PCR analysis of presence and location of *Mycobacterium avium* in a constructed reed bed, with implications for avian tuberculosis control

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Running title: Reed beds as biofilters of *Mycobacterium avium*

ABSTRACT

The potential of reed beds to act as biofilters of pathogenic and environmental mycobacteria was investigated through examination of the fate of mycobacteria in a constructed reed bed filtering effluent from a large captive wildfowl collection. Particular emphasis was made on the presence and location of *Mycobacterium avium* - the causal agent of avian tuberculosis - in an effort to clarify the potential role of reed beds in the control of this disease. Water, sediment and stems and roots of common reed (*Phragmites australis*) and greater reedmace (*Typha latifolia*) were taken from 15 locations within the reed bed plus sites upstream and downstream. Samples were analysed for mycobacteria using PCR and specifically for *M. avium* using nested PCR. Environmental mycobacteria were found throughout the entire reed bed but *M. avium* was not found downstream of the first vegetation growth. The reed bed was found to effectively remove *M. avium* from the water through a combination of sedimentation and adsorption onto vegetation stems. The results of this study show that constructed reed beds composed of a settlement lagoon and one or more vegetation beds can act as valuable and ecologically-friendly tools in the environmental control of avian tuberculosis.

Keywords: Avian tuberculosis, constructed wetland, mycobacteriosis, *Mycobacterium avium*, *Phragmites australis*, reed bed, *Typha latifolia*, wildfowl.

INTRODUCTION

The potential of reed bed technology – the use of constructed wetlands for wastewater treatment – was first realised in the 1960s in the Netherlands (Brix & Schierup, 1989). Reed beds have since been used worldwide for many purposes, including removal of parasitic helminth eggs from wastewaters in Egypt (Stott, *et al.*, 1999), reduction of pathogenic bacteria levels in dairy wastewater (Karpiscak, *et al.*, 2001), removal of viral pathogens from

wastewater (Jackson & Jackson, 2008) and in the treatment of human sewage in many countries (Kadlec & Knight, 1996). Wetlands act as biofilters through a combination of physical, chemical, and biological processes (Brix, 1993). Physical factors may include mechanical filtration by vegetation, adsorption to organic matter, and sedimentation (Wood & McAtamney, 1994). The chemical processes of oxidation and exposure to biocides excreted by some hydrophytes act to reduce bacterial loads (Brix, 1997). Predation by nematodes and protozoa was found to be an important factor in the removal of bacteria from wastewaters in subsurface flow wetlands by Green *et al.* (1997). Attack by lytic bacteria and viruses, and natural die-off in the reed bed are other biological mechanisms thought to play a role in removal of pathogenic bacteria (Gersberg et al. 1989, cited by Rivera, *et al.*, 1995). Several studies have shown the potential of reed bed technology in removing pathogenic bacteria from wastewater (Rivera, *et al.*, 1995, Green, *et al.*, 1997, Ottova, *et al.*, 1997, Karpiscak, *et al.*, 2001, Stenstrom & Carlander, 2001). Constructed wetlands typically remove greater than 90 percent of coliforms (reportedly up to 99.999% in one study (Soto, *et al.*, 1999)) and greater than 80 percent of faecal streptococci (Kadlec & Knight, 1996). Such research has focussed primarily on the removal of common faecal bacteria (reviewed in Edwards, *et al.*, 2005). Consequently, little is known of reed bed filtration efficacy with regard to mycobacteria.

Mycobacteria are ubiquitous environmental saprophytes, found in marshes, ponds and rivers at the interface of air and water, and in soil, particularly that which is rich in organic matter (Grange, 1987). Several species of mycobacteria cause disease in birds, with *Mycobacterium avium*, *M. intracellulare* and *M. genavense* implicated most frequently (Tell, *et al.*, 2001). Avian tuberculosis (ATB) is endemic within captive wildfowl populations at several Wildfowl & Wetlands Trust (WWT) sites in the UK (Cromie, *et al.*, 1991, Painter, 1997, Evans, 2001, Zsivanovits, *et al.*, 2004). This is hampering a range of WWT's conservation programmes, and is the single greatest cause of death of adult birds at WWT Slimbridge

(Thorpe, 2000). In captive wildfowl in WWT collections, ATB is caused principally, but not exclusively, by *M. avium* serotype 1 (Cromie, *et al.*, 1991, Painter, 1997). Evidence that the water flowing through the captive wildfowl pens is the source of infection comes from isolation of *M. avium* from 'soil, mud or muddy water' at WWT Slimbridge (Schaefer, *et al.*, 1973), an epidemiological study of disease spread progressively downstream from the initial case of infection (Cromie, 1991) and studies showing that the pathology of affected birds indicates oral infection (Brown & Cromie, 1996). Attempts have been made to control ATB in WWT collections using a range of approaches including development of diagnostic tests (Cromie, *et al.*, 1993), vaccination (Cromie, *et al.*, 2000), management of the bird collection (Thorpe, 2000) including rotation according to age (R.L. Cromie, *unpublished data*) and through environmental control (Evans, 2001). Reed beds have been used at WWT sites for several years (Billington, 2000, MacKenzie, *et al.*, 2004) but thorough investigations into their effectiveness in removing mycobacteria have until now been lacking.

Although culture is a definitive means of confirming mycobacterial presence, the technique has several practical limitations. Mycobacteria require special culture media and many species grow exceedingly slowly: two to four weeks can be required for visible colonies to form on culture media, and some strains of *M. avium* require up to six months before colonies become identifiable (Matthews, *et al.*, 1978). Polymerase chain reaction (PCR) holds several potential advantages over culture of mycobacteria. Not only is PCR a rapid technique, it can detect very low numbers of organisms and distinguish accurately between species of mycobacteria (Aranaz, *et al.*, 1997). Christopher-Hennings *et al.* (2003) showed nested PCR (nPCR) to be similarly sensitive to culture for the identification of *M. avium* subsp. *paratuberculosis* from bovine faeces; nPCR can thus be considered a valid alternative to culture. Techniques for the recovery of mycobacterial DNA from soil samples have been described (Zhou, *et al.*, 1996).

Mendum *et al.* (2000) successfully used PCR to amplify sequences of mycobacterial nucleic acids extracted from environmental samples.

The aim of this study was to investigate the fate of environmental mycobacteria, with special reference to *M. avium*, in a constructed reed bed that filters effluent from a large captive wildfowl collection, in an effort to clarify the potential role of reed beds in the environmental control of ATB. This was achieved through the application of single-stage PCR and nPCR on samples of water, sediment and vegetation taken from before, within, and after the reed bed. A comparison was made between areas of the reed bed planted with common reed (*Phragmites australis*) and greater reedmace (*Typha latifolia*) as well as between samples taken at the water's surface, from the submerged stems and from the root systems of the reed bed vegetation.

MATERIALS AND METHODS

Sampling locations

The study site was the South Finger Reedbed at WWT Slimbridge, Gloucestershire, UK. Constructed in 1993, the South Finger Reedbed receives around 2000 cubic metres of effluent daily from a large collection of captive wildfowl (approximately 2800 captive birds and a similar number of wild and feral birds), and discharges ultimately into the River Severn. Fifteen sampling sites were selected between the inflow rhine (ditch) and the outflow to the River Severn (Figure 1). Choice of sampling sites was based on results of a preliminary study and a vegetation survey of the study area carried out in 2003. This enabled a known selection of plant species to be sampled, allowing a comparison between the two predominant macrophyte species, common reed and greater reedmace, to be made. Samples were collected on two consecutive days in June 2004.

Sample collection

At each sampling location, three samples were collected: approximately 150 mL each of surface water, mid-depth water, and sediment. Sampling was carried out using sterile 150 mL collection pots held in a telescopic sampling device. A rowing boat was used to obtain samples from the settlement lagoon. Where vegetation occurred at the sampling sites (locations 7 to 14 inclusive), representative plants were sampled using new clean gloves for each sample: sections of submerged stem were collected with mid-depth water, and roots/rhizomes with the sediment samples (Figure 2). In addition, as positive controls, three samples were collected from the white-winged duck (*Cairina scutulata*) enclosure at WWT Slimbridge; these were considered very likely to test positive for *M. avium* using PCR based on the results of a preliminary study conducted in June 2003 and this species' known susceptibility to the disease (Cromie, *et al.*, 1992). The appearance of each sample was noted upon acquisition. Sample containers were labelled, sealed, and stored at 5 ºC for up to 50 days until processed.

DNA extraction

Samples were processed in batches of seven. Negative extraction controls were always included. Preliminary tests using 10 mL of water sample containing suspended solids resulted in too dilute a sample for DNA detection. Therefore, samples were left to settle, the fluid phase pipetted off, and the sediment used. If no sediment was present (e.g. some surface water samples), the water itself was used. Two millilitres of sample were placed in a sterile tube. If visible vegetation was present, roots or stems were scraped using a sterile scalpel blade and the scrapings added to the tube. Tween-20 (approximately 0.2 µL) was added and the tube contents mixed on a vortex mixer for 5 s before being allowed to stand for 20 min. A sample of the liquid phase (300 µL) was pipetted into a sterile 2 mL Eppendorf tube containing 10

glass beads (1.5-2 mm diameter). This tube was centrifuged (13 000 rpm, 5 min) before 250 µl of supernate was discarded (care was taken to retain the deposit). Demineralisation solution (100 µL) (2.28 mL 0.5 mol l^{-1} EDTA, pH 8.0 and 120 µL Proteinase K 20 mg mL^{-1} (Qiagen Ltd, Crawley, UK)) was added, the tube vortexed, then incubated at 56 ºC for 72 h.

Each tube was mixed using a bead beater (2 500 rpm, 50 s). Lysis buffer (250 µL) (10 mL of 10 mol l^{-1} guanidine thiocyanate and 0.1 mol L^{-1} Tris-HCl buffer pH 6.4, plus 1 mL 0.2 mol L^{-1} EDTA pH 8.0 and 0.13 mL Triton X-100) was added, the tube vortexed, then incubated at 56 ºC for 2 h. The tube was then vortexed and centrifuged (13 000 rpm, 5 min). From this stage, a DNeasy Tissue Kit (Qiagen Ltd) was used. The kit protocol for isolation of total DNA from cultured animal cells was followed from stage 3 onwards. Briefly, this involved: addition of 200 µL absolute ethanol; transfer to a spin column; centrifugation (8 000 rpm, 1 min); addition of 500 µL Buffer AW1; centrifugation (8 000 rpm, 1 min); addition of 500 µL Buffer AW2, centrifugation (13 000 rpm, 5 min); placement of spin column in a new 2 mL collection tube; then addition of 100 µL Buffer AE and incubation at room temperature (1 min) followed by centrifugation (8 000 rpm, 1 min) (twice) to elute. The resulting eluate acted as the template for PCR.

Amplification

Use of single-stage PCR to detect DNA of environmental mycobacteria

The target for DNA amplification was a 439 bp fragment of the 65-kDa heat shock protein (*hsp65*) gene common to all *Mycobacterium* spp. (Shinnick, 1987) and other closely-related genera (Steingrube, *et al.*, 1995). Primers Tb11 (5'-ACCAACGATGGTGTGTCCAT-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3') were used (Telenti, *et al.*, 1993). Ten microlitres of eluate were added to each reaction tube. (Preliminary tests comparing the addition of 5 with 10 µL eluate produced clearer bands using the latter quantity.) The PCR

mixture (50 µL) was prepared in the laboratory, and comprised: 10 mmol L^{-1} bovine serum albumin (BSA); 0.15 mmol L^{-1} $MgCl_2$; 200 µmol L^{-1} (each) dATP, dCTP, dGTP and dTTP; 0.5 µmol L^{-1} (each) primer; and 2.5 units hot start *Taq* DNA polymerase (HotStarTaq, Qiagen Ltd). Amplification consisted of: *Taq* activation (15 min at 95 ºC); 45 cycles of: 40 s at 94 ºC, 1 min at 60 ºC, and 20 s + 1 s per cycle at 72 ºC; followed by 1 min at 72 ºC.

Use of nested PCR to detect Mycobacterium avium*-specific DNA*

Primers Av6 (5'-ATGGCCGGGAGACGATCTATGCCGGCGTAC-3') and Av7 (5'-TGTACGCGCTGCACAAACTGCGATCGAACG-3') were used to amplify a 187 bp segment of *M. avium*-specific DNA fragment DT6 (Thierry, *et al.*, 1993). A second set of primers, Av8 (5'-CGTACCGGTCACCGGGATATC-3') and Av9 (5'-CATCGACGTCCGGGGTTGC-3'), were used to bind internally with some overlap to Av6 and Av7 and amplify a 102 bp fragment (H.D. Donoghue, *unpublished data*). Preparation of the single-stage PCR mixture was conducted as described above, except the concentration of $MgCl_2$ was 0.65 mmol L^{-1}, and 0.4 µmol L^{-1} of each primer (Av6 and Av7) was used. Five microlitres of eluate was added to each reaction tube. The single-stage PCR cycle was exactly as that described above. For nPCR, new reaction tubes were used containing pre-aliquoted master-mix (2x Pre-Aliquoted PCR Master Mix; ABgene, Epsom, UK), to which further reagents were added, resulting in a final mix containing: 10 mmol L^{-1} BSA; 2.0 mmol L^{-1} $MgCl_2$; 100 µmol L^{-1} (each) dATP, dCTP, dGTP and dTTP; 75 mmol L^{-1} Tris-HCl (pH 8.8); 20 mmol L^{-1} $(NH_4)_2SO_4$; 0.01 % (v/v) Tween-20; 0.4 µmol L^{-1} (each) primer (Av8 and Av9); and 2.5 units Thermoprime Plus DNA Polymerase. The DNA template was 1 µl of amplicon from single-stage PCR. Amplification consisted of: denaturation (1 min at 94 ºC); 27 cycles of: 40 sec at 94 ºC, 1 min at 58 ºC, 20 s + 1 s per cycle at 72 ºC; followed by 1 min at 72 ºC.

Contamination precautions

Stringent measures were employed throughout to prevent cross-contamination (Kwok & Higuchi, 1989, Donoghue, *et al.*, 1998). Negative and positive controls were included in every PCR.

Gel electrophoresis

PCR product was electrophoresed and visualised as reported previously (Donoghue, *et al.*, 1998) and recorded with a digital camera.

RESULTS

Use of single-stage PCR to detect DNA of environmental mycobacteria

A sample was considered positive for environmental mycobacteria if a 439 bp band was visible following 45 cycles of amplification. The majority of samples from the South Finger Reedbed proved positive for environmental mycobacteria: positive results were obtained in 56 % (25/45) of samples tested (Table 1). Samples taken from the surface, mid-depth and sediment of the inflow rhine tested strongly positive for mycobacterial DNA (Figure 3), as did all three samples collected from the settlement lagoon inlet. All surface-water samples from sampling sites 1 to 6 (inflow rhine and settlement lagoon) tested positive for environmental mycobacteria. From sampling location 7 onwards (reed beds to Kingfisher Pool) no mycobacteria were detected in surface-water samples using the single-stage PCR. Samples of mid-depth water and macrophyte stems yielded mycobacteria throughout the length of the South Finger Reedbed, with the notable exception of the latter two-thirds of the *Phragmites* bed. A similar pattern was seen with sediment and root samples. The only location at which every collected sample tested negative for mycobacteria was the Kingfisher Pool at the South Finger Reedbed outflow.

Use of nested PCR to detect *Mycobacterium avium*-specific DNA

Agarose gel electrophoretograms were produced using PCR product from the first stage of amplification with primers Av6 and Av7. However, myriad visible bands made interpretation difficult. The nPCR product produced clearly visible bands of 102 bp, specific for *M. avium*, in 11 % (5/45) of samples from the South Finger Reedbed (Table 1 and Figure 4). Samples testing positive for *M. avium* came from the settlement lagoon (sediment), throughout the reedmace bed (surface water and *Typha* stems), and the inlet to the common reed bed (*Phragmites* stem). Three of the five positive samples were scraped from macrophyte stems, one was of surface-water (air-water interface) and one contained sediment. *M. avium* was not detected on any macrophyte roots using nPCR. In addition to the South Finger Reedbed samples, *M. avium* was detected in samples of water and mud taken from the white-winged duck enclosure in the main wildfowl collection. All samples collected from the polishing beds and the Kingfisher Pool at the South Finger Reedbed outflow tested negative for *M. avium.*

DISCUSSION

In this study, the potential role of reed beds in the environmental control of mycobacterial diseases was determined through examination of the fate of environmental and pathogenic mycobacteria in a constructed reed bed. PCR analysis of water, sediment and vegetation revealed that reed beds can effectively remove *M. avium* from water through a combination of sedimentation and adsorption onto vegetation stems. Reed beds have valuable role to play in the environmental control of mycobacterial diseases such as avian tuberculosis.

Presence and location of environmental mycobacteria

This study showed environmental mycobacteria to be present in a wide range of locations within the South Finger Reedbed, including in water from the surface and mid-depth, in

sediment and on macrophyte stems and roots. These findings reflect previous reports of the affinity of mycobacteria for, and wide distribution in, fresh water systems (Collins, *et al.*, 1984, Grange, 1987, Falkinham, *et al.*, 2001). All four surface-water samples from the settlement lagoon were found to contain mycobacteria, compared with three out of four mid-depth and sediment samples from the same locations. This matches closely the reports of Grange (1987) who showed the hydrophobic waxy coats of mycobacteria resulted in their inhabiting air-water interfaces preferentially. In the present study, a reduction in samples testing positive for mycobacteria occurred progressively through the South Finger Reedbed and no mycobacteria were detected at the outflow.

Wetlands are frequently regarded as major sources of humic substances (Hemond & Benoit, 1988). Growth of environmental mycobacteria is stimulated by the presence of humic acids (Kirschner, *et al.*, 1999) and extraction of DNA from soils and sediment always results in co-extraction of humic substances (Zhou, *et al.*, 1996). This poses a problem, however, as humic acids are common inhibitors of PCR (Wilson, 1997). Humic acids can inhibit the action of *Taq* DNA polymerase (Smalla, *et al.*, 1993) and reduce DNA hybridisation specificity (Steffan & Atlas, 1988). To help overcome this potential problem, BSA was added to the PCR mixture in this study. BSA has proved effective at overcoming some of the inhibitory effects of humic acids on PCR (Wilson, 1997).

Fate of *Mycobacterium avium* in the South Finger Reedbed

M. avium was found to be unevenly distributed within the South Finger Reedbed, being present in the settlement lagoon and first set of macrophyte beds but not at the South Finger Reedbed outlet. These findings broadly corroborate those of Mwangi (2003) who identified *M. avium* at the South Finger Reedbed inlet and first settling pool but not thereafter, in a preliminary study over a comparable time period to the present study. Taken together, the

results of the present study and those of Mwangi (2003) identified a total of 10/55 (18 %) of samples collected upstream of the final polishing beds as positive for *M. avium* compared with 0/12 (0%) of samples collected downstream of the final polishing beds. The finding of *M. avium* in sediment from the bottom of the settlement lagoon, but not in surface or mid-depth water at this same location, suggests this lagoon is performing its sedimentation role in removing suspended solids from the water. Indeed, measurements taken at time of sampling (not shown) indicated water depth in the settlement lagoon has decreased by 40% in the past 10 years, as a result of sediment accumulation on the lagoon floor. Stenstrom and Carlander (2001) showed that sedimentation of particles and associated micro-organisms is an important factor in reducing the microbial load from water in treatment wetlands. In a three-year study of the South Finger Reedbed, Millett (1997) found suspended solid removal efficiency rarely fell below 70% and was often over 90%. Falkinham *et al.* (2001) found *M. avium* cells to be bound to suspended particles in a water distribution system; many are therefore likely to sediment out in a settling pool. The results of the present study support this hypothesis.

Intensive sampling (of water, macrophytes' stems and roots, and sediment) in this study revealed *M. avium* at the inlet to the *Phragmites* bed, and, perhaps surprisingly, throughout the *Typha* bed. Three of the four *M. avium*-positive samples from the vegetation beds were of macrophyte stems, and one was a surface-water sample. Together with the results from the settlement lagoon, these findings suggest that, for removal of *M. avium*, a constructed reed bed should ideally compose of a settlement lagoon *and* one or more vegetation beds. Such a design would allow macrophytes to remove any *M. avium* that escape sedimentation and ultraviolet sunlight exposure in the settlement lagoon. It may also play a role in removal of other pathogens including viruses which are also predominantly associated with sediment and macrophytes (Jackson & Jackson, 2008).

This study has gone some way into answering the practical question: 'What length of reed bed is required to remove *M. avium* from water?' Since *M. avium* was present in the outflow from the reedmace bed, which is approximately 200 m from the inflow rhine (see Figure 1), it would seem a constructed reed bed (settlement lagoon plus vegetation beds) may need to be at least this long to be effective. *M. avium* removal may occur with equal efficiency in a smaller settlement lagoon than the one that is part of the South Finger Reedbed, but further research (e.g. using seeding of experimental constructed wetlands) is needed to answer this question categorically.

One of the objectives of this study was to compare *M. avium*-removal efficiency of the common reed bed with that of the reedmace bed. *M. avium* was found on macrophyte stems growing at the inlet of both beds. Whereas *M. avium* occurred throughout the reedmace bed, it was not found after the inlet to the common reed bed. These results suggest the common reed may be more efficient than reedmace at removing *M. avium* from water, although since the amount of *M. avium* entering each bed may have been dissimilar it is not possible to conclude this with confidence. The performance of a reed bed may change over time as a consequence of changes in species composition (Brix & Schierup, 1989). Since reedmaces are particularly aggressive invaders that readily colonise beds planted with slower-growing species (Millett, 1997), any deficiency in *M. avium* clearance of reedmaces is potentially extremely significant. A more intensive further study could usefully be conducted to compare these two reed beds, particularly if quantification of the *M. avium* present were to be carried out. Based on the results of the current study, such research should focus on sampling macrophyte stems.

The finding of *M. avium* on macrophyte stems, but not roots, is perhaps unexpected. However, root secretions from the common reed have been shown to kill pathogenic bacteria (*Salmonella*) and faecal indicators (*E. coli*) (Seidel 1976 and Vincent et al. 1994, cited by

Ottova, *et al.*, 1997), offering a possible explanation for the lack of *M. avium* in root samples. Furthermore, the extensive roots and hollow rhizomes of the common reed provide a large surface area for bacterial degradation (Brix & Schierup, 1989). Oxygen leakage by such roots creates oxidised microzones in an otherwise reduced substrate, supporting aerobic and anoxic degradation of organic matter (Brix, 1997). Any *M. avium* in such an environment is likely to be broken down rapidly. It was observed that water flowing through the vegetation beds regularly contacted macrophyte stems that were breaking the water's surface, due to the high density of plants growing in each bed. Thus adhesion or adsorption of *M. avium* onto macrophyte stems may occur readily, explaining why the majority of *M. avium*-positive samples came from macrophyte stems.

It should be appreciated that the capacity of a constructed wetland to treat water is finite. Furthermore, on-going management (e.g. harvesting of reeds, re-direction of water-flow) is essential if wetlands' removal efficiency is to be maintained (Wetzel, 2001). Wood and McAtamney (1994) stated that reed bed technology systems usually function adequately for up to 25-30 years, during which time the wetland 'peat' may double in depth as macrophyte dieback and sedimentation occur. The settlement lagoon preceding the South Finger Reedbed at WWT Slimbridge was designed with a functional 'life expectancy' of at least 25 years (Millett, 1997). It is envisaged that after this time settled sediment will have completely filled it, and emergent plants will have colonised, requiring re-excavation of the lagoon. The functional effects of temporal changes in reed bed composition on *M. avium* removal are unknown.

Having determined the presence and locations of *M. avium* in the South Finger Reedbed in this study, future research in the quantification of *M. avium* present could usefully be conducted. Real-time PCR may prove helpful for this purpose. A problem remains, however,

since PCR does not differentiate viable from dead micro-organisms (Rideout, 2003). Further, culture of mycobacteria is limited by the organisms' fastidious nature and exceedingly slow growth (Matthews, *et al.*, 1978, Aranaz, *et al.*, 1997). One solution would be to employ reverse transcriptase PCR (RT-PCR) using primers specific for *M. avium* messenger RNA (mRNA), since mRNA is unstable and degrades rapidly upon death of a mycobacterium. The genetic sequence of mRNA reflects that of the corresponding DNA being transcribed. Because the currently recognised *M. avium*-specific markers have no known function and are not believed to be transcribed, this approach remains theoretical. Furthermore, validation of such a RT-PCR would involve demonstrating that there are no other common micro-organisms that share part or all of the mRNA genetic sequence (Rideout, 2003). The identification of specific markers for strains of *M. avium* associated with ATB, that are distinguishable from other *M. avium* strains and closely-related species, poses real problems because *M. avium* is an environmental micro-organism. Thus, for the foreseeable future at least, culture is likely to remain the definitive method for distinguishing live from dead *M. avium*.

It can be concluded that the reed bed studied removes *M. avium* from the effluent it receives through a combination of sedimentation and adsorption onto growing macrophyte stems. Water discharging into the River Severn contains a reduced amount of *M. avium.* This study has shown a constructed reed bed to be an effective bioremediator and its design could serve as a useful model for the environmental control of avian tuberculosis in a wide range of situations globally, such as within zoological collections or where poultry and pigs are farmed in close proximity.

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Table 1. Presence and locations of mycobacteria in a constructed reed bed, as detected by single-stage and nested PCR.

Sampling location[a]	Total environmental mycobacteria[b]			*Mycobacterium avium*[c]		
	Surface water	Mid-depth water & macrophyte stems	Sediment & macrophyte roots	Surface water	Mid-depth water & macrophyte stems	Sediment & macrophyte roots
Positive control in wildfowl collection[d]	+	+	+	+	+	+
1. Inflow rhine: by fox-proof fence	+	+	+	–	–	–
2. Inflow rhine: pre-settlement lagoon	+	–	+	–	–	–
3. Settlement lagoon: inlet	+	+	+	–	–	–
4. Settlement lagoon: one third across	+	–	+	–	–	–
5. Settlement lagoon: two thirds across	+	+	–	–	–	+
6. Settlement lagoon: outflow	+	+	+	–	–	–
7. Reedmace bed: inlet	–	+	+	–	+	–
8. Reedmace bed: mid-point	–	+	–	+	–	–
9. Reedmace bed: outflow	–	+	+	–	+	–
10. Common reed bed: inlet	–	+	–	–	+	–
11. Common reed bed: mid-point	–	–	–	–	–	–
12. Common reed bed: outflow	–	–	–	–	–	–
13. Polishing bed: reedmace	–	+	+	–	–	–
14. Polishing bed: common reed	–	+	+	–	–	–
15. Reed bed outflow: Kingfisher Pool	–	–	–	–	–	–

a. Refer to Figure 1 for geographical locations of sampling sites.
b. Total environmental mycobacteria detected using single-stage PCR.
c. *Mycobacterium avium* detected using nested PCR.
d. Samples collected from white-winged duck (*Cairina scutulata*) enclosure.
\+ Mycobacteria detected.
– Mycobacteria not detected.

FIGURE LEGENDS

Figure 1. Plan of the South Finger Reedbed at the Wildfowl & Wetlands Trust, Slimbridge. Water leaving the wildfowl collection enters the reed bed via a rhine (ditch), passes through a large settlement lagoon and filters through one of three treatment reed beds, all of which empty into a small rafted lagoon. After passing over a chalk cascade and through the cascade lagoon, water enters one of two final polishing beds before leaving the reed bed via an outflow into the Kingfisher Pool and thence the River Severn.

Figure 2. A freshly-plucked common reed (*Phragmites australis*) showing the three sampling locations. A = water surface level (air-water interface); B = submerged stem; C = rhizome-sediment matrix.

Figure 3. Agarose (3%) gel electrophoretogram of single-stage PCR product after 45 cycles of amplification with primers Tb11 and Tb12 to detect mycobacterial DNA. Lane 1, inflow rhine (surface water) [1]. Lane 2, inflow rhine (mid-depth water) [1]. Lane 3, inflow rhine (sediment) [1]. Lane 4, settlement lagoon (surface water) [4]. Lane 5, settlement lagoon (mid-depth water) [4]. Lane 6, settlement lagoon (sediment) [4]. Lane 7, common reed bed (*Phragmites australis*) (surface water) [11]. Lane 8, common reed bed (macrophyte stem) [11]. Lane 9, common reed bed (macrophyte root) [11]. Lane 10, Kingfisher Pool at outflow of South Finger Reedbed (surface water) [15]. Lane 11, molecular mass markers. Figures in square brackets refer to sampling locations indicated on Figure 1. The 439 bp band indicates presence of mycobacteria.

Figure 4. *Mycobacterium avium* nested PCR after 45 cycles of amplification with primers Av6 and Av7 followed by 27 cycles with primers Av8 and Av9. Lane 1, settlement lagoon

(sediment) [5]. Lane 2, inlet to reedmace bed (*Typha latifolia*) (macrophyte stem sample) [7]. Lane 3, mid-point of reedmace bed (surface water) [8]. Lane 4, mid-point of reedmace bed (macrophyte stem) [8]. Lane 5, reedmace bed outflow (macrophyte stem) [9]. Lane 6, inlet to common reed bed (*Phragmites australis*) (macrophyte stem) [10]. Lane 7, mid-point of common reed bed (surface water) [11]. Lane 8, mid-point of common reed bed (macrophyte stem) [11]. Lane 9, common reed bed outflow (macrophyte stem) [12]. Lane 10, Kingfisher Pool at outflow of South Finger Reedbed (macrophyte stem) [15]. Lane 11, molecular mass markers. Figures in square brackets refer to sampling locations indicated in Figure 1. The band of 102 bp is *M. avium* specific.

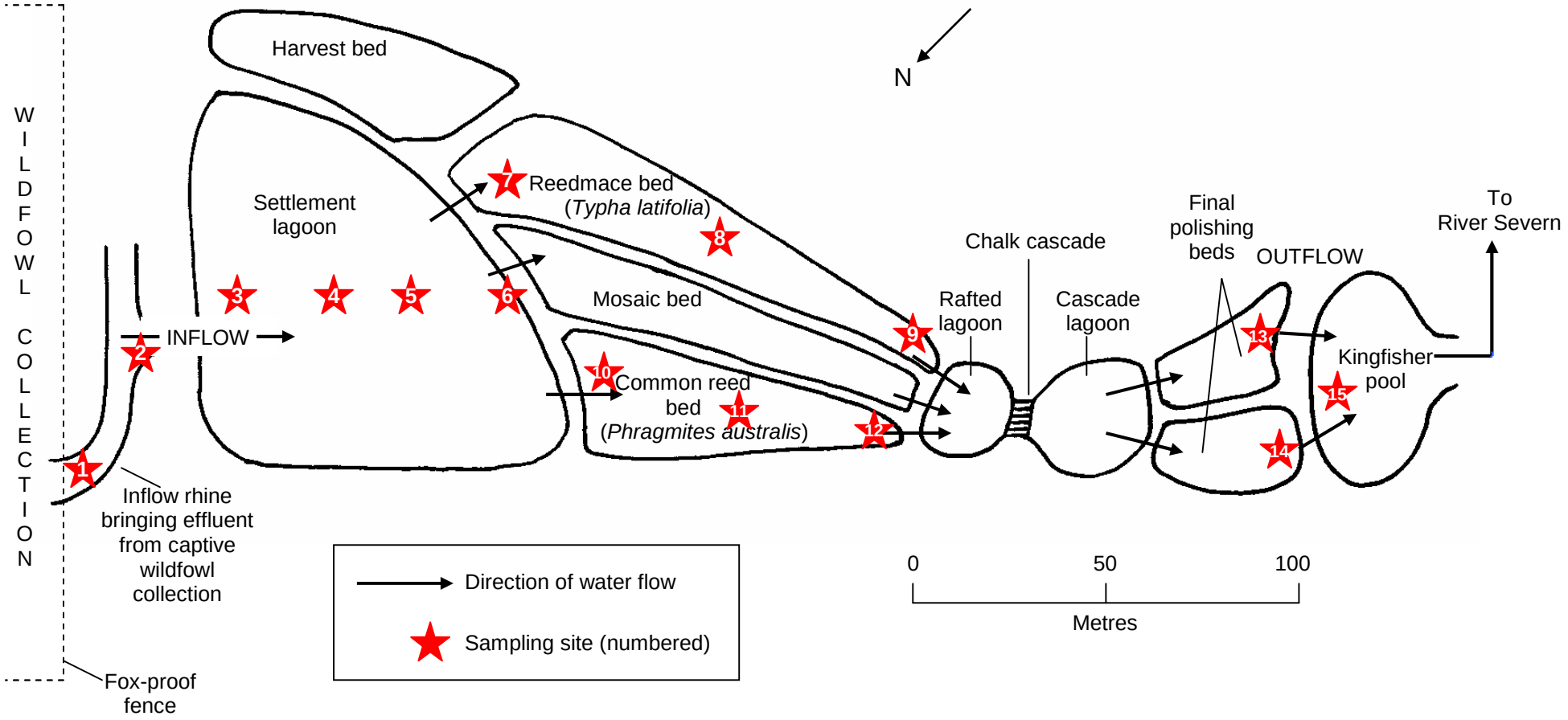
WILDFOWL COLLECTION
Harvest bed
N
Settlement lagoon
Reedmace bed (Typha latifolia)
Mosaic bed
Common reed bed (Phragmites australis)
INFLOW
Rafted lagoon
Chalk cascade
Cascade lagoon
Final polishing beds
OUTFLOW
To River Severn
Kingfisher pool
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Inflow rhine bringing effluent from captive wildfowl collection
Fox-proof fence
Direction of water flow
Sampling site (numbered)
0
50
100
Metres

Figure 1.

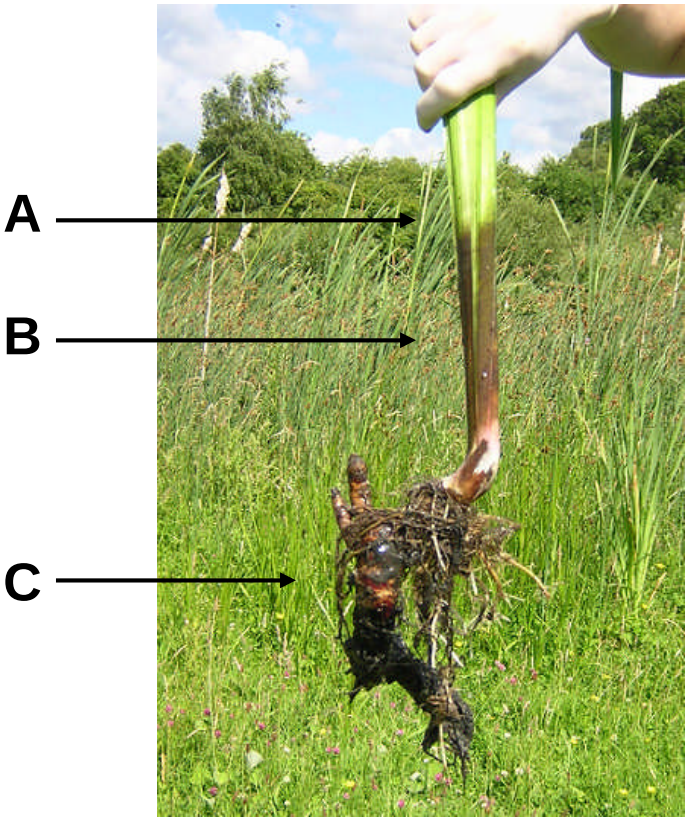

Figure 2.

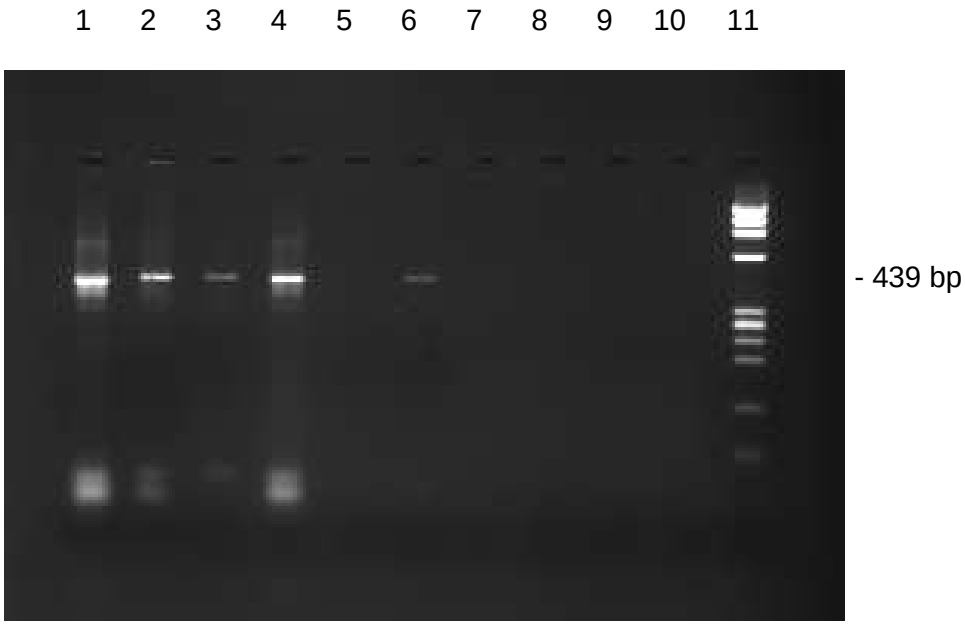

Figure 3.

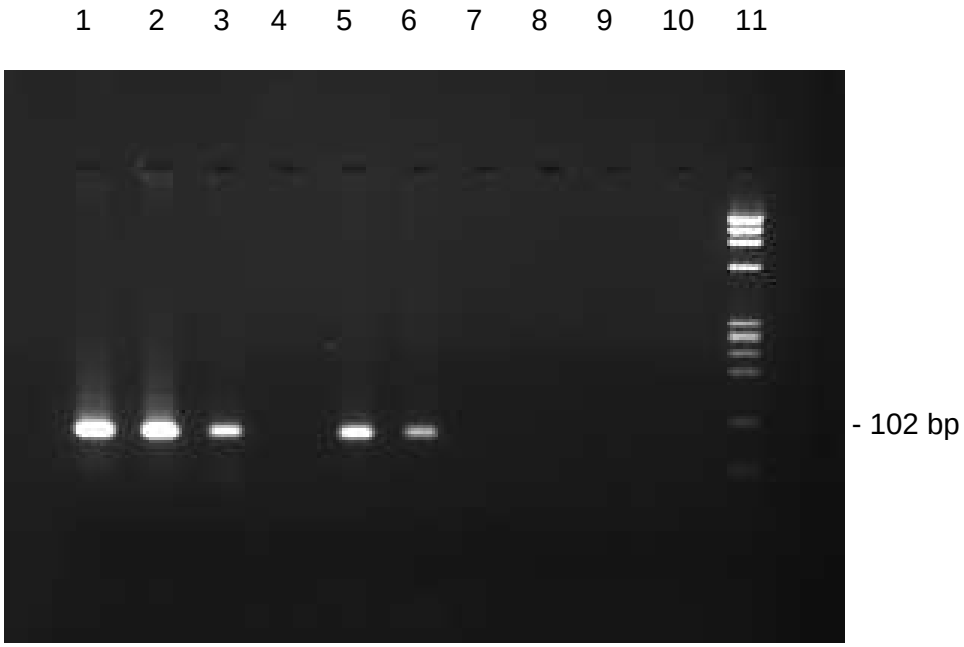

Figure 4.