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Mycobacterium avium subsp. *paratuberculosis* in the Catchment Area and Water of the River Taff in South Wales, United Kingdom, and Its Potential Relationship to Clustering of Crohn's Disease Cases in the City of Cardiff

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In South Wales, United Kingdom, a populated coastal region lies beneath hill pastures grazed by livestock in which *Mycobacterium avium* subsp. *paratuberculosis* is endemic. The Taff is a spate river running off the hills and through the principal city of Cardiff. We sampled Taff water above Cardiff twice weekly from November 2001 to November 2002. *M. avium* subsp. *paratuberculosis* was detected by IS*900* PCR and culture. Thirty-one of 96 daily samples (32.3%) were IS*900* PCR positive, and 12 grew *M. avium* subsp. *paratuberculosis* bovine strains. Amplicon sequences from colonies were identical to the sequence with GenBank accession no. X16293, whereas 16 of 19 sequences from river water DNA extracts had a single-nucleotide polymorphism at position 214. This is consistent with a different strain of *M. avium* subsp. *paratuberculosis* in the river, which is unculturable by the methods we used. Parallel studies showed that *M. avium* subsp. *paratuberculosis* remained culturable in lake water microcosms for 632 days and persisted to 841 days. Of four reservoirs controlling the catchment area of the Taff, *M. avium* subsp. *paratuberculosis* was present in surface sediments from three and in sediment cores from two, consistent with deposition over at least 50 years. Previous epidemiological research in Cardiff demonstrated a highly significant increase of Crohn's disease in 11 districts. These bordered the river except for a gap on the windward side. A topographical relief map shows that this gap is directly opposite a valley open to the prevailing southwesterly winds. This would influence the distribution of aerosols carrying *M. avium* subsp. *paratuberculosis* from the river.

Mycobacterium avium subsp. *paratuberculosis* is a member of the *M. avium* complex (70). *M. avium* strains are widely distributed in the environment as well as in birds, in animals, and in humans (37, 60, 75). They do not usually cause disease unless the host is debilitated or immunocompromised. By contrast *M. avium* subsp. *paratuberculosis* is a specific pathogen with the ability to cause chronic inflammation of the intestine, Johne's disease, in many species, including primates (13, 17, 32, 49). *M. avium* subsp. *paratuberculosis* disease in animals ranges from pluribacillary to paucimicrobial (15), like leprosy in humans. In the United Kingdom, the United States, and the Czech Republic *M. avium* subsp. *paratuberculosis* has been cultured from 1.6 to 2.8% of units of retail pasteurized cow's milk (1a, 23a, 30, 55), and live organisms must be transmitted to humans by this route. These chronic enteric pathogens are implicated in the causation of chronic inflammation of the intestine of the Crohn's disease type (8, 14, 31, 36, 37, 54, 57, 62).

M. avium subsp. *paratuberculosis* can live in the guts of animals for years without necessarily causing clinical disease (61). Infection is widespread in domestic livestock in western Europe and North America (18, 37, 49). Wildlife reservoirs may contribute to its environmental persistence (3, 4, 27) and cycles of reinfection (22, 23). Infected animals, particularly those with the more common pluribacillary form of *M. avium* subsp. *paratuberculosis* disease, excrete huge numbers of these bacilli in their feces (29, 61). *M. avium* subsp. *paratuberculosis* is a robust mycobacterium which can survive for months or even for years in the environment (32, 61), since no upper limit on its environmental persistence has yet been established. To date, very little is known about the ecology, trafficking, and fate of *M. avium* subsp. *paratuberculosis* in the environment (29).

As with other potentially zoonotic pathogens (28, 29), rain falling onto pastures contaminated with *M. avium* subsp. *paratuberculosis* would be expected to wash these organisms into surface waters and rivers (21, 71). There is an unquantified risk that *M. avium* subsp. *paratuberculosis* maybe transmitted to humans where such waters are sourced for domestic supply (46). Where a heavily contaminated fast-flowing river runs through a population center, there is also an unquantified risk of human exposure via aerosols (73). Conditions such as these exist in South Wales in the United Kingdom, where a densely populated coastal plain lies beneath the upland pastures of the Brecon hills to the north, which are grazed by cattle and sheep in which *M. avium* subsp. *paratuberculosis* infection is endemic (12).

The Taff is a major spate river which runs off the Brecon hills in a southeasterly direction and through the city of Cardiff beside the sea. Epidemiological research carried out in Cardiff

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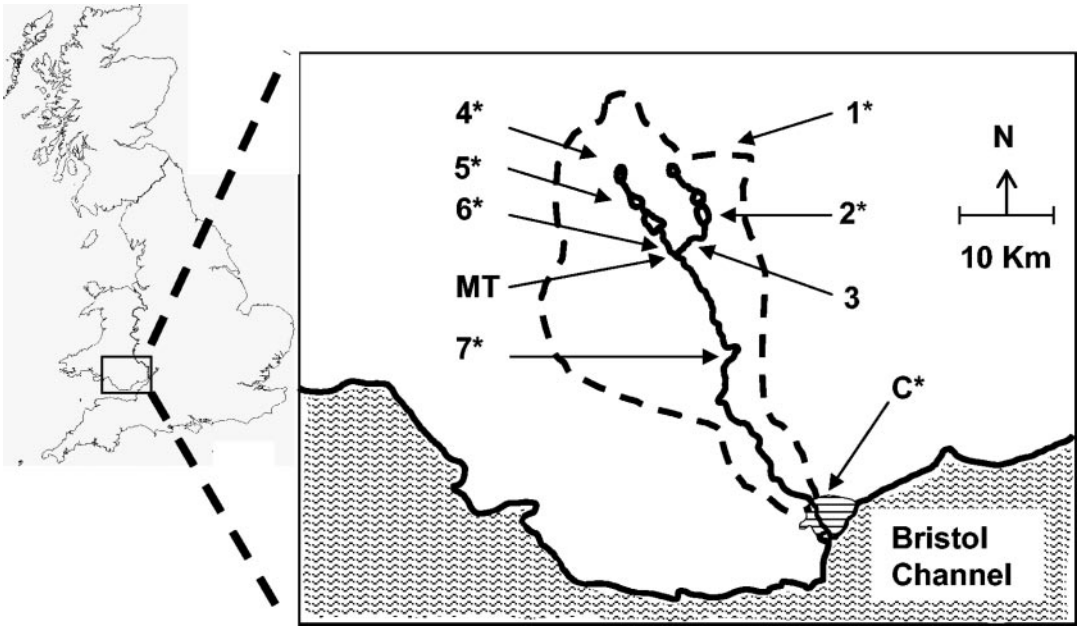


FIG. 1. Location of the study region in South Wales, United Kingdom. The enlargement shows the catchment area (enclosed by the dashed line) of the river Taff, which at the time of the study contained 30,435 beef and dairy cattle and 571,429 sheep, of which 304,443 were breeding ewes (see text). The river Taff itself is a spate river which runs down from the Brecon hills and through the city of Cardiff before entering the Bristol Channel. The prevailing winds are from the Atlantic to the southwest. 1, Upper Neuadd reservoir; 2, Pontsticill reservoir; 3, Taff Fechan River; 4, Brecons reservoir; 5, Llwyn-on reservoir; 6, Taff Faw River; 7, river Taff. MT, town of Merthyr Tydfil; C, city of Cardiff. *, sampling sites.

in the mid-1970s demonstrated a highly significant increase in the incidence of Crohn's disease in 11 of the local electoral city wards (52, 53). Of these 11 high-incidence wards, 8 directly bordered the river Taff, and the 3 that did not were immediately adjacent to the northeast (52, 53). It has been proposed that this is the direction in which aerosols containing *M. avium* subsp. *paratuberculosis* may be carried by the prevailing southwesterly wind (35). The present paper describes the first step in examining this hypothesis experimentally by testing for the presence of *M. avium* subsp. *paratuberculosis* in the catchment area of the Taff and by monitoring the presence of the organism twice weekly in Taff river water from November 2002 over a period of one year. We have also studied for how long *M. avium* subsp. *paratuberculosis* may persist in a model freshwater system.

MATERIALS AND METHODS

Taff catchment area and river sampling. The upper region of the Taff catchment area on the Brecon hills to the north of Cardiff (Fig. 1) was sampled in July 2002. Sediment cores (30 cm in length) were taken from the Pontsticill reservoir (United Kingdom Ordnance Survey grid reference SO056130) and Llwyn-on reservoir (SO009122) at a depth of 20 m, using a Jenkin sampler (56). The cores were themselves sampled from ports situated at the top (0 to 5 cm), middle (15 to 20 cm), and bottom (25 to 30 cm) of each core tube by syringe aspiration with wide-gauge sterile needles. This was carried out on site, with sediment being stored in sterile McCartney tubes as previously described (56). From higher up in the catchment area, sediment samples were also taken from exposed bottom sediment of the drained Upper Neuadd reservoir (SO029158) and from the west bank of the Beacons reservoir (SN986183), using sterile spatulas, and were stored in sealed containers. In addition, a sample was taken from accumulated biofilm material behind a weir in the upper reaches of the river Taff itself (ST081939) (Fig. 1).

Water samples were taken twice weekly from the river Taff in Cardiff, South Wales, at United Kingdom Ordnance Survey grid reference ST138804, from 20 November 2001 to 18 November 2002. At this point the river is 40 m wide. One-hundred-liter samples of river water were taken from 10 cm below the river surface, 5 m from the bank, by using a pipe attached to a telescopic pole and a pump. The 100 liters was collected in four 25-liter sterilized plastic containers and transported directly to the laboratory for processing by tangential flow filtration (TFF) (58). Particles of >0.22 μm in diameter from raw river water were concentrated by TFF with a Pellicon TFF unit (Millipore, Milton Keynes, United Kingdom) fitted with three 0.22-μm-pore-size Pellicon filters (Millipore) (58). The 1-liter concentrates (retentate) (58) were stored at 4°C until processed. Immediately after filtration, the TFF apparatus was thoroughly cleaned with 5 liters of 0.5% sodium dodecyl sulfate, followed by 1 liter of 10% ethanol and washing with 10 liters of water. The filters were maintained in 5% formalin between uses. TFF filters were tested at intervals for contamination by residual *M. avium* subsp. *paratuberculosis* by processing of 100 liters of permeative water (filtered output from TFF) followed by IS*900* PCR on the concentrate. In each case the filters were found to be IS*900* negative. General bacterial contamination was checked by epifluorescence microscopy and was found to be absent. All apparatus (telescopic pole, pump, and Jenkin core and core tubes) was washed with detergent and 70% ethanol and rinsed with distilled water between uses. Sample tubing was similarly treated and then autoclaved at 121°C for 15 min.

DNA extraction. One hundred milliliters of TFF retentate (equivalent to 10 liters of raw river water) was initially filtered through 0.2-μm-pore-size membrane filters (Pall-Gelman, Portsmouth, United Kingdom). Retained material was resuspended in 3 ml of 1 M Tris-HCl (pH 7.4) by vortexing and scraping with sterile plastic loops. The cleaned membranes were discarded, and the remaining sample was aliquoted into 1.5-ml Eppendorf tubes and concentrated by centrifugation at $13,000 \times g$ for 10 min. The supernatant was removed, and the sample was pooled in a final volume of 100 to 500 μl. DNA was then extracted by the procedure described in the Ultraclean Soil DNA kit (Mo-Bio, Solana Beach, Calif.) with one modification. In the manufacturer's protocol samples are disrupted with a bead-beating tube. In the present modification, the bead-beating method was replaced by the use of Lysing Matrix B Ribolyser tubes (QBiogene, Nottingham, United Kingdom). Samples were then disrupted in a FastPrep Ribolyser (QBiogene) at a setting of 6.5 m s^{-1} for 45 s. The remainder of the manufacturer's procedure was unchanged.

IS*900* PCR and amplicon sequencing. PCR was carried out in 0.2-ml thin-walled PCR tubes (ABgene, Epsom, United Kingdom) with the FailSafe PCR system (Epicentre, Madison, Wis.) in a Px2 thermal cycler (ThermoLifeSciences, Basingstoke, United Kingdom). Genomic DNA isolated from *M. avium* subsp. *paratuberculosis* 989 was used as a positive control throughout, and molecular

biology-grade water (Sigma, Poole, United Kingdom) was used as a negative control. A nested PCR strategy designated IS*900*[L/AV] (8) was used to amplify IS*900*. Reagents were defrosted and maintained on ice throughout. Reaction mixtures (50 μl) were prepared so that each received the following: 5 μl of template DNA, 1 μl of each primer (final concentration, 0.2 μM), 25 μl of 2× FailSafe reaction premix (premix G in the first round and premix C in the nested reaction), 0.5 μl of PCR enzyme mix, and 17.5 μl of water. For first-round amplification primers L1 (5′-CTT TCT TGA AGG GTG TTC GG-3′) and L2 (5′-ACG TGA CCT CGC CTC CAT-3′) were used (8). The nested reaction employed primers AV1 (5′-ATG TGG TTG CTG TGT TGG ATG G-3′)and AV2 (5′-CCG CCG CAA TCA ACT CCA G-3′), using 5 μl of the first-round amplification product as the template. In both the first and the nested rounds of amplification, three negative control tubes were incorporated, making six in all after the full amplification. Cycling conditions were the same for both rounds of the process and comprised 1 cycle of template denaturation at 95°C for 4 min (at the start of this stage the machine was paused and tubes were added directly from ice, ensuring a manual hot-start process); 30 cycles of 95°C for 1 min, 58°C for 45 s, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. Reactions were held at 4°C until products could be confirmed to be the correct size (298 bp) by examination under UV after electrophoresis in 1 to 1.5% (wt/vol) agarose gels stained with ethidium bromide. DNA sequencing was carried out twice on both strands of the 298-bp AV1/AV2-derived amplicons by Qiagen (Crawley, United Kingdom). To assess whether PCR-negative results were caused by sample inhibition, two reaction master solutions were prepared. These differed only in that one contained sample to be tested in addition to a spike of 5 μl of positive control DNA.

Culture of sediments from the Taff catchment area and of Taff river water. Samples of sediment from the Taff catchment area and centrifugal pellets from river water were decontaminated by incubation with 2% NALC (NaOH-*N*-acetyl-L-cysteine) for 20 min and then neutralized with phosphate-buffered saline (PBS) and centrifuged at 3,000 × *g* for 20 min. The pellet was resuspended in 0.7% hexadecylpyridinium chloride in half-strength brain heart infusion broth and incubated at 37°C overnight. The mixture was then filtered by centrifugation with 10-μm-pore-size VectaSpin centrifuge tubes (Whatman, Maidstone, United Kingdom), and the filtrate was concentrated by further centrifugation at 3,000 × *g* for 20 min. The pellet was resuspended in 1 ml of sterile water containing 100 μg of vancomycin and nalidixic acid ml^{-1} and 50 μg of amphotericin B ml^{-1} and incubated for 48 to 72 h. The sample was then inoculated on Herrold's egg yolk medium (HEYM) supplemented with mycobactin J at 2 μg ml^{-1} and nalidixic acid, vancomycin, and amphotericin B, all at a final concentration of 50 μg ml^{-1}. Plates were sealed and incubated at 37°C for up to 16 months before subculture. Successful isolates of *M. avium* subsp. *paratuberculosis* were characterized by IS*900* PCR with amplicon sequencing and typed by using the mycobacterial interspersed repetitive unit (MIRU) PCR-based system as previously described (9).

Analysis of suspended solids in river water. The amount of suspended solids in Taff river water was determined as follows. Glass fiber filters (47-mm-diameter; Whatman, Cambridge, United Kingdom) were pretreated at 550°C for 24 h. Each filter was allowed to cool and then weighed to four decimal places and stored in a desiccator at room temperature prior to use. A subsample of 50 ml of retentate (equivalent of 5 liters of river water) was filtered under vacuum. The filters were then dried for 24 h at 125°C and placed in a desiccator to cool to room temperature. They were then reweighed, and the weight of suspended solids was expressed as milligrams per liter of native Taff river water.

Environmental data. Rainfall, height, and flow data for the river Taff were provided by the Environment Agency (Wales). Data on the precise boundaries and stocking densities for the river Taff catchment area were provided by Geographical Information Systems Services, Welsh Assembly, Cardiff, Wales, United Kingdom. The topographical relief map of Cardiff flanking the Taff River as it flows through the city was derived by Memory Map with mapping data sourced, with permission, from the Ordnance Survey.

Statistical methods and analyses. Standard analysis of variance (16) was used to determine whether positive tests for IS*900* on Taff river water were related to significantly different river height, river flow, and suspended solids. These were log transformed to homogenize the residual variance. The mean rainfall values on days of IS*900*-positive river samples and on each of the preceding 7 days were compared with the mean rainfall values on days of IS*900*-negative samples by analysis of variance. Rainfall was square-root transformed to assist the homogeneity of the residual variance. Linear discriminant analysis was used to determine whether river characteristics and rainfall could be combined to form an index with which to predict the presence of IS*900* (51). A randomization method was devised to test for clustering of IS*900*-positive days. For the test statistic, we used the number of days on which one positive result followed a positive result on the preceding sampling occasion. If there was no clustering at all, we would expect positive days to be randomly placed in the series according to their observed frequency. If complete clustering was present, we would expect all positive days to appear together. The method estimated the probability of obtaining the observed number of days from the random assumption. If the probability was small, randomness would be discounted.

Survival of *M. avium* subsp. *paratuberculosis* in water. *M. avium* subsp. *paratuberculosis* strain 989 (kindly provided by D. M. Collins, AgResearch, Upper Hutt, New Zealand) was grown in mycobacterial growth indicator tube (MGIT) liquid medium (Becton Dickinson, Oxford, United Kingdom) supplemented with (final concentrations) 10% (vol/vol) oleic acid-albumin-dextrose-catalase, PANTA (40 U of polymixin B ml^{-1}, 4 μg of amphotericin B ml^{-1}, 16 μg of nalidixic acid ml^{-1}, 4 μg of trimethoprim ml^{-1}, 4 μg of azolacin ml^{-1}), and mycobactin J (2 μg ml^{-1}) (Allied Monitor, Fayette, Mo.). Water samples were collected from the north basin of Windermere, Cumbria, United Kingdom (United Kingdom Ordnance Survey grid reference NY382008). Four sterile-release microcosms were prepared by adding 330 ml of filtered (0.2 μm-pore-size membranes; Pall-Gelman) lake water to 1-liter wide-mouthed polypropylene bottles prior to autoclaving at 121°C. One milliliter of cells from a 4-week-old culture of *M. avium* subsp. *paratuberculosis* 989 in liquid MGIT medium was concentrated by centrifugation at 10,000 × *g* for 5 min and washed three times in sterile filtered PBS (sfPBS). Washed cells were resuspended in 500 μl of fresh sfPBS. One hundred microliters of this suspension was added to each of three microcosms (designated A, B and C). The fourth microcosm (D) received sfPBS only in order to control for contamination throughout the experiment. At each sampling interval, 2 ml was aseptically removed from microcosms A to D. From this, 500 μl was used directly for DNA extraction and IS*900* PCR. Cultivability of *M. avium* subsp. *paratuberculosis* in liquid medium was assessed by adding 500 μl (from the original 2-ml aliquot) to an MGIT supplemented as described above and incubating for up to 12 months. The remaining 1 ml was used in part for culture on solid medium. Serial dilutions were prepared with sterile lake water as the diluent, and 100 μl was spread in triplicate onto Middlebrook 7H11 agar (Becton Dickinson) supplemented with mycobactin J (2 μg ml^{-1}), 10% (vol/vol) oleic acid-albumin-dextrose-catalase, 0.6% (wt/vol) glucose, 0.42% (wt/vol) pyruvic acid, penicillin G (15 μg ml^{-1}), and chloramphenicol (25 μg ml^{-1}). The spread plates were double wrapped in Parafilm (Sigma) and incubated at 37°C in the dark for 4 months. Counts of CFU $milliliter^{-1}$ were determined at several intervals before final numbers were recorded at 4 months, after which no new colonies emerged.

Quantitative real-time PCR of IS*900*. Real-time PCR amplification of IS*900* was also used to quantify the numbers of *M. avium* subsp. *paratuberculosis* cells persisting in the model lake water systems. This was carried out with the ABI Prism 7000 sequence detection system (Applied Biosystems, Warrington, United Kingdom) with previously described primers F2 (5′-AAT GAC GGT TAC GGA GGT GGT-3′) and R2 (5′-GCA GTA ATG GTC GGC CTT ACC-3′) and internal TaqMan probe P2 (5′-TCC ACG CCC GCC CAG ACA GG-3′) (42). The TaqMan probe was labeled at the 5′ end with the fluorescent reporter dye 5-carboxyfluoroescein and at the 3′ end with the quencher dye *N*′,*N*′,*N*′,*N*′-teratmethyl-6-carboxyrhodamine. Primer and probe concentrations were optimized according to protocols specific to the TaqMan Universal PCR Master Mix. Amplification reactions were carried out in individual optical PCR tubes (Applied Biosystems), and the mixtures (50 μl) contained 25 μl of 2× TaqMan Universal Master Mix (AmpliTaq Gold DNA polymerase, AmpErase uracyl-*N*-glycosylase, deoxynucleoside triphosphates with dUTP, and passive reference and optimized buffer components), 5 μl of each primer (F2 at 900 nM and R2 at 300 nM), 5 μl of TaqMan probe P2 (250 nM), 5 μl of DNA, and 5 μl of water. No-template controls received water instead of DNA. The following cycling profile was used: 1 cycle of 50°C for 2 min (for optimal uracyl-*N*-glycosylase activity), 1 cycle of 95°C for 10 min (for activation of the AmpliTaq Gold enzyme), and 40 cycles of 95°C for 15 s and 60°C for 1 min. The standard positive control consisted of *M. avium* subsp. *paratuberculosis* strain 989 genomic DNA quantified spectrophotometrically at 260 nm. The number of genomes and copies of IS*900* in a given volume of this DNA was calculated based on an *M. avium* subsp. *paratuberculosis* genome size of 4.83 Mbp (GenBank accession no. NC002944) and an average 16 copies of IS*900* per genome. Standards for absolute quantification were then prepared by serial dilution. All standard, unknown, and no-template control reactions were run in triplicate. Quantification of the number of IS*900* targets in unknown samples was determined by measuring the threshold cycle value (which equates to the cycle at which a statistically significant increase in the ΔR_n is first detected). Average values were then converted to cells $milliliter^{-1}$ by dividing by 16 (7).

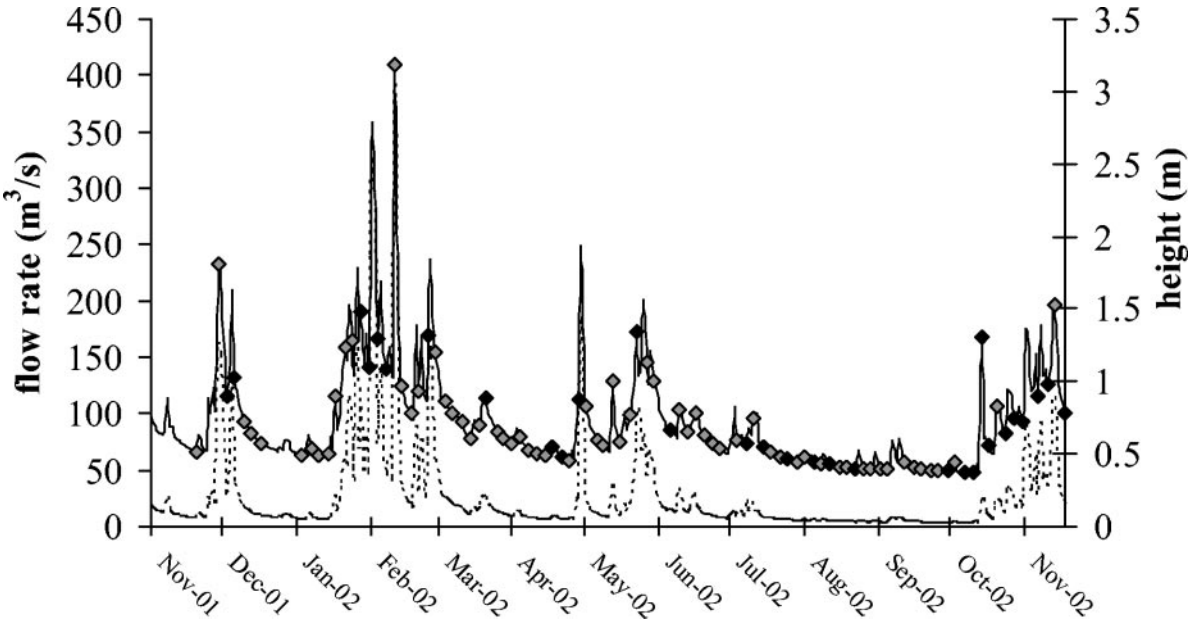


FIG. 2. Relationship between presence or absence of *M. avium* subsp. *paratuberculosis* and flow and river height of the river Taff (November 2001 to November 2002). Data were obtained by using the Hydrolog data management system, version 2.61 (Hydro-Logic Ltd., Bromyard, United Kingdom), from Pontypridd logging station (grid reference ST07938973). Plotted data were recorded at 9:00 a.m. each day. The solid line represents the river height, while the dotted line represents the flow rate. Sample points are shown on the river height graph as grey diamonds, and samples that were PCR positive for *M. avium* subsp. *paratuberculosis* are shown as black diamonds.

RESULTS

Taff catchment area and sediment cores. The river Taff (Fig. 1) is formed by the confluence of the Taff Fechan River from the east, which rises in the Brecon hills and is controlled by Pontsticill reservoir, and the Taff Faw River to the west, which also rises in the Breacon hills and is controlled by Llwyn-on reservoir. These rivers meet at Cefn Coed (United Kingdom Ordnance Survey grid reference SO038073) just north of the town of Merthyr Tydfil to form the river Taff itself (Fig. 1) The longest path length of the river Taff uninterrupted by inflows is 67 km. The river Taff catchment area comprises an area of 510 km^2 with a mean height of 296 m above sea level, a maximum height of 800 m, and an annual rainfall of about 1,800 mm. The catchment area comprises natural grassland grazed by cattle and sheep. During the sampling period the catchment area contained 1,013 farm units with a total of 30,435 cattle and calves (a mix of dairy and beef) and 571,429 sheep and lambs, including 304,443 breeding sheep.

From the highest part of the Taff catchment area sampled, sediment from the Upper Neuadd reservoir drained 2 months prior to sampling was IS*900* PCR negative. A Jenkin core of sediment from the younger Brecons reservoir could not be obtained because the bottom was still composed of a fibrous matt from previous pasture. The sample of sediment taken instead from the west bank of Brecons reservoir was also IS*900* PCR negative, as was the biofilm sample from a weir at ST081939 on the river Taff itself. Jenkin sediment cores from the lower reservoirs of Pontsticill and Llwyn-on, representing the east and west valleys of the catchment area, were both IS*900* PCR positive. Pontsticill was IS*900* positive in the upper 5 cm (top) of the core, and Llwyn-on was positive in the middle section (5 to 10 cm) of the core. The DNA sequences of all IS*900* PCR amplicons showed 100% identity with the sequence in the database.

Taff river water. Figure 2 shows the results of IS*900* PCR tests on river water for the 96 days of sampling throughout the year November 2001 to November 2002, together with the corresponding data on river height and river flow (suspended solids not presented). Thirty-one of the 96 samples (32.3%) of Taff river water were IS*900* PCR positive. Sequence data obtained for 19 of the 31 positive samples showed 100% identity with IS*900* (accession number X16293) in 3 samples and a single-nucleotide polymorphism occurring at nucleotide 214 of the amplicon in 16 samples. This was A to G in 14 samples and a consistently irresolvable A/G in 2 samples. Eleven IS*900*-negative samples were inhibitory to PCR.

Culture of environmental isolates of *M. avium* subsp. *paratuberculosis*. Culture was carried out on 18 of the IS*900*-positive river water samples where sufficient material was available stored as retentate at 4°C and on 10 of the sediment samples on HEYM incubated at 37°C for at least 16 months. Crumbly colonies typical of *M. avium* subsp. *paratuberculosis* (Fig. 3A and B) were obtained from 12 of the 18 PCR-positive Taff water samples (66%) appearing after 8 to 11 months of incubation. We also noticed that colonies often first appeared to form below the surface of the solid HYEM and subsequently appeared to break through the surface with progressive growth (Fig. 3C, D, and E). In addition, colonies of *M. avium* subsp. *paratuberculosis* were successfully cultured from two separate samples of sediment from the Upper Neuadd reservoir which had previously tested IS*900* PCR negative. All isolates from the Taff river water and the sediments were confirmed as *M. avium* subsp. *paratuberculosis* by IS*900* PCR and amplicon sequencing.

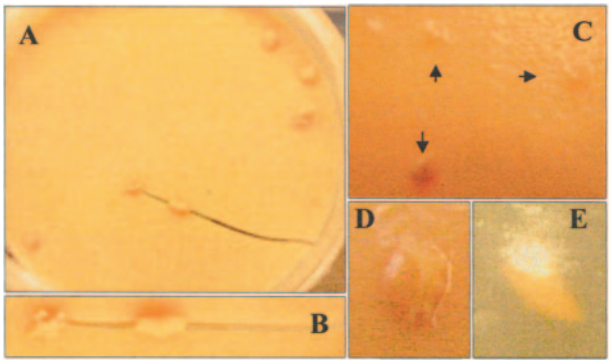


FIG. 3. Culture of *M. avium* subsp. *paratuberculosis* from decontaminated samples of Taff river water and catchment area sediment. Growth was first seen after 8 to 11 months of incubation on Herrold's egg yolk medium. (A and B) Typical crumbly colonies. (C, D, and E) Early colonies forming below the surface of the solid HEYM. All colonies were confirmed as *M. avium* subsp. *paratuberculosis* by IS*900* PCR with amplicon sequencing and by MIRU typing.

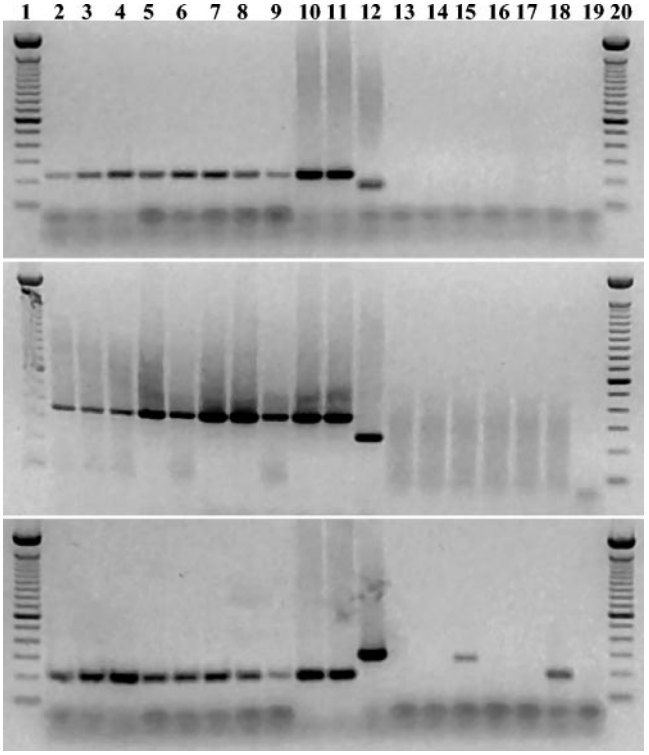


FIG. 4. Products obtained by amplification at MIRU locus 1 (upper panel), MIRU locus 2 (middle panel), and MIRU locus 4 (lower panel) of *M. avium* subsp. *paratuberculosis* isolates from reservoir sediment and Taff river water. Lanes: 1, 100-bp ladder; 2, isolate from sediment from the Upper Neuadd reservoir; 3 to 10, isolates from Taff river water; 11, contemporary isolate of a bovine strain of *M. avium* subsp. *paratuberculosis* from a cow with Johne's disease in the study region; 12, *M. avium* subsp. *avium* ATCC25291; 13, *Mycobacterium* sp. strain 2333 from Sweden, containing a single copy of an IS*900*-related element (25); 14, *M. chelonae*; 15, *M. scrofulaceum*; 16 to 18, three *Mycobacterium* strains with IS*900*-related elements from Australia (20); 19, negative control; 20, 100-bp ladder.

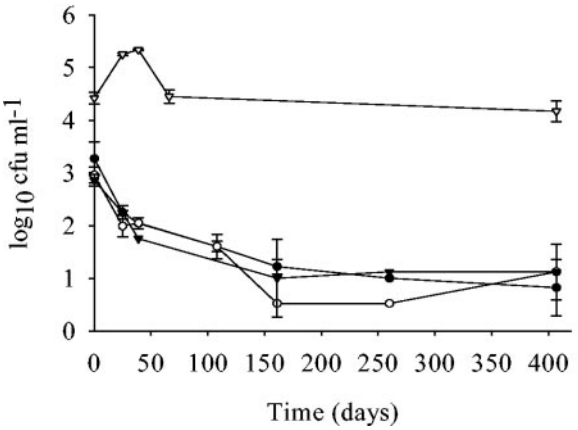
MIRU typing of *M. avium* subsp. *paratuberculosis* isolates from Taff catchment area and river water. MIRUs occur at defined loci throughout mycobacterial genomes (69). MIRU typing can be used to distinguish between strains and to differentiate *M. avium* subsp. *paratuberculosis* from closely related *M. avium* strains. As previously described (9), primer pairs homologous to flanking genomic DNA on either side of MIRU loci 1, 2, and 4, and on some occasions locus 3, were used for MIRU PCR amplification of the cultured isolates. Satisfactory amplification spanning each MIRU was obtained. The product was specific for each MIRU locus and was amplified as a single band. As shown in Fig. 4, the cultured isolates showed the same MIRU profile (3951) as the control bovine *M. avium* subsp. *paratuberculosis* strain recently cultured from a cow with Johne's disease within the study region. The MIRU profiles of the *M. avium* subsp. *paratuberculosis* isolates cultured from reservoir sediment and Taff river water were substantially different from those of all of the other mycobacterial strains tested, including *M. chelonae* and *M. scrofulaceum*, as well as a *Mycobacterium* strain isolated from a cow in Sweden (25) and three *Mycobacterium* strains from Australia (20), all of which contain elements related to IS*900*.

Statistical analysis. We tested the hypothesis that the presence of IS*900*, particularly in periods of apparent clustering, was associated with rainfall and therefore river and catchment area hydrography. A matrix was compiled relating the presence or absence of *M. avium* subsp. *paratuberculosis* on each of the 96 sample days to the values for catchment area rainfall, river Taff height, river flow, and amount of suspended solids on the same day. The matrix was augmented by the addition of the rainfall on each of the preceding 7 days. As expected, river height and flow were closely associated. Linear regression of log-transformed river flow on height detected four occasions when this close association was clearly breached within the data. These four values for flow and height, two with IS*900* present and two with IS*900* absent, were deemed to be in error and were excluded from the analyses.

Higher values for river height and flow were associated with positive IS*900* tests at a significance level of 5%. A relationship between higher values for suspended solids and positive IS*900* tests was not significant at 5% but came close at 8%. The presence of IS*900* in river water from the Taff was clearly associated with higher rainfall on the sample day and on each of the six preceding days (the significance levels were 1% on preceding days 0, 3, 5, and 6 and 5% on preceding days 1, 2, and 4; more than 6 days preceding rainfall had an insignificant influence). From the range of rainfall values, a number of rainfall thresholds were selected. The numbers of sample dates with IS*900* present or absent when rainfall was above or below each threshold on each preceding day were tabulated. No threshold with adequate predictive capability was found for any rainfall day. Similarly, the discriminant analyses of river characteristics and rainfall were unable to identify any index with the ability to predict IS*900*-positive days.

Visual inspection suggested some clustering of IS*900*-positive tests throughout the year of sampling, and on 14 occasions one positive sample day followed another. The randomization method was used to test for clustering. The series was randomized 5,000 times, and the number of occasions (n) on which one positive test followed another was counted for each randomization. A frequency histogram of the values for n was drawn

A



B

day	MGIT culture				L/AV PCR			
	A	B	C	D	A	B	C	D
0	ND	ND	ND	ND	+	+	+	–
25	+	+	+	–	+	+	+	–
39	+	+	+	–	+	+	+	–
66	+	+	+	–	ND	ND	ND	ND
108	+	+	+	–	ND	ND	ND	ND
161	+	+	+	–	ND	ND	ND	ND
260	+	+	+	–	ND	ND	ND	ND
407	+	+	+	–	+	+	+	–
632	+	–	+	–	+	+	+	–
841	–	–	–	–	+	+	+	–

FIG. 5. Persistence of *M. avium* subsp. *paratuberculosis* in sterile lake water microcosms as determined by plating, real-time PCR, and corresponding growth in MGIT tubes and PCR. (A) Persistence (expressed as CFU milliliter^{-1}) of *M. avium* subsp. *paratuberculosis* in sterile lake water microcosms A (▼), B (○), and C (●) and estimated count by real-time PCR in microcosm C (▽) over 400 days. Results for microcosm D (saline inoculated control) are not shown. Error bars indicate standard deviations. (B) Extended study of persistence of *M. avium* subsp. *paratuberculosis* in sterile freshwater microcosms A to D by using MGIT culture and PCR over 841 days (a further 434 days after the last sample was taken for culture). + and −, presence and absence of *M. avium* subsp. *paratuberculosis* culture or PCR signal, respectively; ND, not determined.

up. The median value of *n* was 10 occasions, and the mean value was 10.3. In 345 simulations *n* was 14 or more, giving a significance level of 6.9% for a hypothesis test of random occurrence. Hence, the test was significant at the 10% level but not at the 5% level, and evidence for clustering was not conclusive.

Survival of *M. avium* subsp. *paratuberculosis* in lake water. No recent research on the survival of *M. avium* subsp. *paratuberculosis* in freshwater was available (45, 48, 61), so the potential of a recently isolated *M. avium* subsp. *paratuberculosis* strain to survive long-term in freshwater, a strategic requirement if transport from the catchment area to a human host occurs, was assessed. The survival of *M. avium* subsp. *paratuberculosis* 989 was examined in sterile lake water microcosms (A, B, and C) and an uninoculated control (D) over an 841-day period (Fig. 5). PCR was performed on raw water, before and after sterilization, and in both cases the water was IS*900* negative. Neither sample was inhibitory to PCR. For the purpose of this survival experiment it was hoped that the initial inoculum would be approximately 10^3 to 10^4 *M. avium* subsp. *paratuberculosis* organisms ml^{-1}, as we could not envisage a scenario in a nonsediment freshwater environmental context where concentrations would be likely to exceed this value. However, initial inoculum densities were difficult to enumerate by established methods such as direct cell counts or counts of CFU milliliter^{-1} (59) due to clumping of *M. avium* subsp. *paratuberculosis* and the length of time it takes for CFU to appear on solid media. For these reasons, estimates of initial numbers were obtained retrospectively by using long-term culture and real-time PCR. Culture showed that day 0 counts of CFU milliliter^{-1} were 1.9×10^3, 9.12×10^2, and 7.24×10^2 for microcosms A, B, and C, respectively. No growth was observed for microcosm D. Real-time PCR (carried out on microcosm C only) estimated the day 0 count to be 2.6×10^4 cells ml^{-1}. The survival characteristics from day 0 to 841 are shown in Fig. 5. Culturable numbers in microcosms A, B, and C decreased from the initial levels to around 10^1 cells ml^{-1} by day 160, after which numbers remained stable at this level to day 407. The next two sampling points occurred at days 632 and 841, where no growth was observed on solid medium for any of the microcosms. However, on each of these days samples were IS*900* PCR positive, and growth in MGITs was positive on day 632 for microcosms A and C (Fig. 5B). To date, no growth in MGITs has been observed for day 841 samples. However, real-time PCR detected 8,755 cells ml^{-1} on day 841 (data not shown), thereby demonstrating that cell densities did not fall below 10^3 ml^{-1} for the duration of the experiment. It should be noted that counts of CFU milliliter^{-1} cannot be expected to follow normal assumptions (i.e., that one CFU arises from one cell), due to the clumping of cells. It is just as likely that one CFU arose from a clump of hundreds of cells. This can be supported by the fact that colonies arose at different rates and were of different sizes (data not shown), therefore representing an underestimate of true cell numbers.

DISCUSSION

South Wales in the United Kingdom is well suited to the study of *M. avium* subsp. *paratuberculosis* released from infected livestock into the environment and the potential for exposure of the human population to these pathogens via surface waters. The region is relatively compact, with a mostly industrialized and densely populated coastal strip. This strip lies below the upland pastures of the Brecon hills to the north, which are extensively used for cattle and sheep farming. Infection by *M. avium* subsp. *paratuberculosis* is endemic in these animals. Furthermore, the region has an abundant rainfall mostly carried in from the Atlantic on the prevailing southwesterly winds and running off in a series of spate rivers, such as the Taff, which take a fairly direct course into the sea.

The central question addressed in this initial study was whether *M. avium* subsp. *paratuberculosis* is being carried down the river Taff. This was examined by twice-weekly sampling of the river from the same site just to the north of the city of Cardiff, over the course of a full year. *M. avium* subsp. *paratuberculosis* was identified by IS*900* PCR and amplicon sequencing in 1,00l TFF concentrates of Taff river water on 31 (32.3%) of the 96 days sampled. Since the 11 days, usually when the river was high, on which the processed sample proved to be inhibitory to PCR were recorded as negative, this detection rate may be an underestimate. In 12 of the 31 PCR-positive samples *M. avium* subsp. *paratuberculosis* was also isolated in culture, but, as so often is the case with these difficult organisms, colonies appeared only after incubation of the HEYM plates for about a year. The identity of the isolates was further verified by MIRU typing and IS*900* PCR. All of the isolates typed were bovine strains, which are more easily cultured than ovine strains. However, the amplicon sequences from the all of the cultured *M. avium* subsp. *paratuberculosis* isolates shared 100% identity with the reference IS*900* sequence in the database, whereas such identity was found in only three of the amplicon sequences obtained directly from the river water DNA extract. All of the remaining 16 direct amplicon sequences contained a single-nucleotide polymorphism at position 214, which could not be resolved in 2 sequences and was A to G in the remaining 14. This IS*900* polymorphism has not been described before and would be consistent with the substantial environmental presence of a previously unrecognized strain of *M. avium* subsp. *paratuberculosis* which cannot be isolated in culture. A similar situation has been described for ammonia-oxidizing bacteria (33, 34, 39) and crenarchaea (65), for example, where the ecologically significant organisms are not represented by those obtained from the environment by culture.

An accessory question posed in the present study was how long *M. avium* subsp. *paratuberculosis* might be able to survive in environmental waters. A concept that these robust and versatile pathogens, having limited access to nutrients (especially iron), would die would not be soundly based given general probability of persistence in the environment (41, 45, 48, 74) and the potential for enhanced survival and possible amplification within environmental protozoa (2, 37, 60, 68). The outcome of our studies over nearly 2 1/2 years with the model lake water systems showed that *M. avium* subsp. *paratuberculosis* bovine strain 989 was culturable on solid medium for at least 407 days, was culturable in liquid MGIT medium for at least 632 days, and thereafter remained particulate and persisting in relative abundance in the lake water microcosms to the end of the experiment at 841 days. The survival and persistence of an unculturable and previously unrecognized environmental strain of *M. avium* subsp. *paratuberculosis* is its natural environmental aquatic habitat are of course unknown, but it seems unlikely that they would be less.

Statistical analyses were applied to examine the relationship between the presence of *M. avium* subsp. *paratuberculosis* in the Taff and the catchment area-influenced parameters of river height, river flow, suspended solids, and rainfall. There was a significant association (5 to 1% significance) with rainfall up to 6 days before the sample date, as well as with river height and flow. However, combining the data from these variables in a discriminant analysis did not identify a threshold value for rainfall, river height, or river flow which had an adequate predictive capability for the presence of *M. avium* subsp. *paratuberculosis* in the river. An association with the amount of suspended solids in the river water just fell short of significance. The test for clustering (7% significance) was also just below significance, at the 5% level. Thus, although a period of rain will flush *M. avium* subsp. *paratuberculosis* into the river (43), these organisms may enter from diffuse environmental sources, as well as from point sources such as wastewater treatment outfalls, at a time independent of low or high rainfall (43). Conversely, rivers are nonhomogenous units (72), and failure to detect *M. avium* subsp. *paratuberculosis* organisms during high-rainfall periods may be due to patchiness of their distribution within the river system (24, 72). Future studies using quantitative methods are required to determine the abundance of these pathogens in river water.

At the highest point of the Taff catchment area that we sampled, the superficial sediments taken from the Upper Neuadd and Brecons reservoirs were both negative by IS*900* PCR, without evidence of inhibition of the reaction. However, after long incubation, *M. avium* subsp. *paratuberculosis* was cultured from two Upper Neuadd sediments, with the different results most likely being due to patchy distribution and chance sampling. The result is consistent with the entry of these pathogens into the catchment area at the highest level. This is supported by the positive tests on the Pontsticill and Llwyn-on reservoirs, which are adjacent just lower down in the catchment area. Deposition rates for sediments at the bottoms of the Pontsticill and Llwyn-on reservoirs are not available, but assuming that they may be similar to those found in Windermere (56, 64), the depths in the sediment cores at which *M. avium* subsp. *paratuberculosis* was identified would represent 30 to 50 years of deposition. This is well within the 109 years since the organism was first described as causing chronic inflammatory disease of the intestine in a cow in Germany (40).

The incidence of Crohn's disease in Cardiff over the period from 1976 to 1980 was $5.0/10^5$/year (63). The epidemiological study carried out by Mayberry and Hitchens in the late 1970s (52, 53) examined the incidence and geographical distribution of Crohn's disease and ulcerative colitis in 25 electoral wards in the city of Cardiff. They found a statistically highly significant ($P < 0.001$) increase in the incidence of Crohn's disease, but not of ulcerative colitis, in 11 of the city wards. Although eight of these high-incidence wards bordered the river Taff, their apparent geographical relationship with the river was not statistically significant. Figure 6B shows an enhanced relief map of the city of Cardiff indicating the direction of the prevailing southwesterly winds. The distribution of the wards with a high incidence of Crohn's disease throughout the city is shown in Fig. 6A. The topography of the approaches to the southwest aspect of the river is characterized by hills to the north and south. The valley in between, open to the prevailing winds, is directly opposite the gap in the center of the high-incidence wards on the windward bank of the river. On the leeward side, three additional high-incidence wards lie immediately adjacent to those bordering the river to the northeast. This is the direction in which aerosols containing *M. avium* subsp. *paratuberculosis* would be carried on the prevailing southwesterly winds (35).

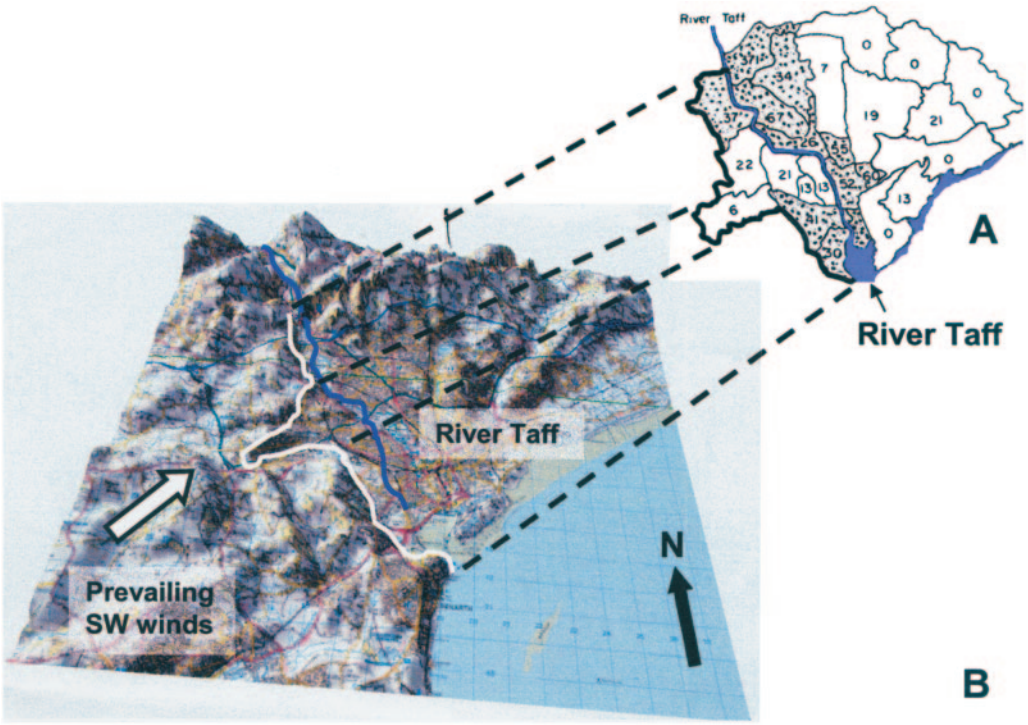


FIG. 6. Relationship between disease clusters and prevailing wind in Cardiff, Wales, United Kingdom. (A) Distribution of the 11 electoral wards in the city of Cardiff that were shown previously (52, 53) to have a highly significant ($P < 0.001$) increase in the incidence of Crohn's disease. (B) Relief map of Cardiff, with the course of the river Taff through the city indicated in blue. The wards with the high incidence of Crohn's disease are seen to lie along the Taff, with the exception of a gap in the center stretch of the windward right bank of the river (facing downstream). This gap directly faces a valley between hills to the north and south, which is open to the prevailing southwesterly winds. (Map reproduced with permission from the Ordnance Survey, Crown copyright NC/2004/29668.)

Although almost 30 years separates our present findings on *M. avium* subsp. *paratuberculosis* in the river Taff from those of the previous epidemiological study, the presence of these pathogens in sediment cores from the upper Taff catchment area suggests that similar conditions may have prevailed at that time. Both the ability of aerosol droplets to concentrate bacteria and the spread of mycobacteria in aerosols are well described (5, 26, 60, 73, 76). Inhalation is a probable route of *M. avium* subsp. *paratuberculosis* infection in cattle (19, 26). *M. avium* demonstrates selective binding to pulmonary surfactant proteins (44). Lung involvement is well described in adults with Crohn's disease (67), and the disease in children often begins with a cough and a mild granulomatous tracheobronchitis (6, 10, 11, 47, 50). *M. avium* subsp. *paratuberculosis* demonstrates a substantial tissue tropism for the gut, so that systemic administration in a range of susceptible hosts eventually results in chronic inflammation of the intestine (37) *M. avium* subsp. *paratuberculosis* in paucimicrobial disease in humans is difficult to detect, but recent data from several laboratories confirm that if the appropriate culture and PCR tests are done correctly, almost everyone with chronic inflammation of the gut of the Crohn's disease type is found to be infected with this chronic enteric pathogen (1, 8, 38, 57, 62, 66). Taken together with our present results, the data suggest that the pattern of clustering of Crohn's disease in Cardiff maybe have been due to the inhalation of *M. avium* subsp. *paratuberculosis* from the river Taff. Further direct studies on aerosols collected from close to the river are indicated.

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