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ORIGINAL ARTICLE

# Use of the modified Robbins device to study the *in vitro* biofilm removal efficacy of NitrAdine™, a novel disinfecting formula for the maintenance of oral medical devices

T. Coenye[1], K. De Prijck[1], B. De Wever[2] and H.J. Nelis[1]

1 Laboratorium voor Farmaceutische Microbiologie, Universiteit Gent, Gent, Belgium
2 MSI Laboratories AG, Vaduz, Liechtenstein

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**Correspondence**
Tom Coenye, Laboratorium voor Farmaceutische Microbiologie, Universiteit Gent, Harelbekestraat 72, B-9000 Gent, Belgium. E-mail: Tom.Coenye@UGent.be

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## Abstract

**Aims:** To evaluate the use of the modified Robbins device (MRD) to test disinfection strategies against biofilms that form on oral medical devices and to test the biofilm removal efficacy of NitrAdine™, a disinfectant for the maintenance of oral medical devices.

**Methods and Results:** Biofilms were grown on discs using the MRD and biofilms formed in this system were used to evaluate the efficacy of NitrAdine™ and to determine the optimal disinfection conditions. Our data indicate that the use of the MRD allows for the rapid and reproducible formation of high-density biofilms. Determination of the efficacy of NitrAdine™ revealed high activity against biofilms tested (e.g. >3 log reduction for *Candida albicans* and *Staphylococcus aureus*) and allowed the determination of the optimal conditions for its use.

**Conclusion:** The high reproducibility and flexibility of the MRD make it an excellent candidate for standardized testing of disinfectants aimed at reducing biofilms on oral medical devices. Using this system, we were able to demonstrate that NitrAdine™ exhibits high activity against biofilms formed by the micro-organisms tested.

**Significance and Impact of the Study:** Our data suggest that our procedure is appropriate for standardized testing of disinfectants aimed at reducing biofilms on oral medical devices.

## Introduction

Biofilms are consortia of micro-organisms that can form on various surfaces, including oral medical devices, and the formation of a biofilm is a multi-stage process in which microbial cells reversibly adhere to the surface, followed by the production of an exopolysaccharide matrix which results in a more irreversible attachment (Stoodley *et al.* 2002; Fux *et al.* 2005). There is growing evidence that *Candida albicans* biofilms play an essential role in the development of denture stomatitis, a multifactorial disease, which has been reported in 11–67% of otherwise healthy denture wearers (Arendorf and Walker 1987; Nikawa *et al.* 1998; Webb *et al.* 1998; Barbeau *et al.* 2003; Ramage *et al.* 2004, 2006). One of the problems associated with *C. albicans* biofilms is their increased antifungal resistance, making the treatment of *C. albicans* biofilm-related oral infections difficult (Chandra *et al.* 2001; Mukherjee and Chandra 2004; Lamfon *et al.* 2005; d'Enfert 2006). Other pathogens potentially involved in denture stomatitis or other oral medical device-associated infections are *Staphylococcus aureus* and *Streptococcus mutans* (see e.g. Barnabe *et al.* 2004; Glass *et al.* 2004; Baena-Monroy *et al.* 2005). In addition, there is increasing concern that oral biofilms can harbour pathogens like *Pseudomonas aeruginosa* and methicillin-resistant *S. aureus* (MRSA). These could cause systemic disease in certain patient groups, especially elderly patients in nursing homes, patients undergoing cancer treatment and HIV-infected patients (Rossi *et al.*

 

1996; Senpuku *et al.* 2003). Sanitation of oral medical devices using disinfectants is considered to be essential, and many effervescent products for routine maintenance of dentures or orthodontic appliances are commercially available (Shay 2000). However, the *in vitro* efficacy of these products is often evaluated using model systems with limited relevance for the *in vivo* situation: as oral surfaces are subject to fluid flow and shear stress, the study of oral biofilms requires an *in vitro* model that incorporates these important aspects (Morgan and Wilson 2000), especially because, it was recently reported that the presence of flow can have a profound effect on the antimicrobial resistance against disinfectants (Buckingham-Meyer *et al.* 2007). However, several systems that do mimic *in vivo* flow conditions are cumbersome to use and are unsuitable for high-throughput analyses, e.g. *in vitro* systems using entire dentures (Leduc *et al.* 1999; Nikawa *et al.* 1999). The modified Robbins device (MRD) is an *in vitro* biofilm model system that allows the formation of various microbial biofilms on diverse substrata under controlled flow conditions (Nickel *et al.* 1985). This model system was previously used to study biofilm formation by the oral pathogens *Streptococcus sanguinis* (Larsen and Fiehn 1995) and *St. mutans* (Honraet and Nelis 2006); it has also been used as an *in vitro* model for cathether-associated biofilm formation by *C. albicans*, *S. aureus* and *Staphylococcus epidermidis* (Raad *et al.* 2003).

In the present study, we investigated the ability of the MRD to function as an *in vitro* model system for microbial biofilm formation on materials often used in the manufacturing of oral medical devices. Biofilms formed in the MRD were subsequently exposed to a novel disinfectant (NitrAdine™) to determine the optimal conditions for treatment and to investigate its efficacy in removing microbial biofilms.

## Materials and methods

### Strains and culture conditions

*Candida albicans* strain SC5314 (ATCC MYA-2876) was routinely cultured in Sabouraud dextrose broth (SDB) (BD, Franklin Lakes, NJ, USA) or on Sabouraud dextrose agar plates (SDA) (BD) for 24 h at 37°C. *Streptococcus mutans* LMG 14558$^T$ (ATCC 25175$^T$) was routinely grown aerobically in brain heart infusion (BHI) broth at 37°C (BD). An MRSA isolate and *P. aeruginosa* ATCC 9027 were grown in tryptic soy agar (TSA) (Oxoid, Drongen, Belgium) at 37°C. All cells were stored frozen at −80°C using the Microbank system (Prolab Diagnostics, Neston, UK). For every experiment, a fresh culture was prepared by taking two beads from a Microbank vial and transferring them into 10 ml appropriate growth medium.

### Substrata for biofilm formation

Biofilms were formed on different substrata. These include poly methyl-methacrylate (PMMA), medical-grade silicone (Q7-4735; Dow Corning, Midland, MI, USA) and hydroxyapatite (HA), in addition to an experimental elastomere used for the manufacture of orthodontic appliances (designated P1). From larger pieces of the aforementioned materials, small discs (height 2 mm, diameter 6·8 mm, surface area available for biofilm formation 36 mm$^2$) were punched. Most experiments were carried out using PMMA, as this is the most relevant material from the point of view of denture decontamination (Frazer *et al.* 2005). *Pseudomonas aeruginosa* was not tested on PMMA as it is not typically associated with denture-related infections. However, *P. aeruginosa* biofilms can form on other medical devices (including silicone-based venous and urinary catheters) and are notoriously difficult to eradicate (Donlan and Costerton 2002).

### Formation of *Candida albicans* biofilms in the MRD

A flow system using six homemade stainless steel MRD (Honraet and Nelis 2006) was used to study the biofilm formation on discs. Before use, these discs were cleaned with 70% ethanol. One MRD contains six individual ports in a linear array along a channel of rectangular cross-section, with dimensions of 10·0 mm (width), 145·0 mm (length) and 3·5 mm (depth). Each port accepts a press-fit plug holding a disc. The six MRD, containing the discs (36 in total) were placed in a custom-made aluminium heating block. Feedback from a Pt electrode placed into the heating block ensured a constant temperature of 37°C in the MRD. The tubing, valves and MRD containing the discs were washed, assembled and autoclaved prior to each run. Assembly was done in a Laminar Air Flow cabinet to prevent contamination. The tubing was connected to a peristaltic pump (Watson-Marlow 505S; Bredel, Wilmington, MA, USA) and bottles either with growth media or inocula. The setup included a bypass to allow rinsing of the tubing at the inlet side of the devices and the removal of air bubbles. At the outlet side, a clamp was placed on the tubing to prevent the drainage of the liquid when the pump was off. Four sterile centrifugation tubes containing 10 ml SDB were inoculated with *C. albicans* and incubated for 17 h at 37°C. The cells were harvested and washed three times with 5 ml 0·9% (w/v) NaCl, and the pellets were combined and resuspended in 5 ml 0·9% (w/v) NaCl. Six bottles

containing 99 ml 0·9% (w/v) NaCl were inoculated with 1 ml of this suspension and were connected to the MRD. After introduction of the inoculated solutions into the MRD, the latter were flipped over to improve the adhesion of the cells to the discs. Once the devices were filled with the suspensions, the tubing at the inlet and outlet side was clamped off and the remaining cell suspension in the tubing at the inlet side was flushed out through the bypass. After 1 h, the devices were flipped back, the clamps were loosened and the pump was started to allow a continuous flow of the growth medium, 0·1 × yeast nitrogen base (BD) + 50 mmol $l^{-1}$ glucose. The biofilms were allowed to develop on the discs for up to 48 h, after which the pump was stopped and the plugs were removed.

### Formation of bacterial biofilms in the MRD

*Streptococcus mutans* biofilms were grown in the MRD as described earlier (Honraet and Nelis 2006). For *S. aureus* and *P. aeruginosa* biofilm formation, four sterile centrifugation tubes with 10 ml TSB were inoculated with the organism and incubated for 17 h at 37°C. The cells were harvested and washed three times with 5 ml phosphate buffer (PB) (0·003 mol $l^{-1}$ $Na_2HPO_4.12H_2O$ and 0·0015 mol $l^{-1}$ $KH_2PO_4$, pH 7·4), the pellets were combined and finally resuspended in 10 ml PB. Bottles containing 99 ml of PB were inoculated with 1 ml of the prepared suspension. The adhesion and the biofilm formation were carried out as described earlier for *C. albicans*. The continuous growth medium consisted of TSB diluted (1 : 500) in PB.

### Treatment of discs with NitrAdine™ and other denture cleansers

Following biofilm formation, the plugs containing the discs with the biofilm were removed from the MRD and were treated with NitrAdine™ (Medical Interporous, MSI Laboratories AG, Vaduz, Liechtenstein), according to manufacturer's instructions. NitrAdine™ was used in the form of an effervescent tablet that needed to be dissolved in lukewarm water. Following treatment, the discs were removed from the solution and the remaining number of culturable cells was quantified. For each experiment, we also included control discs that were treated with water only. For comparison, we also included Polident (GlaxoSmithKline, Uxbridge, UK), Steradent (Reckitt & Benckiser, Slough, UK) and Efferdent Plus (an alkaline peroxide cleanser; Pfizer, Puurs, Belgium). These products were obtained from local stores in Belgium and were used according to the manufacturer's instructions.

### Quantification of culturable sessile cells

After treatment, each disc was transferred to 10 ml 0·9% (w/v) NaCl. The tubes were subjected three times to 30 s of sonication (Branson 3510, 42 kHz, 100 W; Branson Ultrasonics Corp., Danbury, CT) and 30 s of vortex mixing to remove the biofilm cells from the discs. Using this procedure, all cells are removed from the discs and clumps of cells were broken apart (data not shown). The number of sessile *C. albicans* cells was quantified by plating on SDA. The number of sessile *St. mutans* cells was quantified by plating on BHI. The number of sessile *S. aureus* and *P. aeruginosa* cells were quantified by plating on TSA. For all plating experiments, we have used pour plating and used 1 ml, which means our theoretical lower limit of detection is 10 CFU per disc. All plates were incubated at 37°C for 24 h and the number of CFU per disc was calculated by counting colonies on the plates. All experiments were carried out on at least three discs (Table 1).

### Microscopic examination of microbial biofilms on discs

Adherent *C. albicans* cells were stained with Calcofluor white (CFW) (Sigma, Bornem, Belgium) and bacterial biofilms were stained with the fluorescent DNA-binding dye SYTO9 (Invitrogen, Carlsbad, CA, USA). Biofilm structures were visualized by epifluorescence microscopy using an Olympus BX41 microscope (Olympus GmbH, Hamburg, Germany). For confocal laser scanning microscopy, discs with *C. albicans* biofilms were transferred to a 24-well plate, 1 ml of a 250 $\mu$g $ml^{-1}$ stock solution of

**Table 1** Overview of the number of sessile cells recovered from various discs following biofilm formation in the modified Robbins device (MRD) and control treatment (15 min in water of 45°C) and log reductions obtained after treatment with NitrAdine™. Results are expressed in log CFU per disc (average ± standard deviation). Coefficient of variation (CV) is expressed as per cent

| | | | No. of sessile cells | | | Log reduction with NitrAdine™ | | |
|---|---|---|---|---|---|---|---|---|
| Organism | Substratum | *n* | Avg. | SD | CV | Avg. | SD | CV |
| *C. albicans* | PMMA | 65 | 5·89 | 0·68 | 11·6 | 3·27 | 1·10 | 33·8 |
| | Silicone | 5 | 5·55 | 0·36 | 6·5 | 3·41 | 1·01 | 29·6 |
| | P1 | 3 | 5·61 | 0·29 | 5·2 | 3·63 | 0·57 | 15·7 |
| *S. aureus* | PMMA | 6 | 6·32 | 0·91 | 14·4 | 3·23 | 1·47 | 45·5 |
| (MRSA) | Silicone | 6 | 4·81 | 0·34 | 7·1 | 2·88 | 0·61 | 21·2 |
| *St. mutans* | HA | 4 | 9·39 | 0·58 | 6·2 | 0·80 | 0·71 | 88·8 |
| | PMMA | 5 | 8·02 | 0·61 | 7·6 | 2·85 | 0·63 | 22·1 |
| *P. aeruginosa* | Silicone | 3 | 6·45 | 0·22 | 3·4 | 2·73 | 0·40 | 14·7 |

*C. Candida*; *S. Staphylococcus*; *St.*, *Streptococcus*; *P.*, *Pseudomonas*; MRSA, methicillin-resistant *S. aureus*; PMMA, poly methyl-methacrylate; HA, hydroxyapatite.

concanavaline A bound to Alexa fluor 647 (Invitrogen) ($\lambda_{ex/em}$ = 650/668 nm) was added to each well and the plate was incubated for 90 min at 30°C. The biofilm structure was visualized using a Nikon C1si confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon, Melville, NY, USA). After excitation of the samples using the 647 nm laser line from a DPSS laser, images were captured with a 60× water immersion [numerical aperture (NA) 1·4] or a 20× (NA 0·95) Nikon Plan Apochromat objective lens, and the emitted fluorescence was captured using a 655LP detector (Nikon). A nonconfocal transmission image was collected simultaneously with the confocal images.

### Treatment of planktonic cells with NitrAdine™

1·5 ml of cell suspensions containing *c.* $10^8$ CFU $ml^{-1}$ were added to 148·5 ml lukewarm water, to which an effervescent NitrAdine™ tablet was added. After 15 min, 1 ml was used for quantification by pour plating while the remaining 149 ml was used for quantification by membrane filtration.

### Statistical analysis

All data are expressed as log CFU per disc. We used the standard deviation of a measurement across repetitions to indicate repeatability, and based on this repeatability standard deviation, the coefficient of variation was calculated (Pitts *et al.* 2001). Statistical analysis was carried out using SPSS 12·0·0 (SPSS Inc, Chicago, IL, USA).

## Results

### The use of the MRD for microbial biofilm formation

The number of CFU obtained following 24 h of biofilm formation was high and ranged between *c.* $6{\cdot}5 \times 10^4$ (for *S. aureus* on silicone) and $2{\cdot}5 \times 10^9$ (for *St. mutans* on HA) CFU per disc (Table 1). Prolonged incubation (up to 48 h) of the discs in the MRD did not result in a meaningful increase in the cell numbers for any of the micro-organisms investigated (data not shown); for these reasons, biofilms formed following 24 h of incubation were considered as mature and were used for further experiments. In order to determine the reproducibility of our assay, *C. albicans* biofilms were formed on PMMA discs (belonging to three different production batches) by three different operators on multiple occasions. The repeatability standard deviation was 0·68 log CFU per disc (coefficient of variation: 11·6%). Similar low repeatability standard deviations and coefficients of variation were obtained with other organisms and substrata (Table 1). The repeatability standard deviations for observed log reductions were also low (Table 1).

To confirm the presence of their typical three-dimensional structure, 24-h old *C. albicans* biofilms were stained with CFW and visualized using epifluorescence microscopy. CFW is a fluorescent dye that binds to glycans and can be used to detect cell-wall bound polysaccharides and extracellular polysaccharides present in the biofilm matrix. Alternatively, *C. albicans* biofilms were stained with concanavaline A bound to Alexa fluor 647 and visualized using CLSM. It is clear (Fig. 1) that cells had formed a thick three-dimensional structure consisting of a mixture of yeast cells, filaments (hyphae and pseudohyphae) and extracellular matrix containing glycans. Obvious three-dimensional structures were also observed for the bacterial biofilms investigated (data not shown).

### Determining the optimal conditions for testing the efficacy of NitrAdine™ against microbial biofilms

Using biofilms formed in the MRD, the anti-biofilm effect of a novel disinfectant (NitrAdine™) was evaluated. First, we studied the influence of different parameters on the disinfecting efficacy of the NitrAdine™ tablets using

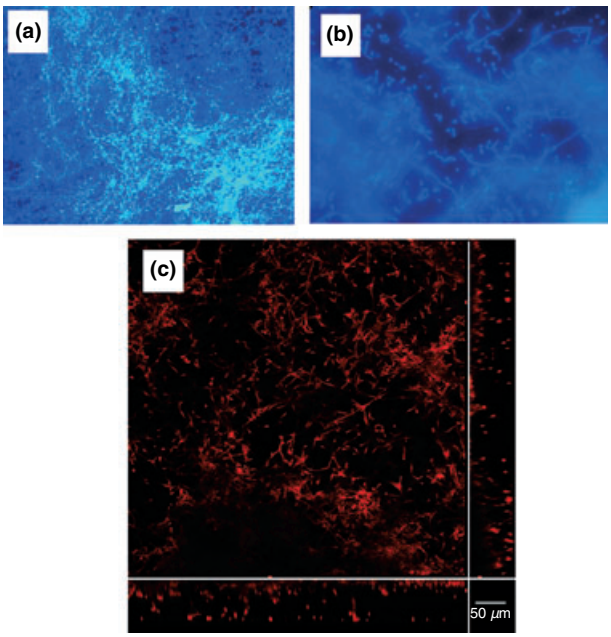


**Figure 1** (a) Mature *Candida albicans* SC5314 biofilms (24 h of incubation) on poly methyl-methacrylate (PMMA) discs, stained with Calcofluor white, low magnification (500×). (b) Close-up of biofilm structure showing yeast cells, filaments and matrix (magnification: 1000×). (c) CLSM image (*x-y* plot) of a mature *C. albicans* SC5314 biofilms (24 h of incubation) on PMMA discs. Shown to the left and below the *x-y* plots are vertical sections through the biofilm.


 

*C. albicans* biofilms formed on PMMA discs. The parameters investigated included the treatment temperature (i.e. the temperature of the water in which the tablet was dissolved), the 'wait time' after tablet dissolution (i.e. the time interval between the complete dissolution of the tablet and the addition of the *C. albicans* biofilm-containing PMMA disc), and the final volume in which the tablet was dissolved. The manufacturer recommended to place the denture or removable orthodontic appliance in 150 ml of lukewarm water and treat it for 15 min with NitrAdine™. The results obtained by varying these parameters indicate that lowering the temperature from 45 to 25°C resulted in a significant decrease in efficacy ($P < 0{\cdot}001$), while the efficacy was maintained at 33°C. Increasing the volume from 150 ml (99·95% reduction), over 450 ml (99·87% reduction) to 900 ml (no reduction) resulted in a gradual decline of efficacy, as did increasing the 'wait time' (simultaneous addition, 99·95% reduction; 15 min wait time, 99·34% reduction; 30 min wait time, 81·8% reduction). For all further experiments, a volume of 150 ml of 45°C water and a treatment of 15 min were used, unless otherwise mentioned.

### Evaluation of the efficacy of NitrAdine™ and other denture cleansers against microbial biofilms

A single 15 min treatment of *C. albicans* biofilms with NitrAdine™ resulted in marked reductions (99·94% for PMMA, 99·96% for silicone and 99·98% for P1) in the number of culturable cells present in the biofilm, irrespective of the substratum on which the biofilm was formed (Fig. 2). High reductions were also seen for the MRSA strain tested (an average reduction of 99·94% and 99·87%, on PMMA and silicone discs, respectively). Slightly lower reductions were observed with *P. aeruginosa* biofilms on silicone discs (average reduction of 99·81%) (Fig. 2). For *St. mutans*, reductions depend heavily upon the surface on which the biofilm was formed, with 99·86% reduction on PMMA and 84·15% reduction on HA (Fig. 2). These differences may be attributed to the differences in the density of the biofilm, but further research will be required to clarify this.

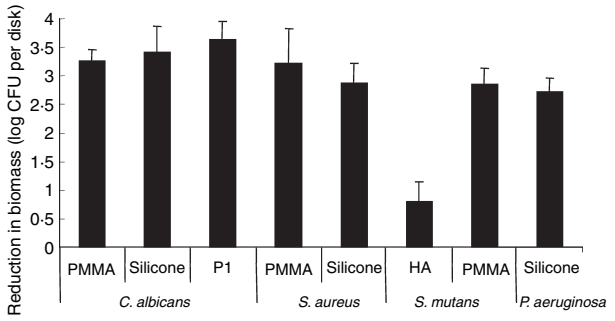


**Figure 2** Efficacy of NitrAdine™ denture disinfectant in removing microbial biofilms from various substrata. Data are presented as reduction in the number of sessile cells (expressed in log CFU per disc) compared with control treatment (15 min in water of 45°C). Error bars indicate standard error.

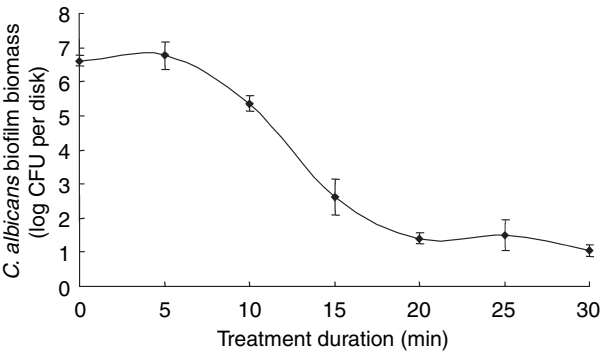


**Figure 3** Time-kill curve for *Candida albicans* biofilms grown on poly methyl-methacrylate (PMMA) and treated with NitrAdine™.

*Candida albicans* biofilms formed on PMMA were much less sensitive to other denture cleansers tested, as no reductions in the number of culturable sessile cells could be observed for Steradent, and only minor reductions were observed for Polident (0·88 log units, 86·82%) and Efferdent Plus (0·19 log units, 35·44%).

We also determined the kinetics of killing for *C. albicans* biofilms grown on PMMA discs. As can be seen from Fig. 3, the time–kill curve has a sigmoidal shape, with only limited killing of biofilms in initial stages (<10 min) and almost complete biofilm eradication when longer treatments are used.

### Evaluation of the efficacy of NitrAdine™ against planktonic cells

Following a single 15 min treatment with NitrAdine™, no culturable planktonic cells could be recovered for any of the isolates tested. Although treating the planktonic cells with lukewarm water for 15 min (control treatment) already resulted in slight reductions in cell numbers (data not shown), our results indicate that treatment with NitrAdine™ resulted in at least 6·5 log units reduction for the bacteria and 5 log units reduction for *C. albicans*.

## Discussion

There is a growing consensus that many oral medical device-related infections, including denture or orthodontic appliance-associated stomatitis, involve microbial biofilm formation, and the high-throughput testing of novel antimicrobial products with antibiofilm properties in relevant *in vitro* model systems is becoming increasingly

 

important. In the present study, we investigated the ability of the MRD to function as an *in vitro* model system for microbial biofilm formation on materials often used in the manufacturing of oral medical devices and evaluated the efficacy of a novel disinfectant (NitrAdine™) against various biofilms formed in the MRD biofilm model system.

*Candida albicans* readily forms biofilms on silicone discs in the Centers for Disease Control Bioreactor (Honraet *et al.* 2005). Previous studies from our research group have also shown that *St. mutans* readily forms biofilms on HA discs in the MRD (Honraet and Nelis 2006). In the present study, we evaluated the use of the MRD for the formation of biofilms by a range of micro-organisms on various materials. As is obvious from the data in Table 1 and Fig. 1, mature, high-density biofilms form rapidly in this system, irrespective of the micro-organism or the substratum used.

Our data also clearly indicate that the number of biofilm cells recovered is very similar between different discs of the same substratum, indicating a high reproducibility. This is attributed to the use of discs with standardized dimensions (instead of, e.g. complete dentures) and the use of plating to unambiguously quantify the number of sessile cells. For log reduction measurements, we also obtained relatively small repeatability standard deviations, which are generally within the range of standard deviations observed for standard suspension and surface disinfection assays (0·2–1·2) (Tilt and Hamilton 1999). The high reproducibility of the procedure as a whole indicates its usefulness in standardized tests.

In contrast to several other *in vitro* biofilm model systems, the use of the MRD allows simultaneous biofilm formation by different micro-organisms, in different growth media and/or on different substrata. Hence, the MRD biofilm model system can be used for high-throughput testing. In addition, discs containing the microbial biofilms can easily be moved from the MRD to a different recipient without loss of cells and the testing of the antibiofilm activity of the disinfectant can be performed 'offline', which means that different products and/or procedures can be tested simultaneously.

In the present study, we have used conventional plating to determine the (remaining) number of culturable cells present on each disc and obtained final results after approximately 48 h (i.e. 24 h of biofilm formation and 24 h of growth on plates to allow quantification). This time could even be further reduced by using surrogate assays for quantification instead of conventional plating. However, although these surrogate assays would allow a rapid estimate of the number of remaining sessile cells, their use precludes the accurate determination of reductions in number of culturable cells.

According to the standards from the Association Française de Normalisation, a product is considered to be biocidal against planktonic cultures, when it results in at least a 10-fold reduction (i.e. one log reduction) in cell numbers, while a minimal reduction of 99·99% (i.e. 4 log reduction) or 99·999% (i.e. 5 log reduction) is required before a product is considered fungicidal or bactericidal, respectively [Association Française de Normalisation (2006a,b)]. However, planktonic test cultures are not very relevant when evaluating the efficacy of disinfecting materials for oral medical devices, as their rough surfaces support the formation of complex multilayered fungal or bacterial biofilms that are sometimes deeply imbedded in the materials used for their manufacturing (Ramage *et al.* 2006).

The high numbers of microbial cells present on each disc (Table 1) allow the potential observation of up to 5 or 6 log reductions for *C. albicans*, MRSA and *P. aeruginosa*, and even higher (up to 8 or 9 log reductions) for *St. mutans* in this system. This high 'dynamic range' allows the testing of disinfectants and/or disinfection procedures with a wide range of efficacies. Using NitrAdine™ as test disinfectant, we have shown that our approach can not only be used to determine the efficacy of the disinfectant in a standardized way, but also to determine the optimal conditions for the application of the disinfectant. Our data indicate that, when using NitrAdine™ as recommended by the manufacturer, the number of *C. albicans* biofilm cells on various materials is reduced by 3–4 log units after a single 15-min treatment, while reductions of *c.* 3 log units were obtained for MRSA and *P. aeruginosa*. Our data also indicate that the contact time needs to be sufficiently long (i.e. at least 10–15 min) in order to obtain meaningful reductions. This may be attributed to the time needed for the disinfectant to penetrate into the biofilm. It should be noted that longer contact times may be required when the disinfectant in used in the presence of interfering susbtances like organic matter.

Other commercially available products (including Polident, Efferdent plus and Steradent) appear to be much less efficient for the removal of microbial biofilms. The substratum-dependent efficacy of NitrAdine™ against *St. mutans* biofilms highlights the need for careful selection of the test surface to mimic the *in vivo* situation as closely as possible.

Testing of disinfectants against biofilm-grown micro-organisms requires appropriate and standardized conditions and guidelines that are not necessarily the same as for planktonic cells. At present only one standardized test (for testing the efficacy of disinfectants against *P. aeruginosa* biofilms, using a rotating disc reactor) has been approved by the American Society for Testing and Materials International (ASTM International 2002), and in general, tests of antibiofilm activity are based on *ad hoc*

methods (Buckingham-Meyer *et al.* 2007). The data from the present study suggest that the procedure outlined earlier may be appropriate for the standardized testing of disinfectants and/or disinfection procedures aimed at reducing biofilms on oral medical devices, including dentures, orthodontic appliances, protective mouth guards or snoring devices.

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