APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1985, p. 1149–1154
0099-2240/85/111149-06$02.00/0

Vol. 50, No. 5

Effects of Metals on *Legionella pneumophila* Growth in Drinking Water Plumbing Systems

STANLEY J. STATES,[1]* LOUIS F. CONLEY,[1] MARIANNE CERASO,[1] THOMAS E. STEPHENSON,[1] RANDY S. WOLFORD,[2] ROBERT M. WADOWSKY,[2] ANN M. McNAMARA,[2] AND ROBERT B. YEE[2]

City of Pittsburgh Water Department, Pittsburgh, Pennsylvania 15215,[1] and Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261[2]

Received 20 February 1985/Accepted 28 June 1985

An investigation of the chemical environment and growth of *Legionella pneumophila* in plumbing systems was conducted to gain a better understanding of its ecology in this habitat. Water samples were collected from hospital and institutional hot-water tanks known to have supported *L. pneumophila* and were analyzed for 23 chemical parameters. The chemical environment of these tanks was found to vary extensively, with the concentrations of certain metals reaching relatively high levels due to corrosion. The effect of various chemical conditions on *L. pneumophila* growth was then examined by observing its multiplication in a series of tap water samples artificially supplemented with various concentrations of metals. Additionally, growth of *L. pneumophila* was examined in a more natural setting by monitoring its multiplication in the chemically analyzed hot-water tank samples after sterilization and reinoculation with *L. pneumophila*. *L. pneumophila* and associated microbiota used in these experiments were obtained from a hot-water tank. These strains were maintained in tap water and had never been passaged on agar. The results of the growth studies indicate that although elevated concentrations of a number of metals are toxic, lower levels of certain metals such as iron, zinc, and potassium enhance growth of naturally occurring *L. pneumophila*. Parallel observations on accompanying non-*Legionellaceae* bacteria failed to show the same relationship. These findings suggest that metal plumbing components and associated corrosion products are important factors in the survival and growth of *L. pneumophila* in plumbing systems and may also be important in related habitats such as cooling towers and air-conditioning systems.

Legionella pneumophila is a common contaminant of plumbing systems, especially hot-water systems, in hospitals, hotels, and apartment buildings (3, 12, 13, 32, 35, 40). It survives in these potable water systems despite the presence of chlorine residuals typically found in municipal water supplies (20). The high frequency of recovery of *L. pneumophila* from hot-water systems in particular implicated hot-water tanks as important propagating sites in these buildings (40). In addition to surviving in plumbing systems, the organism is capable of multiplying in tap water at temperatures typical of those found at the bottom of large institutional hot-water tanks (42).

L. pneumophila appears to have some special metal requirements. Media used for isolation of legionellae are routinely supplemented with iron to enhance recovery (15, 37). Furthermore, while developing chemically defined media for growth of *L. pneumophila*, several investigators have identified additional metals that stimulate its growth in the laboratory (28, 33). Given the metallic nature of plumbing systems, a question arises concerning the effects of metals leached from hot-water tanks and pipes on survival and growth of *L. pneumophila*.

To obtain a better understanding of the ecology of *L. pneumophila* in plumbing systems, we conducted a study to investigate the chemical environment in this habitat and the influence of this environment on the growth of *L. pneumophila* populations. The chemical environment was investigated by the analysis of samples obtained from hot-water tanks for a number of metallic and nonmetallic parameters. The influence of the chemical environment on the growth of *L. pneumophila* and associated non-*Legionellaceae* bacteria was studied by the observation of the multiplication of *L. pneumophila* derived from hot-water tanks and non-*Legionellaceae* bacteria in tap water samples supplemented with specific metals. In an attempt to simulate "real-life" conditions, the *L. pneumophila* used had never been agar passaged and was part of an inoculum that also contained the non-*Legionellaceae* bacteria normally found living with *L. pneumophila* in hot-water tanks. The effects of metals on *L. pneumophila* multiplication were then examined in a more natural setting by monitoring the growth of *L. pneumophila* that had been derived from hot-water tanks and reinoculated into 15 hot-water tank samples collected as part of the chemical survey.

MATERIALS AND METHODS

Bacteria. A hot-water tank sample which contained 160 and 74,000 CFU of *L. pneumophila* and non-*Legionellaceae* bacteria per ml, respectively, served as the initial source of the naturally occurring bacteria used in the growth studies. Direct immunofluorescence testing indicated that the ioslate of *L. pneumophila* in this sample was serogroup 1. It was assumed that the reactions of this isolate would be fairly representative of the behavior of *L. pneumophila* in general. The most prevalent non-*Legionellaceae* bacteria were presumptively identified as a *Flavobacterium* sp. They consisted of yellow-to orange-pigmented, smooth, entire, slightly mucoid colonies on unsupplemented buffered charcoal-yeast extract (UNBCYE) agar with no growth on MacConkey agar. The bacterium was a gram-negative bacillus, asporogenous, nonmotile, and oxidase and catalase positive. Also recovered from the water stock culture were three other unidentified non-*Legionellaceae* bacteria which formed faint purple-, green-, and white-pigmented colonies

* Corresponding author.

TABLE 1. Chemical characteristics of 15 hot-water tank samples

Parameter	Amt (mg/liter)[a]		
	Avg	Maximum	Minimum
Hardness	97	130	72
Ca	22.6	32.0	12.0
Mg	9.9	19.4	5.4
Alkalinity	32	55	8
Cl	22.0	65.0	14.7
TOC[b]	7.97	86.3	1.42

[a] Average pH, 7.62; maximum pH, 7.76; minimum pH, 7.14.
[b] TOC, Total organic carbon content.

on UNBCYE agar. These naturally occurring *L. pneumophila* and associated microbiota were maintained in the laboratory as a water stock culture by periodic transfer into membrane-filter-sterilized tap water collected from a tap in the laboratory. A cellulosic nitrate membrane (Micro Filtration System, Dublin, Calif.) having a pore size of 0.20 μm was used to sterilize the tap water samples which served as growth media for the bacteria. Serial transfers of the naturally occurring bacteria were made into sterile water when the growth of *L. pneumophila* was in the late exponential-to-early stationary phase. This typically occurred between 18 and 21 days after inoculation. These cultures were diluted 1:100 in the membrane-filter-sterilized water and incubated in polypropylene bottles in a room-air incubator at 35°C.

This system of maintaining water stock cultures of *L. pneumophila* permits laboratory study of this bacterium under conditions similar to those of the natural environment. Other investigators have used cultures of *L. pneumophila* that have been maintained on an artificial medium. However, artificial-medium-grown *L. pneumophila* may behave differently from water-grown strains. Ormsbee et al. (23) have shown that prolonged cultivation on an artificial medium reduces virulence of *L. pneumophila* in guinea pigs. Work with *Pseudomonas aeruginosa* indicates that growing a single subculture of this bacterium on an artificial medium reduces its resistance to disinfectants (10) and lessens its ability to grow in distilled water (14). Similarly, tap-water-adapted *L. pneumophila* have been shown to be more resistant to chlorine than are agar-medium-passaged strains (19).

In this study, *L. pneumophila* was enumerated by plating dilutions of water cultures on differential glycine-vancomycin-polymyxin B agar (37). Non-*Legionellaceae* bacteria, which were present in the water stock culture, were enumerated by plating dilutions of the culture on UNBCYE agar (40). UNBCYE agar is prepared the same way as buffered charcoal-yeast extract agar (24) except that L-cysteine and ferric PP_i are not added.

Chemical analysis. Fifteen 500-ml water and sediment samples were collected from the bottom drain valves of hot-water tanks located in hospitals, dormitories, and gymnasiums in Pittsburgh. Each sample was analyzed for organic carbon content with a DC54 total organic carbon analyzer (Dohrmann Envirotech, Santa Clara, Calif.). Total and dissolved concentrations of 16 metals (see Table 2) were measured by flame or graphite furnace atomic absorption spectrophotometry with a spectrophotometer (model 503; Perkin-Elmer Corp., Norwalk, Conn.). For this study, dissolved metals were defined as those passing through a 0.45-μm-pore membrane filter. Total metals were defined as the concentration of metals in an unfiltered sample after vigorous digestion. Alkalinity, pH, and hardness and chloride, sulfate, calcium, and magnesium contents were measured by mercuric nitrate, turbidimetric, sulfuric acid titrimetric, electrometric, or EDTA titrimetric methods as described by the American Public Health Association (2).

Multiplication studies. In the first set of experiments, the hot-water-tank-derived water stock culture containing both *L. pneumophila* and non-*Legionellaceae* bacteria was used to inoculate metal-supplemented tap water samples. The tap water was collected from a laboratory tap. Before inoculation, the samples were amended with either 0.05, 0.5, 1, 10, or 100 mg or one of the following metals per liter: Al, Ca, Cu, Cd, Fe, K, Mg, Mn, Pb, or Zn. In certain cases, several of these metals were combined in various samples. Metal stock solutions were prepared from the following analytical reagent-grade sulfate salts: $Al_2(SO_4)_3 \cdot 18H_2O$, $CaSO_4 \cdot 2H_2O$, $CuSo_4 \cdot 5H_2O$, $3CdSO_4 \cdot 8H_2O$, $FeSO_4 \cdot 7H_2O$, K_2SO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $PbSO_4$, and $ZnSO_4 \cdot 7H_2O$. In the case of iron, solutions were prepared alternately from both sulfate and chloride salts ($FeCl_2 \cdot 4H_2O$) to ensure that observed growth effects were associated with the metal cation rather than the anion. After pasteurization in a water bath (30 min at 60°C), each 100-ml metal-amended sample was inoculated with 1 ml of the water stock culture containing *L. pneumophila* in late exponential phase. Duplicate tap water control samples containing no metal supplement were also prepared. All samples were incubated in the dark in a room-air incubator at 35°C. Samples were cultured weekly on differential glycine-vancomycin-polymyxin B agar for a 5-week period, and population growth was noted. Plates were incubated for 6 days at 35°C in sealed plastic bags to prevent dehydration. Select samples were also cultured on UNBCYE agar to monitor growth of non-*Legionellaceae* bacteria. Each experiment was performed at least twice.

In the second growth study, the effects of metal on *L. pneumophila* multiplication were examined in the hot-water-tank samples themselves. Before use in the study, the samples were stored in the dark at 4°C. At the beginning of the experiment, 10-ml unfiltered aliquots of each of the original 15 hot-water-tank samples were pasteurized and reinoculated with 0.1 ml of the stock culture that had been derived from hot-water tanks, yielding an initial population density of 500 CFU of *L. pneumophila* per ml. These cultures were incubated in the dark in a room-air incubator at 35°C. As in the tap water growth study, all samples were subsequently cultured weekly on differential glycine-vancomycin-polymyxin B agar for a 5-week period.

RESULTS

Chemical survey. Table 1 shows the results of wet analysis and organic carbon analysis of 15 hot-water tank samples. Table 2 shows total and dissolved concentrations of 16 metals measured by atomic absorption spectrophotometry. As the data indicate, chemical conditions varied widely in these samples. In some cases, the concentrations of certain metals reached relatively high levels. The highest concentrations of Fe (69.9 mg/liter) and Zn (7.8 mg/liter) were substantially greater than the typically low levels (< 0.025 mg/liter) of these metals in finished water leaving the municipal water treatment plant. Only a small fraction of the total metals present in the hot-water tank samples was dissolved (Table 2). This was in part due to the relatively high pH values of these waters.

Growth in metal-supplemented tap water. In studies of metal-supplemented tap water, the unsupplemented tap water control samples typically yielded a 2-log growth of *L.*

TABLE 2. Metals in 15 hot-water tank samples

Metal	Total metal concn (mg/liter)			Dissolved metal concn (mg/liter)		
	Avg	Maximum	Minimum	Avg	Maximum	Minimum
Fe	12.412	69.970	0.275	0.190	1.346	0.010
Mn	0.646	6.300	0.026	0.078	0.480	0.002
Al	1.98	10.19	0.46	0.24	0.60	0.06
Zn	0.924	7.756	0.020	0.187	1.212	0.003
Cd	0.004	0.018	0.001	0.002	0.009	0.001
Cu	0.469	2.162	0.012	0.038	0.151	0.006
Cr	0.004	0.024	0.001	0.006	0.032	<0.001
Pb	0.047	0.178	0.006	0.007	0.023	0.005
Ag	<0.001	0.002	<0.001	<0.001	0.001	0.001
Ba	0.082	0.222	0.018	0.048	0.132	0.029
Na	15.20	23.44	11.00	15.10	23.14	10.80
K	2.29	5.50	1.60	2.02	3.50	1.60
As	0.003	0.008	<0.001	0.002	0.005	0.001
Se	0.001	0.007	<0.001	0.001	0.006	<0.001
Ni	0.019	0.124	0.001	0.003	0.024	0.001
Co	0.008	0.060	0.001	0.002	0.009	0.001

pneumophila during the 5-week period. Of the 10 metals studied in this experiment, higher concentrations (10 and 100 mg/liter) of aluminum, cadmium, copper, iron, lead, manganese, and zinc apparently produced toxic effects on *L. pneumophila*. The populations in these samples experienced no growth and in most cases were actually reduced relatively early during the 5-week period. Higher concentrations of calcium, magnesium, and potassium did not appear to be detrimental. Low concentrations of most of the metals exerted no net influence on *L. pneumophila* populations. However, low levels of "total" iron and zinc (0.5 and 1.0 mg/liter) and higher concentrations of potassium (1, 10, and 100 mg/liter) were associated with enhancement of *L. pneumophila* growth which, in some cases, exceeded that observed in the tap water control by as much as 1 log (Fig. 1). Similar results were obtained when the combined effects of these elements were studied in samples containing all three metals.

Additional trials were carried out to examine the individual and combined influence of iron, zinc, and potassium on the growth of accompanying non-*Legionellaceae* bacteria. While higher concentrations of iron and zinc (10 and 100 mg/liter) appeared to be toxic for these organisms, no substantial growth enhancement could be associated with lower levels of these metals. An increase of approximately 2.0 log CFU of the non-*Legionellaceae* bacteria occurred in the unsupplemented control samples as well as in those amended with 0.05, 0.5, or 1 mg of iron or zinc and 0.05, 0.5, 1, 10, and 100 mg of potassium per liter.

Growth in hot-water tanks. In the hot-water tank growth experiment, the 15 samples differed substantially in terms of *L. pneumophila* multiplication over the 5-week period.

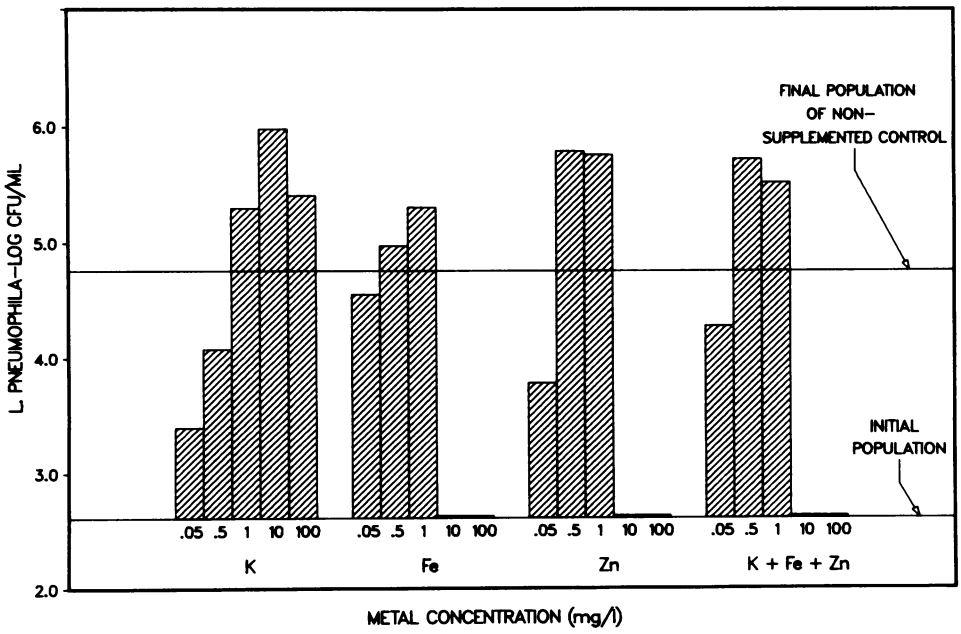

FIG. 1. *L. pneumophila* multiplication in tap water samples supplemented with various concentrations of metals.

TABLE 3. Correlation coefficients for chemical parameters correlated with greatest population size attained among 13 hot-water tank samples inoculated with *L. pneumophila*

Correlation coefficient	Independent parameter	*r*	*R*	Significance of *r*	Significance of *R*
Simple	Fe	0.919		0.001	
	Cl	0.499		0.1	
	Cr	0.494		0.1	
Multiple	Fe, Cl, Cr		0.860		0.001

While the above growth study with metal-amended tap water involved variations in only one metal at a time, the hot-water tank samples differed in a number of chemical properties. To explain the observed differential growth, we preformed a series of computer-assisted linear correlation analyses. A simple correlation coefficient was calculated for the association between each of the 23 chemical parameters originally measured in the hot-water tank survey and the greatest population size attained. Although chloride and chromium showed a mild association with growth, iron was the only parameter of the 23 that was significantly correlated ($P = 0.001$) (Table 3). Similarly, iron was the only one of these three independent parameters to remain significant when the effects of the other two independent parameters were statistically held constant by using partial correlation techniques. This procedure helps to control for the possibility that the influence of Fe on growth is merely due to an indirect association with another key chemical parameter. When the effects of iron, chloride, and chromium were combined in a multiple correlation analysis, the association with *L. pneumophila* growth was again significant ($P = 0.001$).

It should be noted that the results of only 13 of the 15 samples were included in the correlation analysis. One sample containing high concentrations of zinc (7 mg/liter) and another containing high levels of iron (69 mg/liter) were not included, since the apparent toxic effects induced by these metals interfered with the interpretation of factors influencing growth.

Figure 2 graphically depicts the association between total iron and growth of *L. pneumophila* in the hot-water-tank experiment. The sample containing apparently toxic levels of zinc was again eliminated to permit better consideration of iron effects. As the histogram indicates, increasing concentrations of iron were associated with enhanced *L. pneumophila* growth until toxic levels were reached. That the apparent toxic level of iron differed between the metal-supplemented tap water growth study (Fig. 1) and the hot-water-tank growth study (Fig. 2) may be attributed to matrix differences between the two sets of samples. The elevated total organic carbon levels and the relatively high concentrations of metals in the hot-water-tank samples may influence the effect of iron and bacteria.

DISCUSSION

Each of the hospitals included in the hot-water-tank chemical survey had experienced sporadic cases of nosocomial legionellosis during the previous year. All of the hot-water tanks sampled, at one time or another, had been positive for *L. pneumophila*. Each tank was heated by a centrally located steam coil and was part of a recirculating hot-water system. The tanks were constructed of copper-lined steel. Associated plumbing and fixtures were constructed of a variety of materials including copper, galvanized steel, and aluminum. The high concentrations of metals detected in the hot-water-tank water samples as part of the chemical survey were most likely due to corrosion or leaching of the tanks, associated plumbing, and solders. Metal levels were probably further increased by the tendency of water and sediment in the bottom of the tank to become relatively stagnant. Microorganisms inhabiting this part of the water system would be exposed to this chemical environment. A question posed by these data concerns the effects of the varied and, in some cases, high concentrations of metals on *L. pneumophila* growth.

The fact that most of the metals in these samples appeared primarily in the "suspended" rather than the "dissolved" phase does not preclude availability to microorganisms. Although trace elements can exist as different physiochemical species in water, many factors can change the speciation of a given element. In the case of iron, the dominant form in the aquatic environment, ferric hydroxide, is relatively insoluble ($pK_{sp} \approx 38$). This results in extremely low Fe(III) ion activities [at a pH of 7, Fe(III) ions have a concentration of only 10^{-18} M]. Iron siderophores are known to be produced by *L. pneumophila* (W. J. Warren and R. D. Miller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D67, p. 49). As with other siderophores, formation of these virtually ferric-specific compounds is induced by growth in environments low in either absolute or available iron. The siderophores in turn solubilize and transport iron.

Toxicity effects were apparent in both of our growth studies. In the experiment with supplemented tap water, high concentrations of a number of metals (Al, Cd, Cu, Fe, Pb, Mn, and Zn) appeared to be toxic to *L. pneumophila*. Similarly, in the hot-water-tank study, 2 of the 15 samples, containing high levels of iron or zinc, exhibited a complete absence of growth or a diminshed survival rate, apparently due to elevated concentrations of these metals. Toxic effects of metals have been observed previously in many bacteria. High concentrations of metals have been shown to affect metabolic activity (1, 6, 16), survivability (1), diversity, and

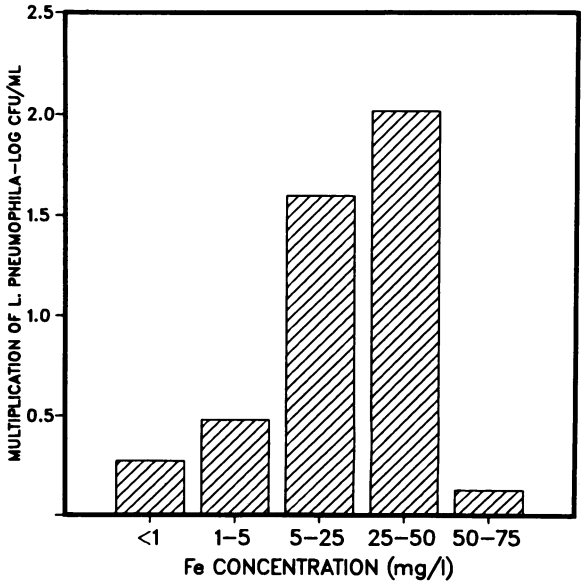

FIG. 2. *L. pneumophila* multiplication and Fe concentration in hot-water tanks.

stability of microbial communities (31). The influence of metals can be attenuated or potentiated by a variety of physiochemical characteristics of the environment (4, 5, 6). Toxic effects result despite the existence of resistance mechanisms, which include selection for resistant strains (6, 29), utilization of extracellular polysaccharides (7), plasmid-specified resistance (8), and a variety of other energy-dependent biochemical mechanisms (41). There is evidence suggesting that bacteria undergo selection for increased metal resistance as they pass through public drinking water treatment and distribution systems (9). Additionally, it appears that *L. pneumophila* in hot-water plumbing systems may generally be more tolerant of increased concentrations of copper than are some other bacteria (17).

In addition of exerting toxic effects, metals also influenced growth. Special metal and material requirements for growth of *L. pneumophila* populations have been suggested by earlier work. Investigators studying *L. pneumophila* multiplication in chemically defined media have shown that *L. pneumophila* requires a greater variety of metals and is more susceptible to the loss of metals by chelation than are *P. aeruginosa*, *Escherichia coli*, or *Salmonella typhimurium* (27, 28, 33). Colbourne et al. (11) demonstrated that certain rubber components of water fittings support *L. pneumophila* growth and may provide an ecological niche for the organism within plumbing systems. Hoekstra et al. (17) observed that, after pasteurization and reinoculation, *L. pneumophila* multiplied in a hospital hot-water system sample but not in a freshly prepared drinking water sample, suggesting the presence of growth factors in hot-water systems not present in other tap sources. The results of our growth studies indicate that these factors may be metals leached from hot-water tanks and associated plumbing. In our experiments, metals significantly influenced the survival and growth of natural populations of *L. pneumophila* derived from hot-water tanks and maintained and studied only in tap and hot-water-tank waters. Iron, zinc, and potassium each enhanced *L. pneumophila* growth in the experiments with supplemented tap water. Metals also stimulated multiplication in the hot-water-tank experiment, thus further suggesting that these effects occur in undisturbed plumbing systems. In the hot-water-tank study, iron was the only metal that appeared to affect growth. A lack of variation in the other metals among the 15 samples may have obscured their potential growth-supporting effects. The fact that metals added to the samples either artificially or by corrosion substantially influenced *L. pneuomphila* survival and growth relative to normal tap water is not surprising considering the relatively low levels of metals typically available in most tap waters after standard municipal water treatment.

Earlier work has suggested that *L. pneumophila* growth is dependent on the support of algae (26, 34), amoebae (30, 36), and non-*Legionellaceae* bacteria (38, 39). Although preliminary studies of the water stock culture used in this study have been unsuccessful in the detection of amoebae (39), previous experiments with this series of isolates derived from hot-water tanks have demonstrated satellitism between *L. pneumophila* and several of the associated non-*Legionellaceae* bacteria (38, 39). This indicates that these bacteria may supply *L. pneumophila* with amino acids (e.g., L-cysteine) required by this bacterium and may thereby be growth supporting. If this is true, the possibility exists that metals indirectly enhance *L. pneumophila* growth by stimulating the growth of these symbionts. However, analysis of the influence of iron, zinc, and potassium on the growth of a *Flavobacterium* sp. in our experiment indicates that the influence of metal on *L. pneumophila* is greater than that on the accompanying non-*Legionellaceae* bacteria. This suggests that metal-induced growth enhancement of *L. pneumophila* may not necessarily occur through non-*Legionellaceae* bacteria.

The specific roles played by the influential metals in the metabolism of *L. pneumophila* have not been determined. Of the more than 100 elements in the periodic table, 30 have been found to be required for microbial life in general. However, not all of these elements are necessary for the growth of each species (41). With the possible exception of the lactic acid bacteria, iron is thought to be a universal requirement for microbial cells whether they be procaryotes or eucaryotes (22). Iron is an important component of oxidation-reduction systems and a cofactor of some important enzymes, as is zinc (18). The significance of potassium for the growth and metabolism of bacteria has also been demonstrated for a number of species (21, 25). However, regardless of the exact biochemical function of these metals in *L. pneumophila* metabolism, the results of this study indicate that metal plumbing components and associated corrosion products are important factors in the survival and growth of *L. pneumophila* in drinking water plumbing systems. These results further suggest that metals may also be important in related habitats such as cooling towers and large air-conditioning systems.

ACKNOWLEDGMENTS

This work was supported in part by the City of Pittsburgh Water Department. It was also sponsored by the Environmental Epidemiology Center of the Graduate School of Public Health of the Universtiy of Pittsburgh under the support of cooperative agreement CR80681-01-2 with the U.S. Environmental Protection Agency.

We thank Monto Ho and John Kuchta for their advice, and Daryl Smith, J. Thomas Bruecken, and Edward Blair for their encouragement.

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