RESEARCH ARTICLE

Efficacy of dental unit disinfectants against Candida spp. and Hartmannella vermiformis

Vanessa Barbot¹, Damien Costa¹, Marie Deborde²,³ & Christine Imbert¹,³

¹ Laboratoire d’Ecologie et de Biologie des Interactions, Université de Poitiers, UMR CNRS 7267, Poitiers Cedex, France
² Institut de Chimie des Milieux et des Matériaux, Université de Poitiers, UMR CNRS 7285, Poitiers Cedex, France
³ Faculté de Médecine et de Pharmacie, Université de Poitiers, Poitiers Cedex, France

This paper describes the antagonistic activity of free-living amoeba on the efficacy of the disinfectant Oxygenal 6© to treat Candida colonizations in dental unit waterlines. This highlights the need to include the determination of the presence of free-living amoeba in such systems.

Keywords
yeasts; free-living amoebae; tap water; biofilm; disinfection.

Correspondence
Vanessa Barbot, Laboratoire d’Ecologie et de Biologie des Interactions, UMR CNRS 7267, Equipe Microbiologie de l’Eau, 6 Rue de la Milétrie, BP 199, 86034 Poitiers Cedex, France.
Tel.: 0 33 549443747
fax: 0 33 549443908
e-mail: barbot.vanessa@hotmail.fr

Received 17 October 2013; revised 19 December 2013; accepted 21 December 2013. Final version published online 31 January 2014.
doi:10.1111/2049-632X.12127

Editor: Patrick van Dijck

Abstract
Human oral commensal Candida yeasts, as well as environmental free-living amoebae (FLA) such as Hartmannella, are known to be direct or indirect human pathogens. These microorganisms may be isolated from dental unit waterlines (DUWL), because of contamination coming from the tap water and/or a patient’s mouth. This study compared the efficacy of commonly used DUWL disinfectants (chlorine, H₂O₂, and Oxygenal 6©) against three species of Candida (C. albicans, C. glabrata, and C. parapsilosis) and one FLA species (H. vermiformis), growing either as single or as mixed biofilms in tap water. Results showed variable efficacies: H₂O₂ had no significant activity, while chlorine was effective but only at the highest doses tested, probably not compatible with DUWL uses. Oxygenal 6© was the most efficacious in preventing the growth of yeasts in tap water. However, in the presence of FLA, Oxygenal 6© displayed a reduced antimicrobial activity against sessile C. albicans. In conclusion, none of the tested disinfectants could eradicate yeasts or FLA. Moreover, the antiyeast activity of Oxygenal 6© was reduced in the presence of FLA. Both sessile or planktonic and mixed or single-species conditions should be considered when evaluating the activity of disinfectants for DUWL maintenance. This study also highlighted that FLA should be included in the testing protocols.

Introduction
Water, which acts both as a coolant for a range of instruments and as an irrigant during dental treatments, is delivered to the dental unit chairs by a network of interconnected narrow-bore plastic tubes called dental unit waterlines (DUWL). The structure of DUWL (plastic tubes, high surface area/water volume ratio, laminar flow, stagnation for extended periods of time, an average temperature of 23°C) encourages microbial proliferation and biofilm formation (Shearer, 1996; Williams et al., 1996; ADA, 1999; Pankhurst, 2003; Szymanska, 2003; Kumar et al., 2010). Biofilms represent a reservoir of microorganisms potentially pathogenic for humans, and consequently, the water delivered to patients may often be contaminated with various microorganisms (bacteria, fungi, protozoa, or viruses) (Walker et al., 2000; Barben et al., 2009). The presence of high levels of microbial contamination may cause a health problem for dentists and patients regularly exposed to these water and aerosols generated by dental equipments, especially for the frailest persons (e.g. elderly people, smokers, HIV+ or cancer patients, people with diabetes, alcoholism) (Coleman et al., 2009; O’Donnell et al., 2011; Barbot et al., 2012a, b).

Microbial contamination of DUWL, known since the sixties (Blake, 1963), originates predominantly from two sources, one of which being mains water (Walker et al., 2000; Szymańska et al., 2008). It can also be the result of a suck-back of microorganisms and saliva from the oral cavity of patients that occurs in the case of an absence or more likely a dysfunction of the antiretraction valves that normally limit re-aspiration of fluid from the oral cavity (Bagga et al., 1984; Kumar et al., 2010). Candida species are commonly regarded as normal constituents of the mucocutaneous microbial communities in healthy humans but they are also considered important...
opportunistic fungal pathogens, causing superficial or systemic infections in immunocompromised patients (Odds, 1988; Hube, 2004). In oral cavity, Candida yeasts contribute to the dental plaque and are also implicated in single-species biofilms developed on dentures, responsible for candidiasis (Cannon et al., 1995). These yeasts have already been isolated in the water from DUWL with saliva traces and other microorganisms commonly found in oral cavity (Walker et al., 2000; Szymaniska, 2005). They may colonize DUWL, grow into a polymicrobial biofilm, disseminate in the water following detachment of sessile yeasts and may be present, mixed with saliva, in water and aerosols generated by dental instruments. The presence of low concentrations of saliva (2% v/v) has been shown to promote Candida spp. survival and proliferation in tap water (Barbot et al., 2011), which may increase the infectious risk associated with dental unit water.

Microorganisms isolated from DUWL can also originate from the main incoming water supply. Free-living amoebae (FLA) such as Hartmannella spp. are ubiquitous in nature and widely distributed (Inoue et al., 1998). These protists are also commonly isolated from recreational water systems and DUWL (Michel & Just, 1984; Barbeau & Buchler, 2001; Kuiper et al., 2006). FLA are considered as opportunistic pathogens for humans. They can represent a rare but serious risk to human health as direct causative agents (Aitken et al., 1996; Kinnear, 2003), but may mainly serve as vector for other microorganisms as it is well known for Legionella (Greub & Raoult, 2003; Kuiper et al., 2004).

Different chemical or nonchemical methods may be used to fight microorganisms associated with DUWL water, such as antiretraction valves, flushing, or disinfectants (Moncarz et al., 2002; Berluti et al., 2003; Schel et al., 2006; O'Donnell et al., 2011; Barbot et al., 2012a, b).

Chemical treatments commonly used by dentists are usually evaluated against bacteria and sometimes yeasts. However, their antiamoebae activity is rarely investigated. The sensitivity and behavior of FLA to treatments cannot be generalized to that of bacteria. Therefore, it seems important to investigate the activity of disinfectants against FLA, especially as these protists are able to internalize bacteria, and thus, amoebae resistance could result in the failure of treatments on bacteria and yeasts (Greub & Raoult, 2003; Kuiper et al., 2004). The aim of this study was to evaluate the antimicrobial activity of three common DUWL disinfectants: chlorine (Karpay et al., 1999), hydrogen peroxide (Linger et al., 2001), and Oxigenal 6C® (Szymaniska, 2006), against three species of Candida and one species of FLA (H. vermiformis), growing as single or mixed planktonic cultures or biofilms in tap water, using experimental conditions mimicking DUWL. Their antimicrobial activity was investigated against both planktonic and sessile yeasts to study experimental conditions as close as possible to those found in DUWL.

Materials and methods

Culture medium

Whole unstimulated saliva was collected on ice from 11 healthy adult volunteers who rinsed their mouths gently with water before sampling to decrease bacterial contamination (Barbot et al., 2011, 2012a, b). Saliva was then pooled and centrifuged (15 min, 3000 g, 4 °C). The resulting supernatant was filtered through a 0.22-µm membrane and stored at −80 °C until used.

Tap water was filtered through a 0.22-µm membrane. It displayed a chlorine concentration < 0.04 µg mL⁻¹ (diethyl-p-phenyldiamine method, APHA, 2000), which would be too low to modulate microorganisms survival. The pH of tap water with or without saliva was approximately 7.7 (Barbot et al., 2011).

Culture of microorganisms

Three fungal species were studied: C. albicans (strain ATCC 31513), C. glabrata (IHEM 9556), and C. parapsilosis (ATCC 22019). Candida species were mostly observed as yeasts forms throughout the study, whereas mycelial forms were rarely produced under the experimental conditions used. Yeasts were cultured on Sabouraud dextrose agar plates at 20 °C for 48 h. A yeast suspension (5 × 10⁶ cells mL⁻¹) was then prepared and incubated in filtered tap water supplemented with a low saliva concentration (2%, v/v), at 20 °C for 360 h, in 96-well microtiter plates. The saliva concentration was chosen because of our previous study which showed an increased survival of Candida in the presence of 1–20% (v/v) (Barbot et al., 2011). Moreover, other authors report that oral fluid sucked back could be up to 1 mL at the level of dental unit handpieces (Bagga et al., 1984; Pankhurst, 2003).

Hartmannella vermiformis (ATCC 50802) was cultured in 75-cm² culture flasks containing PYNFH medium (modified ATCC medium 1034) supplemented with 10% (v/v) of fetal calf serum and antibiotics (streptomycin 2 µg mL⁻¹, penicillin G 500 U mL⁻¹, and gentamicin 4 µg mL⁻¹) (Barbot et al., 2012a, b). Cultures were incubated at 20 °C for 5 days. An amoebae suspension was then prepared by discarding the culture medium, scraping and centrifuging (7 min, 300 g) adherent cells. For the experiments, amoebae were used at a final concentration of 5 × 10⁶ cells mL⁻¹ and were incubated in filtered tap water supplemented with a low saliva concentration (2%, v/v), at 20 °C for 360 h, in 96-well microtiter plates.

Co-culture experiments with amoebae and C. albicans yeasts were also performed, as previously described (Barbot et al., 2012a, b). The multiplicity of infection (MOI) was 1. Amoebae suspension was distributed into wells of a 96-well microtiter plates and incubated for 2 h at 20 °C. Yeasts (v/v) and/or saliva (2%, v/v) were then added and co-cultures were followed up over a time period of 360 h at 20 °C.

Chemical treatments and microorganisms viability

Disinfection experiments were performed on yeasts, FLA, and co-cultures incubated for different times (0, 1, 24, 48, 72, 168, and 360 h) in separate wells in order to study the antimicrobial activity on both planktonic (1 h) and sessile (24–360 h) yeasts. A preliminary study based on both measurements of metabolic activity and scanning electronic microscopy (SEM) showed that the formation of Candida...
spp. biofilm started from the first hours and then increased slowly due to the poor nutritional conditions (data not shown). So, after 1–360 h of incubation in water, microorganisms were treated with different doses of disinfectants: 3–118 μg mL⁻¹ of chlorine (NaOCl, from a solution of 10–15% (v/v) available chlorine, Sigma), 700–9000 μg mL⁻¹ of hydrogen peroxide (H₂O₂, from a solution of 3% (w/v) in distilled water, Sigma), or 500–20 000 μg mL⁻¹ of Oxygenal 6© (from a solution containing 6% (w/v) of H₂O₂ and silver ions, KaVo®). In the case of co-cultures, only the efficacy of Oxygenal 6© was tested (500–20 000 μg mL⁻¹).

Each microbial culture of single species or co-cultures was diluted (1 : 10 and 1 : 100) in fresh tap water 15 min after disinfectant addition. The yeast viability was evaluated by plating 100 μL of diluted suspensions in duplicate on Sabouraud dextrose agar plates containing chloramphenicol. Each experiment was carried out at least twice on different days. Yeast colony-forming units (CFU) were enumerated after 48 h at 37 °C, and results were expressed as survival percentages, calculated as follows: (log CFU mL⁻¹ of sample)/(log CFU mL⁻¹ of inoculum at 0 h) × 100. On the other hand, diluted amoebae suspensions were further diluted (1 : 2) in Trypan Blue (1/400© in distilled water) to evaluate amoebae viability. Trypan Blue stained dead cells in blue, whereas living cells remained uncolored. Total numbers of amoebae (colored and uncolored) were counted in KOVA© grids (Hycore Biomedical Inc.). Each sample was counted in triplicates. Each experiment was carried out at least twice on different days. Results were expressed as survival percentages, calculated as follows: (log amoebae mL⁻¹ of sample)/(log amoebae mL⁻¹ of inoculum at 0 h) × 100.

Statistical analysis
The nonparametric test of Wilcoxon was conducted using Stata® 9.2 to determine the statistical differences between groups.

Results and discussion
Water supplemented with saliva at 0% or 2% (v/v) was still a poor culture media which made yeast filamentation difficult. This explains at least partially that the biofilms obtained were mainly composed of yeast forms and did not have the regular architecture in two distinct layers (a thin basal region mainly composed of yeast forms and an overlying more open hyphal layer) (Douglas, 2003) found in C. albicans biofilms produced in rich culture media, such as YNB (yeast nitrogen base) or RPMI 1640 (Roswell Park Memorial Institute). Moreover, the temperature at which the experiments were carried out (20 °C) could not support filamentation. The efficacy of disinfectants against Candida spp. and H. vermiformis cultured in tap water was studied at both 20 and 27 °C as most DUWL temperatures ranged between these values (Pankhurst, 2003). Because the results obtained were comparable (P > 0.05) regardless of the temperature of incubation (data not shown), only the results obtained at 20 °C are presented herein.

Candida yeasts were studied because of their high occurrence in oral cavity and thus their possible aspiration in DUWL along with saliva and sometimes blood (Cannon et al., 1995; Walker et al., 2000). As previously shown, saliva addition in tap water would provide some essential components to promote Candida survival throughout the 360 h of the study (Barbot et al., 2011). Actually, all yeasts controls cultured in the presence of saliva (named ‘0  μg mL⁻¹ on graphs) were able to maintain a rather stable concentration throughout the experiment, about log 5 CFU mL⁻¹, corresponding to the initial inoculum (% survival ≥ 100%) (Figs 1–4). Moreover, a weak proliferation was observed over time for C. parapsilosis: A survival of 120% was obtained after 360 h in tap water (Figs 1c, 2c and 3c). The FLA survival was previously shown not to be influenced by saliva presence (Barbot et al., 2012a, b). Results showed that H. vermiformis was able to survive in tap water during the 360 h of the study, but its concentration decreased slowly over time: Only 40–60% of amoebae survived after 360 h in tap water (Figs 1d, 2d and 3d).

Chlorine treatment
When used at the highest dose (118 μg mL⁻¹), chlorine completely inhibited the growth of the three tested species of Candida and significantly reduced the growth of FLA (P < 0.0012), regardless of their maturation status before the treatment (1 h to 15 days) or their planktonic or sessile status (Fig. 1). After either 1 or 24 h in tap water with saliva (2% v/v), the treatment with 3 or 6 μg mL⁻¹ chlorine dose induced a slight decrease in C. albicans concentration (Fig. 1a). A stronger effect was observed using a chlorine dose of 12 μg mL⁻¹, regardless of the age of the Candida biofilms: Survival of yeasts was below 38% after 1–168 h of incubation and 62% after 360 h. Moreover, using chlorine at 26 μg mL⁻¹, yeasts incubated up to 168 h before the treatment were completely killed. A low survival (20%) was observed when this treatment was performed on the oldest yeasts (360 h). After a few hours of incubation, Candida spp. started to form a biofilm, thereby acquiring resistance factors. At 360 h of incubation, yeasts were included into a mature biofilm and this status certainly explains the increased resistance of yeasts to chlorine. The greater efficacy of chlorine on planktonic compared to sessile microorganisms has been widely shown with other microbial species (Karpay et al., 1999; Steed & Falkingham, 2006; Liu et al., 2011). Only concentrations of 62 and 118 μg mL⁻¹ of chlorine were shown to be completely effective against C. albicans whatever their age at the time of the treatment, preventing any growth of yeasts (Fig. 1a). Comparable results were obtained with C. glabrata (Fig. 1b). For this species, yeasts were always able to survive to a chlorine treatment used at 3 or 6 μg mL⁻¹, whereas 12 and 26 μg mL⁻¹ chlorine doses were effective on yeasts incubated up to 168 h before disinfectant addition, and only 62 and 118 μg mL⁻¹ chlorine doses were able to completely kill the yeasts regardless of their maturation status before treatment (P < 0.0001) (Fig. 1b). Candida parapsilosis was the most resistant fungal species to chlorine as a dose of...
Fig. 1 Efficacy of chlorine (NaOCl) against Candida albicans (a), C. glabrata (b), C. parapsilosis (c), or Hartmannella vermiformis (d). Yeasts or amoebae were cultured in tap water with 2% of saliva (v/v) at 20 °C for an incubation time of 0–360 h and treated with chlorine (0 to 118 μg mL⁻¹); '0 μg mL⁻¹' corresponds to control untreated yeasts or amoebae. Results are expressed as survival percentage of yeasts or amoebae.

Fig. 2 Efficacy of hydrogen peroxide (H₂O₂) against Candida albicans (a), C. glabrata (b), C. parapsilosis (c), or Hartmannella vermiformis (d). Yeasts or amoebae were cultured in tap water with 2% of saliva (v/v) at 20 °C for an incubation time of 0–360 h and treated with H₂O₂ (0 to 9000 μg mL⁻¹); '0 μg mL⁻¹' corresponds to control untreated yeasts or amoebae. Results are expressed as survival percentage of yeasts or amoebae.
6 μg mL⁻¹ was unable to kill yeasts whatever the age of the treated biofilm: ≥ 86% of yeasts (≤ 72 h old) were alive and cultivable, and even a high growth (≥ 118%) was observed for the oldest (≥ 168 h) (Fig. 1c). A treatment with 26 μg mL⁻¹ reduced the survival of C. parapsilosis to 20% and 38% after 168 and 360 h of incubation, respectively. Only 62 and 118 μg mL⁻¹ doses were able to kill yeasts regardless of their maturation age (P < 0.0001) (Fig. 1c).

Hartmannella vermiformis was more resistant to chlorine than any of the Candida spp. tested certainly due to its ability to form cysts with a double wall (Kuchta et al., 1993; Greub & Raoult, 2004). Chlorine doses of 3 and 6 μg mL⁻¹ had no effect, compared with control (‘0 μg mL⁻¹’, hatched). Using 12 to 62 μg mL⁻¹ doses, the survival of amoebae decreased below 42% up to 360 h of incubation (P < 0.0001) (Fig. 1d). A greater effect was observed using 118 μg mL⁻¹ of chlorine: Only 3–18% of FLA (incubated up to 360 h before treatment) survived. Taken together, our data show that chlorine was an effective disinfectant against Candida spp. and, to a lesser extent, against H. vermiformis, but only at doses > 26 μg mL⁻¹. Unfortunately, these high doses may not be compatible for routine decontamination of DUWL systems.

In other studies, amoebae were eradicated from water samples using 2–4 μg mL⁻¹ (or μg mL⁻¹) of chlorine for 1 h (Dupuy et al., 2011; Wang et al., 2012). However, this duration of treatment would not be compatible with a routine DUWL disinfection protocol. Furthermore, pipe materials and water age could influence the efficacy of disinfectant as chlorine decay was observed within a water aged for several days (Wang et al., 2012).

**Hydrogen peroxide treatment**

The antimicrobial activity of hydrogen peroxide, which is commonly found in water treatment protocols, was also tested with a contact time of 15 min (Fig. 2). The statistical
analysis showed that the antimicrobial activity of hydrogen peroxide on *C. albicans* (Fig. 2a) and *C. parapsilosis* (Fig. 2c) was lower than on *C. glabrata* (Fig. 2b). The weak or absence of activity of H\textsubscript{2}O\textsubscript{2} (% survival > 40%, regardless of the time of incubation before the treatment and the dose of disinfectant) against both planktonic (1 h incubation) and sessile (> 1 h) *Candida* spp. suggested that this disinfectant would not be appropriate to prevent or fight *Candida* spp. colonization into waterlines. The *C. parapsilosis* adaptation to the skin environment, which is less protective than mucosa, may contribute to its increased ability to withstand chemical treatments. H\textsubscript{2}O\textsubscript{2} used at 7000-9000 μg mL\textsuperscript{-1} significantly inhibited (*P < 0.0014*) *C. glabrata* biofilms older than 72 h (Fig. 2b), suggesting that yeasts from the most mature biofilms would be also the most vulnerable. At 168 h, *C. glabrata* survival was decreased between 72% (with 3000 μg mL\textsuperscript{-1} of H\textsubscript{2}O\textsubscript{2}) and 53% (with 9000 μg mL\textsuperscript{-1}), and at 360 h, all yeasts were killed using H\textsubscript{2}O\textsubscript{2} at 7500 or 9000 μg mL\textsuperscript{-1} (Fig. 2b). The reduced survival percentage of yeasts observed for oldest biofilms (168 and 360 h) may be explained by (1) the reduction in nutrients available in water, related to the consumption of both microorganisms and H\textsubscript{2}O\textsubscript{2}, and (2) a higher H\textsubscript{2}O\textsubscript{2} demand of the oldest culture. The presence of saliva certainly enriched the water and prevented the biocide action of H\textsubscript{2}O\textsubscript{2}. A recent study has shown that H\textsubscript{2}O\textsubscript{2} was active against *Candida* spp. biofilms, under the conditions of dialysis systems (which correspond to a poor medium) where a treatment of at least 15 min was needed to obtain ≥ 50% of yeasts reduction. 470 to 930 μg mL\textsuperscript{-1} could inhibit the biofilm formation and 1.87–3.75 g L\textsuperscript{-1} could kill biofilm cells (Pires et al., 2013).

These results suggested that amoebae were more susceptible to H\textsubscript{2}O\textsubscript{2} than *Candida* yeasts. However, H\textsubscript{2}O\textsubscript{2} was unable to completely kill amoebae (Fig. 2d). Even using H\textsubscript{2}O\textsubscript{2} at 9000 μg mL\textsuperscript{-1}, ≥ 10% of *H. vermiformis* were able to survive under the experimental conditions used. Microscopic observations showed that amoeba resistant to H\textsubscript{2}O\textsubscript{2} at 9000 μg mL\textsuperscript{-1} were in cyst form (data not shown). Cysts are well known to be less vulnerable than trophozoites (Kuchta et al., 1993; Greub & Raoult, 2004).

The mechanism of action of H\textsubscript{2}O\textsubscript{2} as a biocide is still not fully understood. It can cause DNA damages or oxidation of proteins and lipids of the cell membrane (Linley et al., 2012). In our experimental conditions, H\textsubscript{2}O\textsubscript{2} had no significant effect against *Candida* spp. and could not eradicate FLA. Higher doses or a longer time of treatment could be tested against these microorganisms but would probably not be compatible with a routine use for DUWL maintenance.

**Oxygenal 6© treatment**

Finally, Oxygenal 6© was tested, and results showed that this chemical was the most effective against *Candida* spp. (*P < 0.0001*) (Fig. 3). *Candida albicans* survival was ≤ 60% (Fig. 3a), regardless of Oxygenal 6© concentration (500–20 000 μg mL\textsuperscript{-1}), the planktonic (1 h) or biofilm (≥ 24 h) growth and the maturation status of biofilms (24–360 h). *Candida albicans* yeasts were all killed using 10 000 or 20 000 μg mL\textsuperscript{-1} of Oxygenal 6©. Similar activities were obtained against *C. glabrata* (Fig. 3b) with all yeasts killed even at low concentrations of Oxygenal 6© (500 μg mL\textsuperscript{-1}), regardless of the growth status and age of the treated yeasts. However, Oxygenal 6© was less effective against *C. parapsilosis* (*P < 0.0001*) (Fig. 3c). Planktonic *C. parapsilosis* (1 h) were killed by a dose of Oxygenal 6© > 500 μg mL\textsuperscript{-1}, but sessile yeasts (> 1 h) were less sensitive to Oxygenal 6©. A yeast survival of 86% (with Oxygenal 6© at 500 μg mL\textsuperscript{-1}) to 17% (with Oxygenal 6© at 20 000 μg mL\textsuperscript{-1}) was observed in the case of treatment of a 360-h-old biofilms. As previously observed, sessile yeasts were less sensitive to chemical treatment than planktonic yeasts.

The antimicrobial activity of Oxygenal 6© on *H. vermiformis* was lower compared to *Candida* spp. (Fig. 3d). Amoeba survival was close to that of the control (decreasing to ≥ 17%) even using 20 000 μg mL\textsuperscript{-1} of Oxygenal 6©, regardless of the time of incubation before treatment.

Using a similar dose of H\textsubscript{2}O\textsubscript{2} (final concentration) than that contained in H\textsubscript{2}O\textsubscript{2} solution or Oxygenal 6©, the microorganisms survival was much more decreased in the case of an Oxygenal 6© treatment. This difference in efficacy may be due to the presence of other components in Oxygenal 6© such as silver ions.

Finally, Oxygenal 6© was also tested against mixed planktonic suspensions and biofilms, including both *C. albicans* and *H. vermiformis* (Fig. 4). The activity of Oxygenal 6© on FLA was similar in both mono- and mixed cultures (data not shown). Regarding the anti-*Candida* activity, results clearly demonstrated that the total efficacy of Oxygenal 6© was maintained against yeasts in mixed planktonic suspensions (1 h); this short co-incubation time was not enough to permit internalization of yeasts by amoebae, as observed by SEM (data not shown), certainly contributing to the absence of any amoeba interference. However, a 24-h co-incubation was enough to permit the internalization, as shown in a previous study (Barbot et al., 2012a, b). Interestingly, co-incubation times ≥ 24 h caused a reduced activity of Oxygenal 6© against *C. albicans*: The percentages of yeast survival were between 37% and 63% for mixed biofilms, and between 0% and 60% for single-species biofilms, regardless of the maturation status of the treated biofilm and the disinfectant dose (Figs 3a and 4). These results highlighted the negative influence of amoebae on the anti-*Candida* activity of Oxygenal 6©. FLA, which represent a form of protection against disinfectants for internalized microorganisms, may furthermore increase the virulence of microorganisms and amoebae, as is well known, for example, in the case of *Legionella pneumophila* (Kuchta et al., 1993; Greub & Raoult, 2004).

To conclude, the three tested disinfectants displayed variable and usually partial efficacy against *Candida* spp. and *H. vermiformis* under our experimental conditions. Increased antimicrobial activity may be obtained with longer disinfectant treatments. Hydrogen peroxide had no significant effect on microbial viability, compared with untreated controls. Chlorine had an effect but only using the highest doses (62 and 118 μg mL\textsuperscript{-1}), which were able to kill all yeasts and decrease the FLA survival below 3%. But
Unfortunately, these effective doses are probably not suitable to the disinfection of DUWL systems as they may be very corrosive. Finally, low doses of Oxygenal 6© (≥ 500 μg mL−1) displayed the best efficacy against single-species biofilms such as C. albicans or C. glabrata. Candida parapsilosis and H. vermiciformis were less susceptible to the treatment and higher doses could be tested in the future although they will probably not be adapted to the daily DUWL maintenance. The reduced antimicrobial activity of Oxygenal 6© against C. albicans biofilms when mixed with H. vermiciformis highlights the need to consider mixed instead of single-species biofilms to better evaluate both the activity of disinfectants and their relevance in DUWL maintenance. In this work, we also demonstrated the variable susceptibility of Candida in tap water, depending on the species with C. parapsilosis being the most resistant species. Regarding H. vermiciformis, the encystment mechanism would explain its ability to survive in severe conditions and thus its resistance, even if moderate, to the three tested disinfectants. Candida has been shown to be able to survive in water (Barbot et al., 2011), even in the presence of FLA (Barbot et al., 2012a, b), and has already been isolated from DUWL (Walker et al., 2000; Szymańska, 2005); its persistence in lines and an inadequate disinfection protocol could be responsible for an infectious risk, especially for the most fragile patients or dentists.

Acknowledgements

This work was supported by the IFRO association (Institut Français de Recherche en Odontologie), by the CNRS (Centre National de la Recherche Scientifique) and by the Poitou-Charentes region. We also thank D. Debail for English revision.

References

dental unit waterlines using disinfectants and filters. Minerva
Odds FC (1988) Candida and Candidosis: A review and Bibliogra-
O’Donnell MJ, Boyle MA, Russell RJ et al. (2011) Management of
dental unit waterline biofilms in the 21st century. Future Microbiol
6: 1209–1226.
Pires RH, da Silva Jde F, Gomes Martins CH et al. (2013)
Effectiveness of disinfectants used in hemodialysis against both
Candida orthopsilosis and C. parapsilosis sensu stricto biofilms.
Schel AJ, Marsh PD, Bradshaw DJ et al. (2006) Comparison of the
efficacies of disinfectants to control microbial contamination in
dental unit water systems in general dental practices across the
Steed KA & Falkinham JO 3rd (2006) Effect of growth in
biofilms on chlorine susceptibility of Mycobacterium avium and
4011.
Szymańska J (2005) Evaluation of mycological contamination of
Szymańska J (2006) Bacterial decontamination of DUWL biofilm
contamination of dental unit waterlines. Ann Agric Environ Med
Walker JT, Bradshaw DJ, Bennett AM et al. (2000) Microbial
biofilm formation and contamination of dental-unit water sys-
tems in general dental practice. Appl Environ Microbiol 66:
3363–3367.
Wang H, Masters S, Hong Y et al. (2012) Effect of disinfectant,
water age, and pipe material on occurrence and persistence of
Legionella, mycobacteria, Pseudomonas aeruginosa, and two
Williams JF, Molinari JA & Andrews N (1996) Microbial contamina-
tion of dental unit waterlines: origins and characteristics. Com-