Letters in Applied Microbiology 2003, **36**, 362–371

Evaluation of biotracers to monitor effluent retention time in constructed wetlands

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2003/004: received 9 October 2002, revised 30 January 2003 and accepted 5 February 2003

ABSTRACT

C.J. HODGSON, J. PERKINS AND J.C. LABADZ. 2003.

Aims: With concern surrounding the environmental impact of chemical tracers on the aquatic environment, this paper presents the initial evaluation of biotracers used to determine the effluent retention time, an important performance indicator, in a Free Water Surface Constructed Wetland.

Methods and Results: Production of the biotracers, coliphage MS2, and the bacteriophage of *Enterobacter cloacae* and antibiotic resistant endospores of *Bacillus globigii* is described in detail. Their subsequent use in three separate tracer experiments – January, March and June (2000) – revealed the variability of retention time with respect to effluent flow. The biotracer MS2 showed the constructed wetland had a retention time of 8–9 h at a mean discharge of 0·9 l s^{-1}, increasing to 10–12 h at a mean discharge 0·3 l s^{-1}. A similar retention of 9–10 h at a mean discharge of 0·3 l s^{-1} was calculated for the *Ent. cloacae* phage. In contrast, use of endospores revealed considerably longer retention times at these mean discharge rates; 12–24 h and 36–48 h, respectively.

Conclusion: Biotracers could provide a useful and environmentally friendly technique to monitor effluent retention in constructed wetlands. At this stage the phage tracers appear particularly promising due to ease of isolation and recovery.

Significance and Impact of the Study: Initial results are encouraging and have highlighted the potential of biotracers as alternatives to chemical tracers, even in microbially-rich waters.

Keywords: bacteriophage, biotracers, constructed wetlands, endospores, retention time.

INTRODUCTION

The adoption of wetland treatment technology has become increasingly popular for the treatment of wastewater in the UK (Cooper and Green 1995). Wetland use has spread to include the treatment of industrial effluent, acid mine drainage, agricultural effluent, landfill leachate, road and airport run-off and primary, secondary and tertiary treatment of sewage effluent. Currently, at least 525 wetlands are now known to operate within the UK (Cooper 2001). They are seen as part of the sustainable development approach to waste management, offering both a low environmental impact and an appropriate ecological option. Constructed wetlands are categorised into two main groups; Free Water Surface (FWS) treatment wetlands used in this study and Subsurface Flow treatment wetlands. In the former type of reed bed effluent passes over the support medium, between the macrophyte stems and through any surface litter. Research effort to date has focused on constructed wetland performance, with particular attention given to changes in key water quality parameters such as biological oxygen demand, chemical oxygen demand, total suspended solids and nitrate and phosphate removal. All these parameters are reliant on effluent retention time within the system, since retention determines the efficiency of treatment.

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Traditional hydrological methods used to establish the passage of water and waterborne pollutants and their retention time in ground and surface waters include the use of fluorescent dyes, lithium salts and radioactive substances. There is environmental concern regarding the impact of chemical tracers on aquatic ecosystems (Rossi 1992). Hence it is important to seek new tracers that have the potential to act as highly efficient indicators of effluent retention in reed beds yet which have low environmental impact. Biological tracers such as bacteriophage and endospores may be both more environmentally acceptable and provide a more useful indication of the dynamics of microbes in hydrological studies (Keswick *et al.* 1982; Pang *et al.* 1998).

Microbial tracers such as bacteria have been used because of their ease of growth and detection (Keswick *et al.* 1982). In particular, coliform bacteria have been used to trace the movement of septic and sewage waste. However, the disadvantage of using bacteria from faecal material is the difficulty of distinguishing the suspected source (Keswick *et al.* 1982; Sinton *et al.* 1997). Another disadvantage of the most commonly used bacterial tracers such as the coliform bacteria is one of die-off, limiting the length of tracer experiments and the quantification of the actual removal processes (Sinton *et al.* 1997). By extending the longevity of biological tracers the quantification of removal processes can be improved. For example, the dormant endospores of *Bacillus subtilis* can survive for long periods in a wide range of environmental conditions (Setlow 1995). Studies have shown the viability of *B. subtilis* endospores in tracing sewage dispersion in marine and freshwaters (Pike *et al.* 1969; Houston *et al.* 1989). In addition to wild type endospores, antibiotic resistant endospores of *B. subtilis* have been applied in groundwater tracing studies (Pang *et al.* 1998).

Biotracers have been used in a wide range of hydrological environments but to the best of the authors' knowledge microbial tracers have not been applied to a FWS constructed wetland to monitor wastewater retention time. The processes which occur in constructed wetlands to remove chemical and biological contaminants, include sedimentation, filtration, adsorption and predation. Effectiveness of these removal processes relies on an adequate contact time with the substrate and vegetation. This contact time can be determined from the retention time; hence the accurate determination of retention time becomes an essential tool in predicting wetland performance. This paper presents the initial research findings of a project designed to evaluate the suitability of different biotracers to determine effluent retention time in a FWS wetland. The microbial tracers used in this study were antibiotic resistant endospores of *B. subtilis* var. *niger* (*B. globigii*), the coliphage MS2 and the bacteriophage of *Enterobacter cloacae*. *Bacillus globigii* has a characteristic morphology with a bright orange pigmentation, when grown on nutrient agar (NA), making it easy to identify from other bacteria.

MATERIALS AND METHODS

The research site

Research was conducted at Yorkshire Water's Crow Edge Sewage Treatment works near Penistone, South Yorkshire, UK. A series of four constructed wetlands (each approx. 15 m long by 6 m wide), operate in parallel to form the tertiary treatment process (Fig. 1). The reed beds were constructed during the summer of 1996, by excavation of the site. The first third of each bed was planted with *Iris pseudacorus* (Yellow Flag Iris) the remaining two-thirds with *Phragmites australis* (common reed). With maturity of the reed beds *Typha latifolia* have self-seeded amongst the *Iris*, although their numbers remain low (only two or three per reed bed). *Glyceria* spp. have become invasive in the two central beds and are now the dominant species at the outflow of these beds.

The effluent inlet for each reed bed is supplied by a 'T' piece constructed from domestic drainpipe. The effluent from each pair of reed beds discharges, via separate capped plastic drainpipes, into an access well situated centrally at the end of each pair of reed beds. The final effluent discharges into Sledbrook Dyke, a tributary of the River Don. These four reed beds are classified as FWS treatment wetlands.

Fieldwork

On three separate occasions – January, March and June 2000 – samples were collected at the clearly defined inflow and outflow of the control reed bed (RB 3) and the tracer inoculated reed bed (RB 4) (Fig. 1). Effluent discharge rate was measured at the combined discharge well, prior to its discharge to Sledbrook Dyke. Discharge was measured using a calibrated bucket and stopwatch. Water samples were collected in presterilized, 500 ml, screw top polypropylene bottles, stored on ice in a cool box and analysed in the laboratory. All biotracers were injected directly to the gully that feeds RB 4 situated at the end of the final settling tank (Fig. 1). Tracers were washed through the injection apparatus using 100 ml of sterile Ringer's solution.

Bacteriophage MS2 and *Ent. cloacae* tracer preparation

Bacterial hosts. *Escherichia coli* DF1100 (University of Huddersfield culture collection), a high frequency of recombinant strain, with the fertility, or F plasmid,

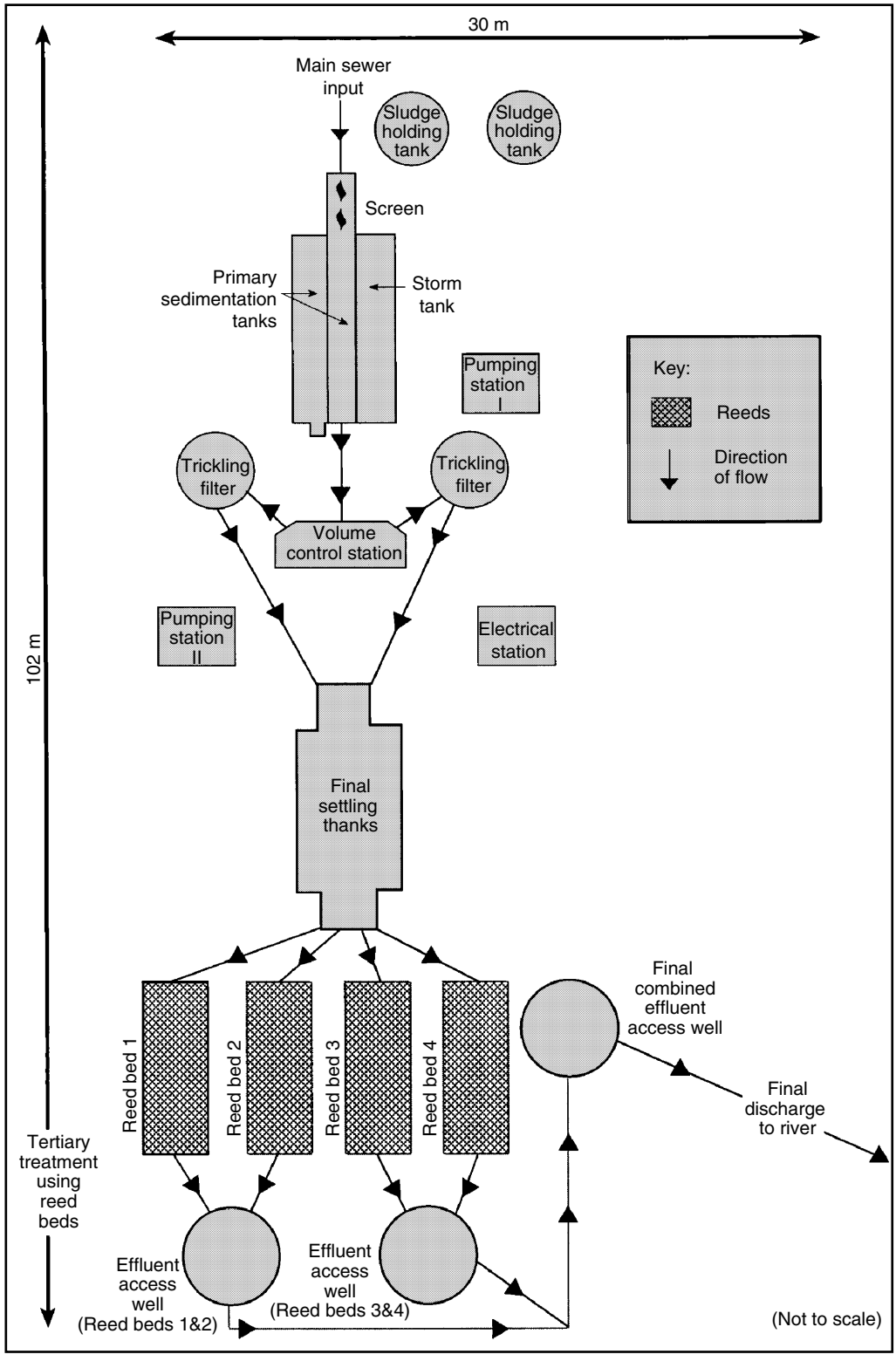

Fig. 1 Schematic representation of Crow Edge Sewage Works indicating direction of effluent flow

encoding for a pilus to which MS2, a male specific bacteriophage, first attaches prior to infecting the host cell. *Enterobacter cloacae* (University of Huddersfield culture collection) this culture was originally obtained in 1989 from Yorkshire Water plc.

Loop inoculated *E. coli* DF1100 was grown overnight in 5 ml of Luria–Bertani (LB) broth that was incubated at 37°C on an orbital shaker at 120 rev min^{-1}. LB broth contained (g l^{-1}): tryptone (LAB M), 10; yeast extract (Difco), 5; sodium chloride (NaCl), 10. The final pH of the medium was adjusted to pH 7·0 (±0·2) using 2 mol l^{-1} NaOH. The overnight culture was then added to 400 ml of LB pre-warmed to 37°C in a 2 l conical flask. At 15 min intervals A_{600} readings were taken from the growing culture. When the absorbance reached 0·15 (±0·05), 0·15 ml of the phage MS2 (titre $1{\cdot}1 \times 10^9$ PFU ml^{-1}) was added. Incubation was continued for a further 270 min, until lysis of the culture was complete. Cell debris was removed by centrifugation at 10 000 *g*, at 4°C for 15 min. The supernatant was then distributed evenly into 3 × 100 ml volumes, to which 2 ml of chloroform was added prior to storage at 4°C.

The *Ent. cloacae* phage tracer was prepared as for the MS2 tracer with the following amendments: *Ent. cloacae* substituted for *E. coil* DF1100 and phage *Ent. cloacae* (titre $4{\cdot}2 \times 10^9$ PFU ml^{-1}) was added at the appropriate absorbance value.

Preparation of rifampicin resistant *B. globigii* endospores

The following methods were adapted from Houston *et al.* (1989). An overnight culture of *B. globigii* (National Collection of Industrial and Marine Bacteria, NCIMB) was grown in 5 ml of nutrient broth and incubated at 30°C on a rotary shaker (120 rev min^{-1}). This culture was then distributed evenly into five sterile eppendorf tubes and microfuged at 25 000 *g* for 2 min. The supernatant was decanted and the remaining pellet was resuspended in 100 μl of nutrient broth (Oxoid), vortex mixed for 60 s and then spread onto NA (Oxoid) plates each containing 100 μg ml^{-1} rifampicin. After incubation at 30°C for 72 h, orange pigmented colonies were observed on all plates. Selected isolates were then subcultured twice on the same medium to obtain a rifampicin resistant clone. A 20% (v/v) glycerol stock of the Rif^r *Bacillus globigii* was prepared and stored at −80°C (±10°C).

An overnight culture was prepared by loop inoculating the glycerol stock into 5 ml of nutrient broth, containing 100 μl ml^{-1} of rifampicin. One millilitre of the overnight culture was used to inoculate 400 ml of nutrient broth supplement medium containing (g l^{-1}): nutrient broth, 25; $MgSO_4{\cdot}7H_2O$, 2; $CaCl_2{\cdot}H_2O$, 8; $MnSO_4{\cdot}4H_2O$, 0·5. The culture was then incubated at 30°C on a rotary shaker (120 rev min^{-1}) for 10 days. The long incubation period allowed the ratio of free spores to entrained endospores and vegetative cells (as viewed under the phase-contrast microscope) to exceed 9 : 1. The endospores were then harvested by centrifugation at 10 000 *g* for 20 min at 5°C. The supernatant was decanted and the pellet was washed in 10 ml of sterile Ringer's solution. The spore solution was made up to 200 ml in Ringer's solution washed and centrifuged and finally resuspended in 40 ml of Ringer's solution. The preparation was stirred vigorously and heated at 80°C for 10 min to break up clumps and destroy any vegetative cells. The concentration of spores was determined by serial dilution and plating on NA containing 100 μg ml^{-1} of rifampicin. Regular quality control ensured no loss of viability or 'loss' of rifampicin resistance between the date of production and the tracer trial.

Preparation of rifampicin and nalidixic acid resistant mutant *B. globigii*

A 25 mg ml^{-1} stock solution of nalidixic acid was prepared; nalidixic acid (Sigma) 50 mg dissolved in 4 ml of 1 mol l^{-1} NaOH, made up to 20 ml and filter sterilized through 0·2 μm membrane filter (Sartorius, Goettingen, Germany). A suitable aliquot of the nalidixic acid stock was added to NA plates to provide a final concentration of 25 μg ml^{-1}. An overnight 5 ml culture of the rifampicin resistant *B. globigii,* was prepared from a glycerol stock. About 100 μl of this culture was spread on plates with 25 μg ml^{-1} nalidixic acid, and incubated at 30°C for 72 h (±4 h). This was performed in triplicate. After 72 h no growth was observed. Using a sterile tooth pick small drops of the mutagens, 2-aminopurine and ethylmethane sulphonate were placed in the centre of separate 25 g ml^{-1} nalidixic acid plates, on which 100 μl of an overnight culture of the Rif^r *B. globigii* had been spread. The plates were incubated at 30°C, for 24 h.

An isolate was selected from each plate and cultured onto 25 μg ml^{-1} nalidixic acid plates on two further occasions. This culture was tested against the original wild type *B. globigii* on NA, NA + 100 μg ml^{-1} rifampicin, NA + 25 μg ml^{-1} nalidixic acid and NA + 100 μg ml^{-1} rifampicin and NA + 25 μg ml^{-1} nalidixic acid. The culture exhibited growth on all media plates, while the wild type showed growth only on the NA plates.

A 20% (v/v) glycerol stock of the double mutant *B. globigii* was prepared and stored at −80°C (±10°C).

Bacteriophage enumeration

Enumeration of all bacteriophage in collected effluent samples used the double agar overlay method of Adams (1959) and Havelaar and Hogeboom (1984). *Escherichia coli* DF1100, was used as the host for the F specific

bacteriophage MS2. In order to confirm that observed plaques were as a result of F-RNA phage infection and not somatic phage, samples were prepared with the addition of 200 μl of RNase solution (0·1% w/v) to the overlay agar. *Enterobacter cloacae* was used as the host for the *Ent. cloacae* phage.

Enumeration of antibiotic resistant endospore tracers

Endospore concentration in collected effluent was determined via spread plates, membrane filtration or pour plates. The medium used was NA containing the appropriate concentration of antibiotic. Plates were incubated at 30°C for 48 h and all orange pigmented colonies were enumerated.

RESULTS

The final titre of the phage tracers were: MS2 $2{\cdot}7 \times 10^{12}$ PFU ml^{-1} and cloacae phage $7{\cdot}95 \times 10^{10}$ PFU ml^{-1}. The final titres of the endospore tracers were; rifampicin resistant strain $2{\cdot}6 \times 10^{8}$ CFU ml^{-1} and rifampicin and nalidixic resistant strain $4{\cdot}1 \times 10^{8}$ CFU ml^{-1}.

The break through curve for January 2000, Fig. 2, includes the combined discharge measured over the sampling period; no rain fell during this period and therefore no rainfall was recorded. There was a notable fluctuation in the combined discharge between time 6 and 9 h, (3–6 PM).

Median concentrations recorded at the outlet and inlet of the control bed were 42 and 6 PFU ml^{-1}, respectively (Table 1a). These values are negligible when compared with the corresponding median concentrations noted at the outlet/inlet of the tracer inoculated reed bed 4, 18 700 and 322 PFU ml^{-1}, respectively. The concentration of tracer recovered over the sampling period is recorded in Table 1b. A standard arithmetic method was used to calculate the concentration of tracer recovered (PFU ml^{-1}) and this value was then expressed as a percentage of the titre of the original injected tracer.

The tracer run in March 2000, shown in Fig. 3, used two biotracers simultaneously (MS2 and rifampicin resistant endospores of *B. globigii*). Inlet and outlet concentrations of both biotracers are indicated. It is clear that the maximum concentration of both tracers were recorded at the outlet of reed bed 4 at the same time, 1 h. Again the background concentrations of biotracers were negligible.

Table 2a gives the descriptive statistics for the combined tracer run in March. Median phage concentration during the study period was 17 713 PFU ml^{-1} at the outflow of the inoculated reed bed. This is approx. 3000 times greater than the concentration of indigenous F-RNA phage (6 PFU ml^{-1}) at the outflow of the control reed bed. The median endospore concentration of 20 CFU ml^{-1} observed at the outflow of the control reed bed was suspected of being indicative of slight cross contamination experienced in sample collection and not an indication of a population of

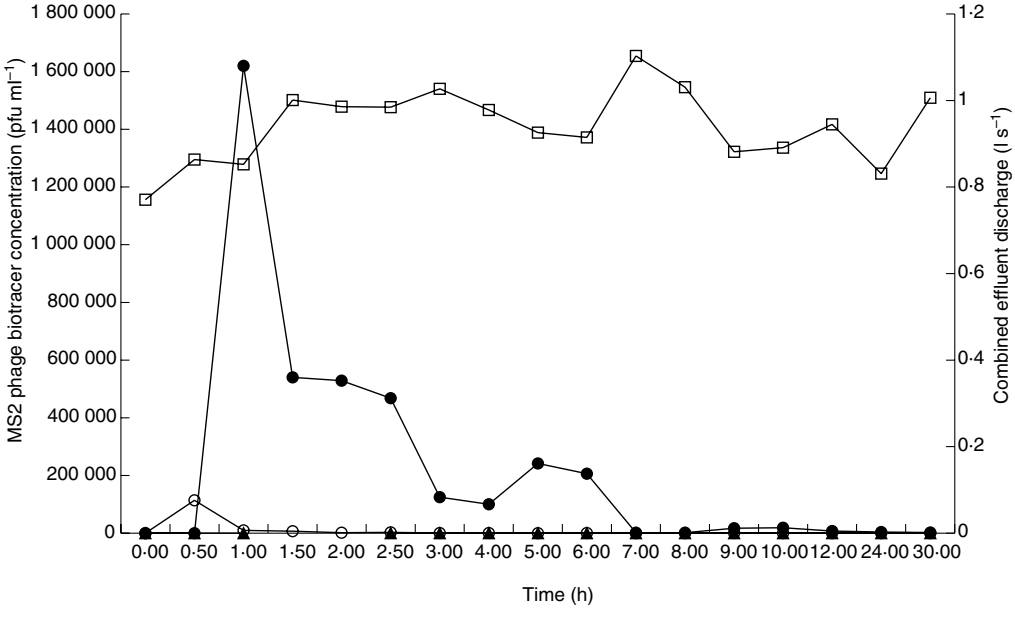

Fig. 2 MS2 biotracer break through curve for reed bed 4. The background F-RNA phage concentration (reed bed 3) and combined effluent discharge rate (l s^{-1}) are also shown. January 2000 – biotracer phage MS2: inflow (○), outflow (●), reed bed 4; background F-RNA phage: inflow (△), outflow (▲), reed bed 3. Combined discharge (l s^{-1}) (□)

Table 1a Descriptive statistics of the biotracer inoculated reed bed 4 and the control reed bed 3 at the inflow and outflow points. All results are expressed as PFU ml^{-1} except outflow discharge ($l\ s^{-1}$). January 2000

Variable	*n*	Min.	Max.	Mean	Median	S.D.	CV (%)
MS2 conc.							
Rb 4 in	17	0	114 000	8092	322	27 426	339
Rb 4 out	17	0	1 620 000	228 388	18 700	407 391	178
F-RNA conc.							
Rb 3 in	17	1	88	37	42	25	68
Rb 3 out	17	2	40	13	6	14	108
Outflow discharge	17	0·8	1·1	0·9	0·9	0·09	10

n = no. of samples; F-RNA = indigenous biotracer concentration; CV = coefficient of variation.

Table 1b MS2 tracer recovery, January 2000

Biotracer	Concentration of tracer recovered	Percentage of tracer recovered
Phage MS2	$1{\cdot}88 \times 10^{12}$* PFU ml^{-1}	69·63

*Figure adjusted for background concentration, as indicated in the control reed bed (RB 3).

indigenous antibiotic resistant endospores. Table 2b shows the concentration of tracer recovered; it is notable that three times as much of the phage tracer was recovered as compared to the endospores. Phage tracer recovery from the tracer runs in January and March (Tables 1b and 2b) are consistent.

Figure 4 is the combined tracer break through curve for the study period June 2000, a period of low combined effluent discharge. Considerably more tracer was recovered at the inlet sampling points on this occasion compared to the January and March tracer runs. The break through curve is presented in the same manner as the previous two curves, both inlet and outlet concentrations of the tracer are plotted. As can be seen the greater peaks are observed at the inlet on this occasion.

The descriptive statistics from this tracer run are shown in Table 3a. Inlet data are displayed to highlight low flow and hence low tracer movement through the system resulting in poor tracer recovery. Combined discharge on this occasion was considerably lower than the previous tracer runs, a mean discharge of $0{\cdot}3\ l\ s^{-1}$, as compared with $0{\cdot}9\ l\ s^{-1}$ in January and $0{\cdot}8\ l\ s^{-1}$ in March. Table 3b shows the concentration and percentage of each tracer recovered, the values include the tracer recovered at the inlet as well as the outlet, which may explain the greater recovery in percentage terms of both the *Ent. cloacae* and endospore tracer compared with the MS2 tracer.

A comparison of retention times for the three tracer runs is shown in Table 4. The increase in retention time with the

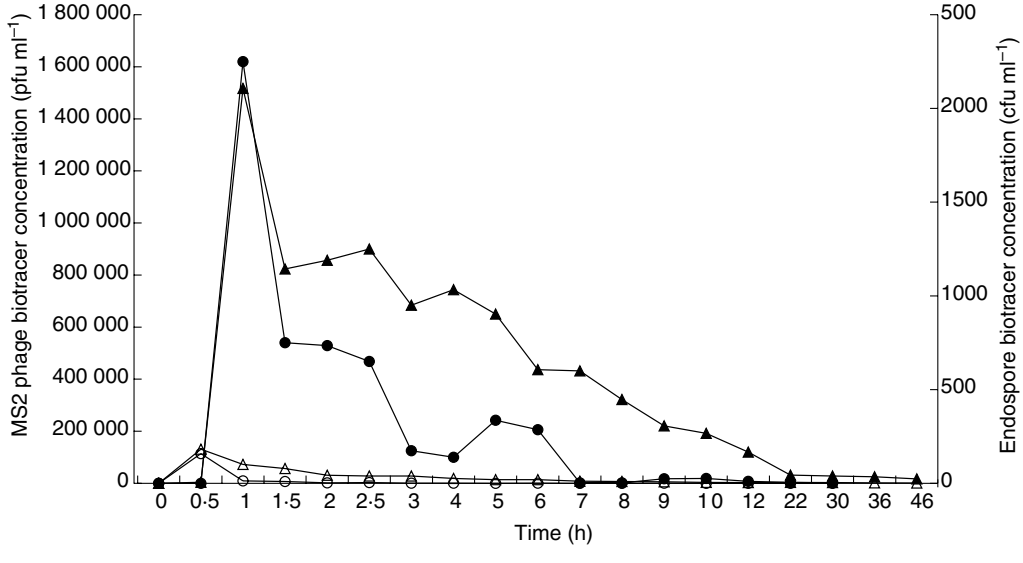

Fig. 3 Biotracer break through curves for reed bed 4. March 2000 – biotracers phage MS2: inflow (○), outflow (●); *Bacillus globigii* endospore: inflow (△), outflow (▲)

Variable	*n*	Min	Max	Mean	Median	S.D.	CV (%)
Spores (CFU ml^{-1})							
Rb 4 in	19	0	910	70	11	205	293
Rb 4 out	19	0	2680	666	450	747	112
Rb 3 in	19	0	0	0	0	0	0
Rb 3 out	19	0	199	44	20	59	134
MS2 (PFU ml^{-1})							
Rb 4 in	17	0	108 000	7665	305	25 978	321
Rb 4 out	17	0	1 610 000	216 330	17 713	385 881	169
Rb 3 in	17	1	88	37	42	25	68
Rb 3 out	17	2	40	13	6	14	107
Outflow discharge (l s^{-1})	19	0·5	1·0	0·8	0·8	0·13	0·16

Table 2a Descriptive statistics of the biotracer inoculated reed bed 4 and the control reed bed 3 at the inflow and outflow points. Results are expressed as PFU ml^{-1} (phage), CFU ml^{-1} (endospores) and l s^{-1}, combined outflow discharge. March 2000

n = no. of samples; F-RNA = indigenous biotracer concentration; CV = coefficient of variation.

Table 2b Biotracer recovery, March 2000

Biotracer	Concentration of tracer recovered	Percentage of tracer recovered
Bacillus globigii endospores	$4{\cdot}86 \times 10^{8}$* CFU ml^{-1}	18·71
Phage MS2	$1{\cdot}74 \times 10^{12}$† PFU ml^{-1}	64·35

*Figure adjusted for background concentration, as indicated in the control reed bed (RB 3).
†Figure adjusted for background concentration, as indicated in the control reed bed (RB 3).

reduction in discharge is evident. At discharge rates of around 1 l s^{-1} there is a 99% retention time of between 8–9 h for the phage tracers and 12–24 h for the endospore tracer. At discharge rates of <0·5 l s^{-1} there is a noticeable increase in retention times of all three tracers within the reed bed, 9–12 h for both bacteriophages and 36–48 h for the endospore.

DISCUSSION

The results reported here form the early work in evaluating the use of biotracers to determine retention time within a FWS constructed wetland. They suggest that the three biotracers provide retention times that fall within those of the original design for the constructed wetlands, 4–24 h (Hiley 2000, personal communication). Current literature uses a number of terms to define retention time; they include detention time, residence time and hydraulic retention time. Irrespective of the term used, all authors have assumed that these terms refer to retention time, which

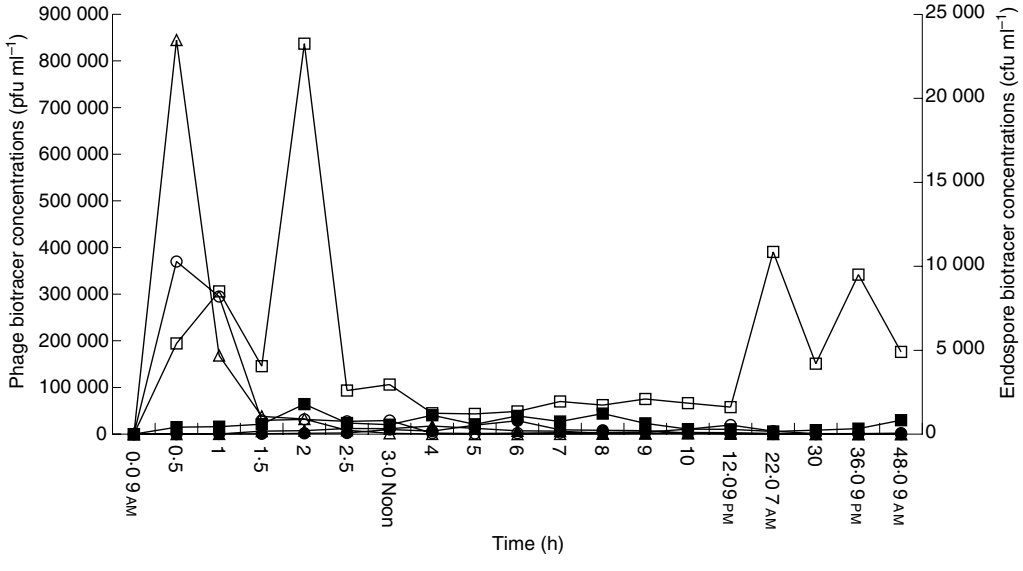

Fig. 4 Biotracer break through curves for reed bed 4. June 2000 – biotracers phage MS2: inflow (○), outflow (●); cloacae phage: inflow (△), outflow (▲); *Bacillus globigii* endospore: inflow (□), outflow (■)

Table 3a Descriptive statistics of the biotracer inoculated reed bed 4 and the control reed bed 2 at the inflow and outflow points. Results are expressed as PFU ml^{-1} (phage), CFU ml^{-1} (endospores) and l s^{-1}, combined outflow discharge. June 2000

Variable	*n*	Min.	Max.	Mean	Median	S.D.	CV (%)
Spores (CFU ml^{-1})							
Rb 4 in	19	0	23 250	4696	2600	5408	115
Rb 4 out	19	0	11 995	631	580	435	69
Rb 2 in	19	0	0	0	0	0	0
Rb 2 out	19	0	0	0	0	0	0
MS2 (PFU ml^{-1})							
Rb 4 in	19	0	370 000	44 192	4100	102 977	233
Rb 4 out	19	0·5	28 800	5178	1905	7630	147
F-RNA (PFU ml^{-1})							
Rb 2 in	19	1	900	84	6	222	264
Rb 2 out	19	0	0·5	0·1	0	0·04	40
Cloacae (PFU ml^{-1})							
Rb 4 in	19	0	845 000	58 158	1255	194 472	334
Rb 4 out	19	0	17 450	4548	1290	5516	121
Rb 2 in	19	0	44	5	2	12	240
Rb 2 out	19	0	1·5	0·1	0	0·4	400
Outflow discharge (l s^{-1})	19	0·12	0·5	0·33	0·34	0·12	0·16

n = no. of samples; F-RNA = indigenous biotracer concentration; CV = coefficient of variation.

Table 3b Biotracer recovery, June 2000

Biotracer	Concentration of tracer recovered	Percentage of tracer recovered
Bacillus globigii endospores	$4{\cdot}30 \times 10^{8}$* CFU ml^{-1}	10·48
Phage MS2	$1{\cdot}82 \times 10^{11}$† PFU ml^{-1}	6·74
cloacae phage	$1{\cdot}72 \times 10^{10}$ PFU ml^{-1}	21·87

*Figure adjusted for background concentration, as indicated in the control reed bed (RB 2).
†Figure adjusted for background concentration, as indicated in the control reed bed (RB 2).

is the length of time the effluent remains in the wetland. Detention times ranging from 3–28 days have been reported in FWS wetlands (Panswad and Chavalparit 1997) and 2–5 days in engineered wetlands (Higgins *et al.* 2000). Chendorian *et al.* (1998) report the mean residence time as 9 ± 3 days in two surface water constructed wetlands, determined using the conservative tracer bromide. These considerably longer residence times are as a result of differences in design, size and constant flow rate. *Bacillus globigii* endospores have been used previously in tracer studies to determine the dispersion of sewage out flows, particularly in marine environments. They have been used because of the relative ease in which large concentrations can be produced and their distinctive orange pigmentation which makes distinguishing them from other bacterial colonies relatively easy. However, a drawback to applying endospores to microbially rich aquatic settings has been that the growth of the recovered endospores tends to be obscured by dense background microflora (Houston *et al.* 1989). Similar dense growth of bacteria was observed in effluent samples from the constructed wetlands in this study. Therefore a practical alternative was to inhibit the growth of indigenous bacteria. This was achieved by the production of antibiotic resistant endospores of *B. globigii*. By using selective media containing an appropriate antibiotic concentration, the recovered endospore tracer was easily isolated and enumerated.

The most likely explanation for the marked difference in recovery rates observed during the March 2000 tracer run, 64% for the MS2 and 19% for the endospores, is their size. Bacteriophage MS2 has an approximate diameter of 26 nm

Table 4 Retention time reed bed 4

Date of tracer run	Type of tracer	Retention time 90% rec. (h)	Retention time 99% rec. (h)	Mean combined discharge (l s^{-1})
26–27 January 2000	MS2	4–5	8–9	0·94
21–23 March 2000	MS2	4–5	8–9	0·81
	Endospore Rif^{r}	7–8	12–24	0·81
20–22 June 2000	MS2	8–9	10–12	0·33
	Cloacae phage	6–7	9–10	0·33
	Endospore Nal^{r}	22–30	36–48	0·33

while the endospores are approx. 1·5 μm (*ca.* 60 times larger). The size of the phage leads it to stay entrained within the water body and at relatively high flow rates little removal is observed. The small quantity removed may be attributed to phage adsorbing to particulate matter (Rossi 1992). The larger endospore, on the other hand, is likely to be acted upon by bacterial removal mechanisms in the reed bed. A wide range of processes such as sedimentation, filtration and predation have previously been associated with prokaryote removal in wetlands (Perkins and Hunter 2000).

During the March tracer run both the bacteriophage MS2 and the rifampicin resistant endospores of *B. globigii* were used simultaneously. A problem in the sampling regime was highlighted by the detection of endospores in the effluent of the control reed bed. It was believed, at the time, that the close confines of the sampling well resulted in the cross contamination of the effluent samples due to the production of aerosols. What is unclear is why no obvious increase in MS2 concentration was observed. The situation was remedied by not using the adjacent reed bed as the control in the June tracer run, confirming that cross contamination was the likely source of error.

The combined tracer run in June produced quite contrasting results to those of the earlier tracer runs. Only 7% of the MS2 was recovered in June compared with 64% in March and 10 and 18% of the endospore, respectively. However, 22% of the *Ent. cloacae* phage was recovered, tentatively highlighting this particular phage as being the most promising of the three biotracers. On all occasions the tracers were injected via an open gully situated at the end of the final settling tanks, (Fig. 1). This gully then leads into a drain pipe approx. 3 m long capped by a 't' piece which allows the effluent to flow into the constructed wetland. The tracer break through curve for the three tracers is likely to reflect the retention time of the effluent as it passes through this 3 m length of connecting drain pipe, during extremely low flow conditions. Results therefore may suggest that the use of biological tracers in these particular wetlands at times of relatively low flow is unsuitable. However, by changing the sampling regime to the outlet only and extending the sampling time to 72 h or more it is anticipated that the suitability of these tracers may be established, in future field trials.

The suitability of the MS2 tracer has been further questioned by a recent study (data not presented) which indicated a substantial increase (6 log units) in concentration of background F-RNA phage during the summer months, probably as a result of elevated temperatures resulting in conditions conducive for host replication and thus phage replication. The possible location of viral replication within the sewage works has not been researched further. The data generated from the June tracer trial indicated a substantial decrease in F-RNA phage from the inflow to the outflow of both the tracer inoculated reed bed (RB 4) and the control reed bed (RB 2), in terms of percentage removal this decrease was greater than 99% for the control reed bed and 93% for the tracer inoculated reed bed. This may help to clarify and highlight the ability of wetlands to remove viruses (Gersberg *et al.* 1987; Scheuerman *et al.* 1987; Chendorian *et al.* 1998). Prior to this study the role of reed beds in viral removal was unresolved.

In conclusion, initial results for the biotracer field trials are extremely positive. Inability to detect indigenous *Ent. cloacae* phage even in microbial-rich waters and satisfactory recovery at low flow rates has revealed this biotracer to be the most promising at this stage. Unlike a recent hydrological study (Daniell *et al.* 2000) this research has revealed that genetically modified biotracers are not required. However, further seasonal field studies 'especially at high flow rates' are necessary to provide a thorough evaluation for each biotracer.

ACKNOWLEDGEMENTS

The University of Huddersfield for funding this research. Yorkshire Water plc. for allowing access to the sewage works at Crow Edge, in particular Allan Fletcher the Area Manager and Ron Farrar the works foreman. The following undergraduates; Laura Chapman for her assistance in the field and Christina Stergeon for her help with production of the endospores.

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