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Microbial Biofilm Formation and Contamination of Dental-Unit Water Systems in General Dental Practice

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Dental-unit water systems (DUWS) harbor bacterial biofilms, which may serve as a haven for pathogens. The aim of this study was to investigate the microbial load of water from DUWS in general dental practices and the biofouling of DUWS tubing. Water and tube samples were taken from 55 dental surgeries in southwestern England. Contamination was determined by viable counts on environmentally selective, clinically selective, and pathogen-selective media, and biofouling was determined by using microscopic and image analysis techniques. Microbial loading ranged from 500 to 10^5 CFU · ml^{-1}; in 95% of DUWS water samples, it exceeded European Union drinking water guidelines and in 83% it exceeded American Dental Association DUWS standards. Among visible bacteria, 68% were viable by *Bac*Light staining, but only 5% of this "viable by *Bac*Light" fraction produced colonies on agar plates. *Legionella pneumophila*, *Mycobacterium* spp., *Candida* spp., and *Pseudomonas* spp. were detected in one, five, two, and nine different surgeries, respectively. Presumptive oral streptococci and *Fusobacterium* spp. were detected in four and one surgeries, respectively, suggesting back siphonage and failure of antiretraction devices. Hepatitis B virus was never detected. Decontamination strategies (5 of 55 surgeries) significantly reduced biofilm coverage but significantly increased microbial numbers in the water phase (in both cases, $P < 0.05$). Microbial loads were not significantly different in DUWS fed with soft, hard, deionized, or distilled water or in different DUWS (main, tank, or bottle fed). Microbiologically, no DUWS can be considered "cleaner" than others. DUWS deliver water to patients with microbial levels exceeding those considered safe for drinking water.

The water obtained from dental units via 3-in-1 syringes, air rotors, and low-speed handpieces may be heavily contaminated with microorganisms and thus may be a potential source of infection for both practice staff and patients (5, 34). The range of microorganisms isolated includes both environmental organisms (e.g., *Moraxella* spp. and *Flavobacterium* spp.) and opportunistic and true human pathogens (e.g., *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Mycobacterium* spp., and *Staphylococcus* spp.) (10, 14, 16, 21, 30). Such organisms may originate from incoming local water supplies, although organisms commonly found in the oral cavity have also been recovered (37, 38), suggesting that some bacteria may be derived from the patient following back siphonage. The most common cause of dental-unit water contamination is believed to be the formation and subsequent sloughing off of microbial biofilms from the surfaces of tubing within dental-unit water systems (DUWS) (22, 36). To date viruses have not been detected in DUWS (37).

Microorganisms persist in DUWS by growing as a multispecies biofilm on the inner surface of the plumbing (14, 16, 21). Biofilms may be difficult to remove from surfaces, and the bacteria within biofilms are more resistant to antimicrobial agents (12, 17, 25) than planktonic cells. This antimicrobial resistance is a result of a number of factors that may include (i) binding of the agent, (ii) a lack of penetration of inhibitors, (iii) the localization of neutralizing enzymes, (iv) the low growth rate of the microbes, and (v) the expression of a resistant phenotype due to surface growth (9). Biofilms may also enhance the survival of fastidious pathogens such as *L. pneumophila* in water distribution systems (32). Up to 25% of DUWS have been shown to be contaminated with this bacterium (8).

There are currently no rational, evidence-based guidelines available to dentists for the control of DUWS contamination. This study is the first to focus on DUWS in typical general dental practices and to compare systematically different types of DUWS (bottle, main, or header tank fed) and units supplied with deionized, distilled, soft, or hard water. Biofilms play an important role in the microbial contamination of water systems; therefore, we have investigated biofilms using conventional viable counting of bacteria, as well as microscopy and image analysis and vital staining, in parallel with equivalent (planktonic) water samples. We also assessed the nature of the microbial contamination by detecting "environmental" and oral organisms as well as a range of potential pathogens, including the hepatitis B virus surface antigen (HBsAg).

MATERIALS AND METHODS

Survey of general dental practices. Fifty-five DUWS were selected for study in 21 general dental practices in southwestern England. The units selected represented units in use and included 32 supplied by bottled water, 20 supplied by mains water, and 3 supplied by header tank systems. Of these, 33 were supplied with hard water, 11 with soft water, 9 with deionized water, and 2 with distilled water. Five of the sampled DUWS (all bottle fed) were reported to have been sanitized. The sanitization regimens in four cases consisted of a weekly 5-min addition of a 1:10 dilution of Milton sterilizing fluid (neat solution contains 2% [wt/wt] sodium hypochlorite and 16.5% [wt/wt] NaCl; Procter & Gamble Ltd., Weybridge, Surrey, United Kingdom). The other sanitized unit was treated by the continuous addition of 1:10 diluted Corsodyl (neat solution contains 0.2% [wt/vol] chlorhexidine gluconate; SmithKline Beecham, Brentford, United Kingdom).

Sampling of DUWS. Samples were taken from four points in each device at approximately mid-morning: (i) the 3-in-1 syringe's (the pistol-like device de-

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signed to deliver air, water, or air/water into the mouth during dental treatment) distal outlet (the water line sample), (ii) a section of the water line tubing supplied to the 3-in-1 syringe for biofilm analysis (the water line biofilm), (iii) the air rotor water line (the air rotor water sample), and (iv) the air line (as a control sample for detection of background contamination of the tubing—the air line biofilm).

Water samples were processed in the following way. Approximately 100 ml of water was passed through a sterile nozzle into a sterile water bottle containing 0.1 g of sodium thiosulfate to remove any residual disinfectant (3) (Abinghurst Ltd., Northampton, United Kingdom). The samples were returned to the laboratory in a cool box (4 to 8°C) within 3 h and then filtered through 100-ml-capacity, 0.2-μm-pore-size analytical test filter funnels (Techware, Poole, United Kingdom) in order to recover the waterborne microorganisms. The membrane was removed from the funnel with sterile forceps and placed in a screw-cap sterile container (Elkay Products Inc., Shrewsbury, Mass.). Organisms were washed from the membrane by vortexing the container for 1 min in 10 ml of sterile phosphate-buffered saline (PBS).

Biofilm samples were taken using the following procedure. External DUWS tubing surfaces were wiped with a sterile alcohol wipe (CS Dental Supplies, Redhill, United Kingdom) and approximately 5 cm of the tubing was cut off with presterilized (121°C for 15 min) scissors. The tubing section was then placed in a bottle containing enough sterile water to cover the samples. All samples were again transferred in a cool box to the laboratory within 3 h. Tubing was sectioned to obtain a specimen representing 1 cm^2. The surfaces were rinsed in nonflowing sterile PBS to remove planktonic cells. Using sterile (121°C for 15 min) dental probes, the surface biofilm was scraped into 1 ml of sterile PBS. The scraping was checked by microscopy to determine that all the biofilm had effectively been removed from the surface.

Viable counts of selected bacteria. Total viable counts were carried out on decimal dilutions of the water and biofilm samples (in sterile PBS) and were used as the definitive measure of total microbial contamination of the water passing through the DUWS. This was compared with both the European Union standard for potable water (3) and the American Dental Association standards for DUWS (1). Samples of appropriate dilutions of biofilm and water samples were plated onto a range of selective and nonselective agar media. The media were (i) Columbia blood agar for oral streptococci, *Actinomyces* spp., and oral anaerobes (incubated anaerobically at 37°C for up to 10 days under a gas phase of 80% [vol/vol] CO_2–10% [vol/vol] H_2–10% [vol/vol] N_2) (colonies were assessed morphologically and by Gram staining); R2A agar for environmental isolates (29), incubated at 37°C for up to 7 days; (iii) CFC supplement SR103 for *Pseudomonas* spp. (27), incubated at 37°C for up to 48 h; (iv) MacConkey agar CM7 for enterobacteria (15), incubated at 37°C for up to 48 h; and (v) Sabouraud dextrose agar for *Candida* spp. (26), incubated for up to 7 days.

For the enumeration of *Legionella* and *Mycobacterium* spp., aliquots of the samples were pretreated either with heat (at 50°C in a water bath for 30 min) or with acid (1 ml of sample added to 1 ml of 1 M HCl for 15 min and then neutralized with KOH [4, 13]). Aliquots of untreated and treated samples were then plated onto BCYE agar for the enumeration of *Legionella* spp. (11), and the plates were incubated aerobically at 37°C for up to 10 days. Colonies morphologically typical of *Legionella* spp. were counted, and the serogroup was determined using a latex agglutination kit (Pro-Lab Diagnostic, Neston, United Kingdom). Similar aliquots were dispensed onto Middlebrook agar plus OADC 7H10 for *Mycobacterium* spp. (24) (incubated aerobically at 37°C for up to 30 days). Representative colonies were assessed using the Ziehl-Neelsen technique for the detection of acid-fast bacilli and examined microscopically.

Detection of viruses. Aliquots of each unfiltered water sample were frozen (−20°C) and transported frozen to the University of Liverpool. The level of HBsAg was measured by the Monolisa Ag HB Plus assay (Sanofi Pasteur, Guildford, United Kingdom). Samples (50 μl) were processed according to the manufacturer's instructions, and the resultant chromophore was read on a Dynex MR7000 plate reader at 420 and 620 nm. Positive and negative controls were included in every batch.

Microscopy and image analysis. The extent of biofouling in DUWS tubing was assessed by using image analysis. Lengths of tubing were aseptically sectioned into thin strips (approximately 2 to 3 mm wide) and stained for 1 min with 50 μl of prefiltered (0.2-μm pore size; Sartorious, Epsom, United Kingdom) propidium iodide (1 mg of stock · ml of sterile distilled $water^{-1}$; Sigma Poole, United Kingdom) before being gently rinsed twice in nonflowing sterile distilled water to remove planktonic and loosely adhered cells. The tubing surface was then examined using a Nikon Labophot 2 microscope with episcopic fluorescence and a 50× water immersion lens (33). Ten representative images were captured as computer images (TIFF) for analysis of percentage of coverage, using Optimas Software (Optimas Datacell, Finchhampstead, United Kingdom).

Viability assay. A 3-cm length of DUWS tubing was aseptically sectioned horizontally into four equal sections, with the control sample immersed in 4% (vol/vol) formalin (Western Solvents, Westbury, United Kingdom) for 10 min. Equal volumes of *Bac*Light (Molecular Probes, Eugene, Oreg.) reagents A and B were added to PBS to prepare the viability probe (final concentration, 3 μg/ml) (20). Control (killed) and test (untreated) samples were assayed with the *Bac*Light live/dead viability probe. Following a 15-min reaction, the samples were rinsed in nonflowing PBS to remove excess stain and assessed using the microscopy system described above. Microscopic counts of the stained cells were also carried out using an improved Neubauer counting chamber (Hawksley, London, United Kingdom) with the results compared against total plate counts.

Statistical analysis. Statistical analyses were carried out using Excel (Microsoft Office) and Statgraphics (STSC Inc., Rockville, Md.). Bacterial loads in different types of water (deionized, distilled, soft, and hard) and from DUWS with different water sources (main, bottle, or tank fed) were compared using a two-way analysis of variance (ANOVA) on log-transformed viable counts. Where significant differences were indicated by ANOVA, individual groups were then compared by the least-significant-difference method. Percentage coverage data were compared using the nonparametric Kruskal-Wallis test. Biofilm and planktonic counts from the same surgeries were also analyzed for correlation using the nonparametric Kendall rank correlation analysis. Statistical significance was assumed at a P value of <0.05.

RESULTS

Survey of general dental practices and sampling of DUWS. Fifty-five water line and air rotor water line fluid (planktonic) samples and 47 water line and air line biofilm samples were taken during the survey. Eleven surgeries used soft (<50 ppm $CaCO_3$) water, 33 used hard (>250 ppm $CaCO_3$) water, 9 used deionized water, and 2 used distilled water. Only 3 surgeries had tank-fed (break tank) DUWS, 32 used bottle-fed systems, and 20 had main-fed systems. Five of the 55 surgeries reported recent disinfection of their DUWS. For the purposes of clarity, each surgery was assigned a number, to which appropriate sections of the results refer.

Bacterial contamination of DUWS. (i) Water line microbial contamination. A geometric mean of 2.9×10^3 CFU of bacteria · ml^{-1} was recovered from the water of the DUWS (range, 7 CFU · ml^{-1} to 6.4×10^4 CFU · ml^{-1}). The number of bacteria recovered varied with the type of water supplied to the DUWS. Viable counts from distilled water were greater than those from hard, soft, or deionized water (Table 1). Similarly, more bacteria were recovered from units supplied by bottles than from those supplied by mains or tanks. However, the differences between DUWS types and between units supplied with different types of water were not significant at the 95% confidence level (two-way ANOVA, $P = 0.28$ and 0.58, respectively).

Surgeries 13, 14, 15, 16, and 43 had units that were reportedly sanitized (Fig. 1). Significantly greater numbers of bacteria were found in the water phase of units treated with disinfectant than in the untreated units (ANOVA, $P = 0.048$). A geometric mean of 1.6×10^4 CFU · ml^{-1} (range, 6.0×10^3 CFU · ml^{-1} to 3.6×10^4 CFU · ml^{-1}) was recovered from treated units' water, compared with a geometric mean of 2.8×10^3 CFU · ml^{-1} (range, 7.0 CFU · ml^{-1} to 6.4×10^4 CFU · ml^{-1}) from the untreated systems.

Notable bacterial species isolated from the water lines of the DUWS were slow-growing *Mycobacterium* spp. from surgeries 21, 22, and 44, *Fusobacterium* spp. from surgery 19, and fluorescent *Pseudomonas* spp. from surgeries 16, 32, 35, 47, and 48.

(ii) Air rotor water line microbial contamination. Similar numbers of bacteria (geometric mean, 3.3×10^3 CFU · ml^{-1}; range, not detectable to 9.5×10^4 CFU · ml^{-1}) (Table 1) were found in the water from the air rotor water line and the water line. The numbers of bacteria in air rotor line water were significantly different for different DUWS types (two-way ANOVA, $P = 0.04$), with the numbers of microorganisms from main-fed units being significantly greater than from the bottle-supplied units ($P < 0.05$). Higher numbers of bacteria were recovered from hard water than from deionized, distilled, or soft water (Table 1), though these differences did not quite reach significance (two-way ANOVA, $P = 0.055$).

The following specific microorganisms were detected from air rotor water line samples: *Candida* spp. from surgery 21 and fluorescent *Pseudomonas* spp. from surgeries 7, 16, 33, 35, 44, 47, and 49.

TABLE 1. Comparison of viable counts from the DUWS water samples

Sample	Viable count (CFU · ml^{-1}) from water samples taken from:						*n*
	Water lines			Air rotor water lines			
	Geometric mean	Minimum	Maximum	Geometric mean	Minimum	Maximum	
Water type							
Soft	2,590	7	31,000	1,224	0	40,000	11
Hard	3,290	32	64,000	5,063	0	95,000	33
Deionized	1,740	68	40,000	2,699	55	30,000	9
Distilled	5,970	4,400	8,100	2,013	1,350	3,000	2
Supply type							
Tank fed	1,550	480	3,700	1,881	360	7,400	3
Bottle fed	3,583	7	36,000	1,802	0	95,000	32
Main fed	2,217	32	64,000	4,914	42	40,000	20
Total	2,874	7	64,000	3,325	0	95,000	55

(iii) Water line biofilm. A geometric mean of 9.2×10^2 CFU · cm^{-2} (range, not detectable to 6.4×10^4 CFU · cm^{-2}) was recovered from the water line biofilm of the DUWS (Table 2). More bacteria were recovered from the distilled-water units than from the hard-, soft-, or deionized-water units; similarly, more were recovered from the main-fed units than from those supplied with either bottled or tank water (Table 2). However, the differences between the numbers of CFU recovered from biofilms in different DUWS types and from units supplied with different types of water were not significant (two-way ANOVA, $P = 0.89$ and 0.91, respectively). The numbers of bacteria in biofilms were significantly associated with the numbers in the corresponding planktonic phase (Kendall rank correlation; $\tau = 0.31$, $P = 0.04$).

The numbers of bacteria recovered from the biofilms in the "sanitized" units (surgeries 13, 14, 15, 16, and 43) were significantly lower than the numbers recovered from the untreated surgeries (ANOVA, $P = 0.04$). *Mycobacterium* spp. were recovered from the biofilms of surgeries 4 and 23.

(iv) Air line biofilm. Significantly lower numbers of bacteria were found in the DUWS air lines than in the water lines. A geometric mean of 5.1×10^1 CFU · cm^{-2} (range, not detectable to 3.0×10^4 CFU · cm^{-2}) were recovered from the air lines, compared with a geometric mean of 9.2×10^2 CFU · cm^{-2} from the water line (paired *t* test, $P \ll 0.0001$) (Table 2).

However, a number of medically important bacteria were detected in air line biofilm samples: *L. pneumophila* in surgery

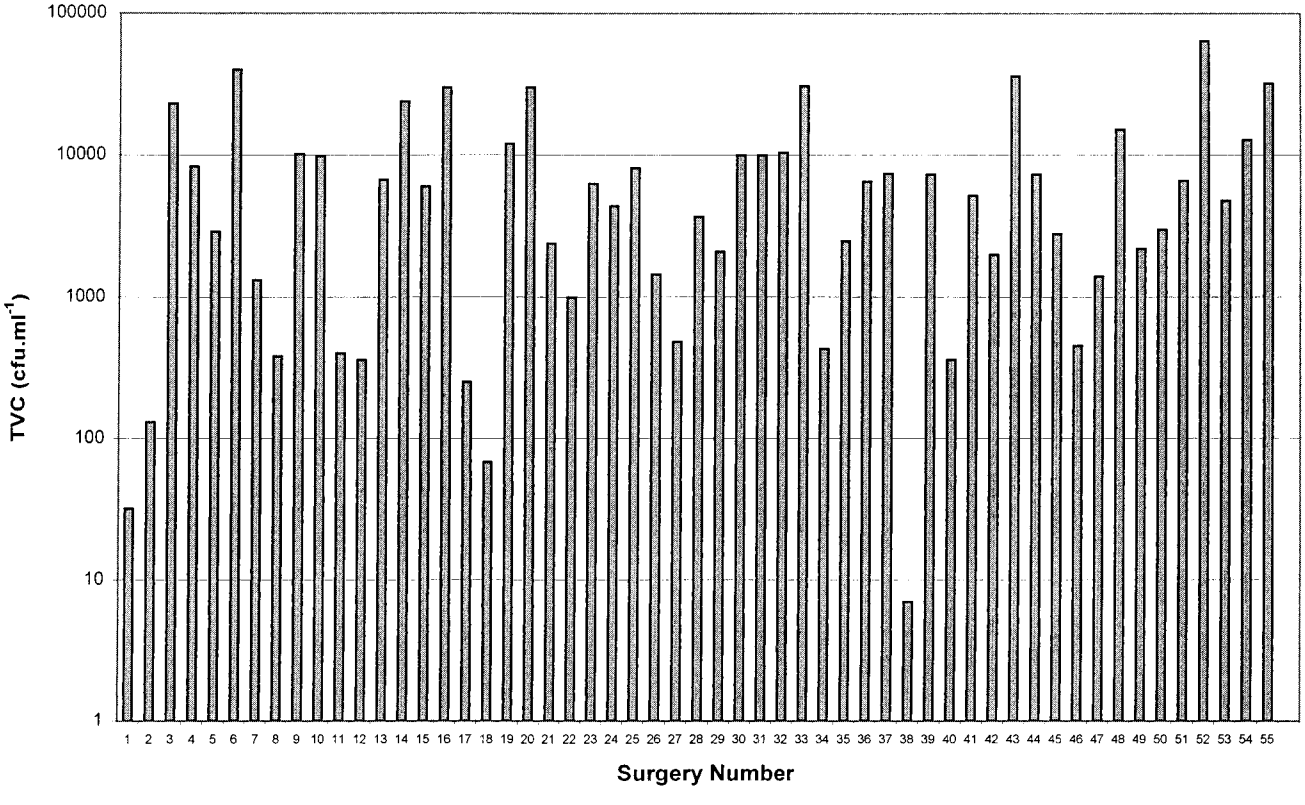

FIG. 1. Total viable counts (TVC) of water samples from DUWS in different dental surgeries.

TABLE 2. Comparison of viable counts from the DUWS biofilm samples

Sample	Viable count (CFU · cm^{-2}) from biofilm samples taken from:						*n*
	Water lines			Air lines			
	Geometric mean	Minimum	Maximum	Geometric mean	Minimum	Maximum	
Water source							
Soft	892	2	42,000	33	0	30,000	10
Hard	1,103	0	64,000	85	0	3,200	27
Deionized	411	5	500	10	0	1,600	8
Distilled	2,200	1,100	4,400	259	12	5,600	2
Supply type							
Tank fed	1,268	40	8,800	33	0	2,000	3
Bottle fed	717	2	42,000	26	0	30,000	29
Main fed	1,383	0	64,000	199	0	30,000	15
Total	917	0	64,000	51	0	30,000	47

24, *Candida* spp. in surgery 30, and *Lactobacillus* spp. and *Streptococcus* spp. in surgery 19.

(v) Percentage of biofilm coverage in water and air lines. The average coverage of water line surfaces was 43% (range, 0.01 to 94%) (Table 3). Significantly less coverage was observed on the DUWS units supplied with soft water than on those supplied with hard, distilled, or deionized water (Kruskal-Wallis test, $P = 0.035$). The highest coverage was found on those units supplied with deionized water, followed by those using hard, distilled, and soft water (Table 3). There was no significant difference among units with different types of water supply, although it was observed that a higher percentage of coverage was recorded in the units supplied by water mains than in bottle- or tank-fed systems.

The results of the percent coverage analysis of the air line demonstrated that the average coverage was 5.2% (range, 0.1 to 74%) (Table 3). A higher degree of biofouling was observed on the tubing surfaces of those DUWS that were supplied with hard water than in those supplied by deionized, distilled, or soft water (Table 3). This difference was statistically significant (Kruskal-Wallis test, $P = 0.04$). In terms of the type of water supply, more extensive biofouling coverage was observed on those units supplied by mains water than on those supplied by tanks or bottles, although this difference was not significant (Kruskal-Wallis test, $P = 0.35$).

TABLE 3. Comparison of percentage of coverage from the DUWS biofilm samples

Sample	% Coverage from biofilm samples taken from:						*n*
	Water lines			Air lines			
	Avg	Minimum	Maximum	Avg	Minimum	Maximum	
Water source							
Soft	21	0.1	62	0.0	0.1	0.1	10
Hard	48	0.01	94	8.8	0.1	74	27
Deionized	54	0.5	94	1.9	0.1	6.4	8
Distilled	44	5	83	0.1	0.1	0.1	2
Supply type							
Tank fed	33	25	50	3.5	0.1	8	3
Bottle fed	41	10	94	2.9	0.1	64	29
Main fed	50	0.1	92	10	0.1	74	15
Total	43	0.01	94	5.2	0.1	74	47

(vi) Assessment of viability. Due to fluorescein binding to the DUWS tubing surfaces, viable cells could not be discriminated against the background; therefore, biofilm counts could not be assessed in situ using the *Bac*Light technique. Resuspended biofilm samples were analyzed and a mean of 68% of the total visible bacterial population was determined to be viable. A mean of 5% (three determinations) of this "viable by *Bac*Light" fraction produced viable colonies on agar plates.

(vii) Detection of viruses. None of the samples taken from the DUWS were positive for HBsAg.

DISCUSSION

Water supplied by 95% of general dental practice DUWS units failed current European Union potable-water guidelines on microbial load (i.e., loads were >100 CFU · ml^{-1}) (2), and 83% failed American Dental Association recommendations for DUWS water quality (<200 CFU · ml^{-1}) (1). The bacterial numbers reported here were comparable to those found in a number of other studies (6, 28) and lower than some (19, 35). These values probably underestimate the true microbial load to which a patient is exposed, since we also demonstrated that only 3% of the microscopically visible bacteria produced colonies on agar plates. Other bacteria may be either in a temporarily nonculturable state or may represent the large fraction of the microflora from many natural habitats which remain "as yet uncultured" (as discussed in the review by Barer and Harwood [7]).

The most common pathogens detected were fluorescent *Pseudomonas* spp. (16% of samples were positive). *L. pneumophila* was isolated on only one occasion, which was far less frequent than reported in previous studies in a dental hospital and in a mixture of institutional and private practices (25 and 6% isolation frequencies, respectively) (4, 8). Larger water distribution systems are known frequently to harbor this organism (C. L. Bartlett, J. B. Kurtz, J. G. Hutchison, G. C. Turner, and A. E. Wright, Letter, Lancet **ii:**1315, 1983). We detected *Mycobacterium* spp. in ca. 5% of surgeries, a lower detection rate than that reported by a previous study (30). These isolates were not identified, so their pathogenic potential is unknown, although several non-*Mycobacterium tuberculosis*, non-*Mycobacterium avium* species of mycobacteria are associated with a variety of infections in humans (18). Presumptive oral streptococci were identified in 7% of DUWS water samples, suggesting the failure of antiretraction devices in these systems and thus raising the possibility of cross-infec-

tion between successive patients. No HBsAg was detected in any sample.

Some dentists perceive that certain types of DUWS may be less prone to microbial contamination than others. We found no significant differences between different DUWS systems, regardless of whether these systems were main, bottle, or header tank fed or whether the water supplied to them was hard, soft, deionized, or distilled. Thus, no DUWS can be considered superior in microbiological terms to any other or can be called microbiologically "clean." Water from air rotor lines and from 3-in-1 handpieces was contaminated to a similar degree, emphasizing that air rotors should not be used as an aid in any dental surgical procedures.

The significant correlation between the numbers of bacteria recovered from biofilms and from water samples from the same units suggests that the biofilms may seed the water with bacteria and vice versa. Strategies developed for the control of DUWS contamination must eliminate both the biofilms and the waterborne bacteria in these systems (23, 31, 36). In addition, although significantly lower levels of biofilm contamination were found in the five units reported to have been recently decontaminated, more bacteria were recovered from the water phase in these systems. Decontamination with detergents or with inorganic acids (including hypochlorous acid) could increase the risk of release of organisms from biofilms and thus increase the numbers of bacteria in the water phase (J. S. Colborne, P. J. Dennis, J. V. Lee, and M. R. Bailey, Letter, Lancet **i:**684, 1987). Inadequate decontamination regimens may thus increase the hazards associated with DUWS water.

In conclusion, improved, evidence-based practical methods for controlling the microbial contamination of DUWS are urgently needed. This is particularly important in view of the increasing numbers of medically compromised and immunocompromised patients receiving regular dental treatment.

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