

BIOFILM AND DENTAL UNIT WATERLINES

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Abstract: Aquatic biofilms, which are well-organized communities of microorganisms, are widespread in nature. They constitute a major problem in many environmental, industrial and medical settings. The use of advanced techniques has revealed biofilm structure, formation and ecology. Special attention was given to the build-up of biofilm in dental unit waterlines (DUWLs), which are small-bore flexible plastic tubing to bring water to different handpieces. They are coated with well-established biofilms. Active biofilm is a source of microbial contamination of DUWLs water. The safety of dental treatment requires a good quality of the water used. The knowledge of nature, formation and the ways to eliminate the biofilm is the first step towards reducing health risk, both for patients and dental personnel. The article reviews these issues.

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The term 'biofilm' refers to the development of microbial communities on submerged surfaces in aqueous environments. The growth of biofilm is considered to be a result of complex processes involving transport of organic and inorganic molecules and microbial cells to the surface, adsorption of molecules to the surface (formation of the conditioning layer) and initial attachment of microbial cells followed by their irreversible adhesion, facilitated by production of extracellular polymeric substances (EPS), often referred to as glycocalyx or slime [9].

The progress of knowledge concerning the formation of biofilm and the course of physiological processes in this specific environment made it possible to expand the definition of biofilm. Donlan [16] defined biofilm as an assemblage of microbial cells that is irreversibly associated (not removable by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix.

Biofilm-associated organisms also differ from their planktonic (freely suspended) counterparts with respect to genes that are transcribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or portable water system piping, or a natural aquatic system.

Donlan and Costerton [17] propose a new definition of biofilm as a microbially-derived sessile community characterized by cells that are irreversibly attached to a substrate or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.

Biofilms represent perhaps the oldest form of life on our planet and can be found in extreme environments. Microorganisms forming biofilm reveal properties different from those typical for monocultures. Advances in light microscopy coupled with developments in microelectrode technology have led to an appreciation that bacterial biofilms consist of microcolonies that bacteria have developed into organized communities with functional

heterogeneity. The complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms [14, 15].

Biofilm provides microbial cells with: (1) easier exchange of genetic material; (2) easier accumulation of nutritive substances from the water phase; (3) protection against an excess of nutritive substances and against drying [16]. Biofilm bacteria are substantially resistant to surfactants, biocides and antibiotics. Three mechanisms are responsible for the biofilm resistance: (1) At least some of the cells in biofilm experience nutrient limitation and therefore exist in a slow-growing or starved state. Slow-growing or nongrowing cells are not very susceptible to many antimicrobial agents. (2) Production of exopolymers - exopolysaccharides, which may constitute over 90% of the biofilm dry weight mass, prevent various agents to penetrate the full depth of the biofilm. (3) At least some cells in a biofilm adopt a distinct and protected biofilm phenotype. This phenotype is a biologically programmed response growth on a surface. They can deactivate some disinfectants or provide a diffusion barrier based on the anionic and hydrophobic nature. Resistance to some disinfectants can be provided to fixed bacteria by deactivation of disinfectants upon contact with underlying surfaces or with deposits mixed in biofilm [11, 15].

Variables important in cell attachment and biofilm formation are: (1) properties of the substratum (texture or roughness, hydrophobicity, conditioning film); (2) properties of the bulk fluid (flow velocity, pH, temperature, cations, presence of antimicrobial agents); (3) properties of the cell (cell surface hydrophobicity, fimbriae, flagella, extracellular polymeric substances [16]. Models of the development of mature biofilm from planktonic cells are described in the papers by Costerton *et al.* [15], and Mills and Karpay [28].

Xu *et al.* [47] using fluorescent probe and gene technology, investigated and visualized physiological gradients within biofilm of *Pseudomonas aeruginosa*. Visualization techniques that indicated protein synthesis, respiratory activity and relative RNA content show that activity was limited in each case to a narrow zone located immediately adjacent to the biofilm/bulk interface. These measurements demonstrated that only the top one-fifth of the biofilm was metabolically active. These authors suggest that the resistance of attached microorganisms within biofilm to antimicrobial agents hinges on the spatial heterogeneity of physiological activity within biofilm. However, spatial heterogeneity activity within the biofilm dose does not derive simply from nutrient limitation. Perhaps cell-to-cell signalling or other mechanisms switch cells into a dormant, and protected phenotype state. Besides, it is quite likely that some antimicrobial-treated cells, though irreparably compromised in terms of their ability to reproduce, continue to consume nutrients for hours or even days beyond the time that they will have been judged to have expired-based on assay for durability.

Free-floating and sessile cells inhabit distinct physiological states even when grown in the same medium, and

this explains the resistance to attached microorganisms within biofilms to antimicrobial agents [11, 12]. The literature supports a general, though not universal, correlation between decreased growth rate in biofilms and increased resistance to killing [11].

DENTAL UNIT WATERLINES BIOFILM

Just as almost all solid surfaces in contact with water are home to remarkable microbial communities known as biofilms, the surfaces of dental unit waterlines (DUWLs) also provide an ideal environment for growth of microorganisms entering dental units from the municipal water supply [3].

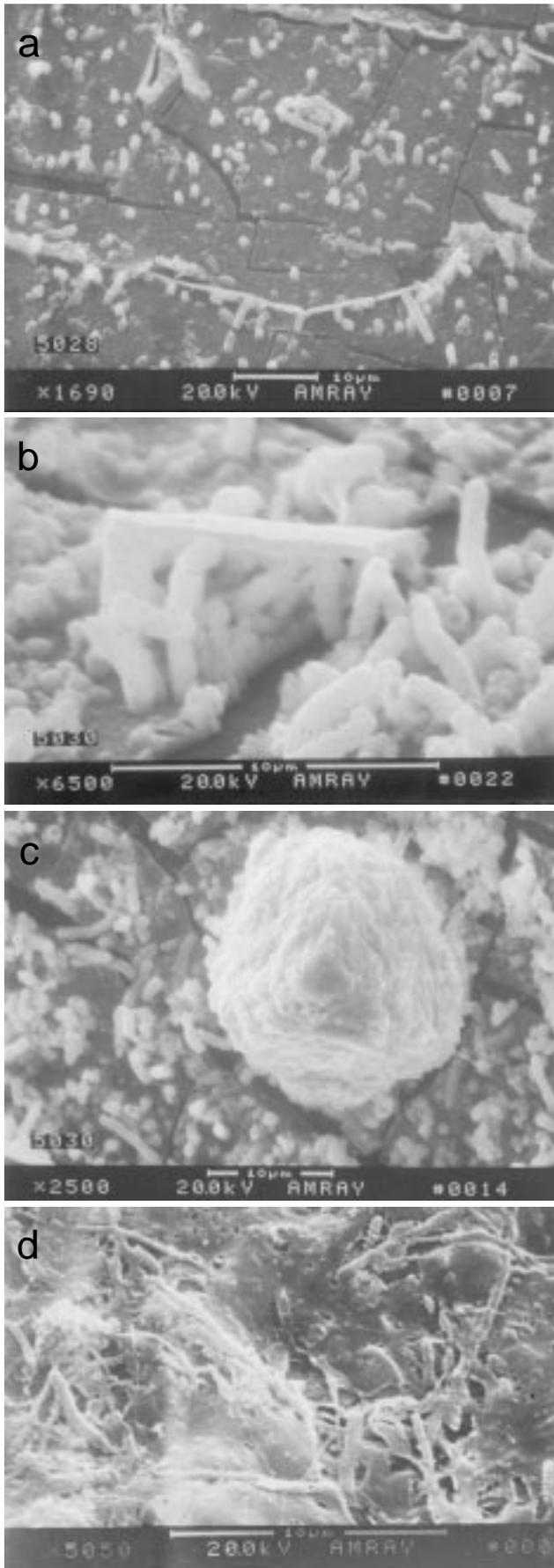
Biofilm formation in dental unit waterlines has been recognized for nearly 40 years [32]. The biofilm formation in DUWLs is a universal problem, which is indicated by research results in many countries. Active biofilm is the primary reservoir for continued contamination within the water supply system [31, 45].

Biofilms are adherent colonies of bacteria, fungi, and protozoa that form along the inner surface of DUWLs. The initial biofilm layer thickens through replication of the organisms that make up the biofilm, as well as through adherence of free-floating microorganisms from the water source. At times, individual microorganisms, as well as pieces of biofilm, can dislodge and pass out of waterlines. It is at this point that the biofilm becomes a potential problem for the dental patient or dental healthcare worker [27, 30, 37].

The nature of DUWLs is such that they will develop a biofilm, and water flowing down the biofilm-coated waterlines will contribute to microbial load in the water as it exits the tubing. Frequent periods of water stagnation in DUWLs (related to the rhythm of work during the day, in the evenings, during the nights, weekends and holidays) and the properties of the plastics used in DUWLs construction can promote the attachment and colonization of biofilm-forming microorganisms. Most plastic dental tubing has an inside diameter of 16 mm (1/16 inch) to 8 mm (1/8 inch). This creates a very large ratio of surface area to water volume of narrow bore tubing. The proportion of the amount of water used in the course of work to the total amount of water in DUWLs tubing seems essential [1, 30, 45, 46].

The physics of laminar flow of water passing through the DUWLs results in maximum flow at the centre of the lumen and minimal flow at the periphery, encouraging deposition of organisms onto the surface of the tubing thus promoting further undisturbed bacterial proliferation [42, 45].

In addition, bacteria adhere more readily to hydrophobic polymeric plastic tubing (polyvinyl chloride, polyurethane) than to tubing composed of glass or steel [43]. SEM illustrates that the area of tube is not smooth but has an undulating surface, which might contribute to biofilm accumulation [22].



NATURE OF DUWLs BIOFILM

In the study of the structure of DUWLs biofilm environment and the processes occurring within it, modern research techniques are used, among others staining, electron microscopy, confocal laser microscopy, genetic and molecular methods. The advanced study techniques make it possible to show the variable nature of biofilms [4, 5, 6, 8, 27, 28, 29, 33]. To evaluate efficiency of disinfectant agents or disinfectant protocols, the presence and condition of biofilm in DUWLs was investigated using SEM [13, 19, 20, 22, 24, 25, 26, 30, 31, 34, 39, 46].

Figure 1 presents scanning electron micrographs that demonstrate a colonization sequence on dental tubing. The lighter coloured material resembling ice floes on which the cells and colonies are growing is probably calcium carbonate deposited by hard water. The underlying darker coloured material is the polyurethane tubing. All are taken at different magnifications, but demonstrate: (a) initial attachment; (b) beginning of elaboration of exopolymer; (c) formation of microcolonies, and (d) mature biofilm with cellular elements.

In the study of Noce *et al.* [29] sections of tubing from waterlines were processed for scanning electron microscopy to identify established biofilm in waterlines. The surface characteristics of section of waterline tubing collected from dental units that had been in use for an extended period showed significant biofilm relative to new tubing samples. New, used tubing was relatively smooth, with no organic biofilm matrix. In contrast, tubing samples from air/water lines and high-speed lines showed a continuous filamentous organic matrix. Embedded randomly throughout this matrix were short and long bacillus-like organisms. In the samples that were examined by Noce *et al.*, no significant differences were found between the air/water and high-speed lines.

Linger *et al.* [24] evaluated the use of a hydrogen peroxide disinfectant as a treatment to reduce the colonization and growth of heterotrophic bacteria, and by using SEM found a variety of biofilm formation in DUWLs, ranging from relatively early stages to well-established organic matrixes containing numerous colonizing microbial forms (Fig. 2a). In contrast, they found few bacteria on the surfaces of tubing taken from units that had been treated for 5 weeks with a peroxide-based disinfectant. However, a residual matrix was evident on these treated samples (Fig. 2b).

Putnins *et al.* [33] showed that all dental waterlines selected for the study were covered with a continuous microbial biofilm (Fig. 3a). The surface layer of this biofilm consisted primarily of filamentous microorganisms with numerous bacilli-like microorganisms interspersed throughout the biofilm structure. In the planktonic phase, few

Figure 1. Scanning electron micrographs of a colonization sequence on dental waterline tubing. Demonstrate: (a) initial attachment; (b) beginning elaboration of exopolymer; (c) formation of microcolonies; (d) mature biofilm with cellular elements. (Micrographs courtesy of the United States Air Force Dental Investigation Service, Great Lakes Naval Station, Illinois.)

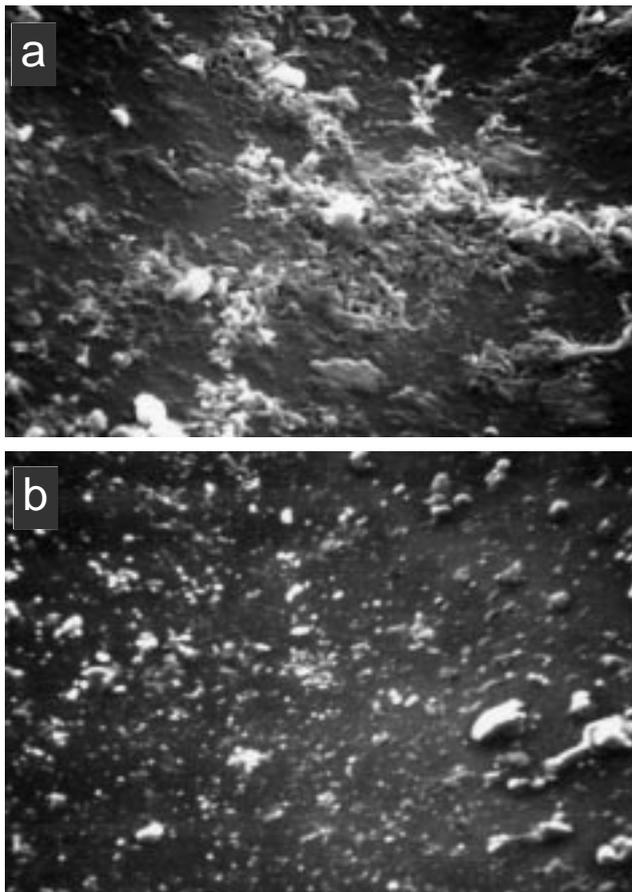


Figure 2. Scanning electron micrograph of: (a) baseline biofilm in a section of the air-water syringe line. Evidence of extensive microbial colonization with extensive organic matrix material is discernible ($\times 1,000$). (b) a representative section of the air-water syringe line after five weeks of periodic disinfectant treatment. Presence of irregular material on the surface was observed with few, if any, microbial forms evident ($\times 250$). (Used with permission from Dr. Jackson B. Linger. Reproduced from Linger JB *et al.* [24].)

individual bacteria were noted, but clumps of bacterial aggregates were seen and shared structural similarities with the sessile biofilm in that they were made up of bacilli and filamentous microorganisms (Fig. 3b).

BIOFILM AND MICROBIAL CONTAMINATION OF DUWLs

The source of bacteria for biofilm in DUWLs may be: (1) municipal water piped into the dental unit and, (2) suck-back of patient saliva into the line due to lack of anti-retraction valves [30].

Bacterial contamination of DUWLs is thought to follow development of biofilms on their inner surface. Frequently, water entering DUWLs is of good microbiological quality, but after shedding of bacteria from the biofilm, it becomes contaminated over the acceptable level [6, 23]. Biofilm can constantly release bacteria [18]. Studies of dental unit water sample have revealed widespread and unacceptably high levels of microbial contamination, with biofilm ranging in thickness from 30-50 μm [44].

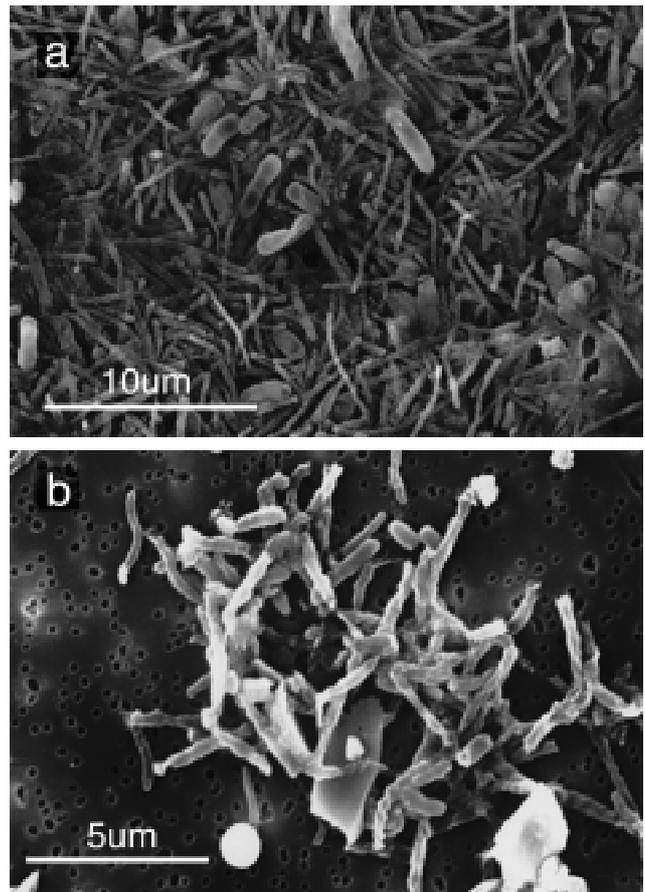


Figure 3. Dental unit waterlines and water collected from dental units contain bacteria and biofilms. (a) Tubing from a dental unit that had been in service for a number of years was removed and sectioned. (b) One ml of water collected from a dental unit was passed through a 0.2 μm pore filter to trap planktonic microorganisms and biofilm. Both samples were processed and viewed using scanning electron microscopy. (Used with permission from Dr. Edward E. Putnins. Reproduced from Putnins EE *et al.* [33].)

Barbeau *et al.* [6] give two mutually nonexclusive explanations which may explain the high levels of bacteria in dental unit waterlines. On the one hand, it can be postulated that most of the bacteria originate from multiplication and shedding of biofilm-associated microorganisms. On the other hand, the metabolic activity of the biofilm could release metabolites locally, and thus create a nutrient-rich interface that planktonic bacteria present in water could use to multiply inside the closed circuit of DUWLs.

Water used for restorative procedures should be of the same quality "as drinking water". A recommendation has been issued by the American Dental Association that by the year 2000, water for non-surgical procedures should contain no more than 200 cfu/ml bacteria in DUWLs [1].

The goal of infection control of DUWLs water is to minimise the risk from exposure to potential pathogens and to create a safe working environment for treatment patients. Water used for cooling the handpieces and flushing is considered as a way of microbiological transmission of pathogens to patients and doctors, and of cross-infections.

Modern methods aiming to reduce DUWLs contamination include: (1) antiretraction valves and retrograde aspiration of oral fluid; (2) filtration; (3) flushing; (4) using biocides and chemical disinfectants; (5) chlorination; (6) peroxide, ozone and ultraviolet light; (7) independent clean water systems; (8) autoclavable systems; (9) electrochemically activated water; and (10) drying.

It was established that using filters on the dental waterlines, has no impact on biofilm formation [31]. Biofilm is difficult to remove; flushing removes only an accumulated planktonic form and a few of the biofilm surface-absorbed microorganisms. It is recognised that flushing provides only temporary reductions in bacterial load and has no effect on the biofilm. As a result of the physics of the laminar forming flushing at different times, a fresh bacterial contamination flow in the waterline, the layer in immediate contact with the biofilm is stationary, even during flushing. After performing flushing at different times, a fresh bacterial contamination was found which seems to result from shedding of bacteria from the biofilm [27, 28, 36, 41].

Biofilm ecosystems are characterised by inner resistance to biocides and chemical disinfectants. Bacteria within the biofilm pose a major stumbling block to the use of biocide. They are 3,000 fold less susceptible to hypochlorite and therefore are not readily degraded, even by concentrated solutions of bleach or of other disinfectants such as glutaraldehyde. Planktonic organisms will be destroyed, but even if the majority of the organisms in the biofilm are eliminated, the architecture of the biofilm survives and acts as a pre-formed matrix for renewal of the biofilm. Inactivation of biocides is further impaired by interaction with organic material and electro-repulsion caused by surface charges on the biofilm [31].

Activities aiming to maintain the quality of water in DUWLs are oriented mainly towards biofilm removal. According Shepherd *et al.* [34], removal of the biofilm is the best approach for controlling the release of planktonic bacteria. In the study of Kettering *et al.* [22], who evaluated the efficiency of five antimicrobial agents, the biofilm was apparently reduced in volume, but not entirely eliminated.

Evaluation of Zerosil, a new waterline-cleaning product, indicates that it is very easy to use and extremely effective in killing the commonly found microorganisms in dental unit waterlines, as well as eliminating existing biofilms [30]. It seems, however, that a long-term evaluation of the product, also combined with other protocols securing an adequate water quality in DUWLs, is necessary.

Fiehn and Larsen's [18] study showed that drying DUWLs did not reduce the number of living bacteria in dental unit water; this procedure therefore has no effect on the biofilm.

BIOFILM MICROBIOLOGICAL COMPONENTS

Tall *et al.* [38] described the growth of biofilm in clean dental unit air-water syringe tubing from 0–120 days.

Using scanning electron microscopy, in the first week a few rods and spiral forms were seen, and by the end of the first month, there were many heterogenous microcolonies. After 6 months, there were multiple layers of different morphologies completely covering the lumen. The author presents the succession of species in order of appearance, as cultured. They were: *Pasteurella pneumotropica*, *Pseudomonas* spp., *Ochrobactrum anthropi*, *Stenotrophomonas maltophilia*, *Pasteurella haemolytica*, *Burkholderia pickettii*, *Pseudomonas stutzeri*, *Pseudomonas acidovorans*, *Aeromonas salmonicida*, *Acinetobacter calcoaceticus*, *Brevundimonas vesicularis*, *Pasteurella* spp., *Burkholderia cepacia*, *Psychrobacter phenylpyruvica*, *Pseudomonas putida*, *Flavobacterium* spp., *Flavobacterium odoratum*, and *Moraxella urethralis*.

It was found that a newly-installed dental unit had up to 2×10^5 cfu/ml count in a week [7]. The initial number of bacteria required to establish a biofilm can be very low; increase in the biofilm mass are due primarily to bacterial replication and growth within the biofilm.

The composition of biofilm can be inferred from the DUWLs microflora. Williams *et al.* [44] list some of the more prevalent microorganisms that have been identified in DUWLs, like other authors who described bacterial contamination many years earlier [21].

Another paper presented a microbial characterisation of predominant organisms representative for dental unit water and biofilms; they were: *Burkholderia pickettii*, *Burkholderia cepacia*, *Psychrobacter phenylpyruvica*, *Moraxella osloensis*, *Sphingomonas paucimobilis*, *Myroides odoratum*, *Brevundimonas vesicularis*, *Achromobacter* spp., *Stenotrophomonas maltophilia*, *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas stutzeri*, and *Alcaligenes faecalis* (*odorans*) [25].

Multiparametric analysis of waterline contamination in dental units made it possible to describe the different bacteria isolated from the dental units. *Sphingomonas paucimobilis* and *Acinetobacter calcaceticus* were the predominant cultivable species found in the microflora of DUWLs. The opportunistic pathogen *Pseudomonas aeruginosa* was isolated from 24% of examined units. Dental units contaminated by *P. aeruginosa* showed significantly higher total bacterial counts than others. Less predominant species obtained in the isolates from dental units were identified as: *S. maltophilia*, *P. putida*, *P. fluorescens*, *B. vesicularis*, *P. acidovorans*, *Actinomyces* spp., and *Bacillus* spp. Some yeasts and amoebae were observed by direct microscopic observation [7].

In Sheperd *et al.* study [34], the types of bacteria recovered on the R2A agar were typical of environmental bacteria associated with water supplies and were predominately Gram-negative rods (*Actinobacter*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, *Sphingomonas*, *Xanthomonas*), as well as Gram-positive rod (*Bacillus*), and gram-positive coccus (*Streptococcus*). An unexpected finding was that approximately 80% of the DUWLs tested harboured streptococci typical of those found in the oral cavity (*S. sanguis*, *S. mutans/sobrinus*, *S. intermedius*, *S.*

mitis, *S. salivarius*), typically located in the mouth in dental plaque, mucosal tissues, tongue and saliva. This indicates that contamination from patient-derived bacteria can occur from the functional end of the line.

In DUWLs biofilm there are always present primarily the bacteria of saprophytic Gram-negative species well adapted to growth in aquatic systems. The organisms colonizing water supplies are generally nonpathogenic environmental bacteria. However, as shown in the literature, microorganisms isolated from the DUWLs, such as *Pseudomonas aeruginosa*, *Legionella pneumophila* and non-tuberculosis *Mycobacterium* species, could pose a risk to immunocompromised patients and dental health care providers [2, 4, 5, 6, 7].

By the PCR-gene probe detection method, *Legionella* spp. were detected in 68% of the dental unit water samples and *L. pneumophila* was detected in 8%. *Legionella* spp. are regularly observed in concentration of 10^2 – 10^5 cfu/ml. Other studies, however, reported lack of *Legionella* in DUWLs [34, 40, 45].

Some dental instruments can considerably increase the concentration of free-living amoebae, some of which are potential human pathogens. *Hartmannella*, *Vanella* and *Vahlkampfia* spp. were most frequently encountered; *Acanthamoebae* and *Naegleria* spp. were also isolated [4].

CONCLUSIONS

The risk of acquiring an infections disease from exposure to dental water is difficult to evaluate. Bacterial load in dental unit water is often excessive and potential pathogens may occur [18]. According Panagakos *et al.* [30], the dental practitioner must be knowledgeable regarding microbial contamination in biofilm formation in dental unit waterlines. Education should stress the need for improvement in the quality of water delivered to patients during treatment. Manufacturers must also play an important role providing training and education regarding the proper use and maintenance of their systems.

Dental facilities, both public and private, need a reliable method to prevent the development of biofilms within DUWLs. These methods must be economical and require minimal effort to use on the part of dental staff. In order for the system to work efficiently, the effluent water produced must be compatible with dental materials and be potentially free from toxic or carcinogenic materials [30].

The prevention strategies which are designed to reduce the impact of the biofilm in DUWLs are a real and continuing problem. Future research into prevention of biofilm proliferation should be promoted by dental organisations and governmental agencies around the world.

In the shorter term, within dental units, disinfectants may have a role to play in controlling the levels of microbial contamination lines to more acceptable levels [35]. New products allowing the control of contamination and disruption of the biofilm should be looked for [18].

According to Bleinkinsop *et al.* [10] the solution of the biofilm formation problem, from the medical point of view, in the future will be “electro-enhancement” of biocides to neutralise the surface charge.

Researchers suspect that residual biofilm is mainly due to the complex design of dental chair equipment resulting in the stagnation of water within the equipment lines and build up of biofilm, and suggest that units should be redesigned to discourage biofilm formation [35].

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