APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1994, p. 1585–1592
0099-2240/94/$04.00+0

Vol. 60, No. 5

Influence of Temperature and Plumbing Material Selection on Biofilm Formation and Growth of *Legionella pneumophila* in a Model Potable Water System Containing Complex Microbial Flora

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Received 24 November 1993/Accepted 10 March 1994

Survival and growth of *Legionella pneumophila* in both biofilm and planktonic phases were determined with a two-stage model system. The model used filter-sterilized tap water as the sole source of nutrient to culture a naturally occurring mixed population of microorganisms including virulent *L. pneumophila*. At 20°C, *L. pneumophila* accounted for a low proportion of biofilm flora on polybutylene and chlorinated polyvinyl chloride, but was absent from copper surfaces. The pathogen was most abundant on biofilms on plastics at 40°C, where it accounted for up to 50% of the total biofilm flora. Copper surfaces were inhibitory to total biofouling and included only low numbers of *L. pneumophila* organisms. The pathogen was able to survive in biofilms on the surface of the plastic materials at 50°C, but was absent from the copper surfaces at the same temperature. *L. pneumophila* could not be detected in the model system at 60°C. In the presence of copper surfaces, biofilms forming on adjacent control glass surfaces were found to incorporate copper ions which subsequently inhibited colonization of their surfaces. This work suggests that the use of copper tubing in water systems may help to limit the colonization of water systems by *L. pneumophila*.

The occurrence of *Legionella pneumophila* between 5.7 and 63°C in natural environments has demonstrated the ability of the pathogen to grow and survive over a wide temperature range (6). In human-made ecosystems, the occurrence of *L. pneumophila* has been responsible for human infection (22) and appears to be particularly linked with warmer water systems, where up to 85% of sites may contain the pathogen (13). Much of the work on the effect of temperature has been concerned with *L. pneumophila* in liquid culture. Optimum growth of the pathogen in a mixed consortium in water occurred at 37°C, and growth was prevented at 42°C (19, 24). However, growth of *L. pneumophila* was found to occur at 45°C when the microorganism was in coculture with a cyanobacterium (17).

Although the organism had been shown to be able to grow and survive in a broader range of temperatures than many aquatic microorganisms, the evidence suggested that there was a maximum temperature for growth. At temperatures above 50°C, the pathogen, when in pure cultures, rapidly declines (4), and at 60°C, the decimal reduction time is 1.3 to 10.6 min (16).

The United Kingdom Department of Health and Social Security Code of Practice issues guidelines for the operations of hot water systems to ensure that systems are not colonized. Hot water should be stored at 60°C, and the returning water should be maintained at 50°C. The water from any tap in the circuit should reach 50°C after 1 min of flushing. Cold water systems should be maintained below 20°C in order to discourage excessive growth within the systems. Most often, cases of infection have appeared to occur when the operating temperature of hot water systems is below that of the suggested guidelines (14). Maintenance of high operating temperatures has been largely successful in the elimination of *L. pneumophila* from hot water systems (1). However, the organism can be persistent despite heat treatment (2): *L. pneumophila* was found in a hospital water system operating at 55°C because of survival of the organism within biofilms on washers. The failure of the heat treatment has also been attributed to the presence of dead ends of pipe work which were not reached by the hot water used for decontamination (7). In addition, some water systems are deliberately operated at low temperatures to avoid patient scalding, to reduce costs, and because older systems cannot attain the desired temperatures (9).

The suggestion that biofilm formation could enhance the potential for survival (2) and the failure of heating to be successful led to investigations of survival of *L. pneumophila* in biofilms (11). This work considered only one plastic material, and further work was required to determine whether *L. pneumophila* could survive in biofilms on different plumbing materials.

The aim of this work was to determine what influence material selection and temperature have on both biofilm development and growth of *L. pneumophila*. Since copper has been shown to inhibit growth of *L. pneumophila* (15, 23), colonization of this material was compared with that on two commonly used plastics, polybutylene and chlorinated polyvinyl chloride (PVCc). Temperatures examined were 20, 40, 50, and 60°C since a cold water system may reach 20°C in summer, and 40, 50, and 60°C cover the operating range of hot water systems.

(Portions of this work were previously presented at the 93rd

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General Meeting of the American Society for Microbiology, 16 to 20 May 1993, Atlanta, Ga. [12a].)

MATERIALS AND METHODS

Model system. The two-stage biofilm model used filter-sterilized tap water as the sole growth medium for a mixed population of bacteria, fungi, and protozoa (12). The inoculum was sludge from the bottom of a calorifier implicated in an outbreak of Legionnaires' disease and contained virulent *L. pneumophila* serogroup 1 Pontiac. Two 500-ml glass vessels were linked in series to simulate conditions found in a water system. The first vessel simulated a storage tank, the flow rate of sterile water into which produced a dilution rate (D) of 0.05 h^{-1}, and to ensure reproducibility, conditions in this vessel remained constant, with no plumbing materials being introduced. The first vessel was a seed vessel which supplied a constant inoculum into the second vessel (which represented a distribution system) and was used to generate biofilms. Additional sterile water was added to this biofilm-generating vessel to produce an overall dilution rate of 0.2 h^{-1}. Effluent from the second vessel was pumped into a waste collection bottle.

Conditions within the vessels were controlled and monitored with Anglicon microprocessor control units (Brighton Systems, Hove, United Kingdom) linked to a personal computer. The temperature of the seed vessel was maintained at 30 ± 0.1°C with proportional integral derivative controllers and heated by an electrical external pad. The temperature was measured with a glass temperature probe inserted into the aqueous phase. The glass galvanic oxygen electrode was temperature compensated, and the dissolved oxygen tension was maintained at (20 ± 0.5)% via proportional control of the stirrer speed. This maintained a fluid velocity of 1 to 2 m s^{-1}. The pH and E_h of the cultures were monitored throughout the experiments. Conditions in the biofilm-generating vessel were controlled in the same manner as those in the seed vessel, with the exception that the stirrer speed was maintained at 300 rpm; therefore, dissolved oxygen tension was monitored but not controlled.

Biofilm generation. Sections of polybutylene, copper, and PVCc plumbing material tube were cut into sections with an inside area of 1 cm^2, and a 1-mm hole was drilled so that coupons could be suspended from copper or titanium (in the case of polybutylene and PVCc) wires. The materials were cleaned in acetone to remove any dirt or oil and suspended on the wire alongside a control glass surface. The tile assemblies were placed in bottles of water and heat sterilized by autoclaving. Visual inspection after autoclaving indicated that the materials appeared to be unaffected by the heating process. Total organic carbon analysis showed that coupons of the plumbing materials prepared this way leached less than 6 μg of total organic carbon when shaken in 10 ml of sterile distilled water for 3 days. This indicated that the cleaning process was sufficient to remove organic contamination from the surfaces. Copper samples were aged for 14 days in 200 ml of sterile tap water so that the surfaces could be oxidized and prevent high levels of copper ions from being released into the model system.

Each chemostat experiment used plumbing materials of only one type, and colonization was investigated at 20, 40, 50, and 60°C. For each of the experiments, the material to be colonized and the control glass surface were aseptically immersed in the aqueous phase of the model on day 0. Tiles were removed after 1, 4, 7, 14, and 21 days from the outset of the experiment. Attempts were made to ensure minimum variability during the sampling of the biofilms. The plumbing materials were washed by complete immersion in 10 ml of sterile water with gentle movement to ensure that all unwanted planktonic bacteria were removed. The biofilm was then removed by scraping the whole of the inside curved surface of the plumbing material methodically with a sterile dental probe. Biofilms were resuspended in 1.0 ml of sterile water and mixed with a vortex mixer for 30 s to disperse bacteria.

Microbiological assessment of biofilm and planktonic samples. Nonlegionella populations were enumerated with a nonselective, low-nutrient R2A medium (10) to avoid substrate shock. These R2A plates were incubated for 7 days at 30°C and at the same temperature as the corresponding chemostat experiment. In all cases, the highest numbers of CFU occurred on those plates incubated at 30°C, and these values were used for determining the total nonlegionella flora. Buffered charcoal-yeast extract agar (BCYE) (8) and buffered charcoal-yeast extract supplemented with glycine, vancomycin, polymyxin, and cycloheximide (GVPC) (3) were used to determine the numbers of the more fastidious bacteria including *L. pneumophila*. These plates were incubated at 30°C for 7 days. Those colonies on BCYE and GVPC which showed the characteristic ground glass appearance of *L. pneumophila* were subcultured onto BCYE and also BCYE lacking cysteine. Organisms were presumptively identified as *L. pneumophila* if they were unable to grow in the absence of cysteine but were capable of growth on BCYE.

One plate of each medium which contained 30 to 100 colonies and on which colony morphology could be distinguished was selected for evaluation of population profiles for each biofilm of each age. Each colony type was subcultured onto R3A or BCYE three successive times before inoculation into the appropriate API (API bioMerieux, Basingstoke, United Kingdom) and Biolog (Biolog, Haywood, Calif.) bacterial identification systems.

Statistical analysis of data. The colonization of the different materials was compared with the Wilcoxon matched-pair, signed-rank test for this type of correlated, nonparametric data. Calculations were performed with "Statistics," a PC-based package by K. B. Smith (Blackwell Scientific Publications, Oxford, United Kingdom, 1990).

Microscopy of samples. For scanning electron microscopy, the tiles of plumbing materials were removed from the culture and planktonic bacteria were removed by gentle rinsing in sterile water. The biofilms were fixed and stained with 1% osmium tetroxide in 0.1 mM phosphate buffer at pH 6.9 for 2 h and then dehydrated through an alcohol series. Tiles were mounted on 0.5-in. (ca. 1.3-cm) scanning electron microscopy specimen stubs with a high-conductivity silver paint (Acheson Colloid Company, Prince Rock, Plymouth, United Kingdom). Specimens were coated with a 20-nm layer of gold in an Edwards 12E6 vacuum coating unit and then examined in a Cambridge Stereoscan S2A scanning electron microscope operated at a 10-kV accelerating voltage.

Biofilms were also examined by light microscopy and differential interference microscopy as described previously (12).

Copper determinations. Copper concentration was determined for planktonic samples with a Philips 9100x Flame Atomic Absorption Spectrophotometer. Samples were aspirated into an air-acetylene flame, and copper concentration was determined by measuring the $A_{325.8}$. A 21-day-old biofilm developed on the control glass surface immersed during the investigation of the colonization of copper surfaces at 40°C was also examined. The biofilm was dissolved in nitric acid, and the copper concentration of the liquid was then determined.

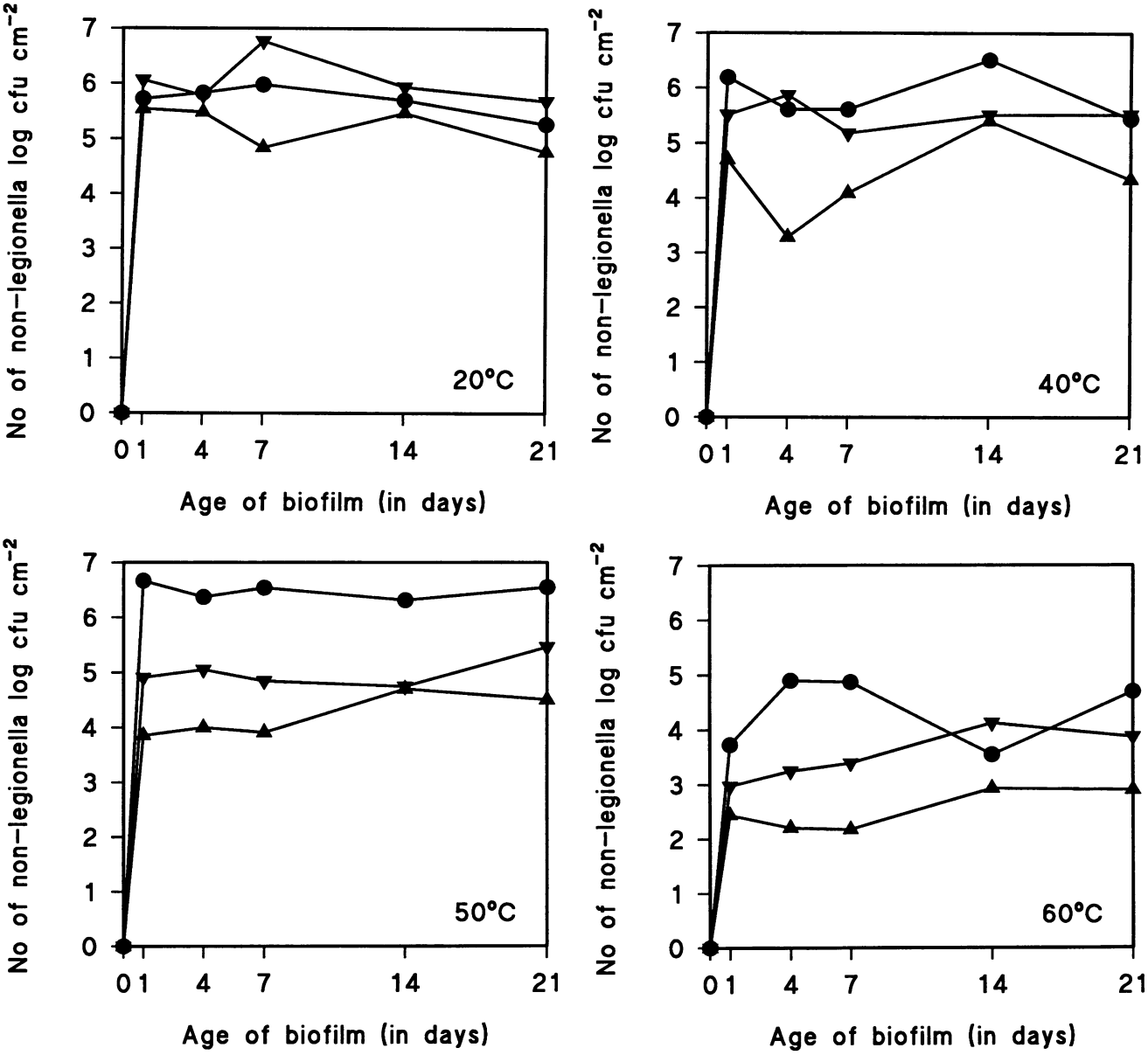

FIG. 1. Total nonlegionella flora colonizing the surfaces of copper (▲), polybutylene (●), and PVCc (▼) at 20, 40, 50, and 60°C. In all cases, the standard deviation is less than $\log_{10}$ 0.15 of the plotted mean datum points.

RESULTS

Growth in the planktonic phase. At 20°C, the planktonic phase contained 1.3×10^4 to 7.56×10^5 CFU of nonlegionella bacteria ml^{-1}, dependent on the materials in contact. Their numbers were least when copper was present in the model system (see Table 2). The presence of copper also reduced the numbers of *L. pneumophila* organisms to below the limit of detection of 10 CFU ml^{-1} (see Table 3). When plastic materials were present, *L. pneumophila* accounted for a low proportion of the planktonic flora, approximately 0.1%.

The planktonic populations of nonlegionella bacteria varied between 1.42×10^4 and 3.90×10^5 CFU ml^{-1} at 40°C, dependent on the composition of the materials present. *L. pneumophila* occurred in the planktonic phase at a concentration of between 2.99×10^2 and 7.50×10^3 CFU ml^{-1} and accounted for up to 12% of the population. Again, the presence of copper in the aqueous phase reduced both the total population and the numbers of *L. pneumophila* organisms at this temperature.

At 50°C, the total nonlegionella bacterial population was between 2.00×10^4 and 3.35×10^6 CFU ml^{-1}. *L. pneumophila* accounted for less than 0.7% of the total population, being between 1.00×10^1 and 3.45×10^2 CFU ml^{-1}. Copper was again observed to reduce both the total planktonic population and the numbers of *L. pneumophila.*

L. pneumophila was absent from the planktonic phase at 60°C. The numbers of planktonic-phase bacteria were low at this temperature, being between 8.45×10^2 and 8.39×10^4 CFU ml^{-1}, with lowest numbers occurring when copper was present.

TABLE 1. Comparison of the colonization of different plumbing materials and inclusion by *L. pneumophila* at different temperatures

Temp (°C) and material	Mean colonization (CFU cm^{-2})		Colonization ratio[a]	
	Total flora	*L. pneumophila*	Total flora	*L. pneumophila*
20				
Copper	2.16×10^5	0	1	
Polybutylene	5.70×10^5	665	2.6	665
PVCc	1.81×10^6	2,132	8.4	2,132
40				
Copper	8.04×10^4	1,967	1	1
Polybutylene	1.18×10^6	111,880	14.7	56.8
PVCc	3.67×10^5	68,379	4.6	34.7
50				
Copper	2.26×10^4	0	1	1
Polybutylene	3.21×10^6	868	142	868
PVCc	1.22×10^5	60	5.4	60
60				
Copper	4.47×10^2	0	1	
Polybutylene	4.25×10^4	0	95	
PVCc	5.19×10^3	0	11.6	

[a] The colonization ratio is the total nonlegionella or legionella population recovered from each material in comparison with the copper data. Means were calculated from all values determined over 1 to 21 days.

Growth in biofilms at 20°C. Polybutylene and PVCc surfaces were colonized by high numbers of nonlegionella bacteria at 20°C (Fig. 1) with mean total numbers of nonlegionella populations of 5.70×10^5 and 1.81×10^6 CFU cm^{-2}, respectively (Table 1). Copper was consistently less colonized than the plastic surfaces, having a mean of 2.16×10^5 CFU cm^{-2}, which was significantly lower than that on the plastic materials (at a 95% confidence limit). Biofilm formation on glass surfaces in coculture with the other materials reflected that on the materials themselves. Indeed, the glass surface that had been suspended alongside a copper tube was the least colonized (Table 2).

TABLE 2. Comparison of total nonlegionellae in biofilms forming on different plumbing materials, on glass control surfaces, and in culture at different temperatures

Temp (°C) and material	Mean total no. of nonlegionellae[a]		
	On material surface (CFU cm^{-2})	On glass control surface (CFU cm^{-2})	In planktonic phase (CFU ml^{-1})
20			
Copper	2.16×10^5	3.08×10^5	3.79×10^4
Polybutylene	5.70×10^5	6.23×10^5	2.87×10^5
PVCc	1.81×10^6	5.05×10^5	2.63×10^5
40			
Copper	8.04×10^4	6.66×10^4	9.18×10^4
Polybutylene	1.18×10^6	4.43×10^5	4.30×10^4
PVCc	3.67×10^5	1.40×10^5	3.68×10^5
50			
Copper	2.26×10^4	1.20×10^5	2.40×10^4
Polybutylene	3.21×10^6	1.5×10^6	8.43×10^4
PVCc	1.22×10^5	3.78×10^5	6.43×10^4
60			
Copper	4.47×10^2	6.29×10^2	1.83×10^3
Polybutylene	4.25×10^4	1.25×10^4	5.84×10^3
PVCc	5.19×10^3	1.78×10^4	6.06×10^4

[a] Means were calculated from all values determined over 1 to 21 days.

L. pneumophila was present in the biofilms on polybutylene and PVCc as a low proportion of the total biofilm flora (Fig. 2) with means of 6.65×10^2 and 2.13×10^3, respectively (Table 3). No legionellae were detected in any of the biofilms sampled on copper (limit of detection was 10 CFU cm^{-2}). The presence of copper inhibited the numbers of *L. pneumophila* organisms colonizing the glass control: there were no detectable *L. pneumophila* organisms over the duration of the experiment (Table 3).

The populations of microorganisms on PVCc were similar in diversity to those occurring on polybutylene at all temperatures examined; therefore, only data for polybutylene are presented in this study as a comparison for copper. The biofilm that developed on polybutylene contained a diverse mixture of gram-negative bacteria, actinomycetes, fungi, and protozoa including amoebae. Pseudomonads were the principal pioneering microorganisms, composing 72% of the total bacterial flora, the most abundant being *Pseudomonas testosteroni* and *Pseudomonas paucimobilis*, with *Pseudomonas maltophilia*, *Pseudomonas mendocina*, and *Pseudomonas stutzeri* also being present. Actinomycetes accounted for 24% of the total biofilm flora, and *Acinetobacter* sp. occurred in low numbers. The biofilm flora was increasingly dominated by the pseudomonads with 96% of the climax community being attributed to seven species of pseudomonads with increased diversity due to the inclusion of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas vesicularis*. The diversity of microorganisms present on the copper surface was significantly reduced in comparison with that on the polybutylene, with only four species of pseudomonads and two other genera of bacteria present on the copper surface. *P. paucimobilis* was the most abundant pioneer, with *P. mendocina*, *P. testosteroni*, and *Pseudomonas xylosoxidans* also being detectable. *P. paucimobilis* remained dominant in the climax community, which had increased in diversity because of the inclusion of *P. testosteroni* and *Flavobacterium* sp.

At 20°C, several species of amoebae and other protozoa could be identified in the model system. The amoebae present included *Verillefera bacillipedes*, *Hartmannella cantabrigiensis*, and *Hartmannella vermiformis*, which is known to be capable of supporting growth of *L. pneumophila* (20). Several other protozoa were also present, including motile and resting stages of *Rotari neptunia* and predatory *Lacrymaria* spp. Although protozoa were detectable within the culture and on the glass surface in the presence of copper at 20°C, none appeared on the surface of the copper tile over the 21-day experiment.

Growth in biofilms at 40°C. Polybutylene showed significantly higher levels of colonization than the PVCc and copper surfaces at 40°C, with a mean of 1.18×10^6 CFU cm^{-2}. The PVCc surface supported high numbers of nonlegionella bacteria with a mean of 3.67×10^5 CFU cm^{-2}. Copper was the least colonized of the materials, having a maximum of 5.05×10^4 CFU of bacteria cm^{-2}, and this was significantly lower than that on either of the plastic surfaces. Glass surfaces suspended in the presence of the plastic materials were less colonized than the plastic materials themselves, with means of 4.43×10^5 CFU cm^{-2} on glass suspended with polybutylene and 1.40×10^5 CFU cm^{-2} on glass suspended with PVCc. The colonization of glass was inhibited in the presence of copper to a mean of 6.44×10^4 CFU cm^{-2}.

L. pneumophila was rapidly incorporated into the biofilms on the plastic surfaces. Up to 48% of the population on

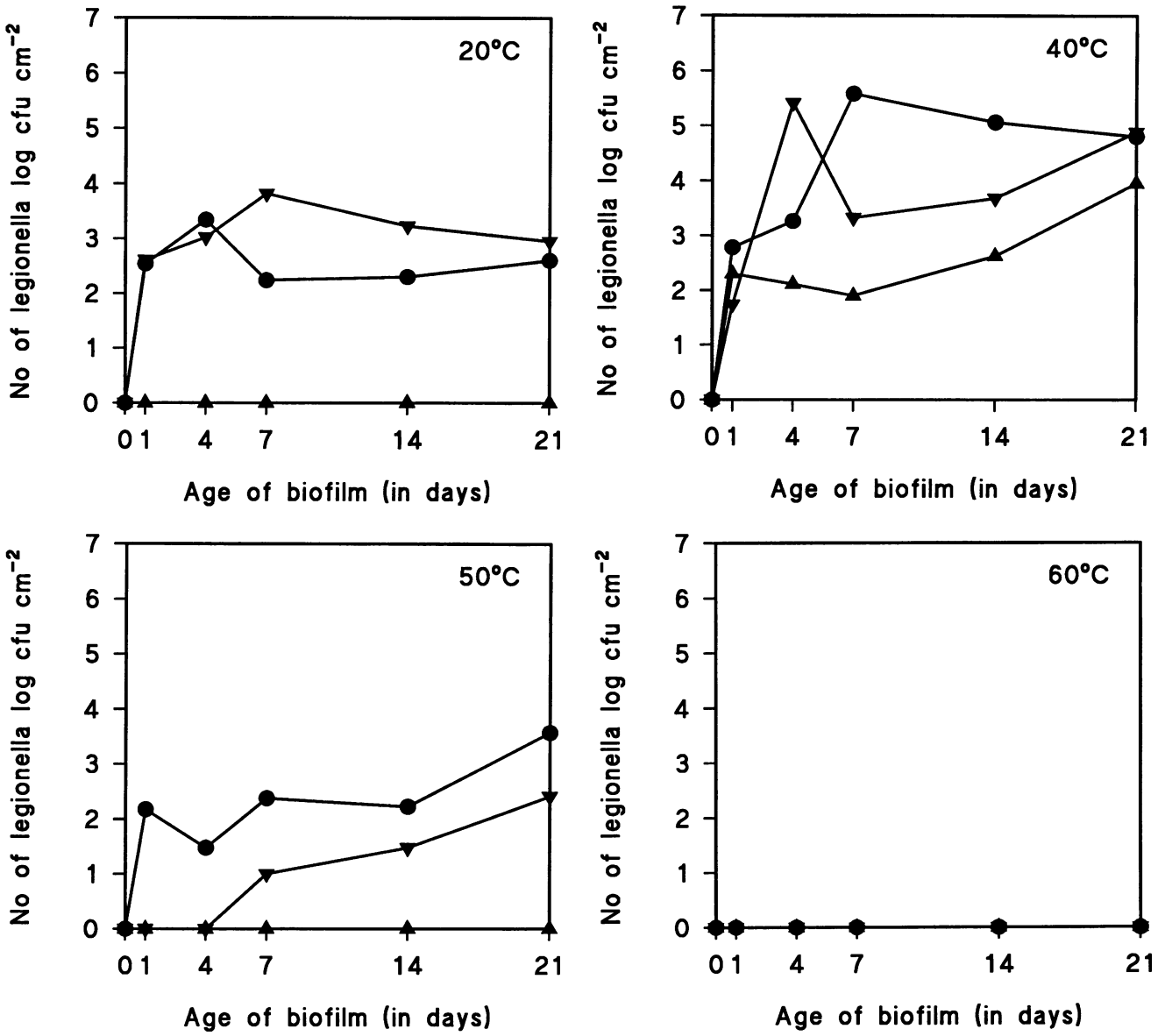

FIG. 2. Numbers of *L. pneumophila* organisms colonizing the surface of copper (▲), polybutylene (●), and PVCc (▼) at 20, 40, 50, and 60°C. In all cases, the standard deviation is less than $\log_{10}$ 0.15 of the plotted mean datum points.

polybutylene was *L. pneumophila*, with a mean of 1.59×10^4 CFU cm^{-2}. The PVCc surface supported a biofilm with a mean population of *L. pneumophila* of 4.64×10^3, accounting for a maximum of 35% of the total flora. The biofilms on the copper surfaces were capable of sustaining *L. pneumophila* after 24 h, but the numbers of the pathogen were significantly lower than those on the plastic surfaces. *L. pneumophila* on glass surfaces incubated with the plastic materials reached a maximum of 10% of the flora on PVCc and 14% of flora on polybutylene. The presence of copper suppressed the inclusion of *L. pneumophila* into the control glass surface, with the pathogen being consistently less than 3% of the population.

The pioneers on the polybutylene surface were a diverse mixture of microorganisms composed of 62% pseudomonads and 38% other gram-negative organisms. The pseudomonads were dominated by *P. testosteroni* and *P. xylesoxidans*, with other species including *P. aeruginosa*, *P. mendocina*, *P. paucimobilis*, and *P. vesicularis*. *Alcaligenes* sp., *Flavobacterium* sp., *Acinetobacter* sp., and one further gram-negative organism that would not grow after subculture were also present. *Alcaligenes* sp., *P. xylosoxidans*, *P. vesicularis*, and *P. paucimobilis* were replaced in the climax community by *P. fluorescens*, *P. mendocina*, *P. stutzeri*, and *Aspergillus* sp. The population on the copper surface was initially dominated by *P. paucimobilis* with the other microorganisms occurring in low numbers. The diversity of microorganisms colonizing the copper surface was much less than that on the plastic surfaces with *L. pneumophila* (2.00×10^2 CFU cm^{-2}), *Methylobacterium* sp., *Alcaligenes* sp., *Aspergillus* sp., and actinomycetes being the only detectable bacteria present. The climax community supported *Flavobacterium* sp. and *Aspergillus* sp. and had a total *L. pneumophila* population of 9.00×10^3 CFU cm^{-2} (29% of the biofilm flora). The amoebae and other protozoa which had been clearly evident at 20°C were not detectable at 40°C.

TABLE 3. Comparison of the inclusion of *L. pneumophila* in biofilms on different plumbing materials, on glass control surfaces, and in culture at different temperatures

Temp (°C) and material	Mean no. of *L. pneumophila* organisms[a]		
	On material surface (CFU cm^{-2})	On glass control surface (CFU cm^{-2})	In planktonic phase (CFU ml^{-1})
20			
Copper	BD[b]	BD	3.00×10^0
Polybutylene	6.65×10^2	2.79×10^2	1.28×10^2
PVCc	2.13×10^3	4.69×10^3	2.81×10^2
40			
Copper	1.67×10^3	7.89×10^2	8.17×10^2
Polybutylene	1.12×10^5	1.59×10^4	2.20×10^3
PVCc	6.84×10^4	4.64×10^3	2.20×10^3
50			
Copper	BD	2.00×10^0	1.70×10^1
Polybutylene	8.58×10^2	3.31×10^2	1.18×10^2
PVCc	6.00×10^1	4.3×10^1	7.00×10^1
60			
Copper	BD	BD	BD
Polybutylene	BD	BD	BD
PVCc	BD	BD	BD

[a] Means were calculated from all values determined over 1 to 21 days.
[b] BD denotes that numbers were below the detection limit of 10 CFU cm^{-2} or 10 CFU ml^{-1}.

Growth in biofilms at 50°C. Both plastic surfaces supported high numbers of bacteria at 50°C, but PVCc was consistently less colonized than polybutylene, and this was statistically significant. The mean colonization of polybutylene was 3.21×10^6 CFU cm^{-2}, while on PVCc the mean number was 1.22×10^5. Copper supported a sparse biofilm with a mean of only 2.26×10^4 CFU cm^{-2}, which was significantly lower than those on PVCc and polybutylene. The glass surfaces incubated in the presence of the plastics also supported high bacterial numbers. Colonization of the glass surfaces incubated in the presence of copper was lower than that on the glass surfaces incubated with the plastics.

Low numbers of *L. pneumophila* organisms were present in the biofilms on the plastic surfaces and their glass controls for less than 0.1% of the biofilm flora. No *L. pneumophila* was detectable on the copper surfaces over the duration of the experiment. *L. pneumophila* was detectable only on one occasion in the biofilms on the glass incubated with the copper, and this was at the limit of detection.

The diversity of the population on polybutylene was greatly reduced at 50°C with the only pioneers being pseudomonads: these were *P. aeruginosa*, *P. paucimobilis*, and the predominant *Pseudomonas hydrogenophaga*. The only microorganisms within the climax community, apart from *L. pneumophila*, were *P. hydrogenophaga* and *Aspergillus fumigata*, both of which could be cultured on agar plates at 50°C. *Methylobacterium* sp. was the predominant pioneer on copper surfaces, accounting for 62% of the total biofilm flora, with *P. hydrogenophaga* and *P. paucimobilis* also being present. *P. hydrogenophaga* became increasingly abundant and represented 99% of the climax community. No thermotolerant protozoa were detected in the model system by either light microscopy or culture.

Growth in biofilms at 60°C. Polybutylene was the most abundantly colonized surface at 60°C, having a mean total flora of 4.25×10^4 CFU cm^{-2}. The PVCc surface was less colonized with a mean colonization of 5.19×10^3 CFU cm^{-2}. Copper was the least colonized of the materials tested, significantly less than the plastic materials, having a mean colonization of 6.29×10^2 bacteria. Glass surfaces incubated in the presence of the plastic materials had a total colonization of 1.25×10^4 and 1.78×10^4 CFU cm^{-2}; on glass in the presence of copper, this was reduced to 6.29×10^2 CFU cm^{-2}. *L. pneumophila* was not detected in the model system at 60°C.

The biofilm developing on polybutylene was dominated by *P. hydrogenophaga* and *A. fumigata*. There were several other species transiently present, including *P. paucimobilis*, *Flavobacterium* sp., and *Methylobacterium* sp., but their low numbers and erratic occurrence indicated that they were survivors from the first chemostat rather than active members of the community. The sparse biofilms on copper were principally composed of *P. hydrogenophaga*, with *P. paucimobilis* and *Methylobacterium* sp. also present at 24 h. The climax community was a unispecies biofilm of *P. hydrogenophaga*.

Leaching of copper into the aqueous phase and accumulation in biofilms on glass. Samples from the aqueous phase of the model system were found to contain negligible levels of copper since the copper concentration was below the limit of detection for the duration of the experimental phase (>1 ppm).

The biofilm developed on the glass surface was found to contain 23.75 μg of total copper cm^{-2}. This suggests that although the planktonic phase contained undetectable levels of copper ions, copper leached from the surface of the suspended copper sample and accumulated in the biofilm on the glass control surface.

Microscopy of biofilm formation. During the initial colonization of polybutylene at 20°C, bacteria formed microcolonies that were isolated over the surface of the plastic. These areas became increasingly dense; by 21 days, bacteria could be observed embedded within extracellular polymer and could be observed to cover the whole of the material surface (Fig. 3a). A similar pattern of colonization occurred at 40°C, at which small but densely colonized regions could be observed after only 24 h of immersion in the bacterial culture (Fig. 3b). After 24 h at 50°C, the plastic supported a layer of biofilm bacteria dispersed over the whole surface in small microcolonies (Fig. 3c). These bacteria were observed to form more-dense biofilms as time proceeded. At 60°C, calcium carbonate scale prevented visualization of biofilm bacteria.

Little evidence of microorganisms was observed on copper because of the crystalline nature of the surface; however, imprints of bacteria could be observed on copper biofilms at 40°C (Fig. 3d).

DISCUSSION

The ability of *L. pneumophila* to grow and survive over a wide range of temperatures has been demonstrated. *L. pneumophila* is incorporated into both the planktonic and biofilm phases of the model system at 20, 40, and 50°C. At 60°C, the pathogen was absent from the model system. The overall trend of growth was temperature related. At 20°C, biofilms and planktonic phases contained low numbers of *L. pneumophila* organisms. Since there were many amoebae present in the culture which were known to be capable of being potential hosts (5, 18) and the bacterial species were diverse at 20°C, the low numbers of *L. pneumophila* organisms within the system were probably due to their low metabolic rate at this temperature.

At 40°C, the *L. pneumophila* organisms were at their most abundant. On the polybutylene surfaces, almost half of the total population was *L. pneumophila*. The high numbers of *L.*

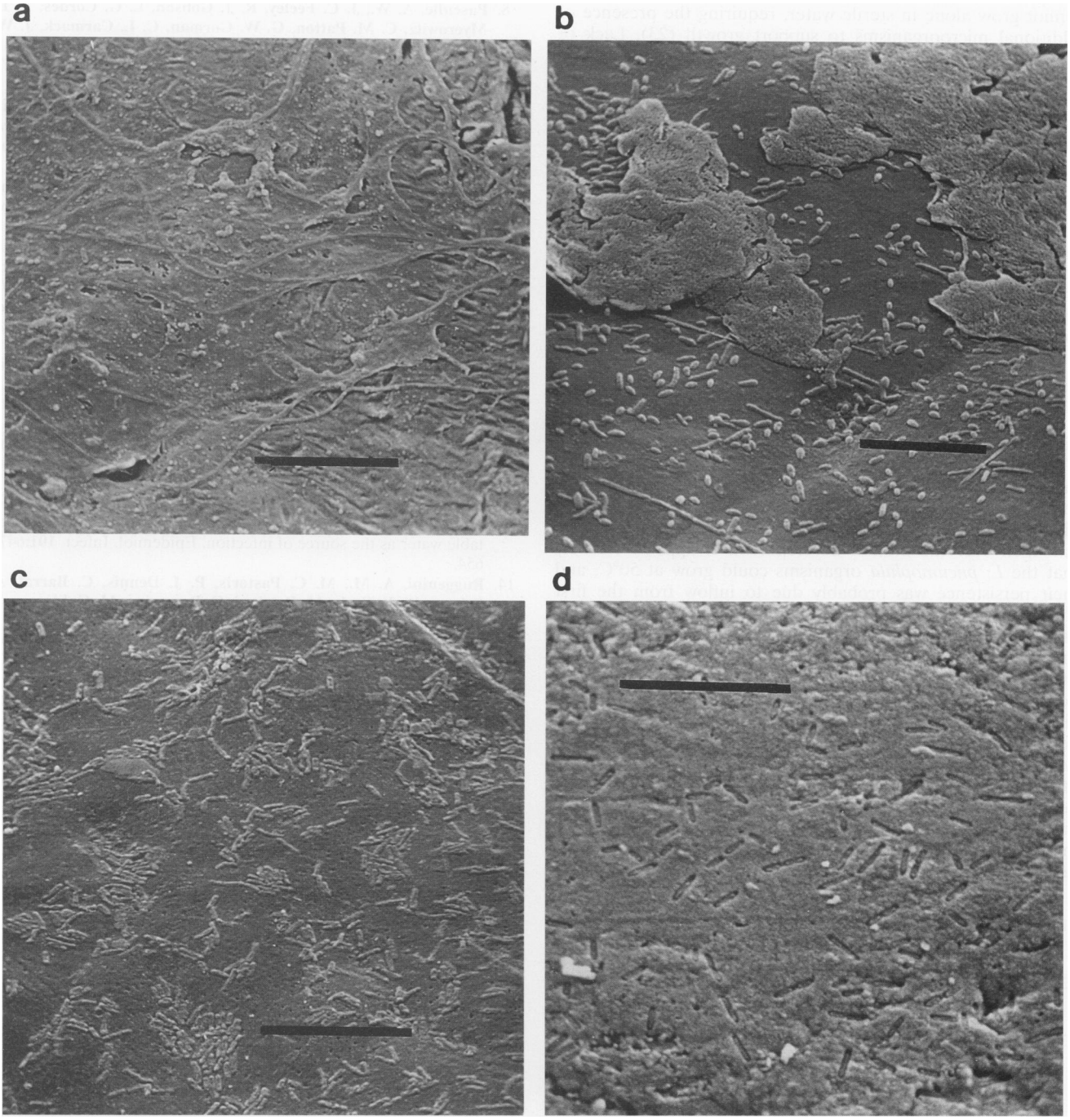

FIG. 3. Scanning electron micrographs showed that dense biofilms formed on the surface of polybutylene after 21 days in culture at 20°C (a). Polymer production was evident on polybutylene after only 24 h at 40°C (b). Initial colonization at 50°C was dominated by one bacterial type which rapidly formed dense microcolonies (c). Copper was less well colonized, but bacteria could be observed to have colonized the surface (d). Scale bar indicates 10 μm.

pneumophila organisms coincided with undetectable numbers of potential hosts, suggesting that the bacterial consortium was amplifying numbers of *L. pneumophila* organisms at this temperature. Many bacterial and cyanobacterial species have been able to support growth of the pathogen extracellularly (17, 21), and microcolonies of *L. pneumophila* within biofilms (12) support the hypothesis that biofilm consortia could encourage growth of the pathogen.

Copper was consistently less generally biofouled than either polybutylene or PVCc at all of the temperatures examined. The numbers of *L. pneumophila* organisms were also consistently lower on the surface of the copper than on that of the plastics at any of the temperatures. Copper ions could be inhibitory to growth of *L. pneumophila* directly, and this could possibly account for the reductions in the pathogen populations when copper was present. However, *L. pneumophila*

cannot grow alone in sterile water, requiring the presence of additional microorganisms to support growth (23). Lack of growth of *L. pneumophila* in the presence of copper could be attributed to the inhibition of these nutrient-supplying populations by the copper. Bacterial diversity was greatly reduced on copper surfaces compared with the plastics, and this lack of supporting populations may have accounted for some of the inhibition by copper.

Copper was found to inhibit both biofilm growth and the inclusion of *L. pneumophila* at all temperatures tested. The copper surface appeared to leach low levels of copper ions into the culture which reduced planktonic populations, and the accumulation of these ions on glass surfaces led to reduced colonization. This suggests that the use of copper tube in plumbing systems not only may prevent colonization of the tube, but could also inhibit colonization of minor noncopper components. The use of copper as a plumbing material may help to minimize the risk of Legionnaires' disease, particularly if plumbing systems are unable to be operated at 60°C.

When temperatures were increased to 50°C, the bacterial diversity was greatly reduced, and the numbers of *L. pneumophila* organisms within the system declined. The plastic surfaces supported numbers of *L. pneumophila* organisms similar to those found at 20°C. The interesting feature of these biofilms was the gradual increase in the numbers of the pathogen over the biofilm development. It appears unlikely that the *L. pneumophila* organisms could grow at 50°C, and their persistence was probably due to inflow from the first chemostat vessel rather than to growth. However, the viability of *L. pneumophila* organisms was maintained over extended time periods, indicating that the biofilm in some way protected them from the high water temperatures. This suggests that hot-water systems operating at 50°C may contain a reservoir of viable *L. pneumophila* in biofilms, along with a supporting population of microorganisms. This may be of little concern if system temperatures can always be maintained, but it may be of greater importance if temperatures were to fall by only a few degrees. The rapid growth rate of *L. pneumophila* at 40°C indicates that high numbers of the pathogen could be attained in the system after a short time period, leading to an increased risk of human infection.

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