–	0.0688	0.0885	0.0903
E. coli	0.0529	–	–	0.7173	0.1002	–
Faecal streptococci	–	0.0087	–	–	–	–
Clostridium perfringens	–	–	–	0.0159	–	0.1803
Coliphages	–	–	–	–	–	–

Only significant regressions are shown. Facultative pond (FP), surface flow wetland (SF) and sub-surface flow wetland (SSF).

significant in some cases. The positive slopes of the linear regressions ranged from 0.0027 to 0.71 m/d, depending on particular organism and type of wetland.

3.2. *Relationship between intestinal parasites and indicator microorganisms*

Significant correlations (*r*-Spearman, $p<0.05$) were found in raw wastewater between pathogenic parasites (*Cryptosporidium*, *Giardia*, and helminths) and faecal indicators as total coliforms, *E. coli* and faecal streptococci (Table 5). However, no other significant correlations were observed for any of the studied sampling points. This absence of correlation could be due to the different rates of survival of the microorganisms in each wetland. On the other hand, significant statistical correlations (*r*-Spearman, $p<0.01$) were reported between the parasites and faecal indicators, except coliphages, when considering all samples (influent and effluents).

4. Discussion

Different processes may be involved in the removal of microorganisms in a natural wastewater treatment system. Their fate, distribution and survival is influenced by the type of wetland and associated factors that cause their removal or inactivation. In this study, we evaluated the removal efficiency of pathogenic and indicator organisms by a combined constructed wetland indicating the main decay factors for each microorganism.

Wetlands system effectively removed all microorganisms studied (Table 1), with reductions ranging from 78% to >99%, which are within the broad ranges previously reported in constructed wetlands treating domestic wastewater (Soto et al., 1999; Greenway, 2005; Morsy et al., 2007). Our findings showed that faecal streptococci and *E. coli*, commonly used as indicator bacteria, were the most reduced microorganisms (approximately 3 log). However, these reductions were not enough to achieve a final effluent that would meet the WHO guidelines faecal bacteria limit and recent revisions for use of treated wastewater in agricultural unrestricted irrigation (Blumenthal et al., 2000).

The type of wetland significantly influenced the removal efficiency of the microorganisms during the study period (Table 2). The facultative pond, due to their long retention time (>75 days), obtained the highest removal of indicator bacteria, both percentage and surface removal rates. Protozoan parasites and helminths were also effectively removed in this pond. These removal efficiencies were 1–2 orders of magnitude higher than previously reported by García et al. (2006) in a similar pond of 15 m^2 surface and 20 days HRT, where TC and FS removal rates of 7.05 and 5.84 log-cfu m^{-2} day^{-1} were found, respectively. Several processes could contribute to the higher bacterial removal observed in the facultative pond. Adsorption onto settleable solids and further sedimentation (Grimason et al., 1996) and solar irradiation (Curtis et al., 1992; Davies-Colley et al., 1997) are thought to be the main bacterial removal mechanisms in the pond although other factors as predation by antagonistic organisms (Manage et al., 2002), physico-chemical conditions (Araki et al., 2000, 2001) and toxins excreted by certain algae (Oufdou et al., 2001) could also affect the removal of microorganisms.

The SSF wetland was significantly more efficient in the removal of coliphages and protozoan cysts than SF and FP. However, the highest surface removal rates for pathogenic parasites were reported in SF wetland, probably due to their smaller surface (Table 3). Comparable coliphage removals, ranging from 90% to 95%, were found by previous authors in similar subsurface flow constructed wetlands (Thurston et al., 2001; Hench et al., 2003). Thurston et al. (2001) also observed in this type of wetlands that *Giardia* cysts and *Cryptosporidium* oocysts were reduced by an average of 87% and 64%, respectively, values slightly lower than those obtained in this study (Table 1). On the contrary, bacterial removal rates found in our wetlands (Table 3) were lower than reported by García et al. (2006) in similar wetlands, 10.23 and 7.29 log-cfu m^{-2} day^{-1} for total coliforms and faecal streptococci, respectively, in a SF wetland (3 days HRT) and 9.32 and 7.70 log-cfu m^{-2} day^{-1}, respectively, in a SSF wetland (3 days HRT). Filtration

Table 5 – Significant correlations (*r*-Spearman correlations coefficients, $p<0.05$) between pathogenic parasites and faecal indicators in the influent and when considering all samples from the influent and effluents

	Parasites	Total Coliforms	*E. coli*	Faecal Streptococci	*Clostridium perfringens*	Coliphages
Influent	*Cryptosporidium*	0.91	0.81	0.8	–	–
	Giardia	0.73	–	0.83	–	–
	Helminths	0.87	0.87	0.71	–	–
All samples	*Cryptosporidium*	0.73	0.67	0.8	0.65	–
	Giardia	0.72	0.69	0.8	0.63	–
	Helminths	0.7	0.68	0.77	0.63	–

and adsorption of microorganisms to root-substrate complexes and associated biofilm (Williams et al., 1995; Gerba et al., 1999; Sleytr et al., 2007) were shown to be the main removal mechanisms of intestinal parasites in these wetlands. Others mechanisms of microbial removal could be sedimentation (Quiñónez-Díaz et al., 2001; Stott et al., 2003; Karim et al., 2004) or predation (Decamp et al., 1999; Stott et al., 2001).

With regards to seasonal differences, some authors have observed higher removal efficiencies during the hot season (El Hamouri et al., 1994; Karathanasis et al., 2003) whereas others have not found seasonal changes (García et al., 2006). In our study, only the removal rates of *Giardia* cysts and faecal streptococci in SF wetland were significantly higher ($p<0.05$) in summer than winter period and no seasonal differences were observed for the rest of organisms. Some authors suggest that the performance of these systems in winter is worse due to reduced dissolved oxygen concentration in the root zone and lower microbial activity (Armstrong et al., 1990; Rivera et al., 1995). However, Quiñónez-Díaz et al. (2001) indicated that shading by vegetation may reduce exposure from UV light decreasing the removal of microorganisms. Mezrioui and Baleux (1992) and Hatano et al. (1993) demonstrated that the effect of temperature and seasonal variation on removal of microorganisms may vary with different pathogen species and type of wetland vegetation.

Previous works have demonstrated that the HLR is an important factor for the design of constructed wetlands (García et al., 2004). However, the lack of good adjustments in the linear regressions showed in the present study for most organisms (Table 4), due to the scarce flow variability in the system, demonstrated a no clear effect on the removal of microorganisms suggesting that other factors as temperature, hydraulic retention time, solar irradiation, physico-chemical characteristics, etc., should be taken into consideration when microorganisms decay models are assumed.

There are few papers at present evaluating the relationships between indicator organisms and pathogenic parasites in natural systems for wastewater treatment (Lemarchand and Lebaron, 2003; Karim et al., 2004; Savichtcheva and Okabe, 2006). Studies carried out in surface, river and marine waters have reported good correlations, especially between the *Cryptosporidium* oocysts concentrations and the levels of faecal bacteria (Hsu et al., 1999; Hörman et al., 2004). In this study, we found significant correlations (r-Spearman, $p<0.05$) between intestinal parasites and indicator bacteria in the influent of wetlands system and also when considering all samples (influent and effluents) demonstrating that these relations may also occur in wastewater (Table 5). Previous studies have shown contradictory results about these pathogen-indicator correlations in wastewater. Lemarchand and Lebaron, (2003) in France, observed significant correlations between *Cryptosporidium* and faecal coliforms (r-Spearman = 0.44, $p<0.05$) in treated wastewater effluents. On the contrary, no significant Spearman correlations between concentrations of indicator organisms and pathogens were observed by Harwood et al. (2005) in several wastewater reclamation facilities in the United States. However, these authors using a binary logistic regression detected weak correlations between faecal coliforms concentrations and *Giardia* presence/absence (r-square = 0.222), and between total coliforms and infectious *Cryptosporidium* presence/absence (r-square = 0.241), suggesting that there was pathogen-indicator interaction. Similarly, Karim et al. (2004) found die-off rates for faecal coliforms (0.256 $\log_{10}$ day^{-1}) approximately 10x faster than for *Giardia* cysts (0.029 $\log_{10}$ day^{-1}) in two constructed surface-flow wetlands demonstrating that pathogens and indicators may have different trends in the aquatic environments. Our results also showed that these relations varied along wetland effluents, in which no significant relationships were reported, probably as consequence of the different behaviour of the microorganisms in the natural systems, which is governed by numerous factors, as for example, type of wetland, water flow, physico-chemical conditions, climatic characteristics, organism survival, sedimentation, filtration, etc.

5. Conclusions

The combined use of different natural wastewater treatment systems removed significant amounts of pathogenic and indicator microorganisms. However, these reductions were not enough for a safety reuse of the final effluent. The type of wetland had a clear effect on the decay rate of the microorganisms being FP more efficient than SF and SSF in bacterial removal and SSF more efficient than SF and FP in protozoan parasites and coliphages removal. With the exception of *Giardia* and Streptococci in SF wetland, no other significant differences were found between winter and summer when considering removal loading rates. In addition, we found significant relationships between pathogenic parasites and faecal indicators in the influent of the wetlands system although these relations varied along the wetlands system due to different behaviour and survival of the microorganisms in each wetland.

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