



UvA-DARE (Digital Academic Repository)

A new strategy in root canal therapy: there is a lot going on in endodontic disinfection

van der Waal, S.V.

Publication date

2015

Document Version

Final published version

[Link to publication](#)

Citation for published version (APA):

van der Waal, S. V. (2015). *A new strategy in root canal therapy: there is a lot going on in endodontic disinfection*. [Thesis, fully internal, Universiteit van Amsterdam]. Boxpress.

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.



Salt marshes, Curaçao

Chapter 2

The effects of hyperosmosis or high pH on a dual-species biofilm of *Enterococcus faecalis* and *Pseudomonas aeruginosa*: an *in-vitro* study

S.V. van der Waal, L.W.M. van der Sluis, A.R. Özok, R.A.M. Exterkate,
J. van Marle, P.R. Wesselink, J.J. de Soet

International Endodontic Journal 2011; 44:1110-1117.

Abstract

Aim To investigate the effect of hyperosmotic or alkaline stress on a dual-species biofilm of *Enterococcus faecalis* and *Pseudomonas aeruginosa*.

Methodology Biofilms were grown on glass cover slips suspended in bacterial inoculate for 96 h, after which the cover slips with attached biofilms were immersed in brain heart infusion broth (BHI-broth) with 6 mol L⁻¹ sodium chloride (NaCl) representing the hyperosmotic group or Ca(OH)₂, pH 12.1, representing the alkaline group. Two per cent sodium hypochlorite and BHI-broth served as positive and negative controls, respectively. After treatment, the biofilms were washed, harvested and plated on blood-agar plates after serial dilution. The bactericidal effect was assessed by determining the colony-forming units (CFU). The effect on the biofilm mass was imaged with confocal laser scanning microscopy (CLSM).

Results Hyperosmosis reduced the CFU of both species significantly after 72 h ($P < 0.0001$). After 168 h, *P. aeruginosa* was eradicated and the *E. faecalis* reduction was more than 99%. High pH could not induce a significant bacterial reduction. CLSM revealed dense flocculation of the biofilms incubated in alkaline broth.

Conclusion Hyperosmosis effectively reduced a dual-species biofilm of *E. faecalis* and *P. aeruginosa*, whilst high pH had limited bactericidal effect in this model.

Introduction

Incomplete disinfection of the root canal system is considered to be a major cause of post-treatment disease^{124,197}. Hence, elimination of infection is one of the main goals of root canal treatment. However, after contemporary nonsurgical root canal treatment, studies confirm the presence of persisting microorganisms^{141,179} likely due to the complex architecture of the root canal system^{67,233,247}. Furthermore, microorganisms live in a biofilm configuration, which renders them less susceptible to endodontic treatment procedures¹⁶⁷. A biofilm is a microbial (bacterial, fungal, algal) community, enveloped by extracellular polysaccharides (EPS) that these microbial cells produce, which adheres to the interface of a liquid or a surface. Biofilms might contain 95–99% EPS and 1–5% microorganisms¹¹¹.

Osmosis is a physical phenomenon whereby solvent (water) moves, without input of energy, across a semipermeable membrane (permeable to the solvent, but not the solute) separating two solutions of different concentrations, until a certain equilibrium is reached. Hyperosmosis reduces the water activity of microorganisms and their environment. Hyperosmotic metabolic stress inhibits growth and eventually kills microorganisms^{21,240}.

Calcium hydroxide ($\text{Ca}(\text{OH})_2$) is used as an intra-canal dressing to prevent regrowth of microorganisms during the inter-appointment period between two treatment sessions and for additional reduction in the microbial load after chemo-mechanical cleaning of the root canal system. The antimicrobial action is based on alkaline stress, because of its pH of approximately 12²⁰³. However, whilst some studies report bactericidal action of $\text{Ca}(\text{OH})_2$ ^{31,84,207}, others report that its antibacterial effect is insufficient^{88,161,162}. The aim of this study was to evaluate *in vitro* the effect of hyperosmosis or high pH on a dual-species biofilm of *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Viability of the biofilms was assessed by conventional plate counting, whilst the effects on the EPS of the biofilms were demonstrated with confocal laser scanning microscopy (CLSM) imaging.

Materials and methods

The biofilm model used here was described by Deng et al. (2009)⁵⁵. In brief, round glass coverslips attached to a custom-made stainless steel lid were suspended in a medium inoculated with bacteria. In this model, biofilm attachment and growth is an active process against gravity.

Microbiology

Cells of *E. faecalis* V583 and *P. aeruginosa* HG 4950, both clinical isolates, were grown and maintained as pure cultures on blood agar plates. *E. faecalis* was cultured under anaerobic conditions (80% N₂, 10% H₂ and 10% CO₂) whilst *P. aeruginosa* was cultured in air, both at 37 °C. Growth media for overnight cultures and biofilms were BHI (Oxoid, Basingstoke, UK) and BHI broth containing 0.5 BHI, 50 mmol L⁻¹ 4-piperazinediethanesulfonic acid, buffer (Sigma, St. Louis, MO, USA), 0.5% saccharose and 1 mmol L⁻¹ Ca²⁺ during biofilm growth. In the experimental phase, 6 mol L⁻¹ sodium chloride (NaCl; Merck, Darmstadt, Germany) was added to the BHI broth (pH 6.9) to raise the osmotic value. Addition of NaCl did not alter the pH. Ca(OH)₂ of 0.02 mol L⁻¹ (to reach saturation) was added to BHI broth for the alkaline treatment (pH 12.1). Two per cent sodium hypochlorite (NaOCl; Merck) was used for the positive control and 2% sodium thiosulphate (STS) in peptone water [1% peptone (Oxoid) in water] for neutralising the NaOCl. Phosphate-buffered saline (PBS; Oxoid) was used to wash the negative controls, as a neutralising agent for NaCl and Ca(OH)₂ and for serial dilution.

Biofilm growth for viability assessment – CFU

Fresh cultures of each microorganism were grown overnight at 37 °C in air. Numbers of colony-forming units (CFU) were estimated by optical density measurement at 600 nm based on previously performed plate counts. One hundred microlitres with approximately 2×10^9 *E. faecalis* and 100 µL with approximately 2×10^7 *P. aeruginosa* were inoculated in wells of a 24-well multiwell plate (Greiner Bio-one, Frickenhausen, Germany) containing 1.5 mL BHI broth. Round glass coverslips (Thermo Scientific, Braunschweig, Germany), diameter 12 mm, mounted on a custom-made stainless steel lid, were suspended in the BHI broth.

In this experiment, 96-h biofilms were used because after 96 h, the biomass in a biofilm increases whilst the quantity of living cells is more or less established²⁴². The broth was refreshed daily. Inoculation of bacteria and the biofilm growth took place at 37 °C in air. Biofilm growth was confirmed after swabbing a coverslip and streaking the biofilm onto a glass microscope slide. After heat fixation of the glass slides, the samples were gram-stained. Biofilm growth was confirmed after examination of the glass slides with a reflected-light microscope (1000× magnification; Zeiss Axiolab, Axiolab A, Carl Zeiss B.V., Sliedrecht, The Netherlands).

Biofilm growth for biofilm matrix assessment – CLSM

The procedure is the same as described above, except that the biofilms used for CLSM imaging were grown for 24 h.

Experiments: CFU

After 96 h of growth, the coverslips with the attached biofilms were immersed in hyperosmotic or alkaline broth and incubated for 4, 24, 48, 72 and 168 h. The negative controls were kept in BHI broth. Where applicable, the incubation fluids were refreshed daily. Immersion in 2% NaOCl for 10 min served as a positive control. After the experiments, the coverslips were removed from the lid and immersed in 20 mL of neutralising agents: 10 min in PBS for the NaCl groups, $\text{Ca}(\text{OH})_2$ groups and negative controls and 30 min in STS for the positive controls. Biofilms were harvested using a sterile cotton swab and resuspended in 1 mL of PBS by vigorously moving the cotton swab up and down fifty times in a 1.5-mL test tube (Standard Micro Test Tube 3810; Eppendorf AG, Hamburg, Germany). Serial dilutions of the suspension were plated and incubated for 48 h in the absence of oxygen. Each group consisted of three specimens, and each experiment was performed in triplicate.

Experiments: CLSM imaging

Twenty-four-hour biofilms were incubated for 4 h in hyperosmotic or alkaline broth after which the biofilms were washed twice in 2 mL PBS to remove nonadherent cells. Biofilms were stained in duplicate for 15 min with Calcofluor White 50 mmol L^{-1} (CW) in a dark chamber at room temperature. CW is a polysaccharide-binding dye, used to stain the extracellular matrix of biofilms formed by bacteria^{145,209}. Of each sample, three standardised sites were chosen for imaging.

For imaging, a Leica DM-IRB (Leica Microsystems CMS GmbH, Mannheim, Germany) inverted micro-scope/SP2-AOBS confocal system was used. Images (512×512 pixels) were acquired with a $40\times/\text{NA } 1.25$ HCX PL APO oil objective. The final pixel size was $0.776 \mu\text{m}$. The pinhole size was set at 1 airy unit, resulting in a Z resolution of approximately $0.5 \mu\text{m}$. Image stacks were acquired with a stepsize of $0.3 \mu\text{m}$. Excitation/detection of CW was achieved with 388-nm excitation and with 440-nm detection. Using gain and offset of the photo-multiplying tube, the images were adapted to the full (8 bit) dynamic range of the system. Presence or absence of EPS was observed by CLSM.

Statistical analysis

Colony-forming units counts were logarithmically transformed. The data were analysed by one-way analysis of variance (anova) to compare the experimental groups with the negative controls. The significance level was set at $\alpha = 0.05$. The analyses were carried out with the statistical package SPSS version 12 (SPSS Inc. Chicago, IL, USA).

Results

Viability of the biofilms

The results are presented as log CFU reduction per biofilm compared with the negative controls (Figure 1). The negative controls contained 8.1 ± 0.6 log (CFU) microorganisms per biofilm, which gradually increased in time. The biofilms used for the CLSM experiment, 24 h, contained on average 3.2×10^7 cells.

The mean log reduction in CFU in the hyperosmotic groups for both *E. faecalis* and *P. aeruginosa* after 72 h and after 168 h was 6 and 8, respectively, and was statistically significant compared with the negative controls.

At 168 h, the control biofilms contained on average 1.5×10^9 CFU of *P. aeruginosa*, of which none could be detected after 168 h of hyperosmotic treatment. *E. faecalis* was reduced to a mean of 40 CFU. The reductions were highly significant for both species ($P < 0.001$) and showed a significant linear trend in time.

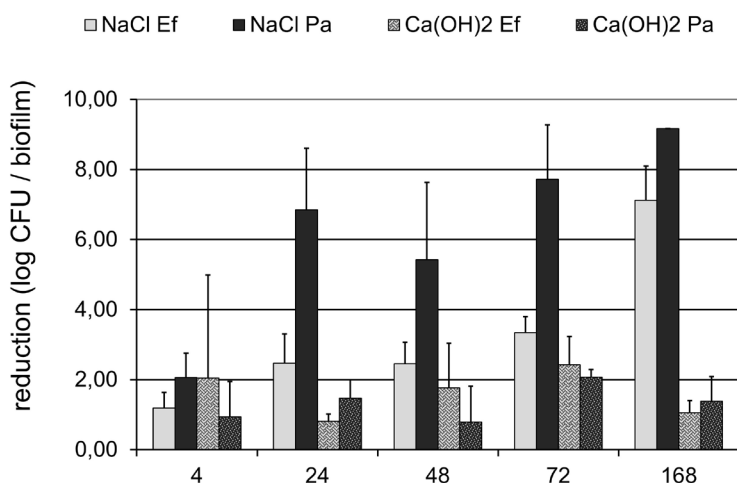


Figure 1 The reduction in colony-forming units (CFU) counts of bacterial species *Enterococcus faecalis* and *Pseudomonas aeruginosa* in log CFU/biofilm after exposure (hours) to hyperosmotic or alkaline broth. The negative controls contained on average 2.0×10^8 CFU *E. faecalis* and 3.5×10^8 CFU *P. aeruginosa*, which numbers increased in time.

Alkaline broth reduced *P. aeruginosa* significantly ($P = 0.02$); however, the reduction did not increase with an increased application time. Reduction of *E. faecalis* by alkali was not significant ($P = 0.09$). After 72 h, a log 2 reduction was determined for both species, but after 168 h, bacterial growth recovered

until a mean log 1 reduction was obtained. The NaOCl groups showed 100% kill after 10-min exposure.

With the naked eye, the glass coverslips exposed to hyperosmotic broth showed no signs of a biofilm plaque after treatment. Increase in biofilm plaque was an obvious observation in the alkaline broth group (Figure 2).

CLSM assessment of biofilm matrix

The CLSM images show the bacterial cells and the EPS. CW does not give an indication of the viability of the cells. The biofilm incubated in hyperosmotic broth (Figure 3) showed reduced amount of biofilm mass compared with the control biofilm (Figure 4). CLSM clearly indicated enhanced biofilm mass production in biofilms incubated in alkaline broth (Figure 5) compared with the control biofilm. This is in line with the

naked-eye observations. Furthermore, the appearance of the cells in the hyper- osmotic group seems altered in size and shape.

Discussion

In this biofilm model, hyperosmotic broth reduced the viability of cells in a biofilm significantly compared with the negative controls (Figure 1). When raising the osmotic value, there was a selective bactericidal effect with *P. aeruginosa* being more susceptible to raising the osmotic value than *E. faecalis* at earlier time points

(Figure 1). This was expected because bacterial species respond differently to environmental stresses. It was reported that *P. aeruginosa* does not tolerate 6.5% NaCl⁸¹ whilst *E. faecalis* was reported to be more resistant to NaCl^{66,119}. The mechanism of hyperosmotic metabolic stress killing of bacteria has been demonstrated earlier^{66,119}. Microorganisms counteract the emigration of free intracellular water owing to the extracellular hyperosmotic value, by restoring the intracellular solute concentration. Restoration occurs through the uptake or synthesis of small ions and molecules, which do not interfere with the cell's metabolism^{47,86,174}. Whether extracellular high osmotic value is lethal depends on the ability of the microorganisms to adapt to the changed osmotic environment, the available resources of compatible solutes and energy and duration of the hyperosmotic change. Storing and synthesising compatible solutes are energy-demanding processes. Once the stocks of compatible solutes or energy resources are depleted, microbes enter the stationary phase, after which eventually bacterial death follows⁴⁸.

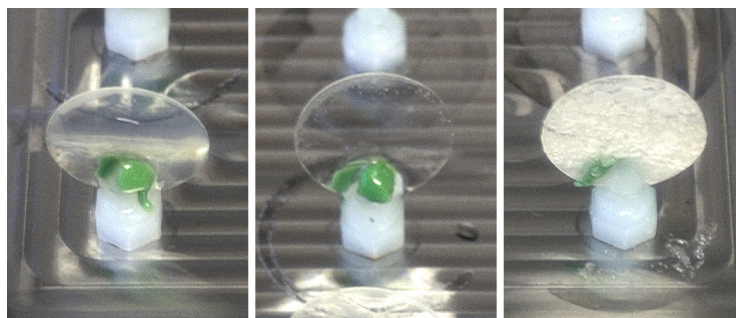


Figure 2 Illustrative photographs of glass cover slips fixed with green silicone impression material (President[®]) in white clamps on the custom-made stainless steel lid, with biofilms after 96 + 24 h control (left), after 24 h incubation in hyperosmotic broth (middle) or 24 h incubation in alkaline broth (right). The thickness of the plaque (right) increased throughout the experiment. The plaque seemed petrified and was firmly attached to the cover slip.

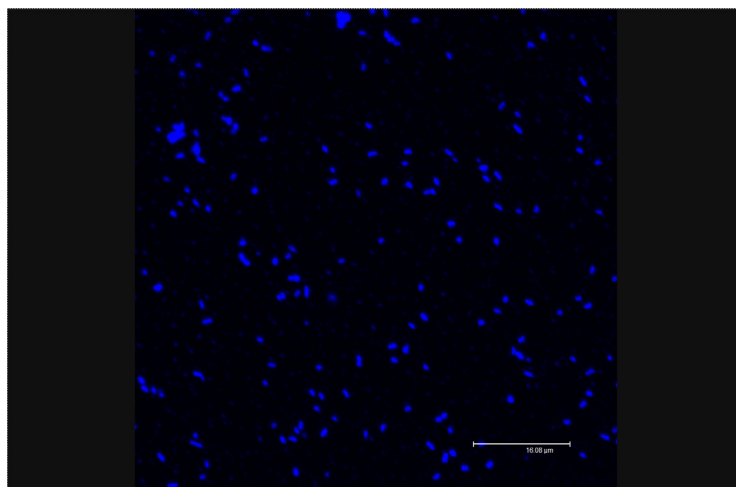


Figure 3 Confocal laser scanning microscopy image of 24 h biofilm after incubation in hyperosmotic broth for 4 h. Cells show more fluorescence which suggests that they are reduced in size and the cells are not embedded in extracellular polysaccharides (EPS). EPS material is reduced to small spots on the surface.

The phenomenon of osmosis is solely dependent on the concentration of solutes. The attraction of solvents by the solutes continues until certain equilibrium has been reached. In theory, a hyperosmotic root canal dressing would raise the osmotic value in the root canal. Because this high ionic concentration will be equilibrated with the surrounding tissues, some effect might perhaps be expected in microorganisms located more distant from the main root canal. The mode of action at a distance was described in a study by Wijnker et al. 2006²⁴⁰ where multispecies bacterial cultures were inoculated inside pieces of intestine. The sample ends were tied, after which the samples were disinfected in 6.0 mol L⁻¹ brine.

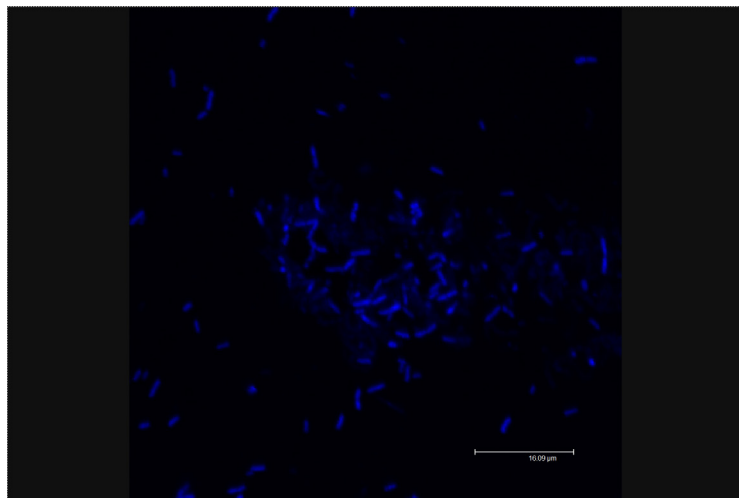


Figure 4 Confocal laser scanning microscopy image of negative control biofilm stained with Calcofluor White. Bacteria are visible as rod- and cocci-shaped structures embedded in extracellular polysaccharides.

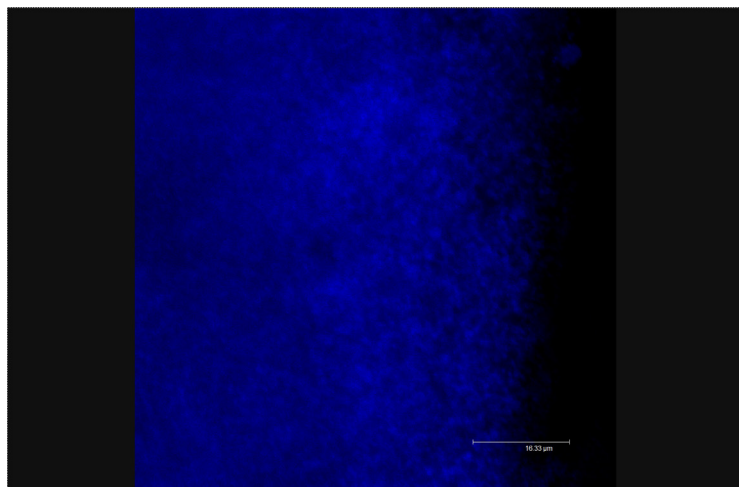


Figure 5 Confocal laser scanning microscopy image of 24 h biofilm after incubation in alkaline broth for 4 h shows a dense cluster of extracellular polysaccharides (EPS). Within the EPS, the cells are difficult to distinguish.

The pH 12.1 did not significantly reduce bacterial numbers in this model. Calcium hydroxide has some bactericidal effect in planktonic cultures^{68,213}.

Microorganisms, however, reside in the root canal system in a biofilm configuration^{35,142,179}. Studies investigating the antibacterial action of Ca(OH)₂ on *E. faecalis* biofilms show that *E. faecalis* is resistant to Ca(OH)₂^{2,36}. Its decreased susceptibility not only might be caused by genetic adaptation of the biofilm cell but might also be a result of increased production of EPS in which the cells can shelter from the alkaline environment.

A dual-species biofilm was used because of its increased resistance to antimicrobials^{103,153}. *E. faecalis*, a facultative gram-positive coccus, was chosen as the test organism because of

its reported association with persistent periapical pathosis^{35,142,179,204,212,214}. Factors facilitating its presence in filled root canals with persistent lesions include the ability to invade dentinal tubules^{88,239}, to tolerate the alkaline

pH of $\text{Ca}(\text{OH})_2$ ^{63,143} and to withstand long periods of starvation with subsequent recovery in the presence of serum⁶⁹. *E. faecalis* grows easily in a laboratory setting. Furthermore, *E. faecalis* is known to efficiently accumulate the compatible solutes from the medium to counteract increased osmotic pressure¹⁶⁴. *P. aeruginosa* is an aerobic gram-negative motile rod. It is a notorious EPS producer and for this reason commonly chosen as model bacterium in biofilm studies^{22,221}. No matter how smooth a surface, *P. aeruginosa* adheres within 30 s²³⁰. Although classified as aerobic, *P. aeruginosa* survives anaerobic conditions²⁵⁰. Its presence in the root canal is associated with failed endodontic treatment^{172,205}.

In pilot studies performed prior to this study, varying ratios of *P. aeruginosa* and *E. faecalis* were cultured together to determine whether and in which composition the species would grow together in a biofilm. The ratio of 1 cell of *P. aeruginosa* to 100 cells of *E. faecalis* yielded a reproducible dual-species biofilm and was therefore used to inoculate this model.

Biocides and disinfectants work by killing microorganisms, a strategy that is rendered ineffective by the reduced susceptibility of biofilm microorganisms to antimicrobial challenges²⁸. This study focused on biofilm killing, measured by the number of viable microorganisms attached to the glass coverslip after treatment. Plate count of CFU, however, combines the effects of biofilm removal and killing of bacterial cells³⁸.

The addition of Ca^{2+} ions to the growth medium is known to enhance the attachment of the biofilm cell to the substrate⁷⁸ and to induce the production of EPS¹⁵⁸ as well as to increase the Young's modulus of the EPS¹¹¹. It has also been demonstrated that raising the pH of the growth medium in the presence of divalent cations has an effect on the EPS of bacteria and yeasts. By raising the pH, divalent cations bind to binding sites of the polysaccharide strands formerly occupied by H^+ ⁷⁷. These findings might explain the increase in biofilm plaque and its petrified appearance in the present model after incubation with $\text{Ca}(\text{OH})_2$ -containing broth (Figure 2). The sterile coverslips were also incubated in alkaline broth and showed very little precipitate on the coverslips after a week's incubation (not reported here). This suggests an active role of the bacteria.

Sodium hypochlorite was used in this study in the positive control group and killed all bacteria in the biofilms. In the complexity of the root canal system, however, NaOCl does not remove all biofilm and microorganisms¹⁴¹. Probably, this is because of the difficulty of dispersing NaOCl during irrigation into the complete root canal system, especially into the apical part of the root canal system²⁶.

Application of an inter-appointment root canal dressing, after chemo-mechanical preparation with NaOCl, with a mode of action based on hyperosmotic, metabolic stress might possibly result in a decrease in the microorganismal load either by killing in a biofilm or by removing the

EPS. This concept needs further investigation in models that resemble the root canal.

Conclusions

Raising the osmotic value of growth medium has a bactericidal effect as well as an antibiofilm effect and might therefore seem promising for future use as an inter-appointment root canal dressing. High pH induced by calcium hydroxide was not effective in this model. Whether a hypertonic dressing affects biofilms in the complexity of a root canal system needs to be investigated in future studies.

Figure 3 Confocal laser scanning microscopy image of 24 h biofilm after incubation in hyperosmotic broth for 4 h. Cells show more fluorescence which suggests that they are reduced in size and the cells are not embedded in extracellular polysaccharides (EPS). EPS material is reduced to small spots on the substrate.