

## Survival of enteric microorganisms on grass surfaces irrigated with treated effluent

J. P. S. Sidhu, J. Hanna and S. G. Toze

### ABSTRACT

Treated effluent can be reused for the irrigation of parks and sports grounds but there is an associated potential public health risks from microbial pathogens present on the grass surface, particularly when used for contact sports. The main aim of this study was to investigate the survival of pathogenic and indicator microorganisms on the grass surface of a sports ground irrigated with treated effluent under differing climatic conditions. Results showed that *Salmonella enterica* serotype typhimurium, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* decayed faster under direct sunlight than MS2 with one log<sub>10</sub> reduction ( $T_{90}$ ) varying from 3 to 11 hours. Rapid decay ( $T_{90}$  3 to 4 hours) of bacterial pathogens occurred in both sunlight and shade during the summer. In contrast,  $T_{90}$  times for the bacteria during the winter varied from 6 to 11 hours in direct sunlight and from 23 to 38 hours in shade. No significant seasonal variation was observed in the inactivation of the bacteriophage MS2. Enteric viruses are expected to show inactivation rates similar to MS2. The results show that rapid inactivation of enteric bacteria can be expected on grass surface irrigated with treated effluent at higher ambient temperatures, in direct sunlight and low moisture content.

**Key words** | bacteriophage, pathogens, pathogen survival, sports grounds, treated effluent, water reuse

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### INTRODUCTION

Increasing water shortages and unsustainable uses are regularly placing a strain on both human communities and the environment. A single use of water is viewed as an under utilization of a valuable resource and as an environmentally unsustainable practice. Large volumes of effluent are generated during wastewater treatment in municipal treatment plants which are commonly disposed off through outfalls into oceans and river environments. One potential alternative to this disposal option is reuse of the treated effluent for irrigation of ovals and parks following appropriate treatment.

Municipal wastewater generally contains a variety of pathogenic viruses, bacteria and protozoa. The majority of these pathogens are enteric in origin, although non-enteric pathogens such as *Staphylococcus aureus*, *Legionella* sp.,

*Mycobacterium* sp. and *Leptospira* can also be detected in wastewaters (Fliermans 1996; Neuman *et al.* 1997). During wastewater treatment, pathogen numbers generally decrease and the amount of the decrease dependent upon the extent and type of treatment. For example, anaerobic digestion of wastewater can achieve a 1 to 2 log<sub>10</sub> reduction of bacterial and viral pathogens (Horan *et al.* 2004). Additional treatment of the resulting effluent, including by chlorination, leads to further reductions (up to 3 log<sub>10</sub>) in pathogen numbers (Hassen *et al.* 2000; Hall & Sobsey 2001). However, it is usually uneconomic to treat wastewater to the extent that complete pathogen removal is achieved prior to reuse as an irrigation source for parks and sports grounds. Although wastewater treatment can significantly reduce pathogen numbers, sustained effluent

doi: 10.2166/wh.2008.029

application can result in potentially high pathogen loadings on grass surface. Consequently, there remain undetermined potential public health risks from microbial pathogens, in particular through the bacterial contamination of skin wounds and abrasions. Thus, the potential longer-term risk to users of effluent irrigated parks and sports grounds will be assessed in this study by determining the survival rates of different pathogens on the irrigated grass surface.

*Escherichia coli* from animal faeces have been reported to survive up to 6 months on the grass surface in winter (Avery *et al.* 2004). *E. coli* O157:H7 has been reported to survive on the lettuce and parsley for up to 77 and 177 days (Islam *et al.* 2004). It is also generally accepted that bacteriophage, enteric virus and bacteria have a better survival at low temperatures in water and on plant surfaces (Badaway *et al.* 1990; Allwood *et al.* 2003; Dawson *et al.* 2005). The decay of pathogens in environments can be due to a number of factors. These include a range of physical, chemical and biological factors which have been shown to influence the inactivation of pathogens in soils and pastures. These factors include temperature, moisture content, solar radiation, relative humidity, pathogen type, adsorption, the presence of utilizable organic matter and interaction with other microorganisms (Hurst *et al.* 1980; Yates & Yates 1988; Lewis-Jones & Winkler 1991; Jiang *et al.* 2002). Pathogen numbers on the grass surface irrigated with treated effluent are expected to decline over time. However, regrowth of coliform bacteria on the grass surface irrigated with effluent have been reported (Manios *et al.* 2006). The public health risks posed by the pathogens on the grass surface can only be determined by quantitative evaluation of survival/inactivation potential of pathogens and indicators on the grass surface irrigated with effluent. However, very little has been documented about the survival of pathogens on grass surfaces irrigated with effluent.

The objectives of this research were to determine whether there was a significant difference in the inactivation of pathogens on the grass surface irrigated with treated effluent during summer and winter and whether there is a difference in inactivation rate of pathogens under sun and shade. In the present study, the grass surface was irrigated with effluent which was seeded with selected

bacteria and the F-RNA phage, MS2 (used as an enteric virus surrogate). The rate of inactivation of each microorganism was then determined under full sun and shade conditions. This approach was used in both winter and summer experiments. Important factors believed to affect survival of enteric pathogen such as temperature, grass moisture content, solar radiation, wind speed and relative humidity were monitored throughout the study.

## MATERIALS AND METHODS

### Sports ground complex and irrigation regime

The sports grounds complex used for this study is located in Perth, Western Australia. Perth's Mediterranean climate is characterised by high temperatures, along with high solar radiations and evaporative indexes in the summer months with the vast majority of the yearly rainfall occurring in the later autumn and winter months. Annual rainfall averages at approximately 600–800 mm/year with 80% generally falling between May and September (Australian Bureau of Meteorology 2006). Average summer temperatures are around 30°C with temperatures above 40°C common in the mid to late summer months. Average winter maximum temperatures are approximately 20°C with minimum temperatures commonly less than 10°C.

The sporting complex consists of a number of grounds which are used for a wide range of training and sporting activities including athletics, several football codes, cricket and hockey. In addition, the complex is used by the local community for recreational walking and fitness training. Irrigation of the sporting complex with recycled water commenced in March 2004 (previously the complex had been irrigated with local groundwater). The irrigation system is supplied by recycled water from the Subiaco wastewater treatment plant. The recycled water is secondary treated effluent which has undergone further rapid sand filtration and chlorination (minimum 30 minute contact time) with a final chlorine residual of approximately 6–8 mgL<sup>-1</sup>. The recycled water is irrigated between the hours of 9 pm and 1 am and is allowed a minimum drying time of four hours (nominally 1 am to 5 am) prior to access by the public.

## Microbial quantification

The microorganisms tested in this study were *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ACM 1803), *Enterococcus faecalis* (ACM 2517), *Staphylococcus aureus* (ATCC 12600) and MS2 (ATCC 15597-B1). *S. typhimurium* and *E. coli* were cultured in nutrient broth (Oxoid), *E. faecalis* and *S. aureus* were cultured in brain heart infusion broth (Merck). All cultures were incubated overnight at 37°C. The bacteriophage MS2 was cultured in tryptone yeast extract broth (Oxoid) with an *E. coli* host HS(pFamp)R (ATCC 700881). MS2 culture was centrifuged at 6,000 rpm for 10 minutes and then passed through 0.2 µm membrane to remove bacterial cells. Purified MS2 culture was stored at 4°C in phage buffer. All the microorganisms were washed twice in sterile phosphate buffer (P-buffer) to remove culture media and then re-suspended in P-buffer prior to use in the survival experiments. All cultures were then acclimatised in sterile P-buffer overnight at room temperature prior to seeding. The suspension was used to seed previously sterilized (autoclaved) effluent to a final concentration of more than  $1 \times 10^5$  cfu mL<sup>-1</sup> of each pathogen.

All analyses for the quantification of pathogens were performed in triplicate. The quantification of MS2 was carried out by the standard double layer agar method (Havelar & Hogeboom 1984). All the bacteria were detected by spread-plating 100 µL of appropriate dilutions on the respective agar plates. *S. typhimurium* was detected on xylose lysine deoxychlorate agar (BBL), *E. coli* on Chromocult™ coliform agar (Merck), *E. faecalis* on Chromocult™ enterococci agar (Merck) and *S. aureus* on Baird Parker agar (Merck). Inoculated plates were incubated at 37°C overnight and then typical colonies were counted to determine the average number of colony forming units (cfu g<sup>-1</sup>) in the grass samples. Representative presumptive colonies for *S. typhimurium* and *E. coli* were confirmed after purification with Crystal non-fermenter ID system (BBL). Similarly, coagulase and catalase tests were performed for the confirmation of presumptive *S. aureus* and *E. faecalis* colonies.

## Survival experiments

The treated grass surfaces were either exposed to continual sunlight or were in full shade during both the winter and

summer tests. Aluminum frame grids (1 × 1 m outer dimensions with 5 × 5 cm internal grids) were laid on the grass surface to facilitate uniform sampling. One frame was used in the study area which was continually exposed to sunlight. A second grid was placed over an area of grass which had been completely shaded from direct sun light by a tarpaulin held approximately 1.5 metres above the ground by tent poles. The tarpaulin used in the study was found to cut up to 90% of solar radiation under bright sun (from 829 to 82 W/m<sup>2</sup>). The grass surface inside the frame was evenly irrigated with sterile effluent (5L) seeded with known numbers of the target microorganisms. For the summer experiment, the final microbial numbers on the grass surface were approximately 10<sup>7</sup> g<sup>-1</sup> while the starting numbers on the grass for the winter experiment were approximately 10<sup>4</sup> g<sup>-1</sup>. During the winter and summer survival experiments background grass samples were analysed and found to be free of microorganisms used in this study.

At each sampling event grass samples were collected from random duplicate grids within the frames in both the sunlit and shaded areas using sterile scalpel blades, with each sample matching the area of the grid square (5 × 5 cm). During the summer experiment each sample was split into the green surface grass blades and the denser thatch (brown portion) underneath. Grass and thatch samples were placed into separate sterile pre-weighed 50 mL polypropylene tubes containing 20 mL sterile phosphate buffer and stored at 4°C prior to analysis. For the winter experiment the grass and thatch were not separated and were tested as one sample. After recording the sample weight, each tube was sonicated in a Branson 3200 sonicator at a frequency of 60 Hz for 5 minutes and then vortexed for one minute. Extraction with phosphate buffer along with sonication gave consistent recoveries of bacterial pathogens from the grass surface in our laboratory studies (data not shown), and hence this method was chosen for the study. The final results were expressed as cfu g<sup>-1</sup> dry grass.

## Physical measurements

Temperature, solar radiation, grass moisture content and relative humidity were monitored during each of the survival experiments. Global solar radiation (direct plus diffused) were recorded both under sun and shade with an

automated Global Radiation Instrument (Unidata model 6501-F/G) connected to data logger (Unidata Starlogger 6004-2). The instrument has an arbitrary response to radiations in the range of 500–1,500 nm (visible to invisible). Grass temperature was recorded with a hand held temperature probe (Hanna HI8752). Moisture content of the grass samples was determined by drying samples in an oven at 100°C overnight and comparing the pre and post drying weights. Relative humidity and wind speed data for the local area during the study period was obtained from the Australian Bureau of Meteorology web site.

### Data analysis

One  $\log_{10}$  removal time ( $T_{90}$ ) in hours for each microorganism was determined from each plot using Equation (1) as described by Gordon & Toze (2003).

$$T_{90} = t/\log_{10}(C_t/C_0) \quad (1)$$

Where  $C_t$  is the final number at day  $t$ ,  $C_0$  is the number at day 0 and  $t$  is time interval. The average  $T_{90}$  was determined from the replicates of each microorganism. A Student's  $t$ -test was performed to compare the inactivation rates of different pathogens under different conditions. The critical  $P$ -value for the test was set at 0.05.

## RESULTS

### Environmental conditions

Measured grass moisture content remained above 70% under both sunlight and shade conditions during the winter sampling whereas rapid drying of the grass from 60 to 47% moisture content was observed during the summer in areas exposed to sunlight (Table 1). Temperatures at the grass surface did not differ between shaded areas and areas exposed to direct sunlight during either winter or summer, although the average temperature was higher in summer than in winter. Total solar radiation under direct sunlight was higher during summer than in winter and peak at solar noon. As anticipated solar radiation under direct sunlight was lower during the winter as compared to summer. Shade cloth was effectively blocking up to 90% of solar radiation during both summer and winter. During the summer, solar radiation in the grass thatch was found to be completely blocked (from 829 reduced to 3 W/m<sup>2</sup>) by the green grass leaves and dense thatch. It was also observed that green grass leaves absorb more than 90% of radiation which shades the thatch from the influence of sunlight.

### Microbial survival on oval surface

All of the tested microorganisms were observed to inactivation on the grass after application but the amount of

**Table 1** | Climatic conditions on the sporting complex ovals during summer and winter sampling events

Climatic condition	Sun				Shade			
	Mean	Median	Max	Range	Mean	Median	Max	Range
<b>Summer</b>								
Temperature (°C)	21.48	21.73	29	14–29	20.75	21.36	29.17	14–29
Solar radiation (W/m <sup>2</sup> )	492	515	771	20–771	50	53	72	7–72
Grass moisture (%)	60	61	72	47–62	62	61	75	56–66
Relative humidity (%)	36	33	62	24–62	–	–	–	–
Wind speed (km/h)	12.44	13	18	9–18	–	–	–	–
<b>Winter</b>								
Temperature (°C)	17.37	17.45	19.04	17–19	17.35	17.40	19.17	17–19
Solar radiation (W/m <sup>2</sup> )	246	243	530	3–530	24	25	48	0–48
Grass moisture (%)	79	80	93	65–93	74	74	80	65–80
Relative humidity (%)	57.50	47.5	86	43–86	–	–	–	–
Wind speed (km/h)	13.28	15	18	5–18	–	–	–	–

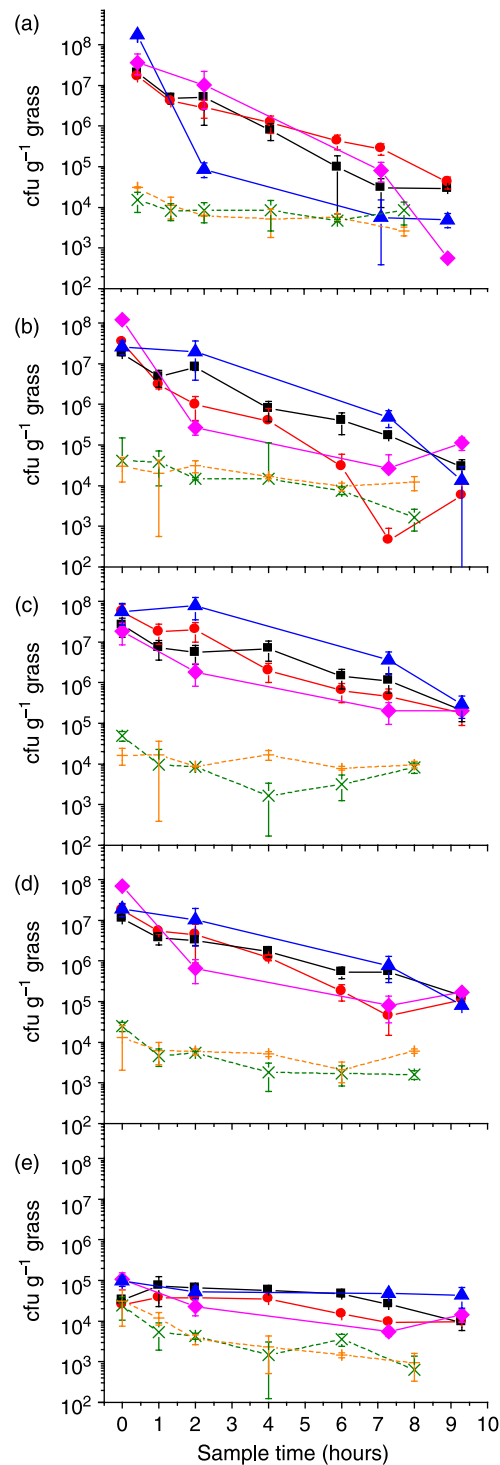
survival depended on the microorganism and the season tested. Inactivation patterns can be seen in Figure 1. The  $T_{90}$ s for each of the tested microorganisms under all of the conditions tested are given in Table 2.

In the summer experiment, the inactivation rates for all the bacteria were the same in both direct sunlight and shade, with all the  $T_{90}$ s being less than five hours (Table 2). The summer inactivation rates were the same both in direct sunlight and in shaded conditions. Similar  $T_{90}$  times were observed for the tested bacterial strains which had moved into the grass thatch beneath the upper green leaves of grass was and were thus less exposed to sunlight and environmental conditions. The viral surrogate MS2 had survived longer on and in the grass than the bacterial strains with  $T_{90}$  values of 9 hours or more. MS2 also differed from the bacterial strains in that its survival in the grass thatch under the shaded conditions was significantly longer (29.5 hours) than on the grass surface under both sunlight and shade conditions or in the thatch in direct sunlight (9–14 hours).

The inactivation of the tested microorganisms was slower in the winter experiment with reduced maximum air temperature and solar radiation along with increased grass moisture and atmospheric humidity. In direct sunlight during the winter experiment the bacteria had  $T_{90}$  values ranging from 6 to 11 hours. When under shaded conditions all of the bacteria had  $T_{90}$  values much greater than in direct sunlight with times varying from 23 to 39 hours which was statistically significant ( $t_{0.025}(6) = 2.44$  lower than calculated value 6.07). As in the summer experiment, the virus surrogate MS2 inactivation was different from the bacteria. The  $T_{90}$  decay times for MS2 in direct sunlight and shade were virtually the same with a sunlight decay time of 13 hours and 12 hours for shaded conditions. Apart from the grass thatch in shaded conditions in the summer experiment, the  $T_{90}$  values for MS2 did not vary significantly between the summer and winter experiments.

## DISCUSSION

The pathogens and indicator microorganisms used in this study are commonly found in the treated effluent. *E. coli* and *E. faecalis* are commonly used indicators to monitor inactivation of pathogens and MS2 is used as a surrogate for



**Figure 1** | Inactivation of (a) *E. coli*, (b) *S. typhimurium*, (c) *S. aureus*, (d) *E. faecalis* and (e) MS2 where (■) were cells on the grass surface in full sun in summer; (●) were cells on the grass surface in shade in summer; (▲) were cells in the grass thatch in full sun in summer; (◆) were cells in the grass thatch in shade in summer; (X) were cells on the grass in full sun in winter; and (+) were cells on the grass in shade in winter.

**Table 2** | One log<sub>10</sub> (T<sub>90</sub>) inactivation time (hours) of enteric microorganisms on the grass during the study

Microorganism	Summer		Thatch		Winter	
	Sun	Shade	Sun	Shade	Sun	Shade
<i>E. Coli</i>	3.3	3.6	3.5	3.3	8.4	35.0
<i>S. typhimurium</i>	2.5	3.3	3.5	3.2	6.6	23.3
<i>S. aureus</i>	3.7	4.4	5.4	4.7	11.7	39.3
<i>E. faecalis</i>	4.2	4.9	4.1	4.5	7.7	27.8
MS2	14.3	9.2	12.2	29.5	12.5	12.5

enteric virus inactivation. *Staphylococcus aureus* is known to cause skin infections and *Salmonella typhimurium* is a leading cause of bacterial gastroenteritis. Results of this study demonstrate that pathogens from recycled wastewater can be expected to survive for only a limited period of time on grass surfaces irrigated with effluent where there is high sunlight, and evaporative index. Short survival times were observed in the higher summer temperatures and sunlight, along with reduced moisture content and humidity. However, significantly longer survival times for bacterial pathogens ( $t_{0.025}$  (14) = 2.14 lower than calculated value 3.49) were observed during the winter season as compared to summer under both sun and shade.

The inactivation rates of seeded enteric bacteria were significantly faster (3 to 4 hours) during the summer months under both sun and shade conditions than in winter conditions (> 6 hours). Inactivation rates of MS2 and *E. coli* in water have been previously shown to increase with an increase in incubation temperature and in the presence of direct sunlight (Allwood *et al.* 2003; Sinton *et al.* 2006). Badaway *et al.* (1990) reported that the rate of inactivation of coliphage on grass irrigated with sewage increased from 0.17 h<sup>-1</sup> (T<sub>90</sub> 5.8 hours) in winter to 0.45 h<sup>-1</sup> (T<sub>90</sub> 2.2 hours) during the summer. In this study, during the winter season under shade, significantly lower inactivation rates, varying from 6 to 11 hours in direct sunlight and 23 to 38 hours in shade, were observed. This is possibly due to the combination of low temperature, high moisture and low intensity of solar radiation as compared to the summer conditions. Sinton *et al.* (2006) observed significantly higher

inactivation rates of *E. coli* and *Salmonella* in both river and sea water during summer as compared to winter under sunlight. They suggested that these differences were due to variations in temperature and solar indexes between the two seasons.

The significant difference between the winter and summer decay rates could also be attributed to the level of moisture present on the grass. No significant differences were observed between the bacterial survival rates during the summer in both shade and under sun indicating that the loss of water through rapid evaporation may be a major factor leading to decay of pathogens. This is supported further by the slow decay of the bacteriophage which is more resistant to desiccation as compared to bacteria. Moist conditions are essential for the viability of metabolically active bacteria. Bacterial cell inactivation is enhanced at less than 50% moisture (Ward *et al.* 1981) whereas, enteroviruses can survive in soil with as little as 10% moisture content (Yeager & O'Brien 1979). During this study, grass moisture content under sun and shade in summer was similar along with bacterial inactivation rates. However, grass moisture content during winter was significantly higher than the summer and a significantly slower inactivation rate of bacterial pathogens was observed during the winter. Consequently, grass moisture content appears to be a significant factor which influences inactivation of bacterial pathogens.

The specific survival times of enteric pathogens on the grass was also influenced by the type of microorganism. On the basis of inactivation rates (T<sub>90</sub>), *S. typhimurium* is least resistant to inactivation whereas, MS2 is significantly more resistant to inactivation as compared to *E. faecalis* ( $t_{0.025}$  (8) = 2.30 lower than calculated value 2.86) under most of the conditions studied. Bacteriophage, MS2 would be expected to survive for longer periods than bacteria cells as virus particles have been shown to be more resistant to adverse climatic conditions. Sinton *et al.* (2002) found F-RNA phage more resistant to inactivation in river water under direct sunlight followed by *E. coli* and *Enterococci*. Similarly, Fujioka & Yoneyama (2002) reported that enteric viruses (poliovirus, echovirus and coxsackievirus) survive better in seawater under sun than *E. coli* and *E. faecalis* and the inactivation rate of *E. coli* is twice that of *E. faecalis*. In this study, during the winter *S. aureus* (12 hours) survived longer than *E. coli* and *S. typhimurium* (6 to 7 hours) at 17

to 19°C temperature. However, at higher temperatures of 29°C (summer) there was no significant difference in the rate of inactivation of bacterial pathogens in either shade or direct sunlight. This was possibly due to the fact that high temperature and low moisture content play a more significant role in the inactivation of a bacterial pathogen than pathogen type. Although the results of published studies are not directly comparable to our study due to the difference in the medium and incubation conditions, however, there is an agreement that the rate of inactivation is influenced by the type of microorganism.

Bacteriophages such as MS2 are generally considered as suitable indicators for the presence and behaviour of enteric viruses due to their similar structure, morphology, composition and size to enteric viruses (Brion *et al.* 2002). However, there is often conflicting information available on the relative stability of MS2 and enteric viruses. F-specific RNA coliphage have reported to be more stable than human enteric virus in environmental water (Sinton *et al.* 2002) whereas, Chung & Sobsey (1993) reported that they are more sensitive to high temperature and die off faster than the enteric viruses. Consequently, a direct estimation of the enteric viruses inactivation rates on the grass surface based on the MS2 inactivation pattern is difficult to make. However, it is expected that enteric viruses will survive better than bacterial pathogens and are expected to show similar inactivation rates to MS2.

Results of this study suggest that exposure to direct sunlight is one of the major factors which affected the inactivation of microorganisms on the grass surface. The inactivation rates of the microorganisms was observed to be 2 to 3 fold faster at low winter temperatures in direct sunlight than in the shade. Sinton *et al.* (2002) reported that at 14°C in direct sunlight, the inactivation of enteric pathogens in fresh water was 10 times higher than in the absence of sunlight. It is expected that the inactivation of pathogens on the grass surface is more rapid due to exposure to direct sunlight. In this study during the summer months no significant difference in the rate of inactivation of pathogens on the grass surface or in the thatch was observed. It is possible that high temperatures along with rapid drying, lead to rapid inactivation of pathogens in the thatch.

While  $T_{90}$ s are useful for comparing the rates of inactivation of enteric pathogens, the decline of pathogen

numbers is not always linear. After an initial rapid inactivation, the decay rate was observed to slow under certain conditions such as low temperature, high moisture content and shade. Consequently, care must be taken in extrapolating the finding of this study to other sites with different climatic conditions. The results of this study suggest that different inactivation rates of enteric pathogens under different climatic conditions are to be expected. In general, pathogens are expected to survive longer on grass surfaces under trees and other shaded areas when ambient temperatures and evaporative indexes are low. Consequently, adequate non-contact drying time of the grass surface prior to sporting activities may remain a requirement to minimize potential public health risks.

## CONCLUSIONS

Our results show that rapid inactivation of enteric bacteria on a grass surface irrigated with effluent can be expected at higher ambient temperature in direct sunlight and where the grass has low moisture content. However, enteric bacteria can be expected to survive for a longer period at lower temperatures under shaded conditions. Thus, where treated effluent is used to irrigate sports ovals, it may be necessary to allow time for the oval to dry before it can be used for contact sports, particularly in the cooler months.

## ACKNOWLEDGEMENTS

The research work presented in this paper is part of a project funded by the Australian National Heritage Trust, The Water Corporation of WA and CSIRO Water for a Healthy Country Flagship.

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First received 1 December 2006; accepted in revised form 7 April 2007. Available online January 2008