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Ecology of *Legionella pneumophila* within Water Distribution Systems

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The reservoir for hospital-acquired Legionnaires disease has been shown to be the potable water distribution system. We investigated the influence of the natural microbial population and sediment (scale and organic particulates) found in water systems as growth-promoting factors for *Legionella pneumophila*. Our in vitro experiments showed that: (i) water from a hot-water storage tank readily supported the survival of *L. pneumophila*, (ii) the concentration of sediment was directly related to the survival of *L. pneumophila*, (iii) the presence of environmental bacteria improved the survival of *L. pneumophila* via nutritional symbiosis, (iv) the combination of sediment and environmental bacteria acted synergistically to improve the survival of *L. pneumophila*, and (v) the role of sediment in this synergistic effect was determined to be nutritional. Sediment was found to stimulate the growth of environmental microflora, which in turn stimulated the growth of *L. pneumophila*. These findings confirm the empiric observations of the predilection of *L. pneumophila* for growth in hot-water tanks and its localization to sediment. *L. pneumophila* occupies an ecological niche within the potable water system, with interrelationships between microflora, sediment, and temperature.

The prevalence of *Legionella pneumophila*, the causative agent of Legionnaires disease, in natural and manmade aquatic environments has been well documented (5, 8, 9, 19, 21, 22). Ongoing surveillance for *L. pneumophila* at the Pittsburgh Veterans Administration Medical Center has demonstrated that the presence of *L. pneumophila* in the hospital water distribution system is epidemiologically linked to the acquisition of Legionnaires disease in susceptible, hospitalized patients (3, 19). Recent experience suggests that hospital-acquired Legionnaires disease not only can be treated but can be prevented with control measures directed at the reservoir (2a, 3, 10, 14). The success of eradication measures directed at water distribution systems will be determined by information obtained from the analysis of the ecology of this organism within the habitat of the water distribution system.

MATERIALS AND METHODS

Media and test organisms. Buffered charcoal yeast extract agar (BCYE) was prepared as previously described (7, 13). *L. pneumophila* was recovered from mixed cultures by using a selective differential medium which is a modification of previously described media for the isolation of *L. pneumophila* (18). This medium, DGVP, is buffered yeast extract agar to which 0.001% bromocresol purple, 0.001% bromothymol blue, 0.3% glycine, 1 μg of vancomycin per ml, 50 U of polymyxin B per ml, and 1.5 g of charcoal are added. One lot of media was used in each experiment to control for sampling variation due to media composition. *L. pneumophila*, serogroup 1, which had been isolated from our hot-water storage tank was used in all experiments. Environmental microorganisms were recovered by plating a water sample from the hot- and cold-water storage tanks on BCYE plates and incubating at 25, 37, and 42°C (Fig. 1). A stereomicroscope was used to screen the plates to differentiate organisms by colony morphology. Single colonies were picked and subcultured to BCYE. Organisms were stocked by making a heavy suspension of 1 to 2 ml of 50% fetal bovine serum–50% (vol/vol) tryptic soy broth mixture and freezing at −20°C. Thirty-two different environmental organisms (16 isolated from cold-water-tank water and 16 from hot-water-tank water) were stocked. Standard methods were utilized to identify these organisms. These included API 20E for the differentiation of non-*Enterobacteriaceae* (Analytab Products) and the UNI-N/F system (Flow Laboratories, Inc.).

One environmental organism, identified as a *Pseudomonas* species by standard methods, was used in certain experiments as a representative environmental organism. This *Pseudomonas* species could not be further identified to the species level by standard morphological and biochemical criteria (G. Gilardi, personal communication).

Sampling and characteristics of hot-water-tank water and sediment. A water sample was collected from the bottom of a hot-water storage tank which supplied our hospital and was divided into two fractions. One fraction was concentrated by centrifugation at 5,000 rpm for 30 min. The supernatant (sediment absent) was removed, and the concentrate (sediment present) was suspended in 1/10 of the original volume of supernatant. The unconcentrated fraction of tank water, sediment, and supernatant were further divided into sterile (microflora absent) and nonsterile (microflora present) fractions. The sediment suspensions were designated 1.0 (undiluted sediment), 0.25 (1:4 dilution of sediment), 0.05 (1:16 dilution of sediment), and 0.0 (supernatant). The supernatant was used as the diluent. Sterilization of the tank water and sediment was accomplished by boiling for 30 min, followed by incubation at 37°C for 24 h. This process was done to detect the presence of spore-forming bacteria and was repeated until sterility was achieved. Sterility was determined by direct plating of 0.1 ml of the suspension onto BCYE plates, followed by incubation at 37°C for 72 h. The supernatant was filter sterilized with a 0.2-μm filter sterilization unit (Nalgene Labware Div., Nalge/Sybron Corp.). Determinations of total organic carbon content, total suspended solids, and volatile residuals as well as atomic absorption spectrophotometric analysis for

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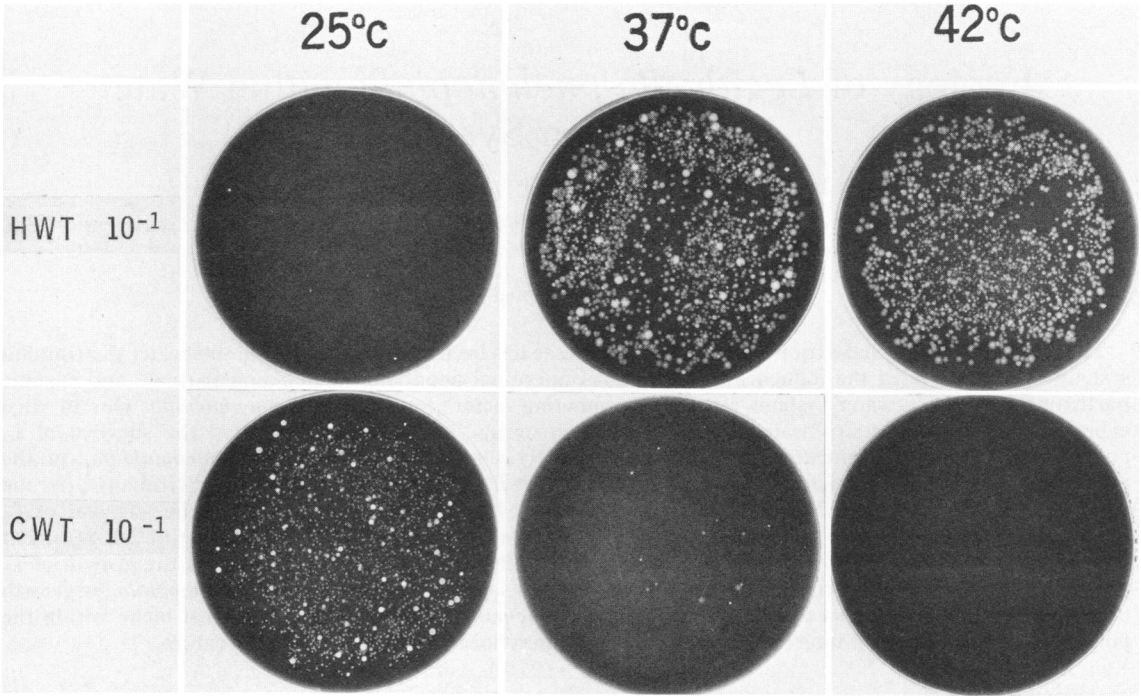

FIG. 1. Isolation of environmental microorganisms from water samples obtained from the hot-water tank (HWT) and cold-water tank (CWT). Plates were incubated at 25, 37, and 42°C. Different microbial populations inhabit each water tank due to differences in their optimal growth temperature.

metals were performed by the standard methods for the examination of water and wastewater (1). Both the nonsterile tank water and sediment were plated on selective media to obtain the number of *L. pneumophila* bacteria present in the sample before incubation or inoculation.

Survival and growth curve studies. Several hypotheses were tested by generating survival and growth curve data for *L. pneumophila* when incubated in suspensions which varied in biotic and abiotic composition. These experiments were conducted as follows. A 10-μl loopful of a freezer stock suspension of *L. pneumophila* was used to inoculate a BCYE plate. After incubation at 37°C for 72 h, a portion of the actively growing culture was used to inoculate a second BCYE plate. This plate was incubated at 37°C for only 24 h to obtain a culture composed of viable *L. pneumophila*. This culture was used to prepare a suspension on *L. pneumophila* in sterile water which contained ca. 10^5 CFU/ml. This suspension (0.5 ml) was used to inoculate 4.5 ml of a test suspension. When experiments required the addition of an environmental organism to the test suspension, 50 μl of an organism suspension (containing ca. 10^9 CFU/ml) was added. The final growth curve experiment utilized nutrient broth (Difco Laboratories) as the test suspension. The nutrient broth was buffered to pH 6.9 with ACES buffer [*N*-(2-acetamido)-2-aminoethane-sulfonic acid] and filter sterilized through a Nalgene 0.2-μm sterilization unit. Two sterile tubes were filled with 4.5 ml of broth and inoculated with 0.5 ml of a suspension of *L. pneumophila* which had a concentration of ca. 10^5 CFU/ml. A suspension (50 μl) of an environmental pseudomonad with a concentration of ca. 10^6 CFU/ml was added to one tube. Broth (5.0 ml) was added to another sterile tube and inoculated only with the pseudomonad.

Nutrient broth was chosen over other possible nutrient sources because it will not inherently stimulate the growth of *L. pneumophila*. All suspensions were incubated at 37°C and sampled at various intervals. The viability of *L. pneumophila* and nonlegionella bacteria in a test suspension was determined by plating 0.1 ml of a given dilution onto DGVP and BCYE plates. All plates were incubated at 37°C for 5 days. All suspensions were vortexed daily and before plating to provide aeration, disperse bacterial aggregates, and suspend sedimented materials. Each experiment was carried out for 28 days.

Demonstration of syntrophy between *L. pneumophila* and other environmental microorganisms (satellitism). Pour plates of *L. pneumophila* (10^9 CFU/ml) were prepared with BCYE lacking cysteine, an essential nutrient for the growth of *L. pneumophila*. A Steers replicator was used to inoculate 32 environmental organisms by placing 0.001 ml of a suspension of each organism containing ca. 10^8 CFU/ml onto each pour plate (17). Plates were incubated at 25, 37, and 42°C for 48 to 72 h. The appearance of satellite colonies of *L. pneumophila* around the peripheral edge of an environmental organism indicated nutritional symbiosis.

Since cysteine is an essential nutrient for the growth of *L. pneumophila*, this test system would determine the ability of other environmental bacteria to provide this essential amino acid (or a metabolic substitute) and thus demonstrate the potential for a symbiotic relationship between these organisms.

RESULTS

Sampling and characteristics of hot-water-tank water and sediment. Quantitative characteristics of the water sample obtained from the hot-water tank and its two fractions, supernatant and sediment, involved determinations of total solids, total organic carbon content, and volatile residuals as well as atomic absorption spectrophotometric analysis (Table 1) and the measurement of viable plate counts for *L.*

TABLE 1. Characteristics of hot-water-tank water, supernatant, and sediment

Sample	Total suspended solids (mg/liter)	Volatile residuals (mg/liter)	Total organic carbon content (mg/liter)	Atomic absorption spectrophotometry					
				Pb (mg/dl)	Cu (mg/dl)	Ca (mg/dl)	Mg (mg/liter)	Fe (mg/dl)	Zn (μl/ml)
Hot-water-tank water	48.0	42.0	ND[a]	6	20	3.8	0.5	210	0.2
Supernatant	NA[b]	NA	ND	4	1	3.7	0.5	6	0
Sediment	2,466.7	316.7	128.0	25	128	2.2	0.5	778	6.6

[a] ND, Not done.
[b] NA, Not applicable.

pneumophila. These tests indicate the relative availability of organic and inorganic compounds for utilization as substrates and energy sources. Total suspended solids and volatile residuals indicate the amount of solid particulate matter in the sample and the portion which is organic in nature, respectively. The sediment has a higher concentration of both total suspended solids and volatile residual when compared with either supernatant or hot-water-tank water (Table 1). The total organic content of the sediment was determined to be 128 mg/liter. Atomic absorption analysis of hot-water-tank water, supernatant, and sediment showed significant differences in the concentration of copper and iron only. The concentration of *L. pneumophila* in hot-water-tank water, supernatant, and sediment was 1.6×10^4, $<1.0 \times 10^1$, and 1.8×10^5 CFU/ml, respectively.

Survival and growth curve studies. *Hypothesis I. Hot-water-tank water supports the growth and survival of* L. pneumophila. Figure 2 demonstrates the response of *L. pneumophila* incubated in nonsterile (microflora and sediment present) hot-water-tank water, sterile supernatant (microflora and sediment absent), and sterile high-pressure liquid chromatography water (microflora, sediment, and dissolved organic nutrients absent). Colony counts of *L. pneumophila* determined on selective agar plates showed *L. pneumophila* to persist at a level equal to or above the initial concentration only in hot-water-tank water. After 21 days of incubation, the concentration of *L. pneumophila* increased in the hot-water-tank water from an initial concentration of 2.0×10^4 to 6.0×10^4 CFU/ml. It is also apparent that the growth-promoting effect of dissolved organic nutrients was minimal, as evidenced by the precipitous decline in the concentration of *L. pneumophila* in both sterile supernatant (containing dissolved organic nutrients) and sterile high-pressure liquid chromatography water (devoid of dissolved organic nutrients) (Fig. 2). To control for the effect of filtration on the dissolved organic nutrient content of the supernatant, sterilization by UV light was also done. The results were virtually identical (data not shown). Thus, *L. pneumophila* cannot multiply in a low-nutrient, aqueous environment.

Hypothesis II. Sediment contributes to the survival of L. pneumophila. There was a direct correlation between the concentration of sediment and growth or survival of *L. pneumophila* (Fig. 3). *L. pneumophila* increased most dramatically when incubated with concentrated nonsterile (microflora present) sediment (Fig. 3A). One-fourth the concentration of sediment had a lesser effect on the growth of *L. pneumophila*, and both the nonsterile supernatant and the suspension of lowest sediment concentration were not capable of supporting the survival of *L. pneumophila*. These data reflect a dose-response phenomenon; as the concentration of nonsterile sediment was increased, the survival of *L. pneumophila* also increased. *L. pneumophila* incubated in sterile sediment, regardless of sediment concentration, did not increase in concentration, but its survival rate correlated with sediment concentration (Fig. 3B). However, the decrease in the concentration of *L. pneumophila* was greater with successive dilutions of sediment. Therefore, sterile sediment had a lesser effect on the survival and growth of *L. pneumophila* than did nonsterile sediment which contained its natural complement of living microflora.

This set of experiments was also performed with a water sample which had been collected from a different hot-water tank. This was done to determine whether the phenomena which we had observed were intrinsic to this particular water sample and whether these observations were reproducible. This set of experiments also demonstrated that as the concentration of nonsterile sediment increased, the survival of *L. pneumophila* improved. The initial concentration of *L. pneumophila* in each suspension was ca. 10^4 CFU/ml. After 28 days of incubation in concentrated sediment (relative sediment concentration, 1.0 Sed), the concentration of *L. pneumophila* was 1.6×10^6 CFU/ml; at one-fourth the sediment concentration (0.25 Sed), 1.6×10^5 CFU of *L. pneumophila* per ml was detectable; in the lowest sediment concentration (0.05 Sed), the concentration of *L.*

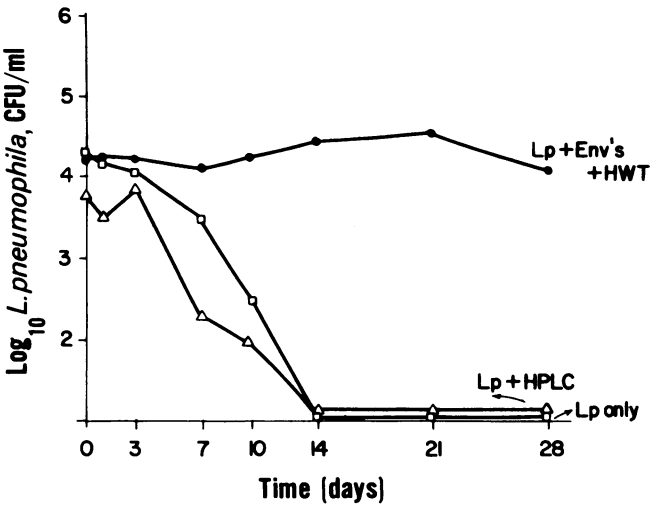

FIG. 2. Hot-water-tank water support of the growth and survival of *L. pneumophila*. Nonsterile hot-water-tank water in which microflora and sediment were present (●) supported the survival of *L. pneumophila*. The growth-promoting effect of dissolved organic nutrients was minimal as evidenced by the decline in viable *L. pneumophila* in both sterile supernatant (□), which contained dissolved organic nutrients, and sterile high-pressure liquid chromatography water (△), which did not contain dissolved organic nutrients.

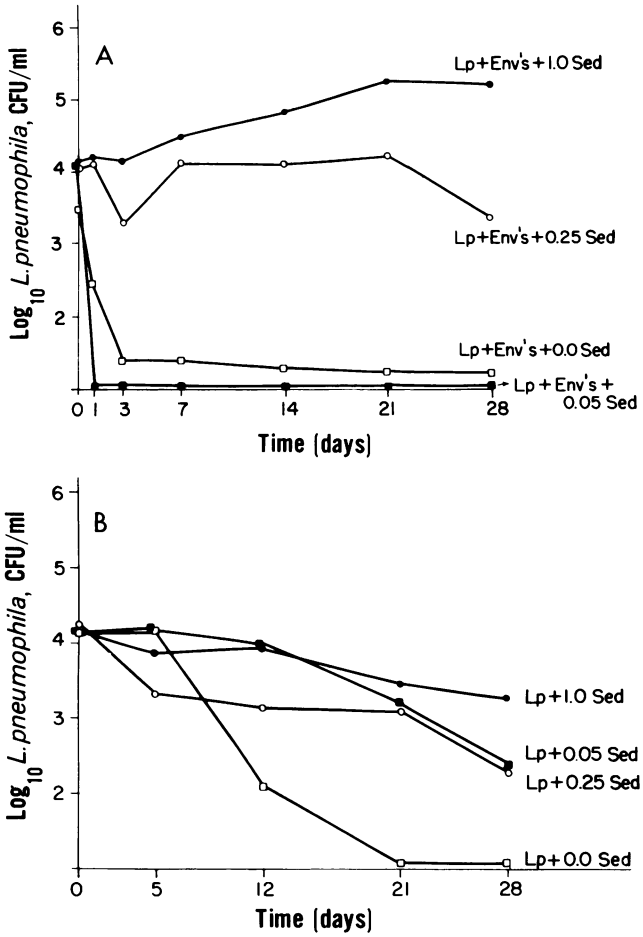

FIG. 3. Sediment contribution to the survival of *L. pneumophila*. (A) Correlation between the concentration of nonsterile sediment in which environmental microflora was present and the survival of *L. pneumophila*. Symbols (in Sed units): ●, 1.0; ○, 0.25; □, 0.0; ■, 0.05. (B) Correlation between the concentration of sterile sediment in which living microflora was absent and the survival of *L. pneumophila*. Symbols are as in (A).

pneumophila was 2.4×10^3 CFU/ml; and in a supernatant suspension totally devoid of sediment, the concentration of *L. pneumophila* at 28 days had fallen to 1.0×10^2 CFU/ml.

Hypothesis III. Environmental microflora within the hot-water tank promotes the growth and survival of L. pneumophila. The first experiment demonstrated syntrophy between *L. pneumophila* and other environmental microorganisms (satellitism). Altogether, 32 environmental organisms were tested for their ability to stimulate the production of satellite colonies of *L. pneumophila* in pour plates of BCYE lacking cysteine. Subsurface satellite colonies were observed at the peripheral edge in 16 of 32 environmental organisms (Table 2, Fig. 4). Although conventional attempts to identify these organisms to the species level were often unsuccessful, organisms were identified to the genus level, including *Flavobacterium*, *Pseudomonas*, *Alcaligenes*, Centers for Disease Control group II, and *Acinetobacter*.

A larger percentage of environmental organisms which produced satellitism were isolated from the hot-water tank rather than the cold-water tank (Table 1). These data support the observed predilection of *L. pneumophila* for hot-water systems.

The second experiment produced survival curves of *L. pneumophila* in sediment-free suspensions containing a mixed population of environmental microflora. *L. pneumophila* decreased more precipitously when incubated in a sterile supernatant suspension devoid of environmental microflora than in a nonsterile suspension which contained environmental microflora (Fig. 5A). This experimental observation indicated that microflora alone can contribute to the improved survival of *L. pneumophila*.

The third experiment was an extension of the second experiment and demonstrated the effect of a single symbiotic organism on the survival curve of *L. pneumophila* in a sediment-free suspension. Incubation of *L. pneumophila* in the presence of a symbiotic pseudomonad improved the survival of *L. pneumophila* when compared with an identical suspension without the symbiote (Fig. 5B). This was observed in a sediment-free suspension (supernatant) and reiterates the observation made in the second experiment that under more controlled conditions, the presence of a symbiotic organism (microflora) can contribute to the improved survival of *L. pneumophila*. Replicate experiments were performed with several different initial concentrations of the pseudomonad, ranging from 10^2 to 10^7 CFU/ml. Again, we observed the same pattern; the viability of *L. pneumophila* decreased more precipitously in the suspensions which contained the lower initial concentrations of the pseudomonad. Growth of *L. pneumophila* was never observed.

Hypothesis IV. Combined effect of sediment and environmental microflora on the survival of L. pneumophila *is greater than either factor alone*. Having already established that neither sediment alone (Fig. 3B) nor microorganisms alone (Fig. 5) was as growth stimulating as hot-water-tank water (Fig. 1), we hypothesized that the combination of sediment and microorganisms would provide the optimal environment for *L. pneumophila*.

The first experiment provided survival curves of *L. pneumophila* and was a comparison of the effect of the combination of sediment and a syntrophic microbial population versus the effect of sediment or the microbial population alone. Throughout the 28-day period, the concentration of *L. pneumophila* in the suspension which contained both sediment and the syntrophic pseudomonad maintained at a higher level than in the suspensions which represented sediment or microflora alone (Fig. 6). In sterile supernatant and sterile supernatant plus the pseudomonad, the concentration of *L. pneumophila* dropped below detectable levels after 21 and 28 days, respectively. This experiment indicated that the combination of sediment and microflora had a greater effect on the survival of *L. pneumophila* than did sediment or microflora (pseudomonad) alone. This experiment was repeated with several different initial concentrations of the pseudomonad, ranging from 10^2 to 10^7 CFU/ml.

TABLE 2. Nutritional symbiosis (satellitism) between *L. pneumophila* and other environmental microorganisms

Source of environmental organisms	No. of strains tested	Satellitism[a]		
		25°C	37°C	42°C
Cold-water-tank water	16	0	2	1
Hot-water-tank water	16	0	13	3[b]

[a] Number of environmental organisms found to stimulate production of satellite colonies of *L. pneumophila* when tested at three temperatures.

[b] Of the 13 organisms which produced satellitism at 37°C, 3 also demonstrated satellitism at 42°C.

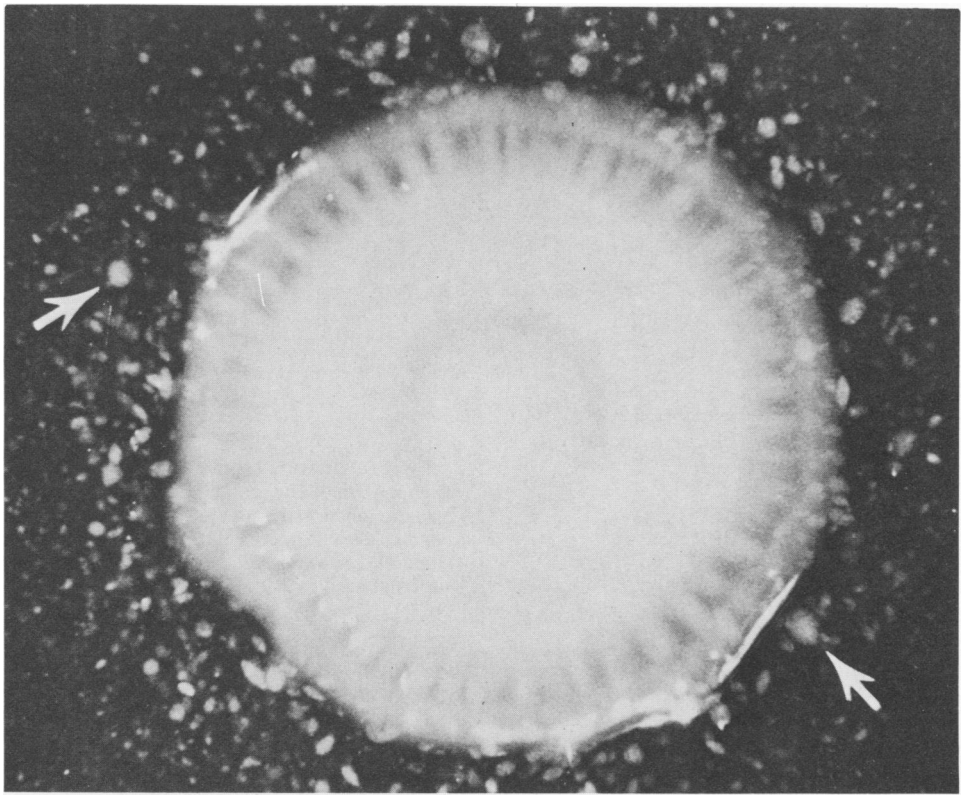

FIG. 4. Nutritional symbiosis of *L. pneumophila* with other environmental microorganisms. Subsurface satellite colonies of *L. pneumophila* (arrows) were observed at the peripheral edge in 16 of 32 environmental organisms.

In each case, *L. pneumophila* maintained a higher concentration in the suspension which contained both sediment and microflora (pseudomonad).

Hypothesis V. Postulated mechanism for the "synergistic" effect of sediment plus environmental microorganisms in promoting the growth of L. pneumophila *derives from the nutritive properties of sediment.* The first experiment demonstrated the growth of environmental microflora in nonsterile sediment. The total microbial population in nonsterile (microflora present) concentrated sediment was ca. 10^4 CFU/ml on day zero of this experiment. After incubation at 37°C for 3 days, the concentration of environmental bacteria had increased to 4.0×10^5 CFU/ml and maintained at this level throughout the 28-day experiment. These data indicate that sediment can support the growth and multiplication of high concentrations of indigenous microbial flora.

The second experiment was a comparison of the growth curves of an environmental pseudomonad in suspensions of various sediment concentrations. The growth rate of the symbiotic pseudomonad was directly related to the concentration of sterile sediment (Fig. 7A). We also performed this experiment with initial concentrations of the pseudomonad of 10^2, 10^4, and 10^7 CFU/ml. The concentration of the psueodmonad always increased with increasing sediment concentration (data not shown). This experiment confirms the growth-promoting properties of sediment, using a single symbiotic pseudomonad.

The third experiment demonstrated the indirect effect of sediment in promoting the growth and survival of *L. pneumophila* by substituting nutrient broth for sediment. The incubation of *L. pneumophila* with the pseudomonad in nutrient broth enabled *L. pneumophila* to survive and even multiply in a liquid medium that normally does not support its growth (Fig. 7B). After 24 h of incubation, the concentration of *L. pneumophila* in both the mixed culture and the suspension of *L. pneumophila* alone had decreased. Over the next 72 h, the concentrations of *L. pneumophila* increased nearly 1 log in the mixed culture but continued to drop in the suspension of *L. pneumophila* alone.

DISCUSSION

Fliermans et al. (9) were able to detect *L. pneumophila* in natural aquatic habitats by using centrifugation and direct fluorescent-antibody staining techniques, although it was noted that the population densities of *L. pneumophila* were extremely low, with serogroups 1 through 4 accounting for less than 1% of the total bacterial population. *L. pneumophila* has also been readily detectable in man-made aqueous environments (5, 19, 21, 22) and shown to be capable of multiplication in water (16, 23, 24). In our institution, the concentration of *L. pneumophila* (as determined by direct culture techniques without the need for concentration by centrifugation or filtration) at numerous sites throughout the water distribution system was alarmingly high, $>3.0 \times 10^3$ CFU/ml (19). Obviously, the conditions which exist in hot-water systems tip the ecological balance in favor of *L. pneumophila*. In the present study, we delineate some of the factors and conditions within a water distribution system which lead to colonization with *L. pneumophila* and, therefore, potential acquisition of Legionnaires disease in exposed susceptible individuals.

Our data demonstrate that the water obtained from a hot-water storage tank provides an environment in which *L. pneumophila* can maintain concentrations exceeding 10^4

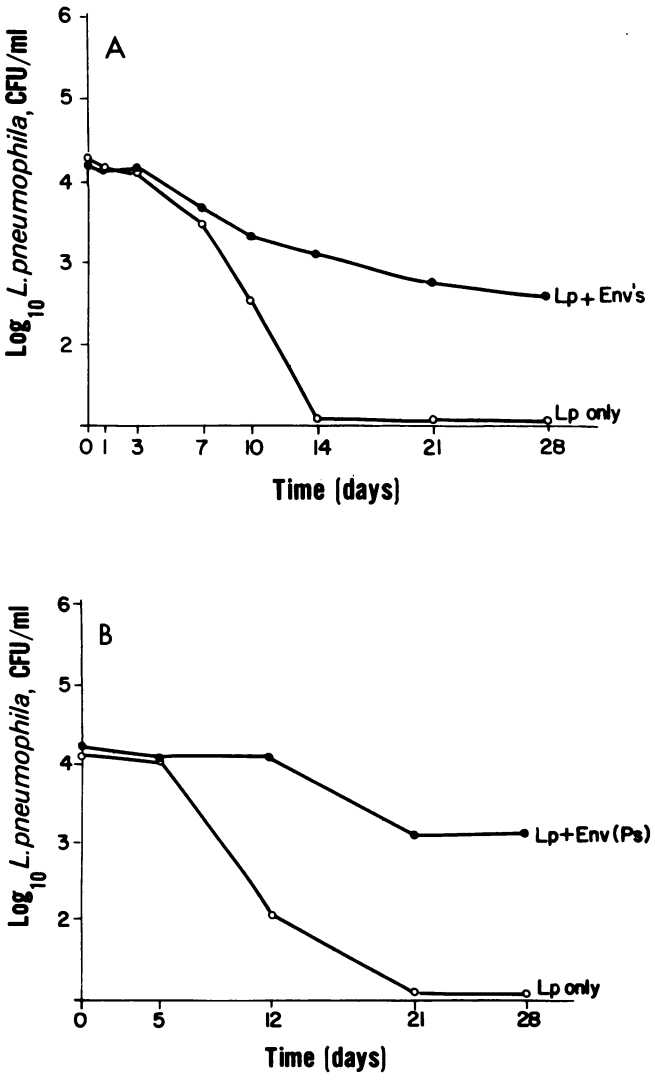

FIG. 5. Contribution of environmental microflora within the hot-water tank to the survival of *L. pneumophila*. (A) *L. pneumophila* growth showed a greater decrease when incubated in a sterile supernatant (sediment absent) suspension devoid of environmental microflora (○) than in a nonsterile supernatant suspension in which environmental microflora was present (●). (B) Incubation of a single symbiotic pseudomonad with *L. pneumophila* in sterile supernatant (●) improved the survival of *L. pneumophila* when compared with the survival in sterile supernatant alone (○).

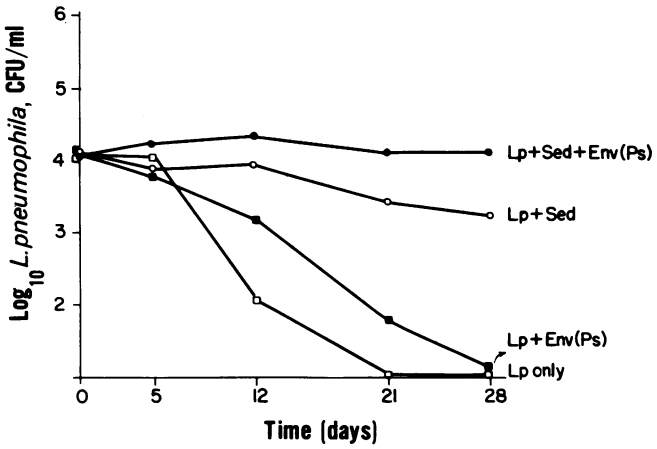

FIG. 6. Effect of an environmental symbiote on the survival of *L. pneumophila*. The combination of sediment plus the symbiotic pseudomonad had a greater effect on the growth and survival of *L. pneumophila* (●) than did sediment (○) or the pseudomonad (■) alone. *L. pneumophila* did not survive when incubated in sterile supernatant only (□).

CFU/ml (Fig. 2). This lends experimental credence to the empiric observations implicating the hot-water storage tank as the principle reservoir for *L. pneumophila*. This is consistent with the theory that the temperature of hot-water tanks (100 to 120°F) provided *L. pneumophila* with a selective temperature advantage, favoring the growth of *L. pneumophila* over that of other organisms. However, we show this theory to be an oversimplification. The population of environmental bacteria found in the warm environment of a hot-water storage tank was demonstrated to be more capable of a syntrophic relationship with *L. pneumophila* than those bacteria found in a cold-water tank (Table 2). These organisms can supply *L. pneumophila* with cysteine (or a metabolic substitute), an essential nutrient for its growth. Although *L. pneumophila* could be shown to benefit from an association with environmental water flora, this relationship was not reciprocal, i.e., improved growth of environmental organisms in the presence of *L. pneumophila* was not observed (data not shown); therefore, this relationship may be considered to be a "commensal" association (2, 6). The organisms shown to stimulate satellite colonies of *L. pneumophila* represent a number of genera and species that can be added to the group of organisms previously reported to benefit *L. pneumophila* by intimate association (*Flavobacterium* species, blue-green algae [cyanobacteria], and amoebae) (4, 15, 20, 21a). Moreover, the association of *L. pneumophila* with water microflora is likely more relevant to the epidemiology of nosocomial Legionnaires disease than its association with amoebae or algae.

L. pneumophila did not survive in a sediment-free (supernatant) suspension regardless of the presence of commensal environmental microflora (Fig. 5). This suggested that microfloras alone were not sufficient to promote growth and would indicate that *L. pneumophila* cannot multiply in circulating water. The low concentration of *L. pneumophila* recovered from water samples collected from distal outlets (before disturbance of sediment by swabbing) is consistent with this observation (19).

The lack of a growth response by *L. pneumophila* in sterile sediment (Fig. 3B) also negated the direct effect of sediment as a growth promoter. Sediment, which is composed of mineral deposits (scale) and decaying plant matter (detritus), can be utilized as a nutrient source by many procaryotic and eucaryotic organisms. Our data indicate, however, that *L. pneumophila* is not a saprophytic organism capable of multiplying on dead or decaying organic matter. Although a growth response was not observed, there was a correlation between the concentration of sterile sediment and the survival of *L. pneumophila*. A plausible explanation for this is provided by the phenomenon of detrital attachment. Water microflora can derive benefit from attachment to detritus (sediment) and solid surfaces via nutritional accumulation on these particles (11, 12).

The combination of sediment and environmental microflora (nonsterile sediment) had a greater effect on the growth and survival of *L. pneumophila* (Fig. 6) than did sediment or microflora alone. We hypothesized that the role of sediment in this synergistic relationship was to indirectly

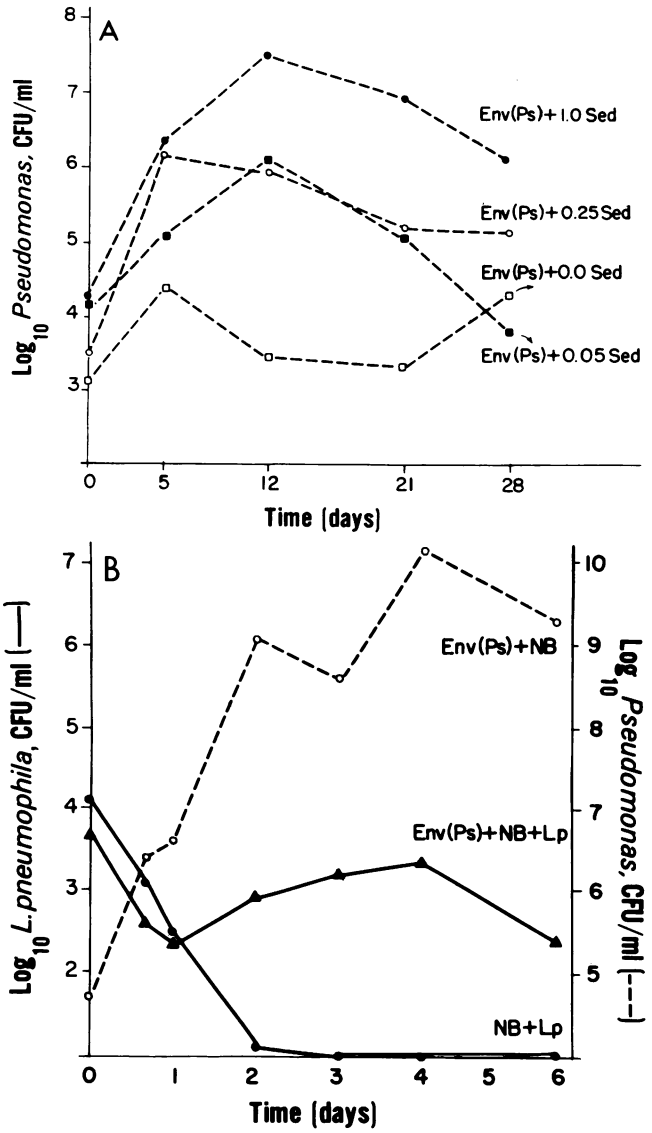

FIG. 7. Mechanism for the synergistic effect of sediment plus environmental microorganisms is dependent upon the nutritive properties of sediment. (A) Growth curves of an environmental pseudomonad in suspensions of various sediment concentrations. The growth rate of a symbiotic pseudomonad was directly related to the concentration of sediment. Symbols (in Sed units): ●, 1.0; ○, 0.25; □, 0.0; ■, 0.05. (B) Substitution of nutrient broth for sediment demonstrating the indirect role of sediment in promoting growth of *L. pneumophila*. Nutrient broth was used to replace sediment since it will stimulate growth of symbiotic microflora; however, nutrient broth alone (●) did not stimulate growth of *L. pneumophila*. The combination of nutrient broth plus a symbiotic population (▲) had a greater effect on the growth and survival of *L. pneumophila*.

promote the growth of *L. pneumophila* by stimulating the growth of environmental microorganisms (Fig. 7A). Their metabolic by-products would, in turn, stimulate the growth of *L. pneumophila* via nutritional symbiosis (Fig. 4, Table 2). To test this hypothesis, we provided a syntrophic population with a different nutrient source (nutrient broth), thereby duplicating the nutritional role of sediment (Fig. 7B). The results indicated that our hypothesis was valid; growth of *L. pneumophila* was observed when incubated in the presence of an actively growing symbiote (regardless of the presence of sediment). In addition, the growth of *L. pneumophila* occurred only after the symbiotic population had reached the exponential growth phase.

With these findings, the ubiquity of *L. pneumophila* within the confines of a hot-water system now becomes less enigmatic. Although the growth requirements of *L. pneumophila* are correctly considered to be fastidious, these requirements are readily fulfilled by the conditions which exist in a hot-water system. Our data suggest that *L. pneumophila* tends to multiply in areas of stagnation in which sediment would accumulate. We emphasize that eradication attempts will likely fail if the presence of *L. pneumophila* in the sediment of distal outlets is not addressed by the eradication protocol. In retrospect, it is not surprising that during our heat eradication protocol, distal water sites remained positive for *L. pneumophila* unless water outlets were systematically flushed with hot water (3). And, finally, it may be a misnomer to refer to water systems as being contaminated with *L. pneumophila* when this organism merely represents one of hundreds of microorganisms which occupy an ecological niche in this environment.

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LITERATURE CITED

1. **American Public Health Association.** 1976. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, Inc., Washington, D.C.
2. **Atlas, R. M., and R. Bartha.** 1981. Interactions between microorganisms, p. 249–283. *In* Microbial ecology. Addison-Wesley, Reading, Mass.
2a. **Beam, T. R., Jr., D. Moreton, T. A. Raab, W. Heaslip, M. Montes, J. Hanrahan, M. Best, and V. Yu.** 1984. Epidemiology and control of *Legionellaceae* in state developmental centers, p. 236–237. *In* C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowski (ed.), *Legionella*: Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
3. **Best, M., V. L. Yu, J. Stout, A. Goetz, R. R. Muder, and F. Taylor.** 1983. Legionellaceae in the hospital water supply—epidemiological link with disease and evaluation of a method for control of nosocomial Legionnaires' disease and Pittsburgh pneumonia. Lancet **ii**:307–310.
4. **Bohach, G. A., and I. S. Snyder.** 1982. Cyanobacterial stimulation of growth and oxygen uptake by *Legionella pneumophila*. Appl. Environ. Microbiol. **46**:528–531.
5. **Dennis, P. J., J. A. Taylor, R. Fitzgeorge, C. Bartlett, and G. Barrow.** 1982. *Legionella pneumophila* in water plumbing systems. Lancet **ii**:949–951.
6. **Doetsch, R. N., and T. M. Cook.** 1973. Bacterial interactions, p. 188–190. *In* Introduction to bacteria and their ecobiology. University Park Press, Baltimore.
7. **Feeley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Mackel, and W. B. Baine.** 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J. Clin. Microbiol. **10**:437–441.
8. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, S. J. Smith, D. L. Tison, and D. H. Pope.** 1981. Ecological distribution of *Legionella pneumophila*. Appl. Environ. Microbiol. **41**:6–9.
9. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, and L. Thacker.** 1979. Isolation of *Legionella pneumophila* from nonepidemic-related aquatic habitats. Appl. Environ. Microbiol. **37**: 1239–1242.
10. **Helms, C. M., R. M. Massanari, R. Zeiter, S. Streed, M. J. R.**

Gilchrist, N. Hall, W. J. Hansler, J. Sywassink, W. Johnson, L. Wintermeyer, and W. J. Hierholzer. 1983. Legionnaires' disease associated with a hospital water system: a cluster of 24 nosocomial cases. Ann. Intern. Med. **99**:172–178.

11. **Jannasch, H. W.** 1978. Microbial ecology of aquatic low nutrient habitats, p. 243–260. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments: report of the Dahlem workshop on strategy of life in extreme environments. Verlag Chemie, Berlin.
12. **Marshall, K. C.** 1979. Growth at interfaces, p. 281–290. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments: report of the Dahlem workshop on strategy of life in extreme environments. Verlag Chemie, Berlin.
13. **Pasculle, A. W., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Myerowitz, C. M. Patton, G. W. Gorman, C. L. Carmack, J. W. Ezzell, and J. N. Dowling.** 1980. Pittsburgh pneumonia agent: direct isolation from human lung tissue. J. Infect. Dis. **141**: 727–732.
14. **Plouffe, J. F., L. R. Webster, and B. Hackman.** 1983. Relationship between colonization of hospital buildings with *Legionella pneumophila* and hot water temperatures. Appl. Environ. Microbiol. **46**:769–770.
15. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. **33**:1179–1183.
16. **Skaliy, P., and H. V. McEachern.** 1979. Survival of the Legionnaires' disease bacterium in water. Ann. Intern. Med. **90**:662–663.
17. **Steers, E., E. L. Foltz and S. B. Graves.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9**:307–311.
18. **Stout, J., V. L. Yu, R. M. Vickers, and J. Shonnard.** 1982. Potable water supply as the hospital reservoir for Pittsburgh pneumonia agent. Lancet **i**:471–472.
19. **Stout, J., V. L. Yu, R. M. Vickers, J. Zuravleff, M. Best, A. Brown, R. B. Yee, and R. Wadowsky.** 1982. Ubiquitousness of *Legionella pneumophila* in the water supply of a hospital with endemic Legionnaires' disease. N. Engl. J. Med. **306**:466–468.
20. **Tison, D. L., D. H. Pope, W. B. Cherry, and C. B. Fliermans.** 1980. Growth of *Legionella pneumophila* in association with blue-green algae (cyanobacteria). Appl. Environ. Microbiol. **39**:456–459.
21. **Tobin, J. O., C. L. R. Bartlett, S. Watkins, G. Barrow, A. D. Macrae, A. Taylor, R. J. Fallon, and F. Lynch.** 1981. Legionnaires' disease: further evidence to implicate water storage and distribution systems as sources. Br. Med. J. **282**:573.

21a. **Wadowsky, R. M., and R. B. Yee.** 1983. Satellite growth of *Legionella pneumophila* with an environmental isolate of *Flavobacterium breve*. Appl. Environ. Microbiol. **46**:1447–1449.

22. **Wadowsky, R. M., R. B. Yee, L. Mezmar, E. J. Wing, and J. N. Dowling.** 1982. Hot water systems as sources of *Legionella pneumophila* in hospital and non-hospital plumbing fixtures. Appl. Environ. Microbiol. **43**:1104–1110.
23. **Wang, S. L. L., M. J. Blaser, J. Cravens, and M. A. Johnson.** 1979. Growth, survival, and resistance of Legionnaires' disease bacterium. Ann. Intern. Med. **90**:614–618.
24. **Yee, R. B., and R. M. Wadowsky.** 1982. Multiplication of *Legionella pneumophila* in unsterilized tap water. Appl. Environ. Microbiol. **43**:1330–1334.