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Influence of Plumbing Materials on Biofilm Formation and Growth of *Legionella pneumophila* in Potable Water Systems

JULIE ROGERS,[1]* A. B. DOWSETT,[1] P. J. DENNIS,[2] J. V. LEE,[1] AND C. W. KEEVIL[1]

Pathology Division, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG,[1] and Spencer House Laboratory, Water and Environmental Science, Thames Water Utilities plc, Reading, Berkshire RG2 0JN,[2] United Kingdom

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A two-stage chemostat model of a plumbing system was developed, with tap water as the sole nutrient source. The model system was populated with a naturally occurring inoculum derived from an outbreak of Legionnaires' disease and containing *Legionella pneumophila* along with associated bacteria and protozoa. The model system was used to develop biofilms on the surfaces of a range of eight plumbing materials under controlled, reproducible conditions. The materials varied in their abilities to support biofilm development and the growth of *L. pneumophila*. Elastomeric surfaces had the most abundant biofilms supporting the highest numbers of *L. pneumophila* CFU; this was attributed to the leaching of nutrients for bacterial growth from the materials. No direct relationship existed between total biofouling and the numbers of *L. pneumophila* CFU.

The colonization of plumbing systems by *Legionella pneumophila* is well documented (6, 10, 11, 31), and the growth of the organism in potable water supplies may lead to human infection (2, 4, 16, 26, 30). The bacterium is incapable of growth in sterile water because it requires nutrients to be supplied by other microorganisms, including bacteria (35), amoebae (25), and cyanobacteria (29). In several investigations, *L. pneumophila* was detected in biofilms on the surface of the plumbing fixtures, including shower heads, spouts, and valve seats (4, 37). The persistence of the pathogen in treated water systems has been attributed to the survival of the organism within biofilms on rubber materials in taps and showers (7).

Before plumbing materials are permitted for use in the United Kingdom, their influence on water quality is examined according British Standard BS 6920 (1). This ensures that the material does not contribute to poor water quality by producing unacceptable taste or odors, by releasing chemicals, or by encouraging microbial growth. The procedure uses a natural river water inoculum and a water sample of known chemistry. Bacterial growth is determined indirectly by measuring oxygen consumption. The test is simple and rapid and has undoubtedly contributed to improving water quality. However, the method does not provide information on the rate of biofilm formation on different plumbing materials or indicate which organisms are present within the biofilm. Of particular interest are the proportions of pathogens that occur in biofilms developing on surfaces. Natural latex is no longer used in plumbing systems because it provides nutrients for the growth of microorganisms. Therefore, this material was used as a positive control surface for biofouling; however, it was not known whether the surface would encourage the growth of pathogens, including *L. pneumophila*.

There are a number of examples of how different materials affect the biofilm growth of different microorganisms. The hydrophobic-hydrophilic nature of the surfaces is known to affect the attachment of aquatic bacterial species to surfaces (17). Biofilm may be encouraged to develop on the surface of a plumbing material if that material is able to supply nutrient for bacterial growth, as is the case for latex (5). Plastic surfaces are known to leach metal ions at a sufficiently low level to prevent a toxic effect but could possibly contribute cations essential for enzyme function. Bacterial cells directly in contact with the materials are more likely to take up the ions. The plasticizers and other components of the pipe material may also be directly utilizable by some of the community of microorganisms in the biofilm and so contribute to the consortium as a whole. It is not understood how these materials (including latex) affect the growth of *L. pneumophila* in biofilms.

Previous work has suggested that surfaces of copper were poorly colonized by cultures of *L. pneumophila* in a recirculating model with tap water as the nutrient source but that rubber components were heavily colonized, forming a dense biofilm with large numbers of *L. pneumophila* CFU (27). The model system recirculated the water for 4 months prior to observation of the *L. pneumophila* in the biofilm by fluorescein-labelled monoclonal antibodies. Other work has indicated that there is little difference in the degrees of colonization of plumbing materials (39); however, the study evaluated the colonization of materials by using pure cultures of *L. pneumophila* in a 10% algal extract solution which was recirculated for 14 days. Use of a more realistic model and enumeration of the total bacterial flora and *L. pneumophila* by culture showed that copper was inhibitory to the colonization and growth of *L. pneumophila* (38).

A two-stage biofilm model system was developed from that used previously (38), to study in greater detail the possible influences of eight different plumbing materials on biofilm development and the growth of *L. pneumophila*. The system was designed to study growth under conditions realistically simulating those encountered within potable water systems.

MATERIALS AND METHODS

Two-stage biofilm model. The growth medium for the model system was from one domestic potable cold water supply and

* Corresponding author. Mailing address: PHLS CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom. Phone: 0980 612310. Fax: 0980 612731.

was filter sterilized by using a 0.2-μm-nominal-pore-size nylon filter. Water sterilized in this way was shown to remain chemically unchanged (8). The inoculum was sludge from the bottom of a calorifier implicated in an outbreak of Legionnaires' disease and contained an indigenous population of *L. pneumophila* along with a diverse range of bacteria, amoebae, and protozoa. To avoid artificial selection of microorganisms for inoculation, microorganisms were not subcultured prior to inclusion in the model. The model system was inoculated at the onset of the work and operated continuously until all experiments had been completed.

The model, which was briefly described previously (23), consisted of two glass vessels linked in series. The first vessel simulated a storage or holding tank, and the second vessel (which was constantly fed by the first) modelled the distribution system. In order to maintain the reproducibility of the model system, the inoculum and conditions within the first vessel remained unaltered throughout the experiment and no plumbing materials were introduced into this vessel. This vessel was used to supply a constant inoculum of microorganisms for the colonization of the various materials. Biofilms were generated in the second vessel by inserting 1-cm^2 coupons of sterile plumbing materials into the culture suspended on titanium wire. The only materials used in the construction of the model were glass, silicon, and titanium to prevent leaching of iron, manganese, and chromium, etc., from metals into the system.

The first vessel had a retention volume of 500 ml, and the flow rate of sterile water into the vessel resulted in a dilution rate of 0.05 h^{-1}. When the retention volume was exceeded, the effluent was pumped via a weir system into the second vessel. This second vessel was also supplied with additional sterile water to maintain a total dilution rate of 0.2 h^{-1}. The effluent from the second vessel was pumped into a waste collection bottle.

The environmental parameters of the chemostats were controlled and monitored with Anglicon microprocessor control units (Brighton Systems, Hove, United Kingdom) linked to a personal computer. The temperature of the vessels was maintained at 30 ± 0.1°C by using proportional integral derivative controllers and warmed by an external heater mat. The temperature was measured with a glass temperature probe inserted into the aqueous phase of the vessel. 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 vessels were monitored throughout the experiments.

Generation of biofilms. Sections of each of the plumbing material tubes were cut open to make coupons. The tube was cut into sections with an inside area of 1 cm^2, and a 1-mm-diameter hole was drilled so that the coupons could be suspended from titanium wires. Materials that are commonly used in the construction of water systems were selected. The materials were all commercially available and were obtained from a local plumbing stockist. These included sections of the elastomeric materials, natural latex and ethylene-propylene copolymer. The plastic tubing that was used was polypropylene, polyethylene, chlorinated polyvinyl chloride (PVCc), and unplasticized polyvinyl chloride (PVCu). The test materials were cleaned with acetone to remove any oil or dirt and were then suspended on the wire beside a control glass surface. The tile assemblies were placed in bottles of water and heat sterilized by autoclaving.

The model system was run continuously for the duration of the work, and the colonization of each material was tested in series in the second vessel of the model system. Each chemostat experiment had plumbing materials of only one type. For each of the experiments, the material to be colonized and the control glass surface were aseptically immersed into the aqueous phase of the chemostat culture at day 0. The tiles were removed after 1, 4, 7, 14, 21, and 28 days from the onset of the experiment. Biofilms were aseptically removed from the curved, inside surface of the pipe or glass tile with a dental probe. The biofilm was resuspended in 1.0 ml of sterile water and vortexed for 30 s to disperse the microorganisms.

Microbiological assessment of biofilm and planktonic samples. The resuspended biofilm or planktonic sample was serially diluted into sterile water, and then 0.1-ml amounts were inoculated onto various selective and nonselective agar media. Heterotrophic non-legionella species populations were enumerated by using a nonselective, low-nutrient R2A medium (21) to avoid substrate shock. Buffered charcoal yeast extract (BCYE) agar (20) was used to culture more fastidious bacteria, including legionellae. BCYE medium was supplemented with glycine, vancomycin, polymixin, and cycloheximide to produce GVPC agar, a selective medium for legionellae (9). All plates were incubated at 30°C for 7 days prior to counting of the CFU on the agar. Those colonies on BCYE and GVPC agars that showed the characteristic ground-glass appearance of *L. pneumophila* were subcultured onto BCYE and BCYE lacking cysteine. Organisms were presumptively identified as *L. pneumophila* if they were unable to grow in the absence of cysteine but capable of growth on BCYE.

One plate of each medium which contained 30 to 100 colonies in which colony morphology could be distinguished was selected for evaluation of population profiles for each biofilm at each age. Each colony type was subcultured onto double-strength R2A (R3A) (21) or BCYE three successive times before inoculation into the appropriate API (bioMerieux, Basingstoke, United Kingdom) and Biolog (Hayward, Calif.) bacterial identification systems.

Statistical analysis of data. The colonization levels of materials were compared by using the Wilcoxon matched-pair, signed-rank test for this type of correlated, nonparametric data. The power efficiency of this test relative to the *t* test for correlated means is reported to be 95.5% (18). Calculations were performed with "Statistics," a computer package by K. B. Smith (27a). Each plotted datum point was the mean value of the number of CFU occurring on two biofilms, each plated in duplicate onto each medium.

Microscopy of samples. For scanning electron microscopy (SEM), the tiles of plumbing materials were removed from the chemostat and gently rinsed in sterile water to remove planktonic bacteria. The biofilm was fixed and stained with 1% (wt/vol) 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. (~1.3-cm) SEM specimen stubs by using 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 SEM operated at 10 kV accelerating voltage.

Biofilms were also examined by light microscopy and differential interference microscopy to determine the structure and depth of biofilms as described previously (24).

Leaching of nutrients from materials. The extent of nutrient release from the materials was assessed by total organic carbon (TOC) analysis of the water. Sections (3 cm^2 in surface area) of the sterile plastic material were inserted into 10 ml of sterile distilled water and shaken for 3 days to allow nutrients to be released into the water. The negative control was a sterile glass

TABLE 1. Comparison of the numbers of microorganisms occurring in the biofilm and planktonic phases of the model system

Organism(s)	Material	No. of microorganisms (mean)[a] in:		
		Biofilm (CFU cm^{-2})	Planktonic phase (CFU ml^{-1})	Biofilm/planktonic ratio
Total flora	Stainless steel	2.13×10^5	2.23×10^5	0.96
	Polypropylene	4.54×10^5	4.48×10^5	1.01
	PVCc	5.14×10^5	6.20×10^5	0.83
	PVCu	6.23×10^5	3.22×10^4	10.35
	Mild steel	1.69×10^6	2.23×10^5	7.58
	Polyethylene	2.75×10^6	1.62×10^6	1.70
	Ethylene-propylene	1.08×10^7	7.45×10^5	14.47
	Latex	5.50×10^7	1.87×10^6	29.4
Legionellae	Stainless steel	1.03×10^4	5.30×10^3	1.94
	Polypropylene	2.10×10^4	3.42×10^3	6.14
	PVCc	2.24×10^4	1.23×10^3	18.21
	PVCu	7.75×10^3	1.06×10^3	7.31
	Mild steel	2.06×10^4	5.30×10^3	3.89
	Polyethylene	6.76×10^3	6.68×10^3	1.01
	Ethylene-propylene	1.44×10^5	1.80×10^3	80
	Latex	2.20×10^5	1.38×10^4	12.2

[a] Means were calculated from all values determined over 1 to 28 days.

coupon which was treated as described previously for the test materials. TOC analysis was determined with a Beckman model 915B Tocamaster TOC computational system. For the determination of total carbon (TC) the syringe-injected liquid sample entered a combustion tube containing oxidizing catalyst maintained at 950°C. The sample carbon was completely oxidized to CO_2, and any water vapor was condensed and removed at the ambient dew point. The resultant sample cloud was conveyed by a continuous flow of dry, carbon-free carrier gas to the integral infrared analyzer for the detection of CO_2.

For the detection of inorganic carbon (IC), the sample was injected into the reaction tube containing a quartz chip wetted with phosphoric acid maintained at 155°C. IC reacted with the acid to liberate CO_2, which was carried via the carrier gas to the infrared analyzer. The TOC was determined by the difference of the TC and the IC. The standards for the calibration of the TC were 100, 50, and 0 mg of sodium bicarbonate per liter. For IC, the standards were 100, 50, and 0 mg of potassium biphthalate per liter. The injection volumes for both TC and IC were 50 μl. The standards and samples were injected in triplicate, and mean values were calculated.

RESULTS AND DISCUSSION

Planktonic microorganisms. The planktonic flora in the model system contained a total microbial flora of 10^4 to 10^6 CFU ml^{-1}, with numbers of *L. pneumophila* CFU between 10^3 and 10^4 ml^{-1} (Table 1) in most experiments. One exception to this was observed following the inclusion of latex and ethylene-propylene in the culture. A slight increase in the total numbers of microorganisms was observed on both occasions, with concomitant reductions in the numbers of *L. pneumophila* CFU. The diversity of microorganisms in the planktonic phase was maintained during the experiments, with all of the organisms that were present initially being maintained in culture (data not shown).

Biofouling of plumbing materials. All of the materials were rapidly colonized by microorganisms following insertion into the aquatic model system; the lowest concentration of flora was 5.24×10^4 CFU cm^{-2} on the stainless steel surface after only 24 h (Fig. 1). Elastomeric surfaces (latex and ethylene-propylene) were the most rapidly biofouled and supported a population of $>1.0 \times 10^7$ CFU cm^{-2} after an equivalent period. Stainless steel and the plastic materials supported biofilms which contained 10^5 to 10^6 CFU cm^{-2} after 24 h.

Latex and ethylene-propylene remained the most heavily colonized materials for the duration of the experiment, with maximum numbers of 8.9×10^7 and 2.9×10^7 CFU cm^{-2}, respectively (Table 2). These were found to be significantly more colonized than the other materials tested (with a confidence limit of 95%). Stainless steel supported the smallest numbers of microorganisms in biofilms compared with the other materials, with a maximum recoverable microflora of 6.45×10^5 CFU cm^{-2}. In contrast, mild steel (which was observed to rust) supported a biofilm which contained up to 4.95×10^6 CFU cm^{-2}.

Of the plastic materials, polyethylene appeared to be most heavily colonized, with a recoverable microflora of 1.3×10^7 CFU cm^{-2} after 4 days in the model system. The total numbers of microorganisms on the surface of the other plastics remained between 10^5 and 10^6 CFU cm^{-2} for the duration of the experiment, with polypropylene, PVCc, and PVCu more heavily colonized in ascending order.

For the duration of the experiments, the elastomeric surfaces supported higher numbers of microorganisms than the control glass surfaces. The glass surface supported less than 1.4% of the microorganisms occurring on the latex surface and less than 7.1% of the microorganisms occurring on the ethylene-propylene surface. Polypropylene and PVCc surfaces supported higher numbers of microorganisms than the glass control surfaces throughout colonization. Although the initial colonization of polyethylene and PVCu was more rapid than colonization of the control glass surface, this was not maintained and there was no significant difference in the colonization levels of the materials and the control glass surfaces over the remaining 27 days.

All of the material surfaces (with the exception of polyethylene and PVCu) supported significantly higher total floras than the control glass surfaces (with a 95% confidence limit). The polyethylene surface had a two-tailed probability of equalling or exceeding the sample statistic of the *t* distribution of 6%, and the PVCu had a two-tailed probability of equalling or exceeding a *t* distribution statistic of 9%.

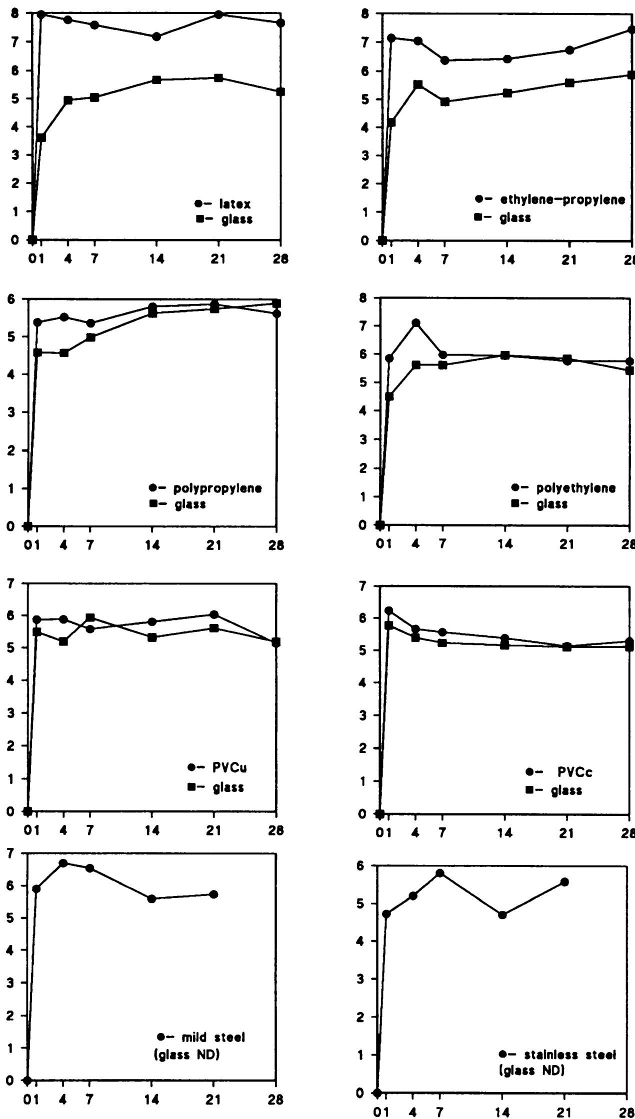


FIG. 1. Colonization by nonlegionella species of plumbing materials and control glass surfaces at 30°C in tap water containing a mixed population of microorganisms. *x* axis, age of biofilm (in days); *y* axis, nonlegionella species $\log_{10}$ CFU cm^{-2}. In all cases, the standard deviation is less than $\log_{10}$ 0.15 of the plotted mean datum points. ND, not done.

Inclusion of *L. pneumophila* into biofilms. *L. pneumophila* was found in the biofilms on all of the surfaces of all of the materials after only 24 h of exposure to the water containing the pathogen (Fig. 2). The numbers of *L. pneumophila* CFU which became included in the biofilms on the surface of the materials were unrelated to the total number of microorganisms present (Table 1). The biofilms on the elastomeric materials were found to contain the largest numbers of *L. pneumophila* for the duration of the experiment, with over 2 × 10^6 CFU cm^{-2}. However, *L. pneumophila* accounted for a maximum of only 3.5% of the total bacterial community on the elastomeric surfaces. In contrast, several materials had a higher proportion of *L. pneumophila* within the biofilm, and most notably large numbers of the pathogen occurred in the biofilm on PVCc after 7 days.

TABLE 2. Comparison of materials for their ability to support biofilm development and colonization by *L. pneumophila*

Material	Colonization (mean CFU cm^{-2})		Colonization ratio[a]	
	Total flora	*L. pneumophila*	Total flora	*L. pneumophila*
Glass	1.90×10^5	1.70×10^3	1	1
Stainless steel	2.13×10^5	1.03×10^4	1.1	6.1
Polypropylene	4.54×10^5	2.10×10^4	2.4	12.4
PVCc	5.14×10^5	2.24×10^4	2.7	13.1
PVCu	6.23×10^5	7.75×10^3	3.3	4.6
Mild steel	1.69×10^6	2.06×10^4	8.9	12.1
Polyethylene	2.75×10^6	6.76×10^3	14.5	4.0
Ethylene-propylene	1.08×10^7	1.44×10^5	56.8	84.7
Latex	5.50×10^7	2.20×10^5	289	129

[a] The colonization ratio is the number of CFU cm^{-2} of the total flora or legionellae recovered from each material compared with that on glass.

The numbers of *L. pneumophila* CFU varied in the biofilms which developed on the plastic surfaces. Polypropylene supported a biofilm containing up to 6.6 × 10^4 CFU of *L. pneumophila* cm^{-2}, which accounted for 10.3% of the total biofilm flora. Polyethylene contained fewer *L. pneumophila* CFU, with a maximum of 2.3 × 10^4 CFU cm^{-2}, and these accounted for 4% of the total biofilm flora. The PVCu material supported a biofilm with a maximum of 1.0 × 10^4 CFU of *L. pneumophila* cm^{-2} in the biofilm, which represented only 1% of the microorganisms. In contrast, the PVCc material supported a biofilm which contained 7.8 × 10^4 CFU of *L. pneumophila* cm^{-2} and accounted for as much as 15% of the total biofilm flora. The metal surfaces contained a high proportion of *L. pneumophila* within biofilms on their surfaces, with as much as 11% of the population on stainless steel and 31% of the population on mild steel being *L. pneumophila.*

The biofilms which formed on the plumbing materials were consistently found to contain more *L. pneumophila* than those on the control glass surfaces. Without exception, the materials tested had significantly larger numbers of *L. pneumophila* CFU in their biofilms than the glass surfaces, with a 95% confidence limit. The glass surfaces were found to contain less than half of the number of *L. pneumophila* CFU occurring on the plumbing material surfaces.

Comparison of colonization levels of different plumbing materials with levels on glass. When mean values were compared, all of the plumbing materials were found to have higher total colonization levels and larger numbers of *L. pneumophila* CFU than glass (immersed into the model system with no other materials) (Table 2). The elastomeric materials supported the largest numbers of total flora and *L. pneumophila* CFU, but the increase in the total biofouling was greater than the increase in numbers of *L. pneumophila* CFU. The plastics supported increases in biofilm floras from 2.4 to 14.5 times that occurring on the glass surfaces. Similarly to the previous planktonic data, the numbers of *L. pneumophila* CFU in the biofilms on the plastic surface were not directly related to the increases in total flora; for example, PVCc had a threefold increase in total flora compared with glass but a disproportionate increase in the *L. pneumophila*, which was 13 times greater. Stainless steel was the least colonized of the plumbing materials, but increased the numbers of *L. pneumophila* CFU within the biofilm above those of the PVCu and polyethylene despite the fact that they supported greater total floras.

The numbers of *L. pneumophila* CFU on the surface of the mild steel coupons were greater than those on stainless steel.

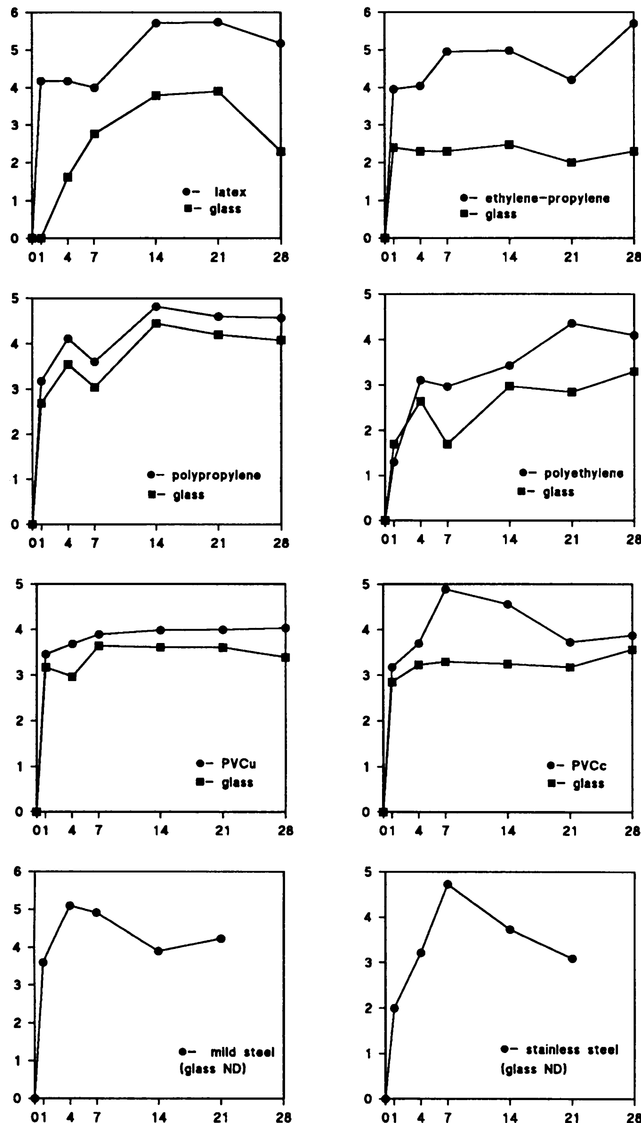


FIG. 2. Colonization by *L. pneumophila* of plumbing materials and control glass surfaces at 30°C in tap water containing a mixed population of microorganisms. *x* axis, age of biofilms (in days); *y* axis, *L. pneumophila* $\log_{10}$ CFU cm^{-2}. In all cases, the standard deviation is less than $\log_{10}$ 0.15 of the plotted mean datum points. ND, not done.

This could possibly be due to the increased availability of iron due to the corroding of the mild steel. Iron is an essential growth nutrient and is usually of limited availability in aquatic systems (22).

Composition of biofilm and planktonic floras. The mean total biofilm floras and mean numbers of *L. pneumophila* CFU in the planktonic phases were compared (Table 1). In most cases, the biofilm was found to contain greater numbers per unit of area than occurred in the planktonic phase per unit of volume. The greatest ratios of biofilm to planktonic microflora occurred in the presence of PVCu, mild steel, ethylene-propylene, and latex. By contrast, the greatest ratios of biofilm-associated to planktonic *L. pneumophila* occurred in the presence of polypropylene, PVCc, PVCu, ethylene-propylene, and latex.

The data suggest that *L. pneumophila* can often be detected in greater numbers in the biofilm than in the planktonic phase, although this varies with time and the material sampled. The determination of water quality in aquatic systems often involves only sampling of water, partially because of the ease of access and processing. However, more accurate determination would be possible if biofilms were also sampled, particularly in those systems with large surface area-to-volume ratios (e.g., cooling towers).

Microorganisms present on material surfaces after 24 h. The microorganisms that colonized the surfaces of the material surfaces varied in species composition (Table 3). After 24 h, the community on the mild steel was dominated by pseudomonads which accounted for 52% of the flora; the principal species were *Pseudomonas testosteroni* and *Pseudomonas paucimobilis*. Other organisms that occurred in large numbers on this material included *Methylobacterium*, *Acinetobacter*, and *Klebsiella* spp. The pseudomonads accounted for 61% of the species present after 24 h on the stainless steel surface, with *P. paucimobilis*, *P. testosteroni*, *Pseudomonas stutzeri* and *Pseudomonas vesicularis* being present. The single most abundant microorganism was an *Acinetobacter* sp. (2.6×10^4 CFU cm^{-2}).

A diverse range of pseudomonads was present on the PVCc surface after 24 h, including *Pseudomonas acidovorans*, *Pseudomonas mendocina*, *P. paucimobilis*, *P. stutzeri*, and *Pseudomonas xylesoxidans*. Actinomycetes were also present. Initial colonization of the polyethylene was also predominantly by pseudomonads. The principal species were *Pseudomonas fluorescens*, *P. acidovorans*, and an *Acinetobacter* sp. In contrast, pseudomonads and the other gram-negative microorganisms occurred in approximately equal proportions in the biofilm forming on the polypropylene. The most abundant organisms were *Pseudomonas diminuta* and an *Acinetobacter* sp., with *P. fluorescens*, *P. mendocina*, an *Alcaligenes* sp., a *Flavobacterium* sp., a *Methylobacterium* sp., and actinomycetes representing a small proportion of the biofilm flora. Biofilms developing on the PVCu surface were composed of a mixture of gram-negative microorganisms (principally *P. acidovorans* and *P. vesicularis*) and of actinomycetes.

The latex surface was initially colonized by a mixed flora, including *P. xylesoxidans*, an *Acinetobacter* sp., and actinomycetes. The community of microorganisms on ethylene-propylene was initially dominated by *Acinetobacter*, *Aeromonas*, *Flavobacterium*, and *Alcaligenes* spp. Approximately 15% of the biofilm was composed of pseudomonads, with *P. diminuta* and *Pseudomonas maltophilia* occurring in equal numbers.

Communities of microorganisms present after 21 days on material surfaces. Pseudomonads continued to dominate in the community developed on the mild steel surfaces, representing 62% of the total flora. However, *Pseudomonas aeruginosa* and *P. vesicularis* replaced *P. mendocina*, *P. testosteroni*, and *Klebsiella* spp. that had previously been on the surface (Table 4). A similar proportion of pseudomonads was present on the surface of stainless steel, where an *Aspergillus* sp. and an *Alcaligenes* sp. replaced a *Flavobacterium* sp. and *P. mendocina* within the community.

The diversity of microorganisms within the biofilm on latex was increased by the addition of *P. paucimobilis* and *P. stutzeri* to the surface; however, pseudomonads accounted for only 11% of the flora. A similarly low proportion of the flora on ethylene-propylene was composed of pseudomonads, and the mature biofilm community included large numbers of *Aspergillus* sp. cells and actinomycetes. In contrast, pseudomonads composed between 37 and 49% of the biofilm communities on the plastic surfaces.

TABLE 3. Microorganisms on the surface of various plumbing materials after 24 h

Organism	Microorganism population[a] (CFU cm^{-2}) on:							
	Mild steel	Stainless steel	Latex	Ethylene-propylene	Polypropylene	Polyethylene	PVCu	PVCc
L. pneumophila	3.95	0.1	15	9	1.5	0.5	2.9	1.5
P. aeruginosa					1.9	260		
P. acidovorans							37	880
P. diminuta				1,000	22			
P. fluorescens					26	380	1	
P. maltophilia	80	10	8,000	100		20	3	
P. mendocina	60	25	4,000	3	3		4.7	210
P. paucimobilis	100	10		1,000		0.2	1.2	300
P. stutzeri	90	1						
P. testosteroni	410	12	9,000		1	80	2	150
P. vesicularis						6.2	59	
P. xylosoxidans			36,000					310
Actinomycetes sp.	10	6	34,000		1		19	200
Aeromonas sp.				8,000				
Alcaligenes sp.	70			2,000	4	0.2		
Flavobacterium sp.	40	7	800	1,000	3	0.3	0.2	290
Methylobacterium sp.	110	4			3		5	
Klebsiella sp.	130							
Acinetobacter sp.	300	26	40,000	10,000	17	440	0.6	

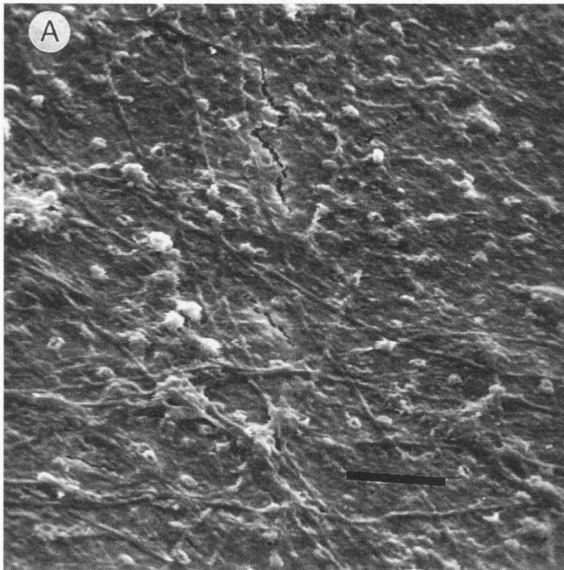
[a] Numbers of non-*Legionella* populations are represented as a sum of the CFU cm^{-2} occurring on R2A, BCYE, and GVPC media. *L. pneumophila* populations are represented as the mean of those CFU cm^{-2} occurring on BCYE and GVPC media.

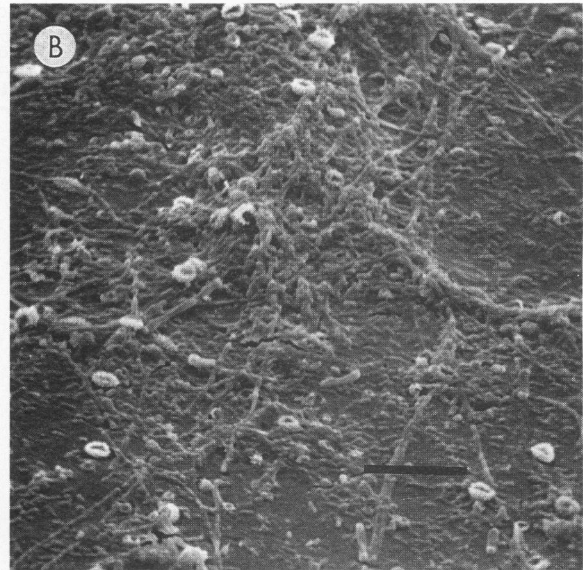
Microscopy of biofilms. Latex and ethylene-propylene were completely covered with a thick layer of biofilm after only 24 h of immersion in the chemostat culture (Fig. 3A and B). The biofilm could be observed without magnification and varied in depth across the surface but was at least 200 μm deep. The biofilm was composed of a range of microorganisms including bacteria of diverse morphologies and amoebae, which were embedded in an extracellular matrix. Cracks in the biofilm were evident following preparation, and the substratum could be observed beneath the biofilm in some regions. In results similar to those for recovery of microorganisms, biofilms on the plastic surfaces appeared to form less rapidly than those on the elastomeric materials. Microorganisms on the plastics initially occurred in small microcolonies on the surface, and these developed into denser areas of biofilm, particularly in crevices on the material surfaces (Fig. 3E and F). As biofilms matured, the space between the microcolonies was reduced and by day 28 most plastic surfaces were covered in a biofilm; however, channels (where water and nutrients could flow) were still evident by the SEM procedure used here or the light microscopy procedure described by Rogers and Keevil (24). Grazing amoebae and other protozoa could be observed in some samples (Fig. 3C and D). Mild steel was heavily corroded after 24 h of immersion in the culture, and few microorganisms could be observed among the corrosion products (Fig. 3G). The accumulation of debris on the surface of stainless steel

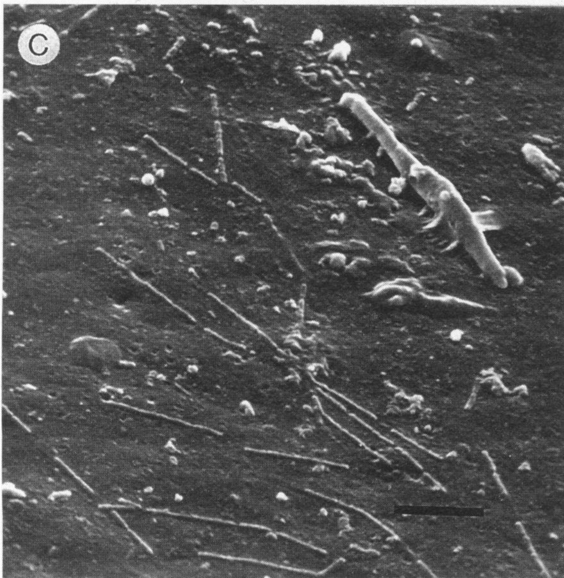
TABLE 4. Composition of communities of microorganisms on the surface of various plumbing materials after 21 days

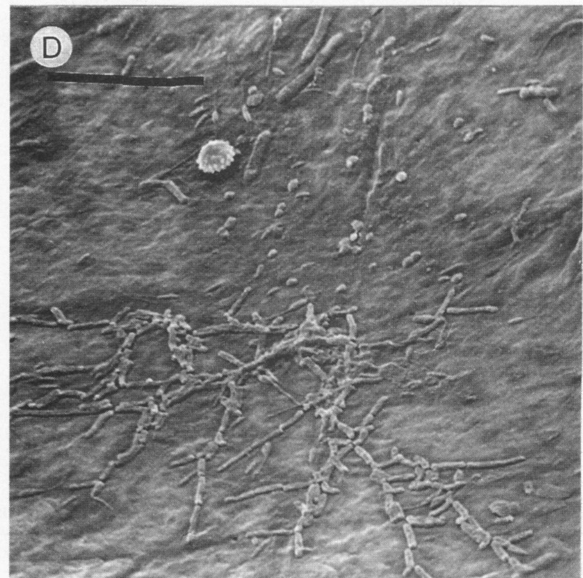
Organism	Microorganism population[a] (CFU cm^{-2}) on:							
	Mild steel	Stainless steel	Latex	Ethylene-propylene	Polypropylene	Polyethylene	PVCu	PVCc
L. pneumophila	17	13	150	500	37	13	11	7.9
P. aeruginosa	30							
P. acidovorans						40		11
P. diminuta						2		
P. fluorescens				1,000				3
P. maltophilia	10	11	3,000			10	10	
P. mendocina			13			40	0.01	
P. paucimobilis	30	36	5,000	1,600	790	170	140	36
P. stutzeri	140	70	2,000					0.1
P. testosteroni		180				20		8
P. vesicularis	250							3
P. xylosoxidans						7		40
Actinomycetes sp.	130	2	7,000	8,000		9	0.01	2.8
Aeromonas sp.			6,000					
Alcaligenes sp.	10	10			320	80		30
Flavobacterium sp.	41		15,000	2,400		0.2	90	50
Methylobacterium sp.	20	150			140	30	60	23
Klebsiella sp.			31,000					
Acinetobacter sp.	70	39	22,000	3,100	400	180	40	60
Aspergillus sp.		0.2		4,400				

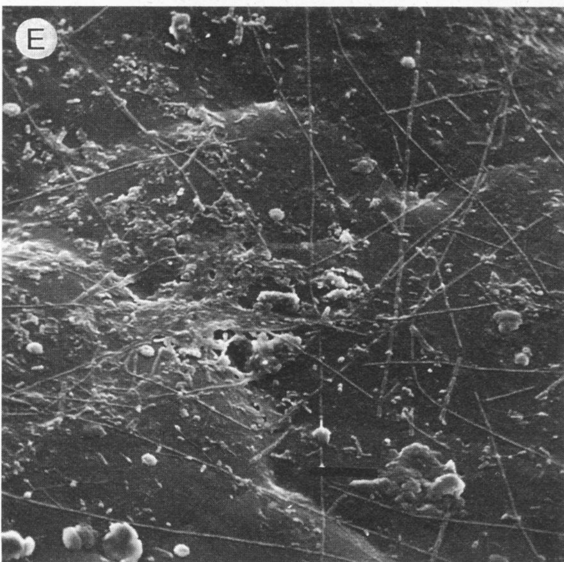
[a] Numbers of non-*Legionella* populations are represented as a sum of the CFU cm^{-2} occurring on R2A, BCYE, and GVPC media. *L. pneumophila* populations are represented as the mean of those CFU cm^{-2} occurring on BCYE and GVPC media.

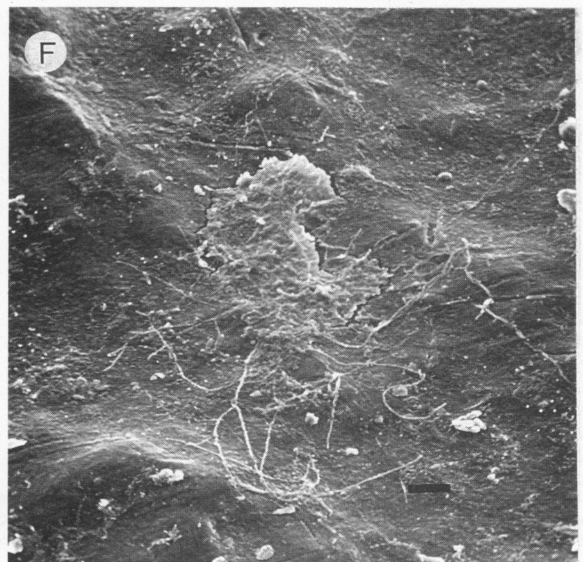

A


B


C


D


E


F

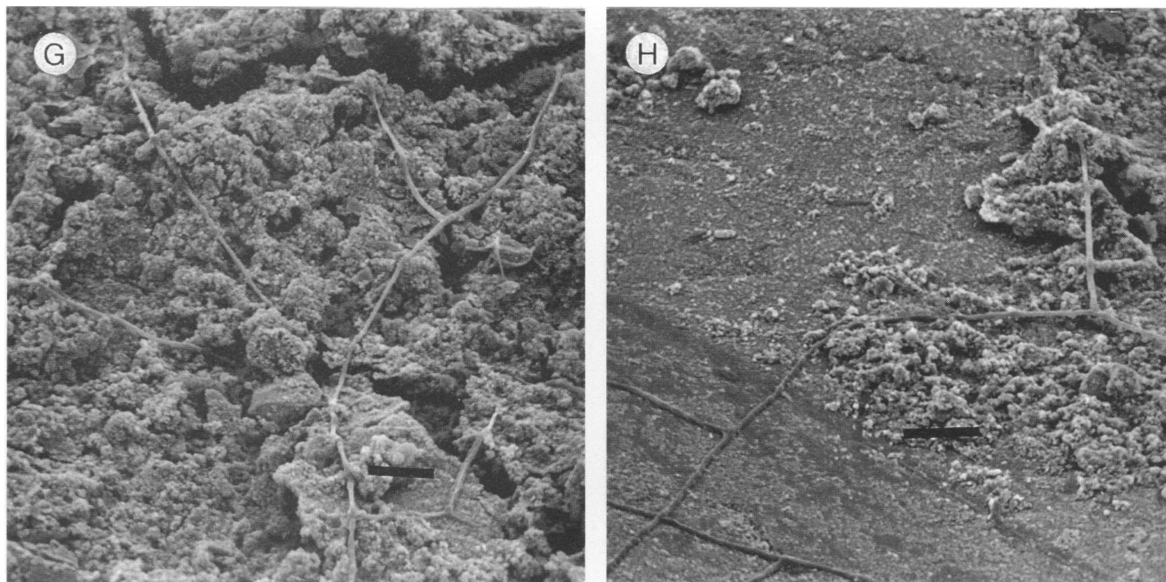


FIG. 3. SEM of biofilms forming on plumbing material surfaces showed that latex (A) and ethylene-propylene (B) were very heavily colonized with thick biofilms which covered the whole material surfaces. The plastic materials were less heavily colonized, but some areas of the polypropylene (E) and polyethylene (F) surfaces were found to have regions where thicker biofilms formed. Amoebae could be observed on the surface of PVCu (D), and other predatory microorganisms were evident on PVCc (C). Some microorganisms were observed on stainless steel (H) and mild steel (G) surfaces despite the accumulation of corrosion products. Bar, 10 μm.

also made observation difficult, but attached growth could be discerned (Fig. 3H).

Amoebae and other protozoa on biofilms. Both resting and motile rotifers which were present were identified as *Rotari neptunia*. Other protozoa included predatory *Lacrymaria* spp., which are known to ingest amoebae. Amoebae isolated from the culture included *Hartmannella vermiformis*, *Hartmannella cantabrigiensis*, and *Verillifera bacillipedes*, which were kindly identified by T. J. Rowbotham (Public Health Laboratory Service, Leeds).

Leaching of nutrients from material surfaces. Incubation of the materials in water produced elevated levels of TOC with all of the materials (Table 5). The latex, ethylene-propylene, and polyethylene surfaces increased the concentration of organic carbon in excess of 150 mg of C liter^{-1}. Copper and the other plastic materials only slightly increased the amount of TOC in the water.

The plumbing materials examined in this investigation were all found to support biofilms, and all of these biofilms contained *L. pneumophila*. Each of the substrata was colonized by different pioneering species, despite the presence of the same planktonic bacterial community, and the biofilm which developed varied in diversity, abundance, and morphology. Materials colonized under the same environmental conditions are therefore influenced by the supporting material. The most extensive biofilms occurred on the surface of the elastomeric materials, and this was attributed to the leaching of nutrient from the material, which increased the total available nutrient in the system. The greatest numbers of *L. pneumophila* CFU were also detectable in the biofilms developing on the elastomeric materials; however, the biofilms were so extensive that the pathogen formed only a minor component of the biofilm flora. It is evident, however, that the materials could indirectly cause an increase in the numbers of the pathogen to proliferate in the aquatic environment by encouraging biofilm growth on the surface.

TABLE 5. TC leached from materials exposed to water for 3 days

Material in water	TOC (mg liter^{-1})[a]
Glass (control)	2.78 ± 0.4
Copper	4.15 ± 0.17
Polybutylene	4.46 ± 0.15
PVCc	6.02 ± 0.11
PVCu	5.42 ± 0.11
Polypropylene	5.98 ± 1.56
Polyethylene	179 ± 0.82
Ethylene-propylene	157 ± 0.84
Latex	320 ± 19.4
Stainless steel	Not done
Mild steel	Not done

[a] Values are the means of three determinations ± standard deviations.

The plastic surfaces were also capable of supplying some additional nutrient to the bacterial flora, but this was not the major cause of enhanced colonization of the plastic surfaces compared to copper. The plastic materials had crevices and hollows on the surface as a result of their manufacture. These hollows were rapidly colonized, and areas of dense biofilm which eventually extended outwards were formed there. The initial colonization of these areas could be due to the protection from shear forces they gave to the colonizing bacteria.

Growth of *L. pneumophila* can be examined in situ in operating cooling or plumbing systems, but since *L. pneumophila* CFU occur in small, fluctuating numbers and since the conditions within any water system vary widely, comparison of results is difficult (12). Previous work modelling the ecology of *L. pneumophila* has principally been concerned with batch culture in the planktonic phase. This work was important in the use of naturally occurring, mixed populations for the simulation of water environments for the determination of factors influencing growth of *L. pneumophila* (34, 40). However,

problems may be encountered when using batch culture; for example, in studies of *L. pneumophila* in coculture with cyanobacteria, the accumulation of algal products resulted in the elevation of pH and consequently prevented the growth of *L. pneumophila*. The use of a continuous culture model system has overcome some of the drawbacks associated with either sampling naturally occurring communities or batch culture experiments.

The biofilm serves as a focal point where bacterial and protozoal populations interact. The pioneering bacterial population will modify the surface conditions to enable bacterial succession to take place. The resultant biofilm may aid colonization by *L. pneumophila* by supporting bacterial floras that provide essential nutrients (24, 28, 29, 32, 36); by removing high inhibitory concentrations of oxygen by respiration (19) or by encouraging protozoal populations which can act as hosts for the pathogen (13, 14, 25). However, the biofilm (or regions of it) may be inhibitory to *L. pneumophila* if bacterial flora produce extracellular products that inhibit growth directly (3, 15, 32) or encourage a protozoal population that uses *L. pneumophila* as a preferential food source (33). This process of modification of the biofilm flora would be continuing and dynamic until a stable climax community was achieved. The material used in the plumbing system would affect this community by influencing the primary colonizing species and subsequent populations.

The data presented here demonstrate that numbers of *L. pneumophila* CFU cannot be predicted from the total numbers of bacteria present in the biofilms. Since the current British standard (BS 6920) only determines the potential for total microbial growth of a material, it is unsuitable for indicating the potential of a plumbing material to encourage growth of this pathogen. It would be preferable to use water system materials that did not encourage the growth of *L. pneumophila* by supporting bacterial populations in the biofilm which aid the growth of the pathogen. This is particularly so in hospitals, where large numbers of susceptible patients are exposed to water which may be at the optimum temperature for the growth of the pathogen.

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