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Mycobacterium avium subsp. *paratuberculosis* in Lake Catchments, in River Water Abstracted for Domestic Use, and in Effluent from Domestic Sewage Treatment Works: Diverse Opportunities for Environmental Cycling and Human Exposure

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***Mycobacterium avium* subsp. *paratuberculosis* from infected animals enters surface waters and rivers in runoff from contaminated pastures. We studied the River Tywi in South Wales, United Kingdom, whose catchment comprises 1,100 km^2 containing more than a million dairy and beef cattle and more than 1.3 million sheep. The River Tywi is abstracted for the domestic water supply. Between August 2002 and April 2003, 48 of 70 (68.8%) twice-weekly river water samples tested positive by IS*900* PCR. In river water, the organisms were associated with a suspended solid which was depleted by the water treatment process. Disposal of contaminated slurry back onto the land established a cycle of environmental persistence. A concentrate from 100 liters of finished water tested negative, but 1 of 54 domestic cold water tanks tested positive, indicating the potential for these pathogens to access domestic outlets. In the separate English Lake District region, with hills up to 980 m, tests for *M. avium* subsp. *paratuberculosis* in the high hill lakes and sediments were usually negative, but streams and sediments became positive lower down the catchment. Sediments from 9 of 10 major lakes receiving inflow from these catchments were positive, with sediment cores indicating deposition over at least 40 to 50 years. Two of 12 monthly 1-liter samples of effluent and a single 100-liter sample from the Ambleside sewage treatment works were positive for *M. avium* subsp. *paratuberculosis*. Since Lake Ambleside discharges into Lake Windermere, which is available for domestic supply, there is a potential for these organisms to cycle within human populations.**

Mycobacterium avium subsp. *paratuberculosis* is a very slow-growing mycobactin-dependent member of the *Mycobacterium avium* complex (10, 27, 62, 73). Unlike other *M. avium* species, *M. avium* subsp. *paratuberculosis* has the specific ability to cause chronic inflammation of the intestine, or Johne's disease (8, 12, 17), which can affect many species, including primates (14, 48, 81). Despite its broad pathogenicity, *M. avium* subsp. *paratuberculosis* can live in animals for years without causing clinical disease. Subclinical infection is widespread in domestic livestock, especially cattle, sheep, and goats. Europe and North America have been particularly affected (19, 30, 35, 38, 44, 69), but infection and disease are now spreading worldwide. Clinically and subclinically infected animals shed *M. avium* subsp. *paratuberculosis* in their milk (70, 71). Research carried out so far in the United Kingdom, the United States, and the Czech Republic shows that *M. avium* subsp. *paratuberculosis* is transmitted from time to time to human populations in retail milk supplies (3, 24, 28, 49). Recent research from several centers using appropriate laboratory methods showed that most people with chronic inflammation of the intestine of the Crohn's disease type are infected with this chronic enteric pathogen (2, 9, 53, 63, 66, 67). Like that of Johne's disease, the incidence of Crohn's disease is increasing (43), particularly in children, with recent data from three sites in Northern and Central Europe and in Australia showing increases in the disease in children under 16 which average fivefold per decade (34, 56, 59).

M. avium subsp. *paratuberculosis* can survive for many months in agricultural slurry and in the environment (41, 62, 77), where it also has the potential to persist within protists (4, 13, 33, 51). Under experimental conditions, we have found *M. avium* subsp. *paratuberculosis* from the human intestine surviving within *Acanthamoeba polyphaga*, even after 4 years of incubation with several cycles of encystment and trophozoite activation (52). *M. avium* subsp. *paratuberculosis* is shed onto pastures in huge numbers from infected cattle (14). No general upper time limit on its environmental survival has yet been established (77). Nonruminant as well as ruminant wildlife reservoirs contribute to environmental contamination (5, 21, 22, 29, 61). The ingestion of pellets containing *M. avium* subsp. *paratuberculosis* from infected rabbits has been shown to establish a cycle of reinfection for grazing livestock (21). Furthermore, the clustering of *M. avium* subsp. *paratuberculosis* isolates in field sites in the Tayside region of Scotland was found to be related to the distribution of rabbits and represented infection hot spots (36, 37).

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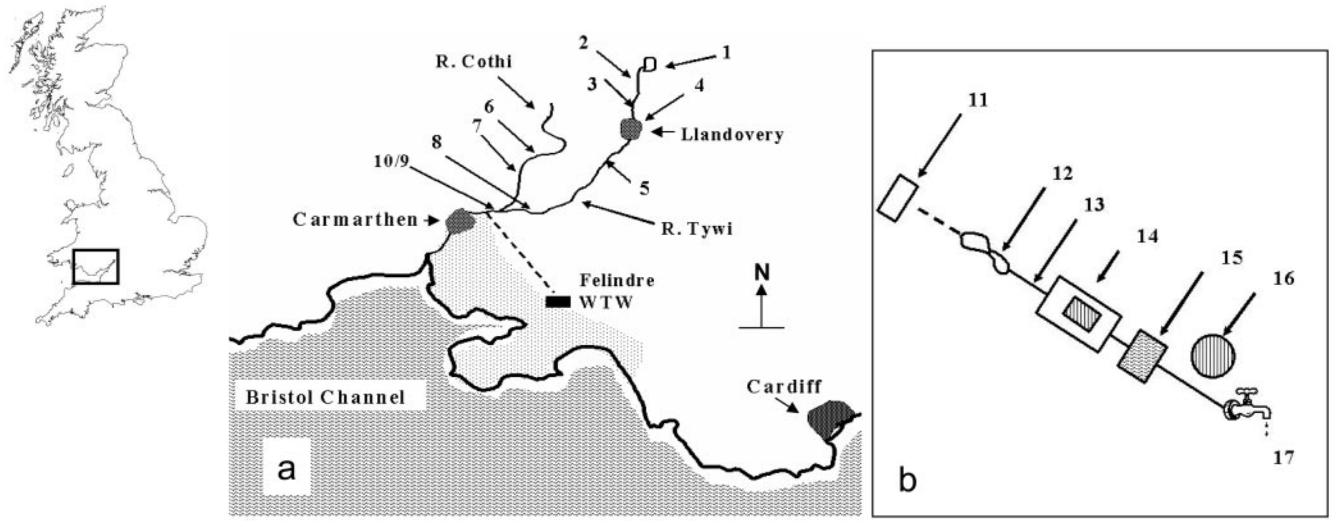

FIG. 1. (a) Map of the study region in South Wales, United Kingdom, showing the River Tywi and its main tributary, the River Cothi, with the regional towns of Llandovery and Carmarthen and the capital of the principality, Cardiff. Also shown (dotted line) is the 26-km abstraction pipeline taking raw river water from the main channel of the River Tywi to the FWTW. Bar, 15 km. Samples and sites: 1, sediment from Llyn Brianne reservoir in the Cambrian mountains at an elevation of 488 m (UK Ordnance Survey grid reference SN796489); 2, epilithon from Gallt y bere on the upper Tywi (SN773459); 3, discharge effluent from human sewage treatment plant (SN766380); 4, epilithon from site at Llandovery bridge (SN761347); 5, discharge effluent from human sewage treatment plant at Llandielo (SN610213); 6, 10-liter water sample from River Cothi (SN525231); 7, 10-liter water sample from River Cothi (SN523229); 8, 10-liter water sample from River Tywi (SN508201); 9, 10-liter water samples twice weekly from 8 December 2002 to 4 October 2003 from Nantgaredig Bridge (SN 494204), which is just downstream of the junction of the River Cothi with the River Tywi; 10, sediment from the River Tywi at the domestic supply abstraction site (SN488205). The dotted area represents the potable water distribution region from the FWTW where domestic properties were sampled. (b) Diagram showing the River Tywi abstraction pumping station (large square), the 26-km pipeline (dotted line [not to scale]), and details of the FWTW (SN649032). Samples and sites: 11, epilithon and sediment from the abstraction pump house screening grill (SN488204); 12, sediment from the Lower Lliw holding reservoir (SN648036); 13, epilithon from the inflow channel leading to the FWTW (SN648036); 14, suspended solids separated by the COCODAFF treatment process in the FWTW; 15, 100-liter sample of finished water product; 16, accumulated suspended solids from sludge lagoon; 17, sediments accumulating in water inflow tanks in domestic properties ($n = 54$).

In previous work in South Wales, United Kingdom (57), we identified *M. avium* subsp. *paratuberculosis* in the catchment and waters of the River Taff, which runs to the southeast off the upland pastures of the Brecon Hills and through the principal city of Cardiff. The presence of the organism in river water correlated with rainfall and river hydrography, but there was also evidence that other influences, such as separate sewage inputs, might also be involved. The depth of the organism in sediment cores from hill reservoirs high up in the River Taff catchment corresponded to an extension back in time of at least 50 years (57). The topography of the southwestern windward approaches to the River Taff within Cardiff itself and the previously reported (31, 47) relationship between the river and city wards with significantly increased incidences of Crohn's disease were consistent with the transmission of infection to humans by inhalation of *M. avium* subsp. *paratuberculosis*-laden aerosols from the river (57).

The River Tywi is another major river in the South Wales region. Its separate catchment lies principally in the Cambrian Mountains but includes inflows from Black Mountain to the west of the Brecon Hills. Important differences between the River Tywi and the previously studied River Taff are that the catchment and course of the River Tywi are predominantly rural and that the River Tywi is a major source of water abstraction and domestic supply. The present study was carried out to test for the presence of *M. avium* subsp. *paratuberculosis* in the catchment area and waters of the River Tywi and to follow the organism along the route from the abstraction point through the water treatment process and down to residues accumulated in domestic water systems. This would provide an initial assessment of the risk of this pathogen being conveyed to domestic outlets in water supplies. To obtain an assessment of the generality of the environmental presence of *M. avium* subsp. *paratuberculosis* in the United Kingdom and the potential for its cycling through human populations, we also studied its distribution in the geographically and metrologically different catchment of Lake Windermere in the Lake District region of northwest England as well as in treated effluent from domestic sewage plants.

MATERIALS AND METHODS

Description of River Tywi catchment and sampling. The source of the River Tywi lies in the afforested undulating moor land of the Cambrian Mountains of Mid Wales (Fig. 1a). The Tywi Fach and the Tywi Fechan join and flow into the northern arm of the Llyn Brianne reservoir, while the Afon Camddwr flows into the northwestern arm of the reservoir. A continual but flow-regulated release of water from the reservoir marks the start of the River Tywi, which falls steeply for 10 miles and then flows out onto a flood plain near the town of Llandovery. It meanders across a 1-mile-wide plain for 30 miles in a southwesterly direction to the town of Carmarthen, below which it enters a 12-mile-long tidal section which then enters the Bristol Channel in a broad estuary. In total, it is 75 miles long, with numerous tributaries.

Details of the sampling of the Llyn Brianne, upper Tywi, and River Cothi as well as inflows of treated water from human domestic sewage plants are given in Fig. 1a and in Table 1. Sediment cores were taken by using a Jenkin corer as previously described (50, 57). Our principal sampling site for the main stream of

TABLE 1. Locations, dates, and characteristics of samples from the River Tywi, FWTW, and domestic properties in South Wales

Sample site	Name	UK Ordnance Survey grid reference	Sample type	Sample date (day/mo/yr or mo/yr)	IS*900* PCR status	SNP at position 216[a]
1	Llyn Brianne reservoir	SN796489	Sediment	29/09/02	−	
2	R Gallt y bere, upper Tywi	SN773459	Epilithon	29/10/02	−	
3	Sewage treatment works, upper Tywi	SN766380	Effluent	29/10/02	−	
4	Llandovery bridge	SN761347	Epilithon	29/10/02	−	
5	Sewage treatment works, Llandielo	SN610213	Effluent	29/10/02	−	
6	River Cothi	SN525231	Water (10 liters)	29/10/02	+	ND
7	River Cothi	SN523229	Water (10 liters)	29/10/02	+	ND
8	River Tywi	SN508201	Water (10 liters)	29/10/02	+	ND
9	Nantgaredig Bridge	SN494204	Water (10 liters twice wkly)	08/02-04/03	+ (48 of 70)	13 sequenced (4-A, 9-G)
10	River Tywi abstraction site	SN488205	Sediment	29/10/02	+	G
11	Abstraction pump house screening grill	SN488204	Sediment	29/10/02	+	ND
	Abstraction pump house screening grill	SN488204	Epilithon	29/10/02	+	
12	Lower Lliw water treatment works holding reservoir	SN648036	Sediment	29/10/02	−	
13	FWTW inflow channel	SN648036	Epilithon	29/10/02	−	
14	FWTW	SN649032	Suspended solids	29/10/02	+	ND
15	FWTW	SN649032	Finished water	29/10/02	−	
16	Felindre sludge lagoon	SN649032	Suspended solids	29/10/02	+	G
17	Domestic properties		Tank sediments	08/03	+ (1 of 54)	ND

[a] ND, not determined.

the River Tywi was at Nantgaredig Bridge (site 9), which is 750 m downstream of the junction of the River Cothi with the River Tywi. For the continuous monitoring of River Tywi water, 10-liter water samples were taken, using a fresh disposable bucket, from the middle of the river off Nantgaredig Bridge twice weekly from 12 August 2002 to 10 April 2003. Samples were sealed in disposable screw-cap plastic containers and sent by fast post directly to the Center for Ecology and Hydrology, Lancaster, United Kingdom.

Sampling the River Tywi abstraction site and water treatment works. Details of the sampling sites are shown in Fig. 1a and b. Sediment samples were taken from the river bank (site 10) opposite the abstraction pumping station on the River Tywi, which is 600 m downstream of Nantgaredig Bridge (site 9), as previously described (57). Samples were also taken from a biofilm adhering to the screening grill of the abstraction pump house and from sediments accumulated on the grill. Water abstracted from the river is carried by a 26-km pipeline in a southeasterly direction to the Felindre Water Treatment Works (FWTW). There it either discharges into the holding Lower Lliw reservoir or directly enters the FWTW. Sediment was sampled from the Lower Lliw reservoir and the channel carrying water into the FWTW. Within the FWTW itself, samples were taken of biofilms on channels conducting raw water to the processing tanks. Within the tanks, the raw water is treated by the counter-current dissolved-air flotation-filtration (COCODAFF) procedure. This separates the suspended solids, which accumulate as a brown surface mat, and this mat was sampled. The solid waste discharges into an adjacent open sludge lagoon to dry, and it was also sampled. Finally, a 100-liter tangential-flow-filtration (TFF) sample was obtained from the finished water product prior to its release into the supply distribution system.

Sampling of domestic water systems. During August 2003, 54 houses supplied with domestic potable water were visited throughout the study region (Fig. 1a). At each site, sediment which had accumulated in the bottom of the cold water header tank or cistern was aspirated using a sterile disposable 50-ml syringe. A total of 70 samples were obtained. Samples were transferred to 50-ml screw-cap centrifuge tubes and stored at 4°C until they were processed.

Lake District sampling. The Lake District National Park comprises an area of 2,292 km^2 in the north of England and contains high fells (hills up to 980 m), 14 major lakes, and numerous small mountain lakes called tarns (Fig. 2). Lowland areas are grazed by cattle, and both highland and lowland areas are intensively grazed by sheep. Samples of sediment or epilithon ($n = 67$) were collected from 29 sites throughout the Lake District region. Sediment cores from the bottoms of major lakes were obtained by using a Jenkin corer as described previously (57). A sterile spatula was used for samples taken from the bank. The Ambleside domestic sewage treatment works (site 9) discharges treated waste into the River Rothay 200 m upstream of its confluence with the River Brathay prior to entry into Lake Windermere. A total of 12 monthly samples comprising 1 liter of treated effluent were taken between November 2002 and November 2003, and a subsequent 100-liter TFF sample was also taken. A single 1-liter sample of effluent was taken from the Windermere sewage treatment works (site 14). Details of the samples and sites throughout the Lake District region and Lake Windermere and its catchment are given in Fig. 2a and b and in Tables 2 and 3.

Processing of samples, DNA extraction, and PCR. The 100-liter and 10-liter river water or sewage treatment effluent samples were concentrated using TFF as previously described (56). DNA extraction and nested IS*900* PCR with amplicon sequencing were performed as previously described (56).

Environmental data. Data regarding rainfall within the catchment as well as height and flow data for the River Tywi were provided by the Environment Agency, Wales, United Kingdom. Rainfall values were obtained using the HYDROLOG data management system V2.61 (Hydro-Logic Ltd., United Kingdom) at the Llandovery logging station (grid reference SN765353). River flow and height were obtained using the HYDROLOG data management system V2.61 at the Dolau Hirion logging station (grid reference SN762362). Data on the precise boundaries and stocking densities for the River Tywi catchment were provided by FEHGIS Services, Welsh Assembly, Cardiff, Wales, United Kingdom.

Statistical methods and analyses. Using data relating to the detection of *M. avium* subsp. *paratuberculosis* and synchronous measurements of catchment rainfall and River Tywi height and flow, a matrix was compiled comprising 67 measurements of four variables, namely, sample date, presence or absence of IS*900*, river height, and river flow. The matrix was augmented by the addition of 11 rainfall variables, namely, rainfall on the sample date, rainfall on the day before sampling, rainfall 2 days before sampling, and so on up to rainfall 10 days before the sample date. Standard analysis of variance (ANOVA) (16) was used to determine whether positive tests for IS*900* on River Tywi water were significantly related to different river heights and river flows, which were log transformed to homogenize the residual variance. Mean rainfall values for days with IS*900*-positive river samples and for each of the preceding 10 days were compared with the mean rainfall on days with IS*900*-negative samples by using ANOVA. Rainfall data were square root transformed to assist in obtaining 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* (45). A randomization method was used to test for clustering of IS*900*-positive days (57).

RESULTS

Tywi catchment and river water. The Tywi catchment comprises 1,100 km^2 containing 3,188 agricultural units. There are 108,142 mixed dairy and beef cattle and calves and 1,388,303

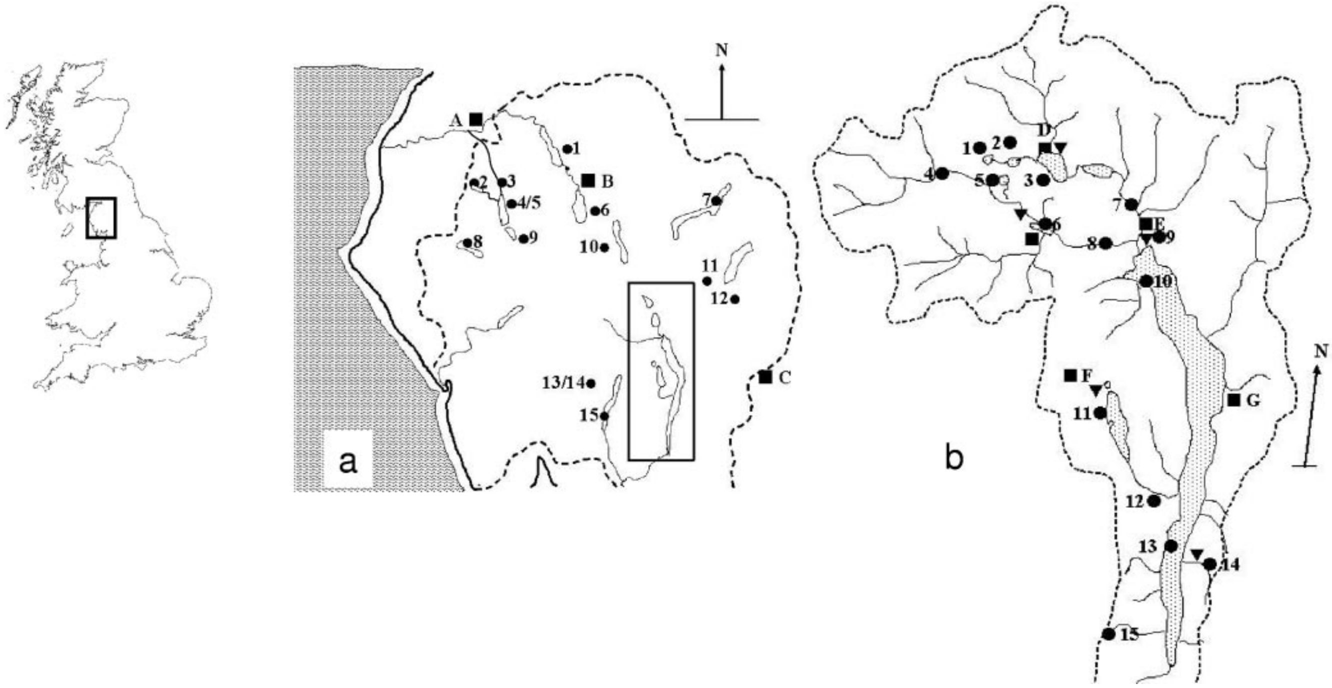

FIG. 2. (a) Map of the study region in the Lake District, United Kingdom, showing the principal lakes and the regional towns of Cockermouth (A), Keswick (B), and Kendal (C). Bar, 10 km. Sediment samples (except where indicated) were taken from the following sites: 1, Bassenthwaite (NY204319); 2, Loweswater (NY122223); 3, River Cocker outlet from Crummock Water (NY150211); 4, Crummock Water (NY149214); 5, epilithon formed due to road runoff from fellside at Hause Point, Crummock Water (NY162183); 6, Derwentwater (NY259219); 7, Ullswater (NY460238); 8, Ennerdale (NY167063); 9, Buttermere (NY188154); 10, Thirlmere (NY306172); 11, Blea Water (NY452108); 12, Small Water (NY457101); 13 and 14, Coniston catchment, comprising Blind Tarn (SD263967) and Goat's Water (SD266978); 15, Coniston (SD310971). (b) Map of the Windermere catchment showing the lake (stippled) and its associated tarns, with the regional towns of Grasmere (D), Ambleside (E), Hawkshead (F), and Windermere (G) indicated. Streams and rivers are indicated by solid lines. Inverted triangles indicate domestic wastewater treatment works. Bar, 1 km. Sediment samples were taken from the following sites: 1, Codale Tarn (NY297088); 2, Easedale Tarn (NY309088); 3, Grasmere (NY334069); 4, Angle Tarn (NY357063); 5, Stickle Tarn (NY288076); 6, Elterwater (NY333042); 7, River Rothay (NY372037); 8, River Brathay (NY372032); 11, Esthwaite (SD359972); 12, Cunsey Beck (SD381936); 15, High Dam (SD363887). Sediment cores were taken from the bottom of Lake Windermere at the following sites: 10, north basin (NY379020); and 13, south basin (SD380897). Samples (1 liter) of treated clean water effluent were also obtained from the human domestic waste treatment works at Ambleside (NY372041) (site 9) and Windermere (SD385973) (site 14).

sheep and lambs, including 717,283 breeding ewes. The average flow of the River Tywi increases from 700 million liters per day (Mliters/day) at Llandovery to 3,850 Mliters/day at Carmarthen (Fig. 1a). The main tributary is the 33-mile-long River Cothi, which lies within the main River Tywi catchment and comprises a subcatchment of 302 km^2. At its confluence with the River Tywi, the average flow of the Cothi is 540 Mliters/day.

The sampling sites in the catchment and from the rivers Tywi and Cothi are shown in Fig. 1a, and the results are summarized in Table 1. *M. avium* subsp. *paratuberculosis* was not detected in Jenkin cores from the bottom of the Llyn Brianne reservoir (site 1) or in biofilms from stones in the river below the dam at sites 2 and 4. The single 1-liter samples of wastewater from the human domestic sewage plants which enter the River Tywi at sites 3 and 5 were also negative for *M. avium* subsp. *paratuberculosis*. Lower down the catchment, *M. avium* subsp. *paratuberculosis* was detected in single 10-liter river water samples from both the River Tywi and the River Cothi at sites 6, 7, and 8.

Twice weekly monitoring of River Tywi water from Nantgaredig Bridge (site 9) yielded 70 daily TFF retentates, of which 48 (68.8%) were positive for *M. avium* subsp. *paratuberculosis* by IS*900* PCR (Fig. 3). We tested the hypothesis that the presence of the organism in the river was associated with rainfall and, therefore, river hydrography. The last three sampling dates in April 2003, comprising two positive and one negative result, had to be excluded because rainfall data were not available for those days. The physical characteristics of river flow and height were closely associated statistically, and there were no occasions when this close association was breached. The differences in the height and flow of the river on days when IS*900* PCR was positive compared with days when it was negative were tested by using ANOVA based on the results from 67 daily samples. For both river height and flow, the difference between positive and negative days was significant at the 0.1% level, with greater average heights and flows being associated with the presence of IS*900* than with the absence of IS*900*. A comparison of the distribution of values (obtained by kernel density estimation) when IS*900* was present and absent showed considerable separation in the range of values of river flow and height. Using a threshold flow of 3.4 m^3 s^{-1} to categorize days, 22 days had low flow and 45 days had moderate to high flow. Of the 22 days with low flow,

TABLE 2. Locations, dates, and characteristics of samples from water bodies in the English Lake District (Cumbria, United Kingdom)

Sample site	Name	UK Ordnance Survey grid reference	Sample type[a]	Sample date (day/mo/yr)	IS*900* PCR status	SNP at 216[b]
1	Bassenthwaite	NY204319	Sediment	12/12/01	+	G
2	Loweswater	NY122223	Sediment	12/12/01	−	
3	River Cocker outlet, Crummock Water	NY150211	Sediment	25/5/2004	+	ND
4	Crummock Water	NY149214	Sediment	25/5/2004	−	
5	Hause Point, Crummock Water	NY162183	Epilithon	25/5/2004	+	G
6	Derwentwater	NY259219	Sediment	12/12/01	+	ND
7	Ullswater	NY460238	Sediment*	21/11/02		
	Ullswater	NY460238	Top (3 cm)	21/11/02	+	G
	Ullswater	NY460238	Mid (7 cm)	21/11/02	+	ND
	Ullswater	NY460238	Bottom (15 cm)	21/11/02	+	G
8	Ennerdale	NY167063	Sediment	25/09/01	−	
9	Buttermere	NY188154	Sediment*	19/11/02		
	Buttermere	NY188154	Top (3 cm)	19/11/02	+	ND
	Buttermere	NY188154	Mid (10 cm)	19/11/02	+	ND
	Buttermere	NY188154	Bottom (15 cm)	19/11/02	−	
10	Thirlmere	NY306172	No sample	19/11/02		
11	Blea Water	NY452108	Sediment	27/11/02	−	
12	Small Water	NY457101	Sediment	27/11/02	−	
13	Blind Tarn	SD263967	Sediment	13/11/01	−	
14	Goat's Water	SD266978	Sediment	13/11/01	−	
15	Coniston	SD310971	Sediment	13/11/01	−	

[a] *, sediment cores from the bottom of the lake.
[b] ND, not determined.

only 8 (36%) had IS*900* recorded as present, whereas of the 45 days with moderate to high flow, 38 (84%) had IS*900* present. Both river flow and height in the River Tywi showed some power to predict the presence of *M. avium* subsp. *paratuberculosis*.

The differences in mean rainfall on a day of sampling when IS*900* PCR was positive and on each of the 10 preceding days were compared with those for the days preceding the days when IS*900* PCR was negative, using ANOVA. The presence of IS*900* 1, 2, 5, 7, and 9 rainfall days before sampling was significantly associated, with 95% confidence. Although the intervening days were not significant, their association was close, at 93%. From the range of rainfall values, a number of rainfall thresholds were selected. The number of sample dates with IS*900* present or absent when rainfall was above or below each threshold on each preceding day was assessed. The only

TABLE 3. Locations, dates, and characteristics of samples from the Windermere catchment in the English Lake District (Cumbria, United Kingdom)

Sample site	Name	UK Ordnance Survey grid reference	Sample type	Sample date (day/mo/yr or mo/yr)	IS*900* PCR status	SNP at 216[a]
1	Codale Tarn	NY297088	Sediment	24/05/02	−	
2	Easedale Tarn	NY309088	Sediment	29/11/01	+	A
3	Grasmere	NY334069	Sediment	27/05/02	+	ND
4	Angle Tarn	NY357063	Sediment	24/06/03	+	ND
5	Stickle Tarn	NY288076	Sediment	29/11/01	−	
6	Elterwater	NY333042	Sediment	07/11/01	+	G
7	River Rothay	NY372037	Sediment	08/11/01	−	
8	River Brathay	NY372032	Sediment	08/11/01	−	
9	Sewage treatment works at Ambleside	NY372041	Effluent	11/01-12/03	+ (2 of 12)	ND
	Sewage treatment works at Ambleside	NY372041	Effluent	26/09/05	+	ND
10	Windermere north basin	NY379020	Sediment	27/11/01		
	Windermere north basin	NY379020	Sediment (top 1 cm)		+	ND
	Windermere north basin	NY379020	Sediment (2 cm)		+	ND
	Windermere north basin	NY379020	Sediment (4 cm)		+	ND
	Windermere north basin	NY379020	Sediment (6-10 cm)		−	
11	Esthwaite	SD359972	Sediment	20/11/01	+	G
12	Cunsey Beck	SD381936	Sediment	20/11/01	+	ND
13	Windermere south basin	SD380897	Sediment	27/11/01	+	G
14	Sewage treatment works at Windermere	SD385973	Effluent	8/11/01	−	
15	High Dam	SD363887	Sediment	16/06/04	−	

[a] ND, not determined.

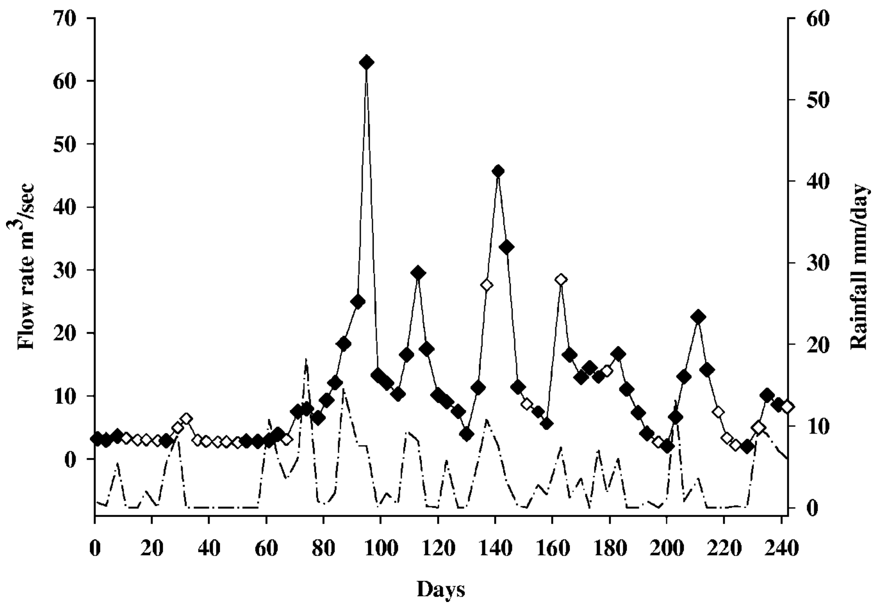

FIG. 3. Relationship between presence or absence of *M. avium* subsp. *paratuberculosis* in river water and the flow and height of the River Tywi and rainfall in the catchment between August 2002 and April 2003. Plotted rainfall data were recorded at 9:00 a.m. each day. The solid line represents the river height data, while the dotted line represents rainfall. Open diamonds are negative results, and black diamonds are positive results for the presence of *M. avium* subsp. *paratuberculosis*.

threshold with any predictive capability for any rainfall day was zero, representing the presence or absence of rain. This analysis showed that the presence of *M. avium* subsp. *paratuberculosis* in river water was associated with the presence of rain, most noticeably with rain falling 5, 6, and 7 days before the sample date.

A visual inspection of the data (Fig. 3) suggested a clustering of daily samples positive for *M. avium* subsp. *paratuberculosis*. The data were randomized 5,000 times, and the number of occasions, *N*, on which one positive sample followed another was counted for each randomization. The frequency histogram of the values of *N* was compiled. The median value of *N* was 31 occasions, and the mean value was 30.9. In 23 simulations, the value of *N* was 36 or more, thus giving a significance level of 23/5,000, or 0.0046, for a test of the hypothesis of no clustering. Clustering of days which were positive for *M. avium* subsp. *paratuberculosis* in the River Tywi was thus significant at the 1% level.

Abstraction site, holding reservoir, water treatment works, and domestic water systems. Sampling sites are shown in Fig. 1b, and the results are summarized in Table 1. Sediment from the river bank opposite the abstraction pump station (site 10) as well as sediment and biofilm on the pump station grill (site 11) was IS*900* PCR positive for *M. avium* subsp. *paratuberculosis*. Sediments from the Lower Lliw holding reservoir at Felindre (site 12) and a biofilm from the open inflow channel to the FWTW (site 13) both tested negative. However, the flocculated suspended solids separated during the COCODAFF treatment process (site 14) and the accumulated extracted waste material drying in the sludge lagoon (site 16) were all strongly positive for *M. avium* subsp. *paratuberculosis.* Of the 54 domestic water systems sampled, sediment from one water cistern was IS*900* PCR positive for *M. avium* subsp. *paratuberculosis*.

English Lake District. Sediment samples were obtained from 14 of 15 sites covering the principal water bodies of the English Lake District (Fig. 2a and Table 2). The major lakes that were positive for *M. avium* subsp. *paratuberculosis* by IS*900* PCR were Bassenthwaite (site 1), Derwentwater (site 6), Ullswater (site 7), and Buttermere (site 9). Sediment cores from the bottom of Ullswater were positive down to a depth of 15 cm, and those from Buttermere were positive down to a depth of 10 cm. Ennerdale (site 8), Blea Water (site 11), Small Water (site 12), and all sites in the Coniston catchment (sites 13, 14, and 15) were negative. No sample was obtained from Thirlmere (site 10) due to low sediment accumulation. Crummock Water (site 4) was IS*900* PCR negative, although the outlet River Cocker (site 3) and a biofilm from Hause Point formed due to road runoff (site 5) were both positive.

The well-defined Windermere catchment, comprising 258 km^2 (Fig. 2b and Table 3), has Lake Windermere as the major receiving body of water. The lake is 16.6 km long running north-south and is divided into the north basin, with a depth of 55 m, and the south basin, with a depth of 38 m, separated by an 8-m shallow underwater ridge.

The north basin receives water from the River Rothay (site 7) and the River Brathay (site 8). These rivers drain two subcatchments, positioned north-northwest and northwest, respectively, and become confluent 200 m before entering the lake. In the north-northwest subcatchment of the River Rothay, Codale Tarn (site 1), the highest sampling site (1,647 m above sea level [asl]), was IS*900* PCR negative, whereas Easedale Tarn (site 2), at 282 m asl, which receives water from Codale Tarn as well as the surrounding hills, was positive. Further down the subcatchment (61 m asl), Grasmere (site 3), which receives water from Easedale, was also IS*900* PCR positive. In the northwest subcatchment of the River Brathay, Angle Tarn (site 4), at 568 m asl, was IS*900* PCR positive, whereas Stickle Tarn (site 5), at 473 m asl, was IS*900* PCR negative. Water from Stickle Tarn runs into Elterwater (site 6), at 53 m asl, which was also IS*900* PCR positive. The single samples of sediment from the River Rothay and River Brathay were both negative. The Ambleside sewage treatment works (site 9) discharges treated waste into the River Rothay. Of the 12 monthly samples of treated effluent, 2 (November 2001 and January 2003) were positive. The 100-liter TFF retentate sample was also positive. The sediment core from the bottom of the Windermere north basin was IS*900* PCR positive to a depth of about 6 cm.

To the west of the south basin of Windermere, Esthwaite Water (site 11) and its outflow, Black Beck (site 12), which runs into the middle of the south basin, were both IS*900* PCR positive. High Dam (site 15), which is a small tarn at 172 m asl feeding directly into the southern end of the south basin, was IS*900* PCR negative. Effluent from the Windermere sewage treatment works (site 14), which is discharged into the south basin, was negative on the one occasion it was tested. Sediment from the bottom of the Windermere south basin was IS*900* PCR positive.

Overall, throughout the Lake District, where some sites were tested twice on different occasions, 21 of 67 samples (31%) were found to be positive for *M. avium* subsp. *paratuberculosis*.

Amplicon sequences. Sequence data obtained for 13 representative IS*900* PCR-positive samples of River Tywi water showed 100% identity with IS*900* (accession number NC_002944) in 4 samples, with a single nucleotide polymorphism (SNP), A to G, occurring at IS*900* nucleotide position 216 (68), within the sequence CTGGAC(A/G)ATGA, in 9 samples. This SNP was also found in the sequences obtained from sediment at the River Tywi abstraction site and from the FWTW sludge lagoon. Of the eight sequences obtained from the Lake District and Windermere catchments, only one sample, from Easedale Tarn (site 2), was identical to IS*900* (NC_002944). All the others, including those from the south basin sediment, showed the A-to-G SNP at nucleotide position 216. All of the sequences containing the SNP at position 216 were also shown to read C at IS*900* nucleotide position 169 (68).

DISCUSSION

In the South Wales study region, we did not detect *M. avium* subsp. *paratuberculosis* in sediment cores from the bottom of the Llyn Brianne reservoir high up in the catchment of the River Tywi or from the upper reaches of the river itself. This probably reflects the smaller number of farm animals higher up in the catchment and a lack of established sediment in the river. Samples from lower down the River Tywi and its principal tributary, the River Cothi, both of which receive runoff from heavily grazed farmland, were positive for *M. avium* subsp. *paratuberculosis*. The main stream of the River Tywi below the confluence of the two rivers was positive for this pathogen in about two-thirds of the samples taken from off Nantgaredig Bridge twice each week from August 2002 to April 2003. This heavy contamination rate of the River Tywi is twice that we found previously in the neighboring River Taff (57). The stocking densities and rainfall in the separate catchments of the two rivers in South Wales are comparable, but the River Tywi catchment is twice the size of the River Taff catchment, with 3 times the number of farm units, 3 times the number of cattle, and 2.4 times the number of sheep.

The presence of *M. avium* subsp. *paratuberculosis* in the River Tywi clustered around periods of rainfall. It was also related to river height and flow, and we showed for the first time that the flow rate (in this case, flow rates of >3.4 m s^{-1}) was a strong predictor of the presence of *M. avium* subsp. *paratuberculosis*. Kistemann and coworkers (39) showed that a substantial proportion of the total microbial load in watercourses is directly related to extreme conditions of rainfall with flooding and extensive runoff. However, in the present study, *M. avium* subsp. *paratuberculosis* was found under normal rainfall conditions for the region, in the absence of extreme events. Entry into the river was likely through leaching or overland flows (75), which have been shown to deliver fecal indicator bacteria into rivers (39, 40, 75).

Aerosolization of mycobacteria from surface waters is well described (1, 6, 26, 79). We previously obtained evidence from the River Taff in Cardiff which was consistent with the transmission of *M. avium* subsp. *paratuberculosis* to humans by this route (57). The heavier contamination of the River Tywi must also be associated with such a potential risk. Furthermore, the not uncommon conditions under which the River Tywi carrying *M. avium* subsp. *paratuberculosis* would be running out strongly against the shallow open waters of its tidal estuary, at 51°44′18″N, 04°24′17″W, and be whipped up by a driving southwesterly wind would generate a plume of estuarine aerosols with the ability to carry this pathogen for substantial distances (7, 46, 54, 58).

The River Tywi is a major source of water to supply the homes of people living in the region. A pipeline links the abstraction site downstream of Nantgaredig Bridge to the FWTW 26 km away. FWTW is capable of processing up to 270 million liters of water per day. *M. avium* subsp. *paratuberculosis* is present in sediment and biofilm on the abstraction site grating. In the water, this pathogen is particularly associated with suspended organic solids, especially when the river is running high. Raw water enters the FWTW, and the COCODAFF process combined with aluminum sulfate treatment removes the suspended solids, which are discharged into the sludge lagoon. The strongly positive PCR tests on this material from both the COCODAFF tanks and the lagoon show that the treatment process substantially depletes the *M. avium* subsp. *paratuberculosis* content of the raw water. Furthermore, we did not detect this pathogen in the single 100-liter TFF retentate sample of finished water we tested. Any *M. avium* subsp. *paratuberculosis* getting through the water treatment process into the finished product would therefore be predicted to be present in high dilution. This robust pathogen is resistant to chlorination, and it is unlikely that any remaining organisms would be killed by this treatment (25, 42, 72). The continuity of domestic water supplies over years carries the theoretical risk that *M. avium* subsp. *paratuberculosis* delivered in high dilution may accumulate in biofilms and sediments and build up in domestic water systems. In the present study, sediment from only 1 of 54 domestic cold water tanks was found to be positive. However, *M. avium* subsp. *paratuberculosis* may have been missed due to the small sample size, as only 50 ml of water containing a few

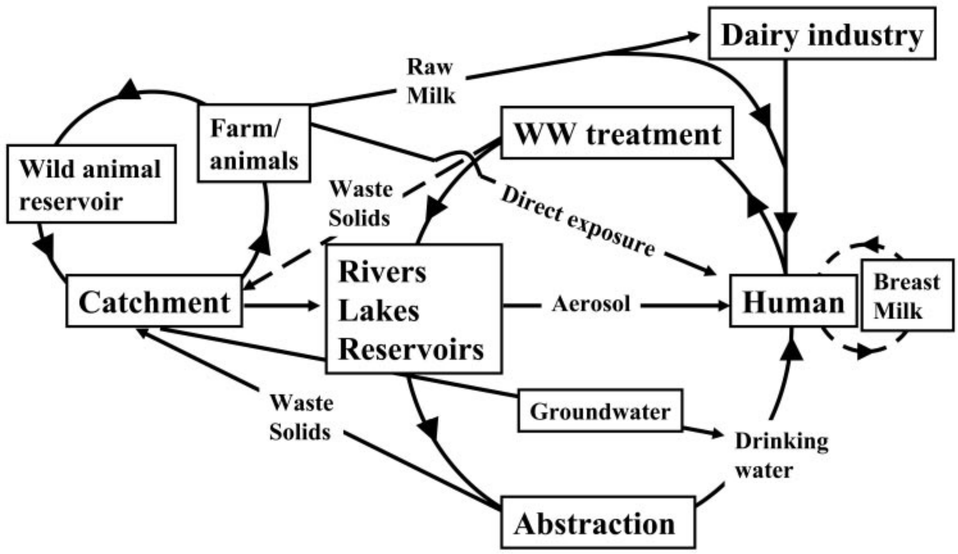

FIG. 4. Model of sources and sinks of *M. avium* subsp. *paratuberculosis* in the environment with respect to human exposure. This model incorporates the established route of transmission of this pathogen to humans via dairy products, especially milk, as well as hypothetical routes of transmission. It also incorporates the catchment cycle, which receives inputs from domestic livestock and wildlife reservoirs as well as agricultural practices such as slurry spreading. *M. avium* subsp. *paratuberculosis* enters water through runoff from the catchment, with aerosols from contaminated rivers and abstraction for domestic supply as potential sources of human exposure. Disposal of waste from water treatment onto catchments and the entry of effluent from sewage treatment works into sources of domestic supply constitute environmental cycles with opportunities for microbial evolution and enhanced pathogenicity.

milligrams of sediment was collected. Much larger studies, particularly sampling biofilms as well as the hot water side of domestic systems and using optimized culture methodologies as well as PCR, are needed before an accurate assessment of this theoretical risk can be made. The slurry containing *M. avium* subsp. *paratuberculosis* extracted by the water treatment process is disposed of by deposition back onto the land, establishing another cycle of environmental contamination. The persistence of the organism in such cycles extends the opportunity for this intracellular pathogen to be taken up by abundant environmental protists (33, 51), such as *Acanthamoeba* sp., where passage of the organism may have a profound effect on the *M. avium* subsp. *paratuberculosis* phenotype and pathogenicity (13).

In the English Lake District, about 300 miles from the South Wales study region, *M. avium* subsp. *paratuberculosis* was detected in mountain areas above 560 m. It was also detected in the sediments of several major lakes, including Bassenthwaite, Derwentwater, Buttermere, and Ullswater. The detection of this pathogen at a 10-cm depth in Buttermere sediment and a 15-cm depth in Ullswater sediment is consistent with deposition over a period of 30 to 40 years and >50 years, respectively (50, 55, 64). In contrast to this apparently widespread distribution in the northern part of the Lake District, tests on Coniston Water and its catchment to the south were all negative. Catchments such as Coniston, the Llyn Brianne reservoir, and the upper reaches of the River Tywi in South Wales were all characterized by low levels of organic sediment. We suggest that the presence of organic sediment is an important feature in the persistence of *M. avium* subsp. *paratuberculosis* in lakes and rivers. Similarly, in the Windermere catchment, the sample sites with high levels of organic sediment were associated with the presence of *M. avium* subsp. *paratuberculosis*.

In contrast to other catchments, Windermere also receives treated wastewater from two human domestic sewage treatment works (STW), namely, Ambleside STW, discharging into the north basin, and Windermere (town) STW, discharging into the south basin. People, especially those with Crohn's disease, have been found to excrete *M. avium* subsp. *paratuberculosis* in their feces (23). Although more work is needed, the present initial study demonstrates that this pathogen is sometimes present in the treated water effluent of human STWs. Recent research has shown that native *M. avium* subsp. *paratuberculosis* obtained directly from the infected human gut can survive and persist through cycles of encystment and trophozoite reactivation for years in *Acanthamoeba polyphaga* (52). STWs discharging into waters which also serve as sources of abstraction and domestic resupply establish the potential for human as well as animal strains of this multihost chronic enteric pathogen to cycle through human populations. This could have a continuing impact on its evolution and virulence (15).

A recent study (68) analyzing IS*900* sequences in 13 cultured bovine isolates reported 100% identity to the published genome sequence of *M. avium* subsp. *paratuberculosis* K10 (GenBank accession no. NC_002944). The authors also reported a mixed-base call, with a predominant G peak and a smaller A peak at IS*900* position 216 (corresponding to IS*900* position 214, described previously [57]), for each of 10 sheep strains grown in culture. Five of these 10 strains also showed a predominant T peak with a smaller C peak at position 169. In the present study, we also observed similar variant base calls of A or G at IS*900* position 216 but not at position 169. No other sequence variations were identified. All other sequences were identical to IS*900* from *M. avium* subsp. *paratuberculosis* K10. These data provide strong evidence that the signals with 100% identity to *M. avium* subsp. *paratuberculosis* K10 were derived from bovine strains and that those which showed variation at position 216 in both this and our previous study of the neighboring River Taff (57) were derived from sheep strains. Our inability to isolate these strains in culture even after a year of incubation is consistent with the long-known difficulty of culturing sheep strains (18). Their presence in Buttermere and Ullswater sediments suggests that they have been around for at least 50 years.

The overall picture obtained in this study is one of the extensive prevalence of *M. avium* subsp. *paratuberculosis* in the environment and surface waters, with a distribution which is patchy rather than ubiquitous and is layered between dynamic cycling and latent persistence. Deep sediments would appear to act as an environmental sink for this pathogen. No general upper time limit on the environmental persistence of *M. avium* subsp. *paratuberculosis* is established, although recent evidence suggests that it is long-term (52, 57, 76).

A model of animal and human interactions with relevant environmental and other compartments is presented in Fig. 4 and is necessarily more complex than previously described (10). Components of the model include the return of *M. avium* subsp. *paratuberculosis* to the catchment from processes involved in both water purification and sewage treatment (Fig. 4). The former returns *M. avium* subsp. *paratuberculosis* to the catchment as soil, and the latter returns it as treated sewage sludge onto the land and as liquid effluent into lakes and rivers. Cycling of this multihost pathogen (15, 80) is indicated on multiple levels, between small and large compartments, and involves farm animals, wildlife, and the environment, waste disposal and the environment, and the potential for environmental involvement in cycling through human populations. Furthermore, aerosols from rivers and estuaries have the ability to convey this pathogen over large distances (7, 54, 58).

It has long been recognized that chronic inflammatory disease in immunocompetent hosts caused by *Mycobacterium avium* complex organisms is acquired not so much by person-to-person transmission, but through environmental exposure (60, 78). The opportunity for environmental exposure of people to *M. avium* subsp. *paratuberculosis* seen in the present and other studies is extensive, particularly in temperate regions, where infection of farm animals is endemic. The outcomes will range between the development of some natural immunity (11, 20, 32) and the cumulative acquisition of a colonizing presence or of infection by this organism in individual hosts (9, 53).The consequences of this in individuals will be determined by the presence of inherited and acquired susceptibilities as well as being influenced by the strain and phenotype of the organism. The minority proportion of people with the wrong set of susceptibilities may eventually come down with chronic inflammatory disease.

The incidence of Crohn's disease in people migrating from regions with low to high incidences rises with that of the host region (65, 74). These and other studies establish a consensus that one or more environmental factors are critically involved in Crohn's disease causation. We propose that the principal environmental factor involved in Crohn's disease causation is exposure to the chronic enteric pathogen *M. avium* subsp. *paratuberculosis*. Central to the resolution of this globally expanding, long-term complex public health problem is the development and use of a series of modern preventative and therapeutic vaccines for animals and people.

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