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An in-vitro dynamic flow model for translational research into dental unit water system biofilms

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1. Introduction

Within a dental office, the dental unit is an essential piece of equipment that provides most services, such as suction, electric power, air and water supply, needed during dental treatments. During treatment, the high-speed rotating instruments and sonic scalers develop heat, which can damage the dental tissue (Siegel and Von Fraunhofer, 2002). To prevent overheating, water is used as a coolant and is usually derived from the municipal water supply. The water is either supplied by a direct connection or by filling a water reservoir in the unit (Walker and Marsh, 2004). During operation, the water is transported to the instruments through an intricate network of narrow bore plastic tubing, valves, and connectors; the so-called dental unit water system (DUWS) (Coleman et al., 2007). Even though the quality of potable water is regulated, within the European Union by the European drinking water directive (Anonymous, 1998), it is not sterile. Microorganisms enter the DUWS and colonize the surface of the tubing and valves either by active or passive adherence mechanisms (Walker and Marsh, 2004). During office hours, the dental unit is in use and water flows for short periods of time, followed by periods of stagnancy. After office hours and during the weekend, the unit is not used and the water is left stagnant. This intermittent use is detrimental to the microbiological quality of the water and gives water-borne microorganisms ample opportunity to build a strong matrix-encapsulated biofilm able to resist antimicrobial treatments (Flemming et al., 2002; Flemming and Wingender, 2010). A recently introduced measure to remove the water during weekends does not kill these biofilm ecosystems. A wide array of bacteria, fungi and protozoa have been isolated from these biofilms including both environmental microorganisms and opportunistic pathogens, such as Pseudomonas spp., Legionella spp. and Mycobacterium spp. (Costa et al., 2015; Pankhurst et al., 1998; Zhang et al., 2018). During patient treatment, these microorganisms may be released from the biofilms and aerosolized. These aerosols can subsequently be inhaled by the patient and dental office staff (Kimmerle et al., 2012; Leggat and Kedjarune, 2001). With an ageing population visiting the dental office, the chance of infecting immunosenescent patients is also increasing (Gavazzi and Krause, 2002; Kline and Bowdish, 2016).

To minimize this risk, the Royal Dutch Dental Association (KNMT) provides guidelines for hygiene and infection prevention (Koninklijke Nederlandse Maatschappij tot Bevordering der Tandheelkunde, 2015).
These guidelines dictate that the water expelled from the dental unit has to meet the same microbiological criteria as the European drinking water directive. Therefore, the water may not contain more than 100 colony forming units per milliliter (CFU mL⁻¹) of chemoheterotrophic microorganisms. The dental practitioner, in general, is willing to meet these criteria and uses prevention protocols to control the biofilm formation (Kamma et al., 2006). A study in the Netherlands, however, revealed that only 73 water samples out of 211 (34%) dental units contained less than 100 CFU mL⁻¹, and 40% of these units contained more than 900 CFU mL⁻¹ of viable bacteria (Exterkate and Crieallaard, 2013). As traditional culturing supports the growth of 0.25% of the microorganisms (Douterelo et al., 2018) and the effluent represents about 5% of the total biomass in a waterline (Flemming et al., 2002), the actual number of microbial cells in the biofilm will be decades higher.

With biofilms being in constant contact with the effluent phase and cells shedding or sloughing off it serves as a permanent reservoir to re-infect the water phase (Flemming et al., 2002). This, in our view, justifies more research into the prevention and control of DUWS biofilm formation. It is, however, unfeasible to use an actual DUWS, as the tubing is not easily accessible and sampling is destructive to the unit. To circumvent this problem, in-vitro biofilm models are used to assess the efficacy of antimicrobials (Lal et al., 2016; Okubo et al., 2019; Patel et al., 2016; Spratt et al., 2004; Walker et al., 2003; Yabune et al., 2008). These studies all vary in their approach and use either single species, multispecies or water samples as an inoculum. As a nutrient source, generally tap water (Lal et al., 2016; Spratt et al., 2004) or R2A (Okubo et al., 2019; Yabune et al., 2008; Yoon and Lee, 2017) is used to enhance the biofilm formation. To mimic the clinical setting, pump drives with various flowrates (12.5–100 mL min⁻¹) are used to pump around the nutrient and often recirculated back into the medium reservoir (Okubo et al., 2019; Spratt et al., 2004), essentially creating a batch system, with mainly planktonic cells. Few models use the intermittent flow pattern typical for a DUWS in which the nutrient is passed once before it is expelled. In an attempt to standardize methods to test the efficacy of antimicrobials on dental unit biofilms, the ISO 16954:2015 directive was developed (International Organization for Standardization [ISO], 2015). This document describes the use of two species to inoculate the system, both lab-strains, and an intermittent flow pattern to simulate daily routine within the dental practice. Even though this choice is understandable, the biofilms formed are not comparable to in vivo and in situ biofilms with regards to complexity in composition, structure and behavior (Bjarnsholt et al., 2013; Douterelo et al., 2014).

Therefore, our aim was to develop a simple in-vitro DUWS model, which (1) uses the intermittent flow pattern typical of a dental unit, (2) supports the growth of an ecologically relevant (thus complex) biofilm using water as nutrient, (3) can support different types of inocula, (4) is sensitive to an antimicrobial intervention, and (5) can recover from such intervention.

2. Materials and methods

2.1. Construction and disinfection of the in-vitro DUWS model

The model consisted of polyurethane (PUR) tubing and connectors and was assembled according to Fig. 1 (Schematic representation and product details). The assemblage was placed in an incubator and sterile silicone tubing was connected to the rear part of the model. To remove air from the tubing, the whole system was primed with autoclaved MilliQ water (Merck Millipore, Carrigtwohill, Ireland) using four calibrated, 4-channel, peristaltic pumps (Ismatec, Metrohm Nederland B.V., Barendrecht, The Netherlands, flowrate 30 mL min⁻¹). The system was disinfected by flushing all tubing for 5 min with 500 ppm active chlorine (Bakta, Ecolab, Nieuwegein, The Netherlands) in autoclaved MilliQ and left static for 15 min. Residual chlorine was removed by

![Fig. 1. Schematic representation of a single water biofilm model.](image-url)

1. Silicone tubing, 2 mm ID x 4 mm OD. (Fisher Scientific, Landsmeer, The Netherlands)
2. Filtropur S plus 0.2 μm filter (Sarstedt, Nümbrecht, Germany)
3. 4 cm segment, 4 mm ID x 6 mm OD, Polyurethane (PUR) tubing (Conrad Electronic Benelux B.V., Oldenzaal, The Netherlands)
4. Norgren Pneu
5. 1 m segment, 4 mm ID x 6 mm OD, PUR tubing (Conrad)
6. T-connector for sampling purposes (Cole Parmer)
7. Swinnex Filter holder, 25 mm, 0.2 μm, nitrocellulose filter (Millipore)

- Flushing all tubing for an additional 5 min with autoclaved MilliQ.

2.2. Water source, inoculation and biofilm growth procedure

To study whether the model can discriminate between different inoculum sources, two water sources were used. Potable water was obtained from a kitchen tap in the town of Zwartsluis, the Netherlands, and non-potable water was collected from the Preventive Dentistry Laboratory of the Academic Centre for Dentistry Amsterdam. Prior to sampling, both taps were flushed for 30 s to remove stagnant water followed by the collection of 2 L water in sterile receptacles. For each water source, 8 models were inoculated by flushing the models for 5 min after which the models were left static for 24 h. at 23 °C ± 2 °C. After this incubation period, a 0.2 μm Filtropur filter (Sarstedt, Nümbrecht, Germany) was placed at the front of each model and connected to 4-channel peristaltic pump with each tube individually supplying non-potable water directly from a cistern (Wisa 500, Gamma, Leusden, the Netherlands) mounted on a steel frame.

To mimic the daily operation of a DUWS, the pumps were programmed, using LabView (V. 5.1, National Instruments B.V., Woerden, the Netherlands), to administer water according to the flow pattern described in ISO-16954:2015. On weekdays, the models were flushed for 30 s, in line with hygiene and prevention guidelines to remove stagnant water. Then water was supplied to the model in 30 cycles (30 s flow, 9.5 min. stagnant) followed by a 19 h. stagnant period. During the weekend the water was left stagnant. The model was operated for a total of 30 days to follow biofilm formation.

2.3. Sensitivity of the model towards an intervention

To study the robustness of the model and its sensitivity towards an intervention, a disinfection protocol, typical for biofilm removal from a dental unit, was carried out after 30 days. Bottles filled with either sterile water (n = 4 per inoculation source) or 1000 ppm active chlorine (1 tablet Bakta/L MilliQ) (n = 4 per inoculation source) were attached to the tubes and were flushed for 30 s to replace the complete lumen volume twice and left static for 5 min. Residual chlorine was...

![Schematic diagram of a single water biofilm model.](image-url)

- Tap
- Pump drive
- Flow direction
- Waste
removed by flushing the model for 5 min with sterile water and another sample was taken similar to the morning sample. After this, the model was operated for an additional 14 days as described above, to monitor possible microbiological regrowth.

2.4. Microbiological sampling of the model

To monitor the microbiological quality of the water in time, three types of samples were taken from the model: (1) a morning effluent sample, (2) a whole-day effluent sample and (3) a biofilm sample.

2.4.1. Morning effluent sample

To monitor the build-up of bacterial growth, on each sampling day, sampling procedures were followed as described in ISO-16954:2015. Prior to the first feed cycle, the models were flushed for 30 s, followed by a 5 min static period. Samples were taken by pumping a 55 mL sample into sterile syringes (Terumo Europe, Leuven, Belgium). Five milliliter of this sample was used directly for the determination of heterotrophic plate counts (HPC). The remaining 50 mL was filtered using a Swinnex filter holder containing a nitrocellulose membrane (both Millipore, Ireland, ø25mm, 0.2 μm pore size). Filters were transferred to 1.0 mL sterile MilliQ, vortexed for 30 s at maximum speed (Vortex-Genie-3, Scientific Instruments Inc., Bohemia, NY, USA) and stored at −80 °C for microbiome analysis.

2.4.2. Whole day effluent sample

To monitor the bacterial composition of the effluent collected during the entire cycling period, immediately after the morning sample, a sterile filter holder (as described above) was placed in-line at the rear of the model. After the 5 h cycling period, representing the daily use of the dental unit (ISO-16954:2015), the filters were collected aseptically and stored at −80 °C for microbiome analysis.

2.4.3. Biofilm sample

To obtain information on the biofilm formation, weekly biofilm samples were taken immediately after the morning sample. To harvest the biofilm, 10 cm tube segments were removed aseptically, and collected in sterile 15 mL test tubes (Greiner Bio-One GmbH, Frickenhausen, Germany). The tubing was drained to remove non-adherent cells and placed in a 2 mL Eppendorf tube. A sterile plunger (2.0 × 13 mm nail (3.8 mm ø nailhead, Gamma) fitted with a 2 × 1 mm silicone, 70 shore, O-ring (Techirub B.V., Zeewolde, the Netherlands) was placed in the tubing and pushed through using a metal rod. Each collected biofilm and plunger were vortexed for 30 s after which the plunger was removed using a magnet. All samples were centrifuged for 5 min at 21,000 ×g, after which the pellet was suspended in 500 μL sterile MilliQ. Samples were then sonicated (52 W, 50 times, 1 s) on ice using a VC130 Ultrasonic processor (Sonic and materials Inc. Newtown, CT, USA) to disrupt biofilm aggregates. To prevent DNA cross contamination between samples, the sonicator tip was cleaned by 30 pulses with 2% (v/v) hydrogen peroxide (Merck, Darmstadt Germany) and rinsed with sterile MilliQ water. Samples were subsequently processed for the determination of heterotrophic plate counts (HPC) and the remaining sample was stored at −80 °C for microbiome analysis.

2.5. Sample analysis

2.5.1. Determination of heterotrophic plate counts (HPC)

To assess the amount of heterotrophic viable cells in both the morning sample (MS) and biofilm (MB) samples, samples were vortexed and 100 μL was used to prepare ten-fold serial dilutions in sterile MilliQ. These dilutions were then plated, in duplicate (Wille et al., 1996), onto R2A agar (BD, Sparks, IL, USA) using an Eddyjet Spiral plater (IUL, Barcelona, Spain). All plates were incubated under aerobic conditions at 23 °C for 7 days. Results are given as CFU/mL ± 1 or CFU cm−2 for the morning sample or biofilm sample, respectively.

The HPC data was Log10 transformed prior to statistical analysis and tested for normality. The data had a non-normal distribution and, therefore, a non-parametric test was used. First, the Kruskal-Wallis test was used and when significant differences were observed, they were identified (pair-wise) using a Wilcoxon Signed rank test. The significance level α was set at 0.05. Statistical analyses were performed using SPSS 24.0 (IBM, Amsterdam, the Netherlands).

2.5.2. Microbiome analysis

To determine the microbial composition of the samples, the stored samples were thawed and transferred to bead-beat tubes. The DNA was then extracted using a PowerBiofilm Kit (Qiagen, Roermond, The Netherlands) according to the manufacturer instructions. Next, the extracted DNA was processed for microbiome analysis according to Koopman and colleagues (Koopman et al., 2016). The DNA concentration was quantified and the V4 hypervariable region of the 16S rRNA gene amplified. The amplicons were equimolarly pooled and paired-end reads of 251 bp were generated using the Illumina MiSeq platform and Illumina MiSeq reagent kit v3 (Illumina, Inc., San Diego, CA) at the Amsterdam Medical University Cancer Center Amsterdam (Amsterdam, the Netherlands). The sequence data were processed and a taxonomic name was assigned to the representative (most abundant) sequence of the operational taxonomic unit (OTU), based on the SILVA ribosomal RNA database, version 128 (Quast et al., 2013). In addition, using BLASTn against the nucleotide collection, the taxonomy was assigned if the genus name was unidentifiable previously and only if the similarity was ≥97% and if the entire query sequence was aligned.

The microbiome data was filtered by removing samples with a DNA yield ≤ 0.4 ng μL−1 and by removing rare OTUs (fraction 10−5). Next, the OTU table was randomly subsampled at 5000 reads per sample and subsequently log2 transformed. Statistical significance between sample type and times was calculated using a Kruskal-Wallis test and when significant differences were observed, pair-wise Wilcoxon Signed rank tests were performed. In addition, the data was ordinated using principal component analysis (PCA). Permutational multivariate analysis of variance (PERMANOVA) was performed using the Bray–Curtis distance for samples per each time point within the same sample types using 9999 permutations. The Shannon diversity index was calculated on the non-log2 transformed data using the function diversity of the microbiome R package. All data handling and statistical analysis was performed in R (v3.6.0) (R Core Team, 2018) using the microbiome (v1.6.0) (Lahti and Shetty, 2017), phyloseq (v1.28.0) (McMurdie and Holmes, 2013) and vegan packages (v2.5–5) (Oksanen, 2017; R Core Team, 2018). Compositional differences in the microbiome and between groups were deemed statistically significant different at p < 0.005 (Ioannidis, 2018).

3. Results

3.1. The model supports the growth of an ecologically complex biofilm

Bacterial community profiling was performed on all morning effluent, whole-day effluent, and biofilm samples. Due to subsampling (at 5000 reads/sample), 32 samples including 14 control samples, were removed resulting in a total of 344 samples for downstream analyses. This filtered table contained, 457 operational taxonomic units (OTUs). Fig. 2 gives an overview of the taxonomic diversity of all samples. The bars represent the cumulative amount of the OTUs over all timepoints for the first 30 days (i.e. before treatment) per sample type and inoculation source. As can be seen in this graph, few taxa were predominant per sample type and the vast majority of the OTUs had a relatively small abundance. Besides bacteria, amoebal mitochondrial DNA (OTU_15) of Verrucomobea vermiformis and its endosymbiont Verrucomithus spp. (OTU_16) were detected in both the whole-day effluent and biofilm samples of non-potable origin indicating a complex
ecological system had formed. Analysis of the Shannon diversity revealed that both the morning and whole-day effluent samples were similar ($p > .005$) in composition. As the morning effluent sample represents the type of sample on which the microbiological quality of the dental unit is judged, for clarity, only the data of the morning effluent sample and biofilm samples will be discussed in this paper.

### 3.2. The model supports the growth of biofilms originating from distinctly different inocula

To determine the growth characteristics of both types of inocula in the DUWS model, morning effluent and biofilm samples were collected and the HPC was determined. The morning effluent samples of both potable and non-potable inocula ($27 \pm /- 22 \text{ CFU mL}^{-1}$ and 1306 $\pm /- 147 \text{ CFU mL}^{-1}$ (avg $+/-$ std., $n = 3$), resp.) showed a typical sigmoidal growth pattern (Fig. 3) even though the inoculum (Day 0) differed 50-fold in initial density. In the effluent, a quasi-steady state was reached of about $10^5$-$10^6 \text{ CFU mL}^{-1}$ from day 16 onwards. Biofilm growth reached this state after day 23 with $10^8$ and $10^7 \text{ CFU cm}^{-2}$ for non-potable and potable water, respectively (Fig. 3). The microbial growth of both types of water sources in the model was reproducible and comparable with HPC ($n = 8$ per inoculation source, $p > .05$ between replicates).

Analysis of the Shannon ($\alpha$-) diversity index (Fig. 4A and C) of biofilms of both inoculation sources revealed that the diversity increased in time for the non-potable water inoculation source resulting in a more diverse microbiome. Biofilms from the potable inoculation source, however, showed a decrease in Shannon diversity indicating a loss in species richness and evenness. The change in Bray-Curtis dissimilarity in time supported the HPC data as it stabilized between day 16 and 23 for both inoculation sources (Fig. 4B and D). While the Shannon diversity and Bray-Curtis dissimilarity stabilized in time the spread in those variables became smaller and closer to zero. This decrease implies an increasing similarity thus high reproducibility between replicate models ($n = 8$). Ordination by PCA showed that the replicates clustered together ($n = 8$) and, even after 30 days, the biofilms grown from the 2 inocula sources clearly differed (Fig. 5). These compositional differences between biofilms from the non-potable and potable water inocula were statistically significant (PERMANOVA, $F = 35.6$, $p < .005$).

### 3.3. The model can recover from an intervention

To determine whether the biofilm in the model was capable of withstanding an intervention, the biofilms from both types of inocula were treated at day 30 for 5 min with 1000 ppm active chlorine. The HPC of the effluent samples ($n = 4$) of both inoculation sources show a biological and statistical significant decrease from about $1.15 \times 10^6 \text{ CFU mL}^{-1}$ $\pm$ 7.41 $\times 10^5 \text{ CFU mL}^{-1}$ (mean $\pm$ standard deviation) and $3.64 \times 10^5 \text{ CFU mL}^{-1}$ $\pm$ 1.95 $\times 10^5 \text{ CFU mL}^{-1}$ to below the detection limit of 100 CFU mL$^{-1}$, immediately after treatment for potable and non-potable inoculation source, respectively. To monitor the resilience of the biofilms against this disinfection the models were operated for an additional 14 days. Effluent samples showed that both types of biofilm were able to re-inoculate the lumen water and HPC of both effluent and biofilm showed similar growth dynamics as compared to the initial 14 days growth. After analyzing the $\alpha$-diversity of the biofilm samples treated with active chlorine at day 37 and 44, the species
richness and evenness of biofilms originating from potable inoculation source did not significantly decrease, whereas biofilms originating from non-potable water did (Data not shown). PERMANOVA revealed that there was no significant difference ($F = 3.5, p > .005$) between biofilm samples ($n = 3, 1$ dropout) of both inoculation sources at day 44 (Fig. 5). Analysis of the predominant species in the biofilm samples (average of samples per day; $\geq 50$ reads per OTU in at least one sample (per inoculation source)) from both inoculation sources, before and after the chlorine treatment, revealed that the abundance of several OTUs decreased post-chlorine treatment (Fig. 6). Amongst these OTUs are the opportunistic pathogen Legionella and an OTU representing the mitochondrial DNA of the amoeba Vermamoeba (previously Hartmannella) and its endosymbiont Vermiphilus spp. (Delafont et al., 2018). However, the abundance of certain OTUs representing species of genus Burkholderia, Sphingomonas, Blastomonas, Microscilla and Methylphilus increased post treatment in both types of biofilms.

### 4. Discussion

A prerequisite to study more complex *in situ* biofilm communities is that samples can be harvested. In many cases, for example, dental plaque, catheters, microbial mats in surface water, samples can easily be obtained and studied. In harder to reach places, such as root canals, hip joints, drinking water distribution systems (DWDS) and DUWS, sampling is virtually impossible without destroying the integrity of the system in which the biofilm grows. An ideal DUWS *in-vitro* model should be made up of tubing and connectors and use water, delivered in an intermittent flow pattern, as a nutrient source. It should also allow for the ability to sample both the effluent and biofilm growth in time. As we aimed to develop a model reproducible in any lab, we opted for tubing and connectors which are easy to obtain. As a water biofilm is a prerequisite for the colonization of bacteria of non-water origin (Spratt et al., 2004), we chose to use “regular” water as an inoculum and nutrient source. The use of water also has drawbacks as this source is subject to seasonal and temporal changes with regards to microbial composition and quality (Potgieter et al., 2018; Prest et al., 2016). This, in combination with a low inoculation density (< 1000 CFU·mL$^{-1}$), could result in non-reproducible biofilm formation, especially with regard to composition. Yet, microbial growth in the effluent samples was in the same order of magnitude with regards to growth time (9–14 days) and maximum HPC counts ($10^4$–$10^5$ CFU·mL$^{-1}$) as compared to previous studies (Lal et al., 2016; Walker et al., 2003; Yoon and Lee, 2017), indicating the effluent HPC counts reach a certain maximum irrespective of inoculation source or the use of R2A or sterile water as a growth medium. Interestingly, the biofilm growth in our model system seemed to be a factor 100–1000 higher as compared to those studies ($10^2$–$10^6$ vs $10^3$–$10^5$ CFU·cm$^{-2}$, respectively) and more in line with the results obtained by Manuel and co-workers (Manuel et al., 2007). A possible explanation for this phenomenon could be the use of dynamic flow, as increased hydrodynamic stress has shown to influence composition, density and structure in both environmental and medical biofilms (Beyenal and Lewandowski, 2002; Chang et al., 2020; Fish et al., 2016; Hart et al., 2019; Manuel et al., 2007). In addition, the microbial composition reached a steady-state, similar to the patterns displayed in the results from the HPC counts for both the effluent and biofilm samples. Interestingly, where the $\beta$-diversity increased in time for non-potable water source samples, the $\beta$-diversity in the potable water source samples decreased, yet did stabilize in time. Nonetheless,
both types of biofilm remained significantly different from each other, even after 30 days. Biofilm formation is a successional process and the α-diversity generally increases in time (Douterelo et al., 2018; Prest et al., 2016). A decrease could be explained by the use of a different water feed source as compared to the inoculum source resulting in a change of water nutrient parameters after the transition from potable and non-potable water (Li et al., 2016). Alternatively, the change from a copper adherence substrate to polyurethane could have resulted in a change in microbiome diversity. However, this hypothesis would require further study. The addition of filter-sterilized water to the system meant no other microorganisms could be introduced into the system, resulting in unique microbiomes, albeit maybe less complex than the original inoculum. This would imply that when using various inocula, one should respect a minimum experimental time of at least 23–30 days to allow the microbiome to adapt to the availability of dietary needs and changes in the adherence substrates. We have also shown that there is a statistically significant difference between the microbiomes of the effluent and biofilm samples. This phenomenon has also been described for drinking water distribution systems (Potgieter et al., 2018). Although not shown, the whole-day effluent samples even had a higher Shannon diversity than the morning effluent and biofilm samples. These whole-day effluent samples contain “effluent” microorganisms and shed-off biofilm microbes and even contained unique OTUs that seem to be in limbo, belonging neither to the biofilm nor the water phase. Specified in national and European norms, the analysis of the water quality is based on conventional culture techniques focusing on a single water phase sample. As stated by Douterelo and co-workers (Douterelo et al., 2018), only 0.25% of the microorganisms present in the drinking water environment can be cultured, resulting in a gross underestimation of the total cell number in the biofilm phase. The current model facilitates monitoring of the conventional effluent sample, but also the whole-day effluent and biofilm samples. As a typical example of this benefit, the presence of Legionella spp. and Vermamoeba verniformis were detected in the biofilm and whole-day effluent samples of non-potable origin, but not in the morning sample taken to monitor the

Fig. 4. Shannon diversity of biofilm samples (n = 8) from non-potable inoculation source (A) and the corresponding Bray-Curtis distances (B). Panels C and D show the respective Shannon diversity and Bray-Curtis distances of the biofilm samples (n = 8) from the potable water inoculation source. Bray-Curtis distances for both inoculation sources illustrate the stabilization of the microbiome after day 16.

Fig. 5. PCA plot of biofilm samples pre (day 30, n = 8) and post 1000 ppm active chlorine treatment (day 44, n = 3, 1 replicate was lost due to quality filtering) for non-potable and potable origin, encircled in blue and red, respectively. Statistical analysis (PERMANOVA) was performed on pre-treatment data sets between non-potable and potable origin (encircled in green) and post-treatment (encircled in yellow). Pre/post treatment microbiomes were significantly different at day 30 (p < .005) but not at day 44 (p > .005). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
microbiological quality of DUWS water. Further analysis of this water revealed the presence of *Legionella anisa* and the visual presence of amoeba. After this confirmation, steps have been taken to reduce and control the biofilm problem in the lab-water distribution system.

Over the last 57 years, scientists have described and studied DUWS effluents and their biofilms (Blake, 1963; Ditommaso et al., 2019) indicating the biofilm problem is still much alive. Like biofilms in the medical field, water biofilms are difficult to eradicate due to their strong encapsulating extracellular matrix which are able to resist antimicrobial treatments (Besemer et al., 2007; de Carvalho, 2018; Flemming and Wingender, 2010; Francolini and Donelli, 2010; Hurlow et al., 2015). An in-vitro biofilm model that can mimic this phenomenon could be of excellent use for translational research into DUWS and DWDS biofilms. In the model described, both the biofilms originating from potable and non-potable water were treated at day 30 for 5 min with 1000 ppm active chlorine. The HPC counts in the effluent plummeted to below the detection limit, indicating an almost complete inactivation of planktonic cells. For DUWS, this would indicate the unit complies with the KNMT guidelines (Koninklijke Nederlandse Maatschappij tot Bevordering der Tandheelkunde, 2015) and does not require further disinfection. However, within 7 days after resuming daily operation, effluent counts increased and the biofilms recovered to near pre-intervention levels. As no new bacteria were introduced into the model, due to the presence of a 0.2 μm filter, it can be concluded that the biofilm was not completely inactivated nor removed. This resilience towards treatment is of importance when studying biofilm disinfection efficacy (Fish and Boxall, 2018; Walker and Marsh, 2007).

Microorganisms that survived treatment have all been linked to possible chlorination resistance and include members of the family Sphingomonadaceae and Microscillaceae in both types of biofilms originating from potable and non-potable water source (Fish and Boxall, 2018; Jia et al., 2015). Although not studied in the present study, the presence of the protective biofilm matrix is also attributable to this resilience (Fish et al., 2016; Fish et al., 2015). The remaining matrix serves as the “house of biofilm cells” (Flemming et al., 2016) introducing a complex level in biofilm control. This in our view indicates the importance of studying both the microbial component as the matrix component of the biofilm.

5. Conclusion

This model is an ideal system for translational research into the hard to access DUWS biofilm and can easily be adapted to study DWDS and veterinary drinking water system biofilms. It was developed using the hydrodynamics of a dental unit water system and is suitable to grow inoculum dependent complex inter-kingdom biofilms. It can be replicated in any lab and used to monitor the disinfection efficacy of an antimicrobial compound of choice on both the effluent as biofilm phase. The relative ease in which biofilm samples can be obtained makes it suitable to analyze both the microorganisms present in the biofilm and their biofilm matrix. Additionally, the tubing material, flow rates, feed sources can be altered with ease, to suit the examiners need. Currently, studies are being performed, using this model, to assess the efficacy of commonly used DUWS disinfection products and their effects on the biofilm and the matrix.

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.

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