Journal of Industrial Microbiology (1995) 15, 384–390

Control of *Legionella pneumophila* in a hospital water system by chlorine dioxide

JT Walker[1], CW Mackerness[1], D Mallon[2], T Makin[2], T Williets[3] and CW Keevil[1]

[1]*Research Division, CAMR, Porton Down, Salisbury, SP4 0JG;* [2]*Department of Medical Microbiology, Duncan Building, University of Liverpool, PO Box 147, Liverpool 169 3BX;* [3]*Broadgreen Hospital NHS Trust, Thomas Drive, Liverpool L14 3LB, UK*

Immuno-compromised patients are particularly susceptible to Legionnaires' Disease. After three cases of the disease occurred in a hospital, a continuous dosing regime using chlorine dioxide was initiated to replace chlorination of the water system. This study identified a number of factors which may have resulted in conditions that would encourage the growth of the water-borne pathogen *Legionella pneumophila*. The residual chlorination was inadequate for microbial control at the taps furthest from the four storage tanks, of which two were found to be in excess for demand. The temperature of the water in the storage tanks was also found to be above 20° C; a temperature that would encourage microbial growth. A back-up calorifier was present and was found to contain *L. pneumophila*, and linseed oil-based sealants that provide nutrients for microbial growth were also prevalent as jointing compounds in the water circuit. Although the shower heads were routinely disinfected, a requirement was identified to also disinfect the shower hoses. No *L. pneumophila* were recovered from the water system after the chlorine reduced dioxide disinfection trial. Biofilm was also dramatically reduced after disinfection; however, small microcolonies were identified and proved to be metabolically active when tested with a metabolic indicator. Using light and fluorescence microscopy, the pipe samples removed from the water system were rapidly analysed for biofouling, complementing existing microbiological methods.

Keywords: *Legionella pneumophila*; chlorine dioxide; water system; biofilms; hospital; copper; fluorescence microscopy

Introduction

Legionnaires' Disease is caused by the inhalation of aerosols contaminated by the bacteria legionellae [4,14]. Although legionellae are responsible for only 2% [8] of all community-acquired pneumonias, the outbreak of the disease can be sudden and often exhibits a high mortality rate (12%) [3]. The disease is a rare form of pneumonia which principally affects the elderly, especially males, smokers and people whose defences are impaired [5], resulting in increased temperatures and shadowing of the lung.

Potable water, particularly in hot water systems, has been proven to act as a reservoir and breeding site for *Legionella pneumophila*, the major causative agent of Legionnaires' Disease [10,18,19]. Plastic, rubber and jointing materials have been demonstrated to encourage the growth of aquatic microorganisms [7]. Potable water systems have traditionally been treated with chlorine, while cooling towers, which have also been implicated in outbreaks of Legionnaires' Disease, have often due to their high pH been treated with bromine-based biocides [20]. This study is concerned with such an outbreak in a water circulation system in a hospital.

Following the occurrence of three cases of Legionnaires' Disease within 3 months in a newly opened nucleus block of the Regional Cardiothoracic Centre in Liverpool in October 1991, the domestic water supply of this nucleus block was found to be heavily colonised by *L. pneumophila* serogroup 1 Pontiac. The nucleus block concerned had been built under the terms of a design and build contract with the Regional Health Authority and Regional Cardiothoracic Hospital Trust. Examination of the facilities in the new nucleus block by ourselves and the hospital engineers at Broadgreen Hospital Trust revealed that the specification of the contract had not been followed. In addition, equipment had not been installed correctly and there had been a failure to comply with the commissioning procedures specified in the contract document. From the point of view of the bacterial colonisation of the water system mentioned above the most important defects were:

i) The two calorifiers were located in the same room in the roof space as the cold water storage tanks. This resulted in the temperature of the water in the storage tank nearest the calorifier being above 20° C. According to DOH guidelines [1], this represents an increased risk of proliferation of legionellae. The cold water in the other three storage tanks was at temperatures between 16–20° C.
ii) The water storage capacity consisting of four tanks each of 5000 L was excessive. Measurement of the water used indicated that only two tanks were required to provide storage for a 24-h supply. All the tanks had well-fitting lids with only a fine layer of silt on the bottom, but otherwise had no defects.
iii) Each of the calorifiers had the capacity to supply hot water to the nucleus block, so that for the purposes of cleaning and maintenance one of the calorifiers could be decommissioned. The backup calorifier when examined was found to be positive for *L. pneumophila*.
iv) Engineers' records demonstrated that when both calorifiers were used at the same time, there was a considerable

Correspondence: JT Walker, Research Division, CAMR, Porton Down, Salisbury SP4 0JG, UK
Received 23 December 1994; accepted 14 August 1995

difference in temperature between the top and bottom of the calorifiers. We were unable to check this at the time of this study but it has to be considered that the over-capacity provided by the simultaneous use of the two calorifiers may have led to stratification, resulting in different temperatures within them.

v) No automatic system of measuring or recording calorifier output and return temperatures had been installed and therefore only manual recording of output temperatures had been carried out before this study commenced. These historical records indicated output temperatures consistently below 60° C, reaching 53° C on occasion.

The specification included the installation of a warning device for calorifier output temperatures less than 60° C. The specifications for this device included a warning panel in the engineering department but the panel was never activated by low calorifier temperatures as the sensor had not been installed.

vi) Calorifier return temperatures were always at least 15° C below output temperatures and on occasion had been as low as 35° C. The inadequate power and capacity of the pump installed in the return part of the circuit was considered likely to be in part responsible for this undesirable difference between output and return temperatures, but dead-legs and more extensive than necessary lengths of pipe work within the system were considered to be likely contributory factors.

vii) Linseed oil-based organic sealing compounds (Bosswhite and Hessian) were found in many of the joints between the pipes in the hot and cold water systems.

In addition to the defects within the new nucleus block there were problems with the supply of the water from the hospital borehole. On emergence from the borehole, water was chlorinated at 0.46 ppm with chlorine and then 80% was passed through a water softener after which it was blended with the 20% that was not softened. The water then passed to a reservoir tank 100 yards away and then to the storage tanks in the nucleus block 400 yards way. The borehole water had a heavy inorganic load which caused the problems of scale deposits in the piping of the nucleus and of other blocks in the hospital. Following the examination and discovery of the defects described above, the following remedies were suggested:

(a) The borehole water should be softened before chlorination.
(b) Residual chlorine concentration of borehole water should be such that effective levels are present at the taps furthest from the storage tank.
(c) The temperature of the storage tanks on the nucleus block should be kept below 20° C.
(d) The storage capacity should be reduced to only 2 × 5000 L.
(e) The backup calorifier when not in use should be disinfected and drained.
(f) All plumbing joints on the nucleus block should be checked for the presence of linseed oil-based organic sealing compounds and where found these should be removed.
(g) Disinfection of shower heads should include disinfection of the extension shower hoses.
(h) Hyperchlorination of the water system of the nucleus block should take place as soon as possible.

Although hyperchorination dramatically reduced the number of legionellae and other organisms present in the water system, bacterial and protozoal organisms were not eliminated and counts of these organisms rose over the next few weeks to alarming numbers. When repeated, hyperchlorination had failed to eliminate legionellae and other organisms from the system and the decision to institute continuous dosing with chlorine dioxide was taken.

Brief description of locations

The nucleus block of the Cardiothoracic Centre contained two wards, A and B, and Intensive Care Units supplied by a water system independent of the rest of the Centre. Room 1 of ward A was chosen as the site for removal of water and pipe samples before chlorine dioxide treatment. After treatment the identically positioned (ie mirror image) room 2 in ward B was chosen as the site of sampling for comparison. In addition, the Audrey Leigh Ward (ALW) was proposed as a control site for the biocide trial as this ward had a water supply very similar to that of the nucleus block. Although commissioned at the same time, it had a separate water supply that had not been colonised by legionellae and therefore provided information for comparison with the Cardiothoracic Centre before and after chlorine dioxide treatment.

Materials and methods

Borehole and mains water supplying the hospital wards

The new Cardiothoracic Centre was supplied with water from a borehole while the rest of the site was supplied by municipal mains water. Two water samples were taken, one from the municipal mains supply and the other from the chlorinated borehole water, for microbiological analysis, hardness and chlorine content determination.

Water samples and pipework

Water samples (approx 5 L) were taken from both the hot and cold water pipes of designated showers before and after treatment, and dispensed into sterile high density polycarbonate bottles. Swabs were taken from the hot and cold faucet of the sink unit in both room 2 (pre-treatment) and room 1 (post-treament). Pipe samples were removed from the hot water dead-leg of the sink unit and from the hot and cold section of the showers (between the temperature control valve and mixer unit). Upon removal the pipe was inspected for signs of linseed oil-based jointing compounds having been used during the original installation before one end was sealed with an end cap. The pipe was then filled with source water to prevent dehydration of the biofilm and sealed with another end cap.

Microbiological analysis

Water samples were filtered through 0.2-μm nylon membranes; material from the filter was resuspended in 10 ml of sterile distilled water. Aerobic heterotrophic bacteria were recovered and enumerated on low nutrient R2A agar [15]

to avoid substrate shock and to enhance recovery of starved heterotrophs or oligotrophic species. Serial dilutions were prepared in duplicate from neat to 10^3 dilution. *L. pneumophila* were recovered and enumerated on BCYE agar [11] and the more selective Glycine, Vancomycin, Polymixin and Cyclohexamide (GVPC) agar [11]. R2A agar plates were incubated at 30° C for 7 days while BCYE and GVPC agar plates were incubated at 35° C for 7 days. *L. pneumophila* were identified by their characteristic morphology followed by analysis using an identification database (Biolog) [13].

Microscopy

After removing endcaps from one end of the pipe two incisions were cut 0.5 cm apart, longitudinally along the tube sample for 1 cm. Another incision was then cut across the tube sample 1 cm from the end of the pipe such that a section of tube with the least amount of curvature could be viewed. Acridine orange [2] (BDH, Eastleigh, UK) was dissolved in sterile water (0.02%; w/v) then filtered (0.2 μm) before being added to the pipe sample and incubated at room temperature for 1 min. Other pipe sections were stained with cyanoditoyl tetrazolium chloride (CTC) (Polysciences Inc, Cambridge, UK, 50 mM) in sterile distilled water, then filtered (0.2 μm) and added to the pipe sample which was then incubated at 30° C for 3 h. Pipe samples stained with either acridine orange or CTC were then rinsed in non-flowing sterile distilled water before examination.

Microscopic examination of the pipe sections was carried out using a Nikon Labophot-2 microscope which combines both epi-fluorescence and espiscopic Normarski differential interference contrast (DIC) optics. Two sections of pipe (1 cm^2) for each stain were rapidly scanned and a minimum of 15 individual areas were examined for the presence of a biofilm. A B-2A filter block was used for the epi-fluoresence. This consisted of a 510-nm dichroic mirror, an excitation filter of 450–490 nm and a barrier filter of 520 nm. An IGS block was used for DIC. Objective lenses were non-contact M Plan Apo (Nikon, UK) 40, 100 and 150× magnification with numerical apertures of 0.5, 0.8 and 0.95, respectively. The light source was a 100-W halogen lamp. Neutral density filters were used where necessary for suppression of high background fluorescence. Photographs were taken using a Nikon F-801 35-mm camera on 320 ASA tungsten balanced transparency film.

Chlorine dioxide treatment of the water supply system

The shock dose treatment of the water supply system was carried out using chlorine dioxide (ClO_2) produced by the reaction of antinium dioxide with a dilute acid to produce a stock solution of 20000 ppm. A feed-back control pump was used to dose the system to give a final ClO_2 concentration of between 50–80 ppm. This concentration of ClO_2 was maintained for 8 h in the storage tanks. All taps and showers in the wards were run to waste until there was between 50–80 ppm ClO_2 issuing from the taps and this concentration was maintained at all outlets for 1 h. The ClO_2 concentration was then maintained at 3–5 ppm and was measured by a modified DPD Lovibond comparator (Tintometer, Salisbury, UK).

Table 1 Borehole results

Borehole	Total hardness (ppm)	Hardness (Ca) (ppm)	Chlorine (free) (ppm)
Before softener	320	190	4–5
Blend (to storage)	180	100	0.6

Table 2 Bacterial counts of water supplying ward A, rm 1 (pre-treatment)

Bacteria	Cold water (CFU per ml)	Hot water (CFU per ml)
Heterotrophic	> 3000	> 3000
Legionellae	3000[a]	2500

[a]Denotes that *L. pneumophila* were detected at 500 CFU per ml (identified using the BIOLOG database of biochemical determinants)

Results and discussion

Water chemistry, temperature and microbiology

Borehole: Wards A and B were the main areas affected by the presence of *L. pneumophila* and were supplied with water from a borehole approximately 400 m from the Cardiothoracic Centre. The automatic dosing meter at the borehole indicated a free chlorine residual concentration of 0.46 ppm but using a Lovibond comparator, the actual concentration was found to be between 4–5 ppm. However, as a result of the water passing through the softener, chlorine residual decreased markedly from 4–5 ppm to 0.6 ppm and therefore the water should have been chlorinated after the water softener. This would also shorten the life of the ion exchange resin in the softening unit (Table 1). From the softening unit the water was blended with 20% of the original unchlorinated borehole water resulting in a free chlorine concentration of 0.45 ppm. After transfer to the hospital reservoir only 0.3 ppm was detected. No bacteria were recovered on the low nutrient R2A agar in the sample before and after chlorine treatment of the borehole water.

Ward A and Ward B: Treatment of both the hot and cold water systems with ClO_2 reduced the total microbial flora of the water supplying wards A and B from >3000 to 70 CFU per ml (Tables 2 and 3). Although *L. pneumo-*

Table 3 Bacterial counts of water supplying ward A, rm 2 (post-treatment)

Recovery media	Cold water (CFU per ml)	Hot water (CFU per ml)
Heterotrophic	70	70
Legionellae	300[b]	100[b]

[A]Denotes a swarmer of *Acinetobacter calco* var *lwoffi* (identified using the API 20 NE database of biochemical determinants)
[b]None were identified as *L. pneumophila*

Table 4 Bacterial counts from swab samples of water supplies in rm 1 ward A (pre-treatment)

Swabs recovery media	Cold tap (CFU per ml)	Hot tap (CFU per ml)	Shower head (CFU per ml)
Heterotrophic	> 3000	> 3000	> 3000
Legionellae	> 3000	> 3000	> 3000

phila were recovered from the cold water supply prior to chlorine dioxide treatment none were detected after treatment. Bacterial counts determined from swabs of the cold tap, hot tap and shower head were >3000 CFU per ml prior to treatment, and <100 after treatment of the taps (Tables 4 and 5). Treatment of the shower heads, however, did not appear to have been successful as >3000 CFU per ml were recovered post-treatment. As the shower hose was not disinfected this may have accounted for the presence of high numbers of non-legionellae bacteria such as *Flavobacteria* and *Alcaligenes* in the shower. As legionella had been recovered from the cold water system, it can be hypothesised that this was the reservoir system for the water-borne pathogen which could be delivered to areas in the water system that would be favourable for multiplication and growth. One such site could be the shower hose pipe which was not only not disinfected but was also made of rubber that would have imparted organic compounds that could be used as bacterial nutrients.

Hot and cold water tap systems: The sink units examined were fitted with a non-return Meynell valve (Meynell Valves Ltd, Wolverhampton, UK) after which there was a short dead-leg up to the tap. Linseed oil-based sealant (Bosswhite) was found in the fittings removed from the pipes in ward B. Linseed oil-based sealant materials should not be used in new water systems as they contain organic ingredients that can support microbial growth and as such contravene DOH guidelines [1]. The presence of linseed oil-based sealants was a surprise to all involved since guarantees had been given previously that this compound had been removed from the system of wards A and B. In the kitchen area of the ward 0.3 ppm of chlorine was measured as there were no intervening blender valves; however there was no detectable residual from the tap furthest from the storage tanks in which blender valves were present.

Showers: Due to the risk of biofilm formation and colonisation by *L. pneumophila* the shower heads were removed weekly and disinfected in 50 ppm hypochlorite and also run to waste at maximum temperatures for 5 min each day. The temperature of the water from the shower was controlled by a temperature-sensitive valve which prevented water flow at temperatures >45° C, to prevent patient scalding. Therefore, water flowing through the shower pipe was always <45° C, as hot water was mixed with cold, and although the shower head was disinfected the extension shower hose between the valve and the shower head was not. When dismantled, linseed oil-based sealant was found on the hot water shower pipe joints in ward B but not in ward A.

Table 5 Bacterial counts from swab samples of water supplies in rm 2 and ward B (post-treatment)

Swabs recovery media	Cold tap (CFU per ml)	Hot tap (CFU per ml)	Shower head (CFU per ml)
Heterotrophic	100	30	confluent
Legionellae	30	20	confluent

Recovery and microscopic appearance of biofilms in the water supplies

Ward A (pre-treatment): The non-return hot water pipe supplying the sink unit constituted a dead-leg in which linseed oil-based sealant was observed and the pipe surface was covered with a dark soot-like layer. The water retrieved from the pipe was cloudy green, indicative of copper corrosion deposits. When viewed microscopically there was patchy film on the surface with evidence of bacteria and exopolysaccharides (EPS) (Figure 1). Large mounds or tubercles were also present on the copper pipe surface. Water from the cold water shower pipe was relatively clear. Many bacteria were observed on the surface. Water from the hot water pipe was very green in appearance and corrosion deposits as well as blackened areas were observed on the internal surface. The surface appeared to possess a thicker layer of scale and a greater amount of EPS in the hot than the cold supply. All microbial cells were metabolically active as detected by the reduction of CTC (Figure 2).

In this case acridine orange was used as a total stain [9], although it has also been used as a differential stain to detect activity, as related to an increase in cellular RNA, under laboratory controlled conditions in a chemostat [22].

Although Zimmerman [22] used iodonitrotetrazolium chloride to determine the number of respiring cells from an aquatic sample this technique could only be used with transmitted light to observe the dark red non-fluorescent intracellular deposit. However, more recently fluorescent

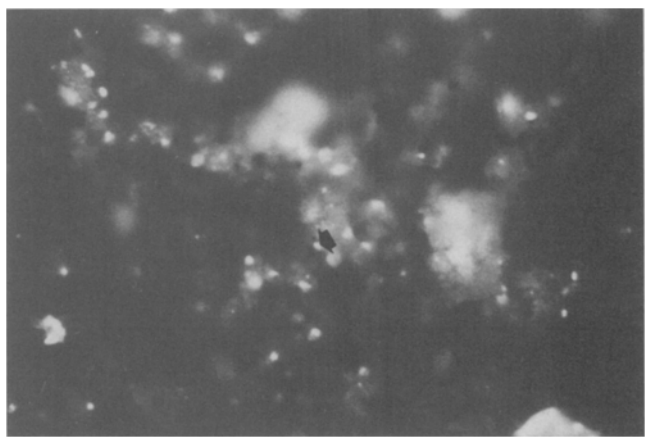

Figure 1 Demonstration of a mosaic and patchy film on the pre-treated copper tube pipe removed from the hot water circuit of the sink-unit in ward A. Bacterial colonies (identified by arrow) are demonstrated on the surface

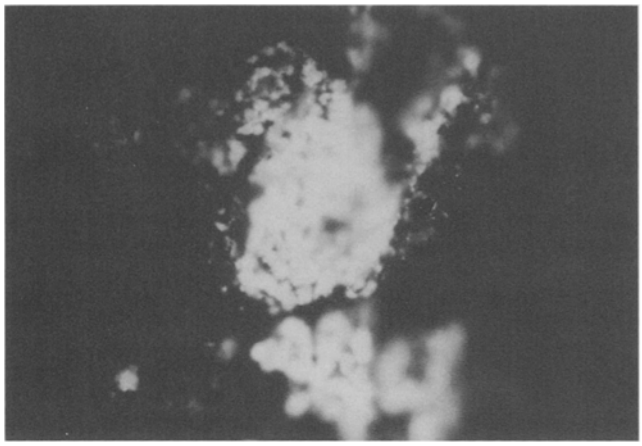

Figure 2 Copper pipe removed from pre-treated ward A hot shower circuit demonstrating a large microcolony in which all the microbial cells present were shown to be metabolically active as detected by the reduction of CTC (as shown by arrow)

metabolic probes for the direct visualisation of actively respiring bacteria have been used [16]. The CTC dye is colourless; however, due to respiratory activity the dye is reduced via the microbial electron transport system to a fluorescent formazan product [17]. Due to the fluorescence produced, actively respiring bacteria can be detected on opaque materials such as copper plumbing tube without having to remove the biofilm for analysis.

Ward B (post-treatment): The water extracted from the cold circuit pipe of the shower unit was only slightly yellow. The internal surface of the pipe appeared very clean and free from corrosion deposits. No linseed oil-based sealant (Bosswhite) was evident on the pipe joints. In contrast with the observations made of the pipe before ClO_2 treatment, there were few bacteria present on the cold water pipe surface (Figure 3). Water from the hot supply to the shower appeared slightly yellow-green and the pipe had a copper corrosion deposit running horizontally along the internal surface. Significant amounts of debris consisting of scale and bacteria were present on the inside surface of

Figure 3 Surface of post-treated copper pipe removed from the cold water circuit of the shower in ward B demonstrating few bacteria present on the cold water pipe's surface

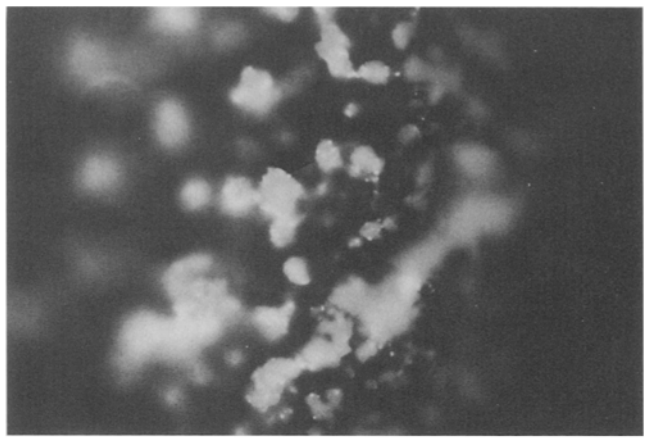

Figure 4 Significant amounts of debris consisting of scale and bacteria were present on the inside surface of the shower unit hot copper pipe of ward B (arrow indicates the position of the bacteria in the microcolonies)

the pipe (Figure 4). When stained for vitality approximately 50% of the microorganisms were metabolically active, suggesting the presence of a thermotolerant population of microorganisms in the shower pipe which was supplied with water originally at 60° C. These microorganisms were presumably resistant to or had been shielded in the biofilm from the effects of the ClO_2 during the time of treatment.

The water from the hot water pipe of the sink unit was slightly turbid. Although there were external signs of corrosion due to seepage from the sealing rings there was no obvious internal corrosion. Scaling was evident by microscopic examination but few bacteria were observed.

Audrey Leigh Ward (control): This ward represented another new complex within the hospital built and commissioned at the same time as the nucleus block containing wards A and B. The former had not previously been reported as being culture-positive for *L. pneumophila*. This new complex was different in the respect that the water storage tanks and calorifier were in separate areas. The water storage tanks were in the roof space above the ward. The water was maintained at approximately 15° C in four plastic tanks of smaller capacity (of 1240 L each) compared

Figure 5 Copious amounts of bacteria were observed, particularly on the surface of the cold water copper pipe from Audrey Leigh control ward (arrow indicates a microcolony)

Table 6 Bacterial counts if water supplying Audrey Leigh Ward

Recovery media	Cold water (CFU per ml)	Hot water (CFU per ml)
Heterotrophic	1700	> 3000
Legionellae	380	300

to wards A and B, such that only 24 h supply of water was held at any one time. Access could not be gained to these tanks for inspection due to the nature of the fittings on the lids. The calorifiers were located in the basement of the building and the temperature of the water from the outlet of the calorifier was measured at 60° C, and 54° C at the return.

A relatively high number of aerobic heterotrophic bacteria were recovered during sampling, particularly from both the hot and cold water supplies (Table 6). No *L. pneumophila* were recovered.

When the shower units were dismantled, linseed oil-based sealant was found in both the hot and cold water systems. Although the water from the pipes was only slightly cloudy there were internal corrosion deposits on both the hot and cold water pipes.

Copious amounts of EPS and bacteria were observed, particularly on the surface of the cold water pipe (Figure 5). EPS has been demonstrated to chelate copper ions and participate in microbially induced corrosion [12]. Bremmer *et al* [6] studied the presence of microorganisms on copper specimens using atomic force microscopy and demonstrated the difficulty of studying copper tube that has not been abraded. Using DIC and fluoresence microscopy demonstrates the rapidity with which field samples can be analysed and viewed to investigate biofouling [21]. The whole internal surface of the pipe from the hot water supply was covered with scale (Figure 6). No *L. pneumophila* were cultured from the water or pipe samples in the Audrey Leigh Ward's water system.

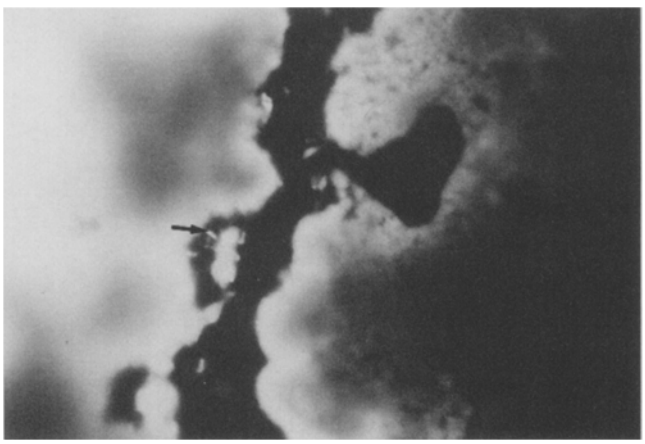

Figure 6 The whole internal surface of the hot water copper pipe from the Audrey Leigh control ward was covered with scale in which bacteria (indicated by arrow) were occasionally observed

Conclusion

L. pneumophila was isolated on one occasion only from the cold water supply before treatment, but conditions that would favour the survival and growth of legionellae were present, ie a low chlorine content of the water, storage in cold water storage tanks at temperatures above 20° C, the presence of banned organic jointing compounds, the hot water supply being below the recommended temperatures and failure to disinfect the shower hoses.

The remedial measure of shock dosing with ClO_2 over an 8-h period lowered the bacterial numbers in the planktonic phase, and although the procedure did not completely remove biofilm and scale during this time there was significantly less bacterial biofilm present after the disinfection programme had been completed. All biofilms found before and after ClO_2 treatment were found to demonstrate vitality by the reduction of the fluorochrome CTC.

Acknowledgements

We are grateful to Mr J Lacey and the Estates Office as well as to Dr John V Lee for their advice and assistance.

References

1 Anonymous. 1990. The control of legionellosis including legionnaires' disease. Health and Safety booklet HS (G) 70. HM Stationery Office, London.
2 Back JP and RG Kroll. 1991. The differential fluorescence of bacteria stained with acridine orange and the effects of heat. J Appl Bacteriol 71: 51–58.
3 Bartlett LR, AD MacRae and JT MacFarlane. 1986. Legionella Infections. Edward Arnold (Publishers), London.
4 Baskerville AR, B Fitzgeorge, M Broster, P Hambleton and PJ Dennis. 1981. Experimental transmission of legionnaires' disease by exposure to aerosols of *Legionella pneumophila*. Lancet ii: 1389–1390.
5 Best M, VL Yu, J Stout, A Goetz, RR Muder and F Taylor. 1983. Legionellaceae in the hospital water supply–epidemiological link with disease and evaluation of a method of control of nosocomial Legionnaires' disease and Pittsburgh pneumonia. Lancet ii: 307–310.
6 Bremmer PJ, GG Geesey and B Drake. 1992. Atomic force microscopy examination of the topography of a hydrated bacterial biofilm on a copper surface. Curr Microbiol 24: 223–230.
7 Burman NP and JS Colbourne. 1979. Effect of non-metallic materials on water quality. J Water Engin Sci 1: 11–18.
8 Colbourne JS and PJ Dennis. 1989. The ecology and survival of *Legionella pneumophila*. J Inst Water Environ Man 3: 345–350.
9 Daley RJ and JE Hobbie. 1975. Direct counts of aquatic bacteria by a modified epifluorescent technique. Limnol Oceanog 20: 875–882.
10 Dennis PJ, JA Taylor, RB Fitzgeorge, CLR Bartlett and GI Barrow. 1982. *Legionella pneumophila* in water plumbing systems. Lancet i: 949–951.
11 Dennis PJ, CLR Bartlett and AE Wright. 1984. Comparison of isolation methods for *Legionella* spp. In: *Legionella*: Proceedings. 2nd International Symposium (Thornsbury C, A Balows, JC Feeley and W Jakubowski, eds), pp 294–296, American Society of Microbiology, Washington.
12 Geesey GG and PJ Bremmer. 1992. Interactions of exopolymers of corrosive biofilm microorganisms with copper ions. In: Proceedings of the NSF-Conicet Workshop on Biocorrosion and Biofouling, pp 36–41, Buckman Laboratories, USA.
13 Mauchline WS and CW Keevil. 1991. Development of the BIOLOG substrate utilisation system for identification of *Legionella pneumophila*. Appl Environ Microbiol 57: 3345–3349.
14 Pasculle AW, JC Feeley, RJ Gibson, LG Cordes, RL Myerowitz, CM Patton, GW Gorman, JW Ezzell and JN Dowling. 1980. Pittsburgh pneumonia agent: direct isolation from human lung tissue. J Infect Dis 141: 727–732.

15 Reasoner DJ and EE Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49: 1–7.
16 Rodriquez G, G Phipps, D Ishiguro and HF Ridgway. 1992. Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. Appl Environ Microbiol 58: 1801–1908.
17 Schaule G, H-C Gleming and HF Ridgway. 1993. Use of 5 cyano-2-3-ditoyl tetrazolium chloride for quantifying bacteria and sessile respiring bacteria in drinking water. Appl Environ Microbiol 59: 3850–3857.
18 States SJ, LF Conley, JM Kuchta, BM Oleck, MJ Lipovich, RS Wolford, RM Wadowsky, AM McNamara, JL Sykora, G Keleti and RB Yee. 1987. Survival and multiplication of *Legionella pneumophila* in municipal drinking water systems. Appl Environ Microbiol 53: 979–986.
19 Wadowsky RM, RB Yee, L Mezmar, EJ Wing and JN Dowling. 1982. Hot water systems as sources of *Legionella pneumophila* in hospital and non-hospital plumbing fixtures. Appl Environ Microbiol 43: 1104–1110.
20 Walker JT, J Rogers and CW Keevil. 1994a. An investigation of the efficacy of a bromine containing biocide on an aquatic consortium of planktonic and biofilm microorganisms including *Legionella pneumophila*. Biofouling 8: 47–54.
21 Walker JT, D Wagner, W Fischer and CW Keevil. 1994b. Rapid detection of biofilm on corroded copper pipes. Biofouling 8: 55–63.
22 Zimmerman R, R Iturriaga and J Becker-Birck. 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl Environ Microbiol 36: 926–935.