An Evaluation of Sampling and Laboratory Procedures for Determination of Heterotrophic Plate Counts in Dental Unit Waterlines

(Évaluation des techniques d'échantillonnage et d'analyse en laboratoire pour la numération sur plaque des organismes hétérotrophes dans les conduites d'eau des unités dentaires)

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Sommaire

Historique : *Le nombre élevé de micro-organismes hétérotrophes dénombrés dans les échantillons d'eau prélevés des conduites des cabinets dentaires a amené certains à craindre qu'il n'excède les seuils proposés. La présente étude avait surtout pour but d'examiner la variabilité des résultats de la numération sur plaque des organismes hétérotrophes et d'examiner en détail les effets de la méthode d'analyse sur les résultats obtenus.*

Méthodologie : *Des échantillons d'eau ont été prélevés des conduites des unités dentaires, au début de la journée de travail ou pendant la journée, puis ont été amenés au laboratoire pour y être analysés.*

Résultats : *Pour déterminer le niveau de contamination par des organismes hétérotrophes dans un cabinet dentaire, il faudrait en tester toutes les unités, car des différences significatives ont été observées entre des unités reliées à un même réseau d'alimentation en eau. De plus, à l'intérieur d'une même unité, le dénombrement moyen des bactéries dans les conduites à grande vitesse a été près de deux fois supérieur à la moyenne pour les conduites air/eau. La méthode d'analyse utilisée en laboratoire a eu également une grande incidence sur le nombre de micro-organismes hétérotrophes récupérés. Ainsi, la température, la durée et le milieu d'incubation, de même que la neutralisation du chlore résiduel, ont tous eu des effets significatifs sur la numération. En revanche, aucune différence significative n'a été observée entre les échantillons étalés sur plaque (gélose R_2A) et ceux en milieu coulé en boîte de Petri (gélose pour dénombrement sur plaque).*

Conclusions : *Les organismes dentaires ont proposé un seuil-limite quant au nombre de micro-organismes hétérotrophes décelés dans l'eau des unités dentaires; ils doivent aussi établir des normes pour l'analyse des échantillons d'eau en laboratoire. Nous proposons ici un protocole pour le prélèvement des échantillons et leur analyse en laboratoire.*

Mots clés MeSH : *colony count, microbial; dental equipment; water microbiology*

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Cet article a fait l'objet d'une révision par des pairs.

Recently, concern has arisen about the quality of water that exits in dental unit waterlines (DUWs). In the past, it was assumed that the quality of water delivered by these waterlines would be comparable to that of the source water supplying the dental unit. However, this may not be the case. Water passing through the tubing of dental units may pick up sloughing bacteria or clumps of bacteria from the microbial biofilm that becomes established in the waterlines.[1] The numbers of microorganisms that have been found in water samples collected from dental units may exceed current limits for water quality and are perceived as a potential health risk to patients and dental personnel.[1-11] The apparent high numbers of heterotrophic microorganisms found in water samples collected from dental units has been the concern. Generally, water should have a heterotrophic plate count (HPC) of < 500 colony-forming units (CFU)/mL.[12,13] However, organized dentistry has suggested that the standard for water from dental units should < than 200 CFU/mL by the year 2000.[14] Current guidelines for managing this problem

include flushing lines at the start of the clinic day and between patients, as well as avoiding heating the water.[14,15]

Numerous studies on the issue of contamination of water in dental units have been published. However, the handling of water samples for analysis differs from one laboratory to another and is not standardized. Different types of media have been used for recovering bacteria from the dental unit water supply.[1,5,6,11,16-22] In addition, incubation temperatures of 20°C to 37°C and incubation times of two to 28 days have been used.[1,6,11,16-21] The selection of incubation time, incubation temperature and culture medium are factors that greatly influence both the number and type of organisms recovered.[22,23] This lack of a standard laboratory protocol for handling water samples from dental units creates confusion and may alter perceptions of water quality. The first objective of this investigation was to examine the tubing wall of DUWs for the presence of biofilm. Second, we aimed to determine the variability in HPC values between and within DUWs. Third, we wanted to determine the degree to which laboratory processing of water samples may significantly alter HPCs (measured as CFU per millilitre). Finally, on the basis of our data, we present a protocol for collecting and analyzing water samples.

Materials and Methods

Scanning Electron Microscopy of Tubing

Two dental operatories were chosen at random from a clinic, and sections of tubing from both the air/water and high-speed lines were collected from each unit. A piece of new tubing served as a control. A 1-cm-long piece of each tube was aseptically sectioned with a sterile scalpel and was prepared for scanning electron microscopy. Tubing specimens to be analyzed were cut longitudinally, to expose the tubing lumen. The tubing specimens were kept on ice, where they were fixed in 2.5% gluteraldehyde in 0.1 mol/L phosphate-buffered saline, pH 7.2, and washed three times with the buffer. They were then dehydrated for 10 minutes, through a series of alcohol washes (50%, 70%, 80%, 95%, 95%, 100%, 100%) at room temperature, critical-point-dried with liquid CO_2, mounted and sputter-coated with gold–palladium in a 60:40 ratio. Samples were viewed with a scanning electron microscope (Cambridge 260, Cambridge, England), and representative photographs were taken.

Collection of Water Samples

Sixteen dental practices participated in the study, and one to nine dental units at each office were sampled. Each dental unit has two narrow-bore tubings that deliver water to the air/water syringe and the high-speed handpiece. On each collection day, samples were collected according to the following protocol. Before sample collection the end of the tubing from which the water exited was disinfected with 70% alcohol. A volume of 25–150 mL of water was aseptically collected into a sterile tube or bottle from the waterlines of the air/water syringe tips and the high-speed handpieces. Water samples were typically collected from units that had been in use for at least one hour during the collection day. If the unit had not been used that day, the line was purged for two minutes before the samples were collected. Samples that had to be transported to the laboratory were kept on ice and were processed immediately when they reached the laboratory. All samples were plated within three hours of collection.

Preparation of Media

To evaluate the number of heterotrophic microorganisms in each water sample, we used the previously established spread plate and pour plate methods.[23] For the spread plate technique, a low-nutrient agar, R_2A (Difco, Detroit, MI), was used. The medium was rehydrated with distilled water and sterilized at 121°C for 15 minutes. Approximately 15 mL was dispensed into each sterile, disposable 100 mm x 15 mm plastic petri dish (Fisher Scientific, Nepean, ON) and allowed to solidify; the plates were stored at 4°C in a sealed container. Plate Count Agar (Difco, Detroit, MI), was used for the pour plate technique. The medium was rehydrated with distilled water, sterilized at 121°C for 15 minutes, tempered in a 44°C to 46°C water bath and maintained at that temperature until required. This medium was used within three hours of preparation.

Sample Dilutions, Plating and Enumeration

To disrupt suspended or planktonic biofilm matrices, samples were vigorously agitated by vortex for 15 seconds. Ten-fold serial dilutions (10^{-1} to 10^{-4}) were then prepared with 10 mmol/L sterile phosphate-buffered saline (pH 7.3). For the spread plate technique, duplicate 1-mL volumes of each undiluted sample were spread with a glass rod on a whole plate, and duplicate 100-μL volumes of each dilution (10^{-1} to 10^{-4}) were spread onto each half of a plate containing R_2A medium (**Fig. 1**). The samples were spread with a sterile glass rod and allowed to be absorbed into the medium. For the pour

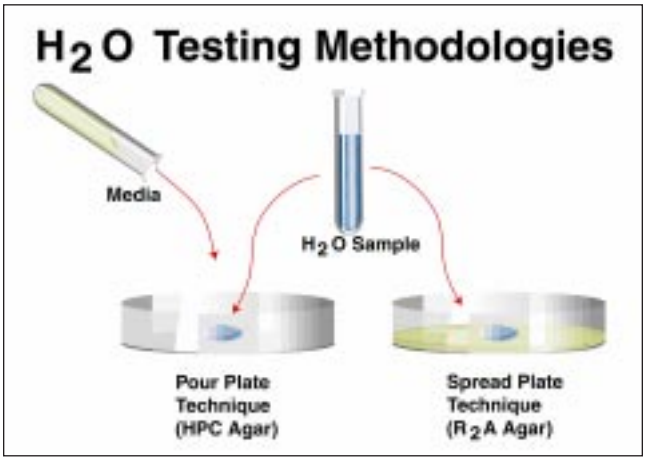

Figure 1: *Two methods were used for culturing heterotrophic bacteria. In the first method, the pour plate technique, aliquots of undiluted and diluted water samples were added to a petri dish, and liquefied Plate Count Agar was poured into the water sample and mixed. In the second method, the spread plate technique, R_2A agar was poured into a petri dish and allowed to solidify. Aliquots of undiluted and diluted water samples were poured onto the agar surface and spread evenly. Plates were then incubated at various times and temperatures.*

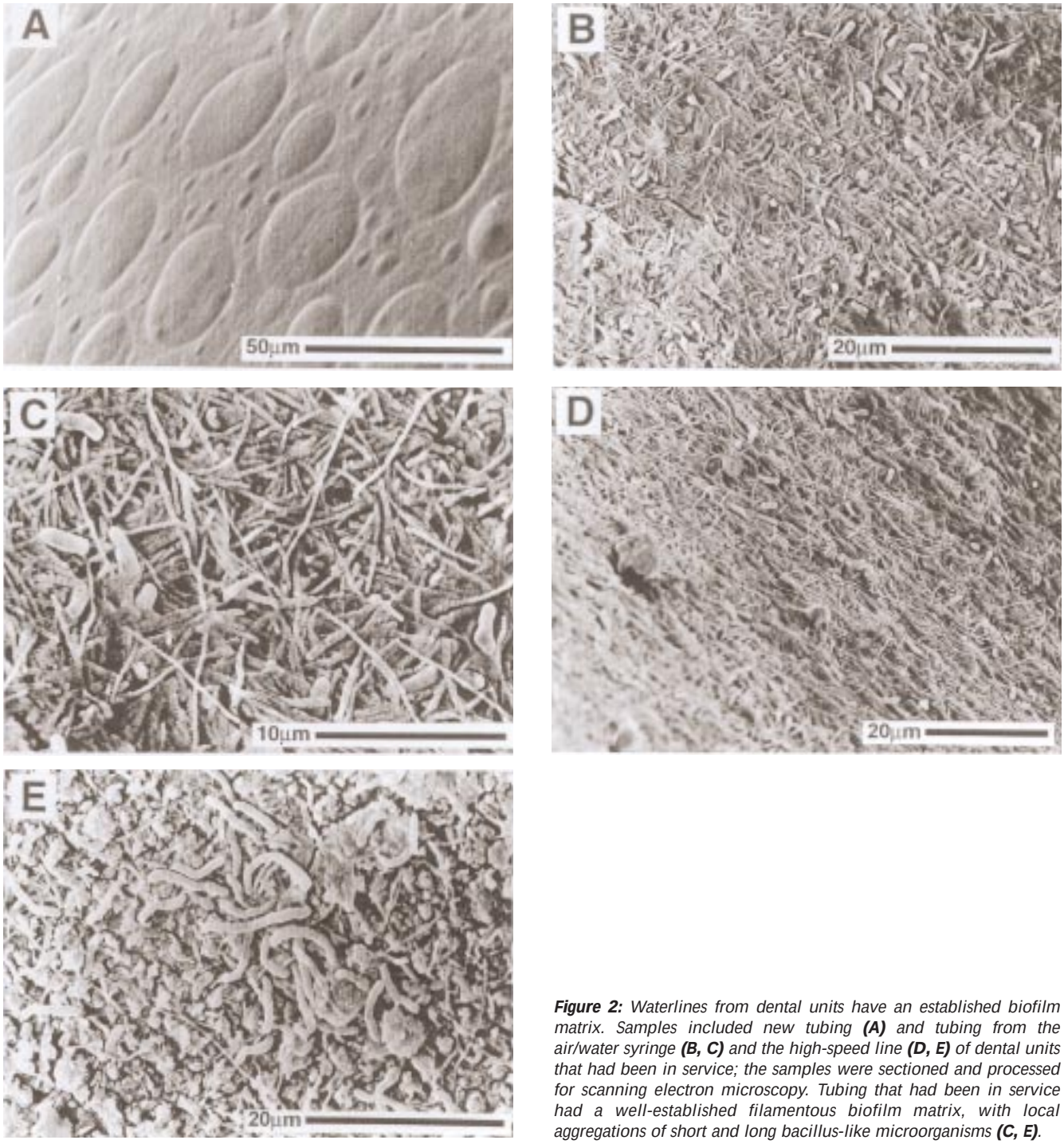

Figure 2: *Waterlines from dental units have an established biofilm matrix. Samples included new tubing* ***(A)*** *and tubing from the air/water syringe* ***(B, C)*** *and the high-speed line* ***(D, E)*** *of dental units that had been in service; the samples were sectioned and processed for scanning electron microscopy. Tubing that had been in service had a well-established filamentous biofilm matrix, with local aggregations of short and long bacillus-like microorganisms* ***(C, E)****.*

plate technique, agitation and dilution of the water samples was performed as described above. Subsequently, 1 mL of each sample dilution was dispensed, in duplicate, into individual sterile, disposable 100 mm x 15 mm plastic petri dishes. Approximately 10 ml of the tempered, liquefied pour plate medium was poured into each dish with the water sample. As each plate was poured, the medium was mixed with the water sample by careful rotation of the dish; the mixture was then left to solidify (**Fig. 1**).

To assess the growth-inhibiting effects of residual chlorine, we divided water samples into two equal portions. Filter-sterilized sodium thiosulphate at a final concentration of 18 µg/mL (Sigma, Missisauga, ON) was added to one half of the divided water samples.[23] Samples were plated on R_2A agar using the spread plate technique. These plates were incubated at 35°C, and colonies were counted at seven days.

For other samples, the spread and pour plates were inverted and incubated aerobically at 21°C, 28°C or 35°C for two, four and seven days. For each water sample, the dilution that was counted contained between 30 and 300 colonies (whole plate) or between 15 and 150 colonies (half plate). The counting process to tabulate the number of colonies was aided by a

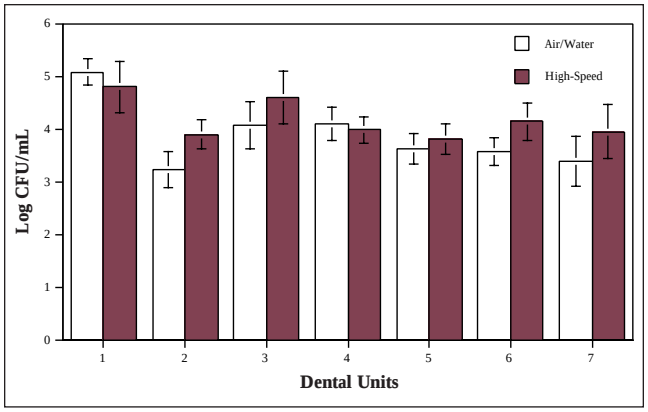

Figure 3: *Dental units from one office may have significantly different heterotrophic plate counts even though they are all connected to the same central municipal water supply. After a two-minute purging flush, water samples were collected (weekly for 12 weeks) from seven air/water lines and seven high-speed lines. Water samples were plated on R_2A agar with the spread plate technique, the plates were incubated at 35°C, and colonies were enumerated at seven days. Values are presented as means ± standard deviation.*

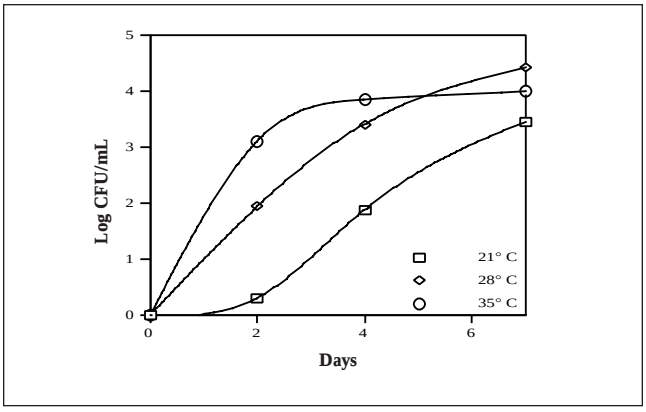

Figure 4: *Incubation time and temperature significantly affect heterotrophic plate counts. After a two-minute purge, water samples were collected, divided in three, and plated on R_2A agar with the spread-plate technique. One set of plates was incubated at each of 21°C, 28°C and 35°C, and the number of colonies were counted at two, four and seven days. Each data point is the mean of nine samples.*

Quebec colony counter. The mean of the duplicate platings was calculated, this value was corrected for dilution, and the data were tabulated as CFU per millilitre.

Data Analysis

Because application of most rigorous statistical techniques requires the assumption of symmetrical distributions, we converted the data to $\log_{10}$ equivalents to achieve a symmetrical distribution resembling the normal distribution curve. The resultant geometric mean corresponds to the antilog of the logarithmic mean and is the best estimate of central tendency.[23] To test for statistically significant differences, analysis of variance (ANOVA) was performed on the $\log_{10}$ values. Values are presented to two significant digits.

Results

Scanning Electron Microscopy Identifies Established Biofilm in Waterlines

The surface characteristics of sections of waterline tubing collected from dental units that had been in use for an extended period showed significant biofilm relative to new tubing samples (**Fig. 2**). New, unused tubing was relatively smooth, with no organic biofilm matrix (**Fig. 2A**). In contrast, tubing samples from air/water lines (**Figs. 2B** and **2C**) and high-speed lines (**Figs. 2D** and **2E**) showed a continuous filamentous organic matrix. Embedded randomly throughout this matrix were short and long bacillus-like organisms (**Figs. 2C** and **2E**). In the samples that were examined, no significant differences were found between the air/water and high-speed lines.

HPCs Vary Significantly with Sampling Method and Source

Each of seven dental units from one clinic showed sample variability in HPCs of 1 to 2 $\log_{10}$ over the 12-week testing period. The counts for samples from air/water lines ranged from 400 ($\log_{10}$ 2.6) to 320,000 ($\log_{10}$ 5.5) CFU/mL. The HPCs from the high-speed lines ranged from 1,300 ($\log_{10}$ 3.1) to 250,000 ($\log_{10}$ 5.4) CFU/mL (data not shown). Even though these dental units were connected to one central municipal supply line, there were significant differences between them in terms of HPCs (ANOVA, $p < 0.0005$) (**Fig. 3**). Unit 1 had a higher number of HPCs than all of the other units analyzed (ANOVA, $p < 0.0005$), and unit 3 had a significantly higher number of HPCs than five of the remaining six units (ANOVA, $p < 0.005$).

For most units examined, the geometric mean HPC from the high-speed line was greater than that from the air/water line (**Fig. 3**). The overall geometric mean HPC for the high-speed lines was 15,000 ($\log_{10}$ 4.17), whereas the geometric mean HPC for the air/water line was 7,000 ($\log_{10}$ 3.86). This difference was significant (ANOVA, $p < 0.0005$).

Laboratory Handling of Water Samples Has Significant Effects on HPCs

Incubation time and temperature had a critical effect on HPC values from samples collected from nine units in one office. In general, longer incubation times and higher temperatures yielded significantly higher HPCs (**Fig. 4**). Incubation time (two, four and seven days) had a highly significant effect on HPCs, with each time point being significantly different from the previous one (ANOVA, $p < 0.001$). Incubation temperature also significantly affected the total recoverable HPCs and the rate at which organisms grew. Incubation of samples at 21°C yielded significantly lower counts than incubation at 28°C and 35°C (ANOVA, $p < 0.0005$). There was no statistically significant difference in HPCs for samples incubated at 28°C and 35°C. The rates at which organisms grew (slope of curve) was different for each temperature, and, by seven days, the geometric mean counts (as CFU per millilitre)

Table 1 **Percentage of nine dental units that met the target for heterotrophic plate counts of < 200 CFU/mL at specified incubation times and temperatures[a]**

Incubation Time (Days)	Incubation Temperature		
	21°C	28°C	35°C
2	89%	56%	33%
4	56%	11%	0%
7	11%	0%	0%

[a] Spread plate method on R_2A media.

Table 2 **Change in heterotrophic plate count with neutralization of chlorine by sodium thiosulphate (18 µg/mL)**

	HPC (CFU/mL, in thousands)[a]	
Dental Unit	Chlorine Not Neutralized	Chlorine Neutralized
1	900	920
2	11	16
3	22	580
4	190	220
5	33	31
6	1,000	4,800
7	200	200
8	48	92

CFU = colony-forming units.

[a] Samples were plated on R_2A agar using the spread plate technique, the plates were incubated at 35°C, and colonies were counted at seven days.

were as follows: 21°C, 2,800 ($\log_{10}$ 3.4); 28°C, 26,000 ($\log_{10}$ 4.4); and 35°C, 10,000 ($\log_{10}$ 4.0). For the various time and temperature combinations, the percentage of dental units that met the HPC target of < 200 CFU/mL varied significantly (**Table 1**).[14] This table clearly shows that the time and temperature selected for plate incubation can dramatically affect perceptions and conclusions concerning the clinical acceptability of water that exits dental units.

To determine the effects of plating media and techniques on HPCs, water samples were divided in half and processed using the spread plate technique on R_2A agar and the pour plate technique on Plate Count Agar. Both of these techniques are established methods of analyzing HPCs.[23] The samples plated on R_2A agar with the spread plate technique tended to have higher mean counts, but no significant differences were found between the two plating techniques for samples collected from the air/water lines and the high-speed lines (data not shown).

To determine the effects of residual chlorine on HPC, we divided water samples into two equal portions. In half of the divided samples, chlorine was neutralized with sodium thiosulphate.[23] The increase in air/water line HPCs after chlorine neutralization varied significantly with each unit (**Table 2**). The geometric mean of the HPCs for the samples in which chlorine was neutralized was 220,000 ($\log_{10}$ 5.34) CFU/mL, nearly double that obtained for the chlorinated water samples, which had a geometric mean of 140,000 ($\log_{10}$ 5.14) CFU/mL. This difference was significant (ANOVA, $p < 0.05$).

HPC Levels in DUWs Depend on Sampling Protocol

We evaluated the mean HPCs from dental office waterlines at the start of and during the clinic day. The HPC level at the start of a clinic day and the decrease after a two-minute flush were determined (**Fig. 5A**). The geometric mean at the start of the day for all offices was 3,200 ($\log_{10}$ 3.5) CFU/mL for plates counted at two days. A two-minute flush reduced the geometric mean HPC to 200 ($\log_{10}$ 2.3) CFU/mL. For plates counted after 7 days of incubation, the geometric mean HPC was 32,000 ($\log_{10}$ 4.5) at the start of the day and 2,500 ($\log_{10}$ 3.4) after the two-minute flush. The average HPC levels for samples collected from 41 dental units in 15 different offices were then examined (**Fig. 5B**). These dental units had been in service for at least one hour or had undergone a two-minute flush. The HPCs were determined on days two and seven. At two days the geometric mean for all offices was 100 ($\log_{10}$ 2.0) CFU/mL, and the average level of contamination for all the offices tested met the year 2000 recommended target limit for dental units of < 200 ($\log_{10}$ 2.3) CFU/mL (**Fig. 5B**). Although the average HPC met the target, 36% of the individual units failed to do so (data not shown). For the longer incubation period, seven days, the geometric mean HPC was 1,600 ($\log_{10}$ 3.2) CFU/mL, which exceeded the recommended HPC target. For this incubation period, 88% of the units exceeded the HPC target.

Discussion

Formation of biofilm occurs principally by growth of the low numbers of viable cells that survive municipal water treatment. Initial adherence of microorganisms to a surface is reversible. With time, the secretion of highly hydrated exopolysaccharides makes the attachment irreversible. As the biofilm begins to mature, adherent microcolonies coalesce and eventually form a continuous biofilm matrix.[24,25] Often, in aquatic environments, the majority of microorganisms in the biofilm are gram-negative rods; however, filamentous microorganisms later become part of the biofilm matrix, after significant extracellular matrix has been deposited.[26,27] The formation

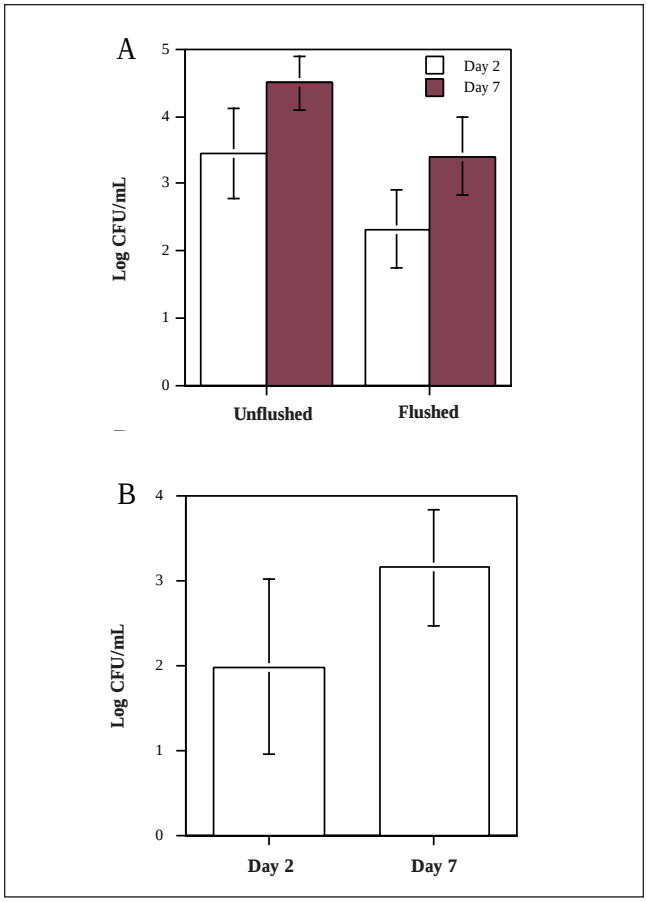

Figure 5: *Evaluation of the heterotrophic plate counts from dental units with a suggested standard technique.* ***(A)*** *Water samples were collected from 12 dental units at the start of a clinic day, before (unflushed) and after (flushed) a two-minute purge. Residual chlorine in the water samples was neutralized, samples were plated on R_2A agar using the spread plate method, the plates were incubated at 35°C, and colonies were enumerated at two and seven days. Data are presented as mean ± standard deviation (n = 12 units).* ***(B)*** *Water samples were collected from 41 air/water syringes in 15 offices. Residual chlorine in the water samples was neutralized, samples were plated on R_2A agar using the spread plate method, the plates were incubated at 35°C, and colonies were enumerated at two and seven days. Data are presented as mean ± standard deviation (n = 41 syringes).*

of biofilms in DUWs appears to occur in a similar manner. The interior walls of the air/water and high-speed lines were covered with a continuous filamentous biofilm matrix, and interspersed on the surface were many short and long bacillus-like microorganisms. It was not surprising to find a continuous matrix in these units, which had been in service for many years, because units in service for a little as six months may have a well-established biofilm.[20] As water flows down the lumen of these biofilm-laden waterlines, the number of microorganisms released into the water increases significantly.[1,5,10,11,16,19,20,22,28-30] The counts may be further increased by the slowing of water flow caused by the presence of a well-established filamentous biofilm matrix, which increases frictional resistance.[27]

The development of a laboratory protocol for processing water samples involves numerous critical decisions. In our study, plate incubation time and temperature, media selection and the inhibitory effects of residual chlorine all affected the number of microorganisms that were cultured. Incubation temperatures that have been used to evaluate the contamination of DUWs have ranged from 20°C to 37°C, and incubation times, from two to 28 days.[1,6,11,16-21] Our data showed that the plate incubation temperature and the incubation period after which the number of colonies was counted are absolutely critical and dramatically affect whether a water sample meets the suggested HPC year 2000 target limit (< 200 CFU/mL). We found no statistically significant difference in HPCs when plates were incubated at either 28°C or 35°C. However, the higher incubation temperature is often used for HPC analysis.[31,32] The significance of plate incubation time and its effects on perceived contamination of DUWs is exemplified by results presented in **Figure 5**. In this study, for plates counted at two days, the mean level of HPCs in DUWs met the target limit. However, the mean HPC level of plates counted at seven days exceeded the target limit **(Fig. 5B)**. It should be pointed out that for both time points, at least some units exceeded the year 2000 HPC target limit.[14] Collectively, plate incubation temperature and time at which colonies were counted are absolutely critical. Both should be standardized, because they directly affect HPC and, indirectly, dramatically affect perception of water quality.

The temperatures and times that have been selected for HPC analysis by other disciplines concerned with HPC densities provide insight into professional methods in current use. For example, the bottled water industry is also concerned about HPCs in water and has used 35°C for 48 hours or 37°C for 24 hours for plate incubation.[32,33] More closely related to dentistry is the protocol used for assessing HPCs from renal dialysis machines. Given that dentistry was planning to adopt the HPC values set up for renal dialysis, it seems reasonable to also adopt the plate incubation times and temperatures that were used for that purpose.[14] A recent seven-year multicentre study that examined HPCs from renal dialysis machines used 48 ± 3 hours at 35°C ± 0.5°C for incubation.[31] The selection of a 35°C, 48-hour incubation appears to be standard and is recommended for dentists who wish to determine if their dental units meet the suggested HPC limit.[14]

A variety of culture media have been used to evaluate bacterial contamination in DUWs. Dilute peptone medium,[11,21,22] brain-heart infusion agar,[17] glucose broth,[5] horse blood agar,[6,18] trypticase soy agar[1,11,16] and tryptose blood agar base with yeast extract[19] have all been used to evaluate contamination of DUWs. We used two well-established media and plating methods for assessing heterotrophic microorganisms and found no statistical difference between the pour and spread plate methods.[23] These data support the use of either of these accepted techniques. The presence of residual chlorine was the last factor to be evaluated. Our results support previous studies, which have suggested that residual chlorine may inhibit bacterial growth and should therefore be neutralized.[23]

The clinical significance of heterotrophic microorganisms in samples from DUWs and the risk they may pose to immunocompromised patients and dental health care providers is a fundamental concern to the profession. Recently, it was shown that heterotrophic bacteria isolated from tap and bottled water exhibited low cytotoxicity and invasiveness in cell culture.[34] The clinical implication of the presence of heterotrophic microorganisms in samples from DUWs is as yet unclear. However, microorganisms such as *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Mycobacteria* have also been isolated from such samples and could pose a risk to immunocompromised patients and dental health care providers.[35] The isolation frequency, pathogenicity and virulence of microorganisms in water samples from dental offices requires further investigation.

Conclusions

1. The nature of DUWs is such that they will develop a biofilm, and water flowing down the biofilm-coated waterlines will contribute to the microbial load in the water as it exits the tubing.
2. Even though the various dental units within an office are connected to the same municipal water supply, each unit may have a different mean bacterial count. Therefore, all dental units within a clinic should be tested. Within a dental unit, water from high-speed lines had a higher mean HPC than water from the air/water lines. This may reflect lower use of and slower flow rates in these lines relative to the high-speed lines.[4,17] Because most high-speed lines have higher counts, it may be possible to test water from this type of line to determine the worst case scenario for a particular unit. A 1 to 2 $\log_{10}$ variability in HPC was consistently found between samples, which suggests that ideally each unit should be tested several times to establish its geometric mean HPC value.
3. In coming to terms with the issue of contamination of DUWs, a target limit for HPCs has been suggested: < 200 CFU/mL by the year 2000.[14] The data presented here indicate that laboratory handling of water samples has a dramatic effect on HPCs. Organized dentistry must establish a standard protocol for laboratory handling of water samples, and this should be done as recommended by *Standard Methods for the Examination of Water and Wastewater*.[23] On the basis of the results presented here and the literature cited, the following analysis method is suggested. After flushing of the line for at least two minutes, a 100-mL sample should be collected into a sterile 100-mL sample bottle containing sodium thiosulphate (to neutralize any residual chlorine). The sample should be held on ice in a cooler for transport to an analytical laboratory. At the laboratory the sample should be plated using either the R_2A/spread plate or Plate Count Agar/pour plate method; plates should be incubated at 35°C, and colonies counted after 48 hours of incubation.
4. With this laboratory method, samples obtained from DUWs in the morning, before use, showed higher bacterial counts than the HPC target limit. However, a two-minute flush of the waterlines reduced mean counts to the HPC target limit (**Fig. 5A**). These data support the recommendation of flushing the waterline in dental units for several minutes at the start of the workday.[14,15] These results differ from those presented in a previous publication, which reported that an eight-minute flush reduced counts to only 500 CFU/mL.[4] However, a seven-day incubation period was used in that study.[4] The suggested testing protocol did identify some individual units that exceeded the target limit. These data support the continued development of commercial options for improving water quality in DUWs.[14] ➧

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