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Multiparametric Analysis of Waterline Contamination in Dental Units

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Microbial contamination of dental unit waterlines is thought to be the result of biofilm formation within the small-bore tubing used for these conduits. Systematic sampling of 121 dental units located at the dental school of Université de Montréal showed that none of the waterlines was spared from bacterial contamination. Multilevel statistical analyses showed significant differences between samples taken at the beginning of the day and samples taken after a 2-min purge. Differences were also found between water from the turbine and the air/water syringe. Random variation occurred mainly between measurements (80%) and to a lesser extent between dental units (20%). In other analyses, it was observed to take less than 5 days before initial bacterial counts reached a plateau of 2×10^5 CFU/ml in newly installed waterlines. *Sphyngomonas paucimobilis*, *Acinetobacter calcoaceticus*, *Methylobacterium mesophilicum*, and *Pseudomonas aeruginosa* were the predominant isolates. *P. aeruginosa* showed a nonrandom distribution in dental unit waterlines, since 89.5% of the all the isolates were located in only three of the nine clinics tested. Dental units contaminated by *P. aeruginosa* showed significantly higher total bacterial counts than the others. By comparison, *P. aeruginosa* was never isolated in tap water remote from or near the contaminated dental unit waterlines. In conclusion, dental unit waterlines should be considered an aquatic ecosystem in which opportunistic pathogens successfully colonize synthetic surfaces, increasing the concentration of the pathogens in water to potentially dangerous levels. The clinical significance of these findings in relation to routine dental procedures is discussed.

Presently, public health standards consider water to be safe for human consumption when it contains a maximum of 500 CFU/ml, when it is free of coliforms, and when its nephelometric turbidity is less than 2 (15). Bacteria in natural aquatic environments have a marked tendency to interact with surfaces (41, 43). The formation of surface biofilms can be regarded as a universal bacterial strategy for survival and for optimum positioning with regard to available nutrients (9). In addition, biofilm bacteria are substantially resistant to surfactants, biocides, and antibiotics (19, 37). Two problems can arise from the presence of biofilms in a distributing aqueous system. First, the biofilm can clog pipes and tubings or interfere with the proper function of mechanical devices. Second, bacterial populations living in this protected mode of growth produce planktonic cells that contaminate fluids and alter their properties or, in the case of pathogens, can result in food poisoning or infections (1, 44). As a result, microbial biofilms constitute major industrial and medical concerns. These concerns are now being realized in the dental profession.

Every dental unit is equipped with small-bore flexible plastic tubing to bring water to the different handpieces, namely the air/water syringe, the ultrasonic scaler, and the high-speed handpiece. Potable municipal water normally supplies dental units, but a few dental units use independent distilled- or sterile-water reservoirs. It has long been known that the water collected at the output of dental unit waterlines (DUWL) is densely populated with microorganisms. Bacterial counts ranging from a few thousand to as high as 10^6 CFU/ml have been reported (2, 3, 5, 12, 13, 20, 22, 27, 28, 35). This dense, planktonic, microbial population is now known to be a reflection of the colonization of the waterlines by biofilms (36, 41). Water stagnation is thought to be in part responsible for the phenomenon (32).

The bacteria isolated from the water in dental units belong to the community of water bacteria (3, 13, 20, 22, 27, 29), but the finding that some potentially pathogenic microorganisms such as *Pseudomonas aeruginosa* (22, 27) and *Legionella pneumophila* (31, 35) may be present in the water used to perform intraoral and sometimes invasive treatments has led to some concern in the dental community (11, 15, 27, 36, 40).

The present investigation was designed to look at bacterial colonization of DUWL from a dynamic standpoint through multilevel analysis and species identification, distribution, and fluctuation in number over time.

MATERIALS AND METHODS

Collection and plating of water samples. Water samples were collected from 123 dental units at the dental school of Université de Montréal for bacterial identification and statistical analyses. Of these 123 units, 30 were included in a multilevel analysis described below. Municipal water supplies all dental units located in all the clinics, where they are connected in parallel; these include the Oral Surgery, Oral Diagnosis and Emergency, Pediatric Dentistry, Removable Prosthodontic, Orthodontic, Team Care Training, Preventive Care, Intra-Mural, and Multidisciplinary clinics. A 2- to 4-ml water sample was collected directly from the outlets of the polyethylene waterlines (internal diameter, 1 mm) of the air/water syringe and the high-speed drill. Samples were collected at the beginning of the work day before the dental unit was used and after a 2-min purge corresponding to an average of 125 ml of water for the high-speed drill and 300 ml for the air/water syringe. All the water samples were vigorously agitated with a vortex for 15 s. The plating was done by inoculating petri dishes with 100 μl of 1:10, 1:100, and 1:1,000 dilutions in duplicate or by using an automatic spiral plating system (Meyer Service & Supply, Lons Sault, Canada) after a 10-fold

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dilution of the sample. The enumeration was done with a magnifying glass and a counting grid.

Control samples (20 ml) were obtained from the nearest taps in each clinic and at the source upstream to the connection to the dental unit in selected units. These samples were filtered through a 25-mm polycarbonate filter (pore size, 0.22 μm; Millipore, Montréal, Canada) with a sterile syringe and a filter holder (Millipore). The filters were then placed on the surface of the culture medium in a petri dish and incubated.

Newly installed dental units (Kavo, Germany) at the dental school were also sampled by the same sampling technique just before their first clinical use.

Culture conditions. A modification of the medium of Reasoner and Geldreich (33) (termed R2Am in this report) was used. The composition is as follows: starch, 0.5 g; yeast extract, 0.5 g; tryptic peptone, 0.5 g; glucose, 0.5 g; K_2HPO_4, 0.3 g; $MgSO_4$, 0.05 g; succinate, 0.25 g; Casamino Acids, 0.5 g; agar, 7.5 g; and distilled water to 500 ml. Tryptone soy agar and sheep blood agar (Difco, Montréal, Canada) were also used. Bacteria were cultivated under aerobiosis and anaerobiosis (10% CO_2–10% H_2–80% N_2 in an anaerobic cabinet [Forma Scientific, Montréal, Canada]) for the determination of their dependency on oxygen and at 25 and 37°C for 24 to 480 h.

Identification and characterization of the microflora in DUWL. The different bacteria isolated from the dental unit water were characterized with respect to Gram stain, catalase and oxidase reaction, API Rapid NFT (nonfermenters), and API 20E (fermenters) (API Laboratories Products, St-Laurent, Canada). The Schaeffer and Fulton spore stain (8) was used to assess the presence of bacterial endospores. *P. aeruginosa* was isolated and cultured on selective PC medium (6) (kindly provided by J. Lagacé, Microbiologie, Faculté de Médecine, Université de Montréal) that contains 30 mg of phenanthroline (Sigma Chemical), 30 mg of 9-chloro-9[4-(diethylamino)phenyl]-9,10-dihydro-10-phenylacridine hydrochloride (C-390; Norwich Eaton Pharmaceuticals, Norwich, N.Y.), and 39 g of Columbia agar (Baxter Canlab, Montréal, Canada) per liter of distilled water. *Pseudomonas* agar P and *Pseudomonas* agar F were used to demonstrate the presence of pyocyanine and fluorescein pigments, respectively. Some of our isolates were sent to the Laboratoire de santé publique du Québec for analysis.

Three dental units were repeatedly sampled at the beginning of the work day, before the unit was used, to investigate the variation in the total number of microorganisms and the proportion of the predominant bacterial species. Sampling and counting were done by the pour plate method with R2Am. Direct counting was done by the direct epifluorescent filter method of Hobbie et al. (21). Briefly, 0.2 ml of sterile 0.1% acridine orange solution in water was mixed with 1.8 ml of a 1:100 dilution of the samples and incubated at room temperature for 2 min. The samples were then filtered through a sterile polycarbonate black filter (13 mm in diameter, 0.2 μm in pore size; Millipore) with a sterile 5-ml syringe. The filter was then placed on a microscope slide, and immersion oil and a coverslip were placed on top. The filters were observed at ×1,000 with a Zeiss Axiophot equipped for epifluorescence optics.

Statistical analysis and multilevel model. Thirty dental units were sampled repeatedly for a total of 208 occasions comprising the explanatory variables T_0 or T_2 (after 2 min of flushing), air/water syringe or high-speed drill, and the presence or the absence of *P. aeruginosa* in the water of the dental unit. Because the CFU per milliliter is more likely to be similar for measurements made on the same dental unit than are measurements made on different dental units, a two-level multilevel model was used for the statistical analyses (17, 39) on 30 randomly selected dental units throughout the nine clinics.

The response variable, y, is the $\log_{10}$ of the CFU per milliliter measured in 208 samples (occasions) on 30 dental units. The explanatory variables are the time at which the samples were taken (x), coded as either T_0 [0] or after 2 min of flushing [1]; the handpiece (z), coded as air/water syringe [0] or high-speed drill [1]; and the presence of *P. aeruginosa* (w), coded as absent [0] or present [1]. Because the samples were taken from a number of different dental units, we must incorporate, explicitly, the hierarchical two-level structure, in which the samples are grouped or clustered into dental units. This allows for the possibility that samples from the same dental unit are more alike in their bacterial count than are samples chosen at random from different dental units.

We use the subscript i to refer to the dental unit, and the subscript j to refer to the samples. There are n_i samples in the ith dental unit and a total of 30 dental units, for a total of 208 samples. The basic model is written as:

$$\gamma_{ij} = \alpha_0 + \alpha_i + \beta_1 x_{ij} + e_{ij} \quad (1)$$

In equation 1, α_0 is an overall constant term, α_i is the contribution of the ith dental unit to the CFU per milliliter, and e_{ij} is the contribution of the jth sample in the ith dental unit. This term is often referred to as a residual, since it represents the difference between what is predicted by the rest of the model and the actual observed value.

As it stands, equation 1 is essentially a single-level model, since the only random variation is between level 1 units (samples), although it does take into account the contributions from level 2 units (α_i). If there were only two or three dental units and we were interested in whether the bacterial count differed between them, equation 1 would be an appropriate model and could be analyzed by any standard multiple-regression or general linear model program. More generally, however, rather than considering the dental units as fixed levels of a factor, we prefer to treat them as a random sample of all the dental units in the population. This implies that α_i are to be thought of as having a distribution over dental units, and our interest will be mainly in the parameters of this distribution, notably, the mean and variance; therefore, we rewrite equation 1 as follows:

$$y_{ij} = \alpha_0 + \beta_1 x_{ij} + \beta_2 z_{ij} + (\alpha_i + e_{ij}) \quad (2)$$

We refer to the term in parentheses as the "random" part of the model and to the remainder of the right-hand side of the equation as the "fixed" part, since it contains no further random terms. Equation 2 contains explanatory variables which are measured at level 1 (samples) only; i.e., they are characteristics of samples rather than dental units. To include the presence of *P. aeruginosa*, which is defined at level 2 (dental unit level), we can specify

$$\alpha_i = \gamma_0 + \gamma w_i + u_i \quad (3)$$

where w_i is a dental unit explanatory variable and u_i is a residual which varies randomly between dental units. If we put equations 2 and 3 together and combine the separate "constant" terms, we get

$$y_{ij} = \alpha_0 + \beta_1 x_{ij} + \beta_2 z_{ij} + \gamma w_i + (u_i + e_{ij}) \quad (4)$$

Equation 4 now incorporates variables from both levels in both its fixed and random parts.

TABLE 1. Multilevel CFU per milliliter as a function of type of handpiece, time, and *P. aeruginosa*

Explanatory variable	Estimated counts[a] ($\log_{10}$ CFU/ml)	Expected mean count on original scale
Fixed		
Constant (T_0, syringe, without *P. aeruginosa*)	5.05 (0.10)	1.13×10^5
After 2 min of flushing (all)	3.78 (0.11)*	6.0×10^3
High-speed drill without *P. aeruginosa*	5.40 (0.09)*	2.52×10^5
High-speed drill with *P. aeruginosa* at T_0	5.54 (0.19)*	3.48×10^5
Random variation		
Samples between dental units (20%)	0.09 (0.04)	
Samples within dental units (80%)	0.36 (0.19)	
No. of dental units	30	
No. of samples	208	

[a] Mean ± standard error. *, significant difference ($P < 0.001$).

RESULTS

Culture conditions and total heterotrophic counts. The highest bacterial recovery was obtained at a temperature of 22 to 25°C on R2Am by either the pour plate or the spiral-platter method. The number of colonies rose sharply during the first 4 days and rapidly reached a plateau after 7 days. Taking 20 days as the time at which we obtained the maximum bacterial counts, we estimated that 99% (range, 94.4 to 100%) of all the cultivable colonies appeared by day 7. We used a 7-day incubation time thereafter to evaluate the counts. No significant growth was observed under anaerobic conditions after 7 days at either 37 nor 25°C. However, small colonies could be observed after 10 to 15 days, indicating some anaerobic facultative growth. No attempt was made to identify the corresponding microorganisms, but none were strict anaerobes.

Quantitative microbial analysis of dental unit water samples. The water in the dental unit lines was heavily populated with bacteria. Our results showed that none of the dental units tested were spared by contamination. At the beginning of the work day, the average bacterial counts for the air/water syringe waterline were 5.05 on the log scale (1.13×10^5 CFU/ml) (Table 1). The high-speed waterlines issued higher counts than the air/water syringe (5.40 and 5.05 on the log scale, respectively). This difference is highly significant ($P = 0.001$). While most of the random variation (80%) occurred between measurements, it was clear from the multilevel analyses that resid-

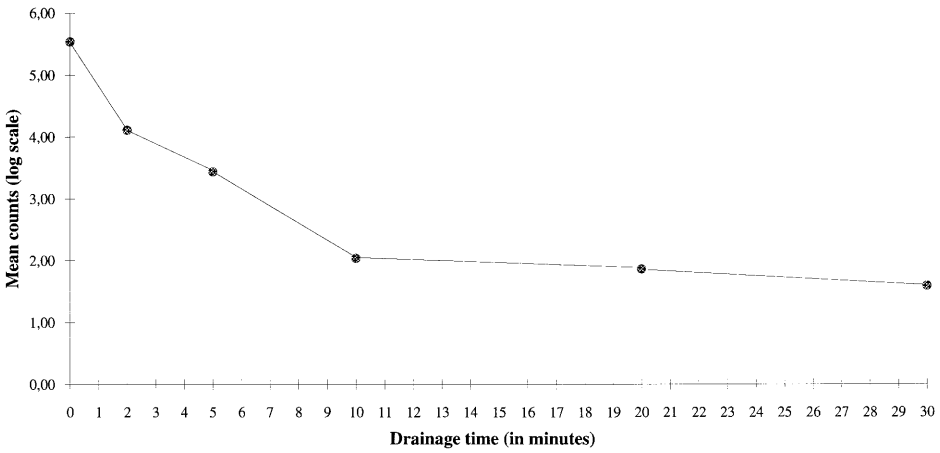

FIG. 1. Influence of drainage on the total bacterial counts. Eight minutes was required to achieve the recommended standard of 500 CFU/ml (2.69 on the log scale).

ual variation also occurred between dental units (20%). A significant difference was also observed between samples taken at the beginning of the day and the samples taken after a 2-min purge (Table 1). On average, cultures on R2Am yield bacterial counts representing 0.3 to 4% of what could be observed by the fluorescent-filter technique. Direct observation by the fluorescent-filter technique showed a good dispersion of microorganisms in the samples, since little microbial aggregation was noted.

Less than a week after their installation, the newly installed dental units at the dental school yielded bacterial counts above 5.3 on the log scale (2×10^5 CFU/ml).

Influence of flushing on the total heterotrophic counts. Purging the lines for several minutes can reduce the bacterial counts. The first 2 min produced a decrease of over 96% (from a mean of 5.53 to 4.11 on the log scale) (Fig. 1). It took at least 8 min to lower the counts to around 500 CFU/ml (2.69 on the log scale). Purges of more than 10 min did not appreciably reduce bacterial counts below those observed at 8 min.

Characterization of dental unit water microflora. The predominant bacterial species recovered from the dental unit water samples were motile gram-negative rods. Further characterization with the Rapid NFT galleries showed that most of these bacteria belonged to the family *Pseudomonadaceae.*

Sphyngomonas paucimobilis (41%) and *Acinetobacter calcoaceticus* (23%) were the predominant cultivable species found in the microflora of DUWL. They were isolated from all dental units. The opportunistic pathogen *P. aeruginosa* was isolated from 24% (29 of 121) of our dental units. However, *P. aeruginosa* was unevenly distributed among the different clinics at the university, with the Oral Diagnosis and Emergency, Oral Surgery, and Removable Prosthodontic clinics having 71% (5 of 7), 67% (6 of 9), and 62% (13 of 21), of their units, respectively, contaminated by this microorganism, whereas only 3.5% (3 of 84) of all dental units located in the other clinics showed the presence of *P. aeruginosa*. When *P. aeruginosa* was isolated from a given dental unit, it was repeatedly recovered from this unit and in all its waterlines over the 1-year period covered by this study. *P. aeruginosa* accounted for 75 to 100% of the cultivable flora of these units. However, dental units not showing any growth of *P. aeruginosa* at the beginning of our study continued to show an absence over the entire period of the study. *P. aeruginosa* could not be detected in any of the tap water samples remote from or near the contaminated dental units after repeated sampling. The isolated *P. aeruginosa* was nonmucoid and produced fluorescein and pyocyanine as shown by growth on the selective media *Pseudomonas* agar P and *Pseudomonas* agar F. *P. aeruginosa* was inhibitory to the growth of certain other bacteria on the same plate, with inhibition zones frequently observed around colonies of this microorganism. The multilevel analysis demonstrated that dental units contaminated by *P. aeruginosa* showed significantly higher total bacterial counts than the others (5.54 and 5.05, respectively, on the log scale; $P < 0.001$). Less predominant bacterial species obtained in the isolates from our dental units were identified as *P. maltophilia*, *P. putida*, *P. fluorescens*, *P. vesicularis*, *P. acidovorans*, *Actinomyces* spp., and *Bacillus* spp. Some yeasts and amoebae were also observed by direct microscopic observations, but no precise attempt was made to identify them. *P. fluorescens* and *P. acidovorans* produced fluorescein on *Pseudomonas* agar F.

Pink-pigmented bacteria were commonly isolated from the DUWL. They constituted about 19% (range, 0 to 97%) of all isolates. These were identified as *Methylobacterium mesophilicum*. These bacteria showed the presence of characteristic vacuoles and volutin granules (Fig. 2). *M. mesophilicum* from DUWL showed a strong resistance to desiccation, since it was the sole bacterial species recovered in culture from lines that were left unused, without exposure to water, for over a year (data not shown).

We also looked at the variation in the proportion of three of the major bacterial species (*S. paucimobilis*, *A. calcoaceticus*, and *M. mesophilicum*) in a given waterline over repeated samplings in culture. Four dental units were used for these analyses. The proportion of each of the three species showed considerable variation over a period of 45 days (14 samplings), with periods of high and low representation (Fig. 3).

DISCUSSION

None of the dental units under study delivered water that could meet the accepted standard for potable water (i.e., less than 500 bacteria per ml of water). The literature reports a very large variability in total bacterial counts obtained from DUWL. These counts ranged from 1.5×10^2 to 1×10^6 CFU/ml (2, 14, 20, 36). Sampling time, culture medium, and time and temperature of incubation, among other variables, are likely to be responsible for some of these variations. As

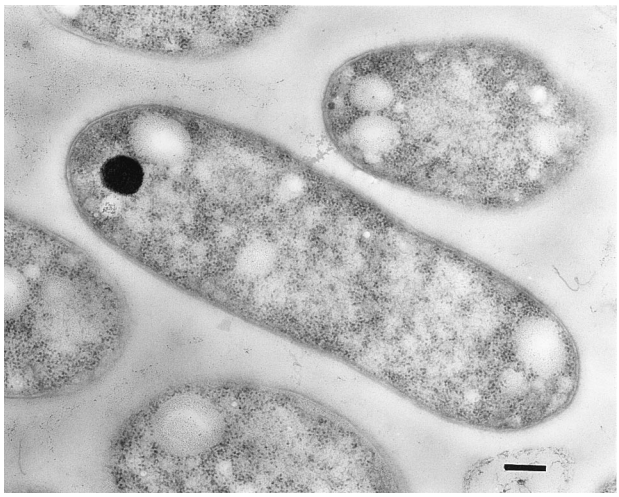

FIG. 2. Electron micrograph of *M. mesophilicum* showing the presence of characteristic vacuoles and volutin granules. Bar, 200 nm.

shown by Williams et al. (42), we report that the best recovery and maximal counts were obtained under aerobiosis, at room temperature, after long incubation periods on a low-nutrient medium. Wide variations between samplings were also reported by others (2, 3, 5, 12, 13, 20, 22, 27, 28, 35). These variations were explained by the heterogeneous distribution of bacterial cells within a given water sample. Bacterial cells in the water obtained from DUWL are thought to be released from the biofilm formed inside the tubing (5, 36, 41). During the sampling, small pieces of biofilm or microcolonies may be released. This is likely to result in a bias toward higher or lower counts or toward the predominance of a given bacterial species in the sample. The fluorescent-filter technique showed that few microbial aggregates were present in our samples. However, the influence of even a few microbial aggregations on the total heterotrophic counts can hardly be evaluated by microbial culture from a small aliquot of water. Other means of cell dispersal, such as an ultrasonic bath, would have to be assessed. Water ecosystems are influenced by several factors: available soluble organic compounds, the presence of heavy metals, and the temperature and level of free chlorine found in municipal water distribution systems. These parameters may change over time, and it may be hypothesized that DUWL microflora will be greatly influenced by these variations, which, in our study, accounted for 80% of the random variation. According to the fluorescent-filter technique, the counts are underestimated by using total heterotrophic counts. Thus, the majority of the microorganisms are either dead, dormant, inhibited by residual chlorine present in the distribution system, or unable to grow on the media used.

The dental unit itself accounted for 20% of the variation. Since all waterlines at the dental school are made of the same material, this finding must be explained by other factors. The air/water syringe-connected waterlines consistently yield lower bacterial counts than the high-speed drill waterlines. As the material and the diameter of the tubing are all the same, the reason for this finding may be explained by different water flow rates or by the fact that the air/water syringe is used more frequently than the high-speed drill on a day-to-day basis.

Since 1981, the large majority of dental units have standardly been equipped with antiretraction valves and check valves that prevent any return of oral material and contamination of the water lines (7). However, Lewis et al. (26) were able to demonstrate by the PCR technique that some oral material and virus particles of human origin could be aspirated back into handpieces and their attached waterlines. We were not able to show the presence of typical oral bacteria in any of our samples. Although we cannot exclude the sporadic presence of oral microorganisms in DUWL, they are not likely to be an important source of water contamination.

The majority of the bacterial species recovered from our samples belonged to the families of aquatic and soil bacteria. Some of our isolates were pigmented bacteria. It has been suggested that pigmented bacteria may be more chlorine tolerant than nonpigmented forms (34). Among our isolates, *P. putida*, *P. aeruginosa*, *S. paucimobilis*, *A. calcoaceticus*, and *M. mesophilicum* are known opportunistic human pathogens (10, 24). Opportunistic pathogens may account for more than 30% of the total bacterial populations in water distribution systems (25), and these bacteria have all been implicated in waterborne nosocomial infections (16, 30).

Since all our dental units were connected in parallel to the same municipal water supply, and since *P. aeruginosa* was the dominant bacterial species in only three of nine clinics, the contamination of a given dental unit by *P. aeruginosa* seems to be under the influence of some as yet unidentified factors. The Oral Diagnosis and Emergency, Oral Surgery, and Removable Prosthodontic clinics may have in common a lower utilization of their waterlines than that of other clinics. It can be proposed that the less often a waterline is used, the greater the chance it

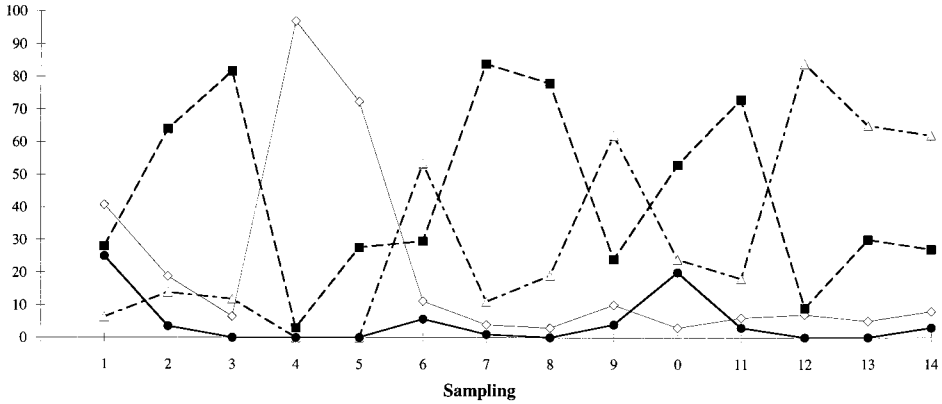

FIG. 3. Variations in the proportion of three of the major bacterial species, *S. paucimobilis* (△), *A. calcoaceticus* (■), and *M. mesophilicum* (◇), and other bacteria (●) over repeated sampling in the same dental unit. One set of four dental unit data sets is shown.

has to be colonized by *P. aeruginosa*. It can also be proposed that operators of the Orthodontic clinic also do not use the water syringe very often and use the high-speed drill only rarely. On the other hand, control samples free of *P. aeruginosa* taken upstream of the dental units ruled out the possibility of contamination of segments of the water distribution system. The stochastic presence of *P. aeruginosa* in particular units thus remains unexplained and needs further investigation.

P. aeruginosa can be recovered from the oral cavity of about 4% of healthy individuals (4). It cannot therefore be excluded that some of these bacteria were aspirated into the waterlines through a defective check valve and were able to colonize the waterlines. The net predominance of *P. aeruginosa* over the other bacterial species in culture could be the consequence of the observed capacity of *P. aeruginosa* to inhibit the growth of other bacteria isolated from the waterlines. It is known that some strains of *P. aeruginosa* produce bacteriocins (18) that can inhibit the growth of other microorganisms (23). This could confer a competitive advantage to *P. aeruginosa* in colonization of the lines. The clinical importance of the presence of nonmucoid strains of *P. aeruginosa* in the DUWL is not known, despite the article by Martin (27) which reported the case of two immunodeficient patients who developed a localized *P. aeruginosa* infection following dental restorations. Immunodeficient patients are also at a greater risk of acquiring opportunistic "non-oral-pathogen-related" periodontitis (38).

Draining DUWL for several minutes reduces bacterial counts significantly (14, 20, 36, 41). Our study showed that to achieve the recommended standard for potable water, namely, 500 CFU/ml (2.69 on the log scale) or less (15), a minimal purge time of 8 min is required. Such times are, however, impractical in a dental practice environment. What, then, could be a realistic efficient drainage time? The reduction from 5.53 to 4.11 CFU/ml on the log scale by a 2 min purge (Fig. 1) is insufficient but perhaps is a respectable attempt toward improving the water quality used for oral irrigation. Drainage may not be the appropriate answer. Obviously, better efforts are necessary to eliminate not only planktonic bacteria but also the biofilm—a source of contamination that can concentrate opportunistic pathogens like *P. aeruginosa.*

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