ANTIMICROBIAL EFFICACY OF DIFFERENT CALCIUM HYDROXIDE CONTAINING PREPARATIONS AGAINST BIOFILMS AT DIFFERENT STAGES OF BIOFILM DEVELOPMENT

by

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Abstract

Objective: To quantify and assess the antibacterial effect of different medicaments on young and aged biofilms, and to modify the medicaments in order to increase their antibacterial effect.

Hypotheses: Microbes in aged biofilms grown from a mixture of oral bacteria are more resistant to the antimicrobial activity of calcium hydroxide than microbes in young biofilms. Biofilms are less resistant to calcium hydroxide combined with other antimicrobial agents than to pure calcium hydroxide.

Methodology: Collagen coated hydroxyapatite disks were immersed in plaque suspension solution and incubated for one and three weeks to grow young and aged biofilms, respectively. The tested medicaments were calcium hydroxide, iodine potassium iodide, cetrimide, and the following combinations: iodine potassium iodide + cetrimide, calcium hydroxide, calcium hydroxide + iodine potassium iodide, calcium hydroxide + cetrimide, calcium hydroxide + iodine potassium iodide + cetrimide. After exposure to the medicaments for one day, one week, and two weeks, biofilms on disks were stained with a LIVE/DEAD viability stain and imaged using confocal laser scanning microscopy. The three-dimensional reconstructions of the images were done and proportions of green and red fluorescence were measured and statistically analyzed.

Results: Aged biofilms were thicker than the young biofilms. All tested medicaments showed reduced antibacterial activity on the aged biofilms compared to young biofilms. Combining iodine potassium iodide to cetrimide had an additive effect and mixed with calcium hydroxide showed stronger antibacterial effect than calcium hydroxide alone.

Conclusions: Aged biofilms are more resistant to antibacterial agents than young biofilms. Combining iodine potassium iodide and cetrimide to calcium hydroxide resulted in an antibacterial effect that was stronger than using calcium hydroxide alone.

Preface

This thesis is an original, unpublished, and independent work by Hadi Alamri. None of the text of the dissertation is taken directly from previously published or collaborative articles. Ethical approval reference: Human ethical study permission code: H12-02430.

This project was performed under the guidance and supervision of Dr. M. Haapasalo and Dr. Y. Shen. Hadi Alamri was responsible for all parts of the research, including the biofilm culturing, tested dressings preparation, confocal imaging, and three-dimensional reconstruction. The relative contribution of the collaborators in this project was: Dr. Hadi Alamri 60%, Dr. Markus Haapasalo 20% and Dr. Ya Shen 20%. Dr. Markus Haapasalo contributed to research design and manuscript editing. Dr. Shen contributed to organization of experimental work.

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List of Abbreviations

3D …… Three-dimensional

AP ……..……. Apical Periodontitis

BHI ……..…. Brain Heart Infusion

C-HA …….. Collagen coated hydroxyapatite disks

$Ca(OH)_2$ ……..…….. Calcium hydroxide

CFU ……..……. Colony Forming Units

CHX ……. Chlorhexidine digluconate

CLSM ……..….. Confocal Laser Scanning Microscopy

CTR …… Cetrimide

EPS …… Extracellular Polymeric Substances

I …… Iodine

IKI ……... Iodine Potassium Iodide

LPS ..... Lipopolysaccharide

NaOCl ……..…….. Sodium hypochlorite

PBS …….……..... Phosphate Buffered Saline

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Dedication

I dedicate this to my parents who supported and guided me through out every step in my life, and to my loving family, who stood by my side when I needed that. Without them, my goals would've still been far from being achieved. And to my mentors, for your guidance and advice kept me going until this journey has come to an end.

Chapter 1: **Literature Review**

1.1 General Introduction

The ultimate goal of the treatment of apical periodontitis is to completely eradicate the causes of infection from the complex root canal system, and to provide a seal for the canal space to prevent reinfection. It has been well established that bacteria are the main cause of infection in the root canal causing apical periodontitis (AP) (Kakehashi 1965; Bergenholtz 1974; Sundqvist 1994; Svensäter & Bergenholtz 2004). The main source of bacteria in the root canal infection is the microflora of the oral cavity. When caries develops and propagates toward the dental pulp, the bacteria invade the pulpal tissue eventually leading to pulp necrosis. The necrotic pulp acts as a reservoir for the microbes, which through complex interaction with the host defense system cause AP (Allard et al. 1979; Möller et al. 1981; Garg & Garg 2010). Further research has shown that the microflora of the infected canal is dominantly occupied by anaerobic and facultatively anaerobic bacteria (Sundqvist 1994). In endodontic infections, bacteria have been found to be in sessile communities and not in a free form; these communities are referred to as biofilms (Nair 1987; Costerton 1999). During endodontic treatment, several procedures aim to reduce the bacterial content in the root canal system by means of chemomechanical preparations (Orstavik 1990; Tanomaru 2003; Vianna 2007; Haapasalo & Shen 2010). In some cases, the use of interappointment intracanal medicaments may be necessary (Martin 1979; Foreman & Barnes 1990; Sjögren & Figdor 1991).

1.2 Biofilms

For many years, researchers have examined the effectiveness of various antimicrobial compounds on planktonic bacteria. This type of research continued until Costerton et al. (1978) described how bacteria adhere to each other and form what they referred to as a "film". The authors continued and described how these films were more resistant to different treatments than planktonic "freely floating" bacteria.

To better understand how the interest in research shifted from planktonic bacteria to biofilms, two main contradicting theories shaped the bulk of research and aided in shifting the interest: "reductionism" and "holism". Reductionism was basically based on the idea that in order to understand the complexity of the whole, one had to break the system into the smallest components to get a better understanding of how the system works (Hargreaves KM, Cohen S 2011). The main drawback of this theory was the fact that microorganisms, when examined alone, had different properties compared to when they were examined as a whole. Contrary to this, holism paid more attention on how these different components combined together determined the properties of the system and how it functions (Hargreaves KM, Cohen S 2011).

The microbial ecology has a certain hierarchy when microorganisms attach to each other to form a population. When these populations adhere to one another, they establish a community. It has been shown that this phenomenon is essential for microorganisms to survive in different environments, in these communities; each population has a certain role "niche" (Siqueira & Rôças 2009). More research followed, examining the formation and structure of biofilms. In the literature, a biofilm is defined as "a microbially derived sessile community characterized by cells that are irreversibly

attached to a substratum, interface, or to each other, embedded in a matrix of extracellular polymeric substances (EPS) it has produced, and exhibits an altered phenotype with respect to growth rate and gene transcription" (Donlan & Costerton 2002).

EPS is comprised of several substances that appear to be in a complex mixture. Polysaccharides are the main and primary biopolymers in EPS; other commonly found substances are proteins, lipids, and nucleic acids (Vu et al. 2009). It has been reported that the EPS may compose 75-90% of a microcolony's volume, with the density of it being mostly concentrated close to the core (Costerton 1999). As the amount of EPS in the biofilm increases, the biofilm becomes more resistant to antibiotics. Furthermore, it may slow down and in some cases prevent other antimicrobial agents from penetrating through the biofilm, acting as a host defense to protect the biofilm (Vu et al. 2009).

The resistance of the biofilm seems to be related to its maturation, it has been reported that it takes up to ten days for the structure of a biofilm to mature (Stoodley et al. 2002). However, a recent study suggested that the maturation of biofilms by oral bacteria might take a longer time. In an *in vitro* study, biofilms were grown using dental plaque suspension from six different donors. The biofilms were cultured for up to eight weeks to measure the antibacterial efficacy of three different irrigant solutions at different maturity stages (Stojicic et al. 2013). It was found that the biofilms became resistant to the solutions at three weeks of maturation and remained resistant since. Interestingly, the resistance development followed the same time dependent pattern for all three antibacterial solutions tested, in all six biofilms collected and grown from different donors (Stojicic et al. 2013).

1.3 Chemomechanical Preparation

The goal of chemomechanical preparation is to eradicate the microbes from the root canal system. Instrumentation is the mechanical cleaning of the root canal space. However, several factors such as complex anatomy (oval shaped canals, fins, isthmuses, lateral canals, etc.) limit the efficacy of instrumentation and prevent access to all surfaces within the system (Peters 2001). In addition, it has been shown that bacteria can penetrate deep into the dentinal tubules from the main root canal. Therefore, even with large preparation sizes bacteria will be left into the tooth structures. Matsuo et al. (2003), in an *in vitro* study, showed that bacteria were found in dentin in 65% of the teeth even after enlargement of the canals was finished. In another study, Peters et al. (2001) using micro computed tomography found that at least 35% of the root canal walls were left untouched, irrespective of the file system used for the instrumentation. These findings should not undermine the importance of instrumentation of the canals, as it is important to create enough space for the irrigant flow, possible interappointment medication, and for the permanent root filling in order to effectively eradicate the remaining biofilms inside the root canal system.

The chemical part of the chemomechanical preparation consists of the use of different solutions with different specific properties to aid in the eradication of the biofilms. An ideal irrigant solution would be one that lubricates the canal during instrumentation to facilitate dentin removal, dissolves organic matter and inorganic tissues, penetrates deep into dentinal tubules, has an antibacterial effect, is not caustic or cytotoxic, and does not weaken the tooth structure (Martin 1979; Orstavik & Haapasalo

1990; Tanomaru et al. 2003; Haapasalo et al. 2010). To date, no single solution has all these characteristics; therefore, several solutions are used in clinical practice to obtain the goals of optimal irrigation and to eradicate and dissolve the biofilms. Sodium hypochlorite (NaOCl) is the most irrigant solution used in endodontic clinical practice; it is used in different concentrations from 0.5% to 6% as part of the final irrigation protocol as well as during instrumentation (Martin 1979; Harrison & Hand 1981; Haapasalo et al. 2010). NaOCl has the advantage of dissolving organic tissue along with its antibacterial effect. However, it does not affect inorganic matter and therefore cannot remove the smear layer if used as the only irrigant (Harrison & Hand 1981). Regarding smear layer removal, the most used solution in clinical practice is 17% EDTA (Aktener & Bilkay 1993). When compared to other chelating agents, 17% EDTA showed effect similar to 10% citric acid (Petrovic & Zivkovic 2005). Moreover, removing the smear layer improves the disinfecting efficacy of irrigants in dentinal tubules, which makes this a critical step in the chemomechanical preparation procedure during endodontic treatment (Haapasalo & Orstavik 1987).

Chlorhexidine digluconate (CHX) has also been used as an irrigant. According to some studies, its antibacterial activity is comparable to that of NaOCl, but it does not have the ability to dissolve tissue, which is considered a major disadvantage of CHX. Nevertheless, the fact that CHX does not cause erosion of dentin after the use of EDTA, gives it some advantage as the final irrigant after smear layer removal (Zamany et al. 2003; Zehnder et al. 2005; Mohammadi & Abbott 2009; Haapasalo et al. 2010; Shen et al. 2011; Wang et al. 2012; Gomes et al. 2013).

Mixing NaOCl with EDTA causes a reduction in the pH of NaOCl, this reduction

is accompanied by a chemical reaction that involves the release of chlorine gas, which has been found to have a hazardous effect (Baumgartner & Ibay 1987). When mixing EDTA or citric acid with NaOCl, the antibacterial effect of the NaOCl is abolished (Zehnder et al. 2005). It has also been found that when combining CHX with EDTA, a precipitate of salts is formed, which advocates the use of each solution alone as a separate step during the final irrigation protocol (Rossi-Fedele et al. 2012). To overcome these complications of mixing irrigants, several products were made to facilitate sufficient smear layer removal and disinfection as a final irrigant; the most common used solutions available are Smear Clear and QMix (Stojicic et al. 2012; Haapasalo et al. 2010).

Smear Clear is an irrigant composed of 17% EDTA and Cetrimide (da Silva et al. 2008), in an *in vitro* study comparing its efficacy against *Enterococcus faecalis* biofilms with other irrigants, it showed less efficacy than 6% NaOCl, however, its antibacterial efficacy against the biofilms was significantly higher than CHX (Dunavant et al. 2006).

QMix is a novel irrigant that was introduced to the field of endodontics, its antibacterial efficacy and its ability to remove smear layer was tested *in vitro*, the results showed that its antibacterial activity was comparable to that of 2% and even 6% NaOCl (Ma 2011; Wang 2012). Moreover, it removes smear layer as well as 17% EDTA (Dai et al. 2011; Stojicic et al. 2012).

1.4 Intracanal Medicaments

Phenol and formaldehyde medicaments have been used for a long time as intracanal medicaments. However, aside from camphorated and paramonochlorophenol

being less effective *in vivo* compared to the results obtained *in vitro* (Byström et al. 1985), it has been well documented that formaldehyde and phenol containing medicaments are potentially mutagenic and carcinogenic (Chong & Ford 1992). Therefore, the interest in using phenolic compounds in endodontics is sharply declining.

An ideal intracanal medicament would be one that has a wide spectrum antibacterial effect, is non-toxic, does not irritate vital structures, has no systemic effect, is easy to administer, penetrates well into the dentinal tubules, and is easily removed from the root canal space (Martin 1979).

Calcium hydroxide ($Ca(OH)_2$) has been the most popular intracanal medicament in endodontics ever since it was introduced (Hermann 1920). $Ca(OH)_2$ is known to have a high pH which is around 12.5, most bacteria cannot survive such high alkalinity, which may be a key factor for the antibacterial property of $Ca(OH)_2$. Several mechanisms of actions have been proposed for the antibacterial effect of $Ca(OH)_2$ (Martin 1979; Foreman 1990; Sjögren 1991; Siqueira 1999). One mechanism of action is related to the high pH of $Ca(OH)_2$, it breaks down ionic bonds that are important for the structure of proteins which leads to their denaturation (Siqueira & Lopes 1999). Two other actions are related to the hydroxyl ions, the lipid peroxidation induced by these ions gives $Ca(OH)_2$ the ability to cause damage to the bacterial cytoplasmic membrane through destruction of the phospholipids found in the cellular membrane (Siqueira & Lopes 1999). Moreover, the hydroxyl ions have the capability to split strands of DNA causing damage to them, compromising cell replication and other cell activities (Siqueira & Lopes 1999).

Another major advantage of using $Ca(OH)_2$ is its effect on bacterial lipopolysaccharides (LPS). LPS stimulates release of cytokines from human cells causing activation of osteoclasts. In an *in vitro* study, it was shown that $Ca(OH)_2$ has the ability to detoxify LPS (Safavi & Nichols 1993). Another major advantage of using $Ca(OH)_2$ is its ability to dissolve necrotic tissues (Foreman & Barnes 1990).

In the literature, the ideal duration of $Ca(OH)_2$ application is controversial. In one study, it was found that dressing the canals with $Ca(OH)_2$ for seven days effectively eliminated bacteria that survived the chemomechanical preparation (Sjögren & Figdor 1991). In another study, where dentin blocks were infected *in vitro* with *E. faecalis*, it was shown that exposure to $Ca(OH)_2$ for at least ten days was required to disinfect the dentinal tubules (Orstavik & Haapasalo 1990). Another study tested the effectiveness of $Ca(OH)_2$ and recommended for it to be applied for 15 to 30 days, the authors stated that application of the medicament for seven days was not sufficient and suggested for it to be applied for a more extended period (Leonardo et al. 2006).

CHX is commercially available as a 2% gel to be used as an intracanal medicament (Gomes et al. 2013). Although it shows superior antibacterial capability compared to that of the $Ca(OH)_2$ (de Souza-Filho et al. 2008), its main drawbacks are that it does not provide a physical barrier within the canal against recolonization and it does not have the ability to detoxify endotoxins as $Ca(OH)_2$ does (Sinha et al. 2013).

1.5 Other Antibacterial Agents

Iodine (I) compounds have also been tested for their antibacterial efficacy. It has been found that iodine is ineffective when used alone, however, iodine potassium iodide (IKI) has antibacterial activity. Two of its disadvantages are that it does not have tissue dissolving capability, and some patients may be allergic to some iodine compounds (Block 1991; Molander et al. 1999; Haapasalo et al. 2010). Nevertheless, IKI has been found to be very well tolerated by tissues (Martin 1979).

Cetrimide (CTR) is a surfactant with bactericidal activity, it is cationic and is known to reduce the surface tension of solutions (Arias-Moliz et al. 2010; Guerreiro-Tanomaru et al. 2014; Claudio et al. 2014). CTR is also known to have a prolonged residual effect (Baca et al. 2012). In an *in vitro* study, CTR was tested in combination with different chelating agents, the results showed that it reduced the regrowth ability of *E. faecalis* for up to 60 days. However, the data in the same study showed that the peak of CTR activity occurred between 15 to 20 days (Ferrer-Luque et al. 2012).

Other agents available to be used as intracanal medicaments are products containing different combinations of antibiotics and steroids such as Ledermix paste (Haupt Pharma GmbH, Wolfratshausen, Germany), Odontopaste (Australian Dental Manufacturing, Brisbane, Australia), and Doxypaste (Ozdent, Castle Hill, Australia) (Chen et al. 2012). Ledermix is a combination of a synthetic steroid (triamcinolone acetonide) and an antibiotic (demeclocycline); which belongs to the tetracycline group of antibiotics (Kaufman et al. 2014). Odontopaste contains clindamycin hydrochloride and triamcinolone acetonide (Chen et al. 2012). Doxypaste contains doxycycline (belongs to the tetracycline group of antibiotics), and triamcinolone acetonide (Chen et al. 2012).

Although these products are used locally, type one allergy to the tetracycline components of these pastes have been reported (Kaufman et al. 2014). Also a major concern to many patients is discoloration these products may cause to both mature and immature teeth (Kim et al. 2000; Chen et al. 2014).

Chapter 2: **Rationale and Hypotheses**

Bacteria and their byproducts inside the root canal system cause an inflammatory reaction in the periapical tissues leading to apical periodontitis. Microorganisms adhering to and accumulating on dentin surface and into the dentinal tubules of root canals as biofilms are the major target for disinfection during endodontic treatment. Recently, most of the research published has focused more on irrigants rather than intracanal interappointment medication. In clinical practice, it is not always possible to complete the root canal treatment in a single visit. In such cases it is preferred to place a medicament into the root canal space for its antibacterial activity. Therefore, detailed knowledge of the effectiveness of intracanal medicaments is needed to effectively eradicate the infection in the root canals.

Hypotheses: Microbes in aged biofilms grown from a mixture of oral bacteria are more resistant to the antimicrobial activity of calcium hydroxide than microbes in young biofilms. Biofilms are less resistant to calcium hydroxide combined with other antimicrobial agents than to pure calcium hydroxide.

Specific Aims of the Research:

(1) To evaluate the antimicrobial effect of short and long term calcium hydroxide dressing on young and aged biofilms, and

(2) To examine the antibacterial effect of three antimicrobial agents used alone and in different combinations on young and aged biofilms using different times of exposure.

Chapter 3: **Materials and Methods**

3.1 Disks Preparation

Collagen coated hydroxyapatite disks (C-HA) were prepared by coating sterile hydroxyapatite disks (0.38-inch diameter by 0.06-inch thickness; Clarkson Chromatography Products, Williamsport, PA) with bovine dermal type I collagen (10 μg/mL collagen in 0.012 N HCl in water) (Cohesion, Palo Alto, CA) overnight at 4°C in the wells of a 24-well tissue culture plate containing 2 mL of collagen solution.

3.2 Biofilm Preparation

Gingival plaque was collected from volunteers after obtaining informed consent (Human ethical study permission code: H12-02430) and mixed in Brain Heart Infusion (BHI) with optical density value of 0.08-0.10 (595 nm, 150 μL; polypropylene microtiter plates, Corning, NY). The prepared C-HA disks were rinsed in potassium buffered solution (PBS) for 1 minute, and then immersed in 1.8 mL BHI and 0.2 mL of the dispersed dental plaque solution in a 24-well plate. Biofilm was incubated under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, UK) at 37 °C for one and three weeks (Fig 3.1). Fresh medium was changed once every week for the aged biofilm.

3.3 Medicament Preparations

All antimicrobial medicaments used in this study were mixed fresh to standard concentrations throughout the study (Table 3.1). The basic agents (iodine potassium iodide, cetrimide, and their combination IKI+CTR) were prepared in 0.5% concentration

by diluting 4% IKI and 1% CTR in distilled water. $Ca(OH)_2$ powder was mixed in distilled water to a 1:2 ratio (w/v) to have the mix in a semi-liquid state. When combined with other agents, all tested medicaments were standardized to a 0.25% concentration.

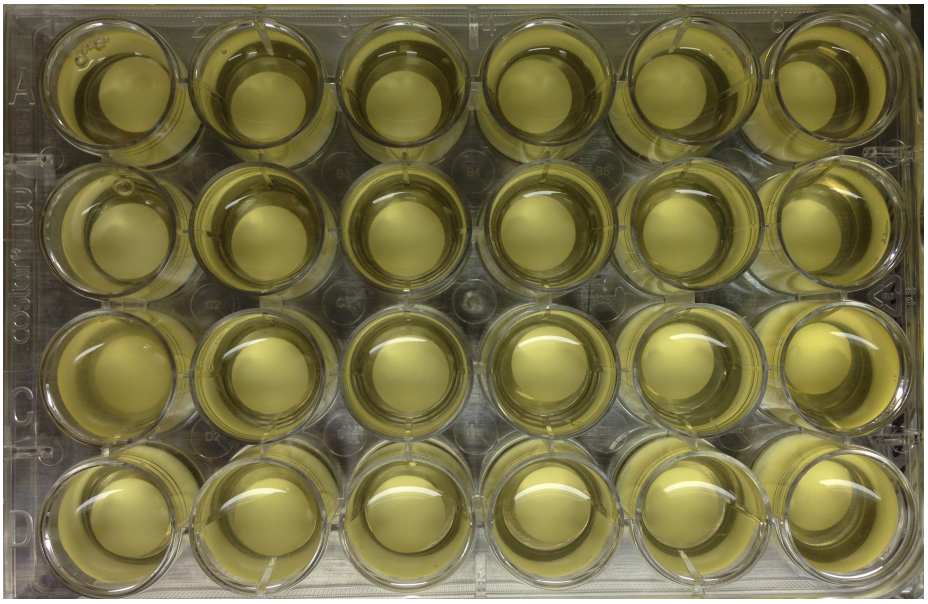


Figure 3.1 Collagen coated hydroxyapatite disks immersed in BHI broth with the suspended gingival plaque bacteria

Medicament	Mixing	Ratio
0.5% Iodine potassium iodide (IKI)	4% IKI + Distilled water	1 : 3
0.5% Cetrimide (CTR)	1% CTR + Distilled water	1 : 1
0.5% (IKI + CTR)	1% IKI + 1% CTR	1 : 1
$Ca(OH)_2$ + Distilled water	$Ca(OH)_2$ powder	1 : 2
$Ca(OH)_2$ + 0.25% IKI	$Ca(OH)_2$ + 0.5% IKI	1 : 1
$Ca(OH)_2$ + 0.25% CTR	$Ca(OH)_2$ + 0.5% CTR	1 : 1
$Ca(OH)_2$ + 0.25% (IKI + CTR)	$Ca(OH)_2$ + 0.5% (IKI + CTR)	1 : 1

Table 3.1 Medicament concentrations and mixing ratios

3.4 Antibiofilm Treatment

Young (one week) and aged biofilms (three weeks) were used to measure the antimicrobial efficiency of the medicaments and their combinations. Two disks were used for each group. The untreated biofilm on C-HA disks were used as negative control, and each medicament (total of seven medicaments shown in Table 3.1) were tested on both young and aged biofilms for different treatment durations. The biofilms were exposed to the medicaments for 24 hours, one week, and two weeks, a total of six groups per tested medicament. Untreated C-HA disks were immersed in the prepared medicaments in a 24-well tissue culture plate (Fig. 3.2). All treatments were incubated at 37 °C under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, UK).

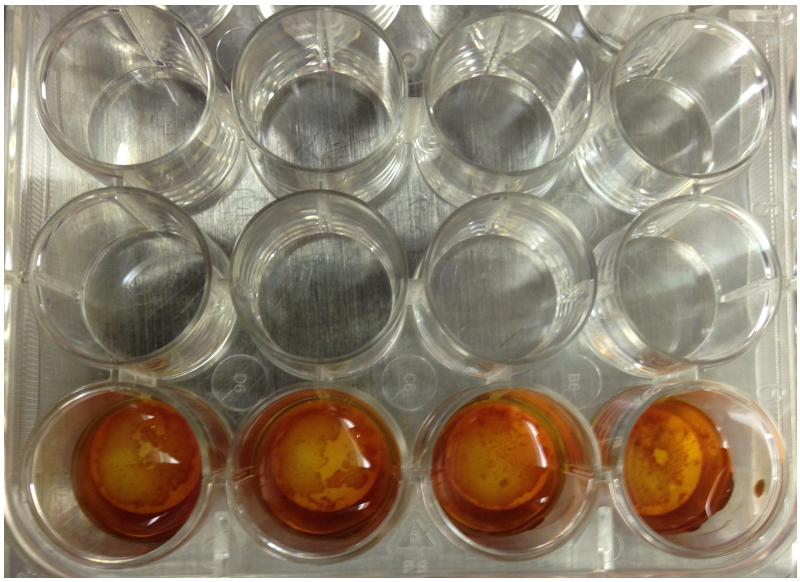


Figure 3.2 Biofilms on collagen coated hydroxyapatite disks immersed in the iodine potassium iodide solution

3.5 Confocal Laser Scanning Microscopy (CLSM)

The biofilms were first exposed to the antimicrobial solutions or pastes for the indicated time periods and gently rinsed with 0.85% NaCl for one minute (Fig 3.3) to remove the medicament before viability staining and confocal microscopy. The disks were then stained using LIVE/DEAD Backlight Bacterial Viability kit (Molecular Probes, Eugene, OR) for microscopy and quantitative essays. The kit includes separate vials of two dyes, SYTO 9 (excitation/emission maxima 480/500 nm) and propidium iodide (excitation/emission maxima 490/635 nm), which were mixed according to manufacturer's instructions. Using a stratified sampling technique to increase the precision of the selection method (Jensen 1991), the areas to be scanned were selected before turning the fluorescence on to avoid any bias in the process of selection in favor of red or green images. Simultaneous dual-channel imaging, green (live) and red (dead) fluorescence from stained cells were viewed using CLSM (Nikon Eclipse C1; Nikon Canada, Mississauga, ON). The images were acquired by the software EZ-C1 v. 3.40 build 691 (Nikon) at a resolution of 512 x 512 pixels. The mounted specimens were observed using 10x lens that covers an area of 1.64 mm^2 per field of view, images of five areas per disk were obtained resulting in a total of ten areas of images per group (Fig 3.4).

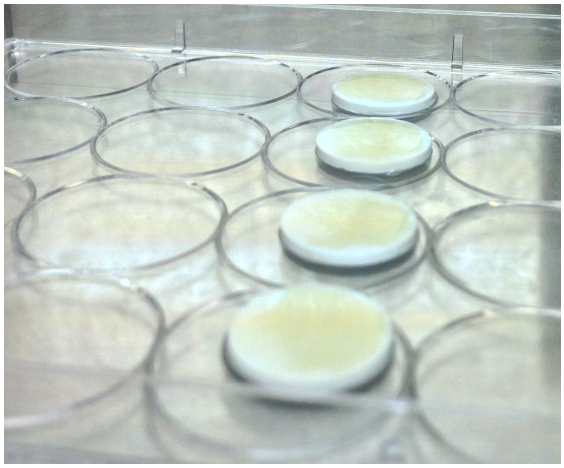


Figure 3.3 Biofilms disks after exposure to an antibacterial substance and rinsing with 0.85% NaCl and before staining with LIVE/DEAD viability stain

3.6 CLSM Images Analysis, Data Interpretation, and Three-Dimensional (3D) Reconstruction

Images obtained from the CLSM were analyzed using Imaris 7.2 software (Bitplane Inc, St Paul, MN). The mean volume of green and red fluorescence was obtained to represent the mean volume of live and dead cells. The biofilm thickness was also calculated from the number of stacks of the images. A 3D biofilm structure was reconstructed using the same software.

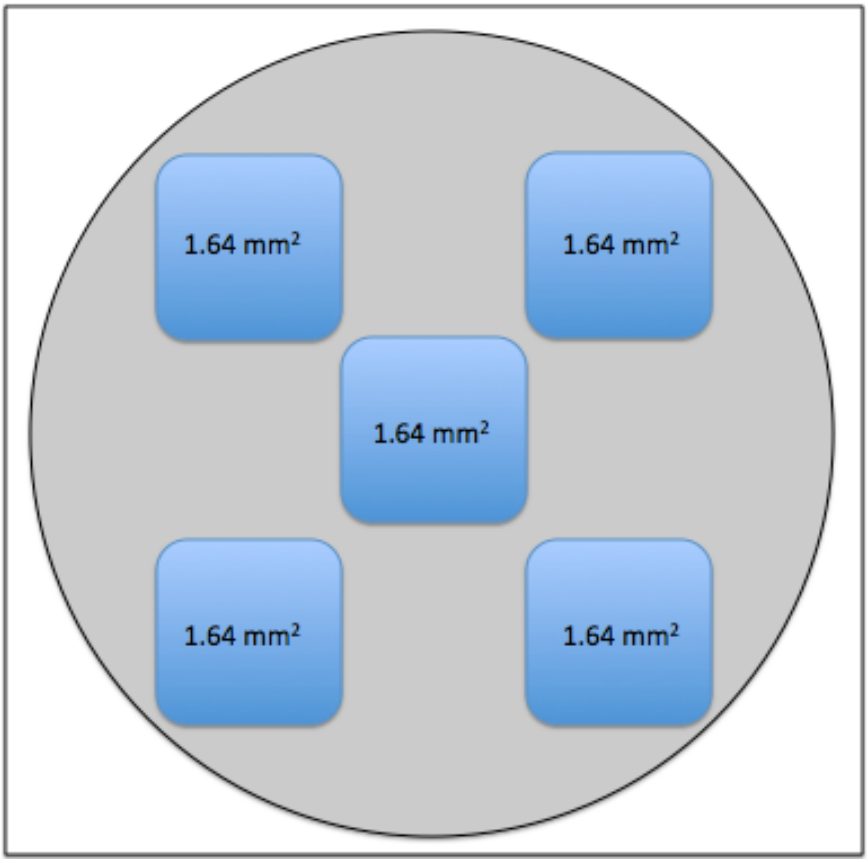


Figure 3.4 Illustration showing the disk (in grey) and the five fields imaged (in blue) using CLSM

3.7 Data Calculations and Statistical Analysis

The percentage of dead cells was calculated from the green and red volumes obtained from CLSM images via Imaris, the percentage of dead cell volume in each group was used to quantify the antimicrobial effect of each dressing, which was calculated for all ten stacks of images obtained in each group. Average volumes and standard deviations were calculated for all the groups.

Using SPSS V.21 (SPSS INC. Chicago, IL), Kolmogorov–Smirnov test and Q-Q plots for the data showed that they were not normally distributed, and non-parametric

tests were performed to test for significance. Kruskal-Wallis was done between groups with dependent variables (for each group comparing different intervals of application), and Mann-Whitney *U* test was done to compare independent variables (between different dressing groups), results were compared at a significant level of $P<0.05$.

Chapter 4: **Results**

4.1 Effectiveness of different antimicrobial regimens, general findings

The mean percentage of dead cells (± S.D.) from the ten stacks of images analyzed for each group was tabulated (Table 4.1) and plotted (Figure 4.1). Kruskal Wallis and Mann-Whitney U tests showed statistically significant differences between one and three weeks old biofilm with regard to biofilm thickness (*P<0.05*). No statistically significant difference was found between the percentage of dead cells between the negative control groups (*P>0.05)*.

	Group	1 week old biofilm			3 weeks old biofilm		
		1 day treatment	1 week treatment	2 weeks treatment	1 day treatment	1 week treatment	2 weeks treatment
1	Contaminated C-HA	3.96% ± 2.42			4.97% ± 2.18		
2	$Ca(OH)_2$ + Distilled water	60.12% ± 5.46	68.57% ± 5.38	74.43% ± 3.12	52.37% ± 2.32	62.32% ± 1.67	69.89% ± 1.33
3	0.5% IKI	51.77% ± 3.35	56.81% ± 1.92	62.22% ± 0.99	43.07% ± 2.52	48.7% ± 2.10	55.05% ± 1.13
4	0.5% CTR	46.09% ± 4.29	56.2% ± 2.27	64.65% ± 2.78	39.19% ± 1.10	50.32% ± 0.88	56.58% ± 0.93
5	0.5% (IKI+CTR)	50.97% ± 2.06	59.31% ± 2.94	65.16% ± 2.64	41.04% ± 2.07	54.42% ± 1.40	59.31% ± 1.26
6	$Ca(OH)_2$ + 0.5% IKI	68.46% ± 6.09	73.13% ± 3.77	77.81% ± 1.62	58.64% ± 2.23	67.8% ± 1.01	71.43% ± 1.56
7	$Ca(OH)_2$ + 0.5% CTR	61.26% ± 3.92	72.8% ± 4.54	77.98% ± 3.95	54.55% ± 1.51	67.08% ± 1.04	71.35% ± 1.30
8	$Ca(OH)_2$ + 0.5% (IKI+CTR)	72.15% ± 6.56	79.93% ± 2.43	83.66% ± 1.25	61.96% ± 2.49	70% ± 1.80	78.12% ± 1.12

Table 4.1 Mean percentage of dead cells ± standard deviation

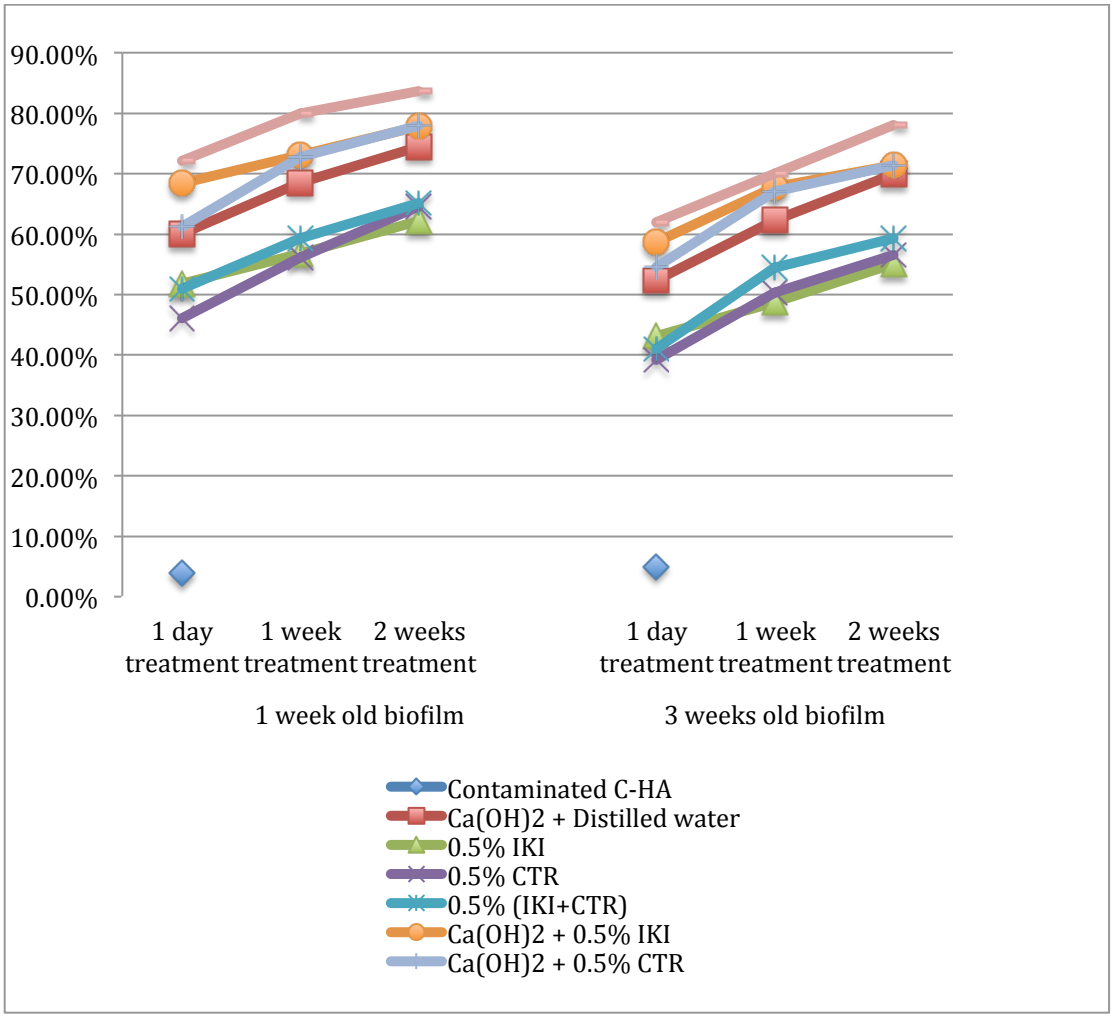


Figure 4.1 Mean percentage of dead cells

4.2 The effect of the medicaments against microbes in 1 and 3 week old biofilms

All treatment groups showed statistically significant increase in the percentage of dead cells with the increase of treatment duration ($P<0.05$), also statistical significance was seen between the one day treatment for the one and three weeks old biofilm ($P<0.05$), mean percentages for each group were plotted (figure 4.2-4.8).

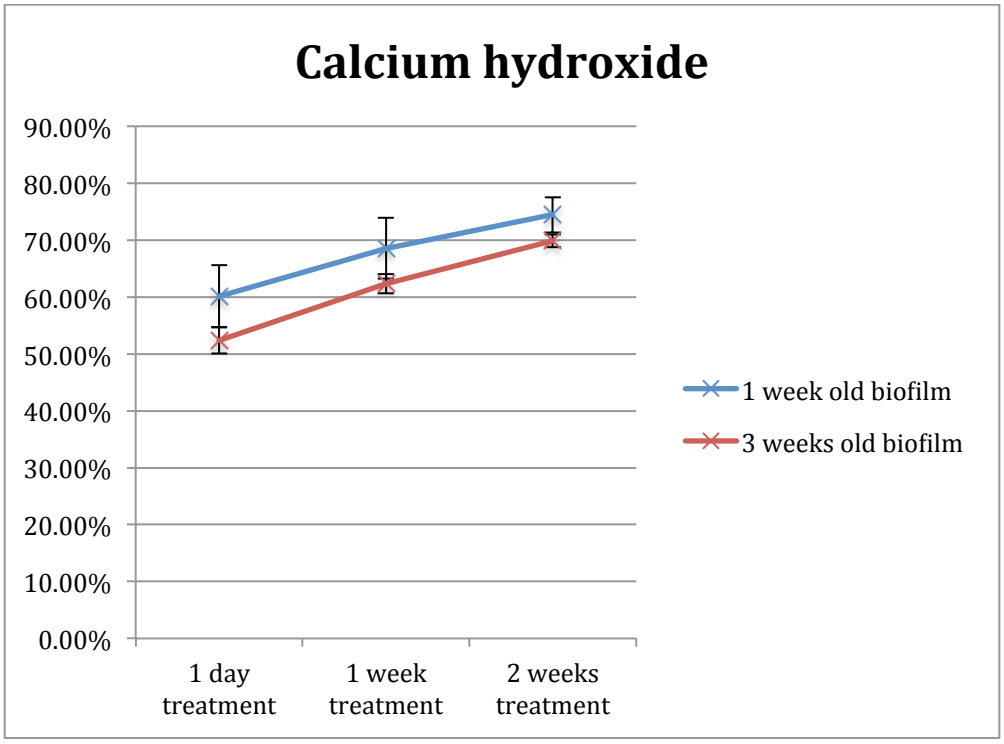


Figure 4.2 Proportion of killed microbes ± S.D. in one and three weeks old biofilms exposed to calcium hydroxide

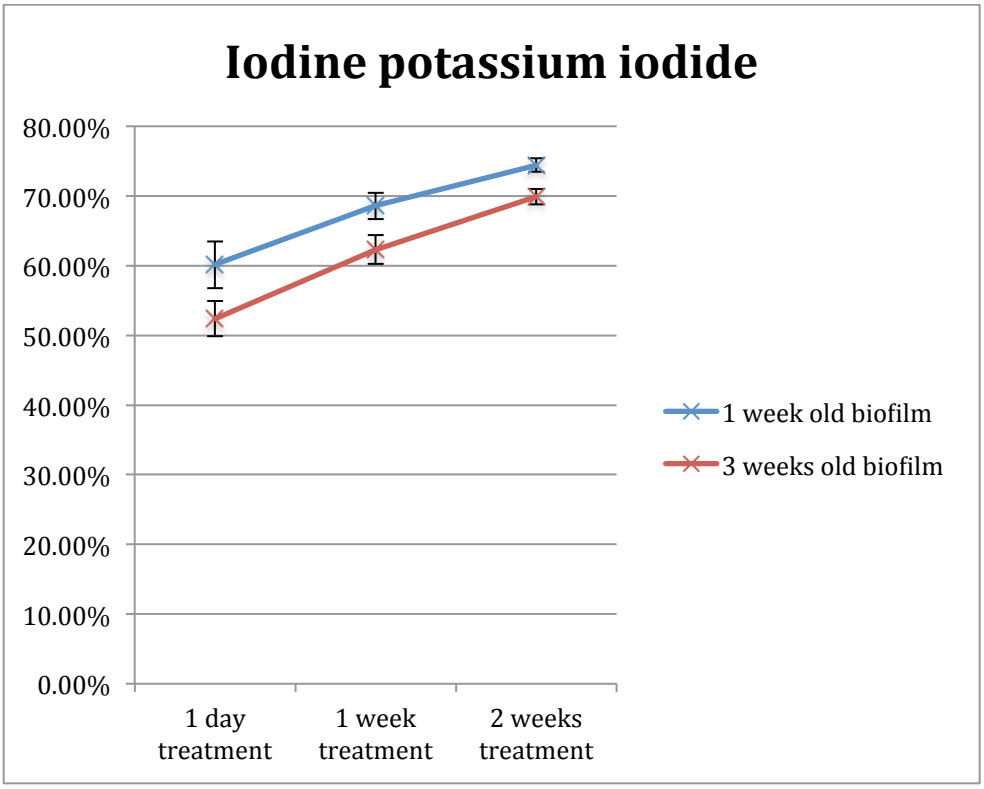


Figure 4.3 Proportion of killed microbes ± S.D. in one and three weeks old biofilms exposed to iodine potassium iodide

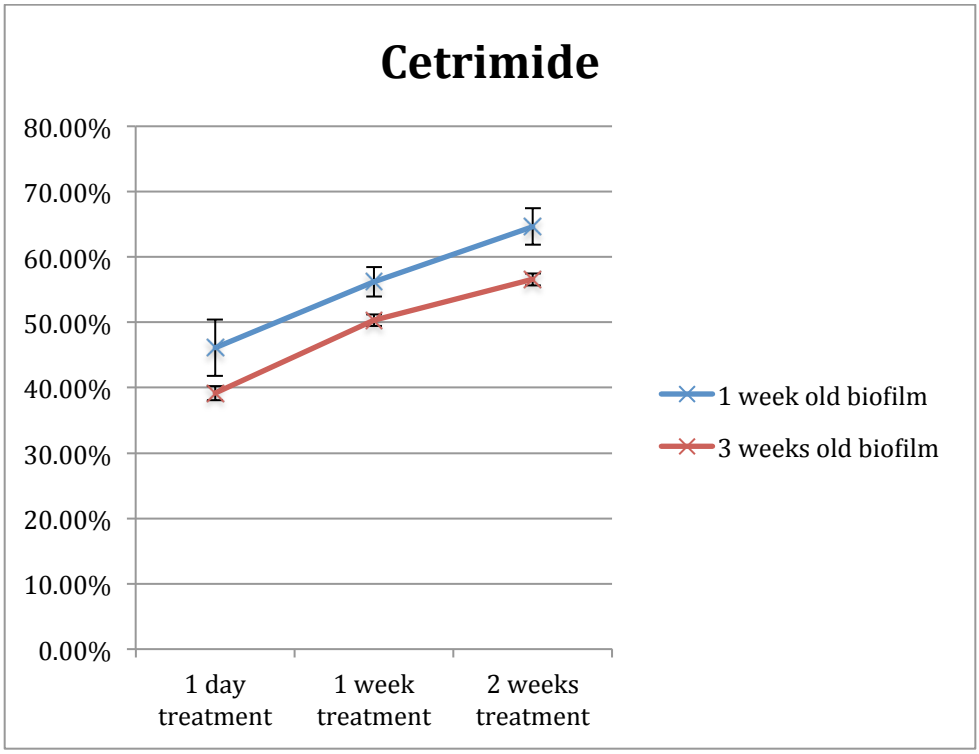


Figure 4.4 Proportion of killed microbes ± S.D. in one and three weeks old biofilms exposed to cetrimide

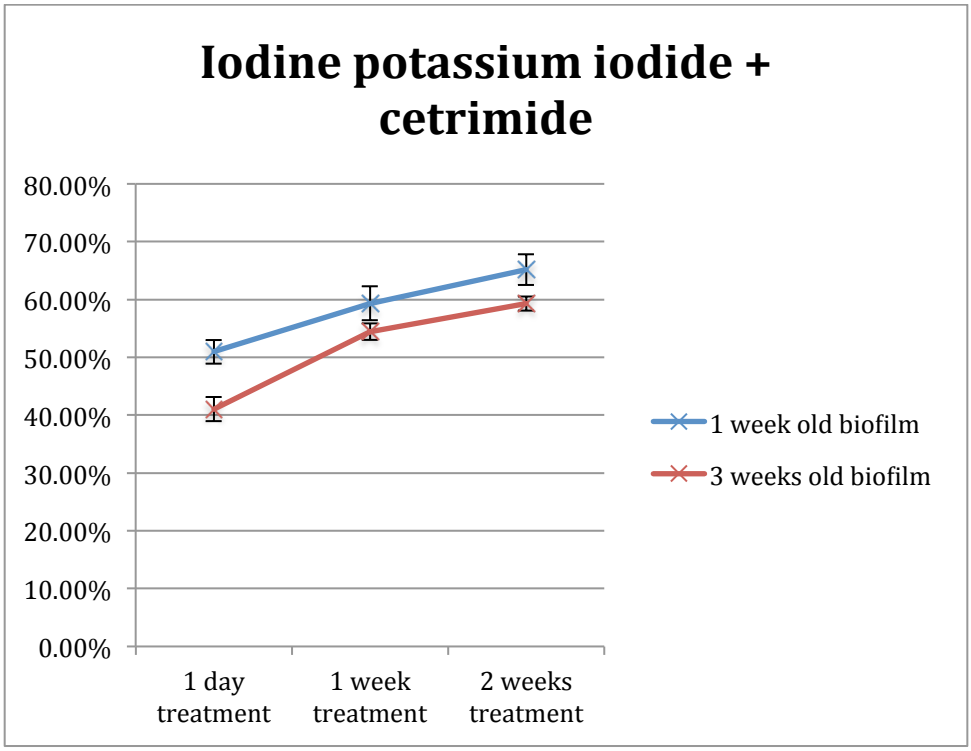


Figure 4.5 Proportion of killed microbes ± S.D. in one and three weeks old biofilms exposed to iodine potassium iodide + cetrimide

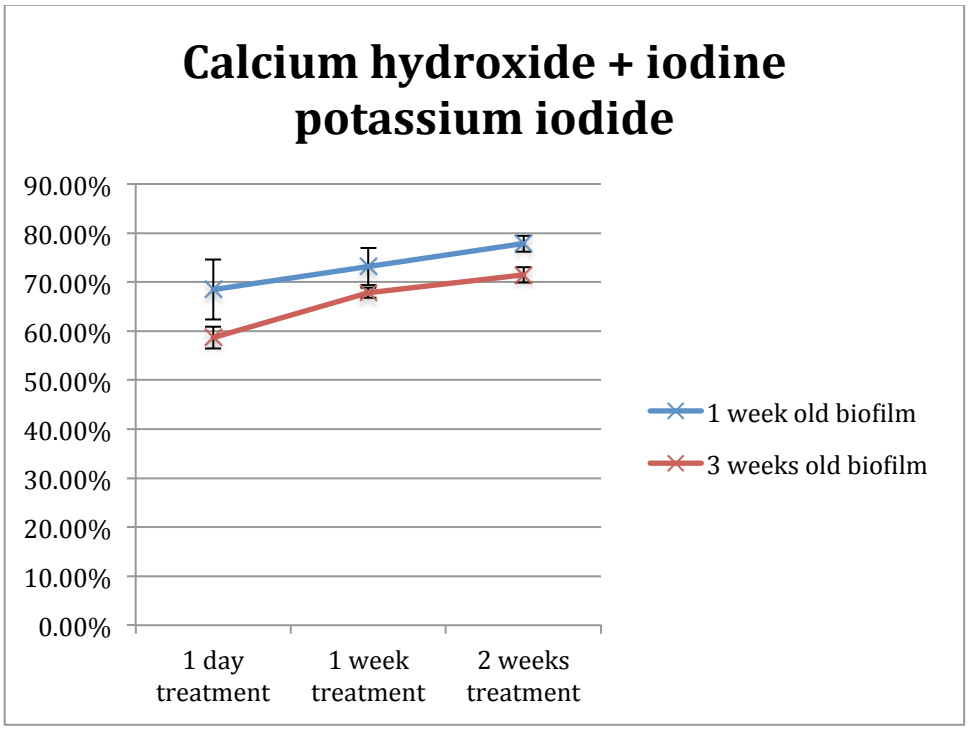


Figure 4.6 Proportion of killed microbes ± S.D. in one and three weeks old biofilms exposed to calcium hydroxide + iodine potassium iodide

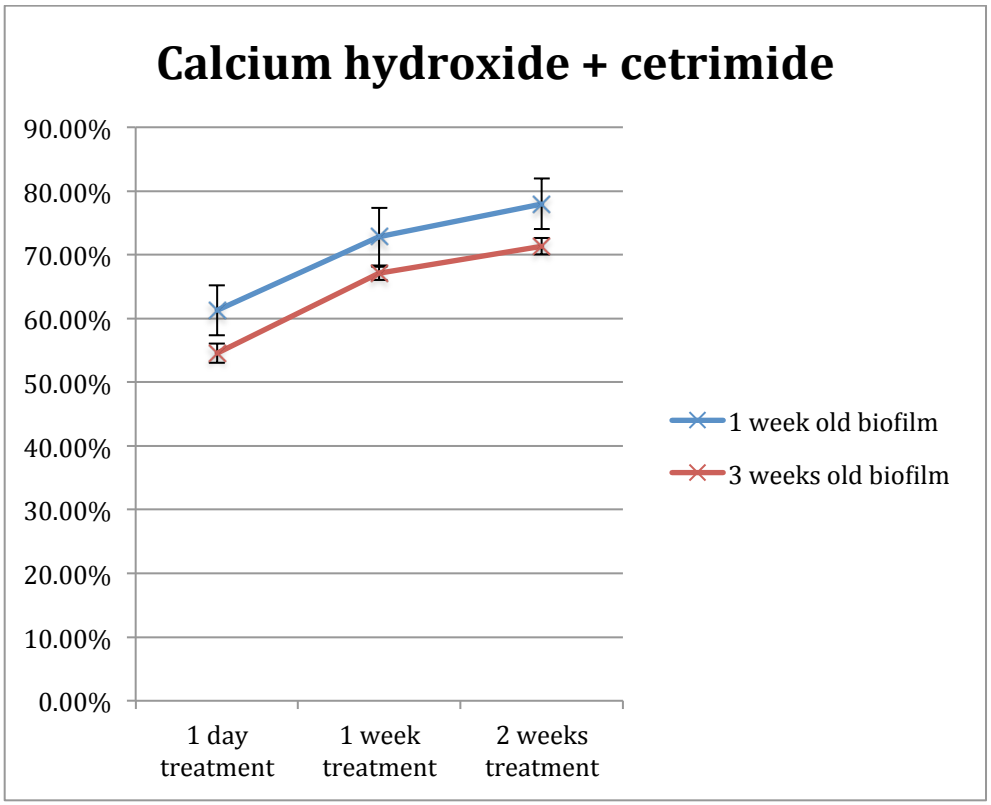


Figure 4.7 Proportion of killed microbes ± S.D. in one and three weeks old biofilms exposed to calcium hydroxide + cetrimide

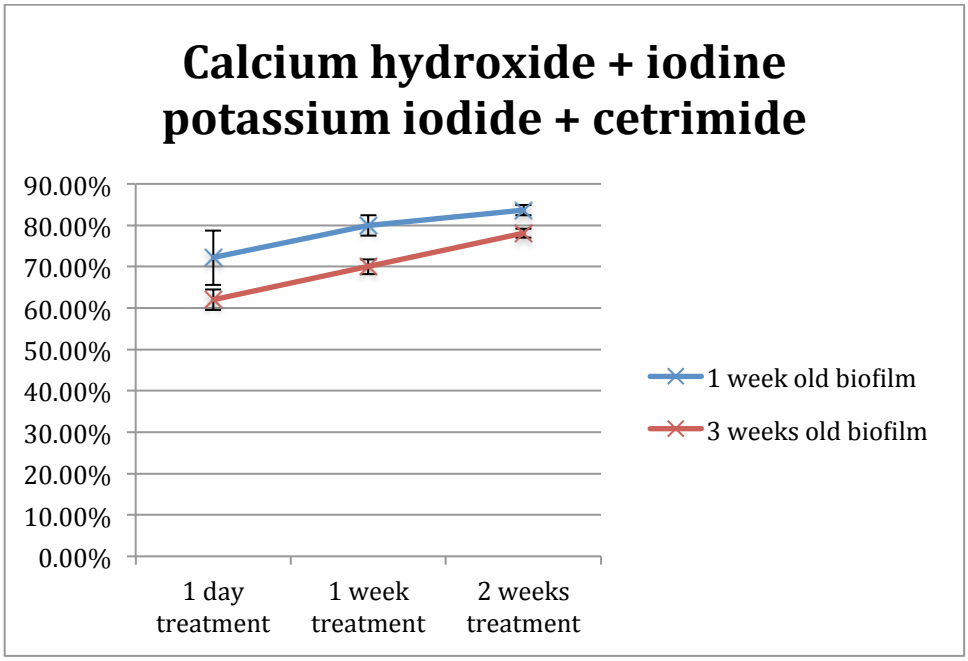


Figure 4.8 Proportion of killed microbes ± S.D. in one and three weeks old biofilms exposed to calcium hydroxide + iodine potassium iodide + cetrimide

4.3 Comparative effectiveness of the different medicaments against microbes in one week old biofilm

All medicament treatments showed significantly higher percentage of dead cells compared to the negative control after one day of medicament exposure ($P<0.05$). The combination of calcium hydroxide, iodine potassium iodide and cetrimide had the highest percentage of dead cells of all treatments, and the difference was statistically significant ($P<0.05$). No statistical significance was found between the percentage of dead cells between the iodine potassium iodide and the iodine potassium iodide + cetrimide groups and between the calcium hydroxide and the calcium hydroxide + cetrimide groups ($P>0.05$). The difference between all other groups was statistically significant ($P<0.05$), (Figure 4.9).

After one week of medicament exposure, all treatments again showed significantly

higher percentage of dead cells than the negative control ($P<0.05$). Calcium hydroxide + iodine potassium iodide + cetrimide had the highest percentage of dead cells, and the difference was statistically significant compared to all other treatment groups ($P<0.05$). No statistical significance was found between the percentage of dead cells in the iodine potassium iodide and the cetrimide, the calcium hydroxide and the calcium hydroxide + cetrimide groups, as well as in the calcium hydroxide + iodine potassium iodide and the calcium hydroxide + cetrimide groups ($P>0.05$). The difference between all other groups was statistically significant ($P<0.05$), (Figure 4.10).

After two weeks of medicament exposure, all treatments showed significantly higher percentage of dead cells compared to the negative control ($P<0.05$). Calcium hydroxide + iodine potassium iodide + cetrimide had the highest percentage of dead cells compared to all other treatment groups and the difference was statistically significant ($P<0.05$). No statistical significance was found between the percentage of dead cells in the cetrimide and the iodine potassium iodide + cetrimide as well as between the calcium hydroxide + iodine potassium iodide and the calcium hydroxide + cetrimide groups ($P>0.05$). The difference between all other groups was statistically significant ($P<0.05$), (Figure 4.11).

No significant difference was found when treating one week old biofilms with calcium hydroxide for two weeks compared to treatment with calcium hydroxide + iodine potassium iodide + cetrimide for one week ($P>0.05$), furthermore, no significant difference was found when treating one week old biofilms with calcium hydroxide for two weeks compared to treatment with calcium hydroxide + iodine potassium iodide + cetrimide for one day ($P>0.05$).

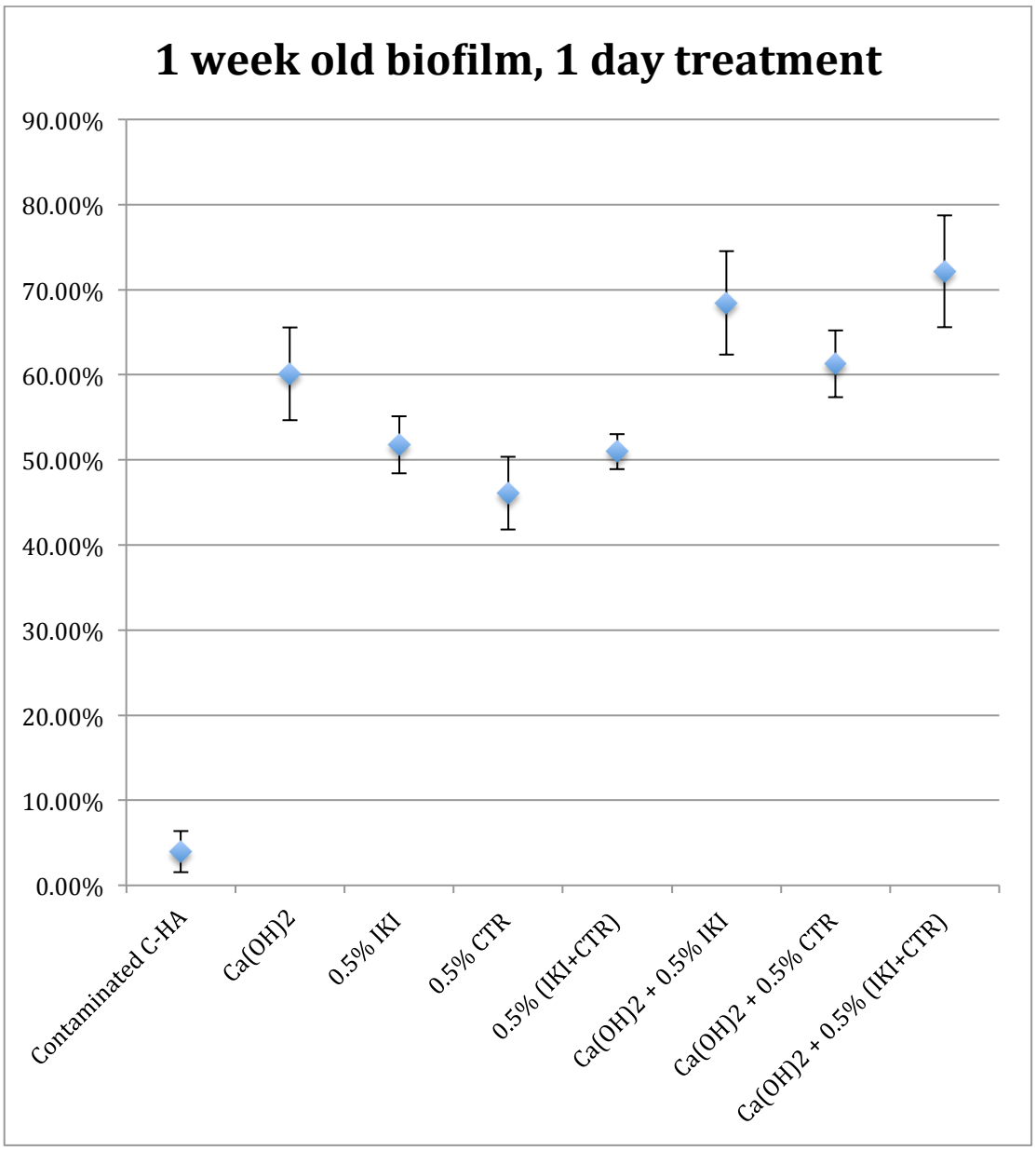


Figure 4.9 Effect of one day exposure to the medicaments against microbes in one week old biofilm ± S.D.

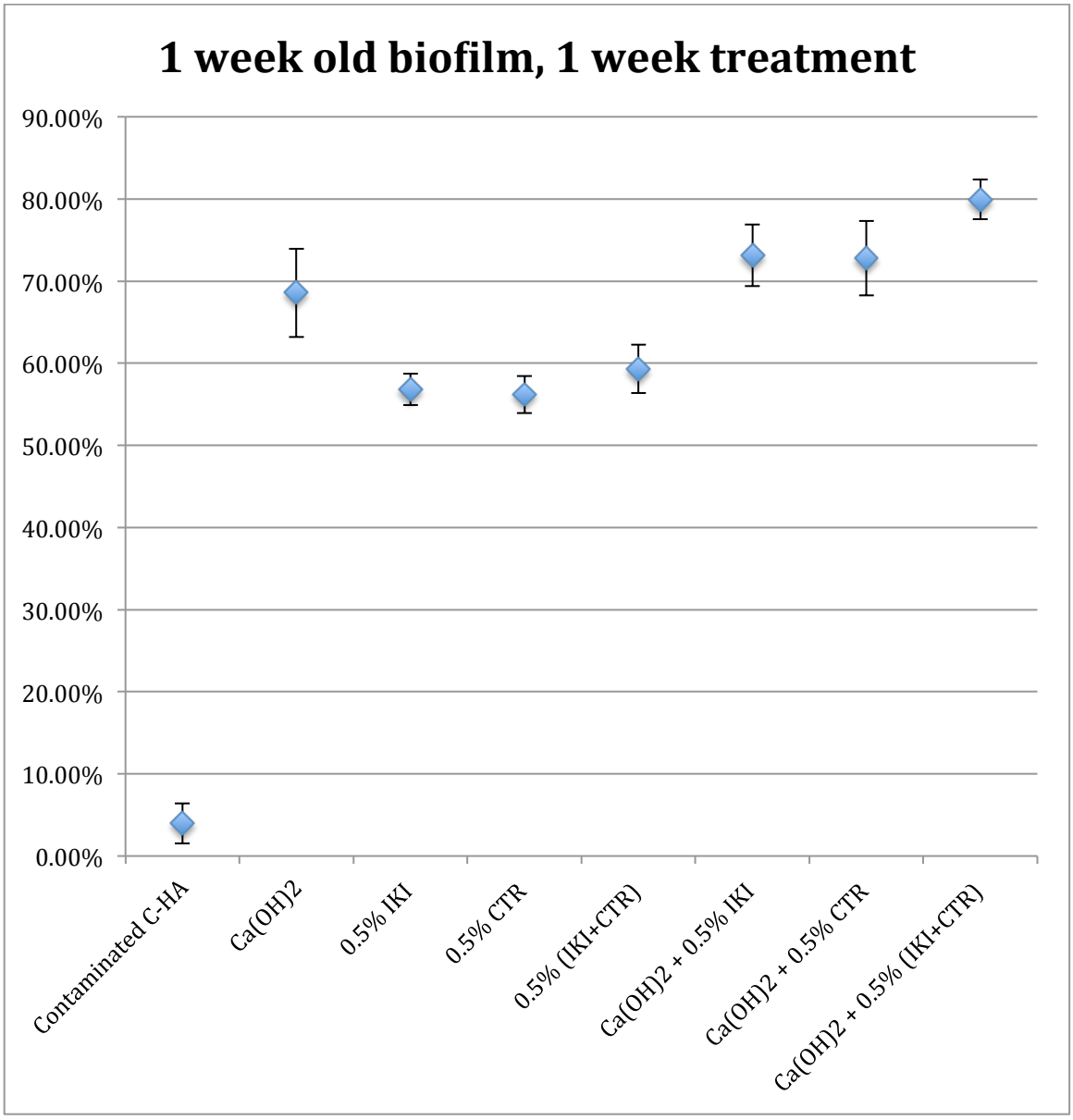


Figure 4.10 Effect of one week exposure to the medicaments against microbes in one week old biofilm ± S.D.

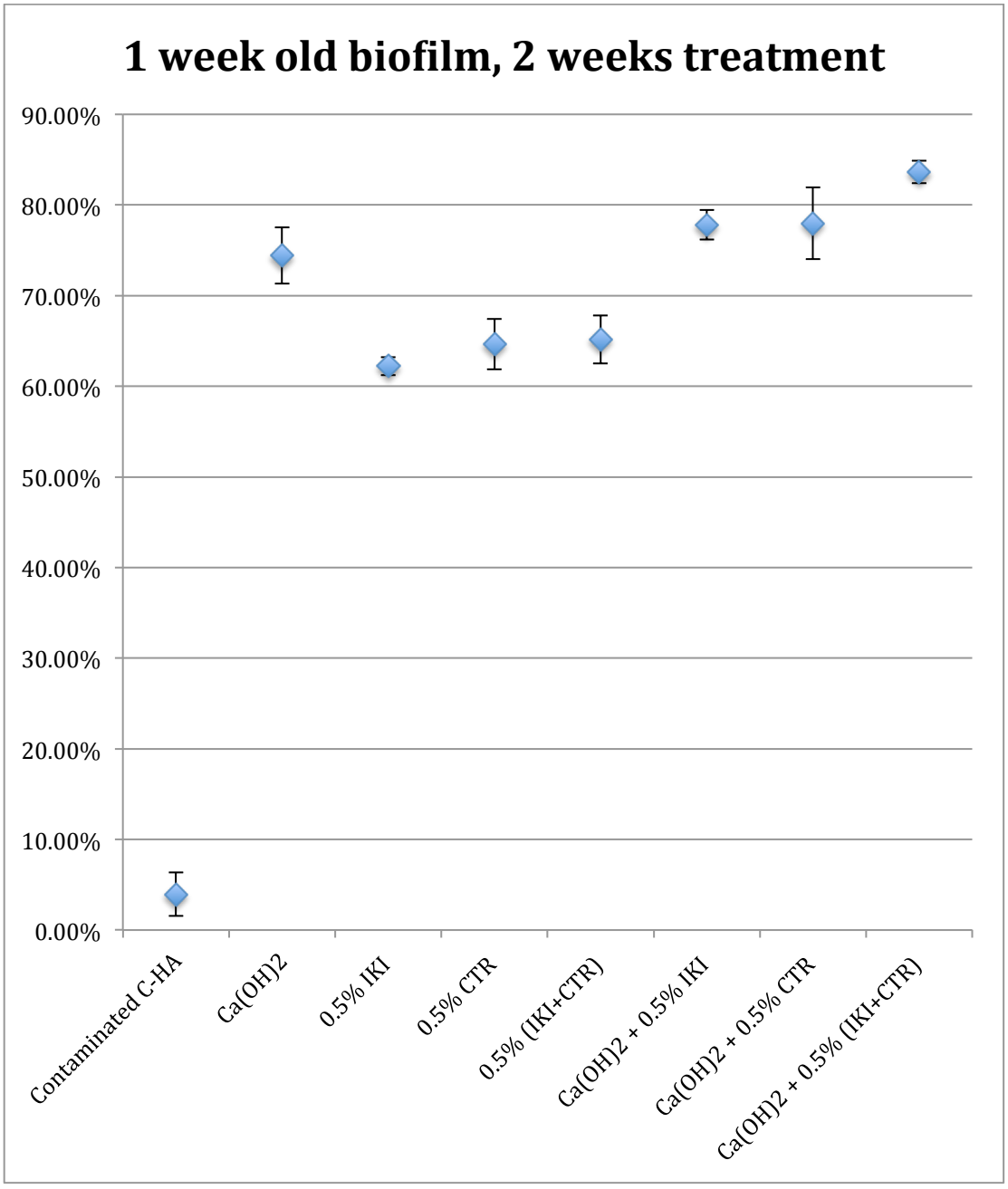


Figure 4.11 Effect of two weeks exposure to the medicaments against microbes in one week old biofilm ± S.D.

4.4 Comparative effectiveness of the different medicaments against microbes in three week old biofilm

In general, the results of medicament effect on microbes in three weeks old biofilms

were comparable to those seen in the one week old biofilm, except for that the percentage of dead cells for all treatments in the three weeks old biofilm was lower and the difference is statistically significant ($P<0.05$).

In experiments where the biofilms were exposed to the antibacterial compounds for one day, all tested treatments showed significantly higher percentage of dead cells compared to the negative control ($P<0.05$). Calcium hydroxide + iodine potassium iodide + cetrimide had the highest percentage of dead cells compared to other tested groups and the difference was statistically significant ($P<0.05$). The difference in the percentage of dead cells between all other groups was statistically significant ($P<0.05$), (Figure 4.12).

In the next set of experiments the biofilms were exposed to the same medicaments for one week, instead of one day. All treatments again showed statistically significant higher percentages of dead cells compared to the negative control ($P<0.05$). Calcium hydroxide + iodine potassium iodide + cetrimide had the highest percentage of dead cells compared to all other treatments tested, and the difference was statistically significant ($P<0.05$). No statistically significant difference was found between the percentage of dead cells in the iodine potassium iodide and the cetrimide, nor between the calcium hydroxide + iodine and the calcium hydroxide + cetrimide groups ($P>0.05$). The difference between all other groups was statistically significant ($P<0.05$), (Figure 4.13).

For the two-weeks treatment group, all treatments showed significantly higher percentage of dead cells compared to the negative control ($P<0.05$). Calcium hydroxide + iodine potassium iodide + cetrimide had the highest percentage of dead cells compared to the other tested treatments and the difference was statistically significant ($P<0.05$). No statistical significance was found between the percentage of dead cells between the

calcium hydroxide + iodine potassium iodide and the calcium hydroxide + cetrimide groups (*P>0.05*). The difference between all other groups was statistically significant (*P<0.05*), (Figure 4.14).

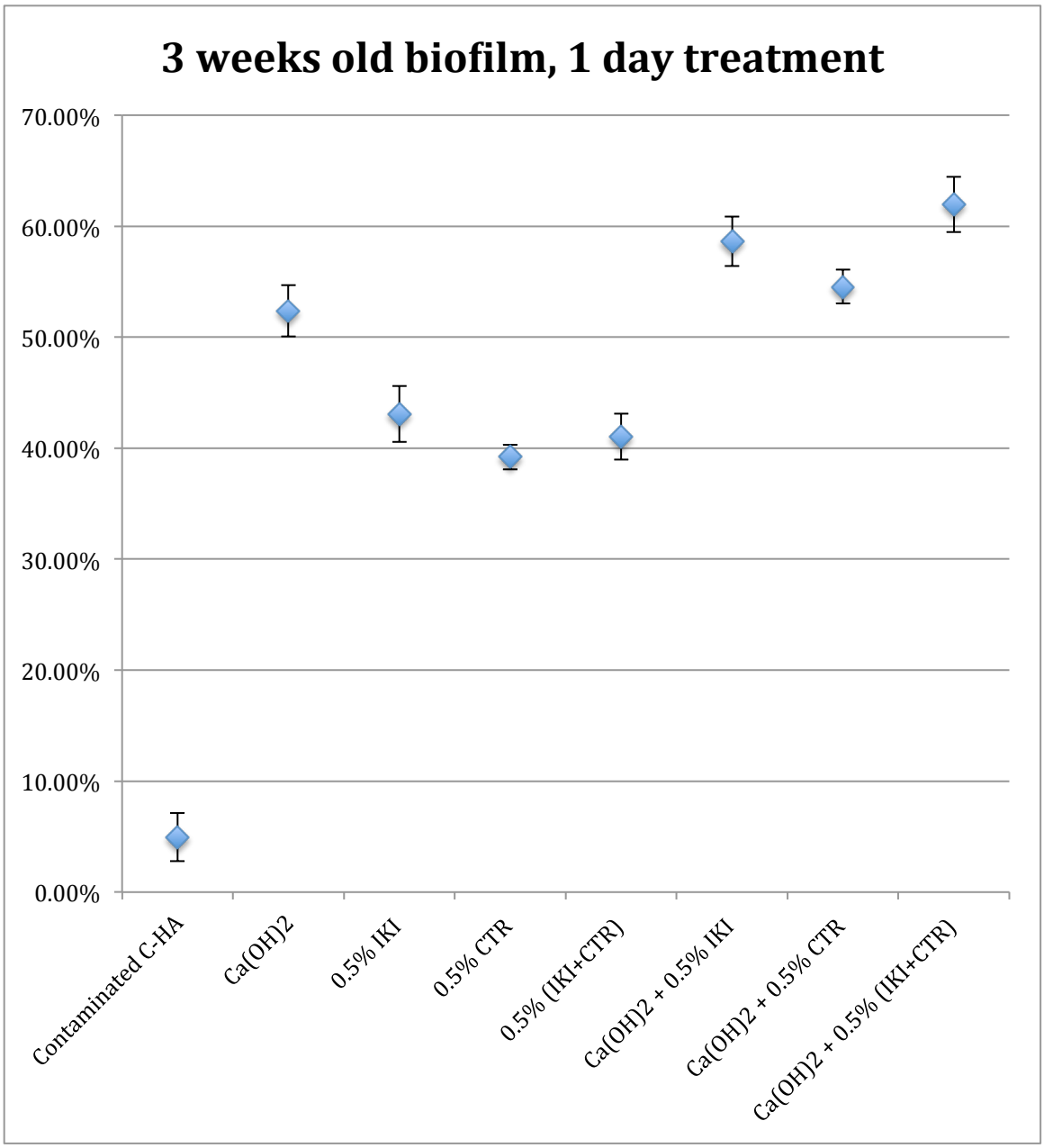


Figure 4.12 Effect of one day exposure of the medicaments against microbes in three weeks old biofilm ± S.D.

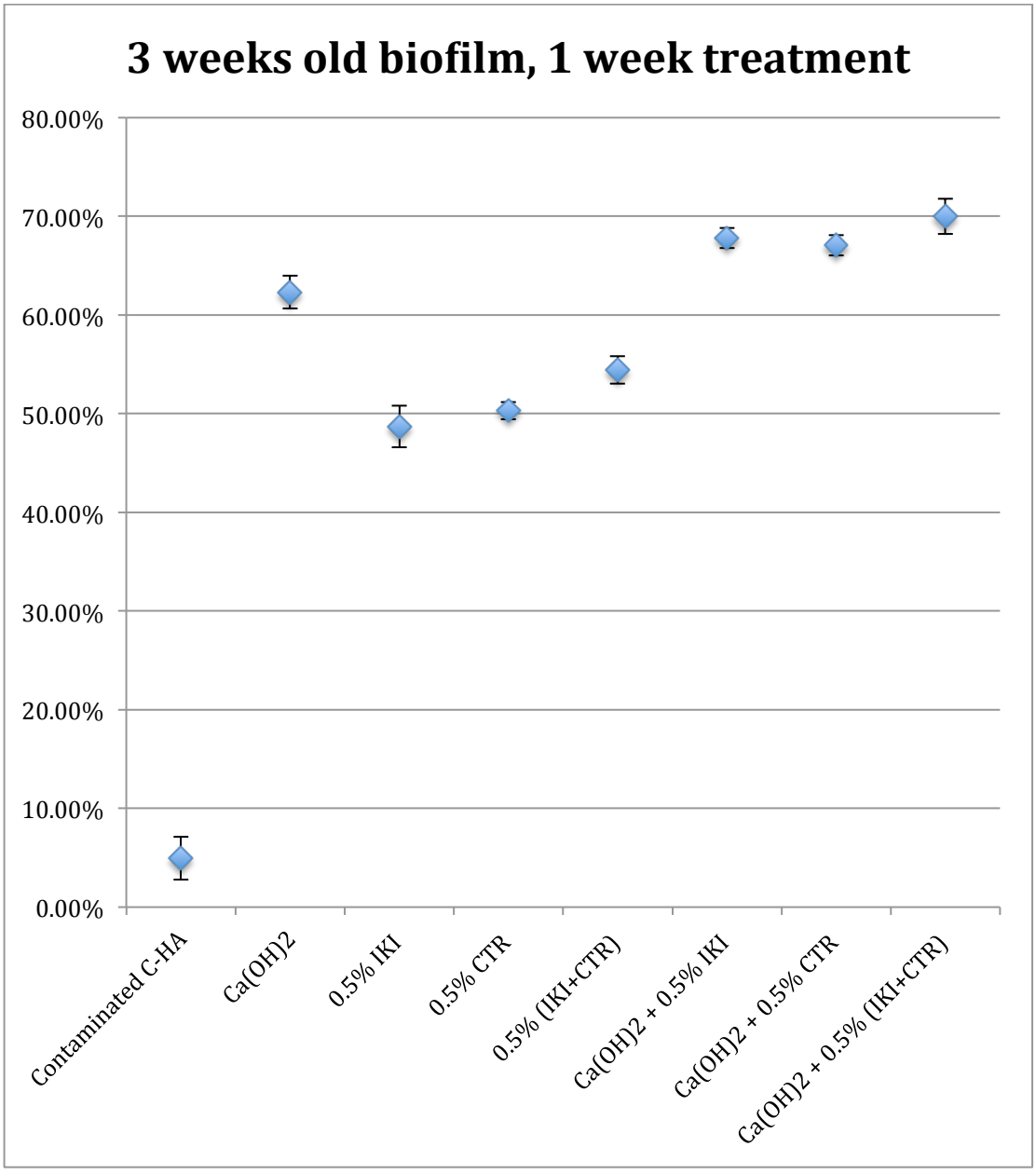


Figure 4.13 Effect of one week exposure of the medicaments against microbes in three weeks old biofilm ± S.D.

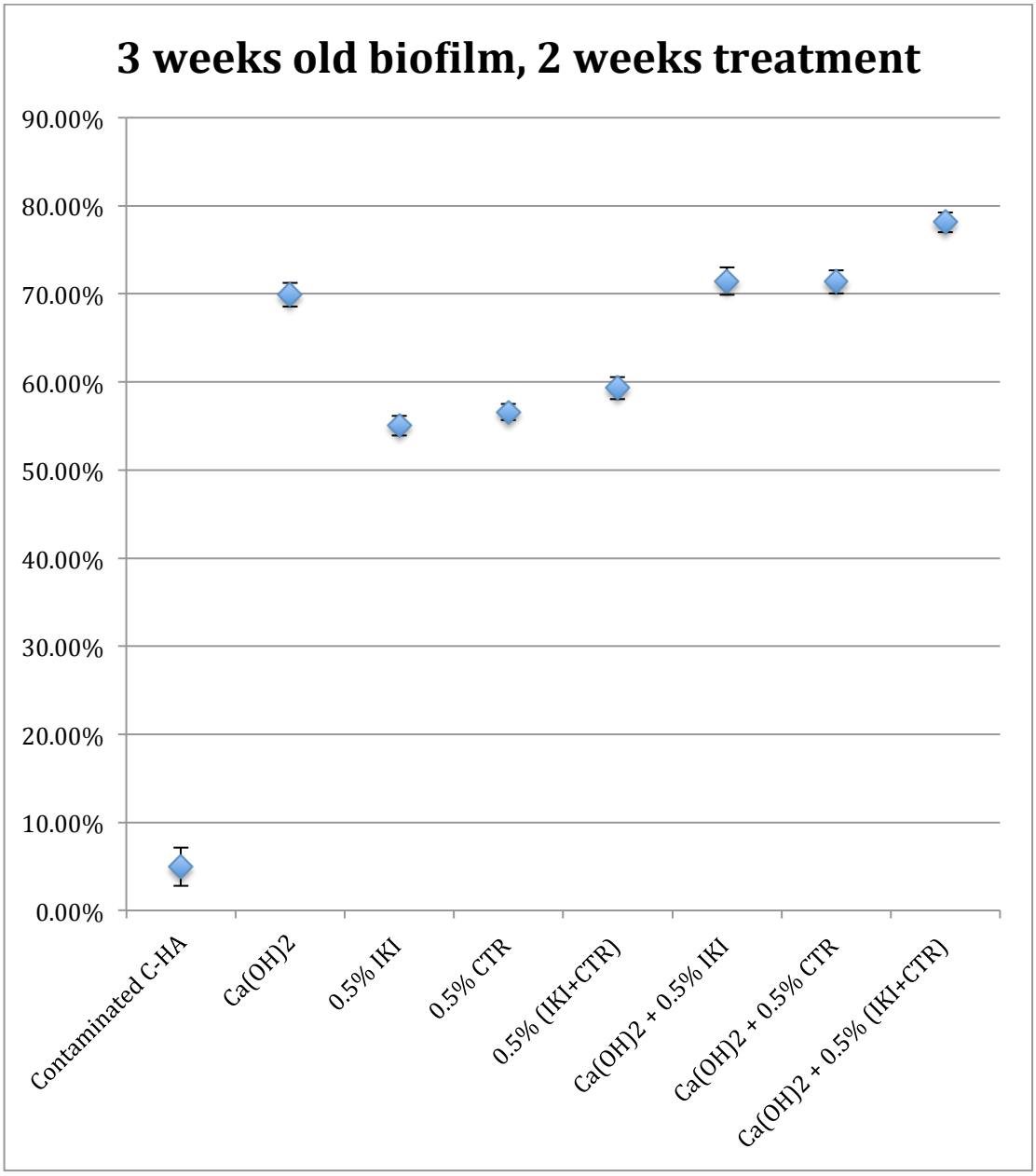
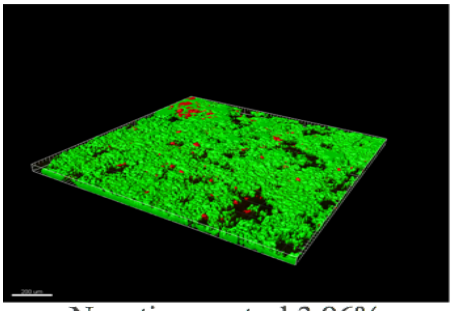


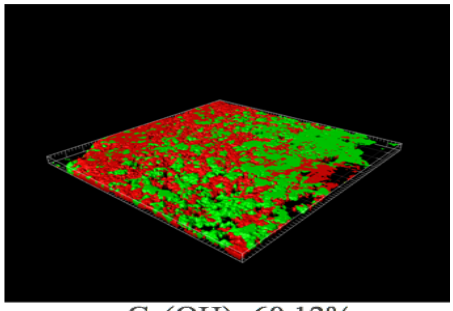
Figure 4.14 Effect of two weeks of exposure of the medicaments against microbes in three weeks old biofilm ± S.D.

4.5 3D reconstruction of the biofilms after viability staining and confocal laser scanning microscopy

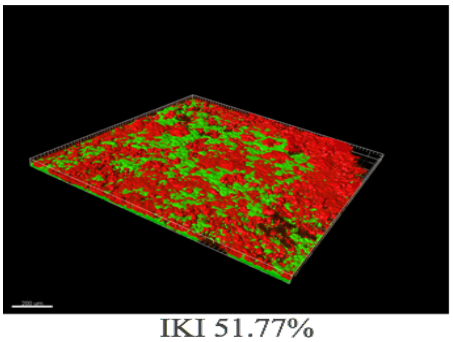
Micrographs from the CLSM were constructed into 3D images using the full stacks obtained from the scans. Areas which fluoresce green represent cells that have intact cell walls which is calculated as living cells or "viable biofilm volume", while the red fluorescence shows cells that have severe damage to their cell walls that was enough for the propidium iodide viability stain to enter the cell and give red fluorescence in CLSM scanning, representing dead cells or "dead biofilm volume". Images from the one-week-old biofilms exposed for one day to the antimicrobial substances are shown in (Figure 4.15), one week old biofilms exposed for one week are shown in (Figure 4.16), and one week old biofilms exposed for two weeks are shown in (Figure 4.17). The three weeks old biofilms exposed for one day is shown in (Figure 4.18), three weeks old biofilms exposed for one week is shown in (Figure 4.19), and the three weeks old biofilms exposed for two weeks is shown in (Figure 4.20).



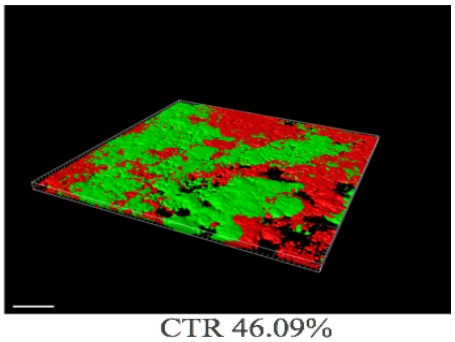
Negative control 3.96%



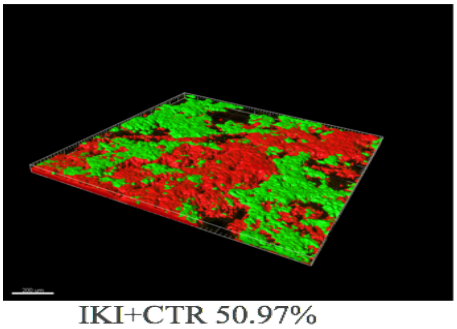
$Ca(OH)_2$ 60.12%



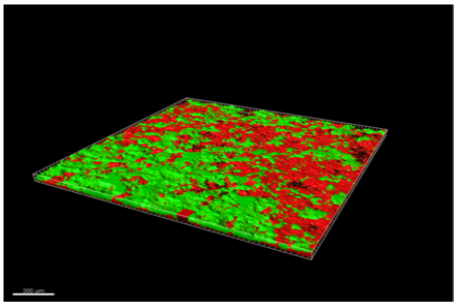
IKI 51.77%



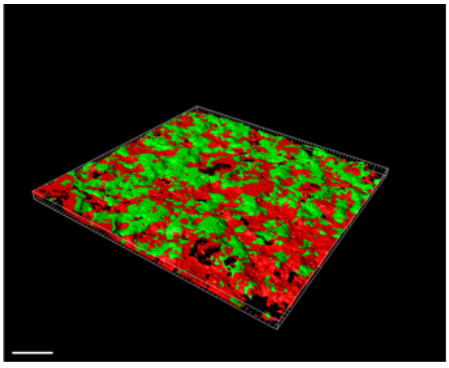
CTR 46.09%



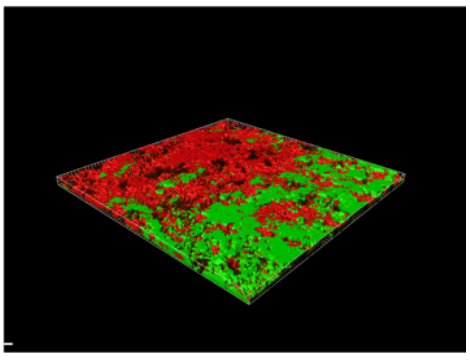
IKI+CTR 50.97%



$Ca(OH)_2$+IKI 68.46%



$Ca(OH)_2$+CTR 61.26%



$Ca(OH)_2$+IKI+CTR 72.15%

Figure 4.15 3D reconstruction of CLSM images of one week old biofilms exposed for one day. The medicament(s) used and the percentage of dead cell volume are shown

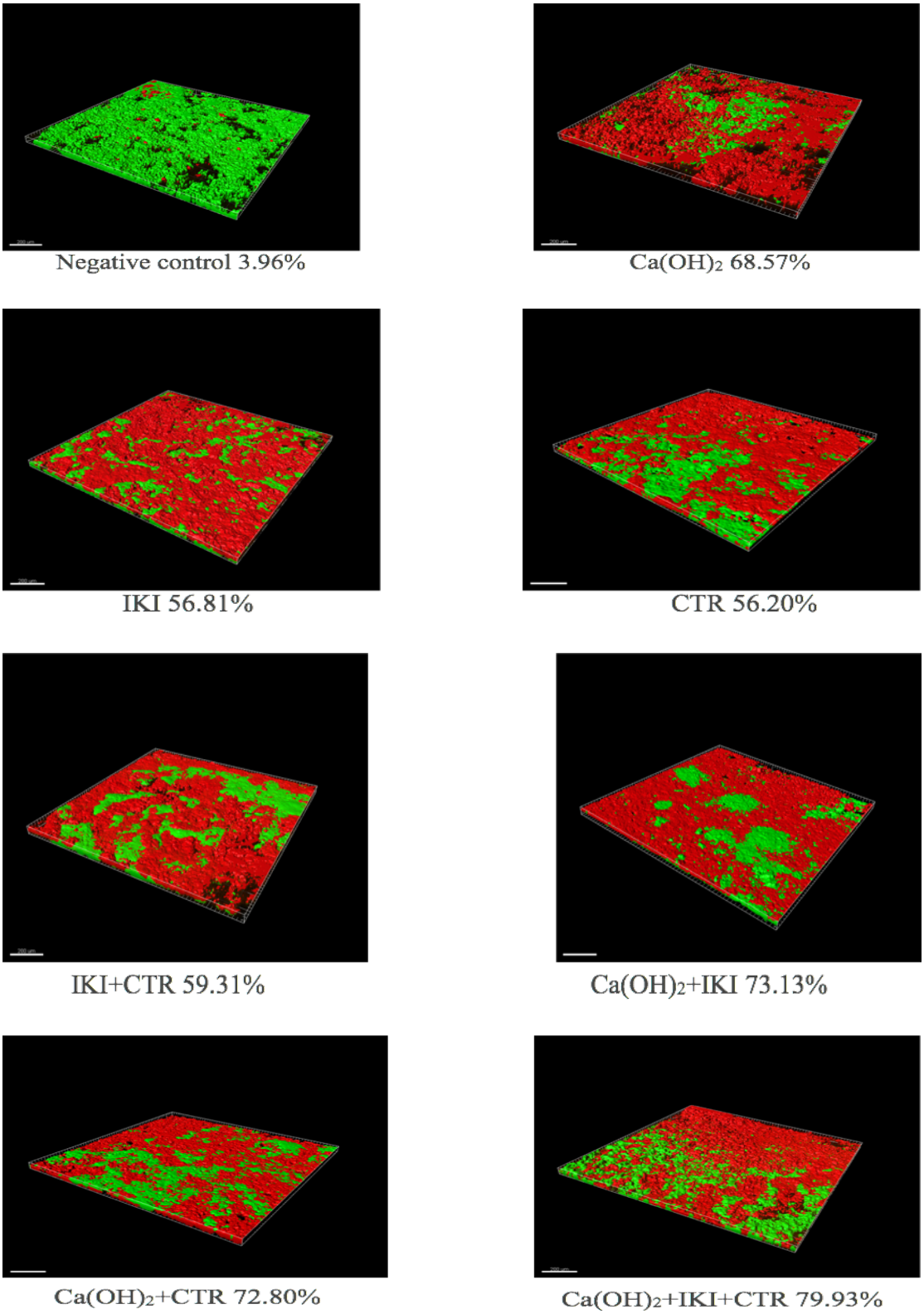


Figure 4.16 3D reconstruction of CLSM images of one week old biofilms exposed for one week. The medicament(s) used and the percentage of dead cell volume are shown

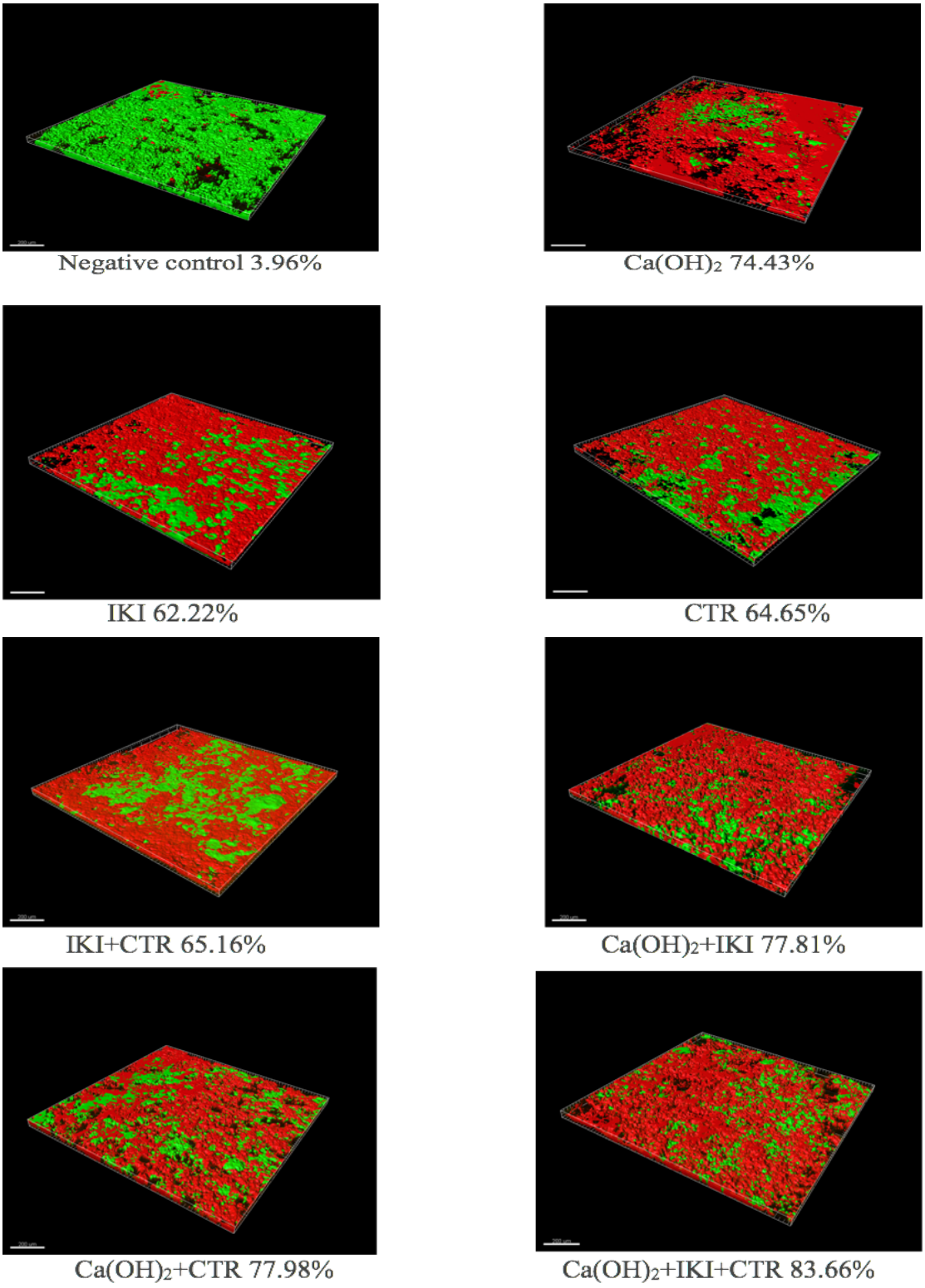


Figure 4.17 3D reconstruction of CLSM images of one week old biofilms exposed for two weeks. The medicament(s) used and the percentage of dead cell volume are shown

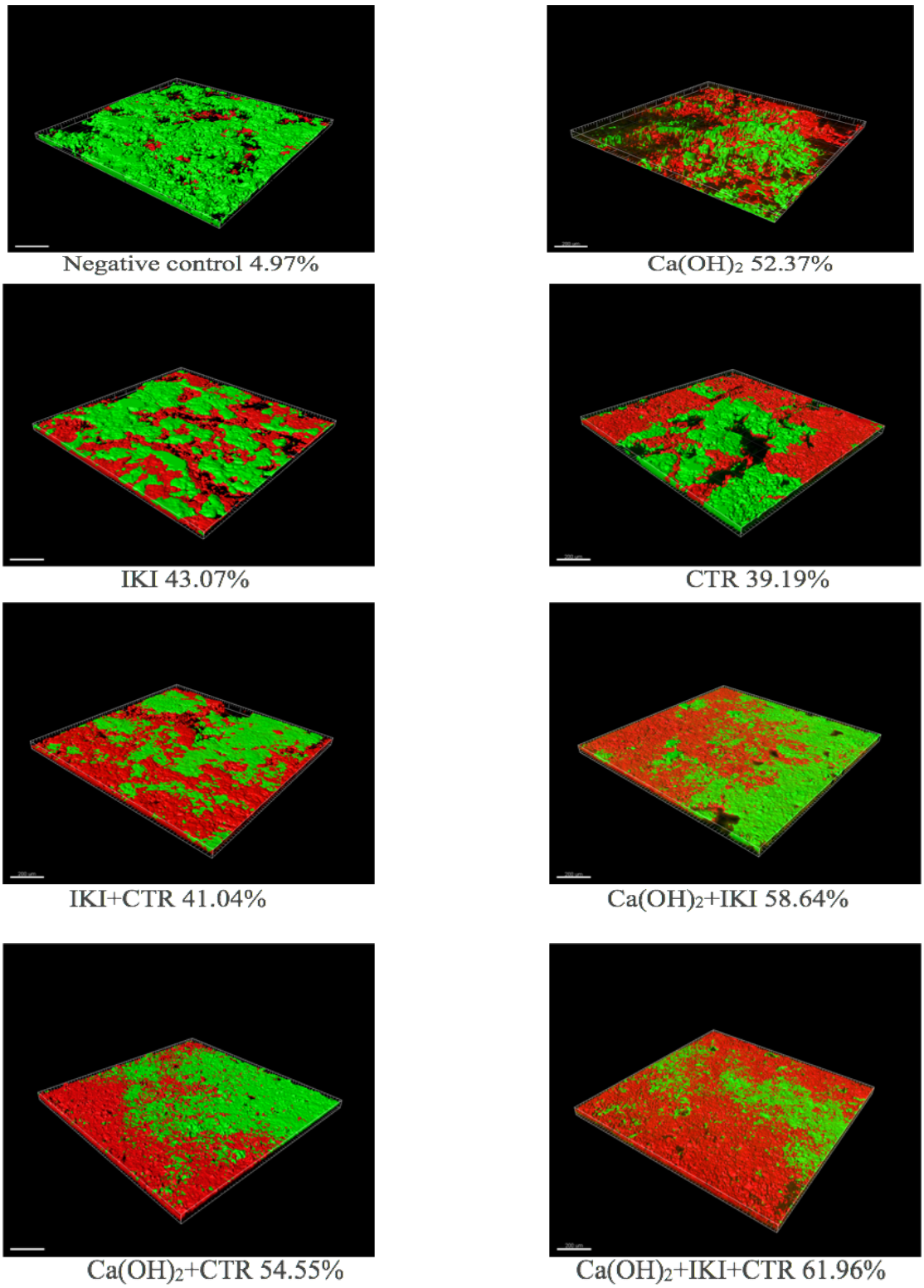


Figure 4.18 3D reconstruction of CLSM images of three weeks old biofilms exposed for one day. The medicament(s) used and the percentage of dead cell volume are shown

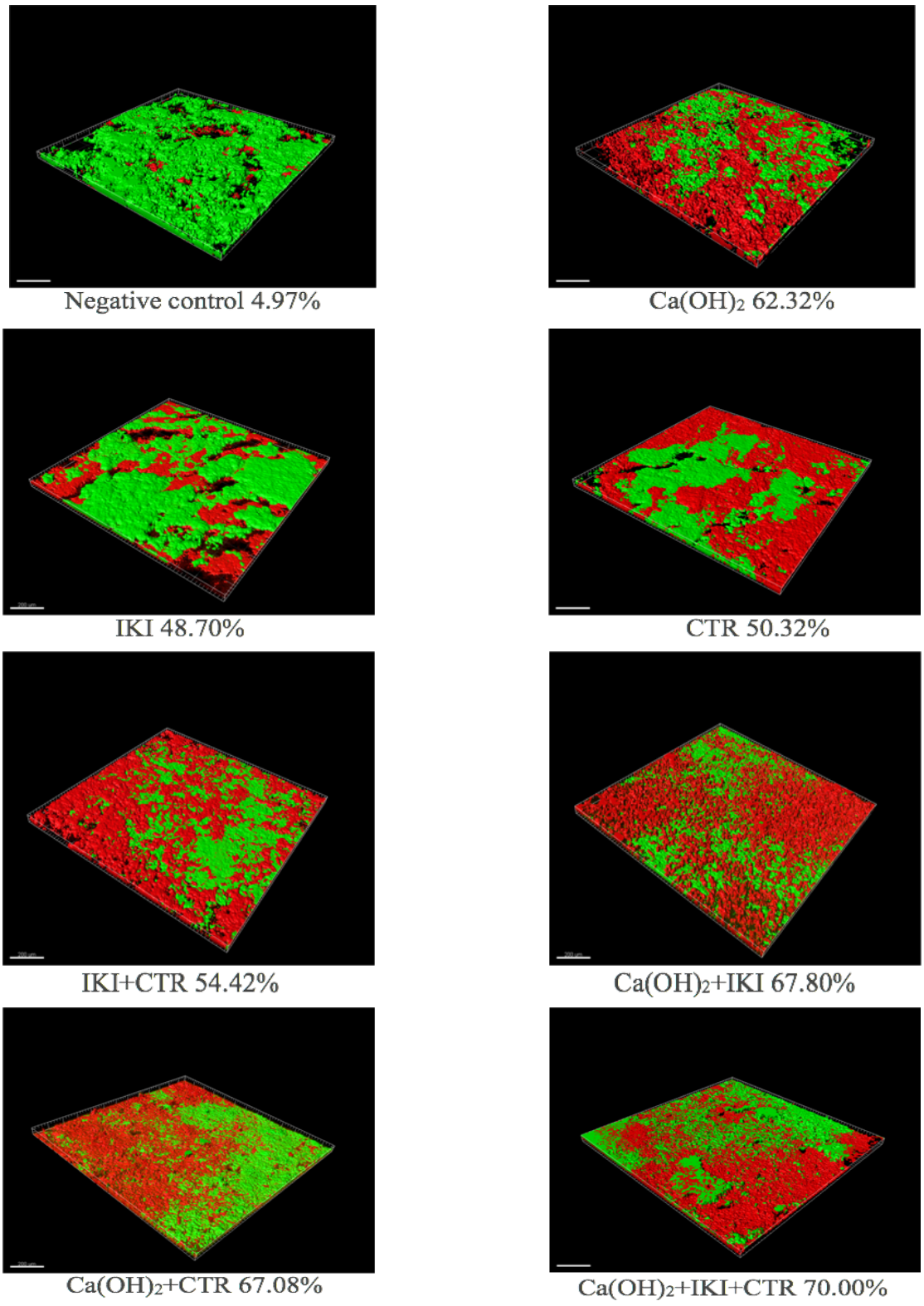


Figure 4.19 3D reconstruction of CLSM images of three weeks old biofilms exposed for one week. The medicament(s) used and the percentage of dead cell volume are shown

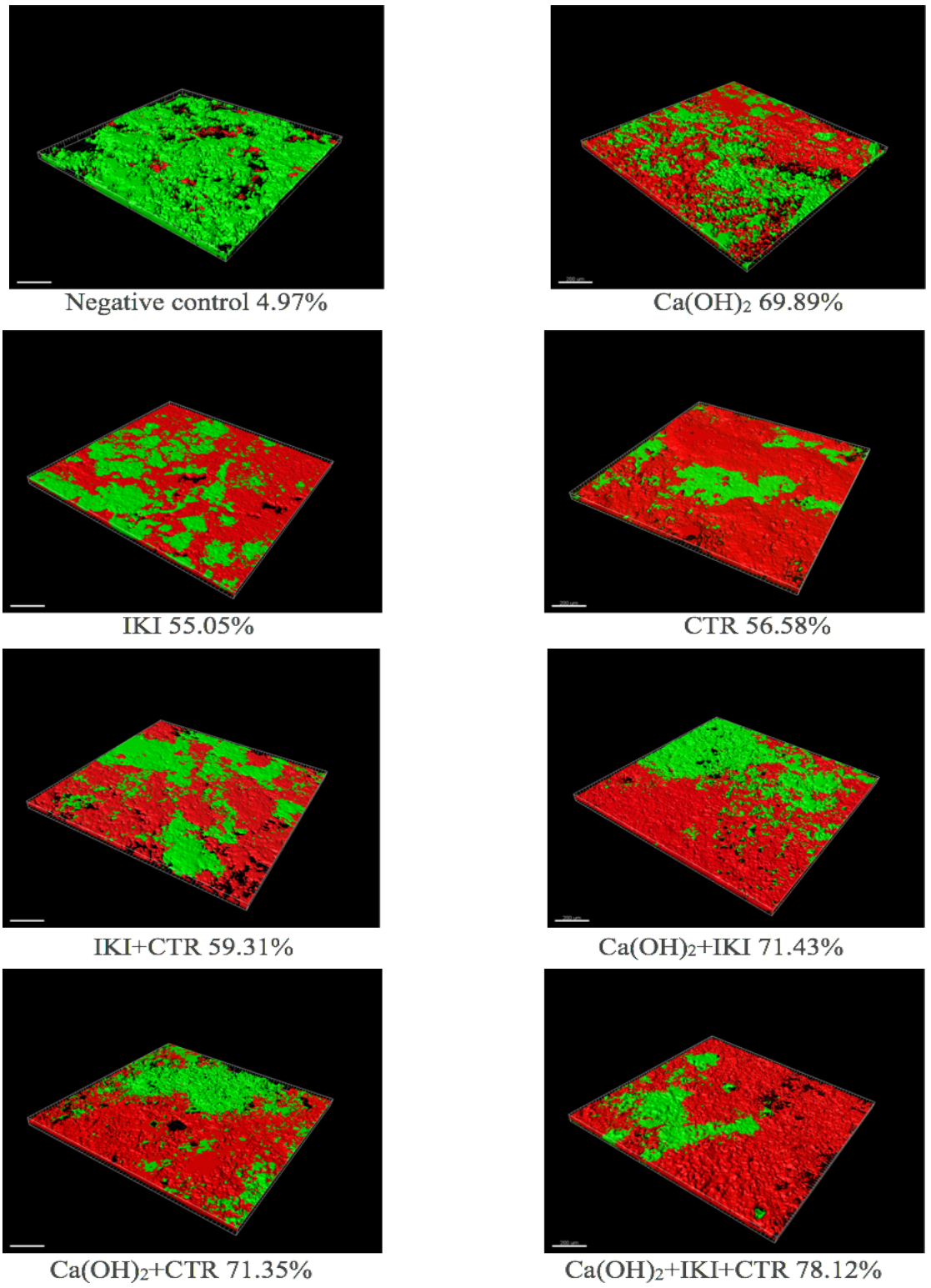


Figure 4.20 3D reconstruction of CLSM images of three weeks old biofilms exposed for two weeks. The medicament(s) used and the percentage of dead cell volume are shown

Chapter 5: **Discussion**

Only one source for the mixed species biofilm was used in the present study. This was done because the numbers of different groups and individual experiments grew very high due to the number of different combinations of the three antibacterial substances, three different exposure times (one day, one and two weeks), and biofilms of two different maturation levels. Nevertheless, lack of other biofilms sources (donor individuals) can be regarded as a potential weakness of the study. However, a recent study by Stojicic et al (2013) showed that biofilms grown from gingival plaque of six different sources (donors) showed surprisingly similar susceptibility behavior to three different disinfecting agents, chlorhexidine, sodium hypochlorite, and iodine potassium iodide. It may therefore be possible that certain universal characteristics of biofilms are more important determinants of biofilm susceptibility and resistance to disinfecting agents used in endodontics than e.g. the detailed species composition of different biofilms. Therefore, although the results of the present study must be interpreted with caution, it is possible that the results are reflective of wider reality than just the reaction pattern of one biofilm.

Different methodologies exist for testing the effect of endodontic irrigating solutions and medicaments in an *in vitro* setting. The use of dentin blocks contaminated with different bacteria and incubated for various periods was proposed by Haapasalo & Orstavik (1986) and widely used in numerous studies. However, despite the benefits of the dentin block model, it also suffers from several shortcomings. These include difficulty to create a standardized presence of microbial cells in all blocks, and the

inaccuracies in the sampling and culturing. Methods using culturing and CFU (colony forming units) for comparing the effect of different disinfecting agents are better suited for testing the killing of planktonic bacteria, whereas in biofilm studies culturing often fails to show differences between different antimicrobial protocols (Kishen & Haapasalo 2010)

A methodology based on viability staining and use of CLSM was recently proposed in order to quantify the effectiveness of dentin disinfection (Ma et al. 2011). After contaminating the dentinal tubules with bacteria using the force of centrifugation in this model, scanning electron microscopy was used to verify a strong presence of bacteria within the tubules, allowing comparisons between different specimens. After exposure to different medicaments or other substances with antimicrobial activity the dentin specimens are stained and imaged using the CLSM. In this model it is possible to quantify the proportion of living and dead cell volume in the samples tested, giving results with far smaller variation and better repeatability than e.g. with the original dentin block method based on sampling and culturing (Molander et al. 1999; Kishen & Haapasalo 2010; Ma et al. 2011).

Another novel *in vitro* model known as the open biofilm model is a design where multispecies biofilm is grown on a sterile collagen coated hydroxyapatite disk for different time periods. Following this, the biofilm can be exposed to a variety of different irrigating solutions used in root canal treatment, interappointment medicaments or other materials used in endodontics (Shen et al. 2009; Ma et al. 2011;Shen et al. 2011; Ferrer-Luque et al. 2012; Wang et al. 2012; Stojicic et al. 2012; Stojicic et al. 2013). The biofilm disks are stained with the viability stain and scanned by CLSM and analyzed for the

proportion of live and dead cells (Shen et al. 2009). This methodology also allows 3D analysis to further visualize and assess the effect of irrigants in different parts of the biofilm's structure. In an attempt to compare the effect of source and biofilm maturations on the susceptibility of the biofilms to several antibacterial agents, a study was made using dental plaque from six different donors and cultured to different maturity stages. The authors concluded that the source of bacteria did not play a role on the susceptibility of biofilms to antibacterial agents; since all biofilms responded similarly to the different irrigants tested. Furthermore, results showed that three weeks of biofilm incubation resulted in a mature and more resistant biofilm compared to one and two weeks incubation, no difference was noted between biofilms incubated for three weeks or more with regard to biofilm resistance (Stojicic et al. 2013).

A limitation of the open biofilm model, which was used in the present study and which uses pure hydroxyapatite is that the possible inhibitory effect of dentin on the antibacterial effect of the irrigants or medicaments tested is not present (Haapasalo et al. 2007). As a consequence, killing may occur at a higher level compared to an *in vivo* situation. Therefore, interpretation of the results must be done with caution.

The model used in the present study used biofilms instead of planktonic bacteria. The reason for this was an effort to mimic the clinical situation as much as possible. In endodontic infections, the bacteria form colonies and are embedded in EPS, the amount of EPS plays an important role in the maturity of the biofilm and its resistance to different antimicrobial agents (Vu et al. 2009). Planktonic bacteria, on the contrary, are far less resistant to antimicrobial agents; therefore the results obtained using planktonic cultures may be misleading when compared to clinical situations. Moreover, the decision to use

CLSM instead of culturing and colony forming units (CFU) counts is that the current methodology using the viability staining makes it possible to identify small differences in the percentage of the killing ability of the tested agents, which is not possible in studies using culturing and CFU counts. Therefore the CLSM method chosen for the present study provides a more accurate evaluation of the efficacy of the agents tested.

Most of the *in vivo* studies have used the same sampling technique, the use of paper points to collect the contents of the root canal, which are then cultured using various methods and culture media depending on the study. Another difference in these studies was the stage at which the samples were taken during the treatment; some compared the bacterial content before and after instrumentation, before and after irrigation, before and after medication (Orstavik et al. 1991; Shuping et al. 2000; Safavi et al. 1985; Kvist et al. 2004; McGurkin-Smith et al. 2005; Peters et al. 2002; Reit et al. 1999). The inconsistency of the findings and the fact that the amount of bacterial reduction could not be properly quantified are the main drawbacks of this methodology. All of these studies reported how many canals were bacteria free but none of them was able to quantify the effectiveness of the tested medications by means of antibacterial efficacy and percentage of reduction in bacterial content.

In the present study, there was a statistically significant difference in the percentage of dead cells for all treatment groups when comparing the same treatment duration for each tested medicament between young and aged biofilms. This confirms that aged biofilms are more resistant to antimicrobial agents than young biofilms, and these findings are in accordance with a previous study using the same model (Stojicic et al. 2013). Interestingly, when comparing the untreated young and aged biofilms, which

served as negative controls, the percentage of dead cells was not statistically different. However, there was a statistically significant difference in the biofilm thickness. It may be therefore suggested from these observations that the increased resistance of the aged biofilms may be partly due to the increase in the biofilm thickness.

Adding CTR to IKI showed an additive effect with regard to the antibacterial activity. In addition, when adding IKI to $Ca(OH)_2$ or combining all three tested agents together, the antibacterial effect increased. Although adding CTR to $Ca(OH)_2$ did not show increase in the antibacterial effect on biofilms compared to $Ca(OH)_2$ alone when applied for one day, the improved effect was statistically significant when the effect of these medicaments was compared after one and two weeks of exposure. This may be due to the residual effect by the CTR, which has been suggested in a previous study (Ferrer-Luque et al. 2012).

Increasing the duration of exposure significantly increased the effectiveness of all medicaments, both when used alone and in the various combinations. The findings also suggest that CTR has a residual effect when combined with $Ca(OH)_2$. When $Ca(OH)_2$ was combined with CTR and IKI, it was equally effective against the biofilm after just one day as pure calcium hydroxide after two weeks of exposure. This may be a significant advantage in clinical practice, since the same antibacterial effect can be obtained in a fraction of time.

Chapter 6: **Conclusion**

Within the limitations of the study, it can be concluded that aged biofilms are more resistant to antibacterial agents than young biofilms. The increased resistance may be partly due to the greater thickness of the biofilm. Combining iodine potassium iodide and cetrimide confirmed an additive antibacterial effect. Moreover, when combining the iodine potassium iodide and cetrimide with the calcium hydroxide, the antibacterial effect was significantly higher than when using calcium hydroxide alone.

Exposure to the combination of the three medicaments of the biofilms for one day resulted in antibacterial effect similar to that when calcium hydroxide alone was applied for two weeks. This may allow reducing the treatment time in clinical practice if the new combination product is used, however, more testing on the new medicament should be done with regards to biocompatibility and its effect on biofilms in the presence of dentin.

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