A THREE-DIMENSIONAL ANALYSIS OF THE EFFECT OF IRRIGANT FLOW VELOCITY ON ENDODONTIC BIOFILMS

by

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B.D.S, King Abdulaziz University, 2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTURAL STUDIES
(Craniofacial Science)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2017

Abstract

Introduction: Irrigation of the root canal system is an essential part of disinfection during root canal treatment. Some irrigants have the ability to dissolve or detach the biofilm from the dentin surface as well as kill residual bacteria. Several chemical and physical parameters affect the effectiveness of the irrigation process.

Aim: The primary aim of this study is to investigate the effect of irrigation flow velocity on the biovolume of the biofilm and antimicrobial effect against biofilm microbes.

Materials and methods: Hydroxyapatite discs coated with type I collagen were used as the biofilm substrate. Mixed supra- and sub-gingival plaque was collected from a single donor and suspended in Brain Heart Infusion broth (BHI). The discs were placed in 24-well tissue culture plates containing BHI-plaque suspension. They were then incubated under anaerobic conditions for three weeks. BHI medium was replaced with a fresh solution once a week. After three weeks of biofilm growth, the discs were placed in a CDC Biofilm reactor placed on a magnetic stir plate. The speed settings used were 60 rpm and 200 rpm both for 30 and 60 seconds. 0.1 % Sodium hypochlorite solutions and sterilized spring water were utilized for the experiment. Control samples were not subjected to any treatment. After treatment, all discs were stained with a viability stain and assessed under a confocal laser scanning microscope. The total biofilm volume and percentage of dead bacteria were calculated using the Bioimage L software.

Results: Sodium hypochlorite at 200 rpm was significantly more effective than control groups in reducing biofilm volume and killing biofilm bacteria. At slower flow velocity (60 rpm), there was no difference when compared to the negative control groups not subjected to

any treatment. Multivariate analysis results revealed that irrigant type and flow velocity had a significant effect on reducing biofilm volume and killing biofilm bacteria.

Conclusion: Irrigation flow velocity is a significant factor in the antimicrobial effectiveness of irrigation procedures. Our findings demonstrate is important to consider irrigation fluid dynamics when studying the antimicrobial effects of different irrigants in the future.

Lay Summary

Irrigants are solutions used for disinfection during root canal treatment. Our investigation aimed to study whether applying these irrigants at higher speeds would enhance the disinfection procedure. We made microbial biofilms in the lab and applied two irrigants, sodium hypochlorite and sterile water. We also had a control group, which was not subjected to any of the irrigants for comparison. Afterwards, the samples were examined by microscopy and analyzed using an imaging software. Our findings showed that when irrigants were applied at higher speeds, the total microbial volume was reduced and more microorganisms were killed than at lower speeds. Using sodium hypochlorite at higher speeds resulted in the highest reduction in microbial volume and the highest proportion of microbial killing.

Preface

All the work presented in this thesis was done by Dr. Lujain Mirdad. The contribution of supervisors in this project was: Dr. Markus Haapasalo 20%, Dr. Ya Shen 10% and Dr. Jolanta Aleksejuniene 5%. Ethical approval for the study was obtained from The University of British Columbia Research Ethics Board (application number H12-02430).

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List of Abbreviations

BHI	Brain heart infusion broth
CDC	Center of disease control
CHA	Collagen coated hydroxyapatite
CLSM	Confocal laser scanning microscope
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
HClO	Hypochlorous acid
MTAD	Mix of tetracycline acid and detergent
NaOCl	Sodium hypochlorite
PCR	Polymerase chain reaction
rpm	Rotations per minute
sec	Seconds
SEM	Scanning Electron Microscope
vs	Versus

Acknowledgements

I wish to thank Dr. Markus Haapasalo and Dr. Ya Shen for their dedication to scientific research. I am grateful to have been a student of such leaders in the endodontic scientific field. They have taught me how to be a researcher.

Dr. Jeffrey Coil for the opportunity to be here in this program. His knowledge of the endodontic literature combined with years of clinical expertise in the field will always be an inspiration in my future carrier.

Dr. Zhejun Wang, Dr. Hazuki Maezono, and Dr. Xiangya Huang for their mentorship and more importantly their friendship.

Dr. Jolanta Aleksejuniene for her advice and help with statistical analysis.

Dedication

I am thankful first and foremost to God for his countless blessings. I dedicate this thesis to my mother, father, and husband. Words cannot describe how much I am grateful for your unconditional love and encouragement. Your prayers and support throughout my graduate studies has given me the strength to follow through. Thank you for being there for me in the hard times and the good.

Chapter 1: Introduction

1.1 Bacteria as the cause of endodontic disease

In endodontics, there is well established evidence that the etiology of pulpal and periradicular disease is of a complex polymicrobial origin. This understanding was developed over decades of intensive research. Miller (1894) was the first one to detect microorganisms in inflamed pulps in stained microscopy specimens and to consider them important for the development of inflammation. The succeeding *in vitro* studies conducted on animal models provided evidence of a causative relationship between microorganisms and pulpal and periapical disease. Kakehashi and associates (1965), found that in germ-free rats, teeth with pulp exposures to the oral cavity did not exhibit signs of pulpal necrosis, apical granulomas, or abscesses, while exposed pulps in conventional rats did. These results proved that the presence or absence of microbial flora has a major impact on the status of exposed pulps. Several studies sampling from the root canals of non-vital intact human teeth emerged later. In one study, by Kantz and Henry (1974), stained bacterial cells were observed in 92 % of the specimens. Another similarly designed study reported infected pulp chambers in 32 out of 40 samples (Wittgow and Sabiston, 1975). In vital pulp chambers, anaerobic bacteria were reportedly not found. However, in non-vital teeth, they comprised 64 % (Keudel et al., 1976). Byström and Sundqvist isolated 88 % anaerobic bacteria from necrotic teeth with apical lesion. They demonstrated the existence of bacteria in all canals in necrotic teeth with a periapical lesion (Byström and Sundqvist, 1983). Baumgartner and Falkler also showed anaerobic

predominance in the apical 5mm of all infected root canals (Baumgartner and Falkler, 1991). The effect of bacterial presence in the pulp on periradicular tissues was explored in intact human teeth necrotized due to trauma by Goran Sundqvist in his much-cited thesis in 1976. He concluded that in necrotic traumatized teeth without apical periodontitis no bacteria were found. Yet, in cases with apical periodontitis, bacteria were present, 94 % of which were anaerobes (Sundqvist, 1976). Later, an experiment by Möller et al. on monkeys showed agreement with earlier findings. The authors showed that in teeth with a sterile necrotic pulp, there was only slight or no development of apical periodontitis. However, in infected necrotic pulps, all the teeth examined histologically revealed the occurrence of apical periodontitis (Möller et al., 1981). These findings established a strong association between cause and effect, providing evidence in both directions, leaving no doubt as to what the etiology of endodontic disease is.

Many studies have attempted to identify a specific causative microorganism. Numerous species were identified in primary endodontic infections. Anaerobic bacteria comprised the majority of organisms, especially in the most apical regions of the root canal. The reasons for their dominance were attributed to scarce nutrient supply and low oxygen tension in those regions (Sundqvist, 1976; Fabricius et al., 1982). Microscopic examination of necrotic pulps had also revealed a mixed species bacterial infection (Sundqvist, 1994). Nair used light and transmission electron microscopes, in combination, to study necrotic teeth with apical periodontitis. The author found clusters of different bacteria on all root canal walls sampled (Nair, 1987). A scanning electron microscopy study by Molven et al. examined the topography of the apical portion of root canals in non-vital teeth with apical lesions microcolonies of cocci, rod-shaped bacteria

and spirochetes. In some of their specimens, the bacteria were covered with a granulomatous material. Similar multi-layered cocci and filamentous bacterial affiliations have been observed in subgingival plaque (Molven, 1991). These microcolonies are what we refer to as a biofilm, which will be discussed in the following section. Although microscopic studies provide useful descriptions of the structure of the flora in the root canal, they cannot identify the specific species of bacteria (Nair, 1987). Bacterial species can be identified using other means. Conventional culturing methods were used in earlier studies. Later, anaerobic bacteriological techniques were developed by Hungate and simplified by the Virginia Polytechnic Institute. It was subsequently realized that the species isolated from root canals were dominated by anaerobic flora (Hungate, 1950; Moor, 1966; Sundqvist and Figdor, 2003). The introduction of molecular methods identified additional species that were not cultivable by traditional techniques (Baumgartner, 2004). A study by Conrads et al. was the first to apply molecular techniques to detect endodontic bacteria. The polymerase chain reaction was used in the study detected *Tannerella forsythia* in infected root canals for the first time (Conrads et al., 1997). Ensuing studies used PCR, DNA-DNA hybridization, Broad range PCR, and other molecular methods. These technologies have led to the discovery of species and strains of bacteria that were uncultivable previously (Siqueira and Rôças, 2005). The specific species forming the biofilms are determined by different ecologic factors such as oxygen, the nutrient supply available, bacterial interactions, and virulence factors (Sundqvist, 1994). Some of the important virulence factors identified in cultivable species include bacterial capsules, the ability for adhesion and invasion, and the release of toxins, proteases, protease inhibitors, collagenases, and immunosuppression (Olsen

and Dahlén, 2004).

1.2 Biofilms

There are multiple routes available for microorganisms to enter the root canal. They could gain access through carious lesions, periodontal communications, open dentinal tubules, or a developmental anomaly such as invagination (Morse, 1981). Upon entering, these bacteria attach to the root canal wall surface and a layer of polysaccharide and protein starts to form. The development of this layer allows bacterial adherence to the surface (Dufour et al., 2010). Bacterial cells attached to this organic layer enable the attachment of other species. With more and more species accumulating, a microcolony is formed, it then matures and can later detach, completely or partially, and migrate to a new location (Dufour et al., 2010). Multiple factors could affect biofilm formation, such as genetic expression, hydrodynamic conditions, surface properties, and environmental conditions including the surrounding medium. Whether they have a positive or negative effect on biofilm formation remains debatable. The formation of biofilms provides advantages for bacteria in terms of their survival and proliferation. These cells are offered protection from hostile external conditions such as shear stress, low nutrient supplies, fluctuations in pH, presence of oxygen radicals, disinfectants and antibiotics. (Kim et al., 2012).

Biofilms have been studied using both static and dynamic models. Shen et al. (2009) developed a static polymicrobial biofilm model that proved to represent both sensitive and resistant phases of development. This *in vitro* model displayed similar

functional and morphological characteristics to *in vivo* biofilms making it legitimate for research in the endodontic field (Shen et al., 2009; Shen et al., 2011; Stojicic et al., 2013). A dynamic model, where growing bacteria is subjected to a continuous flow of nutrient-rich media, was shown to be effective in testing biofilms (Dunavant et al., 2006). These are flow cell models that use a peristaltic pump to control the flow rate of the medium. One of the advantages of this method is that it allows observation of the sequential steps of biofilm formation in conjunction with the use of a microscope, charge-coupled-device camera, or confocal laser scanning microscope (Parvina et al., 2011). It is prudent to acknowledge certain factors when evaluating biofilm studies. The level of biofilm maturity is one factor that has an influence on its behavior. Especially in studies testing the efficacy of antimicrobial agents. Maturation will affect the level of resistance of the biofilm to the disinfecting action irrespective of the source of bacteria used (Shen et al., 2009; Shen et al., 2011). Other parameters to consider include microbial concentration, incubation time, growth conditions, and substrate properties. Culturing bacterial biofilms in the laboratory should ideally aim to reproduce naturally occurring biofilms. There is no single method that could be applied for all experiment models. Thus, the research question determines the culturing method of choice (Kishen and Haapasalo, 2010).

1.3 Advantage of studying biofilms instead of planktonic cultures

In vitro biofilm models were utilized in studies examining the attachment of bacterial cells to the surface of different biomaterials (Kishen et al., 2008; George et al., 2010), the interaction between bacteria in the biofilm and immune cells (Mathew et al.,

2010), and the efficacy of novel antimicrobial agents and irrigation strategies (Pratten and Ready, 2010). Antimicrobial resistance observed in biofilm bacteria may not be generally attributed to classic genetic mechanisms, instead it has been shown to result from certain biofilm characteristics. These characteristics may include the species of inhabiting bacteria, the bacterial cell density, the nature of the adherence to the substrate, physicochemical characteristics of the substrate, thickness of the biofilm, and the amount of EPS (Baumgartner et al., 2008). Microorganisms growing in biofilms are better protected than their planktonic forms (Brown et al., 1993; Chavez de Paz, 2007). Factors affecting the development of biofilms and their characteristics are the associated flora, microbial ecology, nutrient availability, time to maturity, and the canal anatomy. On the other hand, factors that are important in the eradication of biofilms include mechanical instrumentation, irrigant washing effects, irrigant chemical effects, application of external energy to irrigants (e.g. by ultrasound), and the use of filling materials with antimicrobial properties (Haapasalo and Shen, 2011). Chemomechanical canal preparation is essential to achieve microbial disinfection in endodontic therapy (Siqueira et al., 1997).

Mechanical canal instrumentation removes infected tissue, thereby disrupting or detaching biofilms. It also provides access routes for subsequent chemical flushing and disinfection. This is especially beneficial where complex anatomical structures are found. Even with the emergence of new irrigation methods that provide better cleaning of root canals, adequate mechanical preparation is required for their effectiveness. (Peters, 2004; Metzger et al., 2013). Inaccessible areas are likely to harbor microorganisms and their by-products. In these situations, clinicians depend on chemical irrigation (Ricucci and Siqueira, 2010).

1.4 Irrigation with chemically active agents

It is critical to reduce microorganisms in canals to a minimum. That is achieved through providing aseptic conditions for root canal therapy, mechanical instrumentation, irrigation, and the use of antimicrobial root filling materials. Irrigation reduces the number of the microbes by means of physical removal (washing effect) and chemical antiseptic effects (Morse, 1976). Irrigants possess the ability to dissolve or detach the biofilm from the surface as well as kill residual bacteria. Sodium hypochlorite (NaOCl) is the most commonly used irrigating solution in endodontics. Its strong antibacterial effect has long been established (Byström and Sundqvist, 1983; Zehnder et al., 2002).

The antimicrobial effect of hypochlorite is exhibited through ionization forming hypochlorous acid (HClO). Sodium hypochlorite also has the ability to dissolve organic tissues (a property exclusive to it) including the pulp and part of the smear layer (Rosenfeld et al., 1978: Clarkson et al., 2006). The concentration of sodium hypochlorite recommended for use remains equivocal, ranging from 0.5 % to 6 % (Haapasalo and Shen, 2011). Studies on the efficacy of different NaOCl concentrations used were compared to each other and to other irrigants. An *in vitro* study was performed comparing sodium hypochlorite (1 % and 6 %) to Chlorhexidine (2 %), SmearClear, REDTA and BioPure MTAD used against an *E. faecalis* biofilm grown in a flow cell system. Both concentrations of NaOCl showed a significantly better killing potential than all other agents tested. However, no statistical difference was found between the 1 % and 6 % concentrations (Dunavant et al., 2006).

When samples of polymicrobial biofilms were immersed for 15 minutes in 1 %, 3 % and 6 % concentrations of NaOCl in *ex vivo* models, the higher concentrations were more effective against biofilm bacteria. These biofilm samples were evaluated using scanning electron microscopy (SEM). The authors commented that mechanical agitation could have resulted in greater disruption (Clegg et al., 2006). The antimicrobial effects of QMiX, BioPure MTAD, Chlorhexidine (2 %), and NaOCl (1 % and 2 %) were studied on strains of *E. faecalis* and polymicrobial biofilms were compared, with sterile water as a control. QMiX and 1 % NaOCl were superior to the 2 % Chlorhexidine and MTAD in planktonic killing after both 5 and 30 seconds of exposure to the irrigant. QMiX and 2 % NaOCl were the most effective at all tested times. Sterile water had the least percentage (1.65 %) of dead bacteria as detected by viability staining and CLSM (Stojicic, 2013). The main concern regarding the NaOCl concentration was attributed to its possible negative effects on dentinal walls (Ari et al., 2004) and toxicity to the periapical tissues (Gernhardt et al., 2004). Antimicrobial effectiveness *in vivo* was not shown to be as good as in the *in vitro* experiments. The reasons could be attributed to difficulty in reaching the apical third of the root canal and complex anatomical structures such as fins and anastomoses in the root canal system. It may also be that NaOCl is inactivated when the microbial biomass is high or inflammatory exudate is present in the canals. In addition, contact with the pulp tissue or dentinal collagen may contribute to weakening the activity of NaOCl (Haapasalo et al., 2000). Nonetheless, NaOCl is currently the best available irrigant and should be regarded as the main disinfecting solution during root canal treatments (Haapasalo et al., 2012).

1.5 Irrigation dynamics

Irrigation of the root canal system is an essential part of disinfection during root canal treatment. The importance of irrigation in the success of endodontic treatment has been widely discussed in the literature (Haapasalo et al., 2005; Haapasalo et al., 2010). Irrigants used in endodontics are affected by several parameters, including the anatomy of the root canal system, the volume and properties of the irrigant, delivery device, size, and type and insertion depth of the needle. These parameters are important for the effectiveness and safety of irrigation procedures (Moser et al., 1982; Usman et al., 2004; Falk et al., 2005; Gulabivala et al., 2005; Khademi et al., 2006; Vinothkumar et al., 2007; Shen et al., 2010).

The factors affecting irrigation have been considered by several investigators. Huang et al. subjected a bio-molecular film model of collagen to static and dynamic irrigation. Besides acknowledging the importance of the chemical effect and volume of the irrigant used, they found that removal of stained collagen was significantly higher on the side that faced the side vent on the needle, which clearly showed the advantage of physically directing a flow toward the canal walls (Huang et al. 2008). The same findings were concluded by Baker et al. (1975), who found that the flushing effect of the irrigant was more important than that of dissolving the tissues. Pressure tests applied to different types and sizes of needles revealed that larger needles that had been inserted into the whole length of the canal were more successful in removing debris than smaller ones (Moser et al., 1982). Contrary to this, another study found that smaller needles and larger canals contribute to a more mechanically effective irrigation (Chow, 1983). The benefit

of which was to clean the canal with a flow of irrigant that could carry out organic, dentinal debris and microorganisms. He concluded that in order for irrigation to be mechanically effective, the needle must be small enough to reach the apex, induce force needed to start a current against the walls, and remove debris as it flows outside the canal. Therefore, the higher effectiveness associated with smaller needles and larger canals was due to the production of better currents of the fluid inside the canal (Chow, 1983). These forces create shear stresses on the walls that could potentially disturb the structure of biofilms (Stewart and Franklin, 2008). If a certain yield point is surpassed, it would result in expansion or, detachment of the biofilm (Busscher et al., 2003). There is a scarcity of knowledge provided by the literature in regard to the effect of fluid flow on biofilms. This is mainly due to vast differences in biofilm components and physical characteristics. Furthermore, biofilms are produced by living organisms that are capable of adapting to changes in environments. Therefore, the mechanical properties of a biofilm should be studied based on a small timescale, such as minutes (Flemming et al., 2011).

The velocity of irrigant flow is one of the key parameters of mechanical irrigation (Shen et al., 2010(b); Townsend, 1980). A recent study by Gulabivala et al. (2010) acknowledged that the irrigant flow velocity creates shear stresses on the root canal walls. This mechanical property along with the chemical effect is believed to remove the biofilm as well as the smear layer. Increasing the irrigation velocity has not yet been directly studied, despite its potential benefit on the disinfection in root canal treatment. It is difficult to isolate a single factor such as irrigation velocity while conducting a clinical study. Instead, velocity must be separately tested with experiments in order to understand the true nature of its effects. Subsequently, its application to specific clinical situations

can be successfully achieved. *In vitro* models are used in hopes of minimizing variability and enhancing reproducibility (Gulabivala et al., 2010).

The flow of an irrigant inside the small confines of the root canal space can be studied utilizing the principles of microfluidics. An understanding of fluid dynamics and its application in root canal irrigation could further our knowledge of the mechanism by which disinfection takes place (Boutsioukis and Van der Sluis, 2015). The physical properties of the irrigants determine much of their characteristics. Irrigant flow, for example, is largely determined by its density and viscosity. For most endodontic irrigants' used, the irrigant flow is very similar to that of distilled water (Guerisoli et al., 1998). Hence, both irrigants in this study should be similar in that respect. The flow rate of an irrigant is defined as the volume of irrigant delivered within a certain period (Mott, 1999). It is expressed preferably in units of mL/s rather than mL/min, because the former is more realistic to associate clinically (Tilton, 1999)

Over the years, multiple methods have been introduced to enhance irrigation. Yet, syringe irrigation remains the main modality used currently. Therefore, despite its limitations, most studies still use syringe irrigation (Boutsioukis and Van der Sluis, 2015).

A large component of the available literature on fluid flow is obtained from computerized models of root canals and syringes. These studies revealed that there are two types of fluid flow; laminar and turbulent flow. A steady laminar flow results from a low flow rate (0.01 mL/s), with an increase up to 0.26 mL/s the flow remains laminar but becomes unsteady. Turbulent flow results from higher flow rates. However, high flow rates such as 0.5-0.7 mL/s may not be achievable clinically. Boutsioukis et al. (2009)

concluded that with regards to the flow rate, 0.26 mL/s should optimally be used in the root canal.

Irrigation objectives are achieved by virtue of the irrigants' chemical and mechanical effects. Only chemically active irrigants can disrupt biofilms and kill microorganisms. However, both chemically active and inert irrigants can exert mechanical effects on biofilms (Gulabivala et al., 2005). The mechanical force projected on the surface of the canal walls, can detach and/or disrupt biofilms. This force is frictional in nature and is termed wall shear stress. It occurs within a flowing irrigant and exhibits shear stress between the flowing irrigant and the root canal wall. In commonly used irrigants, the wall shear stress is proportional to their velocity gradient (Boutsioukis and Van der Sluis, 2015). Velocity relates to flow rate, where increasing the flow rate, volume of irrigant, or depth of needle insertion in the canal can enhance the solution's renewal throughout treatment (Bronnec, 2010; Sedgley et al., 2004, 2005).

In more recent years, the focus of studies on irrigation has shifted towards the testing of their antimicrobial efficacy. Different methods have been utilized including paper point sampling, radiolabelled bacteria or microscopic observations (Siqueira et al., 2002; Shabahang et al., 2003; Baker et al., 1975). Bioluminescent bacterial and real-time optical imaging have also been applied to quantify the bacteria in root canals before and after experiments (Sedgley et al., 2004, 2005). Although studies were done in teeth, a lot of them utilized planktonic bacteria. Planktonic bacteria may not reflect the actual clinical situation as they are easier to remove than established biofilms (Wilson, 1996). The importance of the biofilm concept cannot be emphasized enough when studying the actions of irrigating solutions (Zehnder, 2006). Furthermore, some methods such as paper

point sampling of root canals may not be as accurate as one would expect (Sathorn et al., 2007). Recent developments such as confocal laser scanning microscopy (CLSM) along with viability staining can be used to measure the effect of irrigation velocity on dental biofilm models *in vitro* (Shen et al., 2012).

Chapter 2: Rationale and Hypothesis

2.1. Objectives

The primary objective of this study is to investigate the effect of irrigation velocity on the antimicrobial effect of the irrigating solutions in an *in vitro* multispecies biofilm model. The specific aims are to:

1. Compare the effect of different irrigation velocities on biofilm volume and bacterial killing.
2. Compare the effect of different times of irrigant exposure on biofilm volume and bacterial killing.
3. Compare the effects of sterile water and NaOCl on the biofilm volume and microbial survival.

2.2. Study hypothesis

Irrigation with a high velocity and a long exposure time will have no significantly greater effect on biofilm volume reduction and bacterial killing when compared to low velocity irrigation and short exposure time to the irrigant.

Chapter 3: Materials and Methods

3.1 Experimental design

3.1.1 Sample preparation

Ethical approval for the study was obtained from The University of British Columbia Research Ethics Board (application number H12-02430). Sterile hydroxyapatite discs which were 9.6 mm in diameter and 1.5 mm thick (Clarkson Chromatography products, Williamsport, PA, USA) were used to culture a mature biofilm in anaerobic conditions (Anaerogen, OXOID, Hampshire, UK). The discs were placed in a 24-well tissue culture plate (Costar, Corning, NY, USA). Then they were coated with 1 ml bovine dermal type I collagen (10 mg/ml collagen in 0.012 N HCL in water) (Advanced BioMatrix, Carlsbad, CA, USA). They were left overnight for incubation at 4 °C. Once 24 hours had passed, the discs were ready for inoculation with the microbial sample. Supra-gingival and sub-gingival plaque was collected from one volunteer. The volunteer was a healthy 28 years old with a periodontal status of healthy to mild gingivitis. The plaque was suspended in Brain Heart Infusion broth (BHI; Becton Dickson, Sparks, MD, USA). From the suspension, 150 µl was withdrawn and added to a 96-well microplate (Costar, Corning, NY, USA). As a control, 150 µl of sterile BHI broth was added in a separate well. A microplate reader (BioRad 3550 model, Hercules, CA, USA) was used to measure the optical density (OD) at 405 nm. The optimal difference in OD between the two occupied wells was aimed at 0.1, with a range of 0.08-

0.15 being considered as acceptable. The plaque suspension in BHI was then diluted in sterile water to 10 times its volume. The discs were submerged in 2 mL of the suspension then incubated in a 24-well tissue culture plate at 37 °C in BHI under anaerobic conditions for 3 weeks. During which, the BHI medium was changed once per week.

3.1.2 Experimental groups

After 3 weeks of biofilm growth, the samples were divided into three main groups.

1. Control; scanned directly without any treatment;

   Group 1: (n=20) 0 rpm for 0 sec.

2. Biofilm exposed to sterile water at different flow velocities and durations;

   Group 2: (n=20) 60 rpm for 30 sec,

   Group 3: (n=20) 200 rpm for 30 sec,

   Group 4: (n=20) 60 rpm for 60 sec,

   Group 5: (n=20) 200 rpm for 60 sec.

3. Biofilm exposed to 0.1 % sodium hypochlorite at different flow velocities and durations;

   Group 6: (n=20) 60 rpm for 30 sec,

   Group 7: (n=20) 200 rpm for 30 sec,

   Group 8: (n=20) 60 rpm for 60 sec,

   Group 9: (n=20) 200 rpm for 60 sec.

3.1.3 Experimental procedure

The 0.1 % NaOCl solution was prepared by diluting a 6 % solution (RW Packaging, Winnipeg, MB, Canada) in distilled water immediately before each experiment. Different speeds were employed via a CDC Biofilm Reactor (BioSurface Technologies Corp., Bozman, MT, USA) (Figure 1). The manufacturer's formula was used to obtain the velocity on the surface of the biofilm. Choosing the 200 rpm setting would produce 0.0838 m/s as can be seen in Table 1. Assuming that we were using those velocities in a hypothetical canal with a diameter of 0.5, 1 or 2 mm, then applying the following equation: Flow rate = ¼ π x $(\text{diameter})^2$ x velocity. The resulting flow rate would be 0.016, 0.065 or 0.263 mL/s respectively. Given these results and the recommended optimal flow rate (0.26 mL/s) used in the root canal cited from the literature, we found using the 60 and 200 rpm settings suitable for the experiment. The flow velocity was produced by the rotation of a magnetic bar on a magnetic stir plate (VDW digital Hotplate/Stirrer, Henry Troemner LLC, NJ, USA). The temperature setting was turned off and only the velocity setting was utilized. The CDC reactor was filled with 400 mL of the selected irrigation solution and the stir bar was placed in the center of the flask. Afterward, the rod sample holder was placed with one Biofilm CHA fixed with a screw to the bottom coupon holder. The reactor was covered with aluminium foil when NaOCl was used. After each 30 sec and 60 sec exposure the rod was removed and the CHA disc with the biofilm was also removed then prepared for staining and examination with a confocal laser scanning microscope.

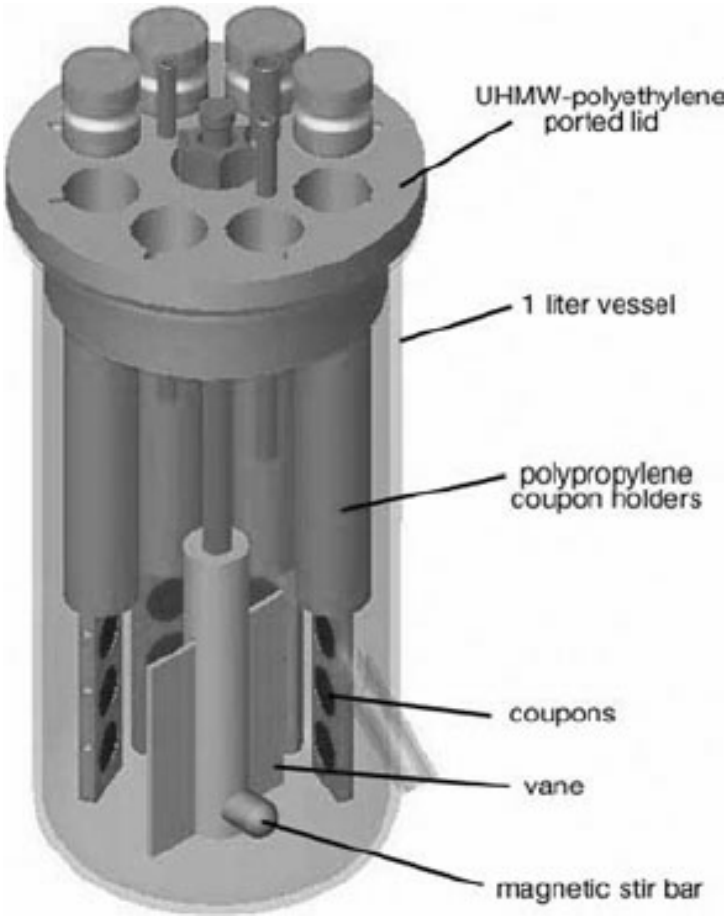

Figure 1. CDC Biofilm reactor. Courtesy of BioSurface Technologies Corp.

Table 1. CDC manufacture's wall shear calculation formula.

omega (rpm)	Re	flow regime	f	velocity* (m/s)	tau (N/m^2)
50	523	transition/turbulent	0.0165	0.0210	0.0036
100	1047	turbulent	0.0139	0.0419	0.0122
150	1570	turbulent	0.0126	0.0629	0.0248
200	2093	turbulent	0.0117	0.0838	0.0410
250	2617	turbulent	0.0111	0.1048	0.0606
300	3140	turbulent	0.0106	0.1257	0.0834
350	3663	turbulent	0.0102	0.1467	0.1092
400	4187	turbulent	0.0098	0.1676	0.1379
* Velocity on the surface of the biofilm coated hydroxyapatite disc					

3.2 Viability staining

All the samples were rinsed by placing the discs in a 24-well plate containing 2 ml of sterilized water for 2 seconds. Then, they were removed from the well and stained with a viability stain for microscopy and quantitative analysis (LIVE/DEAD BacLight Bacterial Viability kit L-7012, Molecular probe, Eugene, OR, USA). The stain consists of two vials, one containing SYTO 9 and the other propidium iodide. As per the manufacturer's instructions, the stains were mixed before the CLSM viewing in a 1:1 ratio, diluted in 1 ml water and kept in the dark. The fluorescence emitted from stained cells was used for evaluation. The excitation/emission maxima for these dyes were between 480/500 nm for the SYTO 9 stain and 490/635 nm for the propidium iodide stain.

3.3 Confocal laser scanning microscopy

The sample was immediately placed in the CLSM (Fluoview, Olympus, Canada) and the top cover was closed creating a dark environment inside for optimal fluorescence. The CLSM was connected to the FV10i 2.1 software with settings adjusted to detect the two fluorescent stains used. Automatic scanning was then initiated and an overall map of the biofilm was acquired. The specimens were observed under live scanning mode and 5 random areas were selected in each CHA disc. The resolution of each scan was set at 512x512 pixels with a pixel resolution of 2.5 μm. For each area scanned, adjustments were standardized so that the laser output was less than 20 % and the photomultiplier

sensitivity was 50 ± 10 %. The depth of the biofilm was captured in every area scanned with an average of 60-70 slices and each was 1-1.5 μm thick. Each of the five areas selected were registered in the same manner, after which they were scanned automatically.

3.4 Image analysis

The obtained images were uploaded to *bio*Image_L software for 2-D analysis (http://www.bioimageL.com/get_bioimage_L) (Chávez de Paz et al., 2009). The 3-D analysis was done by an extended version of *bio*Image_L kindly provided by Dr. Chávez de Paz. The software analyzes CSLM images in two and three dimensions and is based on color segmentation algorithms written in MATLAB (MathWorks, Natick, MA). It quantifies the total biofilm volume, the red and green subpopulations referring to dead and alive microorganisms, and the percentage of dead and live bacteria in each slice of a scanned area in the biofilm. The biovolume (μm^3) and the proportion of the volume of dead bacteria (%) were recorded as previously described (Chávez de Paz et al., 2010). A flow chart of the study design is illustrated in figure 2.

3.5 Statistical analysis

Power calculations were initially completed using an online sample size calculator (Creative Research Systems, http://www.surveysystem.com/sscalc.htm#one). It revealed

that for a confidence level of 95-99 %, the number of samples necessary in each group was 17–18, respectively. In our study, all groups/subgroup consisted of 20 samples, where one scanned area equated to one sample. Each CHA disc provided 5 samples, resulting in the use of four discs per group or subgroup.

Data was analyzed using one-way analysis of variance (ANOVA) and a post hoc Dunnett test to compare test and control groups. The effect of the three predictors studied (type of irrigant, velocity and time on the biofilm volume, and the percentage of dead bacteria) was measured utilizing multivariate analysis. Irrigant types were coded using Dummy tables to utilize the regression model to test the effect each variable exerted without interaction with the others. The significance level was set at 0.05 for all tests done (SPSS, Chicago, IL, USA).

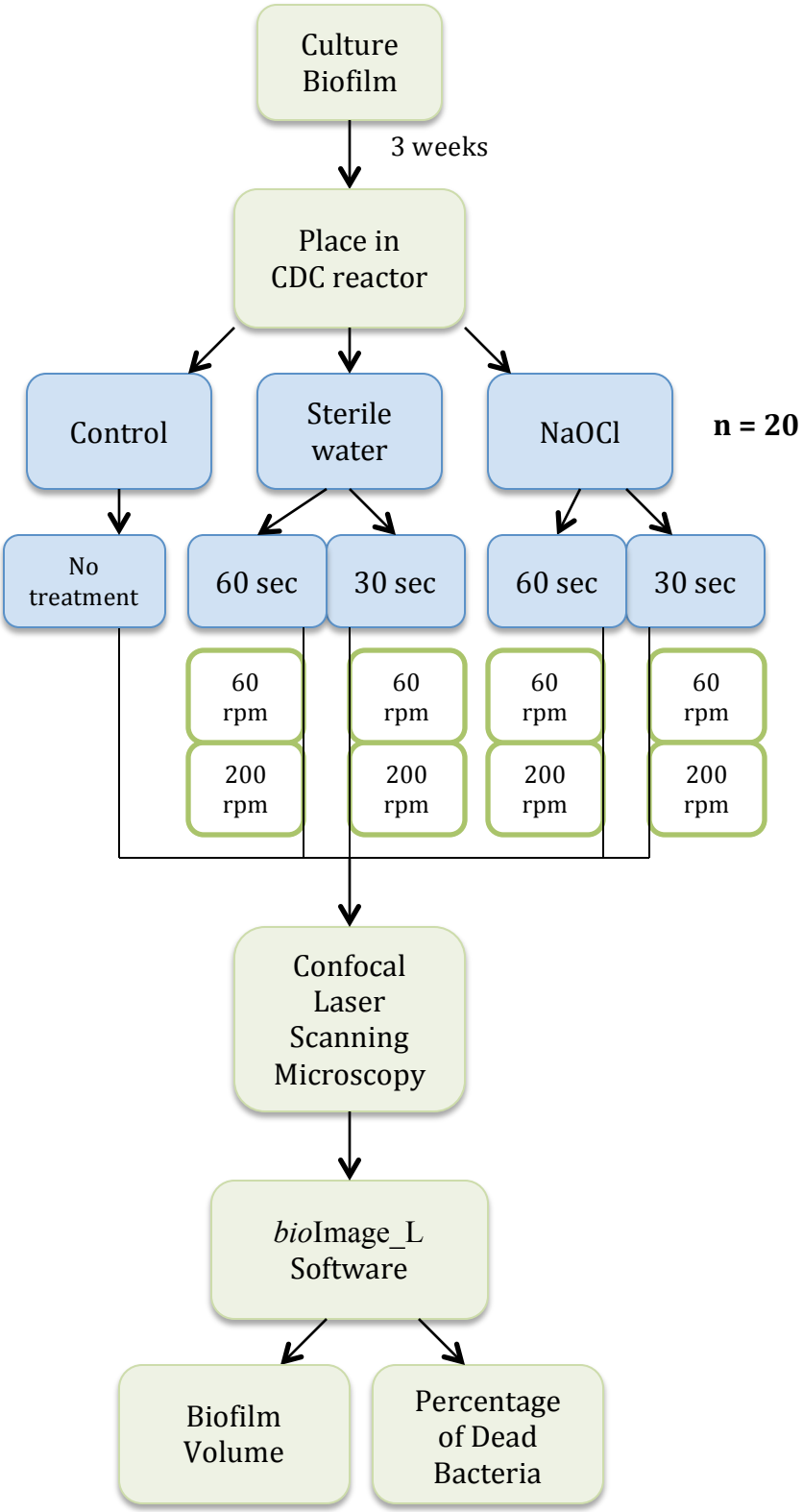

Figure 2. Flow chart of the study's methodology

Chapter 4: Results

4.1 Effect of study conditions on biofilm volume

The biofilm volumes obtained under different conditions are summarized in Table 2-3 and illustrated in Figures 3-5. Untreated control samples had the highest mean biofilm volume. On the other hand, the biofilm volume was the lowest when NaOCl was used as the irrigant and 200 rpm as the flow velocity ($P<0.001$). Utilizing sterile water and 60 rpm resulted in a decrease in the biofilm volume in the control groups, but to a lower extent than that measured with NaOCl and a flow rate of 200 rpm. Subjecting the samples to 30 sec of irrigation resulted in a decrease in biofilm volume more than the 0 and 60 sec applications. Both 30 and 60 seconds of exposures to irrigants had a statistically significant effect on the measured biofilm volume when each was compared to the control group.

4.2 Effect of study conditions on the proportion of dead bacteria

The proportion of dead bacteria under different conditions are summarized in tables 2 and 4 and illustrated in figures 6-8. Specimens in the control groups had the lowest mean percent of dead bacteria. The highest mean percentage of dead bacteria was found when we used NaOCl with the higher flow velocity of 200 rpm and exposure times of 60 and 30 sec.

Table 2. Mean biofilm volume and proportion of dead bacteria of experimental groups 1 to 9 under different study conditions

Group	Irrigant	Velocity (rpm)	Time (sec)	Mean biofilm volume (μm^3)	SD	Mean dead bacteria (%)	SD
1	**Control**	0	0	148,827	41407	3.38	3
2	**Sterile water**	60	30	131,706	42587	2.13	2
3			60	147,050	36844	3.63	3
4		200	30	127,407	44246	5.09	8
5			60	98,867	32031	13.07	9
6	**0.1% NaOCl**	60	30	81,421	44322	10.15	5
7			60	158,700	46692	17.75	16
8		200	30	54,529	44864	39.26	28
9			60	61,709	49699	53.56	21

Table 3. Mean biofilm volume (μm^3) under different study conditions

	Study conditions	n	Mean	SD
Irrigant	No irrigant	20	148,827	41,407
	Sterile water	80	126,258	42,279
	NaOCl	80	89,090*	61,715
Flow velocity	0 rpm	20	148,827	41,407
	60 rpm	80	129,719	51,378
	200 rpm	80	85,628*	51,688
Time	0 sec	20	148,827	41,407
	30 sec	80	98,766*	54,014
	60 sec	80	112,246*	56,935

* Significant at the 0.05 level compared to the control group; Post hoc Dunnett's test

Table 4. Mean proportion of dead bacteria (%) under different conditions

	Study conditions	n	Mean	SD
Irrigant	No irrigant	20	3.1	3.8
	Sterile water	80	6.9	7.0
	NaOCl	80	30.1*	26.6
Flow velocity	0 rpm	20	3.1	3.8
	60 rpm	80	8.4	10.7
	200 rpm	80	28.6*	26.1
Time	0 sec	20	3.1	3.8
	30 sec	80	15.0*	20.5
	60 sec	80	22.0*	23.6

* Significant at the 0.05 level compared to the control group; Post hoc Dunnett's test

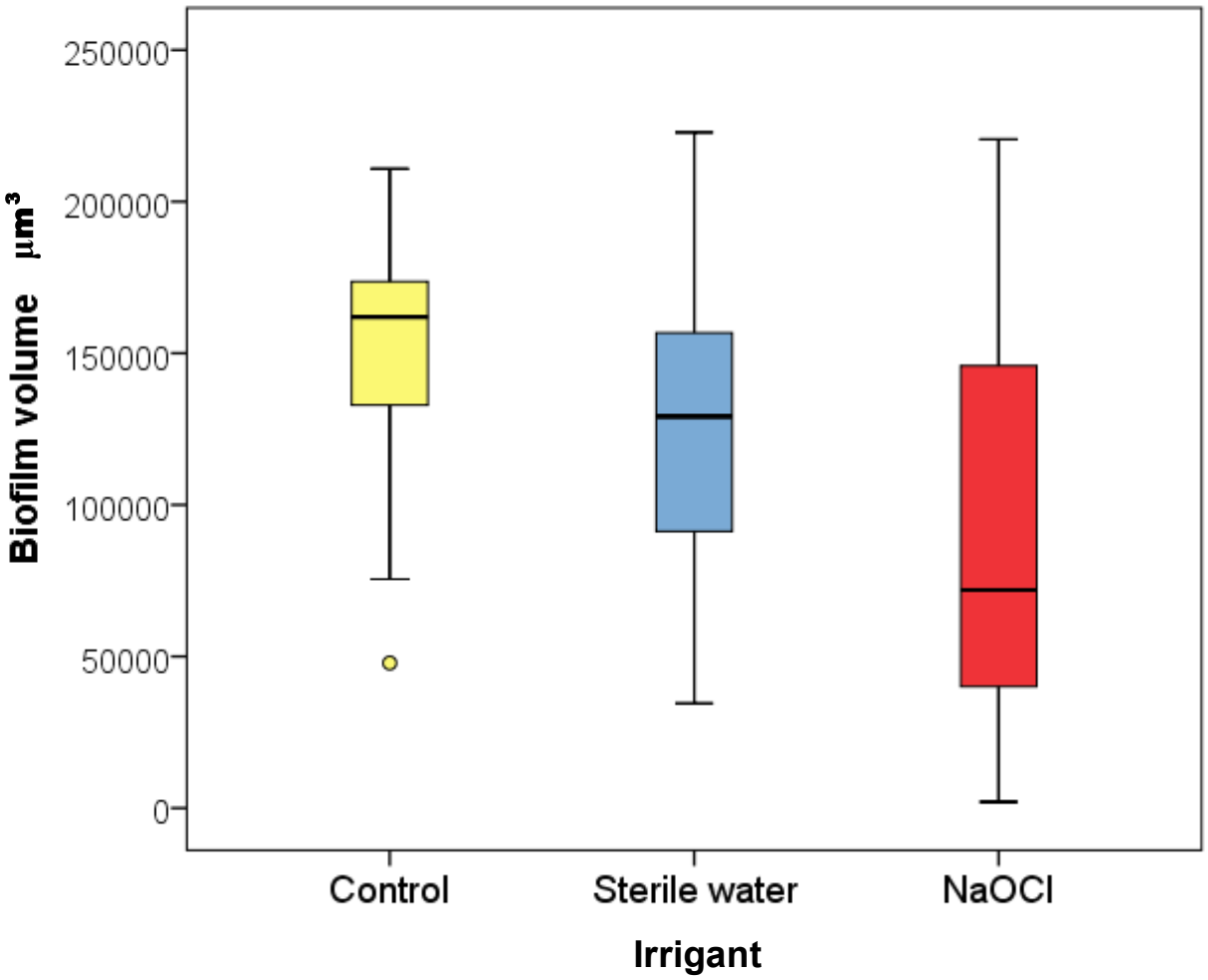

Figure 3. Sample distribution of the biofilm volume after no treatment (control), treatment with sterile water and 0.1 % sodium hypochlorite (NaOCl).

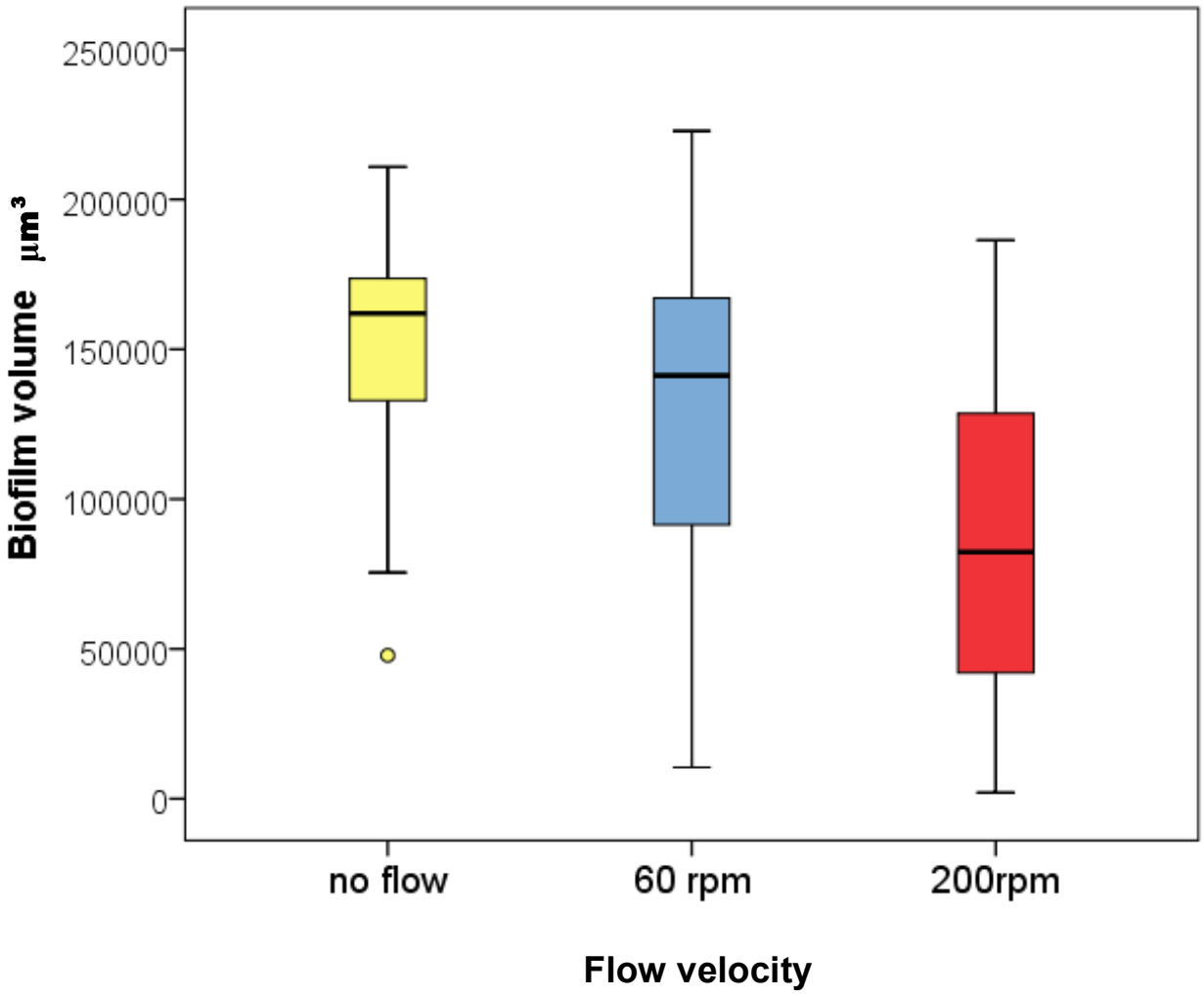

Figure 4. Sample distribution of the biofilm volume using different flow velocities; no flow, 60 rpm and 200 rpm.

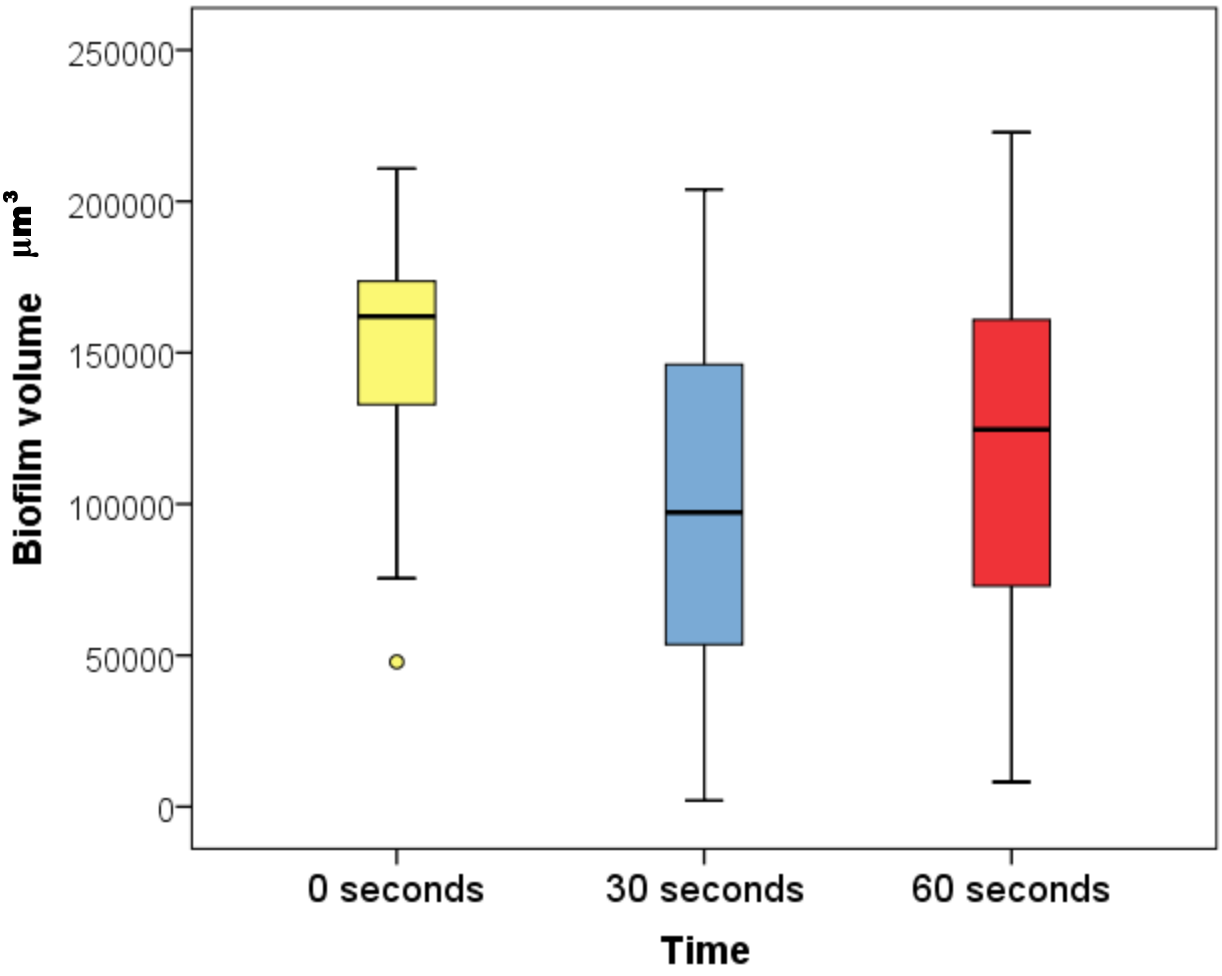

Figure 5. Sample distribution of the biofilm volume after treatment at 0, 30, and 60 seconds.

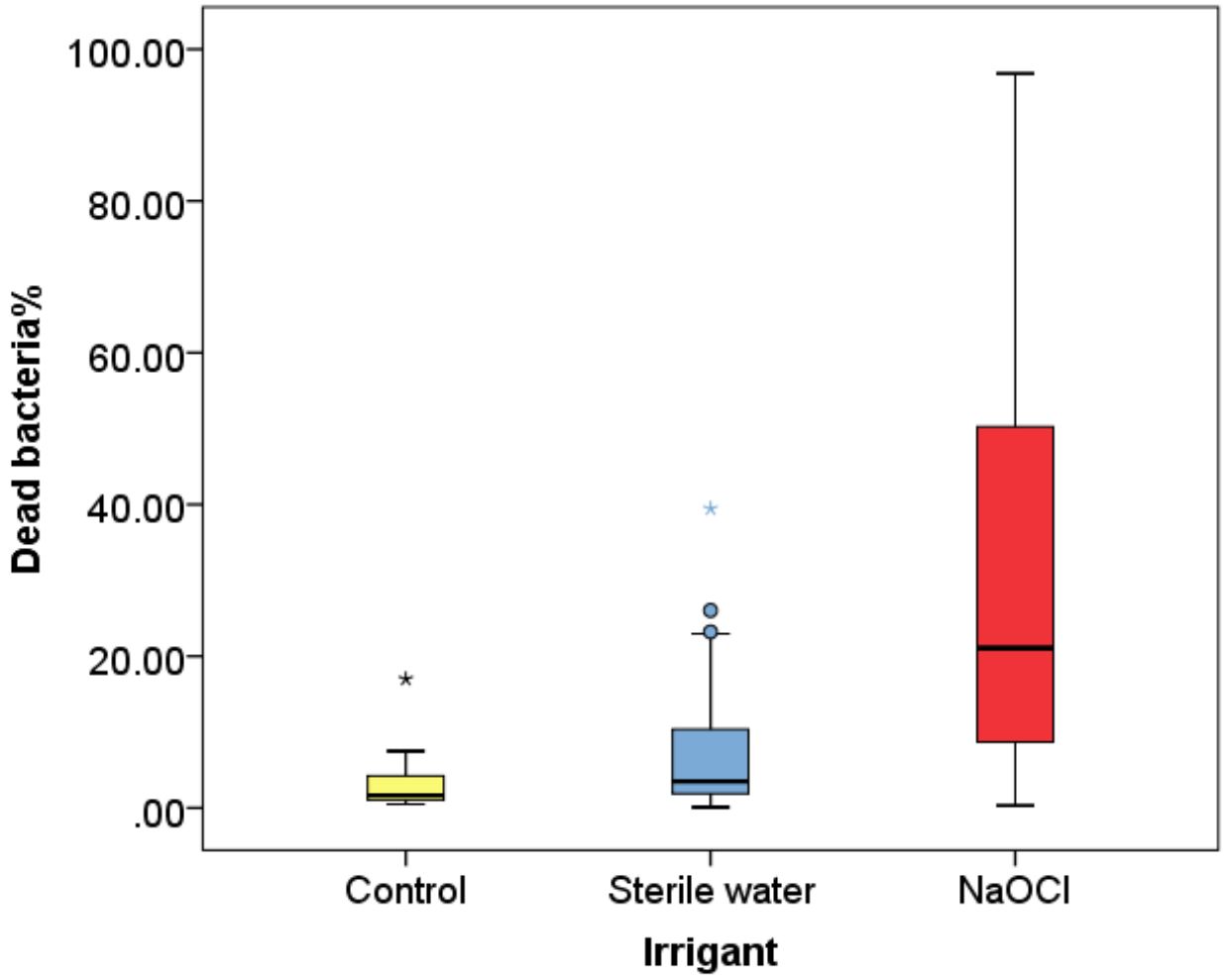

Figure 6. Sample distribution of the percentage of dead bacteria after no treatment (Control), treatment with sterile water and 0.1 % sodium hypochlorite (NaOCl)

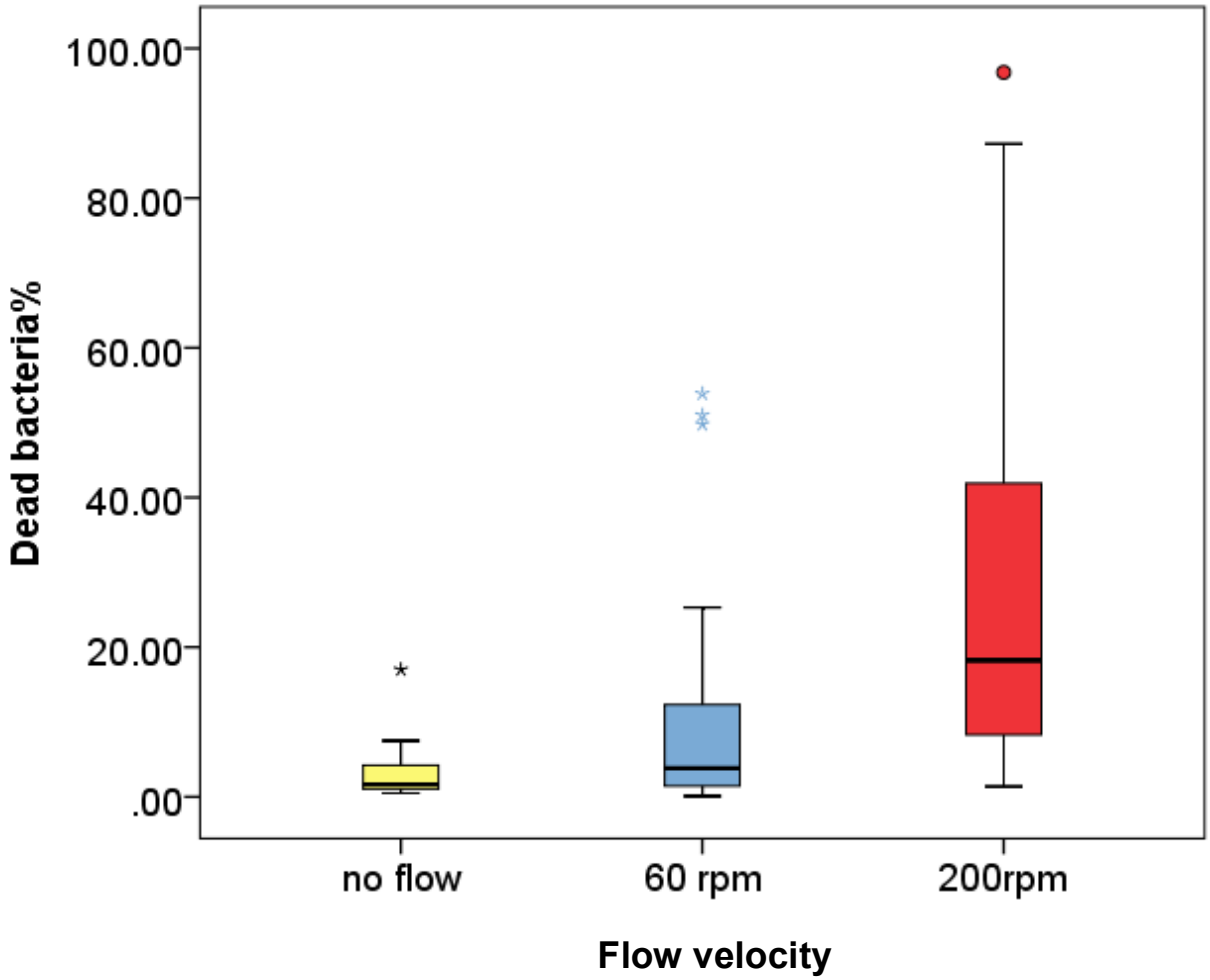

Figure 7. Sample distribution of the percentage of dead bacteria using different flow velocities; no flow velocity, 60 and 200 rpm.

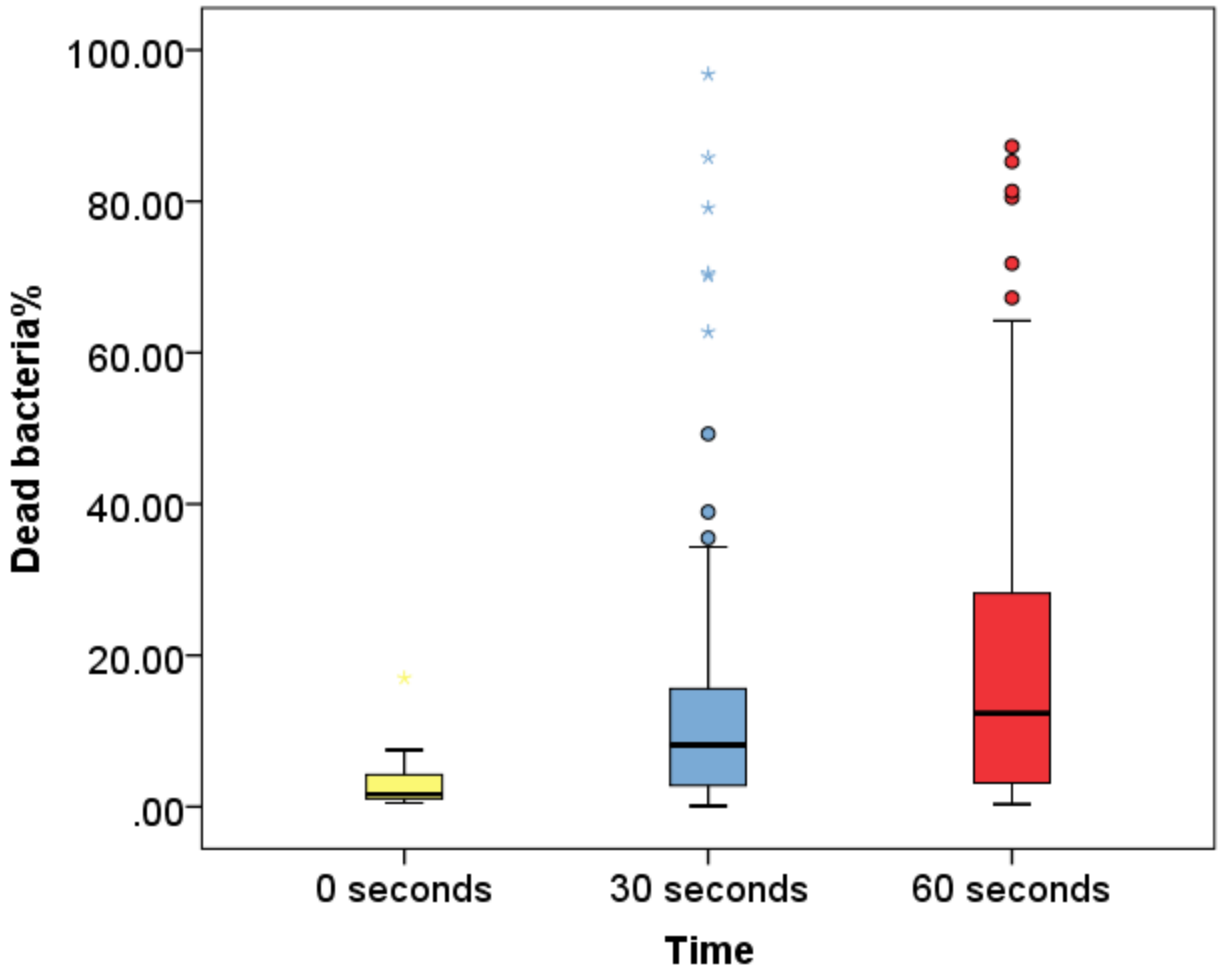

Figure 8. Sample distribution of the percentage of dead bacteria after treatment at 0, 30 and 60 seconds.

4.3 Analysis of individual conditions' effect on biofilm volume

Multiple regression analyses were conducted to test the relationship between several conditions and the biofilm volume. The results are presented in Table 5. After controlling for the other conditions in the model, time of exposure to irrigants had a significant positive regression effect on the outcome. This indicates that the longer exposure time to the irrigant, the higher the expected biofilm volume. Irrigant type and flow velocity had significant negative regression effects on biofilm volume, meaning that when NaOCl and the higher flow velocity were used, a lower resulting biofilm volume can be expected.

4.4 Analysis of individual conditions' effect on the proportion of dead bacteria in the biofilm

Irrigant type and flow velocity had positive regression effects on bacterial killing as shown in table 6. Thus, the more NaOCl and the higher flow velocity (200 rpm) was used, the higher the anticipated percentage of dead bacteria ($p<0.001$). On the other hand, time of exposure to irrigants had a negative regression effect on the proportion of dead bacteria, however it was not statistically significant.

Table 5. Linear multiple regression analysis of the effect of the study conditions on biofilm volume

Study conditions	Outcome: Biofilm volume	
	B coefficient	**Tolerance**
Irrigant	- 0.337*	0.667
Flow velocity	- 0.419*	0.667
Time	0.321*	0.667
Overall model fit: Adjusted $R^2 = 0.279$ * Significant predictors at $p < 0.001$		

Table 6. Linear multiple regression analysis of the effect of study conditions on the proportion of dead bacteria

Study conditions	Outcome: Proportion dead bacteria	
	B coefficient	**Tolerance**
Irrigant	0.408*	0.667
Flow velocity	0.315*	0.667
Time	-0.097	0.667
Overall model fit: Adjusted $R^2 = 0.323$ * Significant predictors at $p < 0.001$		

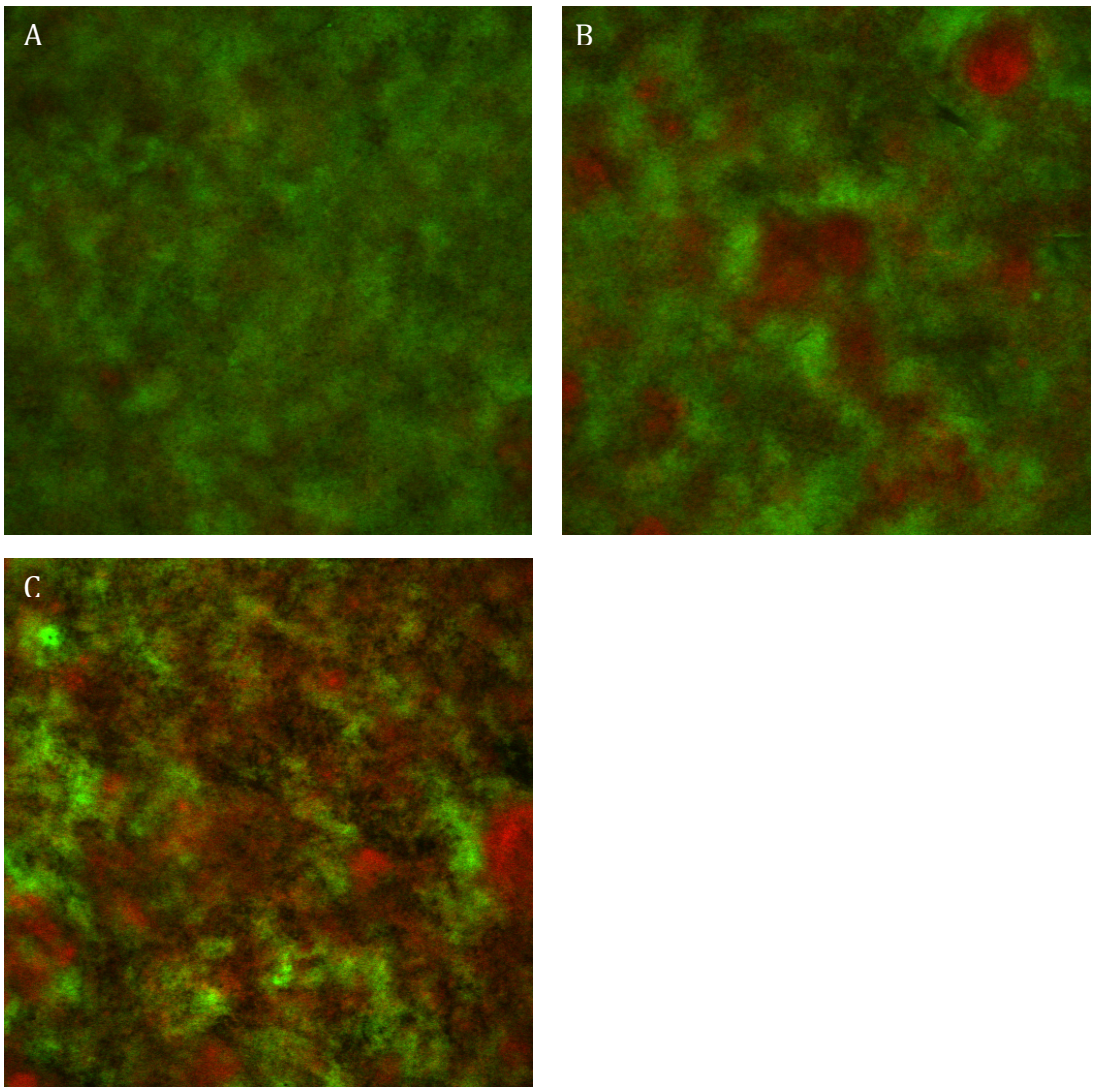

Figure 9. Confocal laser scanning microscopy slices of mature biofilms. (A) Control. (B) Exposure to 0.01% sodium hypochlorite at 60 rpm for 60 sec. (C) Exposure to 0.01% sodium hypochlorite at 200 rpm for 60 sec.

Chapter 5: Discussion

In our study, we used mature biofilm on collagen coated hydroxyl apatite discs (CHA) to test different conditions instead of dentin discs. Although infected dentin discs would be replicate the clinical setting more closely (Haapasalo et al., 1987), CHA discs were used in our study for a variety of reasons. Obtaining dentin from different teeth and various locations would make standardizing samples a tedious task. The diameter of dentinal tubules, and their orientation and density are highly variable within the same tooth, as well as when compared with other teeth (Pashley, 1996). Another reason was the inhibitory effect of dentin on antimicrobial solutions (Portenier et al., 2002). Since our aim was to study the antimicrobial effect of irrigation flow velocity, it was prudent to control for other antimicrobial factors that may affect the results. It was challenging to determine which testing model to use, but preliminary testing using the drip method, flow chamber, and CDC biofilm reactor revealed that the latter was superior: the CDC biofilm reactor had notable advantages with regards to standardization, reproducibility, and control over parameters. The discs would be submerged in solution and a biofilm would be hanging on the main canal walls and in contact with irrigation. Each velocity applied would be directed towards the whole surface of the disc that is placed in an identical position to the previous one.

When no irrigation was used in the control groups, the highest volume of biofilm and the lowest percentage of bacteria killed was seen. This depicts the essential role of irrigation in cleaning root canals and killing root canal bacteria. Baker (1975) related the

flushing action of solvents to the remaining debris in the canals. His study reported that 70 % more debris accumulated in canals that were instrumented without irrigation.

In our study, when sterile water was used as an irrigant, it resulted in lower biofilm volume values and higher bacterial killing than the control group. Yet, when NaOCl was used, it resulted in even lower biofilm volumes and higher proportions of dead bacteria than both sterile water and the control in general. Sterile water was not effective in removing bacteria completely from the root canals, as it had been shown in the literature (Shih, 1970). Although a considerable decline in bacterial counts was registered with water irrigation, its antibacterial effect was still poor (Siqueira et al., 1999; Pataky et al., 2002). It is noteworthy to acknowledge that even with the limitations of earlier sampling methods when compared to more advanced techniques used currently, water remains ineffective in reducing either the biofilm volume or its associated bacteria. Hence, it is valid to state that the mechanical effects of endodontic irrigation cannot solely eliminate microorganisms from the root canal (Ingle and Zedlow, 1958; Nichols, 1962).

Sodium hypochlorite (NaOCl) on the other hand, is a robust antibacterial agent. It has the potential to kill bacteria very quickly even when used in a highly diluted form (Haapasalo et al., 2005). In a study on three isolated species of anaerobic bacteria, NaOCl managed to eradicate them all in 15 seconds (Vianna et al., 2004). Thus, regardless of the very short exposure times of our experiments, it came as no surprise that irrigating with NaOCl had significantly reduced the biofilm volume and showed the highest percentage of bacterial killing compared to no irrigant or irrigation with water. This corroborates results obtained by Byström and Sundqvist (1983) and Siqueira et al. (2002) who

demonstrated the superiority of NaOCl over saline in its antimicrobial effects on both mixed anaerobic flora and *Enterococcus faecalis*, respectively.

In both NaOCl and water groups, the high velocity (200 rpm) used in this study was significantly superior to the low velocity (60 rpm) and the control groups with regards to both measured outcomes. In other words, using 200 rpm in general produced greater reduction in biofilm volume and higher bacterial killing than 60rpm in both sterile water and NaOCl. The reasons behind the enhanced antimicrobial efficacy could be that the increased flow velocity may have created turbulence within the liquid media, which would improve the irrigant's infiltration into the biofilm. However, whether or not turbulence is created within the root canal system has yet to be proven experimentally. Another explanation could be the relationship of the velocity gradient on the wall shear stress. A higher velocity gradient would have created higher shear forces on the surface of the biofilm, which has shown to result in a mechanical force that can detach fragments of biofilm, reduce its overall volume, and eradicate bacteria remaining on the attached biofilm (Boutsioukis and Van der Sluis, 2015). Similarly, investigations of bacterial biofilm development and detachment under various hydrodynamic flows showed that a high velocity of 200 rpm was associated with a higher detachment rate than lower velocities of 50 rpm (Garny et al., 2008). New biofilm has the potential to re-grow in areas stripped off biofilm within days. The secondary biofilm formed was only loosely attached to the original biofilm resulting in heterogeneity within the structure and thickness of the biofilm (Telgmann et al., 2004). Having such topography would very likely disturb flow patterns on its surface and cause detachment of larger magnitude (Stoodley et al., 1999a-b). We did not examine the detached biofilm particles, however,

another study showed that the detached particles displayed similar structures compared to their biofilm source. When subjected to high shear forces, they were found to be as dense as their corresponding biofilms (Garny et al., 2008). Depending on the initial structure of the biofilm and its internal strength, the advantage in increasing irrigation velocity is that it could cause total loss of the biofilm at times. (Telgmann et al., 2004). The study design we used focused on the antimicrobial effects on the biofilm that remains attached to the surface rather than that is detached. Our results support the advantage of using higher irrigation velocity in reducing the residual biofilm volume and the proportion of live bacteria.

Statistical analysis of the effect of the time of exposure to irrigants revealed an unexpected relationship to biofilm volumes. After 60 seconds of NaOCl exposure, the biofilm volume was larger than that after 30 seconds. This finding was significant when each of these groups was compared to the control. The biofilm volume was expected to decrease with longer exposure to irrigants, however, the opposite may be a part of the antimicrobial process. Oral biofilms are expected to expand or, detach if a certain threshold was surpassed (Busscher et al., 2003). The extra polymeric substance (EPS) was an important element that influenced the volume of biofilms according to Garny et al. (2008). They hypothesized that an increased volume of EPS had led to an increase in the mass density of the biofilm. When grown under a constant flow of 200 rpm, the volume of bacteria and EPS was larger than for those biofilms cultivated under lower flow conditions of 50 rpm. Though the differences in study aims and models from our study must be kept in mind, their results emphasized the role of EPS in determining the outcome tested and the effect of flow on the volume of cultured biofilms. In another

study, fluctuations in biofilm volumes were recorded throughout a 45-day test period. The bacterial and EPS volume components seem to incidentally correlate negatively with each other; where one increased, the other decreased. These studies offer a possible explanation for the higher biofilm volume we recorded at 60 seconds, which could be the volumetric expansion of the biofilm exerted by the irrigant flow. This expansion enables irrigant penetration, thereby improving the chemical effect of the irrigants (He et al., 2013). We could also attribute our result to the fairly short exposure times used, 30 sec and 60 sec, that may not be enough to detect further alterations. It is critical to remember that our samples are independent of each other and do not reflect a continuous monitoring process before and after each experiment. We used one sample as control and separate sample as test. Further utilization of the control sample was not feasible because the technique used for confocal laser scanning microscopy disrupts the sample after imaging was complete. Since the original parameters of the biofilms before they were treated are unknown, it is not possible to ascertain if the increase of biofilm volume with time is in fact true. Nonetheless, reflecting on the dynamic nature and complexity of biofilms, these unanticipated outcomes are perhaps logical.

The proportion of dead bacteria, on the other hand, was higher after the 60 sec exposure than after 30 sec of exposure and the control groups. This is in accordance with the information available from previous studies using multispecies biofilms *in vitro*. Shen et al. (2010a) found a significant difference in the proportion of the dead cell volume after 1 and 3 minute exposures, with superior killing detected after 3 minutes. The authors found that mechanical agitation through sonics and ultrasonics augmented the antimicrobial effectiveness of chlorhexidine (Shen et al., 2010a). However, agitation

alone did not affect the viability of bacteria in biofilms. Hence, the effectiveness of biofilm eradication was related to the chemical composition of the irrigant, mechanical agitation, and contact time (Shen et al., 2010a). The duration of exposure was also shown to impact bacterial killing in another study. Biofilms subjected to 10 minutes of irrigation were associated with higher proportions of dead cells than at 1 and 3 minutes ($p<0.001$) (Shen et al., 2009). One could doubt whether or not clinicians would irrigate for 10 minutes with each syringe insertion or more likely be willing to use 1 min. A survey regarding irrigation protocols for smear removal followed by Canadian dentists reported that most respondents spent 60 seconds per canal (Mello et al., 2016). Although the conditions of our study are much more simplified than the complex root canal system, we decided to study shorter units of time in order to also comprehend the early antimicrobial effects of irrigant flow velocity on bacterial biofilms.

Linear multiple regression analysis was done to analyze the effect of each independent variable (irrigant type, flow velocity and time of exposure to irrigants) on the dependant outcomes studied; the biofilm volume and the proportion of dead bacteria. Our results showed that irrigant type and flow velocity had significant negative regression effect on biofilm volume. This means that when NaOCl and a higher flow velocity were used, a lower biofilm volume is expected. Time, on the other hand, had a significant positive regression effect on the biofilm volume. This indicates that the longer the exposure time to any of the irrigants used, the higher the expected biofilm volume. This replicates the findings of ANOVA and Dunnet's Post hoc test discussed previously. While an increase in bacterial killing was shown when a 60 sec exposure time was used *vs*. 30 sec, the time of exposure to irrigants had a negative regression effect on the percent

of dead bacteria. Previous evidence has shown that chemical effects depend on the duration of interaction with targeted material along with the concentration of the irrigant and the area of contact between them (Moorer et al., 1982; Portenier et al., 2002; Boutsioukis and Van der Sluis, 2015). However, the effect associated with the duration of exposure was not shown in our study. The regression model showed that irrigant type and flow velocity had a positive regression effect on bacterial killing. So the use of NaOCl and a higher flow velocity, meant a higher anticipated percentage of dead bacteria ($P < 0.001$). It comes as no surprize that NaOCl was associated with a higher level of bacterial killing than sterile water and no irrigation. However, the antimicrobial effect of irrigation velocity on biofilms has not been demonstrated as clearly as our findings do.

5.1. Limitations of the study

In vitro studies such as ours allow control over different clinical variables, minimizing the effect of confounding factors on the results. Nonetheless, *in vitro* studies are associated with limitations. Although efforts were made to replicate conditions of the root canal environment for bacteria to grow, the bacterial species collected in our study are unknown. Bacterial sampling of supra- and sub-gingival plaque from a single donor is not representative of all possible species that could be found. Including samples from other donors may have been beneficial. Despite that fact, we cultured bacteria on collagen coated hydroxyapatite in a peptide rich medium and low oxygen conditions in order to control these species growth patterns. It is unknown whether *In vitro* biofilms developed are as firmly attached to their substrates as they would *in vivo*. These

speculations could affect the remaining biofilm volume observed in our study. Whether *in vitro* biofilms are the identical to those of endodontic origin requires further verification (Stojicic, 2013).

Chapter 6: Conclusion

Our investigation showed that irrigation flow velocity is a significant factor in the antimicrobial effectiveness of endodontic irrigation procedures. Irrigation with a high velocity showed significantly better antimicrobial effect on biofilm volume reduction and bacterial killing compared to low velocity irrigation. Therefore, it is important to consider irrigation fluid dynamics when studying the antimicrobial effects of different irrigants in the future. The methodology used in our study is a reproducible design that can be utilized to test the effects of flow velocity and potentially other irrigation factors as well. With all the new irrigation systems emerging, understanding the effects of factors such as flow velocity on irrigation antimicrobial effectiveness is essential.

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