Biofilm 5 (2023) 100132

Contents lists available at ScienceDirect

Biofilm

journal homepage: www.sciencedirect.com/journal/biofilm

The consistent application of hydrogen peroxide controls biofilm growth and removes *Vermamoeba vermiformis* from multi-kingdom *in-vitro* dental unit water biofilms

Michel A. Hoogenkamp *, Danuta Mazurel, Elly Deutekom-Mulder, Johannes J. de Soet

Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam, Vrije Universiteit Amsterdam, Amsterdam, the Netherlands

ARTICLE INFO

Keywords:
Amoeba
Biofilm
Legionella
Hydrogen peroxide
Medical device

ABSTRACT

The water systems inside a dental unit are known to be contaminated with a multi-kingdom biofilm encompassing bacteria, fungi, viruses and protozoa. Aerosolization of these micro-organisms can potentially create a health hazard for both dental staff and the patient. Very little is known on the efficacy of dental unit disinfection products against amoeba. In this study we have examined the effect of four different treatment regimens, with the hydrogen peroxide (H_2O_2) containing product Oxygenal, on an *in-vitro* multi-kingdom dental unit water system (DUWS) biofilm. The treatment efficacy was assessed in time using heterotrophic plate counts, the bacterial 16S rDNA, fungal 18S rDNA gene load and the number of genomic units for *Legionella* spp. the amoeba *Vermamoeba vermiformis*. The results indicated that a daily treatment of the DUWS with a low dose H_2O_2 (0.02% for 5 h), combined with a weekly shock dose (0.25% H_2O_2, 30 min) is necessary to reduce the heterotrophic plate count of a severely contaminated DUWS (>10^6 CFU.mL^{-1}) to below 100 CFU.mL^{-1}. A daily treatment with a low dose hydrogen peroxide alone, is sufficient for the statistically significant reduction of the total amount of bacterial 16S rDNA gene, *Legionella* spp. and *Vermamoeba vermiformis* load ($p < 0.005$). Also shown is that even though hydrogen peroxide does not kill the trophozoite nor the cysts of *V. vermiformis*, it does however result in the detachment of the trophozoite form of this amoeba from the DUWS biofilm and hereby ultimately removing the amoeba from the system.

1. Introduction

The dental unit is an important part of the dentist's toolbox for providing dental care. Besides the obvious seating part, it also contains all the instrumentation the dentist uses during treatment. Inside this dental unit, the dental unit water system (DUWS) is situated which supplies cooling and irrigation water to the high and low-speed rotary instruments, ultrasonic scalers and the three-way air water syringe. The DUWS is a vast and complex network of tubing, multiple connectors and valves. Within the European Union, mostly potable tap water is used to fill the DUWS, either directly connected to the watermains or by using a reservoir system [1]. This water meets the microbiological criteria as set in the European Drinking Water Directive [2], but does contain small amounts of micro-organisms. The intrinsic properties of the DUWS, namely; small diameter tubing made of different (plasticized) materials, intermittent low water flow combined with long periods of stagnancy and operated at room temperature, create perfect conditions for microbial growth and biofilm formation. This type of microbial contamination was first described by Blake in 1963 [3] and since, several studies have shown the DUWS contains a multi-kingdom biofilm, encompassing bacteria, fungi, protozoa and viruses [4–6].

Water extruded from these contaminated DUWS, either as a splatter or aerosol can pose a risk to both the patient as the dental staff [7,8]. Even though infections associated with contaminated DUWS water are likely to be underreported [9], some cases have been described for nontuberculous mycobacteria, which are associated with post-treatment oral soft tissue infections in both children and the elderly [10–13]. Additionally, *Pseudomonas* and *Legionella* species have been associated with occupational asthma, Pontiac Fever and even Legionnaire's Disease of dental staff and patients [14–16]. To guarantee safety of both patients and dental staff, dental associations in the USA and Europe recommend adhering to the microbiological standards set by the American Dental Association (<500 CFU.mL^{-1} HPC) and European Union water quality standards (<100 CFU.mL^{-1}), respectively [17]. Within for example The

* Corresponding author. Gustav Mahlerlaan 3004, 1081 LA, Amsterdam, the Netherlands.
E-mail address: m.hoogenkamp@acta.nl (M.A. Hoogenkamp).

https://doi.org/10.1016/j.biofilm.2023.100132
Received 23 January 2023; Received in revised form 24 May 2023; Accepted 24 May 2023
Available online 25 May 2023

Netherlands, the Royal Dutch Dental Association (KNMT) has adopted this European standard [18]. To be able to comply to these standards, dental staff needs to perform disinfection protocols and monitor the microbiological quality of the dental unit water. In daily practice, well studied DUWS treatment agents containing hydrogen peroxide with or without silver ions, sodium hypochlorite, citric acid, or quaternary ammonium chlorides (QAC) are used and are primarily aimed at and tested for reducing the bacterial burden in the effluent water and the biofilm [9,19–21]. Only few studies describe the effect of these agents against the eukaryotic constituents of the DUWS biofilm. Hydrogen peroxide containing agents have for instance shown to be active against fungi [22,23], but do not seem to inactivate amoeba [24,25].

In a recently published study on the microbial load and microbiome of the Dutch dental unit there was however, an indication that hydrogen peroxide might have an effect against amoeba [6,26]. Although not statistically significant, the prevalence of *Vermamoeba vermiformis* (formerly *Hartmannella vermiformis*) in DUWS treated with agents containing hydrogen peroxide seemed to be lower than untreated units or units treated with agents containing for instance QAC or citric acid.

The prevalence of free-living amoeba (FLA) in DUWS is estimated at 40–60% and is mainly represented, but not exclusively, by *V. vermiformis* [5,6,25,27,28]. This protozoa has a two-stage life cycle, switching between the trophozoite form, necessary for division, migration and feeding and the cyst form [29]. The trophozoite encysts when challenged with hostile conditions (e.g. biocide treatment or nutrient depletion) and can excyst when conditions become more favourable [30,31]. The trophozoite form exclusively feeds on microorganisms residing in biofilms [32,33]. Prey-organisms are phagocytosed and digested, but some bacteria such as *Legionella pneumophila*, nontuberculous mycobacteria and *Pseudomonas* spp. have developed mechanisms to avoid digestion and are able to survive and even replicate inside the FLA. Inside the amoeba, accumulated bacteria, plasmids and DNA are concentrated in the food vacuole which can promote horizontal gene transfer, potentially resulting in the development of antimicrobial resistance and increased virulence [24,34–36]. Once released from the amoeba, high numbers of these pathogens can re-occupy the existing biofilm or be aerosolized from the DUWS during dental treatment. Several fungi and respiratory viruses have also developed mechanisms to use the protozoal host for their own proliferation and protection against antimicrobial treatment [24,37–39]. Although *V. vermiformis* itself can be the causative agent for keratitis, it is otherwise rarely responsible for human disease [37]. Indirectly, the FLA can therefore play an important role in the adaptation, transmission and dispersal of pathogenic FLA resistant micro-organisms by acting as a 'biological gym' for future encounters with more evolved hosts [40].

Controlling the presence of FLA could therefore also indirectly prevent an increasing virulence of opportunistic pathogens present in the biofilm. As the results from the Dutch dental unit microbiome [6] have indicated a (possible) effect of hydrogen peroxide containing agents against amoeba, *Legionella* spp. and biofilm in general, in this study we have assessed the effect of such products in a translational *in-vitro* dynamic flow model. For this study we had a threefold aim: 1. To determine which treatment regimen using a hydrogen peroxide containing disinfection agent is most suitable for the disinfection of a severely contaminated unit and long-term overall biofilm control. 2. Whether a hydrogen peroxide based dental unit water disinfection agent is suitable to prevent *V. vermiformis* proliferation and 3. How does hydrogen peroxide interact with *V. vermiformis*?

2. Materials and methods

2.1. Evaluation of the most effective treatment regimen to control DUWS biofilm

To assess the most effective treatment regimen for overall biofilm control and specifically the inactivation of the amoeba *V. vermiformis*, a translational *in-vitro* dynamic flow model was used to simulate DUWS biofilms consisting of an multi-kingdom biofilm, including *V. vermiformis* [33].

2.1.1. Pre-conditioning of the model

In short, 13 models were constructed as described and depicted by Hoogenkamp et al. [33], fitted with polyurethane tubing (internal diameter 4 mm, 1 m length) and subsequently inoculated with non-potable laboratory tap water containing $\sim 2{\cdot}10^3$ colony forming units (CFU).mL^{-1} as determined by heterotrophic plate counts (HPC). This water was left static for 24 h to allow the micro-organisms to adhere to the tubing surface, followed by a dynamic flow protocol (See Fig. 1). On weekdays, this protocol consisted of daily, 30 cycles in which water was flowed (30 mL.min^{-1}) for 30s followed by 9.5 min stagnancy. After the daily cycles and during the weekend, the water was left stagnant. Biofilms in all models, bar one, were allowed to develop for 4 weeks at 23°C (±1°C). From this one tubing, the biofilm was harvested after two weeks and used for BioFlux analysis to analyse how hydrogen peroxide acts on the amoeba present in the biofilm. Of the remaining 12 models, at week 4, a 55 mL baseline effluent sample was taken immediately after an overnight stagnancy period and prior to any hygiene measures ('proxy' biofilm sample). Samples were processed immediately, as depicted in Fig. 1 for the determination of the HPC and the remaining sample was concentrated by filtration and stored at −80°C for further DNA isolation and analysis of the bacterial and fungal DNA load and the amount of Genomic Units (GU) of *Legionella* spp. and *V. vermiformis* by Q-PCR, depicted in exactly as described before [6].

2.1.2. Treatment regimes

After the initial baseline sample was taken, the 12 models were divided into four groups (See Fig. 2., n = 3 per group), (I) no treatment, (II) a daily low dose disinfectant (DLDD, 300 times diluted Oxygenal™ 6; KaVo, Biberach an der Riss, Germany, final concentration 0.02% H_2O_2, present during the cycling period), (III) a weekly high dose biofilm disinfectant (shock dose, 24 times diluted Oxygenal, final concentration 0.25% H_2O_2, 30 min), (IV) a combination of a DLDD and shock dose. Shock dose treatment for groups III and IV (0.25% H_2O_2 for 30 min) were performed straight after taking the baseline sample. The shock dose was washed out, by flushing the models for 5 min with tap water (30 mL.min^{-1}). Subsequently, the DLDD groups (II and IV) received water containing 0.02% H_2O_2 and Groups I and III, tap water during the daily cycles. During weekdays the dynamic flow protocol was used as described above. On weekends the models were left static without any flushing of water.

To monitor the efficacy of the treatment regimes, one week after commencing the treatment regime a 'proxy' biofilm sample was taken on the second weekday (See Fig. 2) and processed as described above for the baseline samples. This procedure was repeated at week 3, 5 and 7.

2.2. BioFlux analysis of the anti-amoebal effect of hydrogen peroxide

2.2.1. Preparation of the inoculum

The biofilm from a single model, as mentioned above, was collected at week two and was used for the BioFlux analysis. The biofilm (containing about $1.5{\cdot}10^6$ CFU of heterotrophic bacteria.cm^{-2}) was resuspended in 5 mL R2A broth (0.5 g.L^{-1} of Yeast Extract (BD), Proteose Peptone No. 3. (BD), Casamino acids (BD), Glucose (Sigma), Soluble Starch (Sigma), 0.3 g.L^{-1} of K_2HPO_4 and sodium pyruvate (both Sigma) and 0.05 g.L^{-1} $MgSO_4.7H_2O$) [41] and vortexed for 2 min to suspend the inoculum. Prior to use, the suspension was corrected to an $OD_{600} \approx 0.2$.

2.2.2. Visualization of the anti-amoebal activity of hydrogen peroxide

For the visualization of a possible anti-amoebal effect of the hydrogen peroxide, the BioFlux Z1000 platform (Fluxion Biosciences, Alameda, CA, USA) was used. This platform is an automated microscopy system, combined with a microfluidics device, which enables for high

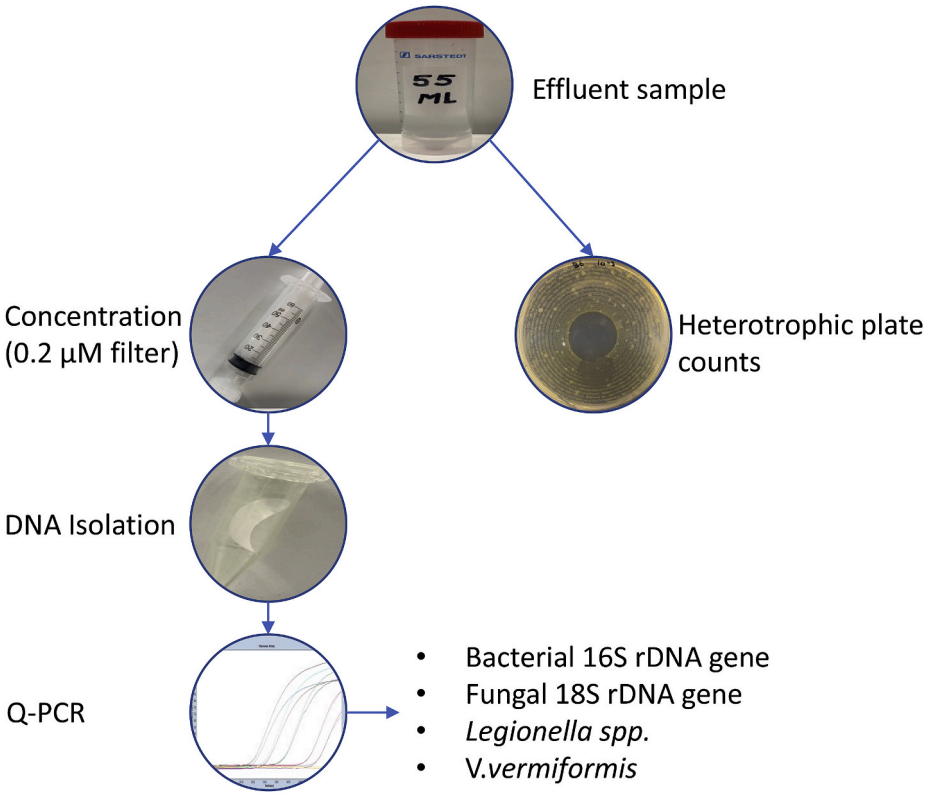

Fig. 1. Flowchart depicting the sample processing.

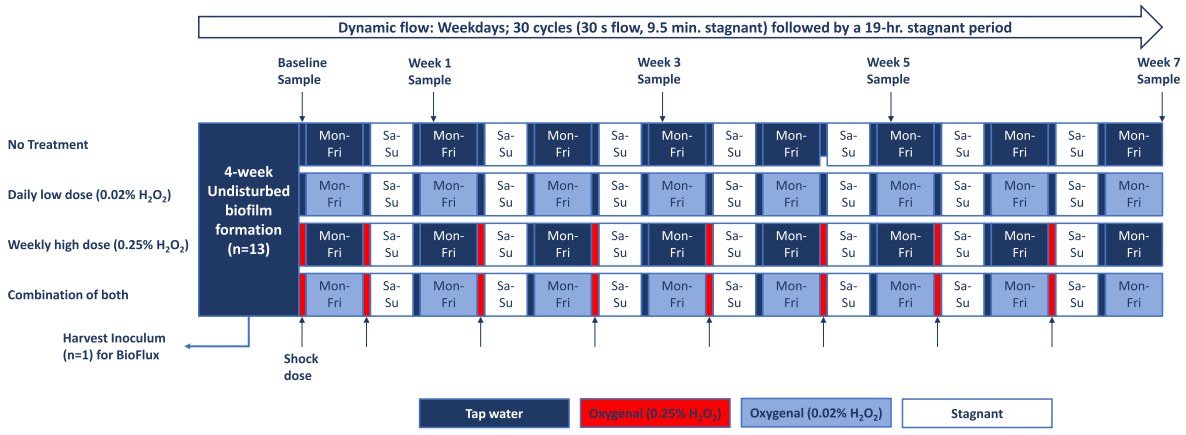

Fig. 2. Depiction of the treatment groups and disinfectant regimens used during the experiment.

throughput shear flow assays using positive pressure fluid displacement and is described in detail by Volgenant et al. [42]. During the entire experiment, both plates and fluids used, were kept at 23°C to avoid air bubble formation. All incubation steps were performed under aerobic conditions. Each channel in the BioFlux plate was seen as an independent sample and each treatment was tested in triplicate in two independent experiments. The detailed sequence of procedures to grow the biofilms is shown in Fig. 3. In short, the microfluidic channels of the BioFlux plates were primed by adding R2A broth to the inlet channel to remove all air from the channels. Then, the channels were seeded using 70 µL of the inoculum and the micro-organisms were allowed to adhere for 24 h. Subsequently, R2A broth was chosen to accelerate bacterial biofilm growth and biofilms were grown for 5 days under the dynamic flow protocol as described in Fig. 2 and similar to the protocol used in the *in-vitro* model described above.

Prior to treatment, the channels were checked for the presence of both the trophozoite and cyst form of *V. vermiformis*. Channels were subsequently flushed with sterilized tap water to remove loosely adhered cells and the proteinaceous growth media components of the R2A broth. Oxygenal solutions were prepared in sterilized tap water to a final concentration of 0.25% H_2O_2. Due to the high oxidizing and proven biofilm disruption potential, hydrogen peroxide (final concentrations 0.25%, 2%, Both Sigma Aldrich, St. Louis, Mo, USA) were included as positive controls [43]. Treatment solutions were added to the appropriate inlet wells and the plate was mounted on the microscope stage. Fixed microscope positions (3 per channel) were chosen, covering almost the entire length of the channel and a real-time image sequence was initiated. Brightfield images were taken every 5 min using a 20x magnification. Immediately after the initial image, the treatments solutions were flowed into the channel using 1 dyne flow for 5 min.

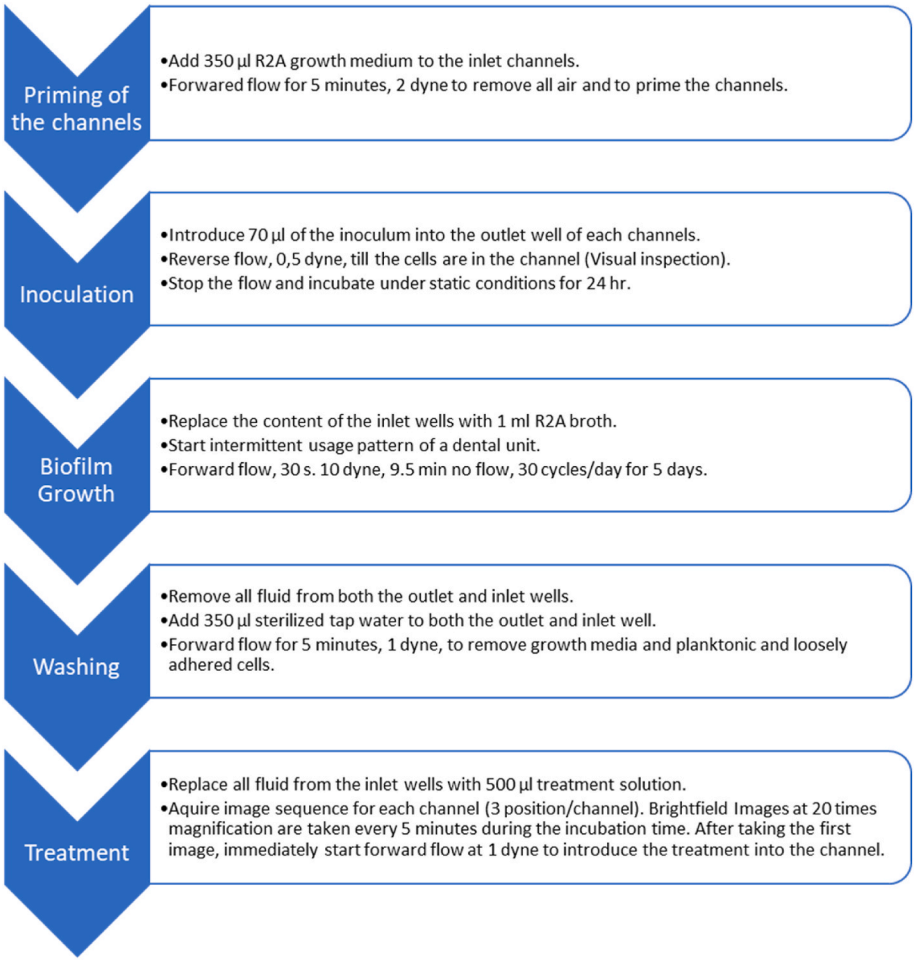

Fig. 3. Workflow of the biofilm growth and treatment protocol to observe the biofilm removal efficacy of the dental unit water disinfectants in the BioFlux system.

Due to the higher oxidative potential, treatments with 2% hydrogen peroxide were imaged every 1 min using a 20x or a 10x magnification for a better overview.

Stacks of each image sequence were created and automatically adjusted for brightness and contrast using Fiji (Image J 1.53c, Java1.8.0_172, 64 bit, http://https://imagej.net/Fiji), and the Bio-Formats import option [44]. Stacks were analysed and specific events were noted, such as the effects on biofilm characteristics and/or amoeba.

2.3. Statistics

Prior to statistical analysis, the HPC, *Legionella* spp. and *V. vermiformis* data were log10 transformed. Values below the detection limit were set a factor 10 lower than the detection limit to allow for successful log10 transformation. For between group comparisons (Difference between treatment groups at baseline or week 7), the Kruskal-Wallis test was used and when significant differences were observed, a Bonferroni post-hoc test was applied. To assess if there was a correlation between the *Legionella* and *Vermamoeba* load, a Spearman's ranks correlation test was performed. Differences were deemed statistically significant at $p < 0.005$ [45]. Statistical analysis was performed using SPSS 26.0 (IBM, Amsterdam, the Netherlands).

Due to the nature of the BioFlux analysis data, the anti-amoebal effect of hydrogen peroxide cannot be tested statistically.

3. Results

3.1. Evaluation of the most effective treatment regimen to control DUWS biofilm

3.1.1. Effect of treatment regimen on heterotrophic plate counts

The HPC data revealed that the 'proxy' biofilm sample contained on average (±SD) log10 6.1 ($\pm 3.2{\cdot}10^{-2}$) CFU.ml^{-1} at baseline. As shown in Fig. 4A, the HPC in the control group remained biologically stable, yet did show to have a statistically significant increase in numbers over time (comparison baseline vs week 7 (log10 6.2 ($\pm 5.1{\cdot}10^{-2}$) CFU.mL^{-1}, $p < 0.005$). Only groups receiving a DLDD in combination with a weekly 0.25% H_2O_2 shock dose had a statistically significant lower ($p < 0.005$) HPC than the control group at week 7 (log10 1.7 (±0.5) CFU.ml^{-1}). After an initial decrease after 3 weeks, the group which received only a weekly shock dose, almost recovered completely after 7 weeks of treatment (log10 5.4 ($\pm 3.9{\cdot}10^{-2}$) CFU.mL^{-1}).

3.1.2. Effect of treatment regimen on the bacterial 16S rDNA gene concentration

Similar to the HPC, the bacterial 16S rDNA gene concentration (See Fig. 4B) in the control group increased significantly ($p < 0.005$) in 7 weeks ($6.1{\cdot}10^{1}$ ($\pm 5.1{\cdot}10^{1}$) and $3.8{\cdot}10^{2}$ ($\pm 1.2{\cdot}10^{2}$) pg.µl^{-1}, respectively). When comparing between treatments at week 7, the group receiving only a DLDD or a DLDD with an additional 0.25% H_2O_2 shock dose contained a significantly lower bacterial 16S rDNA concentration ($2.3{\cdot}10^{-2}$ ($\pm 1{\cdot}10^{-2}$) and $2.1{\cdot}10^{-2}$ ($\pm 8.5{\cdot}10^{-3}$) pg.µl^{-1} respectively, $p < 0.005$).

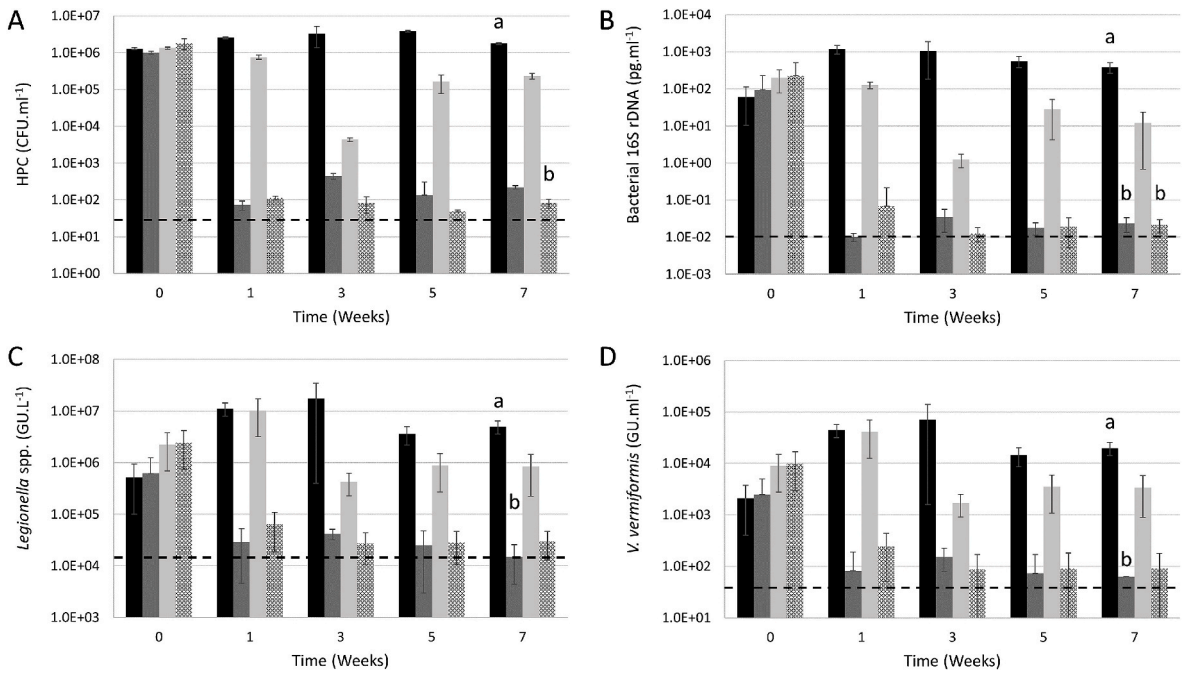

Fig. 4. Effect of four different treatment regimens (n = 3 for each regimen) in time on; (A) the heterotrophic plates count expressed in CFU.mL^{-1}, (B) Bacterial 16S rDNA genes expressed in pg.mL^{-1}, the amount of *Legionella* spp. expressed in GU.L^{-1} and (D) the amount of *Vermamoeba vermiformis* expressed in GU.mL^{-1}. The black bars represent the control group (no treatment), the dark grey bars the daily low dose group (0.02% H_2O_2), the light grey bars the weekly shock dose group (0.25% H_2O_2), the speckled white bars the daily low dose with an additional weekly (0.25% H_2O_2) shock dose group. The dotted lines in each panel represent the detection limit of the respective assay. The black bars marked with an (a) are statistically significant different to the respective baseline value at week 0 ($p < 0.005$). Bars indicated at week 7 with a (b) are statistically significant different to the control group at week 7 ($p < 0.005$).

3.1.3. Effect of treatment regimen on the fungal 18S rDNA gene concentration

The fungal 18S rDNA gene load at baseline fluctuated strongly (range $<2.5{\cdot}10^{-5}$ (detection limit) – $1.4{\cdot}10^{-1}$ pg.µL^{-1}) and was irreproducible within groups in time.

3.1.4. Effect of treatment regimen on the amount of Legionella spp.

As shown in Fig. 4C, within the control group the *Legionella* spp. load a statistically significant increase over time (baseline vs week 7, log10 5.6 ($\pm 4.3{\cdot}10^{-1}$) and log10 6.7 ($\pm 1.3{\cdot}10^{-1}$) GU.L^{-1}, respectively). When comparing treatment regimens at week 7, only the group receiving a DLDD had a significantly lower *Legionella* spp. load as compared to the untreated control group log10 4.0 ($\pm 5.9{\cdot}10^{-1}$) GU.L^{-1} and log10 6.7 ($\pm 1.3{\cdot}10^{-1}$) GU.L^{-1}, respectively $p < 0.005$).

3.1.5. Effect treatment on the amount of Vermamoeba vermiformis

The presence of the amoeba *V. vermiformis* showed identical results as the *Legionella* spp. (See Fig. 4D). The amount of *Legionella* and *Vermamoeba* had a statistically significant ($p < 0.005$) Spearman's ranks order correlation of r_s of 0.996. Within the control group the *V. vermiformis* load increased statistically significant over time (baseline vs week 7, log10 3.2 ($\pm 4.4{\cdot}10^{-1}$) and log10 4.3 ($\pm 1.3{\cdot}10^{-1}$) GU.mL^{-1}, respectively). When comparing treatment regimens at week 7, only the group receiving a DLDD had a significantly lower *V. vermiformis* load as the compared to the untreated control group (<log10 1.1 and log10 3.5 ($\pm 2.8{\cdot}10^{-1}$) GU.mL^{-1}, respectively $p < 0.005$).

3.2. Visualization of the anti-amoebal activity of hydrogen peroxide

Five-day old biofilms containing *V. vermiformis* were treated under flow conditions with both water and Oxygenal (0.25% H_2O_2). Trophozoite and cyst forms of the amoeba were not removed by flowing water over the biofilm. Treatment with Oxygenal appeared to act directly on the trophozoite form of the amoeba as it was removed from the biofilm within 5 min of flow (See Fig. 5). Cyst forms remained unaffected in the biofilm (data not shown).

To determine whether a higher concentration of 2% hydrogen peroxide had an enhanced effect on the biofilm, this solution was also flowed over the biofilm. As shown in Fig. 6 and as real-time footage in Suppl. File 1, treatment with H_2O_2 resulted in incomplete removal of biofilm layers, while complex structures seemed to partially dissolve into streamers. Trophozoite forms of amoeba were removed within 3 min upon exposure to the H_2O_2, while cysts initially shrink (darkening) and eventually swell and lyse.

4. Discussion

In this study we aimed to validate which treatment regimen, with a hydrogen peroxide disinfection agent, is most effective in the overall reduction of the biofilm by reducing the HPC till below the recommended 100 CFU.mL^{-1}, the total bacterial 16S and fungal 18S rDNA gene load and the *Legionella* spp. and *V. vermiformis* load for prolonged time. As a DUWS disinfection agent, the hydrogen peroxide containing, Oxygenal 6 was used as this has proven to be effective against polymicrobial biofilms, both *in-situ* [21,46] and *in-vitro* [19,23].

At baseline, the HPC was $\log_{10}$ 6.1 CFU.mL^{-1} and this would be comparable to heavily contaminated dental units [6,47]. Treating the biofilm with only a weekly shock dose of Oxygenal (0.25% H_2O_2), resulted in an initial reduction in HPC which was effective until week 3. In the weeks after, this biofilm recovered from the applied treatment regimen. This process of survival, adaptation and regrowth has been described by others [33,48,49] and confirms the suggestion made by Walker et al. [19], that 'weekly treatment programs may not be sufficient'. Performing a daily treatment protocol with a daily low dose Oxygenal (0.02% H_2O_2), which is aimed at reducing the microbial load in the effluent water, resulted in a log10 4 reduction in HPC within the first week. This reduction was consistently, although not statistically significant, effective till week 7. This observed biologically significant

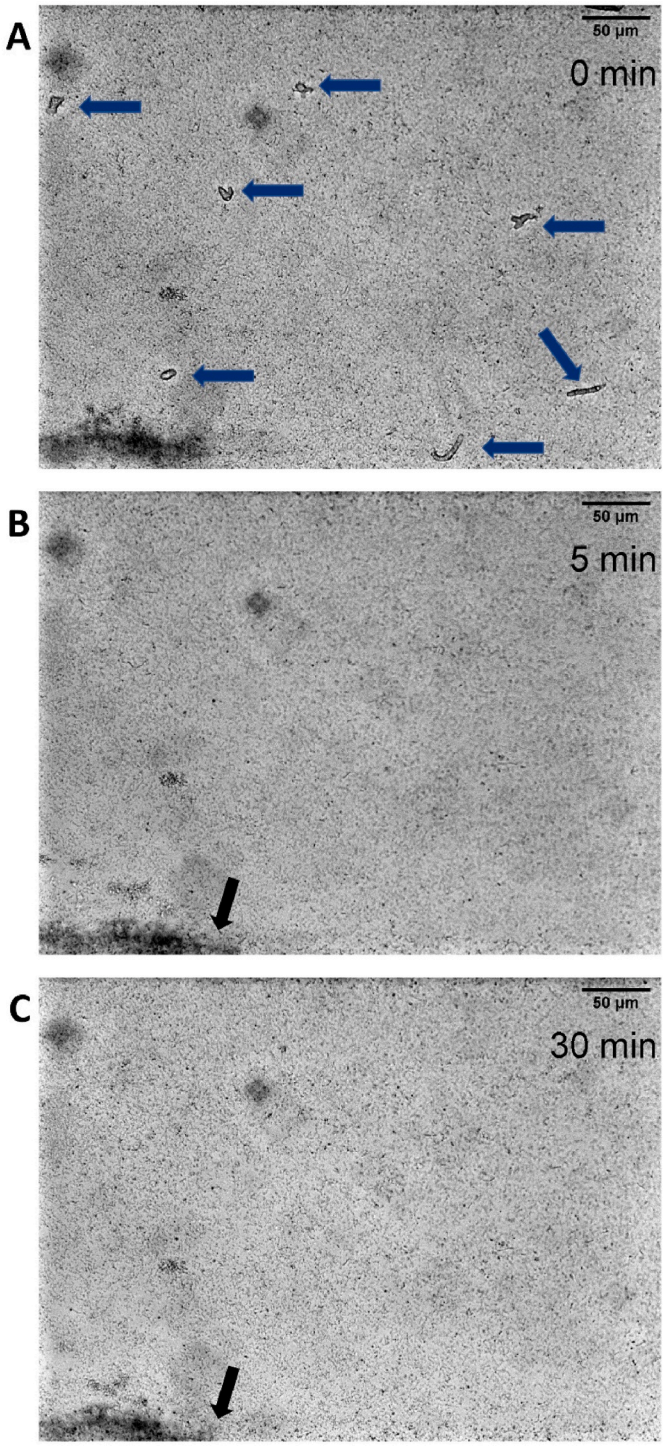

Fig. 5. Photomontage of a single channel location showing the biofilm removal efficacy of Oxygenal (0.25% H_2O_2) under 1 dyne shear force. Panel A shows the presence of the trophozoite form of the amoeba (indicated with blue arrows). Panel B shows the removal of the trophozoite form. Panel B & C show the shedding of the biofilm after 5–30 min (indicated with black arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

effect is consistent with previous studies that have shown similar results using Oxygenal, both in in-situ experiments and using advanced *in-vitro* models [19,21,23,46,50]. As a result, the HPC levels obtained turned out to be lower than those of the input water. It is important to note that this level of decontamination is not exclusive to hydrogen peroxide, as other substances such as tetraacetylethylenediamine (TAED) perborate or electrolyzed water have also been shown to produce similar results in complex *in-vitro* models, inhibiting biofilm growth [51,52]. The current study shows that for a reliable and reproducible inactivation of a biofilm in DUWS using hydrogen peroxide, at least a daily suppression of biofilm growth with Oxygenal (0.02% H_2O_2) was needed in combination with a weekly shock treatment with a high concentration of Oxygenal (0.25% H_2O_2). This finding confirmed results obtained from a field study in the general dental clinic, which indicated that the combination treatment was more effective in reducing the HPC than only treating the biofilms with a daily low dose [6]. From a mechanistic point, this result can be explained, since H_2O_2 only inactivates the outer layer of biofilms as naturally occurring deposits (inorganic salts and biofilm itself) prevent its penetration and subsequent removal of the biofilm [50]. BioFlux data obtained in the current study supports this as a single treatment with an Oxygenal shock dose did not remove the complete biofilm, although more research is needed to study this effect in more detail. Unaffected micro-organisms in the inner layers of the biofilm can therefore recover in the absence of disinfectant, leading to selection and succession towards more H_2O_2 resistant and catalase positive micro-organisms, such as *Pseudomonas* spp. and many more [46,53]. These micro-organisms can subsequently get aerosolized during dental treatment and (in) directly cause a health risk to dental staff and the patient [46]. The combination treatment in which a daily low dose of H_2O_2 inactivates the planktonic cells, combined with a weekly high dose of H_2O_2 which slowly peels the biofilm off the surface, could explain the treatment efficacy of the combined use of a daily low dose and a weekly shock dose tested in *in-vitro* flow models [19,50].

As the growth media used for HPC, does not support the growth of all micro-organisms [49] and lack of growth is not indicative for the absence of *Legionella* spp. [6], we also analysed the bacterial 16S rDNA, *Legionella* spp. and *V. vermiformis* load. Even though *Legionella* spp. were detectable at all timepoints, the use of a daily low dose of Oxygenal alone, contrary to the HPC, was enough for a statistically significant reduction of all DNA parameters. We could speculate that this treatment regimen would also prevent the biofilm formation and *Legionella* and amoebal proliferation as the survival of *Legionella* is dependent on the presence of amoeba and biofilm [54–56]. In the current study also, a strong correlation was found between the presence of *Vermamoeba* and *Legionella* and both organisms showed a similar response to the antimicrobial treatment. Amoeba are known to have a higher tolerance towards antimicrobials and internalized *Legionella* spp. are hence protected against these agents [37]. The biofilms which received only a weekly shock dose, showed a relatively unaffected bacterial 16S rDNA load, while an initial increase in both the *Legionella* and *Vermamoeba* load immediately after the first week of treatment was observed.

It is known that Oxygenal does not irradicate eukaryotes such as amoeba and fungi [24,25,57,58] and antimicrobial treatment only kills the bacterial components in the biofilm, but does not remove them [59]. It can be questioned whether the silver ions in Oxygenal play a part in its efficacy to remove the biofilm? In theory, the silver-ions could de-stabilize the biofilm matrix, resulting in removal [60]. Although not individually tested in the dynamic flow model, previous studies have shown that for instance, Citrisil, a disinfection agent containing the 400 ppm silver ions did not show any biofilm removal effect [61], nor did it remove amoeba from the biofilm (own unpublished data). To assess the effect of Oxygenal on the fungal part of the DUWS biofilm the fungal load was determined. This fungal load, unfortunately, showed a large variation at baseline and was irreproducible within the groups during the study. Hence, no conclusions on the efficacy of the treatment regimens on the fungal constituents of the biofilms could be drawn. *In-vivo* data also shows this high variability of the fungal load [6]. A possible explanation for the variability could be that fungal growth has a longer lag phase as compared to bacterial growth in a water biofilm, possibly indicating that the duration of the current study should be extended to observe fungal growth [49].

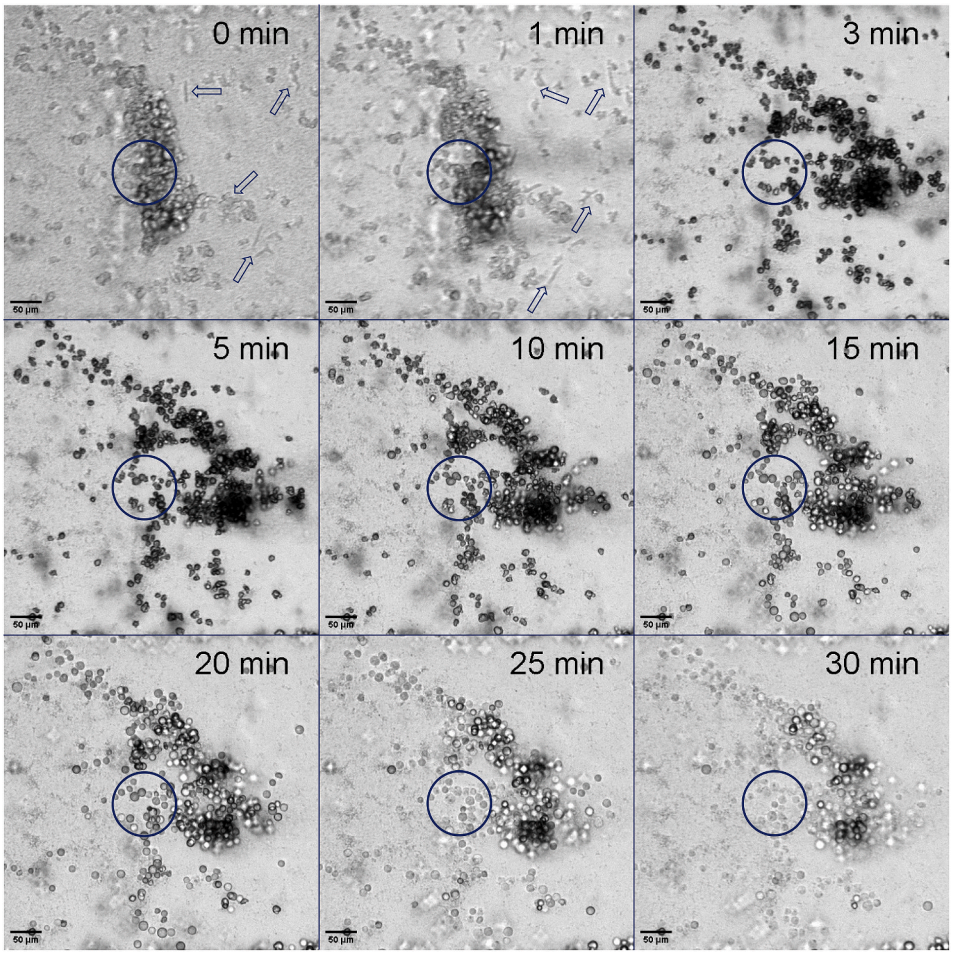

Fig. 6. Photomontage of a single channel location showing the biofilm removal efficacy of 2% H_2O_2 under 1 dyne shear force at 10x magnification. On initiation of fluid flow the biofilm is partially displaced (Blue circle). Removal of the trophozoite form occurs within 3 min (blue arrows), while remaining cysts appear to shrink (darkening) and subsequently swell up before complete lysis (encircled). For real-time footage, please see Suppl. File 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

A possible explanation for the increase of *Legionella* and *Vermamoeba* after shock treatment could be that inactivated biofilm cells remain behind and serve as a nutrient source for the surviving amoeba [55]. Subsequent release of living internalized *Legionella* cells from amoeba, as a part of the natural turnover, could results in an increased *Legionella* presence [62]. As Oxygenal, does not kill *V. vermiformis* [24,25,57], there has to be an alternative mechanism in which the amoeba are removed from the biofilm. One indication could be that the lack of killing efficacy is caused by an experimental artefact in previous studies, as in those studies the biofilms/organisms were given a single treatment under static batch conditions and were subsequently (re-)cultured to test the killing efficacy. In the current study however, all treatments were performed longitudinally for 7 weeks and under flow conditions, the latter flushing out whatever is detaching from the biofilm. To test this hypothesis, the anti-amoebal effect of Oxygenal was visualized under flow conditions using the BioFlux platform. The plates used in this experiment were inoculated with biofilm originating from the dynamic flow model. To enhance bacterial growth in time, R2A broth was used to allow for biofilm development. Although water biofilms fed with R2A, as compared to water as a nutrient source, are likely to differ compositionally and structurally [63], there is no scientific evidence that the behaviour of amoeba is different. The BioFlux biofilms grew heterogeneously with respect to biofilm structure and all contained cysts and trophozoite forms of *V. vermiformis*, either embedded in the biofilm or grazing on the biofilm, respectively. Treatment with Oxygenal resulted in the detachment of the trophozoite forms of the amoeba within 5 min, but not of the cysts. This effect was comparable to treatment of the biofilms with 0.25% 'pure' hydrogen peroxide. A similar effect of hydrogen peroxide on the release of *Neoparamoeba perurans* trophozoites has been documented in relation to the treatment of amoebic gill disease [64]. A possible explanation for the sudden detachment of grazing trophozoites from the biofilm after H_2O_2 could be the oxidative denaturation of the lectins on the protozoal cell. Amoeba use these lectins to bind to the bacteria in the biofilm [32,34] and loosing this grip will likely results in removal from the DUWS. For full removal of both trophozoite and cyst form of *V. vermiformis*, the concentration should be increased to 2% H_2O_2. At this concentration, lysis of the cyst form occurred after initial shrinkage (darkening of the cell) and subsequent permeabilization. This cellular response has been described previous in response to cysts of the amoeba *A. castellanii* to a chlorine treatment [65]. Further research is required to prove the working mechanisms of these oxidative reactions on *V. vermiformis*. Within the context of the dynamic flow model, it was shown that the daily low dose of Oxygenal was sufficient to remove the trophozoite form of *V. vermiformis*. Pathogenic bacteria 'frozen' within the cysts would remain unaffected [38], but can only become virulent when the amoeba excysts. This chemical interaction is, in our opinion, independent on the test model used and therefore continuous treatment with a daily low dose of Oxygenal would subsequently inactivate the planktonic bacteria, including *Legionella* spp. and remove the amoebal trophozoite from the DUWS.

In this study we have proven that, using a translational *in-vitro* dynamic flow model, a contaminated DUWS can be decontaminated within one week. More importantly, the DUWS can be kept within the recommended guidelines over at least 7-week period using a hydrogen peroxide containing disinfection product. This is only achievable by a strict compliance to a treatment regimen with at least a daily low dose (0.02%) preferably combined with a weekly shock dose (0.25%) of hydrogen peroxide. Additionally, we have shown that hydrogen

peroxide in the concentrations recommended by the manufacturers does indeed not kill *V. vermiformis*. It does however, maybe even more importantly, remove the trophozoite form of the amoeba from the DUWS. This research also highlights the need for further evaluation of the efficacy of commercially available DUWS disinfectants and their application protocols for the removal of multi-kingdom biofilms to prevent DUWS-related infectious diseases.

CRediT authorship contribution statement

Michel A. Hoogenkamp: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Danuta Mazurel:** Methodology, Investigation, Formal analysis, Visualization, Writing – review & editing. **Elly Deutekom-Mulder:** Investigation. **Johannes J. de Soet:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2023.100132.

References

[1] Walker JT, Marsh PD. A review of biofilms and their role in microbial contamination of dental unit water systems (DUWS). Int Biodeterior Biodegrad 2004;54:87–98. https://doi.org/10.1016/j.ibiod.2004.03.012.

[2] The European parliament and the council of the European union. Directive (EU) 2020/2184, EU (revised) drinking water directive. Off J Eur Communities 2020 2019:35.

[3] Blake GC. The incidence and control of bacterial infection in dental spray reservoirs. Br Dent J 1963;115:413–6. https://doi.org/10.1038/s41415-022-4972-5.

[4] Lewis DL, Arens M, Suzuki M, Appleton SS, Nakashima K, Ryu J, et al. Cross-contamination potential with dental equipment. Lancet 1992;340:1252–4. https://doi.org/10.1016/0140-6736(92)92950-K.

[5] Spagnolo AM, Sartini M, Di Cave D, Casini B, Tuvo B, Cristina ML. Evaluation of microbiological and free-living protozoa contamination in dental unit waterlines. Int J Environ Res Publ Health 2019;16:2648. https://doi.org/10.3390/ijerph16152648.

[6] Hoogenkamp MA, Brandt BW, Laheij AMGA, de Soet JJ, Crielaard W. The microbiological load and microbiome of the Dutch dental unit; 'please, hold your breath. Water Res 2021;200:117205. https://doi.org/10.1016/j.watres.2021.117205.

[7] Pankhurst CL, Coulter WA. Do contaminated dental unit waterlines pose a risk of infection? J Dent 2007;35:712–20. https://doi.org/10.1016/j.jdent.2007.06.002.

[8] Volgenant CMC, de Soet JJ. Cross-transmission in the dental office: does this make you Ill? Curr Oral Heal Reports 2018;5:221–8. https://doi.org/10.1007/s40496-018-0201-3.

[9] O' Donnell MJ, Boyle MA, Russell RJ, Coleman DC. Management of dental unit waterline biofilms in the 21st century. Future Microbiol 2011;6:1209–26. https://doi.org/10.2217/fmb.11.104.

[10] Moe J, Rajan R, Caltharp S, Abramowicz S. Diagnosis and management of children with *Mycobacterium abscessus* infections in the head and neck. J Oral Maxillofac Surg 2018;76:1902. https://doi.org/10.1016/j.joms.2018.03.016. –11.

[11] Castellano Realpe OJ, Gutiérrez JC, Sierra DA, Pazmiño Martínez LA, Prado Palacios YY, Echeverría G, et al. Dental unit waterlines in Quito and Caracas contaminated with nontuberculous mycobacteria: a potential health risk in dental practice. Int J Environ Res Publ Health 2020;17:2348. https://doi.org/10.3390/ijerph17072348.

[12] Aliano D, Thomson R. The epidemiology of extrapulmonary non-tuberculous mycobacterial infection in a pediatric population. Pediatr Infect Dis J 2020;39:671–7. https://doi.org/10.1097/INF.0000000000002658.

[13] Singh J, O'Donnell K, Nieves DJ, Adler-Shohet FC, Arrieta AC, Ashouri N, et al. Invasive *mycobacterium abscessus* outbreak at a pediatric dental clinic. Open Forum Infect Dis 2021;8. https://doi.org/10.1093/ofid/ofab165.

[14] Dutil S, Veillette M, Mériaux A, Lazure L, Barbeau J, Duchaine C. Aerosolization of mycobacteria and legionellae during dental treatment: low exposure despite dental unit contamination. Environ Microbiol 2007;9:2836–43. https://doi.org/10.1111/j.1462-2920.2007.01395.x.

[15] Schönning C, Jernberg C, Klingenberg D, Andersson S, Pääjärvi A, Alm E, et al. Legionellosis acquired through a dental unit: a case study. J Hosp Infect 2017;96:89–92. https://doi.org/10.1016/j.jhin.2017.01.009.

[16] Martin MV. The significance of the bacterial contamination of dental unit water systems. Br Dent J 1987;163:152–4. https://doi.org/10.1038/sj.bdj.4806220.

[17] Chate RAC. An audit improves the quality of water within the dental unit water lines of general dental practices across the East of England. Br Dent J 2010;209:1–8. https://doi.org/10.1038/sj.bdj.2010.885.

[18] Koninklijke Nederlandse Maatschappij tot bevordering der tandheelkunde. Richtlijn Infectiepreventie in mondzorgpraktijken. Richtlijn Hygiëne in Mondzorgpraktijken 2016;124. https://www.rivm.nl/sites/default/files/2018-11/Richtlijn Infectiepreventie Mondzorgpraktijken DEFINITIEF.pdf. [Accessed 8 February 2021].

[19] Walker JT, Bradshaw DJ, Fulford MR, Marsh PD. Microbiological evaluation of a range of disinfectant products to control mixed-species biofilm contamination in a laboratory model of a dental unit water system. Appl Environ Microbiol 2003;69:3327–32. https://doi.org/10.1128/AEM.69.6.3327-3332.2003.

[20] Walker JT, Bradshaw DJ, Bennett AM, Fulford MR, Martin MV, Marsh PD. Microbial biofilm formation and contamination of dental-unit water systems in general dental practice. Appl Environ Microbiol 2000;66:3363–7. https://doi.org/10.1128/AEM.66.8.3363-3367.2000.

[21] Schel AJ, Marsh PD, Bradshaw DJ, Finney M, Fulford MR, Frandsen E, et al. Comparison of the efficacies of disinfectants to control microbial contamination in dental unit water systems in general dental practices across the European Union. Appl Environ Microbiol 2006;72:1380–7. https://doi.org/10.1128/AEM.72.2.1380-1387.2006.

[22] Szymańska J. Antifungal efficacy of hydrogen peroxide in dental unit waterline disinfection. Ann Agric Environ Med 2006;13:313–7.

[23] Orrù G, Del Nero S, Tuveri E, Laura Ciusa M, Pilia F, Erriu M, et al. Evaluation of antimicrobial-antibiofilm activity of a hydrogen peroxide decontaminating system used in dental unit water lines. Open Dent J 2010;4:140–6. https://doi.org/10.2174/1874210601004010140.

[24] Barbot V, Costa D, Deborde M, Imbert C. Efficacy of dental unit disinfectants against *Candida* spp. and *Hartmannella vermiformis*. Pathog Dis 2014;70:289–96. https://doi.org/10.1111/2049-632X.12127.

[25] Dillon A, Achilles-Day UEH, Singhrao SK, Pearce M, Morton LHG, Crean SJ. Biocide sensitivity of *Vermamoeba vermiformis* isolated from dental-unit-waterline systems. Int Biodeterior Biodegrad 2014;88:97–105. https://doi.org/10.1016/j.ibiod.2013.10.026.

[26] Hoogenkamp MA, Brandt BW, Laheij AMGA, de Soet JJ, Crielaard W. 16S rDNA sequencing and metadata of Dutch dental unit water. Data Brief 2021;37:107221. https://doi.org/10.1016/j.dib.2021.107221.

[27] Barbeau J, Buhler T. Biofilms augment the number of free-living amoebae in dental unit waterlines. Res Microbiol 2001;152:753–60. https://doi.org/10.1016/S0923-2508(01)01256-6.

[28] Khatoonaki H, Solhjoo K, Rezanezhad H, Armand B, Abdoli A, Taghipour A. Isolation and identification of potentially pathogenic free-living amoeba in dental-unit water samples. J Water Health 2022;20:1126–36. https://doi.org/10.2166/wh.2022.097.

[29] Delafont V, Rodier MH, Maisonneuve E, Cateau E. *Vermamoeba vermiformis*: a free-living amoeba of interest. Microb Ecol 2018;76:991–1001. https://doi.org/10.1007/s00248-018-1199-8.

[30] Fouque E, Yefimova M, Trouilhé MC, Quellard N, Fernandez B, Rodier MH, et al. Morphological study of the encystment and excystment of *Vermamoeba vermiformis* revealed original traits. J Eukaryot Microbiol 2015;62:327–37. https://doi.org/10.1111/jeu.12185.

[31] Fouque E, Héchard Y, Hartemann P, Humeau P, Trouilhé MC. Sensitivity of *Vermamoeba* (*Hartmannella) vermiformis* cysts to conventional disinfectants and protease. J Water Health 2015;13:302–10. https://doi.org/10.2166/wh.2014.154.

[32] Pickup ZL, Pickup R, Parry JD. A comparison of the growth and starvation responses of *Acanthamoeba castellanii* and *Hartmannella vermiformis* in the presence of suspended and attached *Escherichia coli* K12. FEMS Microbiol Ecol 2007;59:556–63. https://doi.org/10.1111/j.1574-6941.2006.00224.x.

[33] Hoogenkamp MA, Brandt BW, de Soet JJ, Crielaard W. An *in-vitro* dynamic flow model for translational research into dental unit water system biofilms. J Microbiol Methods 2020;171:105879. https://doi.org/10.1016/j.mimet.2020.105879.

[34] Molmeret M, Horn M, Wagner M, Santic M, Kwaik YA. Amoebae as training grounds for intracellular bacterial pathogens. Appl Environ Microbiol 2005;71:20–8. https://doi.org/10.1128/AEM.71.1.20-28.2005.

[35] Thomas JM, Ashbolt NJ. Do free-living amoebae in treated drinking water systems present an emerging health risk? Environ Sci Technol 2011;45:860–9. https://doi.org/10.1021/es102876y.

[36] Sun S, Noorian P, McDougald D. Dual role of mechanisms involved in resistance to predation by protozoa and virulence to humans. Front Microbiol 2018;9:1017. https://doi.org/10.3389/fmicb.2018.01017.

[37] Cateau E, Delafont V, Hechard Y, Rodier MH. Free-living amoebae: what part do they play in healthcare-associated infections? J Hosp Infect 2014;87:131–40. https://doi.org/10.1016/j.jhin.2014.05.001.

[38] Balczun C, Scheid PL. Free-living amoebae as hosts for and vectors of intracellular microorganisms with public health significance. Viruses 2017;9:65. https://doi.org/10.3390/v9040065.

[39] Dey R, Dlusskaya E, Ashbolt NJ. SARS-CoV-2 surrogate (Phi6) environmental persistence within free-living amoebae. J Water Health 2022;20:83–91. https://doi.org/10.2166/WH.2021.167.
[40] Harb OS, Gao LY, Kwaik YA. From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. Environ Microbiol 2000;2:251–65. https://doi.org/10.1046/j.1462-2920.2000.00112.x.
[41] Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 1985;49:1–7. https://doi.org/10.1128/aem.49.1.1-7.1985.
[42] Volgenant CMC, Hoogenkamp MA, Krom BP, Janus MM, Ten Cate JM, De Soet JJ, et al. Red and green fluorescence from oral biofilms. PLoS One 2016;11:e0168428. https://doi.org/10.1371/journal.pone.0168428.
[43] Hoogenkamp MA, Crielaard W, Krom BP. Uses and limitations of green fluorescent protein as a viability marker in *Enterococcus faecalis*: an observational investigation. J Microbiol Methods 2015;115:57–63. https://doi.org/10.1016/j.mimet.2015.05.020.
[44] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9:676–82. https://doi.org/10.1038/nmeth.2019.
[45] Ioannidis JPA. The proposal to lower P value thresholds to .005. JAMA, J Am Med Assoc 2018;319:1429–30. https://doi.org/10.1017/S0950268814000430.
[46] Costa D, Mercier A, Gravouil K, Lesobre J, Verdon J, Imbert C. Occurrence and diversity of both bacterial and fungal communities in dental unit waterlines subjected to disinfectants. Pathog Dis 2016;74:ftw094. https://doi.org/10.1093/femspd/ftw094.
[47] Ji XY, Fei CN, Zhang Y, Liu J, Liu H, Song J. Three key factors influencing the bacterial contamination of dental unit waterlines: a 6-year survey from 2012 to 2017. Int Dent J 2019;69:192–9. https://doi.org/10.1111/idj.12456.
[48] Tuvo B, Totaro M, Cristina ML, Spagnolo AM, Cave D Di, Profeti S, et al. Prevention and control of *Legionella* and *Pseudomonas* spp. Colonization in dental units. Pathogens 2020;9:305. https://doi.org/10.3390/pathogens9040305.
[49] Douterelo I, Fish KE, Boxall JB. Succession of bacterial and fungal communities within biofilms of a chlorinated drinking water distribution system. Water Res 2018;141:74–85. https://doi.org/10.1016/j.watres.2018.04.058.
[50] Lin SM, Svoboda KKH, Giletto A, Seibert J, Puttaiah R. Effects of hydrogen peroxide on dental unit biofilms and treatment water contamination. Eur J Dermatol 2011;5:47–59. https://doi.org/10.1055/s-0039-1698858.
[51] Okubo K, Ito T, Shiota Y, Kawata Y, Yamamoto T, Takashiba S. Effectiveness and safety of low-concentrated ozonized water for the reduction of contamination in dental unit water lines. Heliyon 2019;5:e02306. https://doi.org/10.1016/j.heliyon.2019.e02306.
[52] Puttaiah R, Svoboda KHK, Lin SM, Montebugnoli L, Dolci G, Spratt D, et al. Evaluation of an automated dental unit water system's contamination control protocol. J Contemp Dent Pract 2012;13:1–10. https://doi.org/10.5005/jp-journals-10024-1087.
[53] Tall BD, Williams HN, George KS, Gray RT, Walch M. Bacterial succession within a biofilm in water supply lines of dental air-water syringes. Can J Microbiol 1995;41:647–54. https://doi.org/10.1139/m95-088.
[54] Lau HY, Ashbolt NJ. The role of biofilms and protozoa in *Legionella* pathogenesis: Implications for drinking water. J Appl Microbiol 2009;107:368–78. https://doi.org/10.1111/j.1365-2672.2009.04208.x.
[55] van der Kooij D, Bakker GL, Italiaander R, Veenendaal HR, Wullings BA. Biofilm composition and threshold concentration for growth of *Legionella pneumophila* on surfaces exposed to flowing warm tap water without disinfectant. Appl Environ Microbiol 2017;83:e02737-16. https://doi.org/10.1128/AEM.02737-16.
[56] Walker JT, Marsh PD. Microbial biofilm formation in DUWS and their control using disinfectants. J Dent 2007;35:721–30. https://doi.org/10.1016/j.jdent.2007.07.005.
[57] Costa D, Bossard V, Brunet K, Fradin B, Imbert C. Planktonic free-living amoebae susceptibility to dental unit waterlines disinfectants. Pathog Dis 2017;75:1–4. https://doi.org/10.1093/femspd/ftx099.
[58] Costa D, Girardot M, Bertaux J, Verdon J, Imbert C. Efficacy of dental unit waterlines disinfectants on a polymicrobial biofilm. Water Res 2016;91:38–44. https://doi.org/10.1016/j.watres.2015.12.053.
[59] Chen X, Stewart PS. Biofilm removal caused by chemical treatments. Water Res 2000;34:4229–33. https://doi.org/10.1016/S0043-1354(00)00187-1.
[60] Chaw KC, Manimaran M, Tay FEH. Role of silver ions in destabilization of intermolecular adhesion forces measured by atomic force microscopy in *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother 2005;49:4853–9. https://doi.org/10.1128/AAC.49.12.4853-4859.2005.
[61] Bowen CG, Greenwood W, Guevara P, Washington MA. Effectiveness of a dental unit waterline treatment protocol with A-Dec ICX and Citrisil disinfectants. Mil Med 2015;180:1098–104. https://doi.org/10.7205/milmed-d-14-00643.
[62] Shaheen M, Scott C, Ashbolt NJ. Long-term persistence of infectious *Legionella* with free-living amoebae in drinking water biofilms. Int J Hyg Environ Health 2019;222:678–86. https://doi.org/10.1016/j.ijheh.2019.04.007.
[63] Li L, Jeon Y, Lee SH, Ryu H, Santo Domingo JW, Seo Y. Dynamics of the physiochemical and community structures of biofilms under the influence of algal organic matter and humic substances. Water Res 2019;158:136–45. https://doi.org/10.1016/j.watres.2019.04.014.
[64] Adams MB, Crosbie PBB, Nowak BF. Preliminary success using hydrogen peroxide to treat Atlantic salmon, *Salmo salar* L., affected with experimentally induced amoebic gill disease (AGD). J Fish Dis 2012;35:839–48. https://doi.org/10.1111/j.1365-2761.2012.01422.x.
[65] Mogoa E, Bodet C, Morel F, Rodier MH, Legube B, Héchard Y. Cellular response of the amoeba *Acanthamoeba castellanii* to chlorine, chlorine dioxide, and monochloramine treatments. Appl Environ Microbiol 2011;77:4974–80. https://doi.org/10.1128/AEM.00234-11.