Microbiological quality of reclaimed water used for golf courses' irrigation

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Abstract Microbial quality of reclaimed water used for irrigation in two golf courses located in the southern Iberian Peninsula (Spain and Portugal) was evaluated. Bacterial indicators for faecal pollution (total and faecal coliforms, *Escherichia coli* and enterococci) were tested by membrane filtration using appropriate selective media. In addition, somatic *E. coli* bacteriophages, enteric viruses (entero-, hepatitis A and rota-) and *Legionella pneumophila* were also analysed. The results obtained showed that all wastewater treatment processes reduced adequately the number of indicator microorganisms although a significant correlation between pathogenic and indicator microorganisms tested was not found. *L. pneumophila* was detected by PCR but not confirmed by culture. Survival experiments of pathogenic microorganisms in aerosols and irrigated turf are conducted to determine the health hazards for the golf practice and to propose a microbial standard for wastewater used for irrigation of golf courses.

Keywords Enteric viruses; golf courses; irrigation; *Legionella pneumophila*; microbiological quality; reclaimed wastewater

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Introduction

Water demand is increasing in arid and semi-arid environments especially in places where numerous recreational resort zones, such as golf courses, have been built. In these areas, it is very important to implement water conservation and recycling plans for a more efficient use of the water (Balogh and Watson, 1992). Turf grass irrigation in golf courses with treated wastewater is one of the most frequently applied strategies (Carrow, 1997) owing to several factors, including water shortage, the rising cost of freshwater and the availability of better quality reclaimed waters (Harivandi, 1997). The applicability of reclaimed water for golf course irrigation depends on the physical, chemical and microbiological quality (Peacok, 1997). Several guidelines and recommended criteria have been established for physical and chemical parameters (USEPA, 1992; Crook, 1997; Bahri *et al.*, 2001) whereas microbiological quality has not been regulated by any general criteria as yet.

The presence of pathogenic microorganisms in reclaimed water used for irrigation creates potential health hazards for the exposed human population. There are several possible routes of exposure to pathogens in reclaimed water, such as (a) the consumption of drinking water and vegetable products contaminated by reclaimed water and (b) the exposure to aerosols generated during spray irrigation with reclaimed water (Yates, 1997). For this reason, it is essential to assess the efficiency of the wastewater treatment and to perform microbiological analyses of the final effluent. Wastewater treatment can ensure 50 to $>99.99\%$ pathogen removal depending on the treatment process. Stewart (1990) reported that tertiary treatment, including disinfection, does not remove

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all pathogens from sewage samples. An important factor for health risk assessment associated with the use of reclaimed water is the infective dose of the organisms in relation to the final concentration. The minimum infective dose is 1 PFU (plaque forming unit) for enteric viruses, from 25 to 100 cysts for protozoa and >100 CFU (colony forming units) for bacterial pathogens (Rose and Gerba, 1991).

The likelihood of human disease occurring through the use of treated wastewater for turf grass irrigation is low (Mancino and Pepper, 1997). However, because a large number of enteric viruses and parasites are found in raw sewage, a potential risk does exist for disease, caused by organisms able to survive the wastewater treatment process, since they can be reintroduced into environments where direct human contact may occur (Moe, 2002). Microorganisms are responsible for numerous waterborne disease outbreaks, making necessary the determination of the microbiological quality of reclaimed water used for specific application (Fields, 2002). Hundreds of different microorganisms may be involved in waterborne disease outbreaks; therefore, the detection of every pathogen potentially present in water samples is impractical, even with the application of molecular techniques currently available. For this reason, the following indicator microorganisms of faecal pollution are routinely used to assess the microbiological safety of waters: total and faecal coliforms, *E. coli*, enterococci and somatic coliphages (Toranzos *et al.*, 2002).

In the present study, the microbiological quality of two golf courses located in the southern Iberian Peninsula was determined by studying the presence of faecal contamination indicators as well as the detection of *Legionella pneumophila* and three enteric viruses.

Materials and methods

Study area and sampling

Samples of water were collected from two golf courses located in the southern Iberian Peninsula. Golf course 1, considered one of the finest holiday resorts in Europe, is located on low-lying, dry and salty soil behind a ridge of sand dunes next to the beach. The area is constantly subjected to moderate to strong breezes, and thus the evapo-transpiration rate is very high. Reclaimed water used for irrigation comes from a wastewater treatment plant nearby. Golf course 2 is also irrigated with treated wastewater. The area is more sheltered than golf course 1, with many umbrella pines and gently undulating ground giving shade from the sun and wind. Water samples were collected in sterilised 500 mL Schott flasks and transported in a refrigerated box to the laboratory, according to Standard Methods (APHA, 1998).

Wastewater treatment

The wastewater plant of golf course 1 has primary treatment of a settling process to remove organic and inorganic solids. Screens vary from coarse to fine and are placed in a slanted receptacle to allow debris to be scraped off and disposed of. After this screening, the sewage passes into a grit chamber where materials (sand, cinders, small stones) settle to the bottom. Settled material is washed and used as landfill. At this point, wastewater still contains undissolved suspended matter that is removed during a primary clarification step. This material gradually settles out of the liquid and forms a mass of raw sludge that is drawn off into a digester and concentrated to be used as landfill. The remaining liquid in the settling tank constitutes the primary effluent. Secondary treatment is composed of aerated lagoons and four stabilisation ponds. The secondary liquid effluent is used as reclaimed water for turf and landscape irrigation, without further treatment.

The wastewater plant in golf course 2 consists of an anaerobic lagoon and a maturation pond. Primary treatment consists of harrowing the solids in suspension in the sewer

followed by a stabilisation pond of about 1 ha. Secondary treatment is carried out in a lake of ~30 ha. The total amount of reclaimed wastewater produced is approximately 3×10^6 m^3 per year.

Bacteriological analysis

The membrane filtration procedure (APHA, 1998) was used to enumerate the bacterial indicators. Appropriate volumes of water samples were filtered through 0.45 μm filters (HA, Millipore, USA). The quantitative analyses by membrane filtration technique were carried out in triplicate. Culture media and incubation conditions used were: (a) m-Endo LES agar (Difco Lab, USA) for total coliforms (36 ± 1 °C, 24 h); (b) m-FC agar (Difco) for faecal coliforms (at 44.5 ± 0.5 °C, 24 h); and (c) m-Enterococcus agar (Difco) for enterococci (36 ± 1 °C, 48 h). *E. coli* counts were carried out by membrane filtration on mFC agar (44.5 ± 0.5 °C, 24 h) with confirmation ß-glucuronidase activity determination according to the *in situ* MUG technique proposed by Gauthier *et al.* (1991).

Volumes of 500 mL treated wastewater were concentrated by filtration through 0.2 μm polycarbonate filters (Isopore GTTP type, Millipore) prior to analysis for *L. pneumophila*. The analyses were performed in duplicate – one filter processed for PCR and the other for culture. Filters were placed into plastic tubes containing 10 mL PBS (APHA, 1998), vortexed for 10 min to dislodge trapped bacteria, and filtered through 0.2 μm fluoropore filters (FGLP type, Millipore) using 13 mm Swinnex disc filter holders. Filters were washed with ethanol and rinsed twice with PBS.

For PCR detection, fluoropore filters were deposited into 1.5 mL Eppendorf vials and extractions performed using 120 μL lysis reagent (Chelex-100, Real, Durviz, Spain) followed by heating at 100 °C (10 min). After cooling, samples were centrifuged and the supernatant containing the DNA was collected and stored at −20 °C until the amplification process. The *L. pneumophila* kit REA40 (Real) was used for the molecular detection according to the manufacturer's instructions. PCR was performed in 100 μL of reaction mixture containing 79.6 μL first amplification solution (RA-LP, Real), 0.4 μL Taq DNA polymerase (R6, Real) and 20 μL extracted DNA. Amplification was performed using one cycle at 94 °C for 2 min, followed by 30 cycles of 15 sec at 94 °C, 15 sec at 50 °C and 15 sec at 72 °C. The final step was at 72 °C for 6 min. In each experiment, negative (R1 buffer) and positive (DNA from *L. pneumophila*) controls were included. Nested PCR was performed using 47.75 μL second amplification solution (RB-LP, Real), 0.25 μL Taq DNA polymerase (R6) and 2 μL first amplification products. Nested PCR generates a 471-bp DNA fragment in agarose gel electrophoresis.

For cultivation of *L. pneumophila*, the concentrated samples were subjected to acid and heat treatment to eliminate non-*Legionella* organisms (ISO, 1996). A volume of 0.1 mL was plated in duplicate onto both selective αBCYE-GVPC (glycine 3 g/L; vancomycine 5 mg/L; polymyxin B 100 IU/mL; cycloheximide 80 mg/L) and non-selective αBCYE media. Petri dishes were incubated for 5–15 d at 37 °C with 2.5% CO_2 in a humid environment. Confirmation was carried out by growth in media without cysteine and immunofluorescent staining (Lye *et al.*, 1997).

Virological analysis

The enumeration of somatic coliphages was performed by direct counting using the double-agar layer technique (Borrego and Romero, 1985) using *E. coli* C as host and modified Scholten agar (1.2 and 0.7% agar) as bottom and top agar layers, respectively (Havelaar and Hogeboom, 1983).

Enteric virus concentration was performed in the field using positively charged filters and filter cartridges (Zeta Plus, MK, AMF/CUNO, Sefiltra, Alcobendas, Spain) without

any amendment of the water (Bosch *et al.*, 1991). Each filter was eluted with 1 L 3% beef extract-0.05 M glycine buffer (pH 7.0). This was kept at 4 °C in contact with the filter during transportation to the laboratory. For viral elution, the pH was raised to 9.5 and the solution was back circulated through the filter twice. Neutralised eluates were concentrated by organic flocculation to a final 30 mL (Katzenelson *et al.*, 1978), and finally kept at − 80 °C.

Hepatitis A virus (HAV), enteroviruses (EV) and rotaviruses (RV) were assayed by RT-PCR (Bosch *et al.*, 2001; Sanchez *et al.*, 2002; Villena et al., 2003) using previously designed primers (Table 1). PCR amplification products were confirmed by Southern-blot hybridisation with digoxigenin-labelled internal oligonucleotide probes (Bosch *et al.*, 2001; Sanchez *et al.*, 2002; Villena *et al.*, 2003).

Results and discussion

In recent years, in arid and semi-arid zones golf courses have been frequently irrigated with reclaimed wastewater. The quality of reclaimed wastewater must be submitted to high quality requirements, both from a public health, environmental and agronomic point of view (Huck *et al.*, 2000; Bahri *et al.*, 2001). Therefore, the aims, procedures, and validation of this specific wastewater treatment should be clearly established to avoid any health hazard during the irrigation (Rose and Gerba, 1991; Asano and Levine, 1996). Physicochemical parameters tested along the wastewater route are shown in Table 2. Dissolved oxygen and pH increased from the inlet of the wastewater treatment plant up to the sprinklers. Minor variations of electrical conductivity and temperature were observed throughout the treatment. However, an important decrease in other chemical parameters, such as potassium, phosphate and ammonium was recorded.

The microbiological quality of the final effluent in both golf courses is shown in Tables 3 and 4. The abundance of all the indicators varied with the season, always being

Table 1 Primer sets used to detect human enteric viruses

Virus	Primer	Sequence	Length*	Reference
HAV	HAV 240	GGAGAGCCCTGGAAGAAAGA		Bosch *et al.* (2001)
	HAV 68	TCACCGCCGTTTGCCTAG	174 bp	Bosch *et al.* (2001)
EV	PV 444	CATTCAGGGGCCGGAGG		Shieh *et al.* (1997)
	P1	CGTTATCCGCTTATGTACTT	236 bp	Bosch *et al.* (1996)
RV	VP 6-3	GCTTTAAAACGAAGTCTTCAAC		Villena *et al.* (2003)
	VP 6-4	GGTAAATTACCAATTCCTCCAG	185 bP	Villena *et al.* (2003)

*Amplified fragment length corresponds to HAV strain HM 175 (M14707), poliovirus type 1 Mahoney strain (VO 1148), rotavirus serotype G1 strain WA (M21843)

Table 2 Physicochemical characteristics of the wastewater treatment plant of golf course 1

Parameter	Mean value			
	Raw wastewater	Primary treatment	Stabilisation pond	Secondary effluent
Temperature (°C)	27.2	27.3	27.7	26.1
Dissolved oxygen (mg/L)	2.20	3.50	12.84	15.50
Saturation (%)	27.66	44.81	164.34	191.42
pH	7.79	8.00	9.00	8.65
Conductivity (dS/m)	2.62	2.73	3.10	2.98
Potassium (mg/L)	0.21	0.20	0.04	0.03
Phosphate (mg/L)	11.60	5.90	0.80	0.90
Nitrate (mg/L)	0	0	0	0
Ammonium (mg/L)	12.10	16.50	0.18	0.05

Table 3 Indicator levels in secondary effluent of golf course 1 wastewater treatment plant

Sample No.	Levels/100 mL				
	Total coliforms (10^{-3} CFU)	Faecal coliforms (10^{-3} CFU)	*Escherichia coli* (10^{-3} CFU)	Enterococci (10^{-3} CFU)	Somatic coliphages (10^{-3} CFU)
1	80.00	1.50	1.50	Not tested	1.50
2	50.00	0.10	0.10	Not tested	1.20
3	72.00	3.80	3.60	0.07	6.60
4	5.00	1.80	1.80	0.20	0.20
5	12.00	1.60	1.50	0.05	2.00
6	21.00	1.00	0.80	0.17	1.50
7	33.00	4.80	4.50	0.82	2.60
8	Not tested	2.05	2.00	0.22	4.10
9	12.00	0.60	0.60	0.28	0.40
10	1.70	1.65	1.65	0.41	0.40

higher in golf course 2 than in golf course 1. The titre of somatic coliphages was similar in both golf courses (arithmetic mean 2.05×10^3/100 mL in golf course 1 vs. 3.35×10^3/100 mL in golf course 2). To establish the possible relationship between the indicators tested in wastewater treatment plants, a Pearson coefficient of linear correlation was applied. In golf course 1 a significant correlation ($p < 0.001$) was only obtained between faecal coliforms and *E. coli*, whilst in golf course 2 a significant correlation was obtained between faecal coliforms and *E. coli* as well as between total coliforms and enterococci.

Detection of *L. pneumophila* and enteric viruses in the wastewater effluent in both golf courses is shown in Table 5. *L. pneumophila* was only detected by molecular methods (PCR) in four samples (17.4%) from golf course 2, being unconfirmed by culture. No detection of the three types of enteric viruses assayed was recorded using the RT-PCR method.

Table 4 Indicator levels in secondary effluent of golf course 2 wastewater treatment plant

Sample No.	Levels/100 mL (all × 10^{-3})				
	Total coliforms (10^{-5} CFU)	Faecal coliforms (10^{-3} CFU)	*Escherichia coli* (10^{-3} CFU)	Enterococci (10^{-3} CFU)	Somatic coliphages (10^{-3} CFU)
1	18.60	0.32	0.30	Not tested	0.07
2	Not tested	0.20	0.20	19.20	0.70
3	90.00	0.68	0.60	18.30	0.17
4	Not tested	3.30	3.30	14.70	<0.01
5	55.50	68.00	60.00	6.00	10.60
6	18.00	80.00	76.00	12.40	15.00
7	20.00	188.00	150.00	16.10	0.49
8	7.50	150.00	150.00	3.55	3.93
9	3.05	28.50	26.00	2.35	4.11
10	49.00	27.00	25.00	0.10	11.50
11	9.50	310.00	300.00	0.18	4.25
12	22.50	7.50	7.50	0.24	3.03
13	7.00	Not tested	Not tested	1.30	0.57
14	0.70	Not tested	Not tested	0.39	0.30
15	1.85	Not tested	Not tested	0.16	0.35
16	Not tested	557.00	500.00	0.23	0.17
17	39.00	2.00	2.00	2.93	0.10
18	Not tested	0.20	0.20	<0.01	0.40
19	Not tested	0.10	0.10	<0.01	<0.01
20	275.00	28.90	27.00	131.00	20.00
21	365.00	2.80	2.80	415.00	1.00
22	137.00	0.10	0.10	219.00	0.25
23	57.20	0.40	0.40	107.00	0.01

Table 5 *Legionella pneumophila* and enteric viruses in secondary effluent

Sample site (*n*)	*L. pneumophila*		Enteric viruses (RT-PCR)		
	PCR	Culture	HAV	EV	RN
Golf course 1 ($n = 10$)	–	<2	–	–	–
Golf course 2 ($n = 3$)	–	<2	–	–	–
Golf course 2 ($n = 4$)	+	<2	–	–	–

The microbiological quality required for the final effluent depends on the irrigation method and on the irrigated crop (Brissaud *et al.*, 1991). However, only a few standards and criteria have been established for the determination of the microbiological quality of reclaimed water used for golf course irrigation. In the present study, the microbiological quality of the reclaimed water used in two golf courses located in the south of the Iberian Peninsula was studied (Tables 3–5).

All wastewater treatments reduced the number of the indicator microorganisms. In golf course 1, the primary treatment removed 74.7% enterococci, 91.8% faecal coliforms and 92.3% total coliforms. The secondary treatment was more efficient, showing removal percentages of 99.7, 99.4 and 98.7%, respectively. In golf course 2, primary treatment gave reductions of 39.6% total coliforms, 74% faecal coliforms and 20.2% enterococci, and in secondary treatment 99.93, 99.98 and 99.94%, respectively. Although the wastewater treatment did not remove all the indicators and pathogens, the data are in accordance with the typical percentages of microbial inactivation (Yates, 1997).

The presence of *L. pneumophila* and enteric viruses in the final effluents used for irrigation purposes in both golf courses was also studied. The results obtained (Table 5) indicated that all samples were negative for enteric virus detection and *L. pneumophila* was never confirmed by culture. Despite recent advances in molecular technology and the development of diagnostic techniques based on PCR, culturing is still the standard method for *Legionella* detection. However, several factors may affect the performance of this method, such as (1) low *Legionella* cell concentration, (2) the presence of *Legionella* organisms in a viable but non-culturable (VBNC) form, (3) growth inhibition by interfering microorganisms, and (4) *Legionella* cells' sensitivity to heat or acid treatment performed during culture procedure (Lye *et al.*, 1997; Bartie *et al.*, 2003). Previous studies have demonstrated the advantages of PCR-based assays for *Legionella* detection especially when compared with viable culture methods from environmental samples (Koide *et al.*, 1993; Martin *et al.*, 1993). Lye *et al.* (1997) demonstrated that 49/58 of ground water samples were positive by PCR but negative by culture. Although the advantage of a plating method is the provision of culturability information, negative results do not necessarily indicate a lack of viability and a lack of risk of infection (Hussong *et al.*, 1987).

Based on reported waterborne outbreak data, the risk of acquiring an illness from contaminated water in the US has been estimated to be approximately 4×10^{-5} /year or 2.8×10^{-3} during a lifetime (Bull *et al.*, 1990). This risk is probably underestimated since many waterborne outbreaks are not reported. The low minimum infective dose recorded for virus particles calls for efforts to control this risk. Increasing evidence suggests that classical bacterial indicators may not be adequate to reveal the presence of pathogenic viruses in water (Yates, 1997; Toranzos *et al.*, 2002). Several authors have proposed the use of bacteriophages as indicators of enteric viruses (Stetler, 1984; Jofre *et al.*, 1986; IAWPRC, 1991). The main reasons to support this proposal are: (1) bacteriophages are virus in nature; (2) they present similarities in size, shape and genetic make-up to human enteric viruses; (3) they are fairly stable in environmental waters and show

similar inactivation kinetics to disinfection as human enteroviruses; and (4) bacteriophage concentration in environmental waters correlates with sewage contamination. However, a lack of correlation between the presence/absence of several enteric viruses (hepatitis A viruses, enteroviruses and rotaviruses) and the somatic coliphages' concentration has been described in the present work (Tables 3–5). Borrego (1995) concluded that the use of somatic coliphages as indicators of enteric viruses is not always appropriate since there are many differences in origin and ecology between both groups of viruses. However, somatic coliphages may be good indicators of both water faecal pollution and water microbiological quality.

In order to minimise health hazards associated with the increasing use of wastewater for irrigation, it is important to monitor the microbiological quality of these reused waters using microbial indicators and reclaimed wastewater quality standards (Crook, 1997). The World Health Organisation (WHO, 1973) recommended that only reclaimed wastewater containing <100 faecal coliforms/100 mL might be used for irrigation without any restriction. The results obtained in the present study showed that only 10% of the wastewater effluent samples examined complied with this. More specifically for golf courses, the EPA guideline (USEPA, 1992) includes a limit of $<1-1{,}000$ faecal coliforms/100 mL depending on the considered state. In the present study, 30% of the samples of golf course 1 met the EPA criterion. In the case of golf course 2, 35% of the water samples tested were in this range although both golf courses fulfilled all the EPA physicochemical requirements (Table 2).

Conclusions

No correlation was found between pathogenic and indicator microorganisms tested in both golf courses; the indicators analysed were not adequate to determine the potential risk derived from the wastewater irrigation. On the other hand, the reclaimed wastewater should be submitted to tertiary treatment and disinfection processes before being used for irrigation purposes on golf courses. Finally, survival experiments of enteric viruses and coliphages on turf irrigated with unchlorinated secondary effluent are needed to propose a standard for viruses in wastewater used on golf courses.

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