Occurance of Legionella pneumophila

(Legionniares' Disease Bacteria)

Completed by

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FWI #81-6

OCCURRENCE OF LEGIONELLA PNEUMOPHILA (LEGIONNIARES' DISEASE BACTERIA) IN NORTHEASTERN U. S. LAKES

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ABSTRACT

Lake George, Lake Champlain, Round Lake and Saratoga Lake, as well as several streams entering Lake George were examined for the presence of bacteria which react with antibodies specific for Legionella pneumophila using the direct fluorescent antibody technique (DFA). All samples examined contained such cells. The range was from several hundred to several million per liter of water. The numbers of DFA positive cells increased during the mid-late summer demonstrating a probable correlation with increased numbers of activity of primary producers in the water column. The results of animal virulence tests showed some samples to have L. pneumophila capable of causing disease in experimental animals.

INTRODUCTION

Approximately 800,000 Americans die as a result of undiagnosed pneumonia annually. Recent estimates suggest that 70,000 of these deaths are due to infection with Legionella pneumophila, the causative agent of Legionnaires' Disease. Extensive investigation of numerous outbreaks and sporadic cases of the disease has suggested an environmental reservoir for the organism (see Eickhoff, 1979). Cells which react with fluorescent antibody specific for L. pneumophila have now been isolated from a variety of environmental habitats, including lakes (Fliermans et al., 1980; Fliermans et al., 1979), streams (Fliermans et al., 1980; Morris et al., 1979; Morris et al., 1979; Tison et al., 1980), creek mud (Morris et al., 1979) and cooling towers (Dondero et al., 1980; Morris et al., 1979; Morris et al., 1979) or evaporative condensers (Cordes et al., 1980; Morris et al., 1979) associated with air conditioning systems in both epidemic and non-epidemic related habitats.

Recent outbreaks of legionellosis at the University of Vermont (Burlington) Medical Center are suspected to be linked to the presence of L. pneumophila in a cooling tower located on top of an adjacent building. Source water for this tower is obtained from

Lake Champlain, leading to speculation that the lake may serve as a reservoir for the organism.

This report summarizes the results of a five month survey of northeastern United States aquatic systems for cells reacting with fluorescent antibody specific for L. pneumophila, including Lake Champlain, Lake George, Saratoga Lake, and Round Lake. Numbers of algal cells, concentrations of chlorophyll a and pheophytin, and numerous physical and chemical parameters were examined in an attempt to explain the occurrence of L. pneumophila at various lake sites.

MATERIALS AND METHODS

Monthly samples were collected from six sites on Lake George, the southern bay of Lake Champlain, Saratoga Lake and Round Lake. Up to seven liters of water were collected at each site and transported back to the laboratory for immediate analysis.

Water was filtered through 0.45 μ membrane filters (Millipore). Trapped material was resuspended in 30 ml of sterile distilled water and centrifuged for 50 min at 15,000 x g in a Sorvall RC-2 centrifuge. The resultant pellet was resuspended in 4 ml sterile distilled water, and dilutions of 1:10 and 1:100 were made in sterile distilled water. Ten μl of each dilution were spotted onto toxoplasmosis slides, air dried, heat fixed, and stained with polyvalent fluorescent antibodies, which includes antibodies specific for serogroups 1-4 of Legionella pneumophila. Pre-immune controls were analyzed for each sample.

Bacterial counts were done with a Zeiss microscope using epi-fluorescence illumination at 1000X magnification. Algal counts were done under similar conditions using phase-contrast illumination.

Water chemistry analyses were performed by the Fresh Water Institute, Lake George, NY using methods as outlined in Standard Methods for the Examination of Water and Waste Water (1976).

Methods used for production and utilization of ^{14}C labeled dissolved organic carbon by L. pneumophila have been reported elsewhere (D.L. Tison, Ph.D. Thesis, Rensselaer Polytechnic Institute, Troy, NY, 1980) and will be furnished upon request.

RESULTS AND DISCUSSION

Tison et al. (1980) demonstrated that Legionella pneumophila could grow on a minimal salts medium in association with a cyanobacterium, Fischerella sp. (Fig. 1). Numbers of bacterial cells reacting with fluorescent antibody conjugates specific for L. pneumophila remained constant after a growth period of approximately 6 h in two parallel experiments. Control cultures incubated in

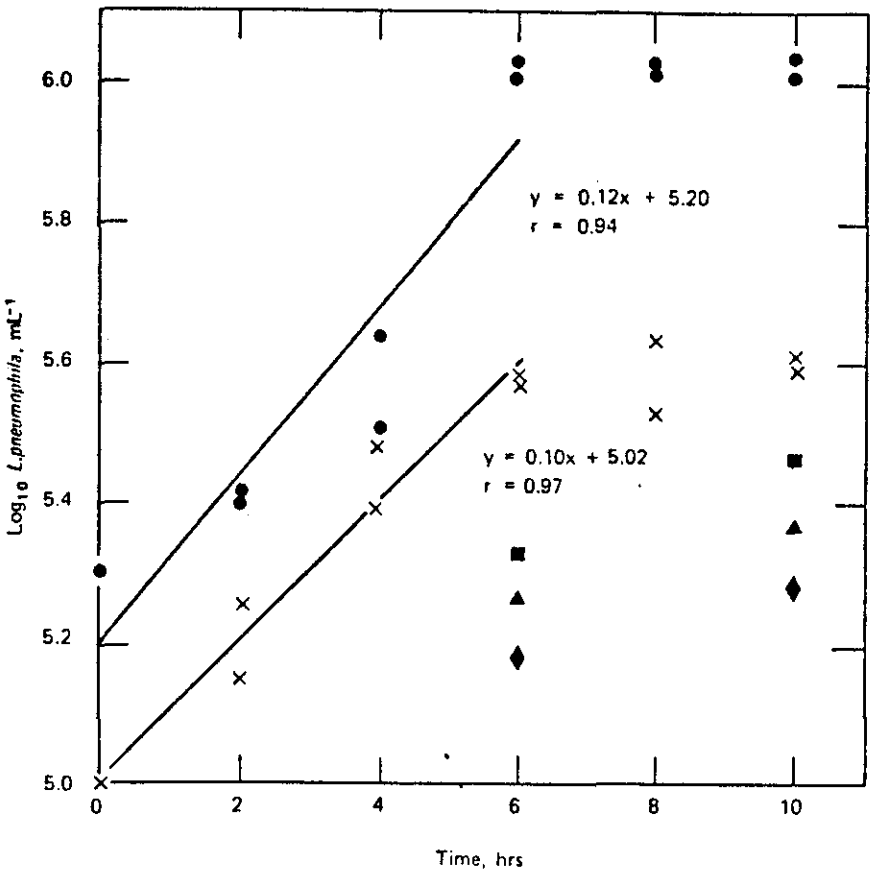

Figure 1. Numbers of L. pneumophila in co-culture with the cyanobacterium Fischerella sp. at 45°C. A and B represent the lines indicated by the equations generated by linear regression analysis of the data from duplicate experiments with different L. pneumophila inoculum concentrations. The coefficient of correlation (r) for each regression line is shown. Controls corresponding to the data associated with regression line A are also shown. Each point represents the mean of triplicate counts from cultures sampled at the time indicated.

Symbols: ● = L. pneumophila in experiment A, X = L. pneumophila in experiment B, ▲ = L. pneumophila in DCMU control, ■ = L. pneumophila inoculum without Fischerella sp. and ◆ = L. pneumophila in dark control.

the dark, without algae, or with 2×10^{-5} M DCMU (3-(3,4 dichlorophenyl) 1-dimethyl urea) showed no increase in numbers of bacteria, indicating that the algae must be actively photosynthesizing in order to support growth of L. pneumophila.

That the organisms are utilizing compounds excreted by Fischerella sp. as a source of energy, rather than previously stored compounds, is demonstrated by showing that growth of L. pneumophila resumed after a second transfer to a new culture. This is illustrated in Fig. 2.

Utilization of algal extracellular products as a source of both carbon and energy for L. pneumophila is shown in Fig. 3. Carbon-14 labeled dissolved organic carbon (DOC) produced by Fischerella sp. was added to an actively growing culture of L. pneumophila at 35°C. An immediate production of $^{14}CO_2$ by the bacteria is observed, with concomitant incorporation of ^{14}C-DOC into macromolecules.

A wide variety of algal types, both green and blue-green, have been tested for their ability to support growth of L. pneumophila on minimal salts media (Table 1). In all cases shown, the algae supported growth of either a clinical or environmental isolate of the organism. Other algae tested, including several diatoms, did not support growth of L. pneumophila (data not shown).

With these data in mind, it seemed logical to examine the number and types of algal cells coincident with the occurrence of L. pneumophila at varous lake sites. Fig. 4 illustrates the numbers of bacterial cells reacting with polyvalent fluorescent antibody specific for L. pneumophila (DFA positive cells) over a five-month period in Lake Champlain, Saratoga Lake, and Round Lake. Relatively few DFA positive cells were observed in June, while a maximum number was attained in August at all sites. The numbers of DFA cells decreased between August and late September. DFA counts ranged between 30 and 2000/ml water in Lake Champlain, 150 and 700/ml water in Saratoga Lake, and 50 and 120,000/ml water in Round Lake.

Six sites on Lake George were sampled at monthly intervals between May and September, 1980. Northern lake basin locations included Hearts Bay, Hague, and Rogers Rock, while Lake George Village, Warners Bay and Huletts Landing were monitored in the southern lake basin. These results are presented in Fig. 5 and 6. As can be seen, the numbers of DFA positive cells remained fairly constant over the five month period at all sites with the possible exception of Huletts Landing, which had an increase between June and July 1 from approximately 100 cells/ml up to 4000 cells/ml water. Lake George Village showed a three-fold increase in bacterial numbers on August 1, while numbers in Hearts Bay increased from 3 cells/ml on July 1 to 1300 cells/ml

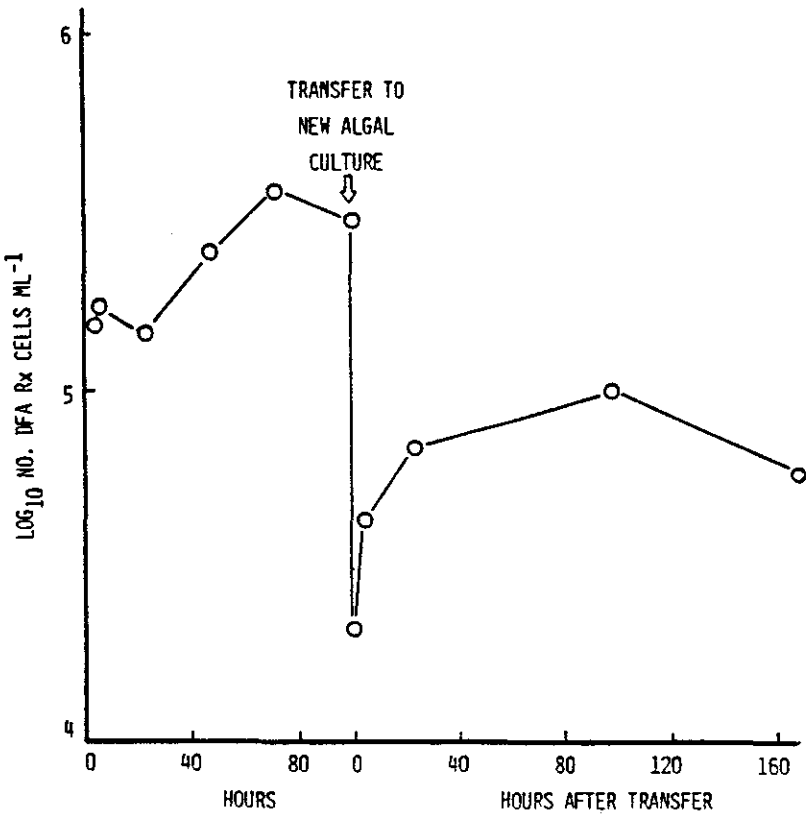

Figure 2. Numbers of L. pneumophila in co-culture with the cyanobacterium Fischerella sp. before and after transfer to a new algal culture. Co-cultures were grown at 35°C in medium D until numbers of L. pneumophila became stationary. Aliquots were then transferred to fresh axenic algal cultures, which were analyzed for numbers of L. pneumophila at the times indicated using immunofluorescent techniques.

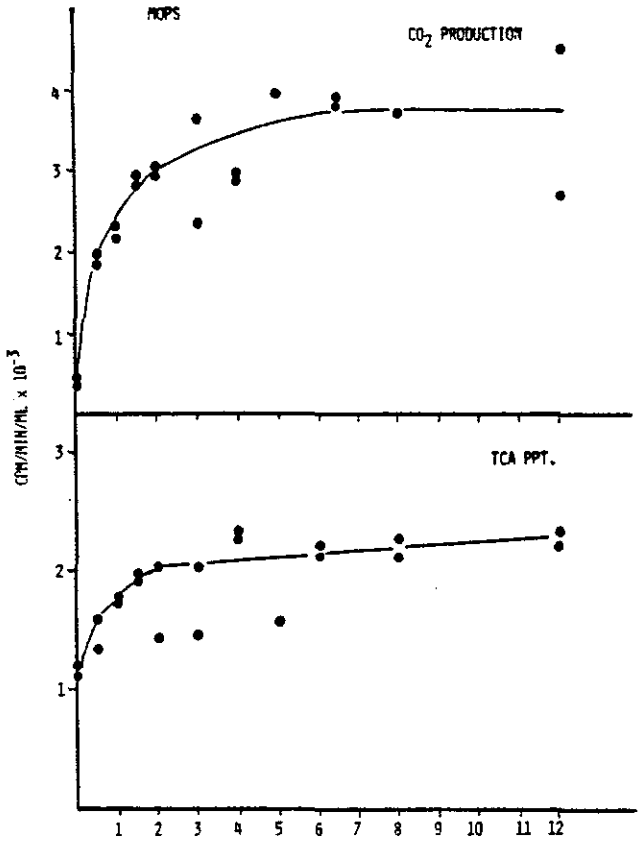

Figure 3. Utilization of Fischerella sp. extracellular products by a Knoxville strain of L. pneumophila. Radioactive algal dissolved organic carbon (DOC) was produced at 45°C by labeling axenic Fischerella sp. cultures with $NaH^{14}CO_3$ for 24 h. Algal cells were removed by filtration, and the filtrate was acidified to remove any inorganic carbon which was not utilized. A Knoxville strain of L. pneumophila was grown aseptically, to mid-log phase, filtered onto 0.45 µm membranes, washed, and resuspended in MOPS buffer, pH = 6.9. One ml each of neutralized ^{14}C-labeled DOC and cell suspension were added to sterile serum bottles stoppered with serum stoppers equipped with plastic cups. CO_2 production was measured at the indicated time points by trapping $^{14}CO_2$ onto PEA: methanol (1:1) saturated filter paper wicks. Incorporation into macromolecules was determined by incorporation into cold TCA precipitable material.

Table 1

Growth of Legionella Pneumophilia Strains in Co-culture with Various Algae

Alga	Algal Medium	Growth of L. pneumophila strain SRP 22	Knoxville Clinical
Chlamydomonas reinhardtii	Allens	+	+
Chlorella vulgaris	Allens	+	+/-
Chroococcidiopsis sp.	D	+	-
Fischerella sp. (ATCC)	D	+	+
Fischerella sp. MAT	D	+	+
Nostoc sp.	D	+	+
Phormidium sp.	D	+	+
Scenedesmus quadricauda	Allens	+	+/-
Synechocystis sp.	D	+	-

+, At least one doubling in the number of L. pneumophila per ml.

+/-, Growth but less than one doubling.

-, No significant increase in the numbers of L. pneumophila per ml.

Table 2

Water Chemistry Parameters Measured for Lake George, New York

Parameters	Range	Mean ± 1 S.D.
Depth	0 - 5 m	
pH	6.94 - 7.53	7.32 ± 0.14
Conductivity (μmhos)	87.0 - 140.8	96.30 ± 9.93
SO_4^- (mg/l)	3.00 - 4.52	3.60 ± 0.36
Cl^- (mg/l)	4.56 - 10.88	5.97 ± 1.13
NO_3^- (mg/l)	.001 - .234	0.031 ± 0.06
NO_2^- (mg/l)	<0.005 - .021	0.008 ± 0.006
NH_4^+ (mg/l)	<.010 - 0.018	0.010 ± 0.002
Total P (mg/l)	0.002 - 0.005	0.003 ± 0.001
Filterable P (mg/l)	0.001 - 0.002	0.001 ± 0.0005
Ortho-PO_4 (mg/l)	< 0.001	< 0.001
Ca^{++} (mg/l)	4.10 - 9.10	7.19 ± 1.24
Mg^{++} (mg/l)	2.13 - 2.32	2.20 ± 0.04
K^+ (mg/l)	0.51 - 0.69	0.56 ± 0.05
Na^+ (mg/l)	3.32 - 4.22	3.58 ± 0.22
Temp (^oC)	7.5 - 26.5	17.7 ± 5.6

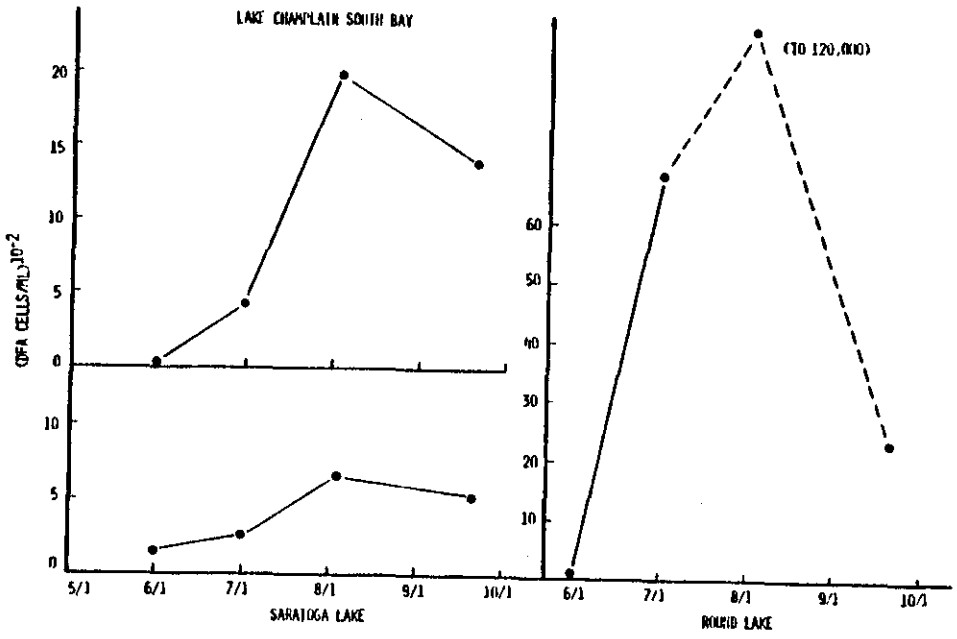

Figure 4. Numbers of bacterial cells reacting with polyvalent fluorescent antibody specific for serogroups 1-4 Legionella pneumophila in lakes of upstate New York. Water samples were collected on a monthly basis, and analyzed as described in the Materials and Methods section.

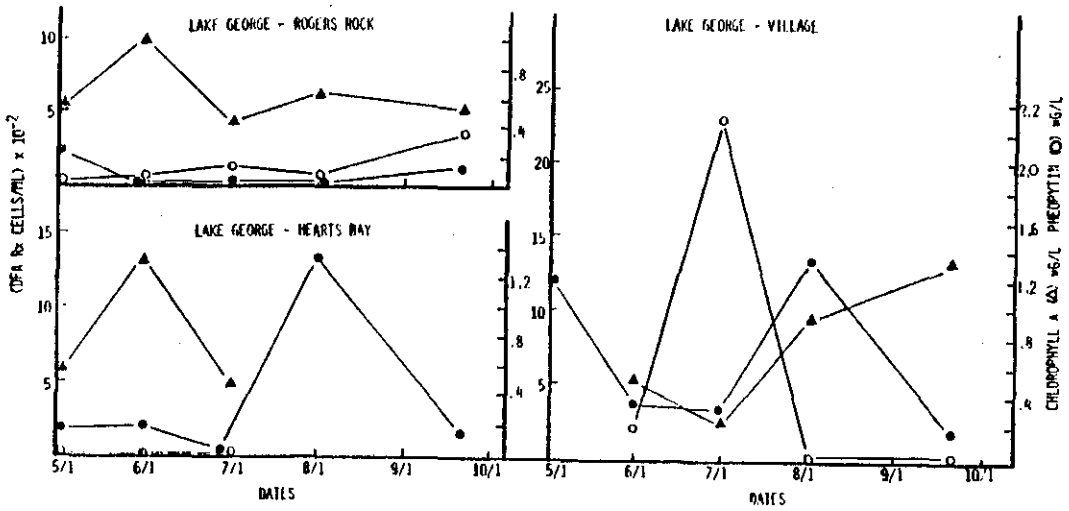

Figure 5. Numbers of bacterial cells reacting with polyvalent fluorescent antibody specific for serogroups 1-4 Legionella pneumophila in 3 sites on Lake George, New York. Water samples were collected on a monthly basis, and analyzed as described in the Materials and Methods section.

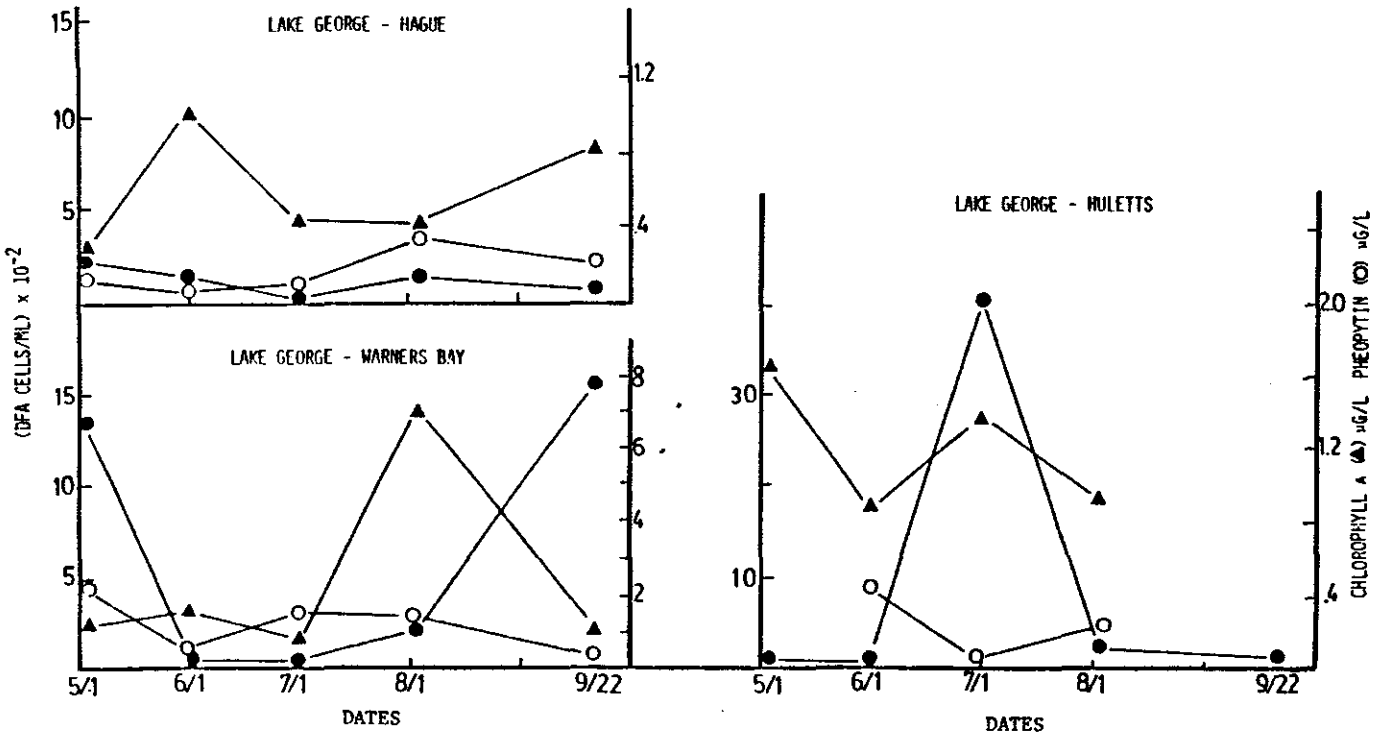

Figure 6. Numbers of bacterial cells reacting with polyvalent fluorescent antibody specific for serogroups 1-4 Legionella pneumophila in 3 sites on Lake George, New York. Water samples were collected on a monthly basis, and analyzed as described in the Materials and Methods section.

on August 1. Hague and Rogers Rock remained at very low levels throughout this summer.

Analyses of both chlorophyll a and pheophytin concentrations were performed in most cases. There is no obvious correlation between these values and the number of DFA positive cells/ml.

Numerous physical and chemical analyses were performed by the Fresh Water Institute on water samples obtained at the test sites on Lake George. Ranges of the values obtained for each analysis are presented in Table 2. There is no apparent correlation between the numbers of L. pneumophila cells and any of the measured parameters.

Table 3. Algal Counts (Cells/ml) at Specific Lake Sites

	Round Lake				Lake George Village				Lake George - Huletts			
Date	Chryso.	Chloro.	Cyano	Total	Chryso.	Chloro.	Cyano	Total	Chryso.	Chloro.	Cyano	Total
5/1/80		ND			442	0	0	442	72	0	0	72
6/1/80	0	22	900	922	0	0	4	4	0	17	3	20
7/1/80	0	0	17,250	17,250	91	0	0	91	0	2	0	2
8/1/80	0	0	432	432	0	3	46	49				
9/22/80	0	144	490	634	0	8	159	167	0	5	229	234

Table 3 illustrates the number and types of algal cells found at three lake sites over the five month period. Algal numbers remained relatively constant at Lake George Village and Huletts Landing. On July 1, however, a large increase in the number of cyanobacteria was noted in Round Lake. This bloom may be related to the increase in numbers of DFA positive cells observed the following month (see Fig. 4).

In conclusion, four lakes located in the Northeastern U.S., including Lake Champlain, Lake George, Saratoga Lake, and Round Lake were found to contain bacterial cells which react to fluorescent antibody specific for Legionella pneumophila, the causative agent of Legionnaires' Disease. Variation in bacterial numbers did not correlate with any of the physical or chemical parameters measured, including numbers of algae and concentrations of either chlorophyll a or pheophytin.

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