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The microbiological load and microbiome of the Dutch dental unit; 'please, hold your breath'

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ABSTRACT

Dental unit water systems are prone to biofilm formation. During use of the dental unit, clumps of biofilm slough off and can subsequently be aerosolized and inhaled by both patient and staff, potentially causing infections. The aim of this study was to determine the microbial load and microbiome of dental unit water, in the Netherlands, and the factors influencing these parameters. In total, 226 dental units were sampled and heterotrophic plate counts (HPC) were determined on the traditional effluent sample. Of all dental units, 61% exceeded the recommended microbiological guidelines of 100 colony forming units per milliliter. In addition, the microbiome, with additional q-PCR analysis for specific species, was determined on an effluent sample taken immediately after an overnight stagnancy period, in which the biofilm is in its relaxed state. These relaxed biofilm samples showed that each dental unit had a unique microbiome. *Legionella* spp., amoeba and fungi were found in 71%, 43% and 98% of all units, respectively. The presence of amoeba was positively associated with nine bacterial biomarkers and correlated positively with bacterial and fungal DNA and *Legionella* spp. concentrations, but not with HPC. Only when adhering to disinfection protocols, statistically significant effects on the microbial load and microbiome were seen. The relaxed biofilm sample, in combination with molecular techniques gives better insight in the presence of opportunistic pathogens when compared to the heterotrophic plate counts. Infection control measures should focus on biofilm analysis and control in order to guarantee patient safety.

1. Introduction

The dental unit is an indispensable piece of equipment for the dentist, supplying water, air and electric power, needed to perform treatment. When using sonic scalers and high-speed rotating instruments, heat is being generated, which can be detrimental to the dental tissue (Siegel and Von Fraunhofer, 2002). To avoid overheating, water is used to cool and irrigate the surgical site. This water is usually derived from the municipal water supply, either by a direct connection or by filling an external reservoir (Walker and Marsh, 2004). Although the quality of this potable water is regulated, in for example the European drinking water directive (The Council Directive of the European Union, 1998), and usually free of pathogens, it is not sterile. During use of the dental unit, this water is transported to the instruments, through the dental unit water system (DUWS); an intricate network of narrow bore plastic tubing, valves, and connectors. The combination of the intrinsic properties of the different materials used, and the intermittent laminar flow of the water, make the DUWS prone to biofilm formation.

Micro-organisms in the water will form, once adhered, a matrix encapsulated biofilm capable of withstanding a range of external stresses such as antimicrobial treatment (Flemming and Wingender, 2010; Walker and Marsh, 2004). With biofilm forming on all surfaces of the DUWS, anti-retraction valves start to fail (Panagakos et al., 2001). Subsequently, patient material, such as blood and saliva, can be sucked back into the DUWS giving oral microorganisms the chance to colonize the aquatic biofilms (Spratt et

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al., 2004; Walker et al., 2004). If left undisturbed, these biofilms can evolve in multi-kingdom biofilms containing (pathogenic) bacteria, fungi, viruses and protozoa (Azeredo et al., 2017; Barbeau and Buhler, 2001; Pankhurst and Coulter, 2007) and will serve as a reservoir for continuous bacterial contamination of the effluent water (Fish et al., 2016). During treatment, this contaminated effluent, including clumps of shedding biofilm, is aerosolized and inhaled by both patient and dental staff (Kimmerle et al., 2012). With increased aging and immunosenescence of the patient population, the chance on infection is also increasing (Kline and Bowdish, 2016).

To control biofilm formation and guarantee patient safety, as required by the European medical aid directive EU 2017/745, DUWS manufacturers have to supply a disinfection protocol (The Council Directive of the European Union, 1993), which is performed through the daily addition of a low dose of an antimicrobial agent to the water. Additionally, a weekly decontamination with a high concentration of antimicrobial agent (shock dose) to reduce the biofilm load in the DUWS can be performed. To monitor DUWS water quality, dental associations, such as the Royal Dutch Dental Association (KNMT), provide guidelines for infection control. These guidelines, advise that dental practices monitor the microbiological quality of the dental unit water twice a year and that the water should not contain more than 100 colony forming units per milliliter (CFU•ml^{-1}), based on heterotrophic plate count (HPC). If more than 10^4 CFU•ml^{-1} are present, an additional test for the presence of *Legionella* should take place. If positive, the practice has to halt all clinical procedures until the HPC and *Legionella* counts are below the prescribed limits. However, while HPC provides a general indication of the water quality, it only supports the growth of 0.25% of the aquatic microorganisms (Douterelo et al., 2018) and is considered a poor indicator of *Legionella* spp. presence (Pierre et al., 2019).

Therefore, to gain insight into the degree of bacterial contamination and the possible presence of opportunistic pathogens, such as fungi, amoeba and Legionella, in the dental unit water, we set out to perform this study to determine the microbial load and microbiome of dental unit water and the factors influencing these parameters.

2. Materials and methods

2.1. Sample collection and processing

A randomly drawn list from members of the Royal Dutch Dental Society (KNMT) was provided by the KNMT ($n = 921$). Practices were sent a sampling kit, to sample individual dental units, and a questionnaire, on the water management protocols in use. To avoid external contamination, dental practices were given clear instructions on how to disinfect and sample the air rotor handpiece. To prevent skewed results, due to residual antimicrobial activity of a possible biofilm disinfection step performed in the weekend, sampling had to be performed on the second day of operation in the week. No neutralizer, to prevent residual antimicrobial activity, was added to the samples as (i) No infection control measures were taken prior to sampling, (ii) Dutch drinking water does not contain chlorine (Smeets et al., 2009) and (iii) a high diversity of antimicrobial agents were expected, needing their own specific neutralizer (Fernández-Crehuet et al., 2013).

To obtain a proxy sample of the biofilm in its relaxed state (relaxed biofilm sample, RBS), a 50 ml effluent sample was taken aseptically from the air rotor handpiece, preceding any flushing or disinfection of the unit. Then, following infection control guidelines, the units were flushed for 30 s and a second 10 ml effluent sample was taken. That same day, the samples and questionnaire were returned for analysis by regular mail. Upon arrival in the laboratory, the questionnaire and samples were coded and blinded to the operator. Transit time was recorded and both the 50 ml and 10 ml samples were processed as described previously (Hoogenkamp et al., 2020). In short, the RBS sample was concentrated by filtration using a 0.2 µm filter (Sarstedt, Nümbrecht, Germany) and the filter was stored at −80 °C till further processing and the 10 ml sample was used to determine the HPC, in duplicate (Wille et al., 1996), on R2A agar (BD, Sparks, IL, USA).

To assess the effect of transit time on the sample quality, throughout the study, non-potable lab water (500 ml) was taken from the Preventive Dentistry lab of the Academic Centre for Dentistry in Amsterdam, the Netherlands and distributed over several sampling sets. Baseline HPC and microbiome of two sampling sets were determined and the other sets were posted, in duplicate, before and after the mail pick up time to simulate the postal transit times of the dental unit samples. Upon arrival, these control samples ($n = 62$) were processed similarly to the dental unit samples.

2.2. Molecular DNA analysis

Microbiome analysis and quantitative PCR (q-PCR) on specific target organisms was performed only on RBS samples. The DNA isolation, 16S V4 rDNA PCR, sequencing and processing of microbiome samples with the inclusion of appropriate kit blanks, filter and PCR controls was performed as described previously (Hoogenkamp et al., 2020). In short, the V4 hypervariable region of the 16S rRNA gene was amplified. Paired-end reads (251 bp) of equimolarly pooled amplicons were generated using the Illumina MiSeq platform and the V3 reagent kit (Illumina, Inc., San Diego, CA). 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. Ordination of the data was performed using principal component analysis (PCA). Permutational multivariate analysis of variance (PERMANOVA) was performed using the Bray–Curtis distance for samples and the Shannon diversity index was calculated on the non-log2 transformed data. All data handling and statistical analysis was performed in R (v3.6.0).

Linear discriminant analysis effect size (LEfSe) of the microbiome data, to detect biomarkers associated with the presence of amoeba, was performed on the processed data with an additional filtering on operational taxonomic units abundance of 0.05% (Segata et al., 2011).

To determine the presence of specific target species, q-PCR was performed using Lightcycler technology and chemistry (Roche, Almere, The Netherlands). All RBS samples were analyzed, in duplicate, using molecular grade water (Thermo Scientific) as a negative control.

The presence of *Legionella* spp., L. *pneumophila* and L. *pneumophila* SG1, was determined according to Collins and co-workers, using 10 µl template DNA (Collins et al., 2015). Calibration curves were constructed using L. *pneumophila* DSM 7513 DNA (DSMZ, Braunsweig, Germany) with a detection limit of 10 Genomic unit numbers (GU) per reaction (2000 GU•l^{-1}) for *Legionella* spp. and 1 GU for L. *pneumophila*. Positive samples were verified by melting point analysis and GU were calculated based on the inclusion of certified genome unit numbers (LGC standards, Wesel, Germany).

The fungal concentration in the RBS sample was determined using the 18S rDNA FungiQuant primer set (Liu et al., 2012), in combination with a Roche Lightcycler protocol (Wagner et al., 2018). Calibration curves were constructed using *C. albicans* SC5314 DNA (detection limit 1 fg•µl^{-1} 18S rDNA).

To detect the presence of the amoeba *Acanthamoeba* and *Naegleria,* a conventional PCR was used (Le Calvez et al., 2012). *Escherichia coli* DH5α containing a pUC54:*kan* vector with the respective target sequences, based on 18S rDNA from *Acanthamoeba*

Table 1
Sample distribution of 213 DUWS water samples based on the heterotrophic plate count.

HPC (CFU•ml^{-1})	n	% of total
Total	213	100
< Detection limit	28	13
2.5–10^2	56	26
10^2–10^3	30	14
10^3–10^4	45	21
10^4–10^5	27	13
> 10^5	27	13

castellanii Castellanii ATCC 50,374 (GenBank: U07413.1) and *Naegleria fowleri* (Genbank: AF338423.1), with 20 bp flanking regions and a *Hin*dIII restriction site on both ends were constructed (BaseClear, Leiden, the Netherlands). Plasmid DNA of both clones was isolated using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Landsmeer, The Netherlands) and served as a positive control. Presence of both amoebal species was determined by DNA gel electrophoresis (60 min, 100 V, 3% Tris-Acetate-EDTA agarose (Fisher Scientific) on the PCR samples. The presence of *Hartmannella* was detected using a q-PCR specific for *H. vermiformis* (Kuiper et al., 2006) with the modification for Roche Lightcycler technology using 5 µl DNA template (Ren et al., 2018).

2.3. Statistics

Overall, the data had a non-normal distribution. Prior to statistical tests, HPC data and q-PCR data for *Legionella* spp. were LOG_{10} transformed. Values below the detection limit were set at the detection limit to allow for successful LOG_{10} transformation. Unless stated otherwise, for among group comparisons, the Kruskal-Wallis test was used and when significant differences were observed, a Bonferroni post-hoc test was applied. To determine the relation between parameters, both the Pearson and Spearman correlation coefficient were determined.

Statistical analysis on HPC and q-PCR data were performed using SPSS 26.0 (IBM, Amsterdam, the Netherlands). Microbiome analyses was performed as described previously (Hoogenkamp et al., 2020). Differences between groups were deemed statistically significantly different at $p < 0.005$ (Ioannidis, 2018).

3. Results

3.1. Unit samples

In total, 226 dental unit water samples were returned, of which most samples (97%) within 2 days. Analysis of the control samples ($n = 62$) revealed that the average HPC of samples, which were in transit less than 3 days, did not increase significantly ($p > 0.005$). Seven samples, which were in transit for more than 2 days, were therefore removed from the study. Two additional samples were removed due to incomplete questionnaires and another 4 samples, were lost for HPC, due to leakage during transit, resulting in a total of 213 samples analyzed for HPC. For sample information on distribution per province, unit brand, the type of disinfectants used, average unit age, see Suppl. File "Data summary". The complete metadata file can be found under NCBI BioProject PRJNA690093.

3.2. Heterotrophic plate counts

As can be seen in Table 1, 39% of the samples contained less than 10^2 CFU•ml^{-1} and are considered safe according to infection control guidelines, while only 28 units (13%) could be considered 'clean' as no colonies were detected on plate.

To determine the influence of the type of disinfectant used, either as a daily low dose disinfectant (DLDD) or as a weekly shock dose treatment, the samples were grouped based on the active ingredient in the disinfectant used. As can be seen in Table 2 and depicted in Fig. 1, dental units which received a DLDD, with or without a shock dose, contained a significantly lower amount of heterotrophic plate counts as compared to units which received no treatment ($p < 0.005$). Only units receiving a DLDD with hydrogen peroxide-based products (with and without silver ions) contained a significantly lower HPC ($p < 0.005$), as compared to units which received no treatment (Data not shown).

3.3. Molecular DNA analysis

3.3.1. Microbiome analysis

In total, all 236 RBS samples (226 samples and 10 PCR controls, filter and kit blanks were processed for microbiome analysis. PCR controls, kit and filter blanks contained, at most, 97 reads (average: 19.7; range: 1 - 97) and had no predominant OTUs. After quality control, filtering at 10^{-5} and subsampling at 8100 reads/sample, 131 samples remained. Next, samples ($n = 7$) which were in transit for more than 2 days were removed from the study. Most RBS samples lost ($n = 79$) contained very few reads (<1000) which correlated to low amounts of DNA after isolation. The paired-end-reads and metadata are available under NCBI BioProject PRJNA690093.

The final OTU table contained 1340 OTUs with an average of 159 ± 122 OTU per sample. A summary of the bacterial taxa and their presence in the dental units analyzed can be found in Suppl. File "Bacterial Composition". The bacterial taxa found in the sampled dental units included genera commonly found in water such as *Burkholderiaceae* and *Sphingomonadaceae*, but also opportunistic pathogens such as *Legionella*, *Mycobacterium*, *Serratia, Pseudomonas (aeruginosa), Coxiella and Staphylococcus*.

Dental units which received a different treatment regime had a significantly different biofilm composition ($n = 124$, PERMANOVA, 9999 permutations, df=4, $F = 2.4$, $p < 0.005$. Pairwise PERMANOVAs of the different treatment regimens showed that the composition of units that were treated with a single agent, either as a DLDD, shock dose or a combination of both with the same disinfectant differed from the non-treated units ($p < 0.005$).

When looking closer, principal component analysis on LOG_2-transformed data revealed clear differences in composition between different disinfectants used as a DLDD with 22.32% and 8.74% of variance being explained by PC1 and PC2, respectively (Fig. 2A). The differences were confirmed, using PERMANOVA, to be statistically significant ($n = 34$, 9999 permutations, df = 5, $F = 2.1$, $p < 0.005$). No significant effect was found when only a shock dose was used ($n = 20$, 9999 permutation, dF = 6, $F = 2.4$, $p = 0.074$).

Further analysis of the Shannon diversity (Fig. 2B), between the different daily low dose disinfectants, used in more than 2 units, revealed that units which received hydrogen peroxide (± silver ions) as compared to no treatment had a statistically significant lower Shannon diversity ($p < 0.005$).

In an attempt to distinguish clusters of OTUs based on units which received a certain treatment regime, a heatmap (Kolde, 2015) was prepared of OTUs present in more than 30% of the RBS samples, As can be seen in Suppl. File Heatmap, no clear clusters could be identified, indicating the uniqueness of each RBS sample and indeed no grouping, based on disinfection treatment regimens, was visible either.

To assess whether the anti-retraction valves of the air rotor handpiece prevented the backflush of patient material, such as saliva, the microbiome data was screened for the presence of human oral bacteria. In 28 units, trace amounts (<8 reads after

Table 2
Mean values (± standard deviation) of heterotrophic plate counts (HPC), bacterial 16S rDNA concentration, fungal 18S rDNA concentration and the amount of *Legionella* spp., grouped on treatment regime. Values marked * differ significantly from the no treatment group. Value marked with # significantly differ from the daily low dose disinfectant group ($p < 0.005$).

Regime	*n*=	LOG10HPC(CFU.ml^{-1})	Bacterial16S rDNA(ng.µl^{-1})	Fungal18S rDNA(pg.µl^{-1})	LOG10*Legionella* spp.(GU.l^{-1})
No treatment	60	3.80 (1.52)	1.76 (2.91)	5.39 (9.30)	5.71 (1.62)
Only DLDD	58	2.34 (1.71)*	1.40 (3.22)	6.89 (18.4)	4.55 (1.29)
Only shock dose	38	3.01 (1.59)	0.95 (2.84)*	13.7 (77.9)*	4.13 (1.52)*
DLDD and shock dose, same agent	38	1.99 (1.45)*	1.04 (4.32)*	2.23 (5.30)*	3.45 (1.12)*#
DLDD and shock dose, different agent	19	1.56 (1.59)*	0.79 (3.21)*	7.81 (29.1)	4.05 (1.64)*

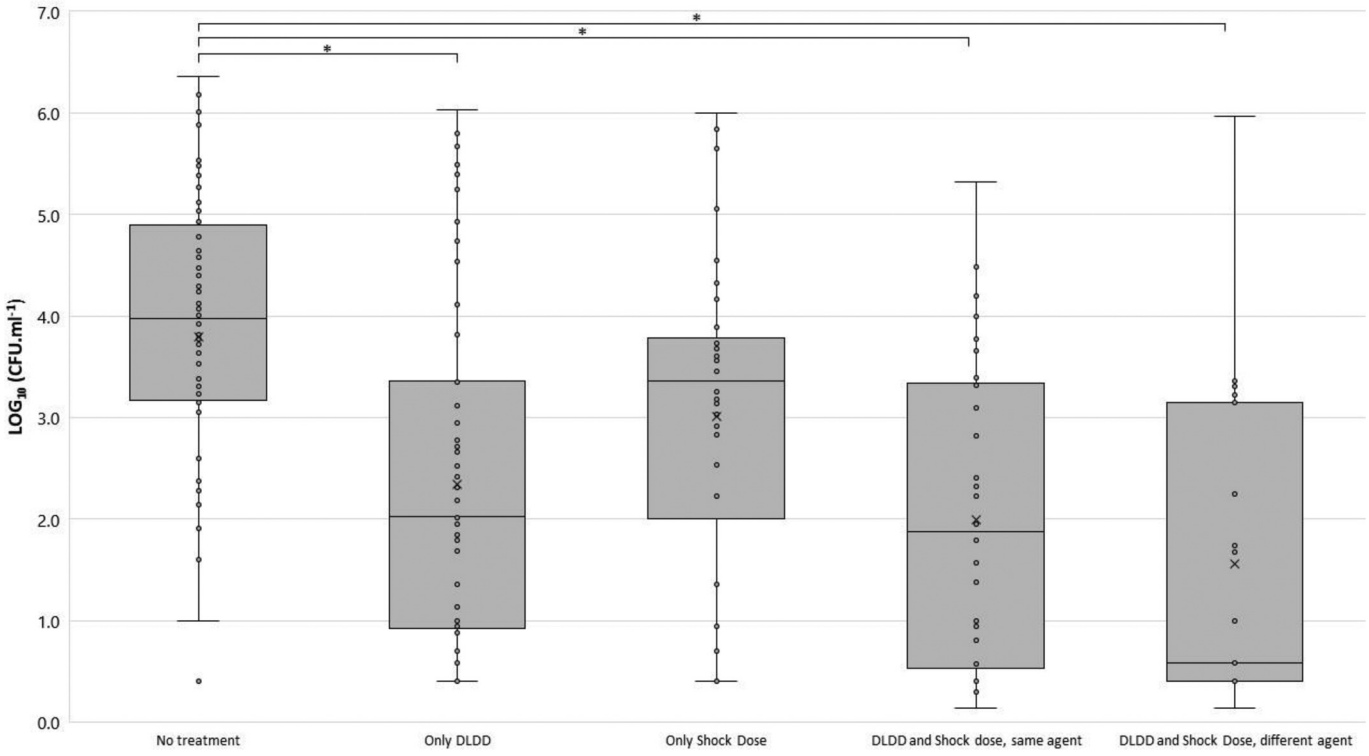

Fig. 1. The effect of a treatment regime with either a daily low dose disinfectant (DLDD), a shock dose treatment, a combination of both with a similar or alternating disinfectant on the microbiological quality of the DUWS effluent water. Results are expressed as the LOG_{10} transformed heterotrophic plate counts in CFU•ml^{-1}. Differences in treatment effect are deemed statistically significant at $p < 0.005$ and marked with an asterisk.

subsampling) of Streptococci, *Porphyromonas* and *Veillonella* were found.

3.3.2. Quantitative PCR

The microbial loads, as determined by q-PCR, were compared between: no treatment applied, DLDD, only a shock dose, both DLDD and shock dose with a similar agent and an alternating agent for DLDD and shock dose.

3.3.2.1. Bacterial 16S rDNA concentration. The bacterial 16S rDNA concentration was determined as part of the sample processing for microbiome sequencing. Overall, 96% of the samples were positive, with an average of 1.3 (± 3.3) ng•µl^{-1} (range 0 - 26 ng•µl^{-1}) bacterial 16S rDNA. A weak to moderate correlation was found between LOG_{10} HPC of the effluent sample and the bacterial 16S rDNA concentration in the RBS samples ($Rc_{pearson}$= 0.451, $Rc_{spearman}$ = 0.708, both $p < 0.005$). As can be seen in Table 2, units receiving a weekly shock dose, contained a significant lower bacterial 16S rDNA concentration when compared to units which did not receive a treatment. No differences were found between different active agents. Only units receiving hydrogen peroxide with silver ions as a DLDD had a significantly lower bacterial 16S rDNA concentration ($p < 0.005$, data not shown).

3.3.2.1. Fungal rDNA concentration. The fungal DNA concentration in the RBS samples was assessed using 18S rDNA as the target. Overall, 98.1% of the samples tested positive, with an average of 6.9 ± 35.6 pg•µl^{-1} (range 0–482pg•µl^{-1}) fungal 18S rDNA. Units which received a shock treatment alone, or combined with a similar disinfectant for the DLDD, contained a lower fungal concentration compared to no treatment ($p < 0.005$), see Table 2. However, no significant differences were found between individual disinfectants.

3.3.2.3. Presence of Legionella spp. *Legionella* spp. were detected in 71% of the 213 samples, containing more than 2000 GU•l^{-1} *Legionella* spp. On average 1.1•10^7 (± 5.7•10^7) GU•l^{-1} *Legionella* spp. (range 2.7•10^3 - 6.3•10^{8}GU•l^{-1}) were detected. Of these samples, 6 units tested positive for L. *pneumophila* of which two belonged to serogroup 1. Samples which were considered safe, based on HPC-counts (n = 84; <100 CFU•ml^{-1}), 45 units contained more than 2000 GU•l^{-1} *Legionella* spp. Units connected directly to the drinking water supply (n = 145) contained significantly more *Legionella*

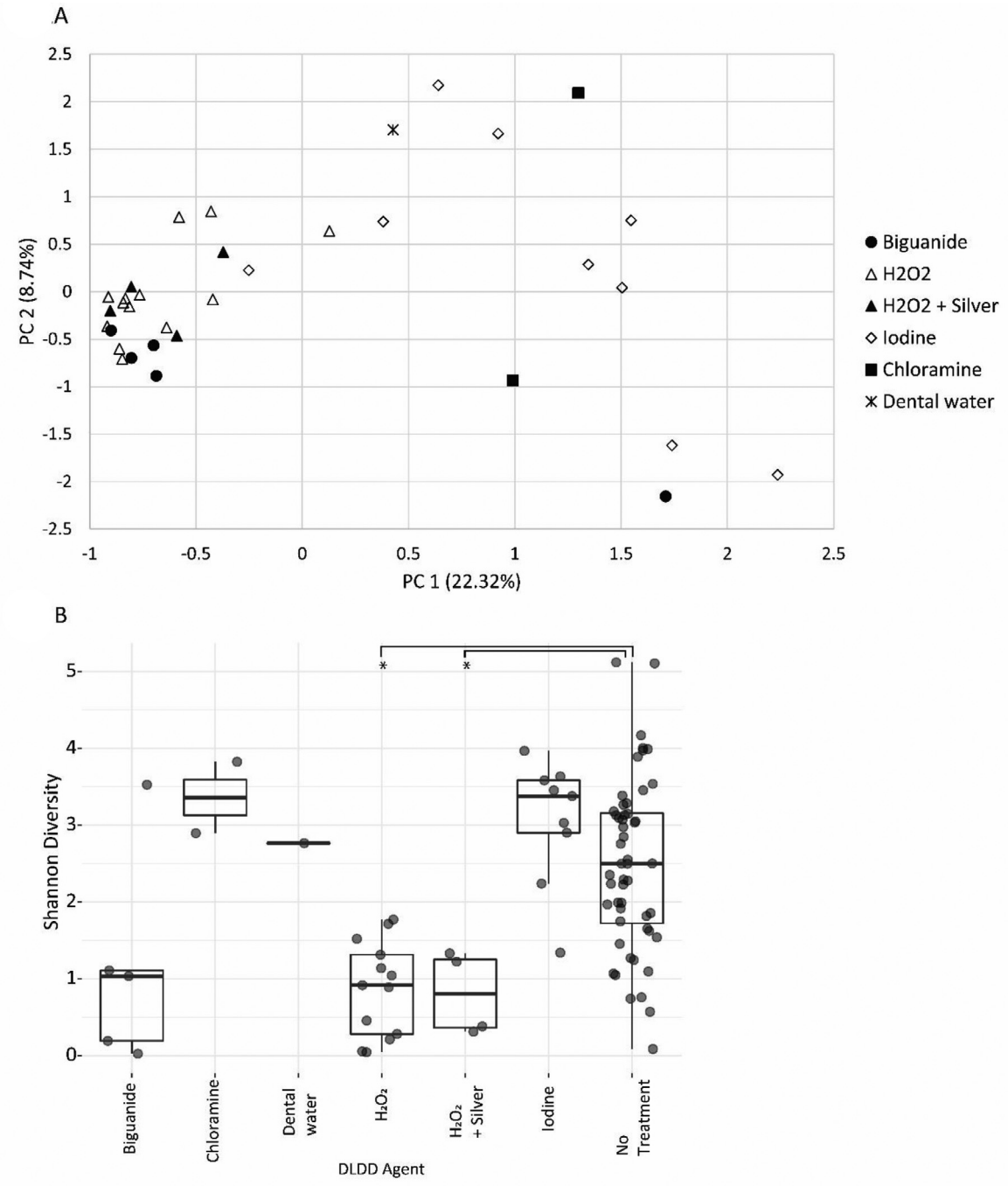

Fig. 2. A) The effect of a daily low dose disinfectant (DLDD) of various active agents on the microbiome composition. Statistically significant differences in composition were found between DLDD active agents ($n = 34$, 9999 permutations, df = 5, F = 2.1, $p < 0.005$). B) Shannon diversity of the different DLDD active agents. Statistical analysis was performed only on actives which were present in more than 2 units. Actives marked with an asterisk had a significant lower ($p < 0.005$) Shannon diversity as compared to the no-treatment group.

spp. as to units which were connected to an external reservoir ($n = 68$) with respectively 4.9 (± 1.5) vs 3.9 (± 1.6) LOG_{10} GU•l^{-1} (Mann-Whitney U test, $p < 0.005$).

As can be seen in Table 2 and depicted in Fig. 3, units which received a DLDD, as compared to no disinfectant, contained an almost statistically significant different *Legionella* spp. load ($p = 0.005$). Units receiving a shock dose (with or without a DLDD) also contained significantly less *Legionella* spp. as compared to units receiving no treatment ($p < 0.005$). Between the different types of disinfectants, units receiving products based on hypochlorous acid both as a DLDD and Shock dose treatment contained a *Legionella* spp. load below the detection limit ($p < 0.005$). Units receiving high doses of silver ions delivered as a weekly shock dose also contained a lower *Legionella* spp. load (2.8 (± 0.7) LOG_{10} GU•l^{-1} as compared to the units which received no treatment ($p < 0.005$).

3.3.2.4. Presence of amoeba DNA. The presence of amoeba was confirmed in 92 out of 213 units (43%) tested. One unit was positive for *Acanthamoeba,* 2 units contained both *Hartmannella* and *Naegleria/Vahlkampfia* and the remainder contained only *Hartmannella.* Bacterial, fungal and *Legionella* spp. concentrations were signifi-

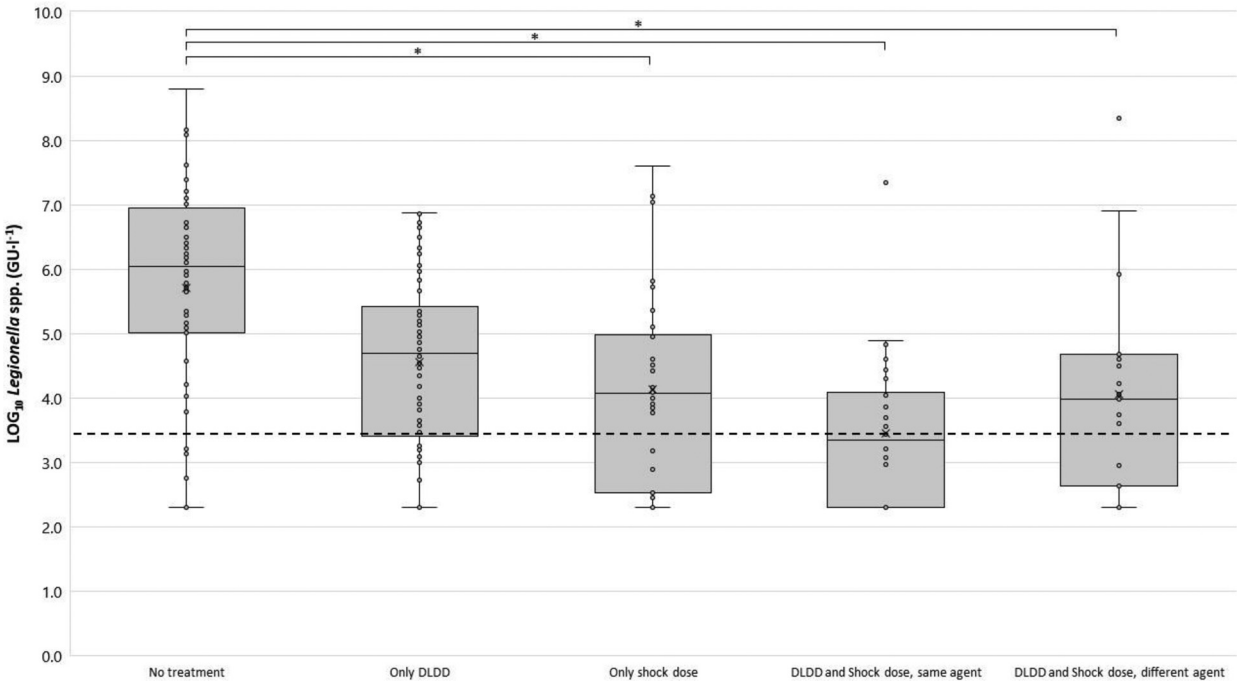

Fig. 3. The effect of a treatment regime with either a daily low dose disinfectant (DLDD), a shock dose treatment, a combination of both with a similar or alternating disinfectant on the LOG_{10} transformed *Legionella* spp. concentration determined by q-PCR and expressed in $GU{\cdot}l^{-1}$. The dashed line represents the detection limit of 2000 $GU{\cdot}l^{-1}$. Differences in treatment effect are deemed statistically significant at $p < 0.005$ and are marked with an asterisk.

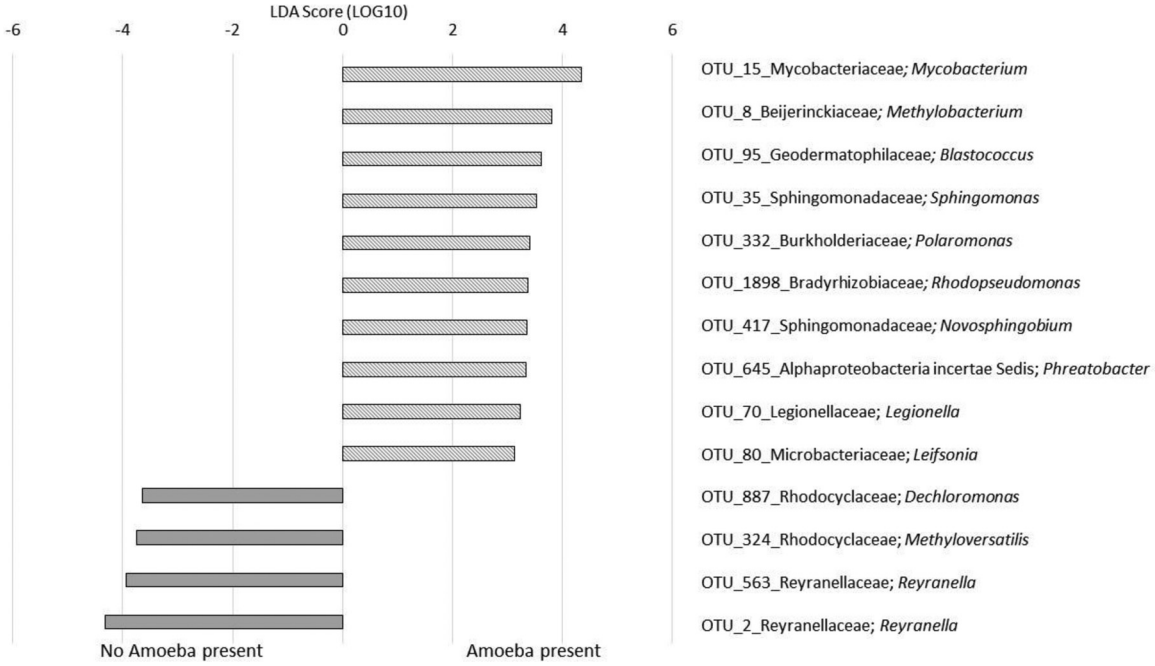

Fig. 4. Linear discriminant analysis effect size (LEfSe) analysis on the microbiome data to detect statistically significant biomarkers associated with the absence (dark grey bars) and presence of amoeba. The representative sequences of the OTU were taxonomically identified using NCBI megablast. Default parameters with the exclusion of models (XM/XP), uncultured/environmental sample sequences. The OTU was annotated with the taxonomic name if the hit had 100% query coverage and ≥99% identity.

cantly higher in the presence of amoeba ($p < 0.005$). Yet, no difference in average HPC ($p = 0.333$) was found between the presence or absence of amoeba.

LEfSe analysis (Fig. 4) revealed that the bacterial families *Burkholderiaceae, Alphaproteobacteria incertae Sedis, Bradyrhizobiaceae, Microbacteriaceae, Legionellaceae, Rhodocyclaceae, Sphingomonadaceae, Reyranellaceae*, were associated with the presence of amoeba. In contrast, *Geodermatophilaceae, Sphingomonadaceae, Beijerinckiaceae* and *Mycobacteriaceae* were positively associated with the absence of amoeba.

4. Discussion

Microbial aerosol formation during dental treatment can pose a risk to both the dental staff and patient (Pankhurst and Coulter, 2007). With an ageing population, the patients' immune sys-

tem gradually deteriorates, making them more vulnerable to infection (Kline and Bowdish, 2016) . Additionally, the current CoVid-19 pandemic creates a whole new population of patients with long-term pulmonary complications making them potentially vulnerable to bacterial and fungal infections (Cox et al., 2020; Koehler et al., 2020). In this study we set out to determine the microbial load and microbiome of Dutch dental unit water and the factors influencing these parameters.

We found that 61% of the dental units sampled contained a higher HPC than recommended. Furthermore, 71% of the dental units sampled contained *Legionella* spp., which could be a potential health issue (Muder and Yu, 2002). Molecular analysis revealed that nearly all dental units (98%) contained fungi and that each dental unit contained a unique microbiome. Off all factors studied, only the treatment regime clearly altered the microbial load and had an influence on the microbiome.

Further analysis showed that, on genus level, *Mycobacterium, Pseudomonas, Legionella* spp., *Coxiella,* and *Staphylococcus* were found in the dental unit water with representatives of these genera being associated with community-acquired pneumonia in the Netherlands (Wiersinga et al., 2018). Case reports have linked legionnaires disease, pneumonia, eye infections and abscess formation to the presence of *Legionella pneumophila, Pseudomonas,* amoeba and non-tuberculous mycobacteria in the dental unit water (Barbeau, 2000; Barbot et al., 2012; Martin, 1987; Petti, 2017).

The high prevalence of *Legionella* spp. found in the microbiome analysis was supported by q-PCR analysis (73% vs. 71% of units positive, respectively). As a primary source of these *Legionella* spp. the watermains is the most likely origin (Leoni et al., 2015) and also in this study we found that dental units which were connected directly to the drinking watermains contained significantly more *Legionella* spp. Even though 6 units (0.03%) tested positive for L. *pneumophila*, and should raise concern, it is a low prevalence when compared to studies reporting a prevalence of 22–78% (Spagnolo et al., 2019; Zanetti et al., 2000). This difference might be explained by higher seasonal temperatures in Italy, as compared to the Netherlands, promoting the growth of *Legionella* and amoeba (Karagiannis et al., 2009). Within the Netherlands, no geographic clustering on the prevalence of Legionella was found (Data not shown), which could be explained by the relatively small size of the Netherlands resulting in small temperature differences.

Proliferation of amoeba and *Legionella* requires a minimum biofilm concentration of about 10^6 CFU•cm^{-2}(van der Kooij et al., 2017). Biofilms grown under hydrodynamic stress can contain 100–1000 times more bacterial cells as compared to the equivalent volume effluent from the same length of 4 mm inner diameter tubing (Hoogenkamp et al., 2020). Thus, units containing more than 10^3 CFU•ml^{-1} in the effluent, could contain about 10^5–10^6 CFU•cm^{-2} biofilm, making 47% of the units eligible as a breeding ground for amoeba. Indeed, in this study, 43% of the units tested positive for the presence of amoeba and is supported by significant positive correlations between the presence of amoeba and high bacterial and fungal DNA load. Amoeba-resistant bacterial families *Burkholderiaceae Alphaproteobacteria incertae sedis, Bradyrhizobiaceae, Microbacteriaceae, Legionellaceae* and *Sphingomonadaceae* and the amoebal prey organisms *Reynanellaceae* (86% of units) and *Rhodocyclaceae* bacterium (80% of units) were more abundant in the presence of amoeba, as delineated by LEfSe analysis. These results are in line with culturing studies showing similar associations (Paquet and Charette, 2016; van der Kooij et al., 2018).

A DUWS biofilm is the causative source of the continuous contamination of the effluent. The microbiological quality of the dental unit water is deemed adequate when the European drinking water standard is met. Traditionally, the unit is flushed prior to sampling and determination of the HPC. However, flushing masks the extent of biofilm contamination in the unit as it temporarily washes out the planktonic bacteria (Rice et al., 2006). Subsequent culturing of the effluent on suboptimal culturing media could then lead to a false sense of security when judging microbial contamination of the unit water (Douterelo et al., 2018; Zanetti et al., 2000). Despite flushing for 30 s, only 39% of the units sampled in this study, complied to the drinking water standard, which is comparable to other studies (Pasquarella et al., 2012; Walker et al., 2004). Alternatively, analyzing the biofilm itself would give the best impression of the presence of possible opportunistic pathogens and the severity of biofilm formation, especially considering that each dental unit has its own biofilm ecosystem. However, taking a biofilm sample from the DUWS is not feasible due to the destructive nature of sampling to the dental unit and collecting a whole-day effluent sample is impossible (Hoogenkamp et al., 2020). Therefore, a proxy sample for the biofilm should be used. In this study an effluent sample was taken immediately after an overnight stagnancy period, prior to any maintenance regime, when the biofilm is in its relaxed state (relaxed biofilm sample). When analyzing the molecular DNA parameters of the RBS sample and correlating this to the HPC, only a weak correlation was found.

Intriguingly, 45 out of 84 units that had an HPC less than 100 CFU•ml^{-1}, contained more than 2000 GU•l^{-1} *Legionella* spp. DNA. In daily practice, the *Legionella* spp. contamination would not have been detected as additional testing for *Legionella* in the Netherlands is only performed when HPC levels exceed 10^4 CFU•ml^{-1}. Growth of *Legionella* is not supported by R2A agar, on which HPC is determined. The presence of *Legionella*, and other (opportunistic) micro-organisms, are more likely to be detected in a biofilm sample (Pierre et al., 2019; Walker et al., 2004). The Relaxed Biofilm Sample, taken in this study, could serve as a proxy as the overnight stagnancy of the unit water results in viscoelastic relaxation of the biofilm in which micro-organisms can easily diffuse or migrate out of the biofilm into the lumen of the tube (Abe et al., 2011; Peterson et al., 2015).

To minimize bacterial growth in the DUWS, manufacturers are obliged to provide a disinfection protocol to disinfect the waterlines of the dental unit (The Council Directive of the European Union, 1993). Units which get administered hydrogen peroxide-based products as a DLDD contained a significantly lower HPC, bacterial 16S rDNA and *Legionella* spp. DNA concentration. Delivering an additional weekly shock dose treatment with hydrogen peroxide, silver ions or hypochlorous acid seemed to further prevent bacterial growth. Hypochlorous acid is a strong oxidizing agent capable of destroying the membrane integrity and silver ions are known to interact with the bacterial membrane and bind to the phosphate groups during DNA replication. While the latter can inhibit *Legionella* replication at relatively low concentrations, intracellularly and biofilm-grown *Legionellae* are less sensitive to silver (Unger and Lück, 2012). Amoeba are known to be more resistant to antimicrobial agents than bacteria and feed on both live and dead biofilms (Cervero-Aragó et al., 2015). Biofilm removal, rather than killing, is of prime importance as no significant difference in the prevalence of amoeba was found between the different types of disinfectant applied.

Currently, the fungal load is not determined to assess the quality of the dental unit water. Being more complex organisms than bacteria, fungi are less sensitive to water disinfecting agents (Hageskal et al., 2012; Nett et al., 2008). This is supported by the finding that a shock dose treatment appears to be necessary to reduce the fungal load. The use of a DLDD disinfectant alone could even be unwanted, as proliferation of fungi has been reported especially in situations where small amounts of prokaryotic organisms were found in the source water (Porteous et al., 2003). This is supported by our finding that all samples with less than 0.5 µg•ml^{-1} bacterial 16S rDNA, always contained detectable concentrations of fungal rDNA (see Data in Brief).

The relationship between the microbiological contamination of dental unit water and the risk for infection remains unclear (Pankhurst and Coulter, 2007). This ambiguity might result from a lack of awareness of a link between an infection in the aftermath of a dental treatment. A strict adherence to an infection control protocol, supported with frequent testing, has proven effective in keeping biofilm growth under control within a dental school setting (Baudet et al., 2020; Volgenant and Persoon, 2019). As only half of the dental offices, screened in this study, applied some sort of infection control measure, it is clear, that they too have a vital role in ensuring water quality and safety. Moreover, as participation in this study was voluntary, it is to be expected that only dental offices which perform active infection control measures responded. It may therefore be possible that other, not tested units, have an even worse outcome than reported here. Further research is needed to assess which disinfectants and treatment regimens are able to remove biofilm from the DUWS in order to help the general dentist practice to control biofilm formation and guarantee patient safety.

5. Conclusion

- The vast majority of dental unit water in the Netherlands exceed infection control guidelines.
- Fungi, *Legionella* spp. and amoeba are frequently detected in Dutch dental units.
- A risk assessment, based only on determining the HPC in the effluent is underestimating the maximum possible biological load and is therefore not sufficient.
- A proxy, relaxed biofilm state sample, in combination with q-PCR techniques, is a valuable tool to monitor the microbiological quality and the presence of opportunistic pathogens in dental unit water.
- The use of generic disinfection protocols are likely to be ineffective in guaranteeing safe dental unit water.
- Infection control guidelines should focus on the presence and control of biofilm formation as this is the source of reinfection of the effluent water.

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.

CRediT authorship contribution statement

Michel A. Hoogenkamp: Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Bernd W. Brandt:** Data curation, Software, Formal analysis, Writing - review & editing. **Alexa M.G.A. Laheij:** Conceptualization, Writing - review & editing. **Johannes J. de Soet:** Conceptualization, Supervision, Writing - review & editing. **Wim Crielaard:** Supervision, Conceptualization, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117205.

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