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Ecological Distribution of *Legionella pneumophila*

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Bacteria were concentrated 500-fold from 20-liter water samples collected from 67 different lakes and rivers in the United States. The data suggest that *Legionella pneumophila* is part of the natural aquatic environment and that the bacterium is capable of surviving extreme ranges of environmental conditions. The data further demonstrate the effectiveness of the direct fluorescent-antibody technique for detecting *L. pneumophila* in natural aquatic systems. Smears of the concentrated samples were screened microscopically for serogroups of *L. pneumophila* by the direct fluorescent-antibody technique. Virtually all of the 793 samples were found to be positive by this method. The 318 samples containing the largest numbers of positive bacteria which were morphologically consistent with *L. pneumophila* were injected into guinea pigs for attempted isolations. Isolates were obtained from habitats with a wide range of physical, chemical, and biological parameters. Samples collected monthly from a thermally altered lake and injected into guinea pigs demonstrated a seasonality of infection, with the highest frequency of infection occurring during the summer months.

The preparation of specific fluorescent antibodies to *Legionella pneumophila* (3, 10, 11, 14) led to the development of techniques for screening environmental samples and for observing and isolating *L. pneumophila* from aquatic habitats (5).

We have previously shown a relationship between nutrients required by *L. pneumophila* for growth and extracellular compounds released by specific blue-green algae (cyanobacteria) (15). This relationship prompted us to screen a larger number of lakes for the presence of *L. pneumophila*-like bacteria as measured by direct fluorescent-antibody (DFA) tests. We then attempted to isolate *L. pneumophila*-like bacteria through guinea pig inoculation and to confirm the identification of the bacterial isolates by cultural, biochemical, and serological testing as previously described (5). *L. pneumophila*-like bacteria are defined as bacteria morphologically resembling *L. pneumophila* and fluorescing strongly with *L. pneumophila* antibodies.

MATERIALS AND METHODS

Water samples (20 liters) were collected from the littoral zone of various lakes and rivers throughout the eastern United States by aseptic techniques and with a peristaltic pump. Samples were brought to a self-contained mobile laboratory and immediately processed (5). Each sample was concentrated by continuous centrifugation (Sorvall SS-3 centrifuge) at 15,000 rpm, at 200 ml/min, and at room temperature. Samples were suspended to a final volume of 40 ml (500-fold concentration) in filter-sterilized (0.22-μm filter) water from the appropriate habitat. Subsamples (0.010 ml) were pipetted into 6-mm-diameter wells on toxoplasmosis slides (Cel-Line Associates, Minotola, N.J.). Smears were air dried, heat fixed, and stained with specific fluorescent antibodies for serogroups 1, 2, 3, and 4 of *L. pneumophila*. Positive and negative controls were included. Samples were viewed by epifluorescence microscopy as described previously (7). Fluorescing cells with morphological characteristics of *L. pneumophila* (3) were counted in 100 fields at a magnification of ×1,600, and the cell count was normalized to the number per liter of unconcentrated sample.

Simultaneously, water from each habitat was measured for designated physical and chemical parameters with a Hydrolab Surveyor Multiprobe Analyzer (Hydrolab Corp., Austin, Tex.). Algal parameters of chlorophyll *a* and pheophytin were determined as described by Strickland and Parson (13). Secchi disk readings, a measure of water clarity, were also taken (1).

Isolation of *L. pneumophila*. Samples positive by DFA staining tests were quantified, and known levels of bacteria (>5 × 10^4) morphologically similar to *L. pneumophila* were injected intraperitoneally into uncompromised Hartley breed guinea pigs. The guinea pigs were observed daily for a rise in temperature and for evidence of overt illness. When the animals became ill or moribund, they were sacrificed and necropsied. Smears were prepared from swabs of the peritoneal wall, the viscera, and the peritoneal fluid. Additionally, freshly cut surfaces of the liver and spleen were imprinted onto microscope slides. Portions of the tissues were frozen and maintained at −70°C for later examination, and others were placed in 10% neutralized Formalin.

Fresh tissue smears were screened by DFA staining with conjugates for serogroups 1, 2, 3, and 4 of *L. pneumophila*. Frozen and formalinized tissues from animals which showed evidence of infection were transmitted for further study to the Center for Disease Control, where they were examined by DFA staining and cultured on charcoal-yeast extract agar (4). When direct culturing was unsuccessful, the results of the DFA staining of *L. pneumophila*-like bacteria in the tissues from each guinea pig were used in the decision of whether a second animal should be injected in an attempt to isolate *L. pneumophila*. When approximately 10^6 *L. pneumophila*-like organisms per ml were observed microscopically, a second guinea pig was injected intraperitoneally with homogenates of spleen, liver, and peritoneal fluid from the first guinea pig. The injected volume was usually 3.0 ml and consisted of a single tissue or a pool of tissues, depending upon the results of DFA screening. These animals were monitored for illness and were sacrificed, necropsied, and examined as described above.

Single-colony isolates of *L. pneumophila* were purified by two additional successive platings on charcoal-yeast extract agar. Their identity was confirmed by methods that included cultural, morphological, biochemical, and serological characteristics (3, 12, 16).

RESULTS AND DISCUSSION

This survey of selected freshwater habitats in North Carolina, South Carolina, Georgia, Florida, Alabama, Indiana, and Illinois is not comprehensive. It is, however, the first extensive survey of freshwater habitats for the presence of *L. pneumophila*, using serospecific fluorescent antibodies. *L. pneumophila*-like bacteria belonging to each of the four published serogroups of *L. pneumophila* were observed at various densities in virtually all of the sampled habitats. The data for 793 samples from 67 different lakes and rivers in the United States are compiled in Table 1, along with the weighted geometric means. By established techniques (5), as few as 9.1×10^3 *L. pneumophila* bacteria per liter of unconcentrated water sample were detected. This is the lower limit of detectability. Because some samples did not contain observable densities of *L. pneumophila*-like bacteria, they were considered to be zero when the geometric mean was calculated. Thus, some densities shown in Table 1 have mean values below the detectable limits. Serogroup 1 was observed most frequently in the natural samples; next in frequency of occurrence were serogroups 4, 2, and 3.

This extensive body of evidence agrees with and extends previous findings (5) that *L. pneumophila* is part of the natural microbial community of aquatic ecosystems. The population densities of *L. pneumophila* are extremely low, and at the highest densities measured, the four serogroups of *L. pneumophila* account for less than 1% of the total bacterial population, as measured by direct epifluorescence microscopy (7; Fliermans, unpublished data).

We have isolated 47 different strains of *L. pneumophila* from guinea pigs injected with concentrates of 318 water samples taken from aquatic habitats not associated with any known outbreaks of legionellosis (Table 2). The isolates encompass the four described serogroups of *L. pneumophila*, as well as the new serogroup 6 (McKinney et al., unpublished data). The tissues from guinea pigs injected with 36 different water samples were tested for *L. pneumophila* of serogroups 5 (3) and 6 by DFA staining. Based on previous experience, we determined that six of these contained potentially culturable densities of *L. pneumophila*-like bacteria (10^6/ml of tissue suspension). The isolates were identified by serogroup as follows: 1, 30 cultures, 63.8%; 2, 1 culture, 2.1%; 3, 1 culture, 2.1%; 4, 13 cultures, 27.7%; and 6, 2 cultures, 4.3% (Tables 2 and 3). Serogroup 5 was not isolated. Among the 47 strains of *L. pneumophila*, two different serogroups were obtained from the same guinea pig on eight occasions. In seven of the eight times, the isolates belonged to serogroups 1 and 4.

It is significant that all water samples injected into guinea pigs contained fluorescing *L. pneumophila*-like bacteria, but compared with the number of samples injected, few isolates were obtained (47 isolates from 318 injected samples, or 15%). The DFA technique is a powerful tool for screening a large number of samples for the presence of specific microorganisms. It does, however, have limitations in that it is not possible to determine by immunofluorescence alone whether a stained bacterium is viable.

There are several possible reasons why more specimens of *L. pneumophila* have not been isolated from the injected samples. One is that the DFA reagents may be cross-staining non-*Legionella* bacterial cells with morphology similar to that of *L. pneumophila*. However, thousands of stock cultures belonging to many species and genera and thousands of colonies picked from water and soil isolation plates have been examined with conjugates for serogroups 1 through 4 of *L. pneumophila*, and we are aware of only two cultures of non-*Legionella* bacteria that cross-react at the 3 to 4+ level (3, 14). These cultures consist of a single strain of *Pseudomonas fluorescens* and one of *Pseudomonas alcaligenes*, and these cultures are related only to serogroup 1 of *L. pneumophila*. Shelton and Tiedje have examined 91 soil isolates for cross-reactions with a labeled polyvalent antiserum for serogroups 1 through 4 (personal communication). Of those isolates, 45 were identified as *P. fluorescens*, and no cross-reactions were observed. Fliermans and Bettinger have examined

TABLE 1. *Selected habitats screened for the presence of L. pneumophila by direct immunofluorescence*[a]

Habitat	State	No. of samples	No. of serogroup conjugates per liter			
			1 (Knoxville 1)	2 (Togus 1)	3 (Bloomington 2)	4 (Los Angeles 1)
Pond B, SRP[b]	S.C.	138	2.4×10^5	3.2×10^4	1.0×10^4	2.0×10^4
Par Pond, SRP	S.C.	208	7.7×10^5	6.3×10^4	2.6×10^4	3.4×10^5
Pond C, SRP	S.C.	109	1.7×10^6	2.4×10^4	2.9×10^5	2.6×10^5
Risher's Pond, SRP	S.C.	35	6.2×10^5	9.2×10^4	6.0×10^4	3.0×10^4
Thermal canal, SRP	S.C.	56	1.6×10^6	5.0×10^{2}[c]	1.4×10^5	1.6×10^5
Gem Lake	S.C.	1	9.1×10^4	9.1×10^3	9.1×10^3	1.8×10^4
Lake Murray	S.C.	11	9.2×10^5	3.2×10^4	5.3×10^4	2.0×10^5
Dick's Pond	S.C.	36	2.4×10^5	2.9×10^5	7.5×10^3	2.1×10^4
Steed's Pond	S.C.	41	6.5×10^5	2.1×10^5	1.2×10^4	1.2×10^4
Lake Hartwell	S.C.	11	4.0×10^5	2.4×10^4	9.1×10^3	1.6×10^5
Lake Keowee	S.C.	13	4.3×10^5	3.1×10^4	7.8×10^4	8.0×10^4
Lake Jocassee	S.C.	12	3.5×10^5	1.1×10^4	3.8×10^4	6.3×10^4
Lake Wylie	S.C.	5	6.7×10^5	8.0×10^4	2.0×10^4	1.1×10^5
Lake Wateree	S.C.	5	7.6×10^5	9.1×10^3	9.1×10^3	1.2×10^5
Lake Katharine	S.C.	1	1.4×10^6	9.1×10^3	9.1×10^3	9.1×10^3
Goose Creek Reservoir	S.C.	3	2.8×10^5	9.1×10^3	9.1×10^3	2.5×10^5
Lake Moultrie	S.C.	4	2.7×10^5	1.7×10^4	4.2×10^4	6.1×10^5
Lake Marion	S.C.	7	3.0×10^6	2.2×10^4	1.6×10^4	3.6×10^5
Lake Robinson	S.C.	3	5.8×10^4	9.1×10^3	9.1×10^3	1.1×10^5
Cooper Creek Estuary	S.C.	4	—	—	—	—
Rocky River	S.C.	1	7.6×10^5	9.1×10^3	9.1×10^3	5.5×10^4
Tyger River	S.C.	1	4.4×10^5	9.1×10^3	9.1×10^3	1.0×10^5
Lake Greenwood	S.C.	3	5.3×10^5	4.0×10^4	9.1×10^3	1.2×10^5
Clark Hill Reservoir	S.C.	4	9.5×10^4	5.7×10^4	9.1×10^3	3.6×10^4
Twin Lakes, SRP	S.C.	4	1.2×10^5	9.1×10^3	9.1×10^3	3.8×10^5
Lake Rutledge	Ga.	14	1.4×10^6	1.7×10^4	4.3×10^4	1.5×10^6
Savannah River	Ga.	7	4.4×10^5	2.6×10^3	5.0×10^3	5.7×10^4
Lake Sinclair	Ga.	5	8.3×10^4	ND	ND	2.9×10^6
Ocmulgee River	Ga.	2	1.0×10^6	1.4×10^4	9.1×10^3	1.1×10^5
Lake Jackson	Ga.	6	3.0×10^6	5.6×10^4	9.1×10^3	1.9×10^5
Oconee River	Ga.	2	1.7×10^5	9.1×10^3	3.2×10^4	3.2×10^4
Magnolia Spring	Ga.	1	1.1×10^5	9.1×10^3	9.1×10^3	1.1×10^5
Okefenokee Swamp	Ga.	1	8.7×10^5	9.1×10^3	5.5×10^4	3.2×10^5
West Point Lake	Ga.	1	9.1×10^3	9.1×10^3	9.1×10^3	9.1×10^3
Jackson Lake	Ga.	1	4.9×10^5	9.1×10^3	9.1×10^3	9.1×10^3
Catawba Reservoir	N.C.	1	1.6×10^5	9.1×10^3	9.1×10^3	8.2×10^4
Catawba River	N.C.	1	3.3×10^5	8.2×10^4	9.1×10^3	1.4×10^5
Mt. Island Lake	N.C.	1	5.5×10^5	9.1×10^3	5.5×10^4	9.1×10^3
High Rock Lake	N.C.	1	8.2×10^4	9.1×10^3	9.1×10^3	1.4×10^5
Lake Tillery	N.C.	1	1.0×10^6	9.1×10^3	2.7×10^4	2.7×10^4
Holtz Lake	N.C.	1	3.3×10^7	9.1×10^3	9.1×10^3	2.7×10^4
Neuse River	N.C.	1	5.7×10^5	9.1×10^3	8.2×10^4	9.1×10^3
Beaufort Inlet	N.C.	1	9.1×10^3	9.1×10^3	9.1×10^3	9.1×10^3
Lake Hickory	N.C.	1	4.6×10^5	1.6×10^5	9.1×10^3	1.1×10^5
Badin Lake	N.C.	1	1.6×10^5	9.1×10^3	9.1×10^3	8.2×10^4
Ocean Pond	Fla.	1	5.4×10^4	9.1×10^3	9.1×10^3	9.1×10^3
Lake Jackson	Fla.	1	4.9×10^5	9.1×10^3	9.1×10^3	9.1×10^3
Lake Talquin	Fla.	1	3.8×10^5	5.5×10^4	9.1×10^2	1.4×10^5
Dead Lake	Fla.	1	9.1×10^3	9.1×10^3	9.1×10^3	9.1×10^3
Lake Seminole	Fla.	1	1.6×10^5	9.1×10^3	9.1×10^3	9.1×10^3
Lake Tholocco	Ala.	1	1.1×10^5	9.1×10^3	9.1×10^3	9.1×10^3
Lake W. F. George	Ala.	1	1.1×10^5	1.1×10^5	9.1×10^3	9.1×10^3
Jordon Lake	Ala.	1	1.9×10^5	9.1×10^3	9.1×10^3	1.9×10^5
Martin Lake	Ala.	1	2.7×10^5	9.1×10^3	9.1×10^3	1.1×10^5
Lake Harding	Ala.	1	1.1×10^5	9.1×10^3	9.1×10^3	9.1×10^3
Lake Vermilion	Ill.	1	5.9×10^5	8.8×10^4	9.1×10^3	9.1×10^3
Lake Holiday	Ill.	1	1.2×10^5	4.1×10^5	3.2×10^5	5.9×10^4
Sangchris Lake	Ill.	3	1.0×10^6	9.3×10^5	9.1×10^3	3.5×10^4
Lake Taylorville	Ill.	1	3.8×10^5	7.9×10^5	9.1×10^3	9.1×10^3

TABLE 1—*Continued*

Habitat	State	No. of samples	No. of serogroup conjugates per liter			
			1 (Knoxville 1)	2 (Togus 1)	3 (Bloomington 2)	4 (Los Angeles 1)
Lake Shelbyville	Ill.	2	3.3×10^5	9.1×10^3	2.7×10^5	9.1×10^3
Lake Coffeen	Ill.	3	2.9×10^6	2.2×10^4	9.1×10^3	7.8×10^5
Lake Lemon	Ind.	1	2.8×10^6	7.1×10^5	9.1×10^3	1.8×10^4
Monroe Lake	Ind.	1	5.3×10^5	9.1×10^3	9.1×10^3	9.1×10^3
Overview Lake	Ind.	1	7.9×10^5	1.8×10^5	9.1×10^3	9.1×10^3
Parkersburk Spring	Ind.	1	9.1×10^3	9.1×10^3	9.1×10^3	9.1×10^3
Racoon Lake	Ind.	1	5.6×10^5	9.1×10^3	9.1×10^3	5.9×10^4
Dietz Lake	Ind.	1	1.3×10^6	5.9×10^5	9.1×10^3	9.1×10^3

[a] Counts are based on microscopic estimations of fluorescing *L. pneumophila*-like bacteria in 0.010 ml of water samples concentrated 500-fold spread over a slide well with a 6-mm diameter. Observations were made on 100 fields at a magnification of ×1,600. —, High background; ND, not determined. Weighted geometric mean values for each serogroup were as follows: 1, 6.18×10^5; 2, 3.87×10^4; 3, 2.85×10^4; 4, 1.01×10^5.

[b] SRP, Savannah River Plant.

[c] The limit of detectability was 9.1×10^3 *L. pneumophila* bacteria per liter. Lower values indicate means of multiple samples from a single source, some of which were calculated as zero.

20 isolates from cooling towers, most of which were *Pseudomonas* spp., without observing any cross-reactions (unpublished data). Minor cross-staining of some strains and species of mycobacteria by *L. pneumophila* conjugates has been reported but should not cause confusion with *L. pneumophila* in either ecological or diagnostic samples (2, 8).

Undoubtedly, other examples of antigenic relationships between *L. pneumophila* and other bacteria will emerge. We believe, however, that the important reasons for the failure to isolate *L. pneumophila* from a higher percentage of DFA-positive water specimens are (i) the lack of a selective isolation medium, (ii) the presence of nonviable but intact cells, (iii) competition and antagonism from the natural biota, and (iv) seasonal differences in the virulence of the organism in guinea pigs.

If nonspecificity were a major cause of false-positive DFA staining tests for *L. pneumophila* in water samples, quantitative microscopic counts of the four serogroups would not necessarily have any relationship to their isolation frequencies. The frequency of isolation compared favorably with the microscopic counts, which suggests that DFA staining validly measures the content of *L. pneumophila* (Table 3).

Selected physical, chemical, and biological characteristics of the habitats from which the strains were isolated were measured to define the conditions under which *L. pneumophila* exists. Individual histograms were prepared for each of the measured habitat parameters. Figure 1 shows the percentage of isolates obtained from habitats with varying conditions of temperature, conductivity, pH, dissolved oxygen, chlorophyll *a*, pheophytin, and water clarity. The data demonstrate the range of habitats where *L. pneumophila* exists and from which it can be isolated. Isolates were obtained from habitats having the following ranges of characteristics: temperature, 5.7 to 63°C; pH value, 5.5 to 8.1; dissolved oxygen, 0.3 to 9.6 ppm (0.3 to 9.6 mg/ml); conductivity, 18 to 106 μS/cm; secchi disk readings, 1 to 4 m; chlorophyll *a*, 0.7 to 24.0 mg/m^3; and pheophytin, 0.2 to 18.8 mg/m^3.

When χ^2 values were calculated for our data, the difference between the frequency of isolation of *L. pneumophila* in the lower 50% and that in the upper 50% of the parameter ranges was significant ($P < 0.05$) only for water temperature; i.e., more isolates were obtained in the 36 to 70°C range (Table 4). In addition, the greatest isolation efficiency (50%) noted in any parameter was observed for samples collected from habitats with a temperature range of 40 to 60°C. The frequency of isolation of *L. pneumophila* in relation to the parameter values in the various habitats can be misleading because of the limited number of water samples examined. Thus, the data should not be extrapolated to predict which habitats support the bacterium. However, the data show the variety of habitats from which *L. pneumophila* can be isolated.

Additionally, the data were examined to determine whether a seasonality existed for the infection of guinea pigs by *L. pneumophila* in water samples from Par Pond (6) (Fig. 2). All water samples were taken monthly at designated sampling stations at the same depths. Concentrated samples (3.0 ml) with known densities of fluorescing *L. pneumophila*-like bacteria, as determined by DFA staining, were injected intraperitoneally into 292 guinea pigs. Except in the November 1978 and December 1979 samples, the injected *L. pneumophila*-like bacteria content was between 5.5×10^5 and 2.5×10^6 cells.

TABLE 2. *Isolates of L. pneumophila from lakes not associated with outbreaks of Legionnaires disease*

Code	Habitat	Serogroup isolated	Temp (°C)	pH	O_2 (mg/liter)[a]	Conductivity (μS/cm)
SRP-2	Par Pond, SRP[b]	Knoxville 1[c]	21.8	8.0	7.3	80
SRP-3	Par Pond, SRP	Knoxville 1	28.0	5.7	2.6	70
SRP-4	Risher's Pond, SRP	Knoxville 1	20.0	8.0	5.6	70
SRP-5	Par Pond, SRP	Knoxville 1	23.2	6.6	7.7	65
SRP-6	Thermal effluent, SRP	Knoxville 1	45.0	6.4	6.0	63
SRP-7	Thermal effluent, SRP	Knoxville 1	45.0	6.2	5.6	60
SRP-8	Lake Wylie, S.C.	Bloomington 2[d]	5.7	6.9	9.6	104
SRP-9	Thermal pond, SRP	Knoxville 1	42.0	7.1	5.3	70
SRP-10	Thermal pond, SRP	Los Angeles 1	42.0	7.1	5.2	70
SRP-11	Thermal pond, SRP	Knoxville 1	41.0	6.6	4.0	70
SRP-12	Thermal pond, SRP	Los Angeles 1[e]	41.0	6.6	4.0	70
SRP-13	Thermal effluent, SRP	Los Angeles 1	53.0	6.5	5.0	52
SRP-14	Thermal effluent, SRP	Knoxville 1	53.0	6.5	5.0	52
SRP-15	Thermal effluent, SRP	Los Angeles 1	53.0	6.4	5.0	52
SRP-16	Par Pond, SRP	Knoxville 1	24.0	5.9	6.2	64
SRP-17	Stream, S.C.	Los Angeles 1	20.0	5.4	7.1	18
SRP-18	Par Pond, SRP	Los Angeles 1	24.7	6.2	6.4	64
SRP-19	Thermal pond, SRP	Knoxville 1	42.0	7.2	4.9	70
SRP-20	Thermal pond, SRP	Los Angeles 1	42.0	7.2	4.9	70
SRP-21	Par Pond, SRP	Knoxville 1	21.0	5.9	0.5	72
SRP-22	Par Pond, SRP	Los Angeles 1	21.0	5.9	0.5	72
SRP-23	Risher's Pond, SRP	Los Angeles 1	25.5	5.7	7.1	28
SRP-24	Risher's Pond, SRP	Knoxville 1	25.5	5.7	7.1	28
SRP-25	Par Pond, SRP	Knoxville 1	24.7	6.2	6.4	64
SRP-26	Par Pond, SRP	Los Angeles 1	14.5	6.3	0.3	82
SRP-27	Par Pond, SRP	Knoxville 1	14.5	6.3	0.3	82
SRP-28	Dick's Pond, SRP	Knoxville 1	21.9	5.5	5.4	20
SRP-29	Lake Keowee, S.C.	Knoxville 1	25.5	6.7	8.1	18
SRP-30	Thermal effluent, SRP	Knoxville 1	63.0	6.2	5.9	71
SRP-31	Par Pond, SRP	Knoxville 1	13.8	6.8	3.3	64
SRP-32	Postthermal pond, SRP	Los Angeles 1	10.3	5.7	1.2	32
SRP-33	Par Pond, SRP	Knoxville 1	26.0	7.3	9.2	61
SRP-34	Postthermal pond, SRP	Knoxville 1	26.0	6.4	7.3	20
SRP-35	Postthermal pond, SRP	Knoxville 1	26.0	6.4	7.3	20
SRP-36	Lake Coffeen, Ill.	Togus 1[f]	28.5	8.1	6.2	ND[g]
SRP-37	Thermal pond, SRP	Knoxville 1	29.0	6.7	5.9	66
SRP-38	Par Pond, SRP	Knoxville 1	16.6	6.6	0.3	106
SRP-39	West Point Lake, Ga.	Chicago 2[h]	26.5	6.7	6.4	70
SRP-40	Steed's Pond, SRP	Chicago 2	12.7	6.9	8.2	35
SRP-41	Thermal pond, SRP	Knoxville 1	40.2	7.1	7.7	64
SRP-42	Par Pond, SRP	Knoxville 1	17.5	6.7	6.0	69
SRP-43	Thermal pond, SRP	Knoxville 1	33.2	6.8	5.8	64
SRP-44	Thermal pond, SRP	Knoxville 1	43.5	7.1	5.1	71
SRP-45	Par Pond, SRP	Knoxville 1	17.6	7.2	8.2	66
SRP-46	Thermal pond, SRP	Los Angeles 1	40.6	6.9	6.8	74
SRP-47	Postthermal pond, SRP	Knoxville 1	16.8	6.3	7.8	20
SRP-48	Thermal pond, SRP	Los Angeles 1	33.2	6.8	5.8	64

[a] Dissolved oxygen.
[b] SRP, Savannah River Plant.
[c] Knoxville 1, serogroup 1.
[d] Bloomington 2, serogroup 3.
[e] Los Angeles 1, serogroup 4.
[f] Togus 1, serogroup 2.
[g] ND, Not done.
[h] Chicago 2, serogroup 6.

Those animals which met the following criteria were considered to be infected with *L. pneumophila*: (i) a temperature increase of 0.6°C above the base line; (ii) lethargy, ruffled fur, and watery eyes; and (iii) the presence of bacteria in the peritoneal fluid, spleen, or liver tissues that stained specifically with one or more of the four described serogroups. The only criteria of infec-

tion used in these experiments were the clinical symptoms and the results of DFA staining of the tissues.

Infection rates ranged from 10% (2 of 20 in December 1979) or below (1 of 21 in November 1978) to 69% (40 of 58) during the months of May, June, and July 1979 (Fig. 2). The injected water samples were all from the same sites within Par Pond, from which many of our isolates have been obtained (Table 2), and except for the injected samples of November 1978 and December 1979, the levels of injected *L. pneumophila*-like bacteria were similar throughout the sampling period (line graph at top of Fig. 2). When these water samples were injected into guinea pigs, they were screened biologically by these animals, which are relatively unsusceptible to contaminants (19). Twenty-one of our cultures were isolated only after a second serial passage through guinea pigs. This could be explained by reduced infectivity, competition from contaminants, effective host response to infection, or chance. Frequently, rather large numbers of fluorescing *L. pneumophila*-like bacteria were seen in the tissues from the first injected animal, but no *L. pneumophila* colonies appeared on the plates even when the contamination was minimal.

TABLE 3. *Comparison of DFA staining and culture isolation results for serogroups of L. pneumophila*

Serogroup	No. of isolates	% of isolates	Weighted geometric mean of microscopic counts (DFA) × 10^5[a]	% of sum of means of microscopic counts (DFA)
1	30	63.8	6.18	78.6
2	1	2.1	0.39	4.9
3	1	2.1	0.29	3.6
4	13	27.7	1.01	12.8
6	2	4.3	ND[b]	ND[b]

[a] See Table 1 for weighted geometric mean values for each serogroup.
[b] ND, Not done.

The number of isolates (47) that were cultured from guinea pigs injected with 318 different water samples demonstrates that *L. pneumophila* can exist in habitats with a wide range of physiochemical parameters, particularly the extensive temperature ranges. The DFA tests indicate the presence of *L. pneumophila*-like bacteria at measurable densities in virtually all of the 793 samples tested. The data do not show whether

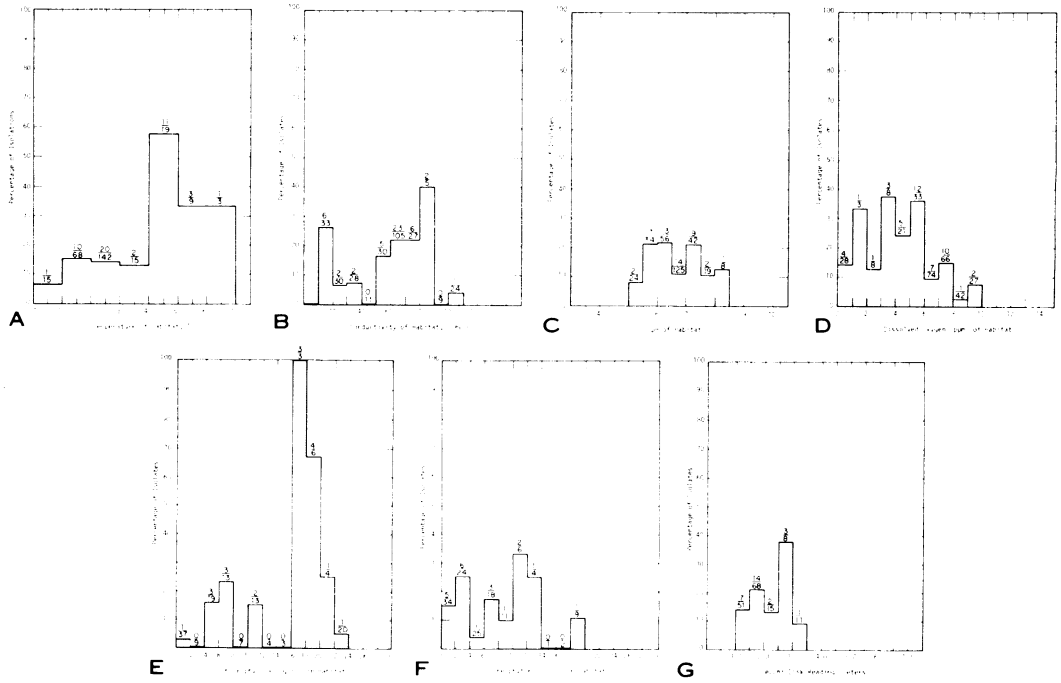

FIG. 1. *Relation of habitat characteristics to the percentage of L. pneumophila isolates. The ratio above each portion of the histograms represents the number of cultures obtained with respect to the number of guinea pigs injected with water samples having that particular parameter. In the conductivity histogram, μmho = μS.*

L. pneumophila can multiply under these natural conditions, but they do show that the bacteria can survive and remain viable. Isolates from water temperatures of 5.7 to 63°C indicate that a great range of habitats is capable of harboring this bacterium. A relationship between *L. pneumophila* and thermal environments is suggested also by its cellular fatty acid composition, which is similar to that of known thermophilic bacteria (12). Thus, frequent isolation of *Legionella* bacteria from habitats found to be positive by immunofluorescence, isolation of five different serogroups, insignificant cross-reactivity, viability data, and seasonality of infection all indicate that *Legionella* bacteria are probably more numerous in natural habitats than the number of isolates suggest. Further, the data suggest that the absence of a selective medium is more restrictive to the ecological study of *L. pneumophila* than are immunofluorescence cross-reactions with antigenically similar bacteria.

We suggest that *L. pneumophila* is part of the natural aquatic environment and that the bacterium is capable of surviving wide ranges of physical and chemical conditions. The effectiveness of the DFA technique for detecting *L. pneumophila* in natural aquatic systems has been demonstrated previously. Further, the relationship between *L. pneumophila* and algal photosynthesis suggests that warm habitats which are open to algal colonization or algal products may be environments for *L. pneumophila*.

TABLE 4. *Relationship of high- and low-parameter characteristics to the frequency of L. pneumophila isolations*[a]

Range of characteristic	No. of samples	No. of isolates	% of isolates	χ^2	*P* value
Temp (°C)					
0–35	233	32	13.9	9.48	0.0021[b]
36–70	38	16	42.1		
Conductivity (μS/cm)					
0–55	120	12	10.0	2.75	0.0971
56–110	185	35	18.9		
pH					
5–6.75	176	29	16.5	0.07	0.7873
6.76–8.5	132	19	14.4		
Dissolved oxygen (ppm)					
0–5	68	14	20.6	0.72	0.2757
6–10	242	32	13.2		
Chlorophyll *a* (mg/m^3)					
0–12	98	9	9.2	2.36	0.1244
13–24	40	9	22.5		
Pheophytin (mg/m^3)					
0–10	112	16	14.3	0.006	0.9371
11–20	22	4	18.2		
Secchi disk (m)					
0–2	119	21	17.6	0.16	0.6857
2.1–4	24	6	25.0		

[a] High 50% of range and low 50% of range. See Fig. 1.
[b] Significant at <5% probability level.

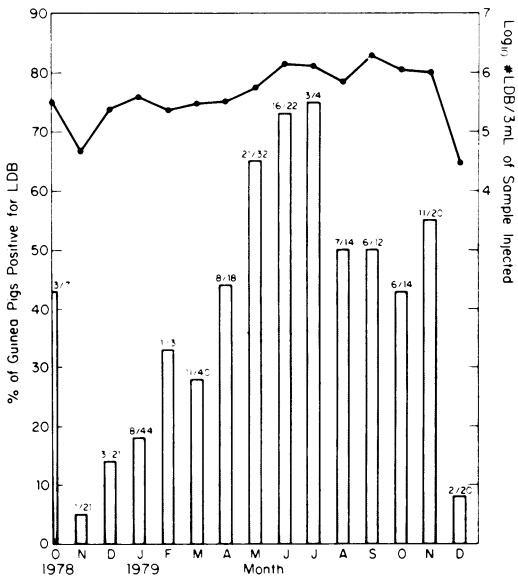

FIG. 2. *Seasonality of guinea pig infection with samples of water from Par Pond containing known levels of L. pneumophila-like bacteria. The line graph represents DFA estimates of the average number of L. pneumophila-like bacteria in samples injected into animals in each month. The ratio above each bar of the histogram represents the number of DFA-positive guinea pigs with respect to the number of animals injected with water samples. LDB, Legionnaires disease bacterium.*

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