JOURNAL OF ORAL MICROBIOLOGY
2023, VOL. 15, 2223477
https://doi.org/10.1080/20002297.2023.2223477

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

OPEN ACCESS Check for updates

First water safety plan approach applied to a Dental Clinic complex: identification of new risk factors associated with *Legionella* and *P. aeruginosa* contamination, using a novel sampling, maintenance and management program

Federica Marino [a#], Marta Mazzotta [a#], Maria Rosaria Pascale [a,b], Carlo Derelitto [a], Luna Girolamini [a,c] and Sandra Cristino [a,c]

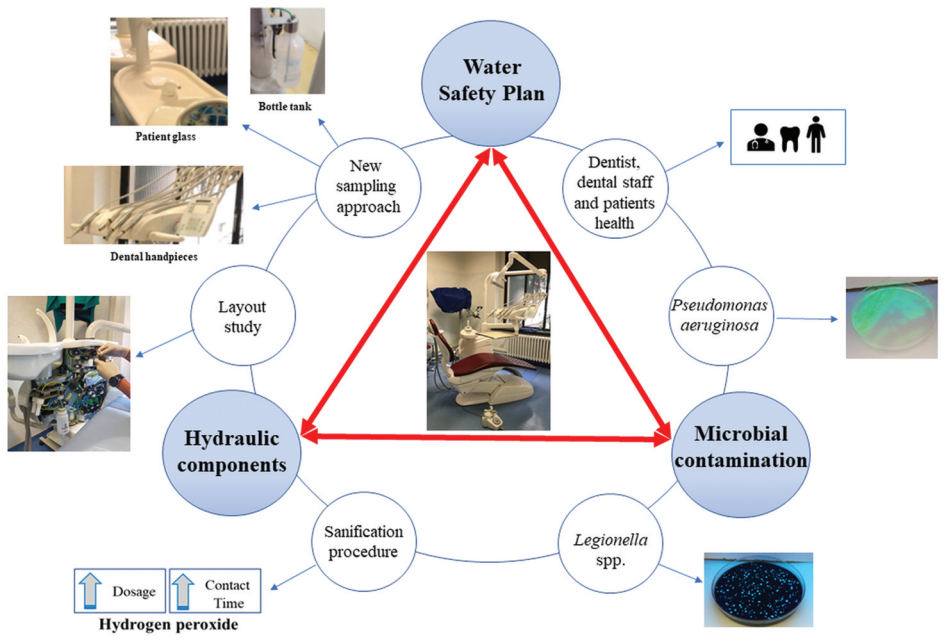
[a]Department of Biological, Geological, and Environmental Sciences, University of Bologna, Bologna, Italy; [b]Department of Civil, Chemical, Environmental and Materials Engineering, University of Bologna, Bologna, Italy; [c]European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for Legionella Infections (ESGLI), Basel, Switzerland

ABSTRACT

Dental unit waterlines (DUWLs) represent a complex environment able to promote microbial contamination, due to functional, mechanical and practical risk factors. According to a water safety plan approach, the main goal is to preserve the health of dentists, dental staff and patients.

The aim of this study is to develop a DUWLs water safety plan that is able to support correct and effective maintenance and disinfection procedures.

Three different water systems serve 60 dental chairs: (i) water that comes directly from municipal water (Type A), (ii) water supplied by municipal water and water bottles (Type B) and (iii) water supplied only via water bottles (Type C). For each type, *Legionella* and *Pseudomonas aeruginosa* contamination was studied, by applying a new sampling scheme, based on separate sampling from water bottles, cup filler and handpieces. Type B DUWL is the only type of DUWL contaminated by *L. pneumophila* (ST 59) and *L. anisa* (mean contamination: 608.33 ± 253.33 cfu/L) detected in cup filler and handpieces, as well as the high presence of *P. aeruginosa* (44.42 ± 13.25 cfu/100 mL). Two subsequent shock treatments and resampling procedures were performed by increasing disinfectant dosage and contact time and removing some DUWL components linked to biofilm growth in DUWLs. A significant reduction of contamination was obtained for both microorganisms (*Legionella* spp.: −100%, $p < 0.001$ and *P. aeruginosa*: −99.86%, $p = 0.006$). The sampling strategy proposed allows us to identify the source of contamination and better focus on the maintenance and disinfection procedures. DUWLs represent an environment that requires a multidisciplinary approach, combining the knowledge of all DUWL components to correct procedures that are able to preserve the health of personnel and patients, as well as guaranteeing DUWLs' safe functionality.



ARTICLE HISTORY

Received 21 March 2023
Revised 18 May 2023
Accepted 6 June 2023

KEYWORDS

Dental unit waterlines (DUWLs); water quality; *Legionella*; *Pseudomonas aeruginosa*; maintenance procedures; water safety plan; dental risk factors

CONTACT Sandra Cristino sandra.cristino@unibo.it Department of Biological, Geological, and Environmental Sciences, University of Bologna, via San Giacomo 12, Bologna 40126, Italy
#These authors contributed equally to this work.

Introduction

Water is an important factor in dental care practices as it is involved in cooling and irrigation equipment during dental treatments. Dentists, dental staff and patients, particularly those who are immunocompromised, smokers or the elderly, are considered vulnerable, and the continuous exposure to contaminated water contact or aerosolization poses a risk to human health. Exposure can occur directly through drinking or splatters as well as through contact with human skin and mucous membranes. In addition, aerosols generated by dental high-speed handpieces and air/water syringes can represent a potential vehicle for indirect infection. For this reason, the water used in dental care must at least comply with drinking water criteria, and therefore infectious risk management is a fundamental aspect to ensure high-quality water [1,2].

Dental Unit Waterlines (DUWLs) may be supplied directly by municipal water, a sterile water bottle, or both; the presence of a water bottle that feeds the dental circuit, if not maintained, can result in water stagnation, especially when dental units are not in use. Moreover, water becomes a source of microbiological contamination, due to several factors including the type of water supply, the back-contamination of biological fluids from patients' oral cavities, especially when the anti-retraction valves fail or are not correctly maintained, and the biofilm formation on the inner surfaces of dental water pipelines [3,4]. In particular, the pipelines characteristics such as the materials used in the manufacturing of tubes (e.g. plastic), the small lumen size (0.5–2 mm), a large area-to-volume ratio (6:1), as well as the age of the waterline system support biofilm development [5].

The biofilm is difficult to remove in DUWLs and represents a reservoir of pathogenic and non-pathogenic bacteria [6,7].

Over the years, several studies have been published on biofilm formation control in dental units. Some of these focused on the development of surfaces with antibiofilm activity [8], the use of sterilized water [9] or chemical–physical treatments (e.g. chlorination or filter systems) [10,11]. However, the implementation of these strategies, due to their high costs, requires the implementation of adequate maintenance programs.

Many Gram-negative bacteria, including environmental microorganisms (*Moraxella* spp. and *Flavobacterium* spp.) and opportunistic human pathogens (*Streptococci*, *Enterococci*, *Staphylococci*, *Pseudomonas* spp., *Legionella* spp. and *Mycobacterium* spp.), have been reported in DUWLs output water samples. In addition, fungi (*Candida* spp.) and protozoa (free-living amoebae) have been isolated from DUWLs [4,12,13].

The first evidence of microbial contamination in DUWLs was described by Blake in 1963 [14]. Several studies have been conducted since this report, focusing on bacterial analyses, microbiological typing and maintenance programs to improve DUWLs output water quality [15,16]. Pathogens from DUWLs were transmitted by aerosolization of water and inhalation of droplets from dental handpieces [17–19], and also by direct transmission through wounds, splatters or ingestion of water [20–22]. Especially in immunocompromised patients, infections caused by the aforementioned pathogens, which are resistant to antimicrobials, can be fatal. A recent fatal case of an elderly Italian patient, who acquired a *Legionella* infection during dental practices, was reported by [23,23]. Despite the low incidence, other 'probable' fatal cases both for patients and dental staff have been reported [24,25], highlighting that the risk associated with dental practices and instruments used should not be underestimated.

Regarding water quality requirements, in the guidelines on infection control in dental health care the US Centers for Disease Control and Prevention (CDC) and American Dental Association (ADA) recommend that the level of heterotrophic plate count (HPC) bacteria should have at most 500 and 200 colony forming units (cfu)/mL, respectively [16,26].

There are no current European guidelines for DUWLs with respect to microbial contamination. Several countries applied the drinking water standard requirements, indicating the absence of pathogenic bacteria (e.g. *Escherichia Coli (E. coli)*, *Pseudomonas aeruginosa (P. aeruginosa)*, Enterococci) in DUWLs, others, applied the suggestions from the CDC and the ADA, which give suggestions concerning the heterotrophic bacteria count at 22°C [27].

In the current Italian National and Regional guidelines for the prevention and control of *Legionella* infection, the contamination limits suggested for dental healthcare settings are referred to healthcare facilities [28,29]. During the COVID-19 pandemic, the ESCMID Study Group for *Legionella* Infections (ESGLI) produced the Guidance for managing *Legionella* in dental practices (20200424 v 01.012019), suggesting a list of control measures to prevent *Legionella* infections [30].

Subsequently, in Italy, a new Covid Report was published containing indications for the prevention of *Legionella* risk in dental units. According to the ESGLI document, the report established the limit of 100 cfu/L as a threshold to undertake corrective measures [31]. Moreover, dental clinics are required to monitor the water quality from DUWLs and to conduct a *Legionella* environmental monitoring at least once a year or whenever a case of disease occurs among dental staff or patients [28,31].

In this study, the microbial contamination of a Dental Clinic complex consisting of 60 dental chairs was evaluated. The aim was to assess the water quality maintenance program developed during the lockdown period that occurred during the global SARS-CoV-2 pandemic in 2020, regarding *Legionella* and *P. aeruginosa* contamination, considering that the presence of *Legionella* in dental units is frequently associated with *P. aeruginosa* contamination [5,32].

Although the Italian Covid Report suggests a sampling approach to apply in DUWLS: collection of a total volume of 1 L from the turbine module, micromotor module, air/water syringes module, scaler module (where present) and cup filler, mixed together, in this study, a new sampling approach was implemented. The DUWLs were separated between dental high-speed handpieces and air/water syringes, water bottles and cup fillers. The main goal was to study the origin of contamination in DUWLs, identifying new potential risk factors. This approach will allow us to elaborate a new risk assessment plan, focused on the optimization of corrective measures and preventive strategies.

Materials and methods

Dental clinic description and characteristics of DUWLs

The Dental Clinic complex is located inside a historical building and consists of 60 dental unit chairs, which are different in terms of model and year of installation (from 2005 to 2022), distributed in 10 dental departments, according to the different dental practices conducted. In the study, the dental chairs were differentiated with regard to the water supply system and their technical characteristics. Therefore, a *Legionella* risk assessment plan, a new sampling program as well as corrective and preventive measures were developed accordingly.

Three types of dental units were classified as follows:

- Dental unit entirely supplied by the municipal water network ($n = 5$), named Type A;
- Dental unit with cup filler supplied by the municipal water network and handpieces supplied by an independent water bottle, with the option, via a bypass button, to switch from the supply tap water system to the water bottle ($n = 43$), named Type B;
- Dental unit with both cup filler and handpieces supplied by an independent water bottle and the dental unit spittoon supplied by the municipal water supply; in these DUWLs, there is no possibility of modifying the supply water system feed ($n = 12$), named Type C.

In DUWLs equipped with a dual water supply system (Types B and C), the independent water bottle has a capacity of 1 to 1.5 L, depending on the model of dental unit chair. Demineralized water plus hydrogen peroxide solution (3% v/v, final volume) was used to fill the water bottle, in accordance with manufacturer's suggestions.

The difference in the number of DUWLS represents the real Dental Clinic equipment.

DUWLs routine maintenance and sanification programs

According to the Center for Disease Control and Prevention (CDC), the European guidelines and the Italian and Regional guidelines [28,66], all DUWLs implemented a *Legionella* risk assessment plan based on routine maintenance program:

- Every morning:
  - Flushing for at least 2 min from handpieces hoses and cup filler;
  - Disinfection of automated water bottle, filled with demineralized water plus hydrogen peroxide solution (final concentration of 3% v/v);
  - Keep the surfaces of the dental unit clean and decontaminated using hydroalcoholic-based disinfectants and detergent solutions.
- Between each patient:
  - Flushing for at least 20–30 s from handpiece hoses and cup filler;
  - Replace with newly sanitized handpieces.
- At the end of the working day:
  - Disinfection procedures were implemented in accordance with the manufacturer's instructions. In detail, for DUWLs belonging to Type A, the disinfection procedure is focused only on the handpieces by autoclave treatment. Types B and C disinfection procedure is carried out via an independent water bottle filled with demineralized water and hydrogen peroxide solution (final concentration of 3% v/v); moreover, at the end of each working day, Type B and C lines present an automatic disinfection program based on hydrogen peroxide (final concentration of 3% v/v) plus Ag^{+};
  - Unplug and autoclave the handpieces, where possible, or unplug and sanitize before reuse;
  - Sanitize and rinse the water bottle with chlorine-based solutions, sterile, distilled or osmotic water. When possible, sterilize the water bottle and store it upside down to allow perfect drying.

To these ordinary maintenance procedures, the following recommendations are advised:

- Eliminate or remove from the (DUWLs) components or pipeline sections excluded by the water flow, to avoid dead water branches.
- Install anti-stagnation devices able to continuously maintain water circulating, especially during work breaks.
- Install filters (≤0.2 µm) capable of retaining microorganisms from inside the circuit, immediately upstream of the handpieces. These should be replaced every 6 months or when the water or pressure flow decreases;
- Monitor the cold-water temperature, coming from the water supply and circulating in the DUWLs, ensure temperature values below 20°C;
- Carry out maintenance and periodic checks of the non-return valves present at the handpiece hoses;
- In case of breakdown or prolonged disuse of the dental unit, ensure its emptiness and disconnection from the main water supply, until the next safe use.

In this study in presence of non-compliant results obtained due to the presence of *Legionella* and/or *P. aeruginosa*, a sanification treatment was undertaken by manufacturer's staff.

The first step of DUWL cleaning was performed using a commercial alcohol-based detergent injected into the DUWL through a pressurized pump. Furthermore, after a rinse with sterile water, a descaling product based on orthophosphoric acid solution (final concentration 7% v/v) in water was applied for 10 min. The circuit was then emptied, washed twice with sterile water and, finally, an extraordinary disinfection treatment with hydrogen peroxide (final concentration 10% v/v) for 10 min was performed.

The dentist and dental staff were informed about the biological risk present, in order to evaluate and carry out functional and maintenance DUWLs programs. Furthermore, each of the activities listed above was recorded on a maintenance register, validated by the health director and the safety manager.

Extraordinary maintenance and sanification program implemented in the study

When non-compliance results were obtained after an ordinary sanification protocol, a second extraordinary sanification procedure was developed, to improve the manufacturing protocol.

Before starting the new extraordinary sanification program, a timely visual inspection of the whole water pipeline system was conducted with manufacturer's staff. Starting from the point of connection with the municipal water, located under the dental chairs, up until the handpieces and cup filler, all components were inspected.

The following activities were performed:

- all the DUWLs were suspended and the contaminated DUWLs were also disconnected from the municipal water connection;
- replacement of two in-line filter pressure reducers (50 and 25 µm), located after the input of the main municipal water, were carried out (Fig. 1a,b);
- all the damaged pieces or anything else that could over time cause water stagnation, flow reduction and biofilm formation, such as the flow sensor (Fig. 1c) or the small pipelines that connect the main water to dental handpieces, cup filler and the spittoon, were replaced (Fig. 1d);
- in accordance with manufacturer's staff instructions, a new cycle of sanification was performed. The applied protocol was changed from the previous one, in terms of the number of rinses (two times after each treatment), use of sterile water and increase in disinfectant concentration,

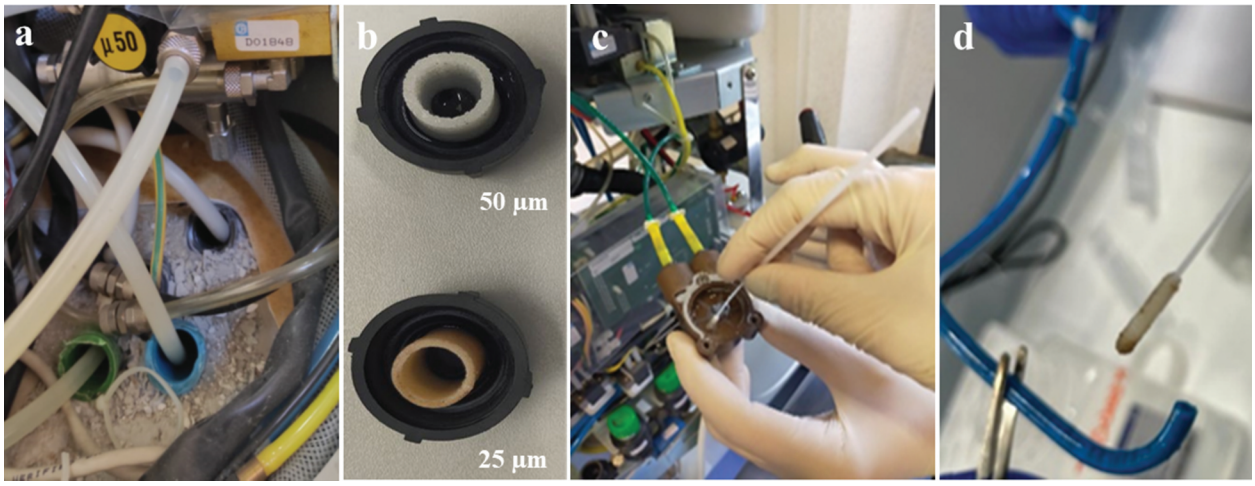


Figure 1.DUWLs components analyzed: a) filter pressure reducers located in the dental chair; b) details of two filter pressure reducers; c) flow sensors and d) FLOQSwabs® minitip samples on part of water pipelines.

hydrogen peroxide solution (final concentration of 35% v/v) and contact time, 20 min instead of 10;
- after these corrective actions, the DUWLs were restored to normal functioning;
- all healthcare workers applied a flushing procedure of 5 min at the beginning, in the middle and at the end of the day from handpieces, patient water glass and spittoon; in particular, for DUWLs without an automatic flushing system from cup filler and spittoon (Type B), a manual flushing activity was implemented every day for 5 min.

Water sample collection and microbiological parameters

A preliminary site inspection was carried out to meet the staff and to analyze the type of dental unit chairs in use, to correctly plan the water sampling with the aim of controlling the compliance of procedures with manufacturer's instructions.

The DUWL monitoring was performed at different times, as follows:

- T0: the first monitoring, to detect the level of DUWL contamination and the compliance with ordinary procedures described in the water safety plan;
- T1: resampling of positive units (*Legionella*, >100 cfu/L) and (*P. aeruginosa*, >0 cfu/L), after the ordinary sanification program;
- T2: resampling of DUWLs that remain positive (*Legionella*, >100 cfu/L and *P. aeruginosa*, >0 cfu/L), even after the ordinary sanification program. Test of efficacy of the extraordinary sanification program was implemented.

During the study, sampling was conducted between 2021 and 2022, considering the different dental chair characteristics and water supply systems.

To obtain exhaustive information on the DUWLs contamination statuses, a representative number of dental unit chairs (50.0%) were chosen from each dental department in relation to the equipment of Dental Complex and the type of supply system (municipal water or water bottle). In particular, Type A DUWLs (connected to the main municipal water) were chosen at the start, the middle and the end of the main water pipelines, while for both Type B and Type C DUWLs (supplied by water bottle), they were randomly chosen. In detail, $n = 3/5$ Type A DUWLs, $n = 22/43$ Type B DUWLs and $n = 5/12$ Type C DUWLs were monitored, respectively.

Regarding the sampling plan, for each dental chair, three different sampling points were chosen: i) dental high-speed handpieces and air/water syringes, ii) water bottle (when it was present) and iii) cup filler, with a total of $n = 90$ water samples. Each of them was separately sampled and analyzed, in order to test the quality of the water pipeline across the dental unit.

In detail, 100 mL was collected from each handpiece and mixed to obtain a total volume of 500 mL, 1 L from the cup filler and 1 L from the water bottle.

The objective of this new sampling approach is to analyze the contamination present in the dental unit, in order to uniquely identify the critical points. In addition, the sampling was carried out at the end of each working day and before ordinary sanification practices, in order to detect the level of contamination present in the operating conditions of the dental unit.

Furthermore, to better understand the possible source of contamination, water samples (1 L) were also collected from the municipal water network connection located at the base of each dental chair (Types A and B) and from the DUWLs water bottle that contained demineralized water (Type C).

The water samples were collected in sterile polytetrafluoroethylene (PTFE) bottles, without flushing according to ISO 19458:2006, and analyzed within 24 h [33]. Water temperatures (°C) were tested for all samples by a digital thermometer coupled with a liquid thermistor probe (XS Temp 7 Vio PT 100 Thermometer from −200 to +999°C; Eutech Instruments Pte Ltd., Singapore).

Microbiological analyses included the detection of *Legionella* and *P. aeruginosa*, in accordance with the Italian and Regional Guidelines and the Italian Regulation on drinking water for human consumption, respectively [28,34,66].

Legionella isolation was performed using culture techniques according to ISO 11731:2017 [35]. For the enumeration of *Legionella*, samples were processed with a membrane filtration technique using polyethersulfone membrane filters with a porosity of 0.22 µm (Sartorius, Bedford, MA, USA). Aliquots of 100 µl of the filtered, heat- and acid-treated sample were seeded on Glycine-PolymyxinB-Vancomycin-Cycloheximide (GVPC) selective agar (Thermo Fisher Scientific, Diagnostic, Ltd., Basingstoke, UK) and then all the plates were incubated at 35 ± 2°C with 2.5% of CO_2. The culture required a minimum of 10–15 days, and the plates were examined every 2 days. The presumptive colonies were enumerated and sub-cultured on Buffered Charcoal Yeast Extract (BCYE) agar with L-Cysteine (Cys+) and without L-Cysteine (Cys-) (Thermo Fisher Scientific, Diagnostic, Ltd., Basingstoke, UK). The detection limit was 100 cfu/L.

The *Legionella* colonies, growth only on BCYE Cys +, were identified using the *Legionella* latex agglutination test kit, differentiating between *Legionella pneumophila* (*Lp*) serogroup 1 (*Lp*1), *Lp* serogroups 2–14 and seven of non-*L. pneumophila* (n*Lp*) species (Thermo Fisher Scientific, Ltd. Basingstoke, UK),

based on manufacturer's instructions. The positive results obtained for n*Lp* species agglutination test were also analyzed by the MALDI Biotyper System (Bruker Daltonik GmbH, Bremen, Germany) and data were interpreted following the manufacturer's instructions [36]. Moreover, the identification of *Legionella* colonies was performed by Sequence-Based Typing (SBT) for colonies belonging to *Lp*1 and by macrophage infectivity potentiator (*mip*) gene sequencing, according to the protocol described by the ESGLI group [37,38]. SBT data were analyzed using the web-based *Lp* SBT database (https://bioinformatics.phe.org.uk/legionella/legionella_sbt/php/sbt_homepage.php). This link is undergoing development and is currently unavailable externally but can be accessed internally by the database curators at UKHSA (legionella-sbt@ukhsa.gov.uk).

P. aeruginosa analysis was carried out according to the UNI EN ISO 16266:2008 standard technique [39]. The analysis was performed on a volume of 100 mL, filtered using a cellulose nitrate membrane filter with a 0.45 µm pore size (Sartorius, Bedford, MA, USA). The membrane was seeded on *Pseudomonas* C-N Selective Agar (Cetrimide Agar) (Thermo Fisher Scientific, Diagnostics, Ltd., Basingstoke, UK) and incubated for 48 h at 36°C. The detection limit was 0 cfu/100 ml.

Blue-green colonies, which produced a characteristic bright green color on the medium and showed fluorescence under a Wood's lamp (ultraviolet light at 365 nm), were identified as presumptive *P. aeruginosa*. The colonies were biochemically identified by indole, oxidase reaction tests and Remel RapID NF Plus system (Thermo Fisher Scientific, Diagnostic, Ltd., Basingstoke, UK), according to the manufacturer's instructions. The results were also confirmed by MALDI Biotyper System (Bruker Daltonics, Germany) according to the manufacturer. Moreover, further analysis was carried out on all changed or removed components of DUWLs by using FLOQSwabs® minitip (Copan, Brescia, Italy). In detail, swab samples were collected on the filter pressure reducers, on the flow sensor and on the changed connecting pipelines, as well as on the handpieces (Figure 1c,d).

The samples with *Legionella* contamination ≤100 cfu/L and *P. aerugionosa* = 0 cfu/100 mL were considered negative, instead when the contamination was >100 cfu/L, a disinfection of DUWLs and a review of correctives measures were required, with a subsequent resampling [31].

Regarding the *P. aeruginosa* results, the reference value is 0 cfu/100 mL, according to the Italian Regulation of drinking water for human consumption [34].

The microbiological contaminations were expressed as the mean value ± standard error (SE).

Moreover, other water samples were taken after non-compliant results were obtained during the study and/or after extraordinary sanification procedures implemented on DUWLs.

Statistical analysis

SPSS software for Windows version 28.0.1.1 (IBM SPSS, Inc., Chicago, IL, USA) and R Statistical Software (version 4.2.3, 'Shortstop Beagle' R Foundation for Statistical Computing, Vienna, Austria) were used to perform statistical analyses. A Shapiro–Wilk test was carried out to assess the normality of variables. A Kruskal–Wallis H test was performed for the comparison of three or more groups, whereas the comparison between the two groups was performed using a Mann–Whitney test. Significance of all statistical tests was set to p value (p) ≤ 0.05.

Results

In the Dental Clinic complex involved in the study, 30 out of 60 DUWLs (50.0%) were sampled, by taking into consideration their number and type of water supply.

The first monitoring aimed to assess the status of water quality (T0) and showed that 12/30 (40.0%) of DUWLs presented contamination of *Legionella* and/or *P. aeruginosa*.

The mean contamination observed was 230.56 ± 95.69 cfu/L for *Legionella* and 17.58 ± 6.02 cfu/100 mL for *P. aeruginosa*.

*Lp*1 was detected in the range from 100 to 2500 cfu/L, with a mean contamination ± SE of 177.78 ± 92.68 cfu/L, n*Lp* in the range of 200 cfu/L to 800 cfu/L (52.78 ± 26.87 cfu/L) and *P. aeruginosa* was present in the range of 27 to 100 cfu/100 mL (17.58 ± 6.02 cfu/100 mL).

Concerning the identification of the *Legionella* species, the SBT profile reported for *Lp*1 was the Sequence Type (ST) 59, whereas *L. anisa* was detected among n*Lp* isolates. The results were confirmed by a MALDI Biotyper System analysis that returned a high confidence identification score for both *Lp* and *L. anisa* isolates.

In Table 1, the data regarding the contamination found for *Legionella* and *P. aeruginosa* are shown, in relation to different DUWL types and sampling points (T0).

The positive dental chairs were disconnected from the municipal water, through a connector located under the floor, and water input was sampled. None of the municipal water samples displayed any contamination. Similar results were also obtained by analyzing the demineralized water used to fill the DUWL water bottles.

All Type A and Type C dental units monitored (n = 3 and n = 5, respectively) showed no microbial

Table 1. *Legionella* and *P. aeruginosa* mean contamination ± SE, in relation to different DUWLs and sampling points during T0.

DUWL type	DUWLs contaminated	Water temperature (°C)	T0 sampling points	Positive samples contamination Mean contamination ± SE (minimum–maximum) *Legionella* spp. (cfu/L)	*Lp*1 (cfu/L)	*L. anisa* (cfu/L)	*P. aeruginosa* (cfu/100 mL)
Type A* (*n*=3)	0/3	18.75±0.40	Cup filler Dental handpieces Tap water	0	0	0	0
Type B (*n*=22)	12/22 (54.55%)		Cup filler	608.33±253.33 (0–2500)	491.67±258.33 (0–2500)	116.67±67.23 (0–800)	44.42±13.25 (0–100)
		23.06±0.22	Dental handpieces	83.33±56.18 (0–500)	41.67±41.67 (0–500)	41.67±41.67 (0–500)	8.33±8.33 (0–100)
			Water bottle	0	0	0	0
Type C (*n*=5)	0/5	21.43±0.17	Cup filler Dental handpieces Water bottle	0	0	0	0

*Type A DUWLs were supplied by the municipal water network; therefore, the water bottle samples are absent.

contamination; instead, all the contaminated samples belonged to Type B.

Regarding the sampling points identified in Type B, the main contamination was found in cup filler points (12/12), followed by dental handpieces (3/12). On the other hand, the water bottle samples never showed contamination for both analyzed parameters.

The temperature measured for each sample (Table 1) showed values ≤25°C, with a mean between 18.75 ± 0.40°C and 23.06 ± 0.22°C.

DUWL contamination after the ordinary sanification protocol

All non-compliance Type B DUWLs ($n = 12$) contaminated at T0, were provisionally closed to patients and dental staff. The positive samples for one or both parameters were then re-analyzed (T1), in order to test the performance of the ordinary sanification protocol undertaken and correctly share the maintenance measures among operators.

A total of 36 samples were collected 24 h after the activity was undertaken, following the same methodology (cup filler, handpieces and water bottle). Moreover, 1 L of water input was again sampled as a control. The results obtained after the ordinary sanification protocol conducted during monitoring time T1, and the comparison with the previous results (T0) are shown in Figure 2.

As shown in Figure 2, after the sanification procedures, a general decrease in *Legionella* and *P. aeruginosa* mean contamination was observed in cup filler. In particular, the following reductions were observed: *Legionella* spp. −59.54% (*Lp*1 −42.53%, *L. anisa* −90.63%) and *P. aeruginosa* −43.29%. These results do not show statistically significant differences. However, in the dental handpieces, the contamination disappeared, and the absence of contamination in the water bottles and water input was reconfirmed.

Results show that, despite the fact that the ordinary sanification protocol led to a contamination decrease, the cup filler, remained contaminated and over the regulation limit.

The Type B DUWLs were again disconnected from water input, and the status of the pipelines was assessed by both visual inspection and collection of swab samples. The small tube connecting the main water pipelines with the cup filler, and the mixed valve connected to the flow sensor were removed. Moreover, using the FLOQSwabs® minitip, a biofilm sampling into the small DUWL pipelines and flow sensor was performed (Figure 1c,d). The two series filters (porosity of 50 and 25 µm), located under the dental chairs, which serve as pressure reducers of the main water flow, were removed and analyzed (Figure 1a,b).

Extraordinary sanification was performed on the DUWLs that remained positive and a resampling (T2) was conducted 24 h after the extraordinary sanification protocol. This showed contamination by *P. aeruginosa* only in the cup filler in 1/12 DUWLs (1 cfu/100 mL). In the other locations of the DUWLs, no contamination was reported. Moreover, the swab samples and pressure reducer filters removed showed a high *Legionella* and *P. aeruginosa* presence.

In order to evaluate the effectiveness of the extraordinary sanification program developed, statistical analysis was performed on cup filler samples that remained contaminated until T2. The analysis of the results is shown in Table 2.

The data obtained confirmed the efficacy of the extraordinary program developed during the study.

Discussion

DUWLs represent an environment susceptible to microbiological contamination. The main risk factors associated with bacterial proliferation are as follows: i) small and thin pipelines; ii) water stagnation inside water bottle and in the DUWLs that promote biofilm formation; iii) inadequate flushing and disinfection procedures; vi) absence or poor maintenance of filter

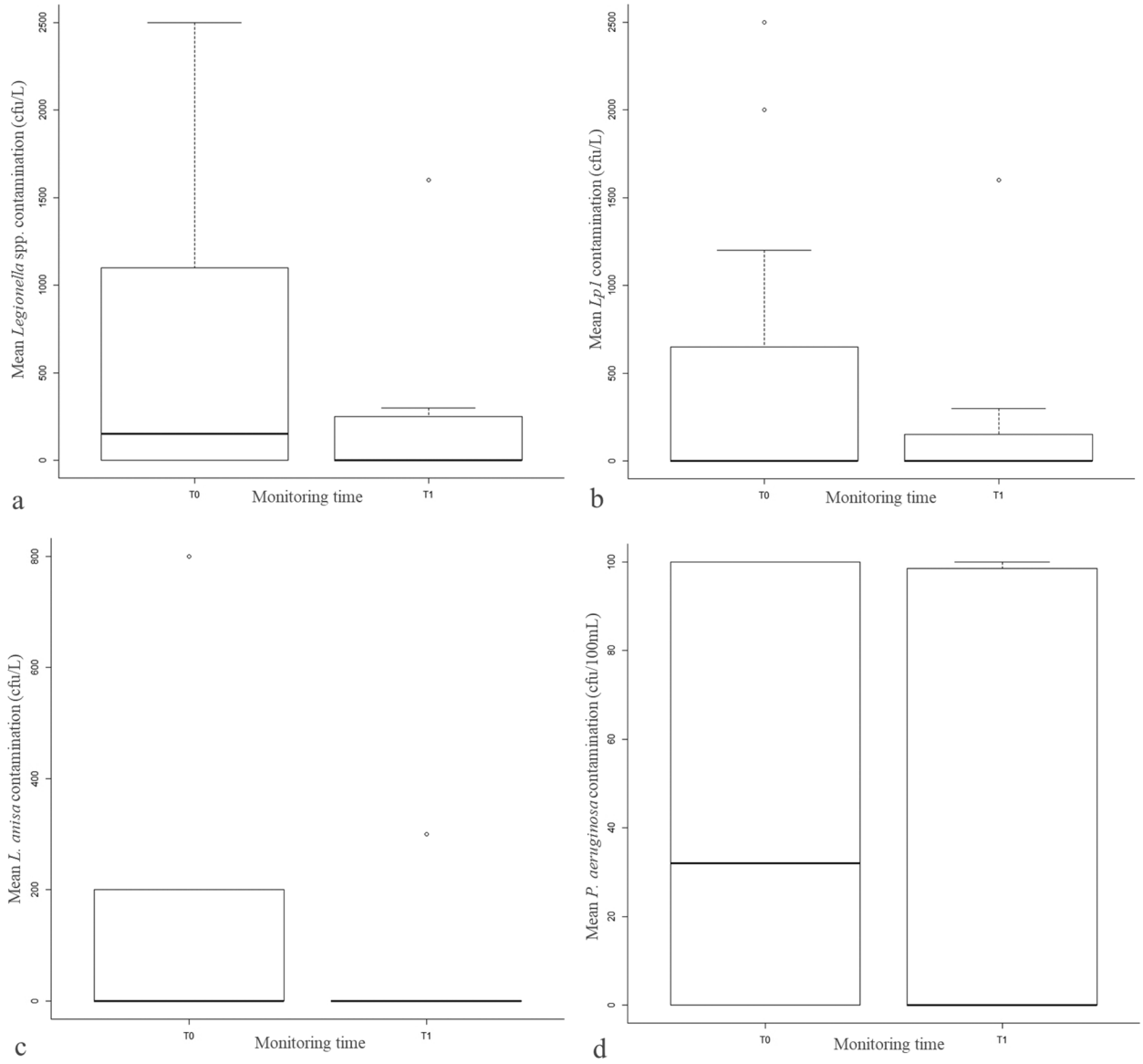


Figure 2. Type B DUWLs cup filler mean contamination: a) *Legionella* spp., b) *Lp*1, c) *L. anisa* and d) *P. aeruginosa*. Contamination comparison between T0 and T1 on 12 Type B DUWLs remained positive. No significant differences were obtained. Detection limits were 100 ufc/L for *Legionella* and 0 cfu/100mL for *P. aeruginosa*.

devices and anti-backflow valves. Moreover, it is estimated that the occupational risk may affect 1 to 2 million healthcare staff worldwide [40], consequently for DUWLs we must develop a risk assessment plan, identifying the main risk factors that can influence water quality [18,32,41].

Generally, to reduce the microbiological risks correlated to DUWLs, routine prevention strategies must be applied, such as using anti-stagnation valves, flushing, supplying DUWLs with sterile water, antimicrobial filter installation and chemical disinfectants provided in continuous or in extraordinary shock treatment modality. All these suggestions associated to a strict compliance with component maintenance could lead to a DUWL microbial contamination control [42,43].

Despite *Legionella* being linked to biofilm growth and handpiece aerosols representing a potential source of infection in DUWLs, until now, it has not been directly regulated in European countries and the USA.

In Europe, the positions among countries with regard to DUWLs water quality are different. While acknowledging the risks represented by DUWLs for workers, no country has developed specific technical guidance concerning water quality. Some countries such as Sweden, the Netherlands, Germany, Ireland, Greece and Hungary have guidelines containing references to the role of flushing activities, the use of sterile water, or the application of continuous or shock chemical treatment. Regarding the microbiological

water quality, they use the heterotrophic bacteria count at 22°C. Other countries do not have any references to water quality or programs for its monitoring [27].

The problems have been emphasized during the SARS-CoV−2 pandemic, with the extended closure of dental units, often without the implementation of maintenance programs. Therefore, the ECDC and National Italian Institute of Health provided suggestions on how to approach dental unit water quality [30,31].

The Italian guidelines on surveillance and control of Legionellosis include dental units among the water distribution systems with a high risk of *Legionella* contamination [28,29], suggesting an important role of water risk assessment plans and surveillance programs once a year. Moreover, the Italian Legislative Decree 09.04.2008, no. 81 concerning the protection of health and safety in the workplace inserts *Legionella* in the level 2 of biological risk and requires *Legionella* surveillance in places where there is a risk for workers, in order to prevent and minimize exposure [44].

In 2020, a new European directive on water intended for human consumption mentions for the first time *Legionella* as a parameter to guarantee the safety of water consumption (Directive (EU) [45]). In Italy, the recent transposition of the European directive, legislative degree 18/2023 [46], requires more attention being paid to *Legionella* in several activities and buildings. In particular, in the recent 'Guidelines for risk assessment and management for water safety in distribution system interiors in priority and non-priority buildings and in some vessels in accordance with Directive (EU) ' Report ISTISTAN 22/32 (https://www.iss.it/rapporti-istisan), published by the National Institute of Health [47], dental units are classified among priority level B buildings. According to the guidelines, these facilities need to appoint a professional figure called a 'GIDI' (Gestore Idrico della Distribuzione Interna dell'acqua), to produce a water safety plan and to perform *Legionella* monitoring at least twice per year.

In this study, the monitoring of *P. aeruginosa* was added considering that it is harmful especially for immunocompromised patients [48]. It is isolated from 2.9 to 50% of water samples collected from dental unit waterlines, and from saliva ejector tubing [49–51]. Its presence could be connected to the retrocontamination inside the tubes, with consequent water stagnation and biofilm formation [52–54]. Moreover, in many studies, its ability to compete with *Legionell*a growth has been reported, with the risk of *Legionella* false negative results [5,52,55]. Although its role is widely recognized, the new European directive on drinking water, as well as the Italian transposition [46] removed it from the indicator parameters, leaving the possibility of testing for it dependent on the risk assessment conducted. This approach may limit its role also as an indicator for maintenance and cleaning procedures, with several implications for hygiene and safety, especially for dental staff and manufacturers.

In this study, a *Legionella* monitoring program was applied for the first time in an extended Dental clinic complex, in several dental unit departments, with maintenance and disinfection programs implemented during the pandemic and lockdown.

The novelty of this study and the results obtained, are firstly due to separately collecting and analysing several sampling points in DUWLs: dental handpieces, water bottles and cup filler. This approach might seem in contrast with the Italian guidelines recommendations, which require collecting a volume of 200 mL from each handpiece and mixing it with samples collected from a cup filler to reach a total volume of 1 L.

This proposed strategy does not permit to accurately identify the critical points present in the DUWLs and check compliance with the disinfection and maintenance program applied.

Of the 30 DUWLs monitored, almost half of them showed a high presence of both contaminants. These findings are in line with data already reported on *Legionella* and *P. aeruginosa* contamination in DUWLs [4,5,51].

In accordance with other studies on water distribution systems, the water input resulted free from contaminants compared to the water output [56]. Our experience suggests that even though the *Legionella* surveillance and control program was frequently applied in DUWLs, contamination control is often not achieved for several reasons.

Considering the water supply system, this study confirms the results obtained by [57], showing that the DUWL supplied by a double feed system, water bottle and municipal water represents a high risk for patient and dental staff [57]. In addition, some new risk factors were discovered during the study: first, the temperature of water, which was higher in Type B than in the other DUWL types, with values over the reference value (<20°C) [58]. Probably the low water flow caused by the narrowness of the pipes combined with the activation of the DUWLs electrical components (e.g. pressure and flow sensors) promote a water temperature increase of over 20–25°C, favoring microbiological proliferation [26,59].

We believe that the source of contamination in type B DUWLs could be explained by also considering several mechanical-functional factors. These

Table 2. Comparison of contaminations of both *Legionella* and *P. aeruginosa* during the monitoring time. The values in bold indicate the statistically significant differences.

	Mean contamination ± SE (Median (Interquartile range (IQR)))			Contamination comparison between monitoring time: percentage reduction and *p* value (Mann–Whitney test)		
Monitoring time	T0	T1	T2	T0 *vs* T1	T1 *vs* T2	T0 *vs* T2
Legionella spp.	608.33±253.33	208.33±131.11	<100	−59.54%	−100%	−100%
(cfu/L)	(150(0,1250))	(0(0,275))	(0(0,0))	$p = 0.224$	**$p = 0.015$**	**$p < 0.001$**
*Lp*1	491.67±258.33	183.33±131.91	<100	−42.53%	−100%	−100%
(cfu/L)	(0(0,925))	(0(0,175))	(0(0,0))	$p = 0.595$	**$p = 0.033$**	**$p = 0.015$**
L. anisa	116.67±67.23	25.00±25.00	<100	−90.63%	−100%	−100%
(cfu/L)	(0(0,200))	(0(0,0))	(0(0,0))	$p = 0.166$	$p = 0.317$	**$p = 0.033$**
P. aeruginosa	44.42±13.25	33.08±14.11	0.08±0.08	−43.29%	−99.75%	−99.86%
(cfu/100mL)	(32(0,100))	(0(0,99.25))	(0(0,0))	$p = 0.371$	$p = 0.103$	**$p = 0.006$**

dental chairs are supplied by a switch system that permits the water feed change. When the water bottle is empty, the switch button changes the water flow that is supplied by municipal water to the DUWLs. This means that it is possible to continuously change the DUWL environment, therefore the microorganisms, during this time, may be delivered from the external environment to the DUWLs, increasing biofilm formation. These findings could also explain the presence of *Legionella* and *P. aeruginosa* contamination found in the cordon handpieces, even if they were sterilized each day. The absence of *Legionella* and *P. aeruginosa* in the municipal water input analysed could support the hypothesis that, in the DUWLs, the bacterial microflora is inside and attached to the pipelines, as biofilm components.

The water bottles are not autoclavable due to their plastic material composition, and often if not fully used, they are not emptied and directly filled with water the next day. Therefore, the disinfection protocol applied is based on good cleaning procedures, using detergent.

Moreover, in the Type B DUWLs, the main contamination was present in the cup filler, and the swab samples performed in this tract of the pipelines showed a higher presence of biofilm. While the contamination found in the handpieces is easy to remove, after ordinary sanification protocols, it disappears.

The high contamination found was linked to the exclusion of this tract of pipelines from automatic and manual disinfection treatments provided in these DUWLs. The water bottle filled with demineralized water plus hydrogen peroxide solution (3% v/v), used for ordinary procedures, is connected only to the handpiece pipelines; therefore, the disinfection procedure does not involve the cup filler and the spittoon. The cup filler and spittoon water line are sanitized only during the ordinary sanification protocol applied by manufacturer's staff that occurs occasionally. Additionally, visual inspections and interviews with workers showed how these two lines are not flushed.

Our data showed how even though the ordinary sanification protocol is able to control general contamination, with effect on *L. anisa* (T1 monitoring), the presence of biofilm is associated with an observed lower reduction in *Lp*1 and *P. aeruginosa*, that remain in the cup filler pipelines.

During the extraordinary sanification, the high concentration of disinfectant in the DUWLS, and especially the increase in contact time between disinfectant and pipelines from 10 to 20 min, followed by rinsing and high flow into the pipeline of the cup filler, provides an effective reduction in contamination (T2). In line with previous studies, *Legionella*, as well as other bacteria during shock treatment, are able to become free living planktonic cells, with a release in the water or aerosol, becoming detectable in the water sample [60–63].

The increase in rinsing and flushing, combined with disinfection and component replacement (e.g. whole parts of cup filler pipelines, cordons and the retro-flux valves), leads to a complete removal of both contaminants.

The analysis of swab samples on filter pressure reducers, located under the dental chair, strictly after water input, confirms the role of this component in biofilm growth. Indeed, neither of them have been changed from the Type B DUWLs date of installation and the dental staff did not know of their existence.

The absence of contamination in Type A and Type C DUWLs could be explained considering their main characteristics. Type A is directly fed by the mains municipal water and the absence of a water bottle, means that there is no limit on the water volume or time used during the flushing. Type C did not show contamination due to the presence of water supply directly from autoclavable bottles, without the possibility of switching to the mains municipal water. Moreover, they are supplied by a continuous disinfection system, which through the water bottle feeds all the DUWL components, including the spittoon, handpieces and cup filler. These DUWLs do not show contamination still to this day. The low number of these types of DUWLs involved in the study cannot influence the results obtained, as they are also in line

with previous analyses performed on other dental chairs [57], and represent the real equipment present in the Dental complex.

The evaluation of microbiological contamination of DUWLs carried out in this study indicates how a low adherence to good practices contributes to lower water quality, as has been widely demonstrated by a recent meta-analysis study performed by Khajedzadeh et al. [32]. Moreover, the presence of the ST 59, an ST with low prevalence in Italy, as well as in the other countries (ESGLI database), shows how the DUWL's environment is critical for its characteristics, requiring more attention, especially during the sampling stage.

The distribution of STs in the environment, as well as in man-made water distribution systems, needs to be investigated considering the selective pressure induced by climate change, environmental stress and disinfection treatments [64]. These risk factors can induce resistance to routine antibiotics treatments. This becomes even more important in light of the absence of a standardized method to determine the antibiotic sensitivity of *Legionella* [65].

The limitation of this work is the lack of longitudinal sampling for monitoring the maintenance of water quality in the Dental complex. This was due to the need to quickly reopen the complex for care and clinical practices, and to the high cost of DUWL maintenance. We are planning to test the proposed new disinfection protocols and maintenance strategies implemented on a set of dental chairs (chosen ad hoc) in order to better investigate the microbial contamination found and implement further procedures.

Conclusions

Until now the dental unit environment was relatively unknown, and a lot of work needs to be done to combine the functionality, performance and health of dental staff and patients. Therefore, DUWL contamination can change in relation to the dental unit characteristics, not only in terms of water supply and good practices but also in relation to DUWL mechano-functional components (e.g. filters, valves and flow sensors).

The 'take-home messages' are as follows:

- Replace the filter pressure reducers under the dental chair at least every 6 months;
- Increase the daily flushing activity for components that are excluded by automatic flushing and disinfection systems;
- Choose DUWLs with water bottles that are able to reach the cup filler and spittoon as well, in order to avoid dead pipeline creation;
- Replace the parts of pipelines that are degraded and obstructed by biofilm or with low flow;
- Perform a microbial investigation collecting samples from handpieces, cup filler and water bottle, separately, to better understand the origin of contamination, assuring that all these components are linked to the main disinfection system. This activity should be performed at least every 6 months.

These findings suggest that to preserve the DUWL water quality and ensure their health and safety for patients and operators, all DUWLs need to be better understood and investigated. A multidisciplinary and integrative approach among manufacturers, dental staff and public health institutions needs to be promoted.

Abbreviations

DUWLs: dental unit waterlines; CDC: Centers for Disease Control; ADA: American Dental Association; HPCs: heterotrophic plate counts; cfu: colony forming units; *E. coli: Escherichia Coli; P. aeruginosa: Pseudomonas aeruginosa;* PTFE: polytetrafluoroethylene; GVPC: Glycine-PolymyxinB-Vancomycin-Cycloheximide; BCYE: Buffered Charcoal Yeast Extract; Cys+: with L-Cysteine; Cys-: without L-Cysteine; *Lp: Legionella pneumophila; Lp*1: *Legionella pneumophila* serogroup 1; n*Lp*: non-*L. pneumophila;* SBT: Sequence-Based Typing; *mip*: macrophage infectivity potentiator; SE: standard error;

Acknowledgments

The authors would like to thank Prof. Ada Dormi for her help in the statistical analysis and the students of the Biological Science degree: Rocco Caleca, Mattia Rizzoli and Eleonora Mezzopera, for their involvement in the sample analysis.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

F.M., S.C. and M.M. conceived the study, designed the experiments and wrote the paper. L.G. performed data analysis. F.M., M.M., M.R.P., C.D. and L.G. performed sample collection and experiments. All authors have read and agreed to the published version of the manuscript.

ORCID

Federica Marino http://orcid.org/0009-0007-8824-9423
Marta Mazzotta http://orcid.org/0000-0002-6966-3115

Maria Rosaria Pascale http://orcid.org/0000-0001-6333-3492
Carlo Derelitto http://orcid.org/0000-0002-5240-8425
Luna Girolamini http://orcid.org/0000-0001-5916-9634
Sandra Cristino http://orcid.org/0000-0002-5084-2615

References

[1] Ji XY, Fei CN, Zhang Y, et al. Evaluation of bacterial contamination of dental unit waterlines and use of a newly designed measurement device to assess retraction of a dental chair unit. Int Dent J. 2016;66:208–214. doi: 10.1111/idj.12225
[2] Volgenant CMC, Persoon IF. Microbial water quality management of dental unit water lines at a dental school. J Hosp Infect. 2019;103:e115–e117. doi: 10.1016/j.jhin.2018.11.002
[3] Baudet A, Lizon J, Martrette JM, et al. Efficacy of BRS® and Alpron®/Bilpron® disinfectants for dental unit waterlines: a six-year study. Int J Environ Res Public Health. 2020;17. 1–9. doi:10.3390/ijerph17082634
[4] Spagnolo AM, Sartini M, Cristina ML. Microbial contamination of dental unit waterlines and potential risk of infection: a narrative review. Pathogens. 2020;9:1–11. doi: 10.3390/pathogens9080651
[5] Tuvo B, Totaro M, Cristina ML, et al. Prevention and control of Legionella and Pseudomonas spp. Colonization in dental units. Pathogens. 2020;9:1–12. DOI:10.3390/pathogens9040305
[6] Coleman DC, O'Donnell MJ, Shore AC, et al. Biofilm problems in dental unit water systems and its practical control. J Appl Microbiol. 2009;106:1424–1437. doi: 10.1111/j.1365-2672.2008.04100.x
[7] Odonnell MJ, Boyle MA, Russell RJ, et al. Management of dental unit waterline biofilms in the 21st century. Future Microbiol. 2011;6:1209–1226. doi: 10.2217/fmb.11.104
[8] Luo J, Porteous N, Sun Y. Rechargeable biofilm-controlling tubing materials for use in dental unit water lines. ACS Appl Mater Interfaces. 2011;3:2895–2902. doi: 10.1021/am200576q
[9] Kettering JD, Stephens JA, Muñoz-Viveros CA, et al. Reducing bacterial counts in dental unit waterlines: tap water vs. distilled water. J Contemp Dent Pract. 2002;3:1–11. doi: 10.5005/jcdp-3-3-1
[10] Liaqat I, Sabri AN. Effect of biocides on biofilm bacteria from dental unit water lines. Curr Microbiol. 2008;56:619–624. doi: 10.1007/s00284-008-9136-6
[11] Stone ME, Kuehne JC, Cohen ME, et al. Effect of iodine on mercury concentrations in dental-unit wastewater. Dent Mater. 2006;22:119–124. doi: 10.1016/j.dental.2005.04.009
[12] Costa D, Mercier A, Gravouil K, et al. Pyrosequencing analysis of bacterial diversity in dental unit waterlines. Water Res. 2015;81:223–231. DOI:10.1016/j.watres.2015.05.065
[13] Szymańska J, Sitkowska J, Dutkiewicz J. Microbial contamination of dental unit waterlines. Ann Agric Env Med. 2008;15. 173–179. doi:10.1080/02615470120107068
[14] Blake GC. The incidence and control of bacterial infection in dental spray reservoirs. Br Dent J. 1963;115:412–416.
[15] Fujita M, Mashima I, Nakazawa F. Monitoring the decontamination efficacy of the novel Poseidon-S disinfectant system in dental unit water lines. J Microbiol Immunol Infect. 2015;50:270–276. doi: 10.1016/j.jmii.2015.05.006
[16] Kohn WG, Harte JA, Malvitz DM, et al. Guidelines for infection control in dental health care settings - 2003. 2004. doi:10.14219/jada.archive.2004.0019
[17] Cristina M, Spagnolo AM, Sartini M, et al. Evaluation of the risk of infection through exposure to aerosols and spatters in dentistry. Am J Infect Control. 2008;36:304–307. DOI:10.1016/j.ajic.2007.07.019
[18] Lauritano D, Nardone M, Gaudio RM, et al. Risk assessment of colonization of *Legionella* spp. in dental unit waterlines. Oral Implantol. 2017;10:283–288. doi: 10.11138/orl/2017.10.3.283
[19] Pankhurst CL. Risk assessment of dental unit waterline contamination. Prim Dent Care. 2003;10. 5–10. doi:10.1308/135576103322504030
[20] Castellano Realpe OJ, Gutiérrez JC, Sierra DA, et al. Dental unit waterlines in quito and caracas contaminated with non tuberculous mycobacteria: a potential health risk in dental practice. Int J Environ Res Public Health. 2020;17:1–11. DOI:10.3390/ijerph17072348
[21] Cicciù M. Water contamination risks at the dental clinic. Biology (Basel). 2020;9:12–16. doi: 10.3390/biology9030043
[22] Pankhurst CL, Coulter WA. Do contaminated dental unit waterlines pose a risk of infection? J Dent. 2007;35:712–720. doi: 10.1016/j.jdent.2007.06.002
[23] Ricci ML, Fontana S, Pinci F, et al. Pneumonia associated with a dental unit waterline. Lancet. 2012;379:684. doi:10.1016/S0140-6736(12)60074-9
[24] Atlas RM, Williams JF, Huntington MK. *Legionella* contamination of dental-unit waters. Appl Environ Microbiol. 1995;61:1208–1213. doi: 10.1128/aem.61.4.1208-1213.1995
[25] Schönning C, Jernberg C, Klingenberg D, et al. Legionellosis acquired through a dental unit: a case study. J Hosp Infect. 2017;96:89–92. DOI:10.1016/j.jhin.2017.01.009
[26] Walker JT, Marsh PD. Microbial biofilm formation in DUWS and their control using disinfectants. J Dent. 2007;35:721–730. doi: 10.1016/j.jdent.2007.07.005
[27] ECDC. Infection prevention and control in dentistry. 2023. Available from: https://www.ecdc.europa.eu/en/publications-data/directory-guidance-prevention-and-control/core-requirements-healthcare-settings-1 [Consultato aprile 26, 2023].
[28] Italian National Institute of Health. Italian guidelines for prevention and control of legionellosis. 2015. Available from: http://www.salute.gov.it/imgs/C17pubblicazioni2362allegato.pdf
[29] Emilia-Romagna Region (2017). Regional guidelines for surveillance and control of legionellosis. Delibera Della Giunta Regionale 12 Giugno 2017, N. 2017; 828.
[30] ESGLI. ESGLI Guidance for managing Legionella in dental practises during the COVID-19 pandemic. 2020. 1–5. Available from: https://www.escmid.org/fileadmin/src/media/PDFs/3Research_Projects/ESGLI/COVId_buidling_water_system_guidance_27_3_20_v4_DS_pk.pdf
[31] Ricci M, Rota M, Scaturro M, et al. Indications for the prevention of *Legionella* risk in dental unit waterline

during COVID-19 pandemic. Version May. 2020;17:1–9. *Rapp. ISS COVID-19 n. 27/2020*.
[32] Khajezadeh M, Mohseni F, Khaledi A, et al. Contamination of dental unit water lines (DUWL) with *Legionella pneumophila* and *Pseudomonas aeruginosa*; a Middle East systematic review and meta-analysis. Eur J Microbiol Immunol. 2022;12:93–99. doi: 10.1556/1886.2022.00023
[33] EN ISO 19458:2006. Water quality—sampling for microbiological analysis. 2006.
[34] Legislative decree No. 31, Feb 2001 Implementation of the water quality directive 98/83/EC relative to water quality intended for human consumption. 2001. pp. 1–21. Gazzetta Ufficiale n.52 of March 3rd 2001, supplemento ordinario n.414. Gazzetta Ufficiale n.52 of March 3rd 2001, supplemento ordinario n.414: https://www.gazzettaufficiale.it/eli/id/2001/03/03/001G0074/sg
[35] ISO 11731:2017. Water quality — Enumeration of Legionella. 2017.
[36] Pascale MR, Mazzotta M, Salaris S, et al. Evaluation of MALDI–TOF mass spectrometry in diagnostic and environmental surveillance of *Legionella* species: a comparison with culture and *Mip*-Gene sequencing technique. Front Microbiol. 2020;11:1–12. doi:10.3389/fmicb.2020.589369
[37] Gaia V, Fry NK, Afshar B, et al. Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of Legionella pneumophila. J Clin Microbiol. 2005;43:2047–2052. DOI:10.1128/JCM.43.5.2047-2052.2005
[38] Ratzow S, Gaia V, Helbig JH, et al. Addition of neuA, the gene encoding N-acylneuraminate cytidylyl transferase, increases the discriminatory ability of the consensus sequence-based scheme for typing *Legionella pneumophila* serogroup 1 strains. J Clin Microbiol. 2007;45. 1965–1968. doi:10.1128/JCM.00261-07
[39] UNI EN ISO 16266:2008. Water quality—detection and enumeration of Pseudomonas aeruginosa—Method by Membrane Filtration. 2008.
[40] Petti S, Vitali M. Occupational risk for *Legionella* infection among dental healthcare workers: meta-analysis in occupational epidemiology. BMJ Open. 2017;7. 1–10. doi:10.1136/bmjopen-2016-015374
[41] Mills SE. The dental unit waterline controversy: defusing the myths, defining the solutions. J Am Dent Assoc. 2000;131:1427–1441. doi: 10.14219/jada.archive.2000.0054
[42] Association AD. ADA statement on dental unit waterlines. J Am Dent Assoc. 1996;127(2):185–186.
[43] Department of Health (DH), England. Health Technical Memorandum 01-05: Decontamination in primary care dental practices. 2013, 1–86.
[44] Legislative Decree No. 81. Implementation of article 1 of the Law of 3 August 2007, n. 123, concerning the protection of health and safety in the workplace. OJ. General Series n.101 of 30-04-2008—Suppl. Ordinary n. 108 April 9 2008 http://www.normattiva.it/atto/caricaDettaglioAtto?atto.dataPubblicazioneGazzetta=2008-04-30&atto.codiceRedazionale=008G0104¤tPage=1
[45] Directive (EU) 2020/2184. Directive (EU) 2020/2184 of the European Parliament and of the Council of 16 December 2020 on the quality of water intended for human consumption, 431/1. 2020. Gazzetta Ufficiale dell'Unione Europea del 23.12.2020.
[46] Legislative Decree No. 18, Feb 23. Implementation of Directive (EU) 2020/2184 of the Parliament European Parliament and of the Council of 16 December 2020 on the the quality of water intended for human consumption. Gazzetta Ufficiale - serie Generale. 2023;55:3–81. https://www.gazzettaufficiale.it/atto/serie_generale/caricaDettaglioAtto/originario?atto.dataPubblicazioneGazzetta=2023-03-06&atto.codiceRedazionale=23G00025&elenco30giorni=false
[47] Istituto Superiore di Sanità. Guidelines for the assessment and management of risk associated to water safety in internal plumbing systems in priority and non-priority buildings and in certain vessel according to Directive (EU) 2020/2184. Ad hoc working group on water safety in intern internal water distribution systems in buildings and certain ships 2022. Rapporti ISTISAN 22/32 (in Italian). 2022;xiv:161.
[48] Araujo M, Andreana S. Risk and prevention of transmission of infectious diseases in dentistry. Quintessence Int. 2002;33:376–382.
[49] Barbeau J, Tanguay R, Faucher E, et al. Multiparametric analysis of waterline contamination in dental units. Appl Environ Microbiol. 1996;62:3954–3959. DOI:10.1128/aem.62.11.3954-3959.1996
[50] Barbeau J, Ten Bokum L, Gauthier C, et al. Cross-contamination potential of saliva ejectors used in dentistry. J Hosp Infect. 1998;40:303–311. doi: 10.1016/S0195-6701(98)90308-5
[51] Barben J, Schmid J. Dental units as infection sources of *Pseudomonas aeruginosa*. Eur Respir J. 2008;32. 1122–1123. doi:10.1183/09031936.00072808
[52] Kimura S, Tateda K, Ishii Y, et al. *Pseudomonas aeruginosa* Las quorum sensing autoinducer suppresses growth and biofilm production in *Legionella* species. Microbiology. 2009;155:1934–1939. DOI:10.1099/mic.0.026641-0
[53] Ouellet MM, Leduc A, Nadeau C, et al. *Pseudomonas aeruginosa* isolates from dental unit waterlines can be divided in two distinct groups, including one displaying phenotypes similar to isolates from cystic fibrosis patients. Front Microbiol. 2014;5:1–11. doi: 10.3389/fmicb.2014.00802
[54] Tuttlebee CM, O'Donnell MJ, Keane CT, et al. Effective control of dental chair unit waterline biofilm and marked reduction of bacterial contamination of output water using two peroxide-based disinfectants. J Hosp Infect. 2002;52:192–205. DOI:10.1053/jhin.2002.1282
[55] Walker JT, Bradshaw DJ, Fulford MR, et al. 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;69:3327–3332. doi: 10.1128/AEM.69.6.3327-3332.2003
[56] Ryan MP, Pembroke JT, Adley CC. *Ralstonia pickettii*: a persistent Gram-negative nosocomial infectious organism. J Hosp Infect. 2006;62. 278–284. doi:10.1016/j.jhin.2005.08.015
[57] Lizzadro J, Mazzotta M, Girolamini L, et al. Comparison between two types of dental unit waterlines: how evaluation of microbiological contamination can support risk containment. Int J Environ Res Public Health. 2019;16:1–14. doi: 10.3390/ijerph16030328
[58] World Health Organization. Guidelines for drinking-water quality; 2011. pp. 876–883. doi:10.1007/978-1-4020-4410-6_184

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

[60] Abu Khweek A, Amer AO. Factors mediating environmental biofilm formation by *Legionella pneumophila*. Front Cell Infect Microbiol. 2018;8:1–10. doi: 10.3389/fcimb.2018.00038

[61] Assaidi A, Ellouali M, Latrache H, et al. Role of biofilms in the survival of *Legionella pneumophila* to sodium chloride treatment. Iran J Microbiol. 2021;13:488–494. doi: 10.18502/ijm.v13i4.6973

[62] Berlanga M, Guerrero R. Living together in biofilms: the microbial cell factory and its biotechnological implications. Microb Cell Fact. 2016;15:1–11. doi: 10.1186/s12934-016-0569-5

[63] Buckingham-Meyer K, Miller LA, Parker AE, et al. Harvesting and disaggregation: an overlooked step in biofilm methods research. J Vis Exp. 2022;1–11. doi:10.3791/62390-v

[64] Mazzotta M, Salaris S, Pascale MR, et al. Occurrence of *Legionella* spp. In man-made water sources: isolates distribution and phylogenetic characterization in the Emilia-Romagna region. Pathogens. 2021;10:552. doi: 10.3390/pathogens10050552

[65] Portal E, Descours G, Ginevra C, et al. *Legionella* antibiotic susceptibility testing: is it time for international standardization and evidence-based guidance? J Antimicrob Chemother. 2021;76:1113–1116. doi:10.1093/jac/dkab027