# The Effectiveness of Heated Sodium Hypochlorite against *Enterococcus faecalis* in Dentinal Tubules

by

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## Abstract

**Introduction:** The goal of chemical irrigation in endodontics is to maximize the reduction of microbes and necrotic tissue remnants in the root canal system. *Enterococcus faecalis* is most frequently associated with persistent endodontic infections. Sodium hypochlorite (NaOCl) is the most commonly used root canal irrigant. Heating NaOCl has a positive effect on the tissue dissolving abilities of the solution.

**Objective:** The aim of this study was to compare the killing effectiveness of two different sodium hypochlorite concentrations (2 % *vs*. 5.25 %) at two different temperatures (20 °C *vs.* 60 °C) against two strains of *E. faecalis* biofilm at different ages of maturation (3 days *vs*. 3 weeks), in a previously described dentin block model.

**Hypotheses:**

Temperature, concentration, the exposure time, the age of the biofilm nor the strain of *E. faecalis* have no effect on the killing efficacy of NaOCl on *E. faecalis.*

**Methods:** Dentin blocks were prepared from human root dentin. Two *E. faecalis* strains were introduced into dentinal tubules by centrifugation and incubated to form biofilms. After 3 days and 3 weeks of growth, the *E. faecalis* dentin biofilm samples were exposed to sterile water, 2 %, and 5.25 % NaOCl for 3 and 10 minutes at room temperature (20 °C) and at 60 °C. After the exposure, the proportions of killed bacteria in dentin canals were assessed by viability staining and confocal laser scanning microscopy.

**Results:** The killing efficacy of *E. faecalis* in dentin tubules was affected by an increase in temperature of the NaOCl solution. The concentration of the NaOCl solution and exposure time to the irrigant played a role in the killing efficacy of NaOCl. Overall, 5.25 % NaOCl

demonstrated a greater effect on the killing efficacy on *E. faecalis*, except when NaOCl is used at 60 °C with an exposure time of 10 minutes. There was no significant difference ($p>0.5$) between the two different strains of *E. faecalis* or between the 3-day and 3-week old biofilms in their sensitivity to the bactericidal effect of NaOCl.

**Conclusion:** Bacterial killing by NaOCl is enhanced by an increase in temperature and concentration.

## Lay Summary

Sodium hypochlorite (NaOCl) is the most commonly used root canal irrigant to maximize the reduction of microbes and necrotic tissue remnants in the root canal system. Of the microbes known to cause endodontic disease, *Enterococcus faecalis* is most frequently associated with persistent endodontic infections. Heating NaOCl has a positive effect on the tissue dissolving abilities of the solution. However, very few studies have investigated the effectiveness of a heated NaOCl solution on the removal of *Enterococcus faecalis* from dentinal tubules. The aim of this study was to compare the killing effectiveness of two different sodium hypochlorite concentrations (2 % *vs*. 5.25 %) at two different temperatures (20 °C *vs.* 60 °C) and against two strains of *E. faecalis* biofilm at different ages of maturation (3 days *vs*. 3 weeks), in a previously described dentin block model using confocal laser scanning microscopy together with viability staining.

## Preface

This thesis is the final proof of competence for obtaining the Master of Science (MSc) degree in Craniofacial Science from the University of British Colombia in Vancouver, Canada. The research question was proposed by Dr. Stefanie Nio and progress was accomplished with careful revision by Dr. Ya Shen and Dr. Markus Haapasalo. The research has been executed on site at the University British Colombia, Faculty of Dentistry, Department of Endodontics in Vancouver, Canada. The study model used was designed in Dr. Haapasalo's laboratory at UBC and first published by Dr. Jingzhi Ma et al in 2010. Collection and preparation of the samples and performance of the research were carried out by Dr. Stefanie Nio. The viability staining and confocal scanning microscopy and analysis were done by Dr. Stefanie Nio with assistance from Dr. Xiangya Huang, Dr. Hazuki Maezono and Dr. Zhejun Wang. Statistical analysis was performed with help from Shenyi Pan (UBC Statistical Consulting and Research Laboratory). Ethics approval was required and granted from the University of British Columbia Clinical Research Ethics Board (certificate number H12-02430). This study was supported by the Canadian Academy of Endodontics, by start-up funds provided by the Faculty of Dentistry, University of British Columbia and by Canada Foundation for Innovation (CFI fund; Project number 32623).

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## List of Abbreviations

CLSM----------Confocal Laser Scanning Microscopy

NaOCl----------Sodium Hypochlorite

## List of Symbols

°C----------Degree Celsius

## Acknowledgements

*"History of all great works is to witness that no great work was ever done without either the active or passive support of a person's surroundings and one's close quarters."*

*-Unknown*

I am grateful to my supervisor, Dr. Ya Shen, whose expertise, understanding, generous guidance and support made it possible for me to work on a topic that was of great interest to me. She was able to guide me through every challenge I encountered during my research. I would also like to express my gratitude to Dr. Markus Haapasalo for being ever so kind to show interest in my research and taking his precious time to give me valuable advice. His prominent role as an educator and his dedication to research is remarkable and admirable. I am thankful to Dr. Clive Roberts for serving on my committee and for his thoughtful suggestions and honest words of wisdom.

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## Dedication

*"There is only one happiness in this life, to love and be loved."*

- George Sand

To my family for their immense support throughout this journey, for their unconditional love and believing in me every step of the way.

To Rudchen for his support and great patience at all times. For allowing me to dream and the encouragement to pursue my dreams. I could not have made it through without you by my side.

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## 1. Introduction

In the past several decades it has been unequivocally proven that apical periodontitis results from etiological agents of endodontic origin. Bacteria and their byproducts cause a chronic inflammatory disorder of the peri-radicular tissues (1-4). Pulp tissue, when infected, will become necrotic and thus offers a favorable environment for the microbiota (5). Bacteria will have the ability to form a biofilm; a community of microorganisms embedded in an exopolysaccharide matrix, adhered onto a moist surface, whereas planktonic organisms are free-floating single microbial cells in an aqueous environment (6,7). When a biofilm is formed, the resistance to antimicrobial agents is up to a thousand times greater compared to bacteria when present in their planktonic form. This results from a metabolic diversity in a biofilm, thereby allowing shared nutritional sources and waste products and thereby resulting in greater overall resistance (8,9).

The ultimate goal in endodontics is to prevent or heal apical periodontitis (10). This is currently done by mechanical preparation of the root canal walls using hand and rotary instrumentation, supplemented with irrigation and chemical disinfection (11). Mechanical instrumentation is known to reduce the microbial load. However, it is unable to completely eradicate bacteria from the system, leaving around 40-50 % of the root canal walls untouched, thereby rendering irrigation imperative (12).

Several methods have been explored to enhance the efficacy of irrigation with chemically active solutions. Various solutions and delivery methods have been proposed. Nowadays, sodium hypochlorite is most commonly used amongst dental practitioners due to its favorable tissue-dissolving capacity and its potential to disinfect dentin (13). As it currently seems impossible to

completely eradicate all the microorganisms present from the root canal system, there has been a continuous pursuit to seek improvements to further enhance the means of disinfection in endodontics (14-15).

### 1.1 Root Canal Infection

Bacteria are the etiological factors of periapical disease (1-3). However, it is unknown how many bacterial cells left in the root canal system after endodontic treatment may prevent complete healing. In most instances, infection of the pulp space occurs in a late stage of caries development (16). Another pathway to the pulp may arise from cracks in the crown extending further into the root canal system (17). Despite the different bacterial pathways, inflammation and infection of the pulp should be differentiated between vital and non-vital cases (18).

In cases of pulpal necrosis with radiographic signs of peri-radicular pathosis, the pulp space always harbors bacteria (19). In contrast, vital pulp has the ability to defend itself against bacteria; therefore, it is for the most part non-infected (although it may be widely inflamed). However, necrosis is inevitable if the pulp tissue cannot withstand the microbial challenge (17). Due to the difference in entity, the aim in vital cases is to remain *aseptic* throughout the endodontic procedure, whereas *antiseptic* measures are taken during the treatment of necrotic root canals (13).

- *Asepsis: the prevention of infection entering a primarily sterile environment*
- *Antisepsis: the attempt to remove all micro-organisms*

In non-vital teeth, the host defense system has lost the ability to control the conditions in the root canal system, due to lack of circulation, which results in a low-oxygen environment with

subsequent harsh ecological conditions (20). Remnants of the organic pulp tissues offer substrates for bacterial populations to thrive on (21,22). Ergo, bacterial communities in non-vital teeth and teeth with failed endodontic treatments are most likely found in the apical third of the root canal system, where they can survive and find nutrients e.g. from tissue fluids entering the apical canal (20). Studies have also shown the ability of micro-organisms to invade the adjacent dentin through the dentinal tubules (23,24).

### 1.2 Micro-organisms in Root Canal Infections

Primary root canal infections are polymicrobial and are characterized by a dominant obligately anaerobic bacterial community (21). Of the bacteria cultivated from these cases prior to root canal therapy, Gram-negative anaerobic rods, Gram-positive anaerobic cocci, Gram-positive anaerobic and facultative rods, *Lactobacillus* species and Gram-positive facultative *Streptococcus* species are typically found (21). During endodontic treatment, the root canal system will be exposed to oxygen which may contribute to the elimination of obligate anaerobes, as opposed to facultative bacteria. The latter group includes non-mutans Streptococci, Enterococci, Lactobacilli and other facultative Gram-positive bacteria; some of these bacteria in particular are known to be more resistant to chemo-mechanical endodontic therapy and intracanal medicaments (25). The focal point of many studies has been on *Enterococcus faecalis,* as recurrent infections or failed endodontic treatment has been associated with its presence (25-27).

### *1.2.1 Enterococcus faecalis*

Enterococci are often a causative factor in human infections, ranging from infections of the urinary tract (28), bloodstream, abdomen (29), endocardium (30), biliary tract (31), burn wounds (32), and in foreign objects placed in the body (33).

In endodontics *E. faecalis* plays an important role, as it has been shown to be the most dominant enterococcus species (34,35) and is commonly found in asymptomatic, persistent endodontic infections (35,36). These findings could lead many to believe that *E. faecalis* plays an important part in persistent infections and failed responses to endodontic treatment.

*E. faecalis*, a Gram-positive facultative anaerobe bacteria, presents with its own virulence factors, some of which have been shown to account for its resistance to intracanal medicaments, such as calcium hydroxide (37-39). It can also survive in a root canal system as a single organism in a monoculture, with no support from other species (40). *E. faecalis* also has the ability to invade dentinal tubules (38,39,41). Therefore, it is plausible for these bacterial cells to recolonize and re-infect the obturated root canal system after surviving the chemomechanical challenge.

### *1.2.2 Enterococcus faecalis – Survival and Virulence Factors*

*E. faecalis* has certain characteristics which enhances its survival and generates a challenge for complete eradication. It has been shown to adhere to host cells (42), it can express proteins to ensure cell survival as the nutrient supply changes and as it competes with other bacterial cells (43-48). *E. faecalis* also possesses the ability to share virulence factors with other species, thereby further increasing its odds of survival (49).

Although *E. faecalis* carries several virulence factors, it relies more on its ability to survive as a pathogen in the root canal system (50). The key characteristics of *E. faecalis* are listed in Table 1.

**Table 1 Virulence Factors of *E. faecalis* (51)**

| |
|---|
| **Endures prolonged periods of nutritional deprivation** |
| **Binds to dentin and proficiently invades dentinal tubules** |
| **Alters host responses** |
| **Suppresses the action of lymphocytes** |
| **Possesses lytic enzymes, cytolysin, aggregation substance, pheromones, and lipoteichoic acid** |
| **Utilizes serum as a nutritional source** |
| **Resists intracanal medicaments** |
| **Competes with other cells** |
| **Forms a biofilm** |

### 1.3 Endodontic Irrigants

The necessary characteristics of an endodontic irrigant are that it kills micro-organisms present in the necrotic root canal and dissolves the necrotic tissue. It should have the ability to penetrate into dentin and disinfect its tubules. It would ideally provide a long-term antibacterial effect after the irrigant had been used, also known as substantivity (52). The irrigant should be able to remove the smear layer, which is created during mechanical instrumentation. As the irrigant solutions will come in close contact with vital tissues, it is desired that they are systemically non-toxic, non-caustic and non-carcinogenic to peri-radicular tissues and pose minimal risk of inducing an anaphylactic reaction. It is also of great importance that they do not have an adverse effect on the properties of dentin or on the sealing ability of restorative materials. In addition, endodontic

irrigants should be easily accessible, user-friendly, and relatively inexpensive. One of the most desirable features for an ideal irrigant includes the capacity to inactivate harmful endotoxins and to dissolve vital pulp tissue and pulp tissue remnants (13,52). Finally, irrigating solutions should not cause tooth discolouration (13,52). The characteristics of an ideal endodontic irrigant are summarized in Table 2. Over the past several decades, a wide variety of compounds dissolved in aqueous solutions have been proposed as chemical irrigants for endodontic use. These included inert solutions such as sodium chloride (NaCl) or highly toxic and harmful substances such as formaldehyde (CH2O) (53).

**Table 2 Characteristics of an Ideal Endodontic Irrigant (52)**

| |
|---|
| **1. Effective germicide and fungicide** |
| **2. Non-irritating to the periapical tissues.** |
| **3. Stable in solution.** |
| **4. Prolonged antimicrobial effect and a sustained antibacterial effect after use.** |
| **5. Active in presence of blood, serum and protein derivatives of tissue.** |
| **6. Able to completely remove the smear layer.** |
| **7. Low in surface tension.** |
| **8. Able to disinfect the dentin/dentinal tubules.** |
| **9. Does not interfere with repair of periapical tissues.** |
| **10. Does not stain tooth structure.** |
| **11. Inactivation in a culture medium.** |
| **12. Does not induce a cell-mediated immune response. Is non-antigenic, non-toxic, and non carcinogenic to tissue cells surrounding the teeth.** |

| |
|---|
| **13. Has no adverse effects on the physical properties of exposed dentin.** |
| **14. Has no adverse effect on the sealing ability of filling materials.** |
| **15. Easy to use/apply.** |
| **16. Inexpensive.** |

### 1.4 Sodium Hypochlorite

*Sodium hypochlorite* (NaOCl) is a chlorine compound often used as a disinfectant or bleaching agent. It is currently the irrigant of choice in endodontics due to its antibacterial and lubricating properties, as well as its ability to dissolve organic tissue (86). Furthermore, it is inexpensive, easily available, and remains well preserved over time if adequately stored (54). A summary of the characteristics of NaOCl is found in Table 3. Chlorine can only be found in combination with sodium, potassium and magnesium (55). In human physiology chlorine compounds can also be found as a part of the non-specific immune defense. Chlorine is produced by neutrophils through the myeloperoxidase-mediated chlorination of a nitrogenous composition (56).

**Table 3 Characteristics of NaOCl (52)**

| Characteristics of NaOCl | Limitations of NaOCl |
|---|---|
| **Current irrigant of choice** | **Toxic (risk of hypochlorite accident)** |
| **Effective organic tissue solvent** | **No long term antibacterial activity (substantivity)** |
| **Lubricates** | **Corrosive, unpleasant odor** |
| **Effective fairly quickly** | **Removes only the organic part of the smear layer** |

### *1.4.1 History of NaOCl*

First produced in the late 18$^{th}$ century in Javel near Paris, NaOCl was initially known as "Eau de Javel". In World War I, NaOCl was used as a buffered 0.5 % sodium hypochlorite solution by the chemist Henry Dakin and the surgeon Alexis Carel to irrigate open wounds. Due to the favorable features of NaOCl, the use of aqueous sodium hypochlorite advanced to be the main irrigant in endodontics by 1920 (57).

### *1.4.2 Mechanism of Action*

Sodium hypochlorite is a strong base with a pH above 11. The high pH of sodium hypochlorite allows it to impede in the integrity of the cytoplasmic membrane. This occurs through irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism, and phospholipid degradation observed in lepidic peroxidation (58). Sodium hypochlorite exists in a dynamic equilibrium as shown in Figure 1 (58). Sodium hypochlorite dissolves organic tissues and degrades fatty acids. It can convert them into fatty acid salts (soap) and glycerol (alcohol) (52). This so-

called saponification reaction will reduce the surface tension of the remaining solution (52). The reduction of surface tension is desired to increase the contact surface area to the solution.

$$NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$$

**Figure 1 Mechanism of Action of NaOCl**

Sodium hypochlorite can neutralize amino acids, forming water and NaCl, resulting in a decrease in the pH (52). Hypochlorous acid is formed when chlorine dissolves in water and is in contact with organic matter. Hypochlorous acid (HClO) is an oxidizer, as well as a weak acid (52). It is responsible for the antibacterial activity of NaOCl as it releases chlorine, which will form chloramines when combined with a protein amino group. Chlorine is a strong oxidant which has antimicrobial properties since it is able to inhibit bacterial enzymes (52,58,59). Hypochlorous acid and hypochlorite ions (OCl-) results in amino acid degradation and hydrolysis (52), however the $OCl^-$ ion is less effective than the undissolved HClO (59,60).

### *1.4.3 Concentrations*

Up to the present day there is no consensus on the ideal concentration of NaOCl in endodontics. Concentrations varying between 0.5 and 10 % are currently used, while Henry Dakin originally used an aqueous 0.5 % NaOCl solution. Eventually, NaOCl solutions of higher concentrations were supported for disinfection during endodontic therapy (61). The concentration of NaOCl solutions has been shown to have an impact on its antibacterial efficacy (62), its tissue dissolution capacity (63), and also impacts its caustic effect (64). Case reports regarding serious hypochlorite accidents are regularly found in the literature; these occur when concentrated hypochlorite

solutions are forced into the peri-radicular tissues causing immediate severe pain, edema and ecchymosis (65,66).

Some clinical reports have demonstrated both low and high concentrations to be equally effective in reducing the bacterial load from the root canal system (15,67). Some *in vitro* studies have shown that NaOCl in higher concentrations is more effective against *E. faecalis* and *C. albicans* (62,68,69). NaOCl in higher concentrations has a better tissue-dissolving capacity (67); however, even lower concentrations have shown to be effective when frequently refreshed and used in high volumes. (70,71). Higher concentrations of NaOCl are known to be more toxic than lower concentrations (72). However, with careful application, ensuring the solution stays within the confines of the root canal system, higher concentrations are successfully used during root canal therapy with a low incidence of complications (66). In general, it would be safe to say that if lower concentrations are used for root canal disinfection, higher volumes and use of more frequent intervals of fresh solution are required. Such use could negate the possible drawbacks of low concentrations (71).

### *1.4.4 Exposure Time*

It is commonly accepted that NaOCl has an excellent antimicrobial effect in endodontics, however the literature shows a wide variation in the extent of its antibacterial effect (73). Published reports have shown that NaOCl, even at lower concentrations, can eradicate planktonic bacteria within seconds (62,68,69). On the contrary, other authors reported considerably longer exposure times necessary for the eradication of the same species (74). Confounding factors are responsible for these differences in the literature and it may be concluded that the presence of organic matter during the killing experiments has a significant effect on the antibacterial activity of NaOCl (73).

The presence of dentin has been shown to result in a considerable delay in the killing of *E. faecalis* by 1 % NaOCl. When such confounding factors are excluded, it has been reported that hypochlorite is a powerful medium for rapid eradication of bacteria, even at low concentrations of less than 0.1 % (74,75). Nevertheless, *in vivo* the presence of organic substances (in the form of inflammatory exudate, tissue remnants, and microbial biofilm) is inevitable. These organic substances will interact with NaOCl, thereby weakening its antibacterial effect. Therefore, it is prudent that NaOCl be used in high volume and repeatedly refreshed, in combination with increased exposure time, to increase its efficacy (73).

### *1.4.5 Temperature*

In a quest to improve on the effectiveness of hypochlorite, reports on increasing the temperature of the solution can be found in the literature. A heated hypochlorite solution allows for improvements in its immediate tissue dissolution capacity (76-78). Furthermore, heated hypochlorite solutions show increased capacity to remove organic debris from dentinal shavings more efficiently than unheated hypochlorite solutions (79).

Heating NaOCl also allows for an effect on the antimicrobial properties. Studies on the effect of NaOCl temperature on *Mycobacterium tuberculosis* survival was demonstrated in 1936 (80). A study by Dychdala showed increased bactericidal rates for sodium hypochlorite solutions (nearly double) for each 5 °C rise in temperature in the range of 50 °C to 60 °C (55). Another study using planktonic *E. faecalis* cells demonstrated increased effectiveness of NaOCl, by a factor of 100, when the temperature was raised by 25 °C (81). The same study also showed that a 1 % NaOCl solution at 45 °C was equally as effective in dissolving human dental pulps as a 5.25 % solution at 20 °C (81). From the above studies, it may be concluded that raising the temperature of sodium

hypochlorite may have some benefit in killing bacteria, however there are only a few published articles on this topic (78,81,82,) and there are no clinical studies available at this point to support the use of heated sodium hypochlorite in endodontic practice.

### 1.5 Study Models

Extensive studies have been performed (83-87) on the eradication of infectious microbes in root canal systems, both *in vitro* and *in vivo. In vivo* studies are affected by a number of challenges; for example, there is broad diversity in the anatomy, size, and shape of teeth in the various canals and canal systems (83). Furthermore, the type and number of micro-organisms present can vary considerably between different teeth and obtaining a standardized bacteriologic sample from representative areas of the root canal is difficult. *In vitro* studies, however, have their own flaws. Using *in vitro* planktonic cultures, for example, represents an oversimplified experimental design which may not properly mimic the true *in vivo* circumstances.

*In vitro* research of the bacterial presence in dentinal tubules dates back to 1980 (88). Most of the studies involving bacterial invasion models are based on culturing methods. This allows bacteria to grow in a culture medium in the root canals of extracted teeth or prepared dentin blocks after mechanical instrumentation of the canals (83,89-91). However, the accuracy of using this method is uncertain. In addition, conventional microbiological staining methods which have been used to investigate the distribution of bacteria within the tubules, fail to provide information on bacterial viability (23,92,93).

Today, it is possible to use methods which are less invasive and can differentiate between viable and dead bacteria. These methods involve confocal laser scanning microscopy (CLSM) combined with viability staining (94-96).

### *1.5.1 Dentin Block Model: Ma et al 2011 (83)*

When conducting a study, it is essential to perform individual experimental designs on similar specimens. A dentin block model, as described by Haapasalo and Ørstavik in 1987, allowed for bacterial growth into dentinal tubules for up to 500 mm (97). However, one of the challenges encountered in culturing methods was obtaining an equal, heavy presence of bacteria in all the dentin specimens (98). In 2011, an innovative dentin infection model was introduced, capable of consistent and deep penetration of *E. faecalis* into dentinal tubules, using the power of centrifugation (83). This allowed for standardized measurements of the numbers of live and dead bacterial cells using confocal laser scanning microscopy (discussed below), which was beneficial in the study of the effectiveness of disinfection solutions (83).

### 1.5.2 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) allows a series of sections as thin as 0.3 µm of intact undisturbed biological samples to be obtained in a non-invasive manner. CLSM analysis is frequently used with vital staining techniques, allowing for investigation of the bacterial viability, architecture, and spatial distribution of bacterial biofilms (99). CLSM techniques can be useful as a complement to the established microbiological, histological, electron microscopy, and PCR-based techniques for the identification of viable bacteria (94).

## 1.6 Rationale

The main objective in root canal disinfection is to eliminate the bacterial infection in the root canal system to allow subsequent healing of the peri-radicular tissues (13,26). Irrigation is imperative in chemomechanical preparation as complete eradication of bacteria by solely mechanical

instrumentation is unattainable (15). Irrigation solutions may also prevent a build-up of dentinal debris in the apical region of the root canal system (100). Irrigation is a key factor for disinfection of the root canal system, especially in areas beyond the reach of mechanical instrumentation (101).

Although NaOCl has its disadvantages, e.g. its unpleasant taste and relative cytotoxicity (72,100,103), it is currently the most commonly used irrigant in endodontics. Of all the currently used solutions for irrigation, NaOCl seems to fulfill the most requirements of an ideal disinfecting agent in endodontics (Table 4).

**Table 4 Overview on the Features of Aqueous Solutions Frequently Recommended for Endodontic use. (13)**

| Compound (recommended concentration) | Type | Action on Endodontic Taxa Biofilm | Tissue Dissolution Capacity | Endotoxin Inactivation | Action on Smear Layer | Caustic Potential | Allergic Potential |
|---|---|---|---|---|---|---|---|
| Hydrogen peroxide (3%–30%) | Peroxygen | + | − | − | − | D. o. c. | − |
| Sodium hypochlorite (1%–5.25%) | Halogen-releasing agent | ++ | +++ | + | ++ on organic compounds | D. o. c. | + |
| Iodine potassium iodide (2%–5%) | Halogen-releasing agent | ++ | − | N. i. a. | − | − | ++ |
| Chlorhexidine (0.2%–2%) | Bisguanide | ++ | − | + | − | D. o. c. | + |
| Dequalinium acetate (0.5%) | Quaternary ammonium compound | N. i. a. | − | N. i. a. | + | − | ++ |
| Ethylenediamine tetraacetic acid (10%–17%) | Polyprotic acid | + | − | − | ++ on inorg. compounds | − | − |
| Citric acid (10%–50%) | Organic acid | − | − | − | +++ on inorg. compounds | − | − |

−: absent or minor, +: reported, ++: definitely present, +++: strong, D. o. c.: depending on concentration, N. i. a: no information available.

To further enhance the disinfection of root canal systems additional research is required to increase the efficacy of hypochlorite preparations. It has been reported in the literature that heating NaOCl has a positive effect on its tissue dissolving ability (70,76,103). However, the bactericidal

efficiency of heated NaOCl solutions using *Enterococcus faecalis* strains has not yet been studied. Currently, there is very limited data available on heated hypochlorite solutions which is of relevance to endodontists.

### 1.7 Objectives

The objectives of the study were:

i) to compare the killing efficacy of high and low concentrations of NaOCl on two different strains of *E. faecalis* at different temperatures.

ii) to compare the killing efficacy of NaOCl on young and established *E. faecalis* mono-species biofilms at different temperatures.

iii) to compare the killing efficacy of NaOCl at different exposure times and at different temperatures.

### 1.8 Null Hypotheses

The null hypotheses ($H_0$) were:

1. Temperature has no effect on the killing efficacy of NaOCl on *E. faecalis*
2. Concentration of NaOCl has no effect on the killing efficacy of NaOCl on *E. faecalis*
3. Exposure time has no effect on the killing efficacy of NaOCl on *E. faecalis*
4. Age of the biofilm has no impact on the efficacy of NaOCl to kill *E. faecalis* in biofilms
5. Different *E. faecalis* strains show no difference in their susceptibility to NaOCl

## 2. Materials and Methods

### 2.1 Sample Size Calculation

The sample size was determined utilizing a statistical power analysis software (G*Power 3, v.3.0.5). The program was set to the F family of tests, a one-way ANOVA and 'A Priori' setting. The alpha-type error was specified to be 0.05 and the power beta was specified to be 0.95. The appropriate sample size for each group was determined at 6.89. The sample size in this study per group was 20.

### 2.2 Sample Selection

Forty intact, single-rooted, extracted teeth were selected for this study. The teeth were collected from various dental offices in Vancouver, British Columbia, Canada and were extracted for reasons unrelated to the present study. Upon extraction, the teeth were stored in a 0.05 % NaOCl solution at room temperature (20 °C). The inclusion criteria for the samples were: single-rooted, caries-free teeth. The exclusion criteria for the samples were: teeth with visible cracks, resorptive defects, horizontal or vertical root fractures, and teeth with previous endodontic treatment (Table 5).

**Table 5 Inclusion & Exclusion Criteria of the Samples**

| Inclusion criteria | Exclusion criteria |
|---|---|
| **Single-rooted teeth** | **Teeth with visible cracks** |
| **Maxillary central, lateral incisors, maxillary canine teeth** | **Teeth with resorptive defects** |
| **Caries-free teeth** | **Horizontal or vertical root fractures** |
| | **Previous endodontic treatment** |

The study was approved by the University of British Colombia Clinical Research Ethics Committee Review Boards (No. H12-02430)

### 2.3 Specimen Preparation

A total of 80 dentin block specimens were prepared following a previously described protocol by Ma et al. in 2011 (83). Cylindrical root dentin blocks were prepared by horizontally sectioning each single-rooted tooth at 1 mm below the cemento-enamel junction by using a 0.6 mm thick precision diamond saw (Isomet 5000; Buehler Ltd, Lake Bluff, IL) at 1000 rpm to a length of 4 mm (Fig. 1, 2A).

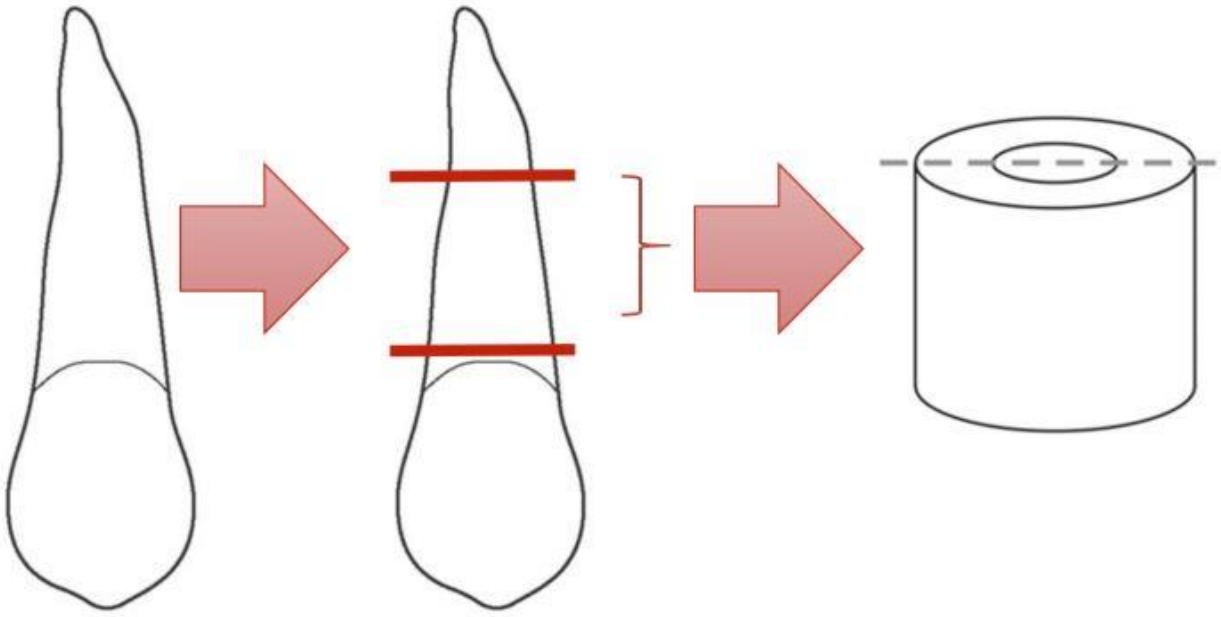


**Figure 2 Schematic drawing of a cylindrical dentin block. The coronal part of a single-rooted tooth is used to create samples. (Courtesy of Ma et all 2011)**

The root canals inside the blocks were enlarged to 1.5 mm with a Gates Glidden drill (size # 6; 1.5 mm; Tulsa Dentsply, Tulsa OK) at 300 rpm. A thin groove was made in the middle of the cylindrical specimen with the precision diamond saw, then each cylindrical dentin block was fractured into 2 semi-cylindrical halves using a blade and a hammer. The outer surface of the halves was ground using the diamond coated precision saw to remove the surface root cementum and to standardize the thickness of each block to 2 mm (Fig. 2B). In order for the dentinal specimen to fit into the inner wall of a Nanosep microfiltration tube (0.45µm pore size;Pall Corporation, Ann Arbor, MI), the dentin specimens were shaped using a low-speed handpiece with a fine carbide bur (Tulsa Dentsply) at 300 rpm. At the end of the procedure, the finalized sample specifications were approximately 4 x 4 x 2 mm (Fig.3, 7B).

The samples were rinsed with 5.25 % NaOCl (4 min; Clorox Company of Canada Ltd, Brampton, ON, Canada), followed by a 6 % citric acid solution (pH 4.0; 4 min; Sigma-Aldrich,

St Louis, Mo), utilizing an ultrasonic bath (Sankei Giken Industry Co Ltd, MIE, Tokyo, Japan) after each rinse in order to remove the smear layer. The samples were then immersed in sterile water (2 min). Each semi-cylindrical sample was placed in a Nanosep microfiltration tube with the canal side up (Fig. 3). To seal any gaps between the specimen and the inner wall of the Nanosep microfiltration tube, composite resin (Kerr Co, Orange, CA) was used and light-cured for 20 seconds.

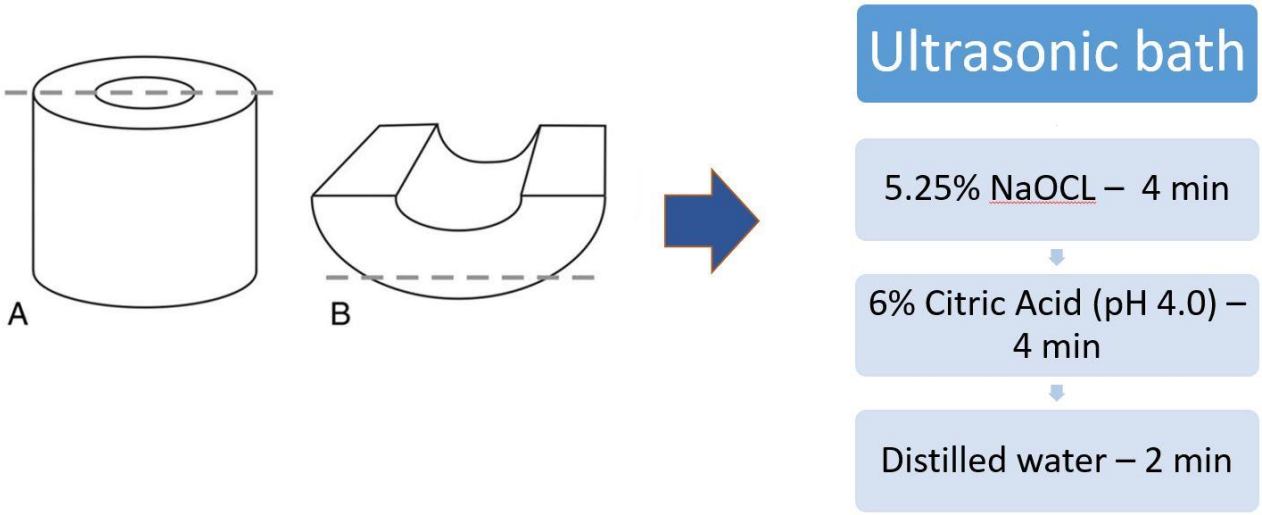



**Figure 3 Schematic drawings of sample preparation (83)**
**A) A cylindrical specimen is obtained after sectioning the coronal part of a single-rooted tooth**
**B) The cylindrical specimen cut in half. The dotted line indicates the cemental layer which is removed**

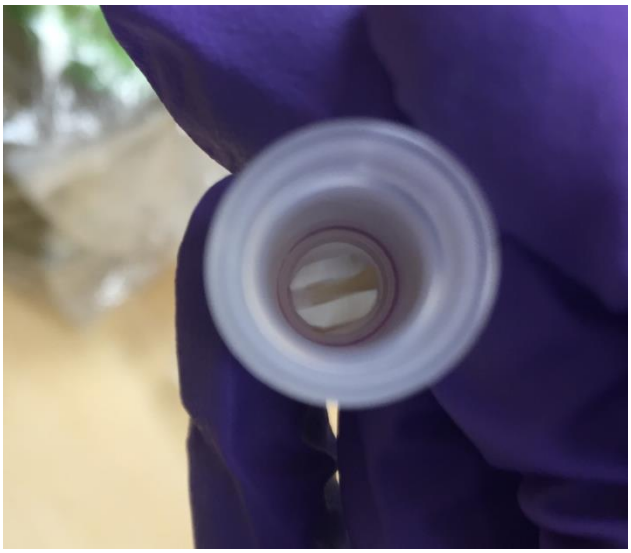


**Figure 4 Specimen placement inside Nanosep filter tube prior to gap sealing**

### 2.4 Dentin Canal Infection with E. faecalis

Two strains of *E. faecalis* (VP3-181 and Gel 31) originally isolated from persistent apical periodontitis cases (104) were grown overnight on brain-heart infusion (BHI) agar (Becton-Dickinson, Sparks, MD) plates in aerobic conditions at 37 °C. The bacteria were collected and suspended in BHI broth (Becton-Dickinson). The cell density was standardized to an optical density of 0.05 (150 µL, 405 nm; ELx808 Absorbance Reader, BioTek Instruments, Inc., Winooski, VT) in BHI broth.

Half of the 80 specimens were randomly assigned to be inoculated with *E. faecalis* VP3-181 and the other half with *E. faecalis* Gel 31. Centrifugation was used to force the bacteria into the dentinal tubules, following a previously described protocol (83). A bacterial suspension (500 µl) and one semi-cylindrical dentin piece were transferred into each filter tube (top compartment). The tubes were subsequently centrifuged at 5000, 6000, 8000 and 10000 rpm, each cycle lasting 5 min, for a total of 2 complete rounds.

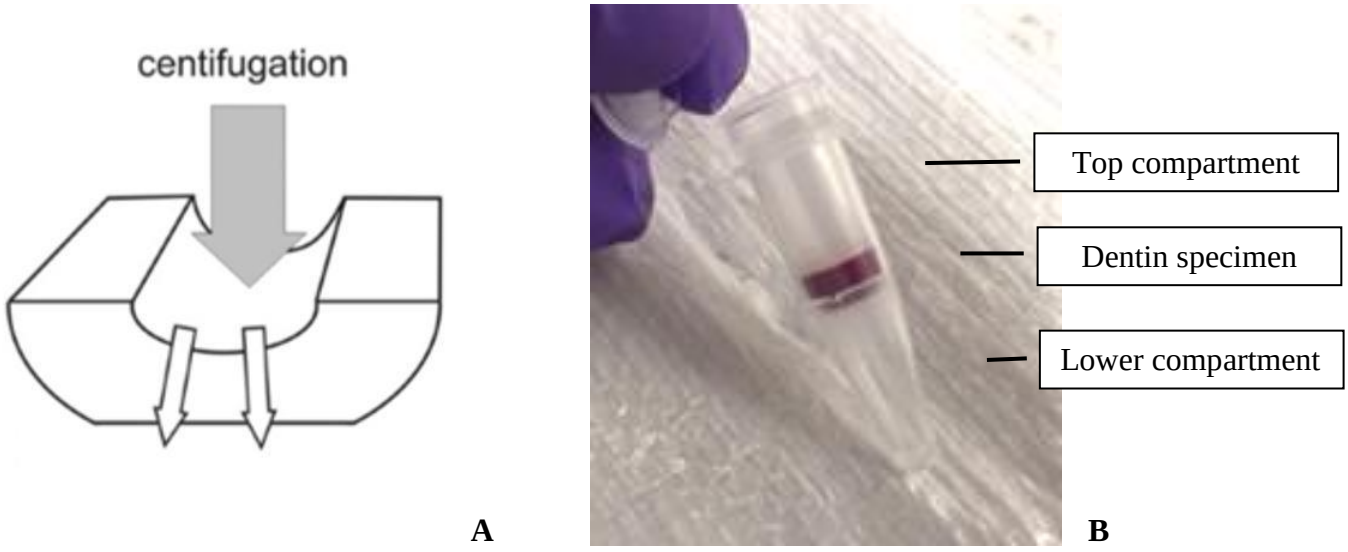



**Figure 5 Mode of centrifugation. A) The side view of a dentin block is shown; the root canal is visible in the middle. With the power of centrifugation the bacterial suspension is forced into the dentinal tubules. B) A Nanosep microfiltration tube is shown with a dentin specimen in place; the top compartment is filled with the bacterial suspension prior to centrifugation. The bacterial suspension is collected in the lower compartment after centrifugation. (83)**

The bacterial suspension that penetrated the dentin piece during centrifugation moved into the lower compartment and subsequently discarded, then a new 500 µL volume of bacterial suspension was added to the top compartment for the second cycle of centrifugation. Half of the inoculated filter tubes (of both strains) were randomly selected and were incubated in sterile BHI broth in air at 37 °C for 3 days, whereas the other half were incubated under the same conditions for 3 weeks to allow biofilm growth and maturation in the dentinal tubules. In the case of the samples that were incubated for 3 weeks, the BHI medium was refreshed weekly.

### 2.5 Disinfection of Dentin

After incubation (either 3 days or 3 weeks), the infected dentin samples were removed from each filter tube. The surrounding composite was removed, and the samples were washed with sterile

water (1 min) and air dried. The outer, cemental side of each dentin sample was sealed using nail varnish to close the open dentinal canals.

A total of 80 dentin specimens were included in the study. These were categorized into 2 bacterial groups: VP3-181 and Gel 31. Each of these groups was further categorized based on the age of their biofilm maturation (3 days or 3 weeks; 20 dentin specimens per group). Finally, the dentin specimens were randomly assigned to 1 of 8 experimental groups (3 min or 10 min; exposure at 20 °C or 60 °C; using 2 % or 5.25 % NaOCl solutions; or a control group using sterile water at 20 °C or 60 °C).

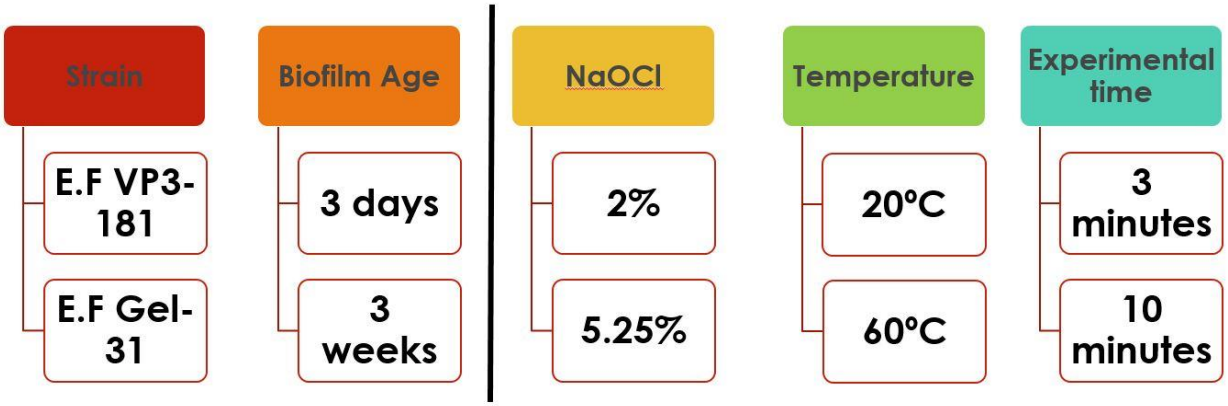



**Figure 6 Experimental factors are shown. Two strains of *E. faecalis* are incubated (VP3-181 and Gel 31) to create a young and old biofilm (3 days and 3 weeks). The dentin specimens are treated with NaOCl (2 % or 5.25 %) at 20 °C or 60 °C for 3 minutes or 10 minutes.**

The dentin specimens were submerged in a 40 mL glass beaker filled with 20 mL of either 2 % or 5.25 % NaOCl solution at 20 °C (room temperature) or 60 °C, at either 3 or 10 minutes (Figure 7B). During the experimental procedures, all glass beakers were covered with aluminium foil to minimize exposure to light.

## 2.6 Temperature Control

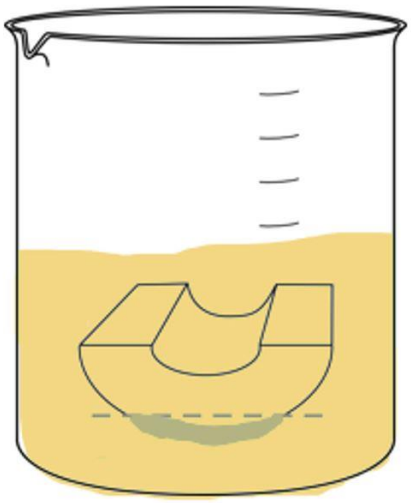
Fresh NaOCl (2 % and 5.25 %; Clorox Company of Canada Ltd) was used for the experimental procedure. For the experimental groups at 20 °C, no further adjustments to the temperature was made after the container of NaOCl was opened. NaOCl was heated to 60 °C using a water bath for the other experimental groups. NaOCl was heated in a test tube and immersed in a preheated water bath for 30 min. In the meantime, a 40 mL glass beaker covered with aluminium foil, was heated on a heating plate. After the NaOCl in the test tube had reached 60 °C, 20 mL was then transferred to the preheated glass beaker, while remaining on the heating plate during the experiment. The aluminium foil covered the glass beaker throughout the experiment. A pilot study had been done to ensure the correct temperature for NaOCl after immersion in the water bath. Sterile water, used in the control groups was heated in a similar manner.

**Table 6 Experimental design for *E. faecalis* VP3-181 & *E. faecalis* Gel 31 dentin biofilm exposed to 2 % and 5.25 % NaOCl (G: group; GC: control group)**

| | | |
|---|---|---|
| 3-day old biofilm | G1 | 20°C – 3 min. |
| | G2 | 20°C – 10 min |
| | G3 | 60°C – 3 min |
| | G4 | 60°C – 10min |
| | GC1<br>GC2 | 20°C – Control<br>60°C – Control |
| | | |
| 3-week old biofilm | G5 | 20°C – 3min |
| | G6 | 20°C – 10min |
| | G7 | 60°C – 3min |
| | G8 | 60°C – 10min |
| | GC3<br>GC4 | 20°C – Control<br>60°C – Control |

### 2.7 Experimental Design

After exposure to the hypochlorite solution at different conditions, each specimen was washed with sterile water (1 min). The specimen was then fractured vertically through the center of the root canal into two halves to expose a fresh surface of longitudinally fractured dentin tubules. A total of 160 fractured dentin pieces were stained with viability staining before confocal laser scanning microscopic examination and analysis.



**A.** **B.**

**Figure 7 A) Dentinal specimens prior to the experiment; B) Specimen shown during the experiment (83) (scale not representative of actual size)**

### 2.8 Confocal Laser Scanning Microscopic Examination

Fractured dentin specimens for confocal laser scanning microscopic imaging (Nikon Eclipse C1; Nikon Mississauga, ON, Canada) were stained using the LIVE/DEAD BacLight Bacterial Viability Kit L-7012 (Molecular Probes, Eugene, OR) containing SYTO 9 (Molecular Probes, Eugene, OR) and propidium iodide (PI) per manufacturer's instruction, followed by phosphate buffered saline rinse (1 min) (83). Bacteria with intact cell membranes stain fluorescent green with SYTO 9, whereas bacteria with damaged membranes stain red with PI. Five areas (318.9 µm x 318.9 µm) at the edge of the root canal and extending into the dentin were randomly selected, then scanned for 3D reconstruction. Thus, a total of 800 scans were performed in the study.

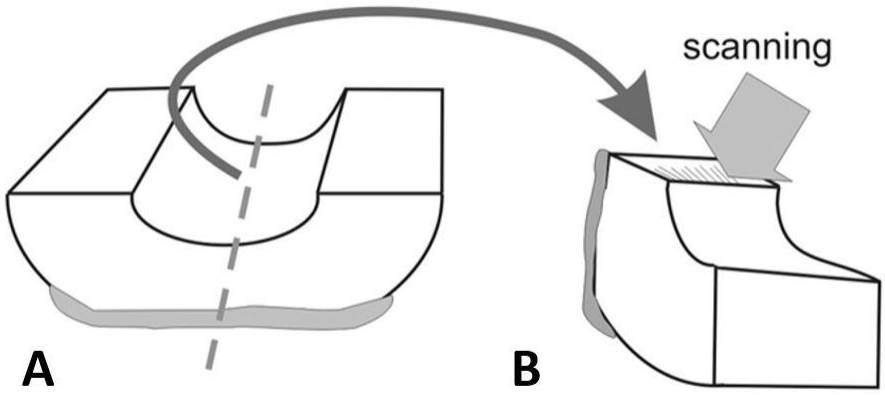



**Figure 8 Fractured specimens indicating the exposed area for confocal laser scanning microscopy (CLSM) (83). A) Dentin block after experimental procedures; the backside of the specimen is covered with nail varnish. B) The dentin block is sectioned through the center of the root canal; the longitudinal dentinal tubules are exposed and can be viability stained prior to CLSM.**

A stack of 20 slices with a 0.5 µm step size were acquired for each confocal laser scanning microscopic scan using the EZ-C1 v.3.40 build 691 software (Nikon Canada) at a field resolution of 512 x 512 pixels. The volume of dead cells (red fluorescence) and live cells (green fluorescence) were calculated using Imaris 7.2 software (Bitplane Inc., St Paul, MN). The thresholds of the red and green fluorescence were manually set according to the raw intensity of the confocal files, and the 3D reconstruction and volume calculations were performed automatically by the software. The proportion of dead cell to live cell volumes were calculated with the volume ratio of red fluorescence to green and red fluorescence.

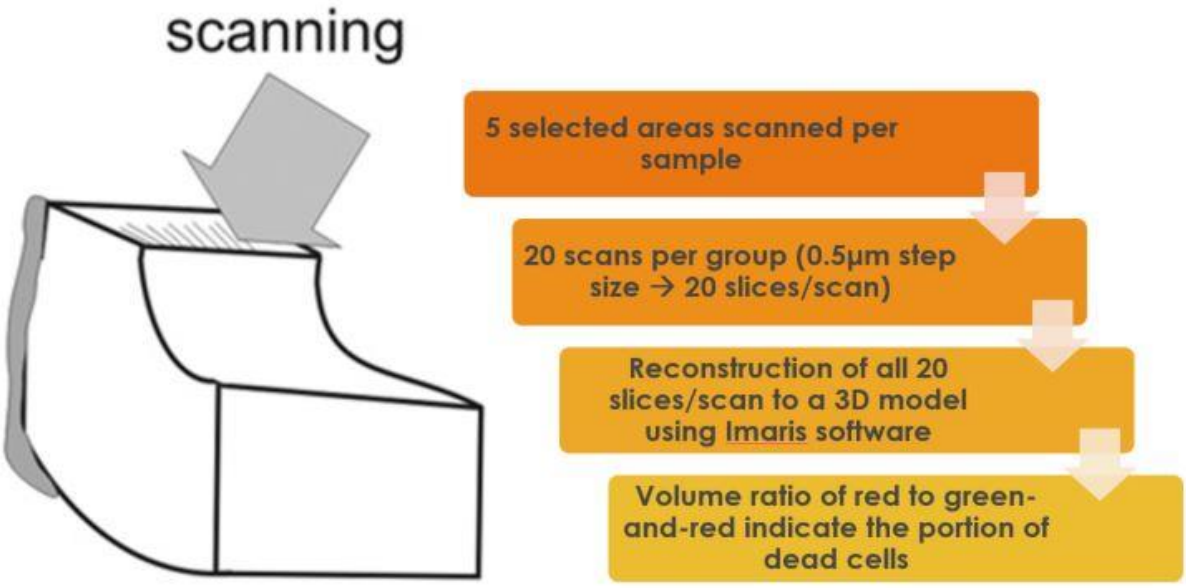



**Figure 9 Description of the CLSM procedure (83)**

### 2.9 Data Analysis

The response variable of this study is the average percentage of the bacteria killed, which can be treated as a continuous variable. The different strains and the age of the biofilm are blocking factors; the three treatment factors include: NaOCl, temperature, and the exposure time. These five factors are all binary variables taking two values. The strain and the age of the biofilm can be treated as blocking factors since these two factors define the experimental environment. Treating strain and age biofilm as blocking factors accounts for possible variability between the blocks. The 32 possible combinations of the two blocking factors and the three treatment factors correspond to the 32 treatment groups in this experiment. Additionally, there are also 12 control groups under 8 different combinations of strain, age, biofilm, temperature, and experimental time.

### *2.9.1 Paired T-Test*

The paired t-test was used to analyze the effect of NaOCl concentration compared to the control groups which were not exposed to NaOCl. Paired t-tests typically consist of matched pairs of measurements and examine the difference between the population mean responses between the two sets of measurements.

### *2.9.2 Linear Model*

The linear model was used to further investigate the effects of different concentrations of NaOCl as well as its interaction with other treatment factors. R (v.3.3.3), an open-source software for statistical computing (https://cran.r-project.org/), was used for the analysis. For further information on statistical analysis, please refer to Appendix A.

# 3. Results

The previous chapter described the methodology in comparing the killing effectiveness of two different sodium hypochlorite concentration (2 % *vs*. 5.25 %) at two different temperatures (20 °C *vs.* 60 °C) and against two strains of *E. faecalis* biofilm at different ages of maturation (3 days *vs*. 3 weeks), in a dentin block model using confocal laser scanning microscopy together with viability staining. The influence of the maturity of the biofilm, the concentration of NaOCl and the exposure time was also investigated. The findings of the experimental procedures will be described below. All the main effects (concentration, temperature, and exposure time) and the pairwise interaction effects of the three treatment factors (concentration, temperature, exposure time) have an effect on the killing efficacy of NaOCl ($p<0.05$). The complete statistical report is enclosed as Appendix A.

### 3.1 Comparison of Sensitivity Between *E. faecalis* Strains VP3-181 and Gel 31

The linear model was used to compare the sensitivity of the two E. faecalis strains (VP3-181 and Gel 31) to NaOCl at two concentrations (2 % and 5.25 %) at two different temperatures (20 °C vs. 60 °C). No significant difference was found between E. faecalis 181 and Gel 31 in their sensitivity to NaOCl at the different experimental conditions ($p>0.05$) (Refer to Appendix A for the statistical report).

### 3.2 Comparison Between *E. faecalis* Biofilms of Different Ages (3 days vs. 3 weeks)

The linear model was used to compare the effect of a young and old E. faecalis biofilm in their sensitivity to NaOCl at two concentrations (2 % and 5. 25 %) at two different temperatures (20

°C vs. 60 °C). Three-day biofilms and 3-week biofilms showed a similar response in their sensitivity to NaOCl at the different experimental conditions ($p > 0.05$).

### 3.3 Effect of NaOCl Concentration (2 % and 5.25 %) on *E. faecalis* Biofilms

The paired t-test was used to evaluate the effect of NaOCl concentration (2 % and 5.25 % on E. faecalis biofilms). On average, the mean percentage of the volume of dead bacteria was 33.3 % higher when 2 % NaOCl is used compared to the use of sterile water ($p<0.05$) (Table 7). When 5.25 % NaOCl was used, the results confirmed that 5.25 % NaOCl effectively killed bacteria. Compared with the control groups, 5.25 % NaOCl is 50.47 % more effective than sterile water ($p<0.05$) (Table 7).

**Table 7 Results between 2 %, 5.25 % NaOCl and sterile water**

| | Mean difference in killing | 95 % confidence interval | p-value |
|---|---|---|---|
| **2 % NaOCl vs negative control** | 0.3332254 | 0.1958707 | 0.001248 |
| **5.25 % NaOCl vs negative control** | 0.5046871 | 0.4716765 | <0.001 |

**Table 8 Results between 2 %, 5.25 % NaOCl and sterile water at 20 °C and 3-minute exposure time**

| | Mean difference in killing | 95% confidence interval | p-value |
|---|---|---|---|
| **2% NaOCl vs control** | 0.1445889 | 0.1215648 | 0.000336 |
| **5.25% NaOCl vs control** | 0.4709702 | 0.4356691 | <0.001 |

**Table 9 Results between 2%, 5.25% NaOCl and sterile water at 60 °C and 10-minute exposure time**

| | Mean difference in killing | 95% confidence interval | p-value |
|---|---|---|---|
| **2 % NaOCl vs control** | 0.5218619 | 0.4591468 | 0.0001455 |
| **5.25 % NaOCl vs control** | 0.538404 | 0.4893864 | <0.001 |

Both concentrations of NaOCl killed more bacteria than water when the temperature and exposure times were considered (Tables 8 and 9). 2 % NaOCl eradicated 14.45% more E. faecalis than sterile water at an exposure time of 3 minutes at 20 °C (Tables 8), and 52.18% more when the temperature was increased to 60 °C and the exposure time to 10 minutes (Table 9). NaOCl of higher concentration (5.25 %) also showed a higher number of eradication when compared to sterile water (control group). 5.25 % NaOCl showed 47.09 % more dead E. faecalis

bacteria with an exposure time of 3 minutes at a temperature of 20 °C (Table 8). The number of dead bacteria increased to 53.8 % when 5.25 % NaOCl was compared to sterile water with an exposure time of 10 minutes at 60 °C (Table 9). All tests confirmed that compared with the control groups, the bactericidal effect of NaOCl on the eradication of E. faecalis was significantly larger when 2 % and 5.25 % NaOCl were used, showing that both concentrations are effective in killing bacteria ($p<0.05$).

### 3.4 The Interaction Effects Between the Experimental Factors

The linear model was used to further investigate the effects of different concentrations of NaOCl as well as its interaction with other experimental factors (exposure time and temperature). The multiple R-squared was 0.89, which indicates a close fit of the data to the fitted regression line.

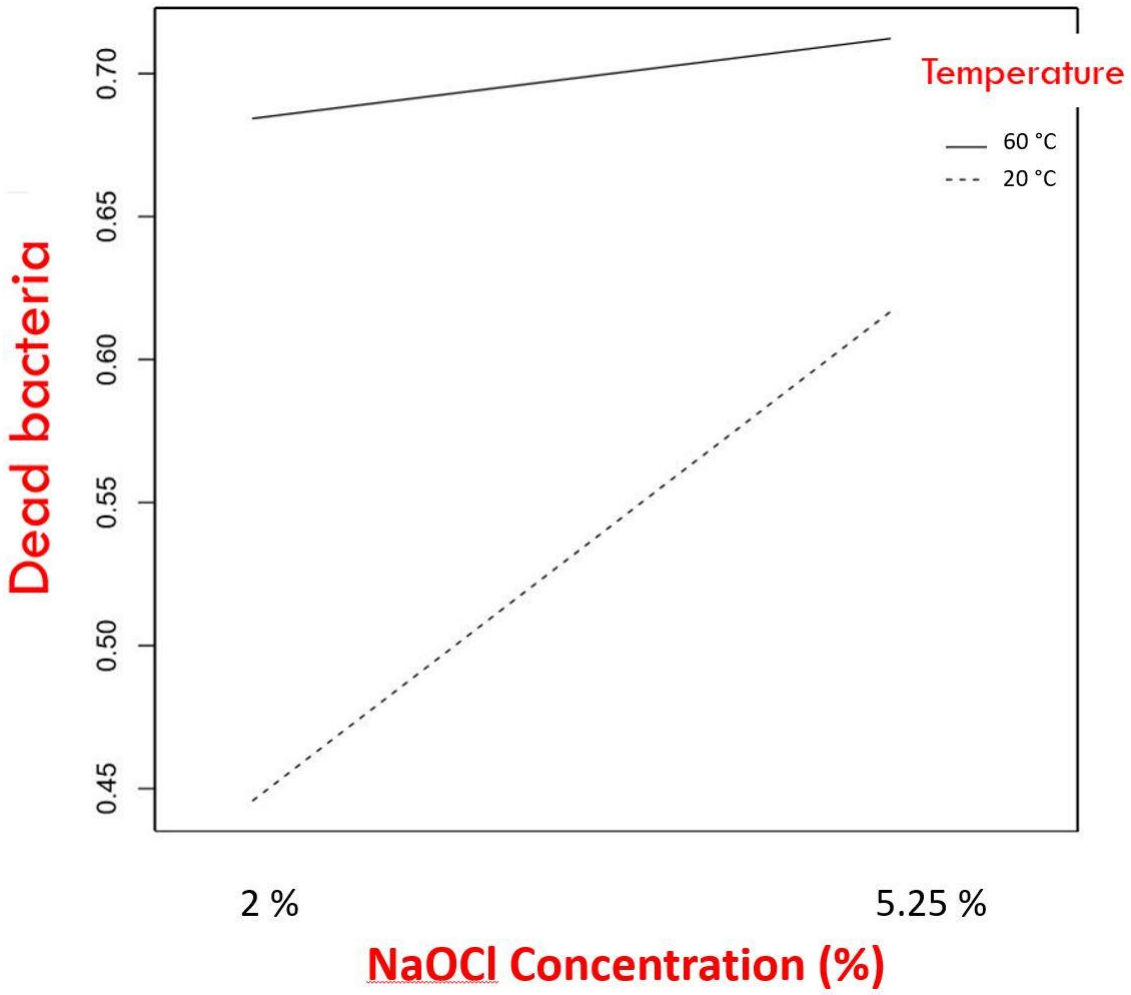



**Figure 10 Interaction plot between the concentration of NaOCl (2 % and 5.25 %) and 2 temperatures of NaOCl (20 °C to 60 °C). The mean number of dead bacteria (y-axis) increases as the temperature of NaOCl increases from 20 °C to 60 °C for both concentrations of NaOCl (2 % and 5.25 %).**

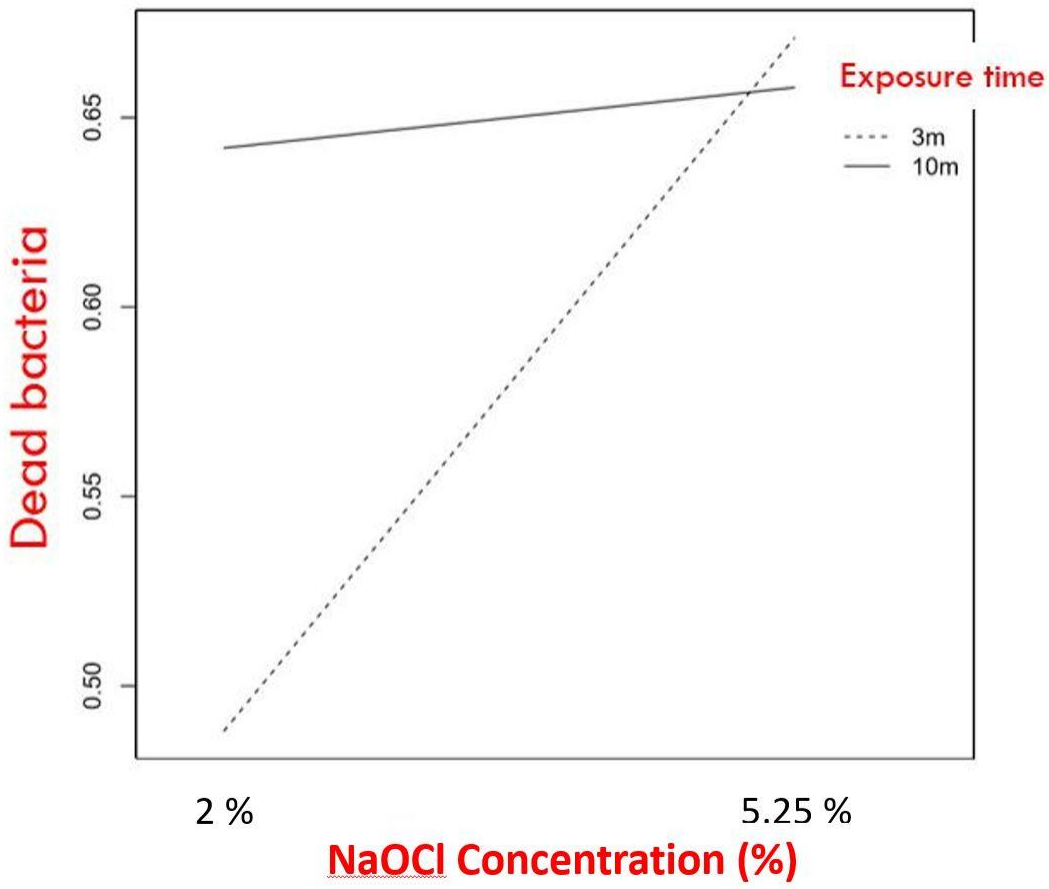



**Figure 11 Interaction plot between the concentration of NaOCl and the exposure time of NaOCl to *E. faecalis*.**

The mean percentage of killed bacteria increased as the concentration was changed from 2 % to 5.25 % (Fig. 10-11). 2 % NaOCl at 20 °C exposed to an *E. faecalis* biofilm for 3 minutes resulted in 37.75 % dead bacteria. The percentage increased to 59.36 % when 2 % NaOCl was used at 20 °C for 10 minutes ($p<0.05$). When 5.25 % NaOCl was used at 20 °C for 3 minutes the bacterial killing was 62.41 %, the number of dead bacteria was 60.9 % when the exposure time was increased to 10 minutes ($p>0.5$). The increase in dead bacteria was smaller when the exposure time was increased from 3 minutes to 10 minutes and the effect of a longer exposure time when 5.25 % NaOCl was used at 20 °C was not significant. A combination of 5.25 % NaOCl at 60 °C and an exposure time of 3 minutes resulted in a 71.77 % dead bacteria.

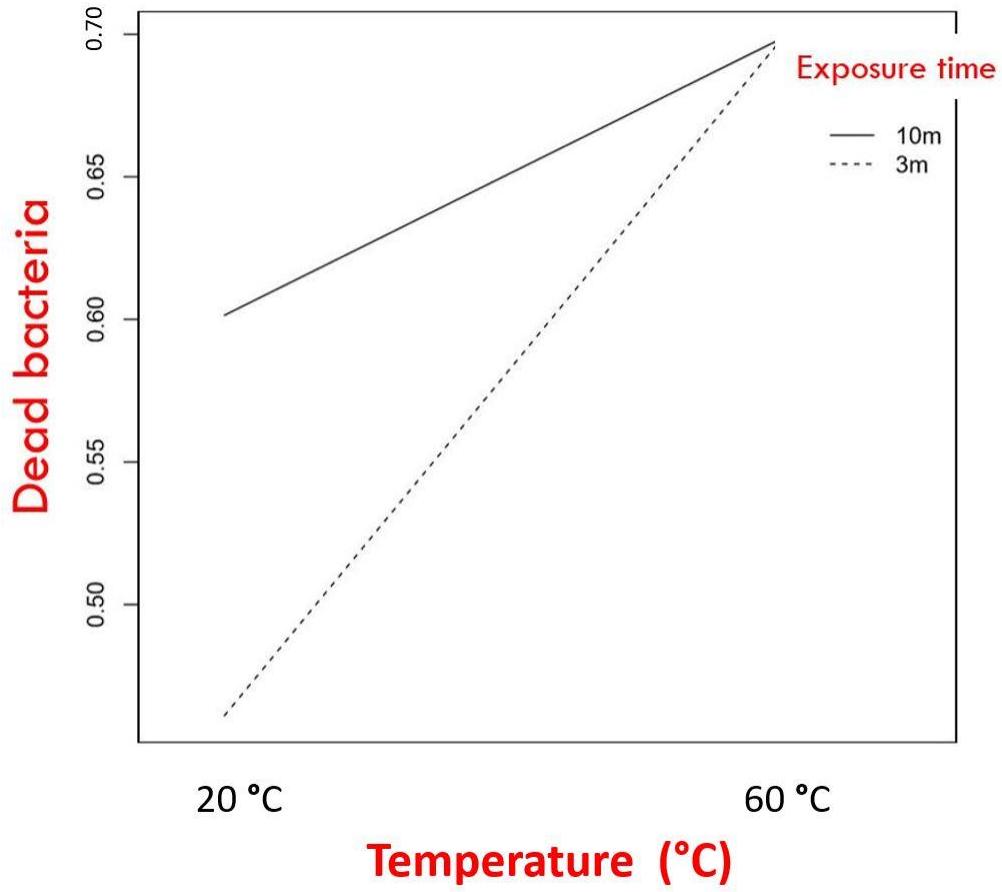



**Figure 12 Interaction plot between temperature of NaOCl and the exposure time of NaOCl to *E. faecalis***

The mean percentage of killed bacteria increased as the temperature was changed from 20 °C to 60 °C (Fig. 12). An *E. faecalis* biofilm exposed to 2% NaOCl at 20 °C for 3 minutes resulted in 37.75 % dead bacteria. When the temperature was increased to 60 °C, the killing increased to 67.89% ($p<0.05$). When 2% NaOCl was used for 10 minutes at 20 °C the killing was 59.36 %; the killing was 60.91 % when 5.25 % NaOCl was used ($p>0.05$). 2 % NaOCl at 60 °C and an exposure time of 10 minutes, resulted in 69 % dead bacteria. This number was lower, compared to the group of 5.25 % NaOCl at 60 °C used for 10 minutes (70.61 %) ($p<0.05$). The mean number of killed bacteria (%) for the experimental groups are listed in Table 10, the two different strains of *E. faecalis* and the age of the biofilm are not considered as they do not

influence the number of killed bacteria. The effect of temperature was not significant when 5.25 % NaOCl was combined with an exposure time of 10 minutes. The complete results of mean killed bacteria (%) per experimental groups (including the strain of *E. faecalis* and the age) is listed in Appendix B and C.

**Table 10 Mean dead bacteria (%) for specific experimental groups**

| NaOCl (%) | Temperature | Exposure time (min) | Mean dead bacteria (%) |
|---|---|---|---|
| **2 %** | **20 °C** $^{a, b, f}$ | **3'** | **37.75 %** |
| **2 %** | **20 °C** $^{A, c, g}$ | **10'** | **59.36 %** |
| **5.25 %** | **20 °C** $^{d}$ | **3'** | **62.41 %** |
| **5.25 %** | **20 °C** $^{e}$ | **10'** | **60.91 %** |
| **2 %** | **60 °C** $^{B}$ | **3'** | **67.89 %** |
| **2 %** | **60 °C** $^{E}$ | **10'** | **69.00 %** |
| **5.25 %** | **60 °C** $^{D, F}$ | **3'** | **71.77 %** |
| **5.25 %** | **60 °C** $^{C, G}$ | **10'** | **70.61 %** |

Superscript small and corresponding capital letters indicate statistically significant differences between groups ($p<0.05$)

### 3.5 The Effect of NaOCl Concentration on Bactericidal Effect

A one-sided hypothesis test was performed to evaluate the effect of different concentrations of NaOCl (2 % *vs.* 5.25 %) on the bactericidal effect of NaOCl against *E. faecalis*. Table 11 demonstrates that the higher concentration (5.25 %) is more effective in eradicating *E. faecalis*, except when NaOCl is used at a temperature 60 °C and exposed for 10 minutes.

**Table 11 The significance of 2 % vs 5.25 % concentration on the killing effectiveness**

| Temperature | Experiment time | $D$ | Estimate of $D$ | S.D. of $D$ | t statistic | Two-sided p-value | Significance at 5% level |
|---|---|---|---|---|---|---|---|
| 60 | 10 | $\beta_1 + \beta_{12} + \beta_{13}$ | -0.05548 | 0.0313137 | -1.7717 | 0.0897 | No |
| 60 | 3 | $\beta_1 + \beta_{12}$ | 0.11145 | 0.0313137 | 3.5591 | 0.00167 | Yes |
| 20 | 10 | $\beta_1 + \beta_{13}$ | 0.08743 | 0.0313137 | 2.7921 | 0.0104 | Yes |
| 20 | 3 | $\beta_1$ | 0.25436 | 0.0313137 | 8.1230 | 3.298e-08 | Yes |

### 3.6 The Effect of the Exposure Time on Bactericidal Effect of NaOCl

A one-sided hypothesis test was performed to evaluate the effect of different exposure times of NaOCl on the bactericidal effect against *E. faecalis*. Table 12 illustrates that the exposure times influences the bactericidal effect of NaOCl in all experimental groups, except when NaOCl is used at 5.25 % at a temperature of 20 °C.

**Table 12 The Significance of the Exposure Time on the Killing Effectiveness**

| NaOCl | Temperature | $D$ | Estimate of $D$ | S.D. of $D$ | t statistic | Two-sided p-value | Significance at 5% level |
|---|---|---|---|---|---|---|---|
| 5.25% | 60 | $\beta_3 + \beta_{13} + \beta_{23}$ | -0.08312 | 0.0313137 | -2.6544 | 0.0142 | Yes |
| 5.25% | 20 | $\beta_3 + \beta_{13}$ | 0.05694 | 0.0313137 | 1.8184 | 0.0821 | No |
| 2% | 60 | $\beta_3 + \beta_{23}$ | 0.08381 | 0.0313137 | 2.6765 | 0.0135 | Yes |
| 2% | 20 | $\beta_3$ | 0.22387 | 0.0313137 | 7.1493 | 2.790e-07 | Yes |

### 3.7 The Effect of Temperature on the Bactericidal Effect of NaOCl

A one-sided hypothesis test was performed to evaluate the effect of temperature of NaOCl on the bactericidal effect. The results shown in Table 13 indicate that the temperature has a significant effect on the killing efficacy of *E. faecalis* ($p<0.05$), except when a high concentration of 5.25 % is used combined with an exposure time of 10 minutes ($p>0.05$). A detailed explanation of Table 13 can be found in Appendix A.

**Table 13 The Significance of Temperature on Bacterial Killing**

| NaOCl | Experiment time | $D$ | Estimate of $D$ | S.D. of $D$ | t statistic | Two-sided p-value | Significance at 5% level |
|---|---|---|---|---|---|---|---|
| 5.25% | 10 | $\beta_2 + \beta_{12} + \beta_{23}$ | 0.0255 | 0.0313137 | 0.8143 | 0.424 | No |
| 5.25% | 3 | $\beta_2 + \beta_{12}$ | 0.16561 | 0.0313137 | 5.2887 | 2.286e-05 | Yes |
| 2% | 10 | $\beta_2 + \beta_{23}$ | 0.16846 | 0.0313137 | 5.3798 | 1.827e-05 | Yes |
| 2% | 3 | $\beta_2$ | 0.30852 | 0.0313137 | 9.8526 | 1.011e-09 | Yes |

### 3.8 Volume of Dead *E. faecalis* After Experimental Procedures

Following the experimental procedures, the numbers of dead *E. faecalis* (VP3-181 and Gel 31) were calculated after LIVE/DEAD staining and confocal imaging. The volume (%) of dead *E. faecalis* is summarized in Figures 13-16.

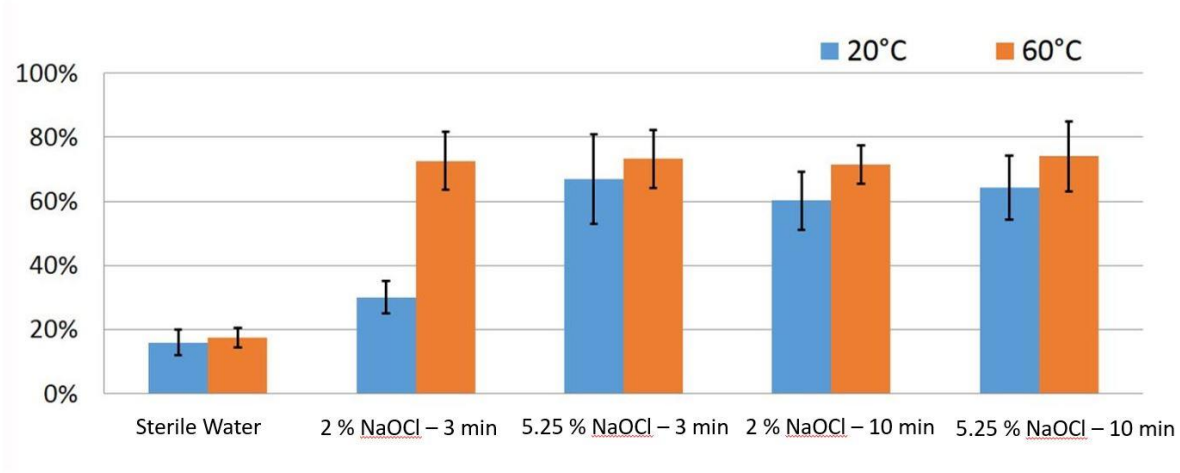



**Figure 13 The volume (%) of dead *E. faecalis* VP3-181 cells in a 3-day old biofilm after exposure to NaOCl (2 % and 5.25 %) at 3 minutes and 10 minutes**

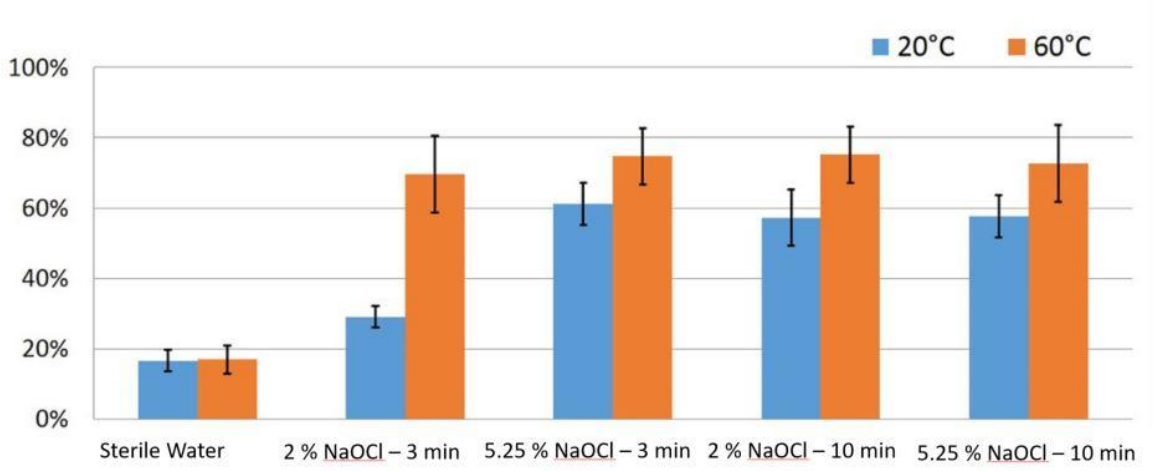



**Figure 14 The volume (%) of dead *E. faecalis* VP3-181 cells in a 3-week old biofilm after exposure to NaOCl (2 % and 5.25 %) at 3 minutes and 10 minutes**

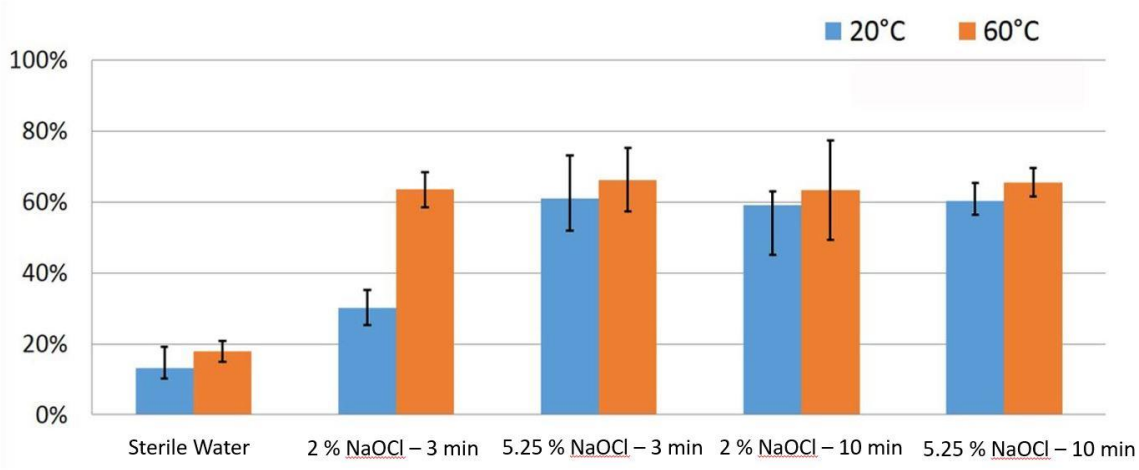



**Figure 15 The volume (%) of dead *E. faecalis* Gel 31 in 3-day old biofilm after exposure to NaOCl (2 % and 5.25 %) at 3 minutes and 10 minutes**

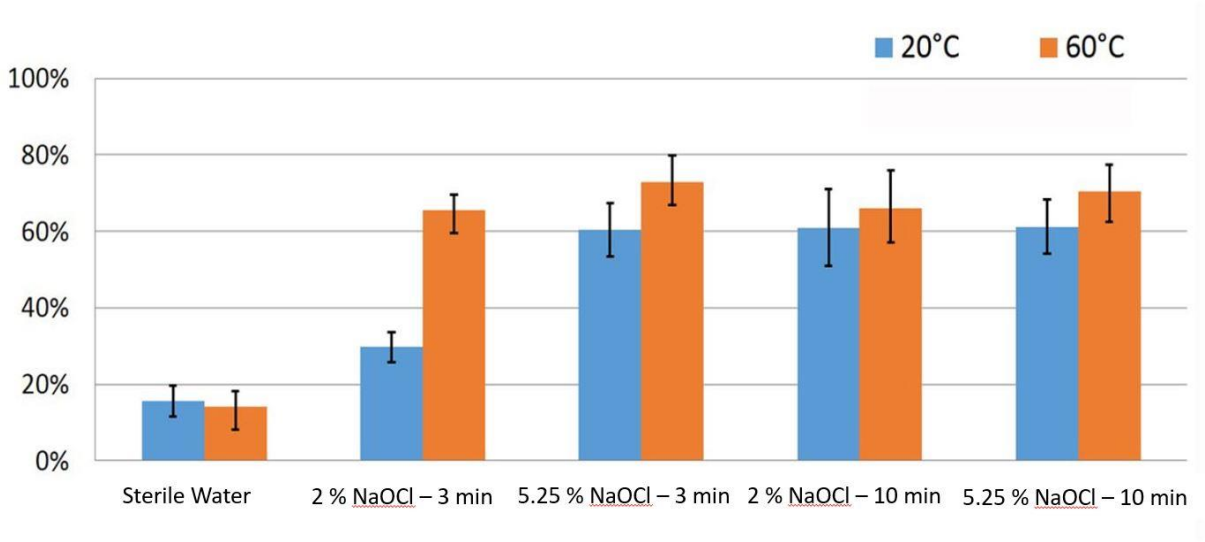



**Figure 16 The volume (%) of dead *E. faecalis* Gel 31 in 3-week old biofilm after exposure to NaOCl (2 % and 5.25 %) at 3 minutes and 10 minutes**

### 3.9 Confocal Imaging of Stained *E. faecalis* in Dentin

*E. faecalis* present in the dentinal tubules after centrifugation was visualized after using confocal laser microscopy scanning after LIVE/DEAD staining. The staining allowed the living bacteria to appear green and the dead bacteria will appear red. The volume ratios of LIVE and DEAD bacteria after the various experimental procedures were then used to calculate the eradicated numbers of *E. faecalis*. The confocal imaging below show the results after some of the experimental procedures. The top side of the image illustrates the surface contact of the dentin blocks with the experimental solutions (NaOCl 2 %, 5.25 %, sterile water). It can be seen that in all cases a thick layer of *E. faecalis* is present on the outside of the dentin block and that great numbers of bacteria penetrated the tubules.

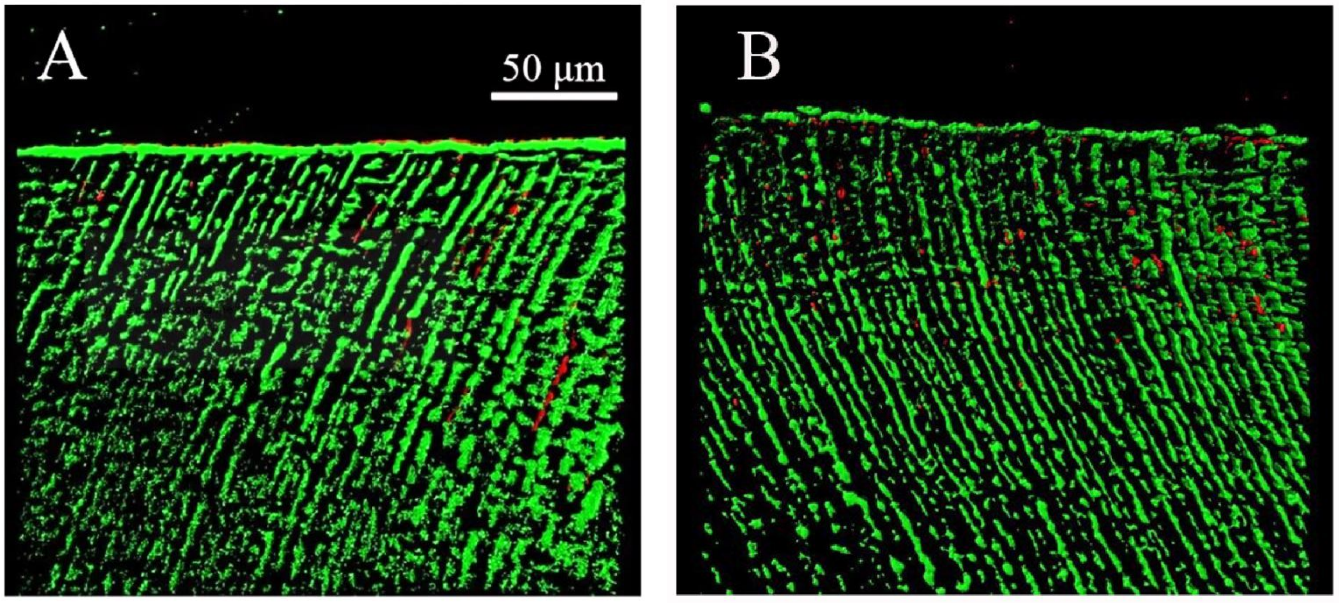



**Figure 17 Confocal imaging (CLSM) of viability stained *E. faecalis* in dentin A) Exposure of *E. faecalis* strain VP3-181 to sterile water at 20 °C for 3 minutes B) Exposure of *E. faecalis* strain VP-181 to sterile water at 60 °C for 3 minutes**

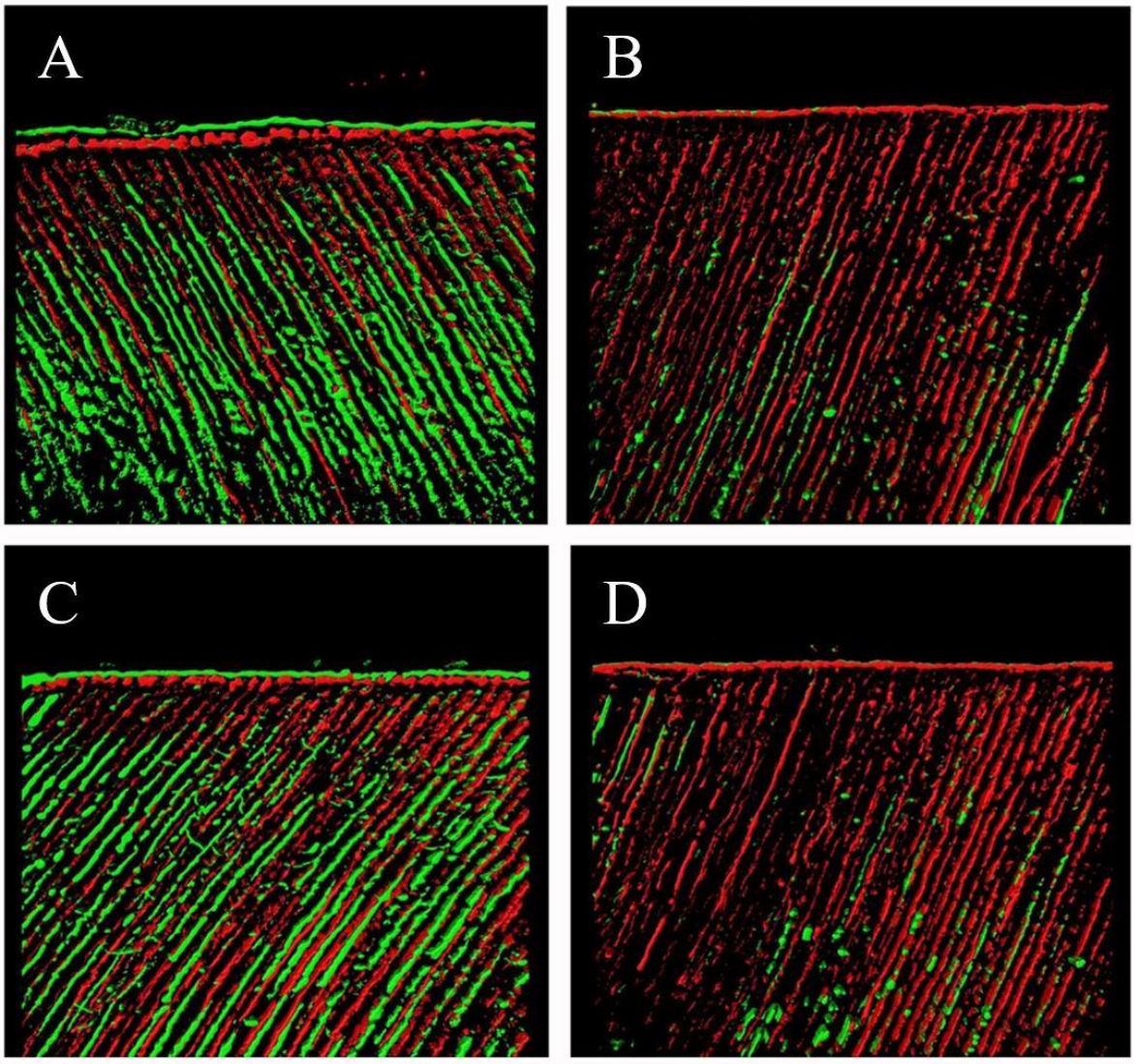



**Figure 18 Confocal imaging (CLSM) of viability stained *E. faecalis* strain VP3-181 in dentin A) Exposure of *E. faecalis* strain VP3-181 to 2 % NaOCl at 20 °C for 3 minutes B) Exposure of *E. faecalis* strain VP3-181 to 2 % NaOCl at 60 °C for 3 minutes C) Exposure of *E. faecalis* strain VP3-181 to 2 % NaOCl at 20 °C for 10 minutes D) Exposure of *E. faecalis* strain VP3-181 to 2 % NaOCl at 60 °C for 10 minutes**

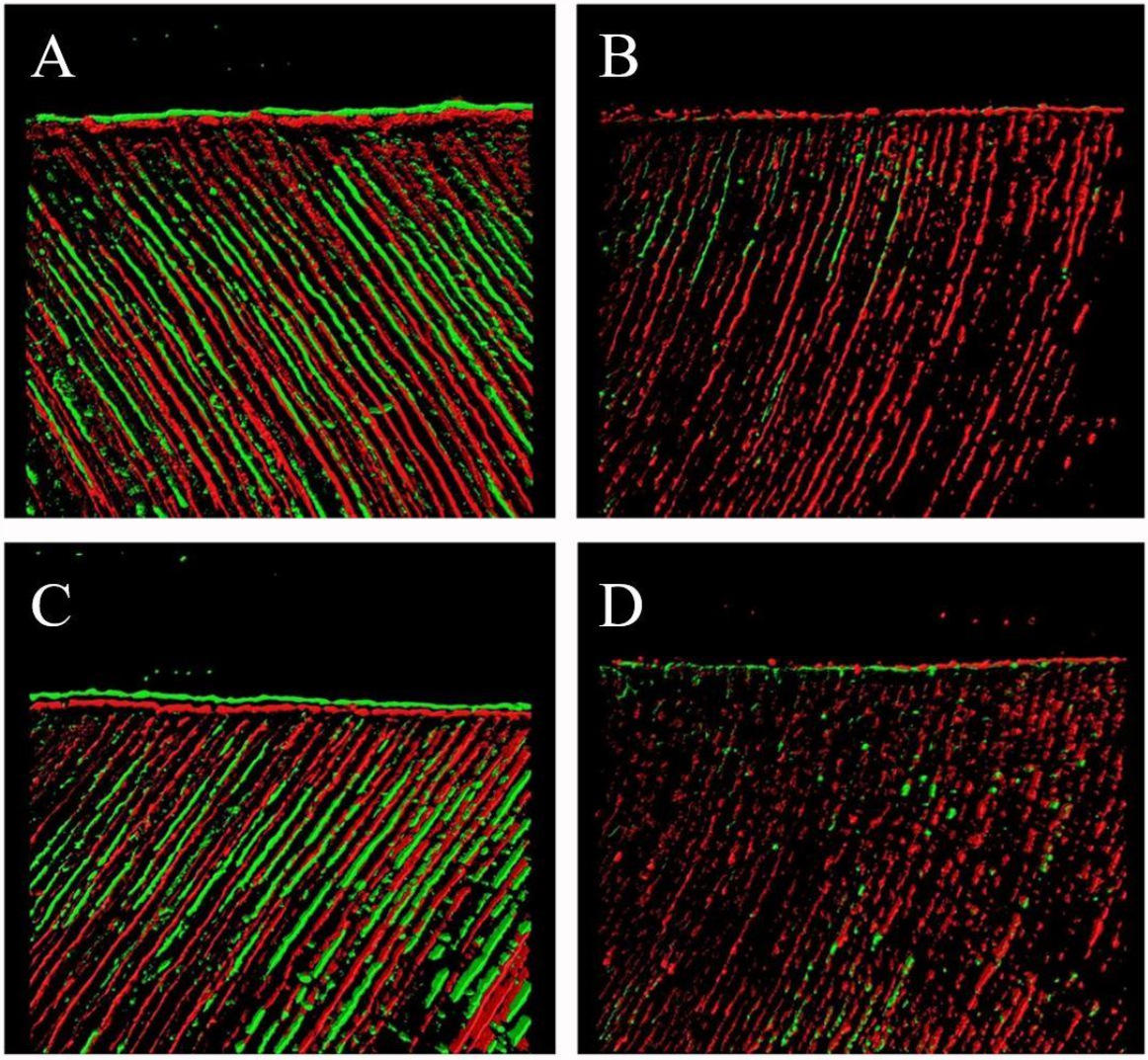



**Figure 19 Confocal imaging (CLSM) of viability stained *E. faecalis* strain VP3-181 in dentin A) Exposure of *E. faecalis* strain VP3-181 to 5.25 % NaOCl at 20 °C for 3 minutes B) Exposure of *E. faecalis* strain VP3-181 to 5.25 % NaOCl at 60 °C for 3 minutes C) Exposure of *E. faecalis* strain VP3-181 to 5.25 % NaOCl at 20 °C for 10 minutes D) Exposure of *E. faecalis* strain VP3-181 to 5.25 % NaOCl at 60 °C for 10 minutes**

## 4. Discussion

The success of endodontic therapy depends on the ability to clean the root canal system by using a combination of chemical and mechanical debridement and disinfection. The most important properties of an ideal endodontic irrigant solution are that it possesses broad antimicrobial efficacy, the ability to remove necrotic and vital pulp tissues, remove the smear layer, and have low to no systemic toxicity (13). The unique ability of sodium hypochlorite to dissolve tissue, in conjunction with its effective antiseptic properties and its relatively low caustic effects, has resulted in it being the most popular endodontic irrigant currently used around the world. To maximize the beneficial properties of sodium hypochlorite, various techniques have been developed and explored. Studies have looked at various concentrations, exposure times and the use of heat (55,83,84). However, to date, there has not been a study examining the effectiveness of heated NaOCl in killing *E. faecalis* in dentin canal biofilms.

Many studies found in the literature report the removal of bacteria from the root canal system by endodontic treatment measures to be challenging. Complete eradication has yet to be achieved and the challenge in eliminating all bacteria is multifactorial (10-12). The formation of multilayered biofilms on the root canal walls is one of the reasons for the reported difficulty (105). *E. faecalis* strains isolated from clinical infections demonstrated a formation of biofilm growth *in vitro*. This allows for enhanced resistance to endodontic disinfectants and an increased pathogenicity (22,106). Another reason for the challenge in disinfection is the chemical environment of the root canal itself, which may alter the antimicrobial efficacy of disinfectants (97,107). Previous studies have illustrated that dentin suppresses the desired antibacterial responses of calcium hydroxide, iodine potassium iodide, and sodium hypochlorite (107,108). Although antimicrobial irrigants are

effective in killing bacteria in the root canal system, some bacteria can find a strategic position in the lateral canals, long isthmuses and dentinal tubules where the irrigants have limited penetration or the bacteria are protected from endodontic disinfectants e.g. by disinfectant inactivation. (109).

Conventional methods described in the past to introduce bacteria into dentinal tubules are based on culturing methods where bacteria are suspended in a liquid medium inside the main root canals of extracted teeth. These research designs have been reported to have encountered challenges in securing bacterial colonization of high magnitude, and therefore they have many limitations (85,110,111). It has been difficult to produce a constant, high number of bacteria in the dentin canals as only a few tubules were invaded by bacteria. A longer incubation period has not necessarily proved to be advantageous. Consequently, achieving standardized dentin infection level is problematic and a great variation in samples has been reported (94,96,97). Creating comparable and strong dentin infection levels in reproducible samples is imperative for performing experiments where bacterial viability is quantitatively measured.

In the present study, a series of centrifugation rounds was used to introduce bacteria into dentinal tubules. To achieve a standardized infection deep into the dentinal tubules, a new dentin infection model was recently developed and introduced (83). This method is reported to be effective and produces reliable numbers of viable bacterial colonies ready for incubation (83). Recent studies have been successful in examining dentin biofilms, in non-invasive models, utilizing confocal microscopy and viability staining (84,112,113). Prior to centrifugation (but after the use of NaOCl), a 6 % citric acid rinse was used in this study to remove the smear layer. The irrigation created a smooth and exposed dentin surface optimally allowing for bacterial invasion (114,115).

This dentin infection model maintains the natural environment (microanatomy and chemistry) of dentin canals for bacterial growth. It also provided a predictable and standardized method to quantitatively determine the numbers of killed bacteria after exposure to different disinfection protocols. The present study used fluorescent viability staining and CLSM to identify and measure the proportions of living and dead bacteria after exposure to disinfecting solutions under different circumstances.

Earlier studies using the same model have looked at various disinfecting agents against *E. faecalis* in infected dentin. The effect of the concentration of NaOCl, the exposure time, and the age of the biofilm have also been studied recently (84,85,876). However, the effect of a heated solution in the dentin infection model has not yet been researched. As heating NaOCl has been reported to have positive effects on its ability to dissolve pulp tissue and on the bactericidal action (103,118), it is interesting to investigate the effect of increased temperature on the killing efficacy of bacteria in combination with the previously studied parameters. This information is of great importance since the quest for an endodontic disinfectant to completely eradicate bacteria from the root canals is still ongoing.

### 4.1 Comparison of *E. faecalis* Strains VP3-181 and Gel 31

In the present study, there was no difference between the two different species of *E. faecalis* in their sensitivity to NaOCl. *E. faecalis* is suitable for the experimental model because in addition to being frequently detected in persistent infections, *E. faecalis* cells are physically strong. They can also be easily centrifuged into the dentinal tubules due to their convenient round shape and small dimensions. Based on the numbers of viable cells in the negative control groups with sterile

water, *E. faecalis* has demonstrated that it is strong enough to survive the impact of centrifugation (110). Incubation time in BHI broth for 24 hours after centrifugation allowed for the bacteria to recover from any damage sustained and also allowed for maturation of the biofilm (83).

### 4.2 Comparison of Different *E. faecalis* Biofilms at Different Ages

In the present study, no significant difference in killing was detected between the young and the old (3 days *vs.* 3 weeks) biofilms. These finding contradict previous studies that have shown a greater resistance to disinfecting solutions between mature biofilms and young biofilms (84,110,118). These studies, however, conducting their experimental procedures using an open biofilm model, using a 1-day-old biofilm and a 3-week-old biofilm which could account for the difference in findings. This study used a 3-day-old biofilm as the young biofilm, which allowed the biofilms to mature for an additional two full days, which could explain the lack of difference in killing compared to the old biofilm. Another reason for the difference in results could be attributed to the NaOCl concentration used. In the present study 2 % and 5.25 % NaOCl solutions were used, whereas only 1% NaOCl was used in the earlier study by Stojicic et al. (118). It may be possible that the bactericidal effect related to the concentration of NaOCl reaches a critical point, where the age of the biofilm plays a minor role in its sensitivity to the antimicrobial irrigant after a certain concentration.

### 4.3 The Effect of Two Different Concentrations of NaOCl on *E. faecalis* Biofilm Compared to Sterile Water

High concentrations of NaOCl has been demonstrated to produce the strongest antibacterial effect against young and old biofilms (83-85). The results of the present study support these findings, as

5.25 % NaOCl showed the highest effectiveness in killing *E. faecalis,* followed by the lower 2 % concentration and sterile water, respectively. Although the mean percentage of the bacteria killed increased as the concentration of NaOCl changed from 2 % to 5.25 %, the extent of the increase was dependent on the temperature and showed a negative interaction effect. The effect of the NaOCl concentration on the killing efficacy decreased when the temperature was increased from room temperature (20 °C) to 60 °C. This indicated that the increase in temperature had an additional positive effect on the killing of *E. faecalis*.

The results of previous studies of the antibacterial effect of NaOCl in various concentrations were inconsistent (68,71,111). Some studies have concluded that the concentration of sodium hypochlorite had no influence on the antibacterial properties in a root canal *in vivo.* However, confounding factors play a role when an *in vivo* experiment is conducted. Differences in canal morphology, apical size, and depth of needle penetration need to be accounted for. Furthermore, a considerable variation in the volume and composition of the intracanal microflora could impact the results. The current dentin infection model used in this study is able to provide *in situ* data points, which allows repeatable results with reasonable standard deviations and statistically significant differences. Another advantage of this model is that it provides the ability to observe the dynamics of killing at different depths of the infection in the dentinal tubules.

In the present study, the paired t-test was done to compare the effect of sterile water to NaOCl with a concentration of 2 % and 5.25 %. This test was limited as the additional experimental factors (temperature and exposure time) may influence the interaction. Therefore, an additional paired t-test on the subset of data was done, pairing 20 °C with 3 minute and 60 °C with 10-minute exposure times.

### 4.4 The Effect of NaOCl Concentration on the Killing of *E. faecalis*

The present study demonstrated the superiority of 5.25 % NaOCl (over 2 % NaOCl), in killing dentin biofilm bacteria. This is in accordance with previous studies following a similar dentin infection protocol and CLSM (83-85,87). They showed that a higher concentration of NaOCl was more effective against both young and old biofilms. However, in our study no significant difference was seen between the two concentrations when the temperature was set at 60 °C combined with an exposure time of 10 minutes. Sodium hypochlorite has been shown to penetrate at least 100-300 µm into dentinal tubules, depending on the time of exposure (112,113,116). The limited penetration depth, inactivation of the solutions by dentin, microbial biofilm mass, and resistant subpopulations of bacteria in the biofilms may be reasons for incomplete killing (98,108,110,). In 2015, Cullen et al (103) published a study using 8.25 % NaOCl. They focused on the effects on dental pulp dissolution and the effects on the flexural strength and modulus of dentin. They demonstrated that increasing the concentration of NaOCl resulted in an increase of pulp dissolution. The authors also concluded that NaOCl does not have a significant effect on the dentin flexural strength or modulus when frequently refreshed. The killing efficacy, however, was not assessed in that study (103).

### 4.5 The Effect of Exposure Time on the Killing of *E. faecalis*

This study showed more killed bacteria after 10 minutes of exposure to NaOCl than after 3 minutes of exposure to NaOCl. This is in accordance with studies found in the literature (87,119,120). Interestingly, however, when 5.25 % NaOCl was used at either 20 °C or 60 °C no significant difference in killing between a shorter or longer exposure time was seen. Previous studies have demonstrated the positive effects of a longer exposure time to endodontic disinfecting solutions

on killing bacteria in biofilms. Retamozo et al. (119), demonstrated that the most effective killing of *E. faecalis* in dentin biofilms used a culture method with an exposure time of 40 minutes using 5.25 % NaOCl. Another study by Hecker et al (120) used exposure times of 0.5, 1, and 10 minutes and concluded that the antimicrobial effect of NaOCl depends on the length of exposure. Recently, Du et al (87), using a similar dentin infection model, showed greater bacterial eradication after 10 minutes (*vs*. 3 minutes) of exposure to 2 % and 6 % NaOCl, although the rate of killing was significantly reduced after 3 minutes and even more so after 10 minutes

In regards to the effect of the exposure times, the findings of this study contrast with our previous study findings when a smear layer was incorporated (86). The smear layer was shown to reduce the effectiveness of disinfectants against *E. faecalis* (86). No increase in the killing of *E. faecalis* was seen when sterile water, 2 % NaOCl and 6 % NaOCl was used and the exposure time was increased from 3 minutes to 10 minutes. However, these samples were all tested after a smear layer had been produced on the root canal walls. The smear layer is formed on the root canal walls after mechanical preparation and consists of organic and inorganic debris (121,122). The smear layer creates a barrier, which physically blocks the entrance of disinfectants into the dentinal tubules. This negatively affects the dentin permeability and may inhibit disinfectants from reaching the bacteria in the infected dentinal tubules (122). In the present study, citric acid (6 %) was used to remove the smear layer prior to bacterial centrifugation and no additional smear layer was created. This could explain the difference in findings.

### 4.6 The Effect of Temperature on the Killing of E. *faecalis*

The influence of temperature on the efficacy of NaOCl to dissolve pulpal tissue and to eradicate bacteria has been reported in different studies (13,65,76,78,79,81,117,118,123). The results of the

present study showed a higher killing rate in *E. faecalis* when NaOCl was used at 60 °C compared to NaOCl at room temperature (20 °C). These findings support previous studies that demonstrated a positive effect on the antimicrobial activity of a heated NaOCl solution (81,117). A recent study by de Hemptinne et al (124) investigated the thermodynamic behavior of irrigant solutions in root canals. They concluded that temperatures are buffered at a rapid pace reaching an equilibrium at 35.1 °C (± 1 °C) in the apical part of the root canal when an irrigating syringe is used. In this study, the use of a syringe was eliminated by submerging the contaminated dentin blocks in a preheated NaOCl solution, which was kept at a steady temperature, therefore eliminating any confounding factors regarding temperature variations. However, in the present study, the effect of temperature was not significant when NaOCl was used at 5.25 % and the experimental time was set at 10 minutes. These findings also confirmed that complete eradication of bacteria from the root canal system was not possible and could possibly be explained by inhibition of NaOCl by the dentinal structures, persistent biofilm bacteria, or the inability of NaOCl to reach the full length of the dentinal tubules (98,108,110).

## 5. Limitations of the Study

As this study is the first using heated disinfectants in the new dentin infection model described by Ma et al (83), direct comparison to previous studies is not possible. Studies described in the literature differ from the present one in methodology.

Although the present study looked at two different strains of *E. faecalis* during the experiments, the biofilm model contained a single species in each experiment, thereby forming a mono-infection instead of a polymicrobial biofilm model, which often does not reflect the *in vivo* conditions. *E. faecalis* is frequently identified in persistent endodontic infections; it has been proven that it has the capacity to endure harsh ecological conditions (108). *E. faecalis* was chosen for the present study in the dentin infection model because of the small dimensions of the bacteria which allows the cells to be forced them into the dentinal tubules. In addition, *E. faecalis* is ecologically strong, as evidenced by high survival rates in negative controls exposed to water only. A goal for the dentin infection model for future purposes is to use multispecies biofilms, thereby also allowing the visualization of gram-negative bacteria. Even though the dentin infection model displays some shortcomings, it does offer the opportunity to perform experiments as close to conditions *in vivo* as currently possible, whilst providing the opportunity for a high level of standardization and accurate sampling surpassing the level possible *in vivo.*

The results of this *in vitro* study cannot be extrapolated to a clinical setting. Further research is needed to determine the effect of heated disinfectant solutions on a polymicrobial biofilm. In addition, the thermodynamic behavior of irrigant solutions need to be considered to ensure a constant heating temperature of the solutions in future research. A recent *in vivo* study using

heated NaOCl in root canals observed loss of heat during their experimental process (124). The authors demonstrated a decrease in temperature while the solutions were in the syringe and a further reduction while the disinfectant was passed from the syringe into the root canal (124). Therefore, it was concluded that the initial temperature of the irrigant in the syringe should be set at 65 °C.

Background fluorescence can be a problem when dentin is examined with CLSM and fluorescent dyes, as described in earlier studies (83-87). In the current dentin infection model the bacteria are introduced into the dentin canals in great numbers by serial centrifugation. This results in a strong fluorescent signal from the bacteria, which allows the use of a low gain setting in the CLSM scanning. However, some scattered background fluorescence can occasionally be seen from the dentin. The signal of the background fluorescence appeared to be weak and it did not provide a challenge during interpretation of the scans, and the findings of the present study corresponded well to those of earlier observations (83).

## 6. Conclusion

Within the limitations of this study it can be concluded that the killing of bacteria in infected dentin tubules is affected by an increase in temperature of the sodium hypochlorite solution. In addition, the exposure time of the irrigant to the dentin tubules and the concentration of the NaOCl solution all influence the killing efficacy of NaOCl. Both concentrations (2 % and 5.25 %) of NaOCl used in the present study were effective against the two different strains of *E. faecalis* biofilms when compared to sterile water; with the higher concentration NaOCl (5.25 %) displaying a higher efficacy. The longer exposure time resulted in greater numbers of dead bacteria. The three parameters, exposure time, concentration and temperature, had a positive effect on the killing. However, the killing rate decreased as the exposure time was prolonged or as the concentration was increased. It appears that the age of the biofilm did not affect the bacterial killing and complete eradication of the bacteria was not achieved despite the use of high concentration, heated NaOCl, and a long exposure time of 10 minutes.

It is currently impossible to completely eliminate bacteria from the root canals. Although sodium hypochlorite is the most popular disinfectant solution used, there has been no suggestion in the literature on an ideal temperature nor an ideal concentration for use in endodontic irrigation. More research is required to eventually formulate an irrigant that satisfies all the criteria required to optimize endodontic disinfection.

The present study is the first one to compare the killing efficacy of NaOCl against *E. faecalis* in infected dentin canals using solutions at different temperatures. Despite the limitations of this study, the findings may be of interest for future research in endodontic disinfection as it appears that the bacterial killing by NaOCl is increased by an increase in temperature and concentration.

## References


1. Nair PN. (2006). On the causes of persistent apical periodontitis: a review. International Endodontic Journal. 2: 249-281.

2. Kakehashi S, Stanley HR, Fitzgerald RJ. (1965). The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surgery, Oral Medicine, Oral Pathology. 20: 340-349.

3. Bergenholtz G. (1974). Micro-organisms from necrotic pulp of traumatized teeth. Odontologisk Revy. 25(4): 347-358.

4. Sundqvist G. (1976). Bacteriological studies of necrotic dental pulps. Umae University Odontological Dissertations.

5. Fabricius L, Dahlèn G, Holm SC, Möller Ă Jr. (2006). Influence of combinations of oral bacteria on periapical tissues of monkeys. Scandinavian Journal of Dental Research. 90: 200-206.

6. Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C. (2003). The application of biofilm science to the study and control of chronic bacterial infections. Journal of Clinical Investigations. 112(10): 1466-1477.

7. Nair PNR. (1987). Light and electron microscopic studies of root canal flora and periapical lesions. Journal of Endodontics. 13: 29-39.

8. Wilson M. (1996). Susceptibility of oral bacterial biofilms to antimicrobial agents. Journal of Medical Microbiology. 44: 79-87.

9. Costerton JW, Stewart PS. (2000). Biofilms and device-related infections. In: Nataro PJ, Balser MJ, Cunningham-Rundels S, eds. Persistent Bacterial Infections. Washington, DC: ASM Press, pp. 423–39.

10. Sirén EK, Haapasalo MPP, Waltimo TMT, Ørstavik D. (2004). In vitro antibacterial effect of calcium hydroxide combined with chlorhexidine or iodine potassium iodide on *Enterococcus faecalis*. European Journal of Oral Sciences. 112: 326-331.

11. Haapasalo M, Shen Y, Qian W, Gao Y. (2010). Irrigation in endodontics. Dental Clinics of North America. 54: 291-312.

12. Peter OA, Laib A, Gohring TN, Barbakow F. (2001). Changes in root canal geometry after preparation assessed by high-resolution computed tomography. Journal of Endodontics. 27: 1-6.

13. Zehnder M. (2006). Root canal irrigants. Journal of Endodontics. 32: 389-398.

14. Baker NA, Eleazer PD, Averbach RE, Seltzer S. (1975). Scanning electron microscopic study of the efficacy of various irrigating solutions. Journal of Endodontics. 1: 127-135.

15. Byström A, Sundqvist G. (1981). Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scandinavian Journal of Dental Research. 89: 321-328.

16. Langeland K. (1987). Tissue response to dental caries. Endodontics & Dental Traumatology. 3: 149-171.

17. Cameron CE. (1964). Cracked-tooth syndrome. Journal of the American Dental Association. 68: 405-411.

18. Zehnder M, Gold SI, Hasselgren G. (2002). Pathologic interactions in pulpal and periodontal tissues. Journal of Clinical Periodontology. 29: 663-671.

19. Hahn Cl, Best AM, Tew JG. (2000). Cytokine induction by *Streptococuccus mutans* and pulpal pathogenesis. Infection & Immunity. 68: 6785-6789.

20. Nair PN. (2004). Pathogenesis of apical periodontitis and the causes of endodontic failures. Critical Reviews in Oral Biology & Medicine. 15: 348-381.

21. Sundqvist G. (1994). Taxonomy, ecology, and pathogenicity of the root canal flora. Oral Surgery, Oral Medicine, Oral Pathology 78: 522-530.

22. Love RM. (2001). *Enterococcus faecalis* – a mechanism for its role in endodontic failure. International Endodontic Journal. 34: 399-405.

23. Shovelton DS. (1964). The presence and distribution of microorganisms with nonvital teeth. British Dental Journal. 117: 101-107.

24. Armitage GC, Ryder MI, Wilcox SE. (1983). Cemental changes in teeth with heavily infected root canals. Journal of Endodontics. 9: 127-130.

25. Chavez De Paz LE, Dahlèn G, Molander A, Möller Ă, Bergenholtz G. (2003) Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. International Endodontic Journal. 36: 500-508.

26. Engström B. (1964). The significance of Enterococci in root canal treatment. Odontologisk Revy. 15: 87-106.

27. Haapasalo M, Ranta K, Ranta H. (1983). Facultative Gram-negative enteric rods in persistent periapical infections. Acta Odontologica Scandinavica. 91: 458-463.

28. Felmingham D, Wilson APR, Quintana AI, Grüneberg RN. (1992). Enterococcus species in urinary tract infection. Clinical Infectious Disease. 15: 295-301.

29. Graninger W, Ragette R (1992) Nosocomial bacteremia due to *Enterococcus faecalis* without endocarditis. Clinical Infectious Disease. 15: 49-57.

30. Eliopoulos GM, Kaye D, ed. (1992). Enterococcal endocarditis, Infective Endocarditis. New York, USA: Raven Press. pp. 209-223.

31. Khardori N, Wong E, Carrasco CH, Wallace S, Patt Y, Bodey GP. (1991). Infections associated with biliary drainage procedures in patients with cancer. Reviews in Infectious Disease. 13: 587-591.

32. Jones WG, Barie PS, Yurt RW, Goodwin CW. (1986). Enterococcal burn sepsis: a highly lethal complication in severely burned patients. Archives of Surgery. 121: 649-652.

33. Richet H, Hubert B, Nitemberg A, Andremont A, Buu-Hoi P, Ourbak P et al. (1990). Prospective multicentre study of vascular-catheter-related complications and risk factors for positive central-catheter cultures in intensive care unit patients. Journal of Clinical Microbiology. 28: 2520-2525.

34. Molander A, Reit C, Dahlen G, Kvist T. (1998). Microbiological status of root-filled teeth with apical periodontitis. International Endodontic Journal. 31: 1-7.

35. Sundqvist G, Figdor D, Persson S, Sjögren U. (1998). Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics. 85: 86-93.

36. Dahlèn G, Samuelsson W, Molander A, Reit C. (2000). Identification and antimicrobial susceptibility of enterococci isolated from the root canal. Oral Microbiology and Immunology. 15: 309-312.

37. Byström A, Sundqvist G. (1985). The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. International Endodontic Journal. 18: 35-40.

38. Haapasalo M, Ørstavik D. (1987). *In vitro* infection and disinfection of dentinal tubules. Journal of Dental Research. 66: 1375-1379.

39. Ørstavik D, Haapasalo M. (1990). Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. Endodontics and Dental Traumatology. 6: 142-149.

40. Fabricius L, Dahlèn G, Holm SE, Möller ÅJR. (1982). Influence of combinations of oral bacteria on periapical tissues of monkeys. Scandinavian Journal of Dental Research. 90: 200-206.

41. Akpata ES, Blechman H. (1982). Bacterial invasion of pulpal dentin wall in vitro. Journal of Dental Research. 61: 435-438.

42. Kreft B, Marre A, Schamm U, Wirth R. (1992). Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. Infection and Immunity. 60: 25-30.

43. Giard J-C, Hartke A, Flahaut S, Benachour A, Boutibonnes P, Auffray Y. (1996). Starvation-induced multiresistance in *Enterococcus faecalis* JH2-2. Current Microbiology. 32: 264-271.

44. Giard J-C, Hartke A, Flahaut S, Boutibonnes P, Auffray Y. (1997). Glucose starvation response in *Enterococcus faecalis* JH2–2: survival and protein analysis. Research in Microbiology. 148: 27-35.

45. Gálvez A, Maqueda M, Mártinez-Bueno M, Valdivia E. (1991). Permeation of bacterial cells, permeation of cytoplasmic and artificial membrane vesicles, and channel formation on lipid bilayers of peptide antibiotic AS-48. Journal of Bacteriology. 173: 886-892.

46. Miyazaki S, Ohno A, Kobayashi I, Uji T, Yamaguchi K, Goto S. (1993). Cytotoxic effect of hemolytic culture supernatant from *Enterococcus faecalis* on mouse polymorphonuclear neutrophils and macrophages. Microbiology and Immunology. 37: 265-270.

47. Rosan B, Williams NB. (1964). Hyaluronidase production by oral enterococci. Archives of Oral Biology. 9: 291-298.

48. Hase CC, Finkelstein RA. (1993). Bacterial extracellular zinc containing metalloproteases. Microbiology Reviews. 57: 823-837.

49. Jett BD, Huycke MM, Gilmore MS. (1994). Virulence of *Enterococci.* Clinical Microbiology Reviews. 7: 462-478.

50. Rôças IN, Siqueira JF, Santos KRN. (2004). Association of *Enterococcus faecalis* with different forms of periradicular diseases. Journal of Endodontics. ;30: 315-320.

51. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. (2006). *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. Journal of Endodontics. 32: 93-98.

52. Basrani B, Haapasalo M. (2012). Update on endodontic irrigating solutions. Endodontic Topics. 27: 74-102.

53. Harrison JW. (1984). Irrigation of the root canal system. Dental Clinics of North America. 28: 797-808.

54. Frais S, Ng YL, Gulabivala K. (2001). Some factors affecting the concentration of available chlorine in commercial sources of sodium hypochlorite. International Endodontic Journal. 34: 206-215.

55. Dychdala GR. Chlorine and chlorine compounds. In: Block SS, ed. Disinfection, sterilization and preservation. Philadelphia: Lea & Febiger. 1:131-151.

56. Test ST, Lampert MB, Ossanna PJ, Thoene JG, Weiss SJ. (1984). Generation of nitrogen chlorine oxidants by human phagocytes. Journal of Clinical Investigations. 74: 1341-1349.

57. Crane AB. A predicatable root canal technique. Philadelphia: Lea & Febiger, 1920.

58. Estrela C, Barbin E, Spanó J, Marchesan M, Pécora J. (2002). Mechanism of action of sodium hypochlorite. Brazilian Dental Journal. 13: 113-117.

59. Barrette WC Jr, Hannum DM, Wheeler WD, et al. (1989). General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. Biochemical Journal. 28: 9172.

60. McKenna SM, Davies KJA. (1988). The inhibition of bacterial growth by hypochlorous acid. Biochemical Journal. 254: 685-689.

61. Grossman LI, Meiman BW. (1941). Solution of pulp tissue by chemical agents. Journal of the American Dental Association. 28: 223-225.

62. Radcliffe C, Potouridou L, Qureshi R, Habahbeh N, Qualtrough A, Worthington H. (2004). Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms *Actinomyces israelii, A. naeslundii, Candida albicans* and *Enterococcus faecalis*. International Endodontic Journal. 37: 438-446.

63. Zehnder M, Grawehr M, Hasselgren G, Waltimo T. (2003). Tissue-dissolution capacity and dentin-disinfecting potential of calcium hydroxide mixed with irrigating solutions. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics. 96: 608-613.

64. Spangberg L, Enstrom B, Langeland K. (1973). Biologic effects of dental materials. 3. Toxicity and antimicrobial effect of endodontic antiseptics *in vitro*. Oral Surgery, Oral Medicine, Oral Pathology. 36: 856-871

65. Hulsmann M, Hahn W. (2000). Complications during root canal irrigation—literature review and case reports. International Endodontic Journal. 33: 186-193.

66. Serper A, Ozbek M, Calt S. (2004). Accidental sodium hypochlorite-induced skin injury during endodontic treatment. Journal of Endodontics. 30: 180-181.

67. Hand RE, Smith ML, Harrison JW. (1978). Analysis of the effect of dilution on the necrotic tissue dissolution property of sodium hypochlorite. Journal of Endodontics. 4: 60-64.

68. Gomes B, Ferraz C, Vianna M, Berber V, Teixeira F, Souza-Filho F. (2001). *In vitro* antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. International Endodontic Journal. 34: 424-428.

69. Waltimo T, Ørstavik D, Siren EK, Haapasalo M. (1999). *In vitro* susceptibility of *Candida albicans* to four disinfectants and their combinations. International Endodontic Journal. 32: 421-429.

70. Moorer W, Wesselink P. (1982). Factors promoting the tissue dissolving capability of sodium hypochlorite. International Endodontic Journal. 15: 187-196.

71. Siqueira JF Jr, Rôças IN, Favieri A, Lima KC. (2000). Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite. Journal of Endodontics. 26: 331-334.

72. Spångberg L, Engström B, Langeland K. (1973). Biologic effects of dental materials. 3. Toxicity and antimicrobial effect of endodontic antiseptics *in vitro*. Oral Surgery, Oral Medicine, Oral Pathology. 36: 856-864.

73. Haapasalo M, Shen Y, Qian W, Gao Y. (2010). Irrigation in endodontics. Dental Clinics of North America. 5: 291-312.

74. ME, Gomes BP, Berber VB, Zaia AA, Ferraz CC, de Souza-Filho FJ. (2004). *In vitro* evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics. 97: 79-84.

75. Portenier I, Waltimo T, Ørstavik D, Haapasalo M. (2005). The susceptibility of starved, stationary phase and growing cells of *Enterococcus faecalis* to endodontic medicaments. Journal of Endodontics. 31: 380-386.

76. Cunningham WT, Balekjian AY. (1980). Effect of temperature on collagen-dissolving ability of sodium hypochlorite endodontic irrigant. Oral Surgery, Oral Medicine, Oral Pathology. 49: 175-177.

77. Thé SD. (1979). The solvent action of sodium hypochlorite on fixed and unfixed necrotic tissue. Oral Surgery, Oral Medicine, Oral Pathology. 47: 558-561.

78. Abou-Rass M, Oglesby SW. (1981). The effects of temperature, concentration, and tissue type on the solvent ability of sodium hypochlorite. Journal of Endodontics. 7: 376-377.

79. Kamburis JJ, Barker TH, Barfield RD, Eleazer PD. (2003). Removal of organic debris from bovine dental shavings. Journal of Endodontics. 29: 559-561.

80. Costigan SM. (1936). Effectiveness of hot hypochlorites of low alkalinity in destroying Mycobacterium tuberculosis. Journal of Bacteriology. 32: 57-63.

81. Sirtes G, Waltimo T, Schaetzle M, Zehnder M. The effects of temperature on sodium hypochlorite short-term stability, pulp dissolution capacity, and antimicrobial efficacy. Journal of Endodontics. 200;31:669-71.

82. Rossi-Fedele G, De Figueiredo JA. (2008). Use of a bottle warmer to increase 4% sodium hypochlorite tissue dissolution ability on bovine pulp. Australian Endodontic Journal. 34: 39-42.

83. Ma J, Wang Z, Shen Y, Haapasalo M. (2011). A new noninvasive model to study the effectiveness of dentin disinfection by using confocal laser scanning microscopy. Journal of Endodontics. 37: 1380-1385.

84. Wang Z, Shen Y, Haapasalo M. (2012). Effectiveness of endodontic disinfecting solutions against young and old *Enterococcus faecalis* biofilms in dentin canals. Journal of Endodontics. 38: 1376-1379.

85. Wang Z, Shen Y, Ma J, Haapasalo M. (2012). The effect of detergents on the antibacterial activity of disinfecting solutions in dentin. Journal of Endodontics. 38: 948-953.

86. Wang Z, Shen Y, Haapasalo M. (2013). Effect of smear layer against disinfection protocols on Enterococcus faecalis – infected dentin. Journal of Endodontics. 39: 1395-1400.

87. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. (2014). Effect of long-term exposure to endodontic disinfecting solutions on young and old enterococcus faecalis biofilms in dentin canals. Journal of Endodontics. 40: 509-514.

88. Miller WD. (1980). The micro-organisms of the human mouth. Philadelphia: SS White Manufacturing Co.

89. Heling I, Chandler NP. (1996). The antimicrobial effect within dentinal tubules of four root canal sealers. Journal of Endodontics. 22: 257-259.

90. Perez F, Calas P, Rochd T. (1996). Effect of dentin treatment on in vitro root tubule bacterial invasion. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics. 82: 46-51.

91. Peters LB, Wesselink PR, Moorer WR. (2000). Penetration of bacteria in bovine root dentine in vitro. International Endodontic Journal. 33: 28-36.

92. Chirnside IM. (1958). The bacteriological status of dentine around infected pulp canals. New Zealand Dental Journal. 54: 173-183.

93. Love RM. (1996). Regional variation in root dentinal tubule infection by *Streptococcus gordonii*. Journal of Endodontics. 22: 290-293.

94. Zapata RO, Moraes IG, Bernardineli N, et al. (2008). Confocal laser scanning microscopy is appropriate to detect viability of *Enterococcus faecalis* in infected dentin. Journal of Endodontics 34: 1198-1201.

95. Parmar D, Hauman CH, Leichter JW, McNaughton A, Tompkins GR. (2011). Bacterial localization and viability assessment in human *ex vivo* dentinal tubules by fluorescence confocal laser scanning microscopy. International Endodontic Journal. 44: 644-651.

96. Nagayoshi M, Kitamura C, Fukuizumi T, Nishihara T, Tershita M. (2004). Antimicrobial effect of ozonated water on bacteria invading dentinal tubules. Journal of Endodontics. 30: 778-781.

97. Haapasalo M, Orstavik D. (1987). *In vitro* infection and disinfection of dentinal tubules. Journal of Dental Research. 66: 1375-1379.

98. Shen Y, Stojicic S, Haapasalo M. (2010). Bacterial viability in starved and revitalized biofilms: comparison of viability staining and direct culture. Journal of Endodontics. 36: 1820-1823.

99. Zaura-Arite E, van Marle J, ten Cate JM. (2001). Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. Journal of Dental Research. 80: 1436-1440.

100. Haapasalo M, Endal U, Zandi H, Coil J. (2005). Eradication of endodontic infection by instrumentation and irrigation solutions. Endodontic Topics. 10: 77-102.

101. Hasselgren G, Olsson B, Cvek M. (1988). Effects of calcium hydroxide and sodium hypochlorite on the dissolution of necrotic porcine muscle tissue. Journal of Endodontics. 14: 125-127.

102. McComb D, Smith DC, Beagrie G. (1976). The results of in vivo endodontic chemomechanical instrumentation: a scanning electron microscopic study. Journal of the British Endodontic Society. 0: 11-18.

103. Cullen JK, Wealleans JA, Kirkpatrick TC, Yaccino JM. (2016). The effect of 8.25% sodium hypochlorite on dental pulp dissolution and dentin flexural strength and modulus. Journal of Endodontics 41: 920-924.

104. Peculiene V, Balciuniene I, Eriksen HM, et al. (2000). Isolation of *Enterococcus faecalis* in previously root-filled canals in a Lithuanian population. Journal of Endodontics. 26: 593-595.

105. Distel JW, Hatton JF, Gillespie MJ. (2002). Biofilm formation in medicated root canals. Journal of Endodontics. 28: 689-693.

106. Flahaut S, Frere J, Boutibonnes P, Auffray Y. (1996). Comparison of the bile salts and sodium dodecyl sulfate stress responses in *Enterococcus faecalis*. Applied and Environmental Microbiology. 62: 2416-2420.

107. Haapasalo HK, Siren EK, Waltimo TM, Orstavik D, Haapasalo MP. (2000). Inactivation of local root canal medicament by dentine: an in vitro study. International Endodontic Journal. 33: 26-31.

108. Portenier I, Haapasalo H, Rye A, Waltimo T, Orstavik D, Haapasalo M. (2001). Inactivation of root canal medicaments by dentine, hydroxylapatite and bovine serum albumin. International Endodontic Journal. 34: 184-188.

109. George S, Kishen A, Song KP. (2005). The role of environmental changes on monospecies biofilm formation on root canal wall by Enterococcus faecalis. Journal of Endodontics. 31: 867-872.

110. Shen Y, Stojicic S, Haapasalo M. (2011). Antimicrobial efficacy of chlorhexidine against bacteria in biofilms at different stages of development. Journal of Endodontics. 37: 657-661.

111. Harrison JW, Hand RE. (1981). The effect of dilution and organic matter on the anti-bacterial property of 5.25% sodium hypochlorite. Journal of Endodontics. 7: 128-132.

112. Kara Tuncer A, Tuncer S. (2012). Effect of different final irrigation solutions on dentinal tubule penetration depth and percentage of root canal sealer. Journal of Endodontics. 38: 860-863.

113. Kuga MC, Gouveia-Jorge E, Tanomaru-Filho M, et al. (2011). Penetration into dentin of sodium hypochlorite associated with acid solutions. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics. 112: 155-159.

114. Pashley DH, Michelich V, Kehl T. (1981). Dentin permeability: effects of smear layer removal. Journal of Prosthetic Dentistry. 46: 531-537.

115. Wang Z, Sa Y, Sauro, et al. (2010). Effect of desensitising toothpastes on dentinal tubule occlusion: a dentin permeability measurement and SEM in vitro study. Journal of Dentistry. 38: 400-410.

116. Zou L, Shen Y, Li W, et al. (2010). Penetration of sodium hypochlorite into dentin. Journal of Endodontics. 36: 793-796.

117. Cunningham WT, Joseph SW. (1980). Effect of temperature on the bactericidal action of sodium hypochlorite endodontic irrigant. Oral Surgery. 49: 175-177.

118. Stojicic S, Zivkovic S, Qian W, et al. (2010). Tissue dissolution by sodium hypochlorite: effect of concentration, temperature, agitation, and surfactant. Journal of Endodontics. 36: 1558-1562.

119. Retamozo B, Shabahang S, Johnson N, et al. (2010). Minimum contact and concetration of sodium hypochlorite required to eliminate *Enterococcus faecalis.* Journal of Endodontics. 36: 520-523.

120. Hecker S, Hiller KA, Galler Km, et al. (2013). Establishment of an optimized *ex vivo* system for artifical root canal infection evaluated by use of sodium hypochlorite and the photodynamic therapy. International Endodontic Journal. 46: 449-457.

121. Pashley DH, Tao L, Boyd I, et al. (1988). Scanning electron microscopy of the substructure of smear layers in human dentine. Archives of Oral Biology. 33: 265-270.

122. Sen BH, Wesselink PR, Turkun M. (1995). The smear layer: a phenomenon in root canal therapy. International Endodontic Journal. 28: 141-148.

123. Bukiet F, Soler T, Guivarch M, et al. (1998). Chemical stability of heated sodium hypochlorite endodontic irrigants. Journal of Endodontics. 24: 432-434.

124. De Hemptinne F, Slaus G, Vandedael M, Jacquet W, De Moor RJ, Bottenberg P. (2015). *In vivo* intracanal temperature evolution during endodntic treatment after the injection of room temperature or preheated sodium hypochlorite. Journal of Endodontics. 41: 1112-1115.

# Appendix A – Statistical Report

## STCS Report for Stefanie Nio

Shenyi Pan

February 12, 2017

In this report, I summarize the proposed statistical methods to address the objectives of your study: which treatment factors have a significant effect on the average percentage of the bacteria killed.

### 1 Introduction to the Experiment

The response variable of this study is the average percentage of the bacteria killed, which can be treated as a continuous variable. There are two blocking factors (strain and age biofilm) and three treatment factors (NaOCl, temperature, and experimental time). These five factors are all binary variables taking two values. You can treat strain and age biofilm as blocking factors since these two factors define the experiment environment. Though you are not interested in their effects, there could still be variability in responses between blocks. Treating strain and age biofilm as blocking factors accounts for such variability. For the three treatment factors, you are interested in their main effects and interaction effects. In statistical analysis, blocking factors are treated in the same way as treatment factors. The only difference is that one focuses more on the interpretation of the effects of treatment factors.

The 32 possible combinations of the two blocking factors and the three treatment factors correspond to the 32 treatment groups in this experiment. Therefore, the treatment groups have a $2^5$ factorial design. Additionally, there are also 12 control groups, where sterile water is used instead of 2% or 5.25% NaOCl.

### 2 Proposed Statistical Methods

In this section, I illustrate how to use R, a free and open-source software for statistical computing, to perform analyses on the data. To make it easier to read into R, I have transformed your data and saved them as `data.csv`. Note that for simplicity, I use 5% to represent 5.25% NaOCl in the data file..

In this experiment, there are 12 control groups under 8 different combinations of strain, age biofilm, temperature, and experimental time. That is, for some combinations of strain, age biofilm, temperature, and experimental time (for example, temperature 20 degrees with experimental time 10 minutes), there are no control groups available. Therefore, it is not appropriate to use a single linear model described in the SOS summary to analyze the treatment groups and control groups simultaneously. Instead, I propose splitting the analysis into two steps. The first step compares the control groups where sterile water is used with the treatment groups where NaOCl is used. If the mean percentage of the bacterial killed in the treatment groups is found significantly different from that in the control groups, one investigates the effects of the treatment factors in the second step.

## 2.1 Paired t-Test

To compared the control groups with the treatment groups, I propose the paired t-test to check whether the mean response when NaOCl is used is significantly different from that when sterile water is used. Paired t-test typically consists of matched pairs of measurements and examine the difference between the population mean responses between the two sets of measurements. In your study, the responses measured when strain, age biofilm, temperature, and experimental time are the same can be considered a pair of measurements. Note that there are two control groups with temperature 20 degrees and experimental time 3 minutes given certain strain and age biofilm with only one treatment group. I take the average of the two responses of the control groups and treat it as a single response of the control group and pair it with the corresponding response of the treatment group. For the comparison between the sterile water and 2% NaOCl, the data transformation and selection work can be done using the following R code:

```
> setwd('D:/Dropbox/study (ms)/yr2 sem2/stcs/stcs')
> dat=read.csv('data.csv',header=T)
>
> install.packages('plyr') # install the plyr packages
> library(plyr)
> naocl2=dat[dat$NaOCl=='2%',]
> naocl5=dat[dat$NaOCl=='5%',]
> ctr=dat[dat$NaOCl=='DW',]
```

```
> ctrnew=ddply(ctr,~strain+NaOCl+age.biofilm+temperature+experimental.time,
summarise,mean=mean(mean.dead))
>
> mergedata2=merge(naocl2,ctrnew,by=c('strain','age.biofilm','temperature',
'experimental.time'))
> names(mergedata2)[6]='mean.dead.exp'
> names(mergedata2)[8]='mean.dead.ctr'
> print(mergedata2)
    strain age.biofilm temperature experimental.time NaOCl.x mean.dead.exp
  1    181          3d          20                3m      2%     0.3006312
  2    181          3d          60               10m      2%     0.7146141
  3    181          3w          20                3m      2%     0.2908841
  4    181          3w          60               10m      2%     0.7517913
  5  GEL31          3d          20                3m      2%     0.3024567
  6  GEL31          3d          60               10m      2%     0.6342368
  7  GEL31          3w          20                3m      2%     0.2974430
  8  GEL31          3w          60               10m      2%     0.6600980
    NaOCl.y mean.dead.ctr
  1      DW     0.1591448
  2      DW     0.1745931
  3      DW     0.1667676
  4      DW     0.1698925
  5      DW     0.1312149
  6      DW     0.1791372
  7      DW     0.1559320
  8      DW     0.1496698
```

The R object `mergedata2` stores the data of all the control groups along with the paired observations in treatment groups. The hypotheses of the paired t-test for comparing sterile water with 2% NaOCl are:

**Null hypothesis** $H_0$**:** the underlying true mean of the response (in your experiment the response is the mean percentage of the bacteria killed) when sterile water is used is the same as the

underlying true mean of the response when 2% NaOCl is used.

**Alternative hypothesis** $H_1$: the underlying true mean of the response when 2% NaOCl is used is greater than the underlying true mean of the response when sterile water is used.

The paired t-test can be done in R using:

```
> t.test(mergedata2$mean.dead.exp, mergedata2$mean.dead.ctr, paired=T,
alternative='greater')

Paired t-test

data:  mergedata2$mean.dead.exp and mergedata2$mean.dead.ctr
t = 4.5963, df = 7, p-value = 0.001248
alternative hypothesis: true difference in means is greater than 0
95 percent confidence interval:
0.1958707       Inf
sample estimates:
mean of the differences
0.3332254
```

It can be seen that the p-value of the test is 0.001248, which is smaller than 0.05, which suggest there is a significant different between the mean responses when sterile water is used versus when 2% NaOCl is used. It is estimated that on average the mean percentage of the bacterial killed is 33.32% higher when 2% NaOCl is used compared to when sterile water is used. The lower bound of the 95% confidence interval for this difference is 19.59%, which is greater than 0, suggesting statistically significant difference.

The limitation of paired t-test approach is that it only compares the effect of sterile water with 2% or 5.25% NaOCl, and assumes that there are no effects of other factors or interactions effects between factors. However, the data seem to suggest that the difference between the responses of the control group and the treatment group is larger when the temperature is higher and the experiment time is longer. But it is worth noting that in control groups, temperature 20 degrees and experimental time 3 minutes always show up together, and temperature 60 degrees and experimental time 10 minutes always show up together. This makes it impossible to distinguish the effects of

high temperature from long experiment time. Nevertheless, you could select a subset of the data with temperature 20 degrees and experimental time 3 minutes setting, and performs a paired t-test on this subset of data (4 pairs of observations). The hypotheses for this test are:

**Null hypothesis** $H_0$: With temperature 20 degrees and experimental time 3 minutes, the underlying true mean of the response when sterile water is used is the same as the underlying true mean of the response when 2% NaOCl is used.

**Alternative hypothesis** $H_1$: With temperature 20 degrees and experimental time 3 minutes, the underlying true mean of the response when 2% NaOCl is used is greater than the underlying true mean of the response when sterile water is used.

The test can be done in R using:

```
> mergedata2lowshort=mergedata2[mergedata2$temperature==20,]
> print(mergedata2lowshort)
strain age.biofilm temperature experimental.time NaOCl.x mean.dead.exp
1    181          3d          20                3m      2%     0.3006312
3    181          3w          20                3m      2%     0.2908841
5  GEL31          3d          20                3m      2%     0.3024567
7  GEL31          3w          20                3m      2%     0.2974430
NaOCl.y mean.dead.ctr
1      DW     0.1591448
3      DW     0.1667676
5      DW     0.1312149
7      DW     0.1559320
> t.test(mergedata2lowshort$mean.dead.exp, mergedata2lowshort$mean.dead.ctr,
paired=T, alternative='greater')

Paired t-test

data:  mergedata2lowshort$mean.dead.exp and mergedata2lowshort$mean.dead.ctr
t = 14.779, df = 3, p-value = 0.000336
alternative hypothesis: true difference in means is greater than 0
```

```
95 percent confidence interval:
0.1215648       Inf
sample estimates:
mean of the differences
0.1445889
```

The test result still shows statistically significant difference between sterile water and 2% NaOCl, though the estimate for such difference is smaller.

You can perform the same test for the paired data of sterile water and 2% NaOCl when temperature is 60 degrees and experiment time is 10 minutes using:

```
ghlong=mergedata2[mergedata2$temperature==60,]
> print(mergedata2highlong)
strain age.biofilm temperature experimental.time NaOCl.x mean.dead.exp
2    181          3d          60               10m      2%     0.7146141
4    181          3w          60               10m      2%     0.7517913
6  GEL31          3d          60               10m      2%     0.6342368
8  GEL31          3w          60               10m      2%     0.6600980
NaOCl.y mean.dead.ctr
2      DW     0.1745931
4      DW     0.1698925
6      DW     0.1791372
8      DW     0.1496698
> t.test(mergedata2highlong$mean.dead.exp, mergedata2highlong$mean.dead.ctr,
paired=T, alternative='greater')

Paired t-test

data:  mergedata2highlong$mean.dead.exp and mergedata2highlong$mean.dead.ctr
t = 19.583, df = 3, p-value = 0.0001455
alternative hypothesis: true difference in means is greater than 0
95 percent confidence interval:
0.4591468       Inf
```

```
sample estimates:
mean of the differences
0.5218619
```

All the above-mentioned three tests indicate that compared with the control groups, the mean response is significantly larger when 2% NaOCl is used, suggesting that 2% NaOCl is effective in killing the bacteria.

You can perform similar tests to compare the 5.25% NaOCl with sterile water using:

```
> mergedata5=merge(naocl5,ctrnew,by=c('strain','age.biofilm','temperature',
'experimental.time'))
> names(mergedata5)[6]='mean.dead.exp'
> names(mergedata5)[8]='mean.dead.ctr'
> t.test(mergedata5$mean.dead.exp, mergedata5$mean.dead.ctr, paired=T,
alternative='greater')

Paired t-test

data:  mergedata5$mean.dead.exp and mergedata5$mean.dead.ctr
t = 28.966, df = 7, p-value = 7.522e-09
alternative hypothesis: true difference in means is greater than 0
95 percent confidence interval:
0.4716765       Inf
sample estimates:
mean of the differences
0.5046871
```

for the overall comparison and

```
> mergedata5lowshort=mergedata5[mergedata5$temperature==20,]
> t.test(mergedata5lowshort$mean.dead.exp, mergedata5lowshort$mean.dead.ctr,
paired=T, alternative='greater')

Paired t-test
```

```
data:  mergedata5lowshort$mean.dead.exp and mergedata5lowshort$mean.dead.ctr
t = 31.397, df = 3, p-value = 3.55e-05
alternative hypothesis: true difference in means is greater than 0
95 percent confidence interval:
0.4356691       Inf
sample estimates:
mean of the differences
0.4709702

>
> mergedata5highlong=mergedata5[mergedata5$temperature==60,]
> t.test(mergedata5highlong$mean.dead.exp, mergedata5highlong$mean.dead.ctr,
paired=T, alternative='greater')

Paired t-test

data:  mergedata5highlong$mean.dead.exp and mergedata5highlong$mean.dead.ctr
t = 25.849, df = 3, p-value = 6.35e-05
alternative hypothesis: true difference in means is greater than 0
95 percent confidence interval:
0.4893864       Inf
sample estimates:
mean of the differences
0.538404
```

for tests for two different settings of temperature and experiment time. The test results indicate that compared with the control groups, the mean response is also significantly larger when 5.25% NaOCl is used, suggesting that 5.25% NaOCl is also effective in killing the bacteria.

## 2.2 Linear Model

In the previous section, paired t-test is used to show the effective in using 2% or 5.25% NaOCl to kill the bacteria in comparison to the control groups where sterile water is used. In this section, I introduce using linear model to further investigate the effects of different concentrations of NaOCl as well as its interaction with other treatment factors. Note that strain and age biofilm are treated as blocking factors. Therefore, it is not of interest to investigate their interaction effects with the treatment factors. Additionally, the interaction effects of order higher than two is often omitted since the residual sum of square explained by these high order interaction terms is usually small, and it is often difficult to interpret the effect of high order interaction terms in the model. Hence, if we treat the interaction effects of order higher than two as negligible, the linear model representation of this $2^5$ factorial experiment can be written as:

$$Y_i = \alpha + \beta_1 X_{i,1} + \beta_2 X_{i,2} + \beta_3 X_{i,3} + \beta_{12} X_{i,1} X_{i,2} + \beta_{13} X_{i,1} X_{i,3} + \beta_{23} X_{i,2} X_{i,3} + \gamma_1 B_{i,1} + \gamma_1 B_{i,2} + \epsilon_i,$$

where

- $Y_i$ is the $i$-th response under one set of experimental condition.
- $\alpha$ represents the expected mean percentage of the bacterial killed of the reference group. The reference group is the group where all the $X_{i,k}$ and $B_{i,k}$ are set to 0.
- $X_{i,1}$, $X_{i,2}$, and $X_{i,3}$ are the binary dummy variables for the three treatment factors NaOCl, temperature, and experimental time respectively. I choose 2% concentration, 20 degrees, and 3 minutes as the reference levels for $X_{i,1}$, $X_{i,2}$, and $X_{i,3}$. That is, 2% concentration, 20 degrees, and 3 minutes will be coded as 0, while 5.25% concentration, 60 degrees, and 10 minutes will be coded as 1.
- $X_{i,1}X_{i,2}$, $X_{i,1}X_{i,3}$, and $X_{i,2}X_{i,3}$ represent the pairwise interactions between the treatment factors.
- $B_{i,1}$ and $B_{i,2}$ are the binary dummy variables for the two blocking factors strain and age biofilm. I choose GEL31 and 3 weeks as the reference levels for $B_{i,1}$ and $B_{i,2}$.
- $\beta_1$, $\beta_2$, and $\beta_3$ represent the main effects of the three treatment factors NaOCl, temperature, and experimental time. When the interaction effects are present in the model, the main effects

are intertwined with the interpretation of the interaction effects and it is difficult to merely interpret the main effects.

- $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ represent the interaction effects of the terms $X_{i,1}X_{i,2}$, $X_{i,1}X_{i,3}$, and $X_{i,2}X_{i,3}$.
- $\gamma_1$ and $\gamma_2$ represent the main effects of the two blocking factors strain and age biofilm.
- $\epsilon_i$ is independent and identically distributed random errors following normal distribution.

This linear model can be fitted in R using:

```
> expdat=dat[dat$NaOCl!='DW',]
> expdat$strain=factor(expdat$strain, levels=c('GEL31',181))
> expdat$NaOCl=factor(expdat$NaOCl)
> expdat$age.biofilm=factor(expdat$age.biofilm)
> expdat$temperature=factor(expdat$temperature)
> expdat$experimental.time=factor(expdat$experimental.time, levels=c('3m','10m'))
> print(expdat)
strain NaOCl age.biofilm temperature experimental.time mean.dead
1   GEL31    2%          3d          20                3m 0.3024567
2   GEL31    5%          3d          20                3m 0.6106397
3     181    2%          3d          20                3m 0.3006312
4     181    5%          3d          20                3m 0.6689312
5   GEL31    2%          3d          20               10m 0.5910916
6   GEL31    5%          3d          20               10m 0.6036258
7     181    2%          3d          20               10m 0.6017223
8     181    5%          3d          20               10m 0.6435365
9   GEL31    2%          3d          60                3m 0.6356285
10  GEL31    5%          3d          60                3m 0.6628566
11    181    2%          3d          60                3m 0.7265407
12    181    5%          3d          60                3m 0.7326488
13  GEL31    2%          3d          60               10m 0.6342368
14  GEL31    5%          3d          60               10m 0.6554737
15    181    2%          3d          60               10m 0.7146141
16    181    5%          3d          60               10m 0.7403253
```

```
23  GEL31    2%          3w          20                3m 0.2974430
24  GEL31    5%          3w          20                3m 0.6047931
25    181    2%          3w          20                3m 0.2908841
26    181    5%          3w          20                3m 0.6125763
27  GEL31    2%          3w          20               10m 0.6097714
28  GEL31    5%          3w          20               10m 0.6124525
29    181    2%          3w          20               10m 0.5723988
30    181    5%          3w          20               10m 0.5770148
31  GEL31    2%          3w          60                3m 0.6549548
32  GEL31    5%          3w          60                3m 0.7279442
33    181    2%          3w          60                3m 0.6964501
34    181    5%          3w          60                3m 0.7478333
35  GEL31    2%          3w          60               10m 0.6600980
36  GEL31    5%          3w          60               10m 0.7041344
37    181    2%          3w          60               10m 0.7517913
38    181    5%          3w          60               10m 0.7269752
> lmfit=lm(mean.dead~strain + NaOCl + age.biofilm + temperature + experimental.time
+ NaOCl*temperature + NaOCl*experimental.time + temperature*experimental.time,
data=expdat)
> summary(lmfit)

Call:
lm(formula = mean.dead ~ strain + NaOCl + age.biofilm + temperature +
experimental.time + NaOCl * temperature + NaOCl * experimental.time +
temperature * experimental.time, data = expdat)

Residuals:
Min       1Q   Median       3Q      Max
-0.08565 -0.02495 -0.00029  0.03712  0.06812

Coefficients:
                                    Estimate Std. Error t value Pr(>|t|)
```

```
(Intercept)                          0.31637    0.02712  11.666 3.87e-11 ***
strain181                            0.03358    0.01808   1.857 0.076108 .
NaOCl5%                              0.25436    0.03131   8.123 3.30e-08 ***
age.biofilm3w                        0.00141    0.01808   0.078 0.938522
temperature60                        0.30852    0.03131   9.853 1.01e-09 ***
experimental.time10m                 0.22387    0.03131   7.149 2.79e-07 ***
NaOCl5%:temperature60               -0.14291    0.03616  -3.952 0.000633 ***
NaOCl5%:experimental.time10m        -0.16693    0.03616  -4.617 0.000121 ***
temperature60:experimental.time10m -0.14006    0.03616  -3.874 0.000770 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.05114 on 23 degrees of freedom
Multiple R-squared:   0.89,     Adjusted R-squared:  0.8518
F-statistic: 23.27 on 8 and 23 DF,  p-value: 2.792e-09
```

In the above R outputs, the coefficients have one-to-one correspondence to the parameters in the linear model. For example, `(Intercept)` corresponds to $\alpha$, `strain181` corresponds to $\gamma_1$, `NaOCl5%` corresponds to $\beta_1$, etc. At 5% significance level, the main effects of the two blocking factors are not significant, which is expected since the setup of the experimental environment should not affect the results. In addition, all the main effects and the pairwise interaction effects of the three treatment factors are significant with p-values smaller than 0.05. It can be seen that the estimates of all the three main effects are positive while the estimates for all the interaction effects are negative. Such result can be interpreted with the interaction plots generated in R using:

```
interaction.plot(expdat$NaOCl,expdat$temperature,expdat$mean.dead)
interaction.plot(expdat$NaOCl,expdat$experimental.time,expdat$mean.dead)
interaction.plot(expdat$temperature,expdat$experimental.time,expdat$mean.dead)
```

The three pairwise interaction plots can be found in Figures 1 to 3. I will use Figure 1 as an example to illustrate how to interpret the result. In Figure 1, it can be seen that the mean percentage of the bacteria killed increases as the concentration of NaOCl changes from 2% to 5.25% (positive main effect). However, the magnitude of such increase depends on the temperature. When

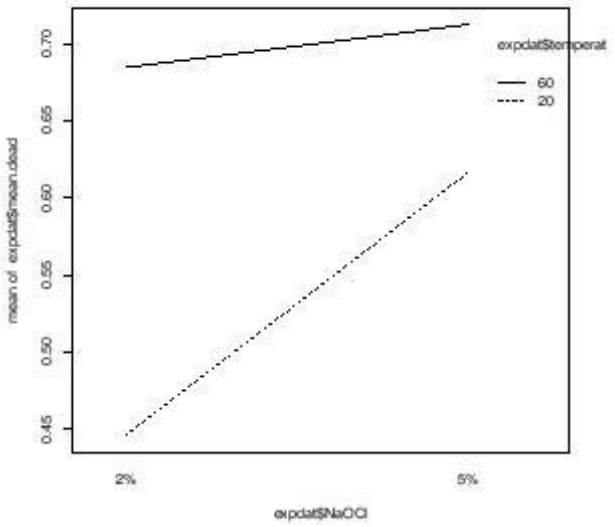



Figure 1: Interaction plot between NaOCl and temperature.

temperature is higher, is magnitude of the increase is smaller (negative interaction effect). The other two interaction plots can be interpreted in a similar way.

It might also be of interest to predict the response given a certain combination of factors. For example, assume that you are interested in the response when the experiment conditions are starin 181, 2% NaOCl, 3 days, 60 degrees, and 3 minutes. This corresponds to the 12th row in the `data.csv` file. Then you can use the following R functions to generate the prediction intervals for the expected response and individual observation under this condition. Note that the expected response is the mean level of the mean percentage of the bacteria killed under this condition, while the individual observation is a single observed value. Since there is random error $\epsilon_i$ for each individual observation, the prediction interval for individual observation is wider than the prediction interval for the mean response.

```
> predict(lmfit,expdat[12,],interval='confidence',level=.95)
        fit       lwr       upr
12 0.7699163 0.7138175 0.8260151
> predict(lmfit,expdat[12,],interval='prediction',level=.95)
```

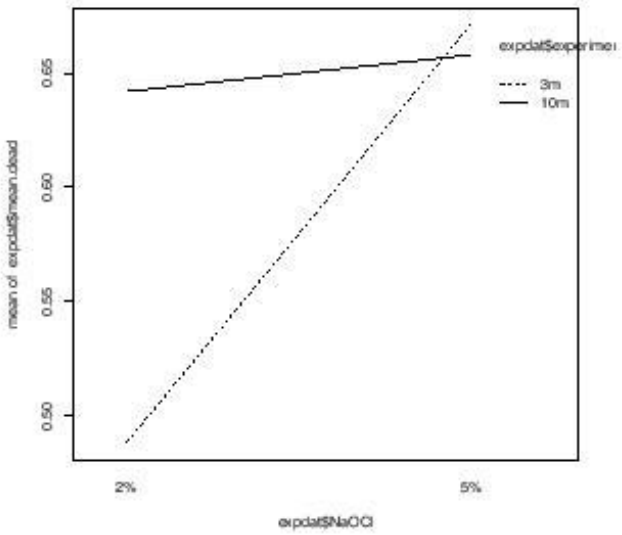



Figure 2: Interaction plot between NaOCl and experiment time.

```
        fit       lwr       upr
12 0.7699163 0.6501804 0.8896522
```

The results indicate that given starin 181, 2% NaOCl, 3 days, 60 degrees, and 3 minutes, we are 95% confident that the mean of the response falls within [71.38%, 82.60%]. If we conduct one more experiment with this setting, we are 95% confident that the observed response falls within [65.02%, 88.97%].

# Additional Note

Shenyi Pan

February 17, 2017

Consider the case that you are interest in examining the difference between high and low temperatures when the concentration of NaOCl is 5.25% and the experiment time is 10 minutes. Using the notation in page 9 of the report, the expected response when temperature is 60 degrees, the concentration of NaOCl is 5.25% and the experiment time is 10 minutes is

$$\mathrm{E}(Y_1) = \beta_1 + \beta_2 + \beta_3 + \beta_{12} + \beta_{13} + \beta_{23} + \gamma_1 B_{1,1} + \gamma_2 B_{1,2},$$

and the expected response when temperature is 20 degrees, the concentration of NaOCl is 5.25% and the experiment time is 10 minutes is

$$\mathrm{E}(Y_2) = \beta_1 + \beta_3 + \beta_{13} + \gamma_1 B_{2,1} + \gamma_2 B_{2,2}.$$

The method on page 13-14 of the report gives the predicted value and the confidence interval for $\mathrm{E}(Y_i)$ under certain combinations of treatment factors and when the levels of blocking factors are known with

```
predict(lmfit,...,interval='confidence',level=.95)
```

and gives the predicted value and the confidence interval for an observed $Y_i$ under certain combinations of treatment factors and when the levels of blocking factors are known with

```
predict(lmfit,...,interval='prediction',level=.95).
```

To examine the difference between high and low temperatures, you are interested in $\mathrm{E}(Y_1) - \mathrm{E}(Y_2)$. When the levels of blocking factors are the same, this difference is

$$D = \mathrm{E}(Y_1) - \mathrm{E}(Y_2) = \beta_2 + \beta_{12} + \beta_{23}.$$

Based on the R output on page 12, the estimates for $\beta_2$, $\beta_{12}$, and $\beta_{23}$ are 0.30852, -0.14291, and

-0.14006. Hence, the estimated value for the difference D is:

$$\hat{D} = \hat{\beta}_2 + \hat{\beta}_{12} + \hat{\beta}_{23} = 0.30852 - 0.14291 - 0.14006 = 0.0255.$$

Further, the variance of D is

$$\begin{aligned} Var(D) &= Var(\beta_2 + \beta_{12} + \beta_{23}) \\ &= Var(\beta_2) + Var(\beta_{12}) + Var(\beta_{23}) + 2Cov(\beta_2, \beta_{13}) + 2Cov(\beta_2, \beta_{23}) + 2Cov(\beta_{13}, \beta_{23}). \end{aligned}$$

The variance-covariance matrix of all the parameter estimates in the linear model can be obtained using the R code

```
> covmat=vcov(lmfit)
```

where `lmfit` is the R object storing the fitted linear model mentioned in the report. Then the estimate of the variance of D can be obtained using:

```
> varD=covmat['temperature60','temperature60']+covmat['NaOCl5%:temperature60',
'NaOCl5%:temperature60']+covmat['temperature60:experimental.time10m',
'temperature60:experimental.time10m']+2*covmat['temperature60',
'NaOCl5%:temperature60']+2*covmat['temperature60',
'temperature60:experimental.time10m']+2*covmat['temperature60:experimental.time10m',
'NaOCl5%:temperature60']
> print(varD)
[1] 0.0009805479
```

Therefore, to test whether $D$ is 0, the t-test statistic is

$$T = \frac{\hat{D}}{\sqrt{\widehat{Var}(D)}} = \frac{0.0255}{\sqrt{0.0009805479}} = 0.8143.$$

From the R output on page 12 of the report, we know that the degrees of freedom for the residuals is 23. Therefore, the p-value for testing the significance of $D$ is

```
> 2*(1-pt(0.8143,df=23)).
[1] 0.4238244
```

Since the p-value is much greater than 0.05, we cannot reject the hypothesis that $D$ is 0. Hence, our conclusion is that there is no significant difference in the response between high and low temperatures when the concentration of NaOCl is 5.25% and the experiment time is 10 minutes.

Tests under other combination of treatment factors can be done following the same procedure.

# Additional Note

Shenyi Pan

February 18, 2017

Consider the case that you are interest in examining the difference between high and low temperatures when the concentration of NaOCl is 5.25% and the experiment time is 10 minutes. Using the notation in page 9 of the report, the expected response when temperature is 60 degrees, the concentration of NaOCl is 5.25% and the experiment time is 10 minutes is

$$\mathrm{E}(Y_1) = \beta_1 + \beta_2 + \beta_3 + \beta_{12} + \beta_{13} + \beta_{23} + \gamma_1 B_{1,1} + \gamma_2 B_{1,2},$$

and the expected response when temperature is 20 degrees, the concentration of NaOCl is 5.25% and the experiment time is 10 minutes is

$$\mathrm{E}(Y_2) = \beta_1 + \beta_3 + \beta_{13} + \gamma_1 B_{2,1} + \gamma_2 B_{2,2}.$$

The method on page 13-14 of the report gives the predicted value and the confidence interval for $\mathrm{E}(Y_i)$ under certain combinations of treatment factors and when the levels of blocking factors are known with

```
predict(lmfit,...,interval='confidence',level=.95)
```

and gives the predicted value and the confidence interval for an observed $Y_1$ under certain combinations of treatment factors and when the levels of blocking factors are known with

```
predict(lmfit,...,interval='prediction',level=.95).
```

To examine the difference between high and low temperatures, you are interested in $\mathrm{E}(Y_1) - \mathrm{E}(Y_2)$. When the levels of blocking factors are the same, this difference is

$$D = \mathrm{E}(Y_1) - \mathrm{E}(Y_2) = \beta_2 + \beta_{12} + \beta_{23}.$$

Based on the R output on page 12, the estimates for $\beta_2$, $\beta_{12}$, and $\beta_{23}$ are 0.30852, -0.14291, and

-0.14006. Hence, the estimated value for the difference D is:

$$\hat{D} = \hat{\beta}_2 + \hat{\beta}_{12} + \hat{\beta}_{23} = 0.30852 - 0.14291 - 0.14006 = 0.0255.$$

Further, the variance of D is

$$\begin{aligned} Var(D) &= Var(\beta_2 + \beta_{12} + \beta_{23}) \\ &= Var(\beta_2) + Var(\beta_{12}) + Var(\beta_{23}) + 2Cov(\beta_2, \beta_{13}) + 2Cov(\beta_2, \beta_{23}) + 2Cov(\beta_{13}, \beta_{23}). \end{aligned}$$

The variance-covariance matrix of all the parameter estimates in the linear model can be obtained using the R code

```
> covmat=vcov(lmfit)
```

where `lmfit` is the R object storing the fitted linear model mentioned in the report. Then the estimate of the variance of D can be obtained using:

```
> varD=covmat['temperature60','temperature60']+covmat['NaOCl5%:temperature60',
'NaOCl5%:temperature60']+covmat['temperature60:experimental.time10m',
'temperature60:experimental.time10m']+2*covmat['temperature60',
'NaOCl5%:temperature60']+2*covmat['temperature60',
'temperature60:experimental.time10m']+2*covmat['temperature60:experimental.time10m',
'NaOCl5%:temperature60']
> print(varD)
[1] 0.0009805479
```

Therefore, to test whether $D$ is 0, the t-test statistic is

$$T = \frac{\hat{D}}{\sqrt{\widehat{Var}(D)}} = \frac{0.0255}{\sqrt{0.0009805479}} = 0.8143.$$

From the R output on page 12 of the report, we know that the degrees of freedom for the residuals is 23.

The hypotheses of the two-sided test are:

**Null hypothesis** $H_0$**:** $D = 0$;

**Alternative hypothesis** $H_1$**:** $D \neq 0$.

The p-value for testing the significance of $D$ is

```
> 2*(1-pt(0.8143,df=23)).
[1] 0.4238244
```

Since the p-value is much greater than 0.05, we cannot reject the hypothesis that $D$ is 0.

However, in your study, since you suspect that high temperature should be more effective in killing the bacteria than low temperature, one-sided hypothesis test is more relevant. The hypotheses are

**Null hypothesis** $H_0$: $D \leq 0$;

**Alternative hypothesis** $H_1$: $D > 0$.

The p-value for testing the significance of $D$ under one-tailed setting is

```
> 1-pt(0.8143,df=23).
[1] 0.2119122
```

Again the p-value is much greater than 0.05, we cannot reject the null hypothesis. Hence, our conclusion is that there is no significant difference in the response between high and low temperatures when the concentration of NaOCl is 5.25% and the experiment time is 10 minutes.

Tests under other combination of treatment factors can be done following the same procedure. The results are summarized in the following table.

| NaOCl | Experiment time | $D$ | Estimate of $D$ | S.D. of $D$ | One-tailed t statistic | p-value | Significance at 5% level |
|---|---|---|---|---|---|---|---|
| 5.25% | 10 | $\beta_2 + \beta_{12} + \beta_{23}$ | 0.0255 | 0.0313137 | 0.8143 | 0.2119 | No |
| 5.25% | 3 | $\beta_2 + \beta_{12}$ | 0.16561 | 0.0313137 | 5.2887 | 1.143e-05 | Yes |
| 2% | 10 | $\beta_2 + \beta_{23}$ | 0.16846 | 0.0313137 | 5.3798 | 9.137e-06 | Yes |
| 2% | 3 | $\beta_2$ | 0.30852 | 0.0313137 | 9.8526 | 5.055e-10 | Yes |

# Additional Note

Shenyi Pan

February 20, 2017

Consider the case that you are interest in examining the difference between high and low temperatures when the concentration of NaOCl is 5.25% and the experiment time is 10 minutes. Using the notation in page 9 of the report, the expected response when temperature is 60 degrees, the concentration of NaOCl is 5.25% and the experiment time is 10 minutes is

$$\mathrm{E}(Y_1) = \beta_1 + \beta_2 + \beta_3 + \beta_{12} + \beta_{13} + \beta_{23} + \gamma_1 B_{1,1} + \gamma_2 B_{1,2},$$

and the expected response when temperature is 20 degrees, the concentration of NaOCl is 5.25% and the experiment time is 10 minutes is

$$\mathrm{E}(Y_2) = \beta_1 + \beta_3 + \beta_{13} + \gamma_1 B_{2,1} + \gamma_2 B_{2,2}.$$

The method on page 13-14 of the report gives the predicted value and the confidence interval for $\mathrm{E}(Y_i)$ under certain combinations of treatment factors and when the levels of blocking factors are known with

```
predict(lmfit,...,interval='confidence',level=.95)
```

and gives the predicted value and the confidence interval for an observed $Y_1$ under certain combinations of treatment factors and when the levels of blocking factors are known with

```
predict(lmfit,...,interval='prediction',level=.95).
```

To examine the difference between high and low temperatures, you are interested in $\mathrm{E}(Y_1) - \mathrm{E}(Y_2)$. When the levels of blocking factors are the same, this difference is

$$D = \mathrm{E}(Y_1) - \mathrm{E}(Y_2) = \beta_2 + \beta_{12} + \beta_{23}.$$

Based on the R output on page 12, the estimates for $\beta_2$, $\beta_{12}$, and $\beta_{23}$ are 0.30852, -0.14291, and

-0.14006. Hence, the estimated value for the difference D is:

$$\hat{D} = \hat{\beta}_2 + \hat{\beta}_{12} + \hat{\beta}_{23} = 0.30852 - 0.14291 - 0.14006 = 0.0255.$$

Further, the variance of D is

$$\begin{aligned} Var(D) &= Var(\beta_2 + \beta_{12} + \beta_{23}) \\ &= Var(\beta_2) + Var(\beta_{12}) + Var(\beta_{23}) + 2Cov(\beta_2, \beta_{13}) + 2Cov(\beta_2, \beta_{23}) + 2Cov(\beta_{13}, \beta_{23}). \end{aligned}$$

The variance-covariance matrix of all the parameter estimates in the linear model can be obtained using the R code

```
> covmat=vcov(lmfit)
```

where `lmfit` is the R object storing the fitted linear model mentioned in the report. Then the estimate of the variance of D can be obtained using:

```
> varD=covmat['temperature60','temperature60']+covmat['NaOCl5%:temperature60',
'NaOCl5%:temperature60']+covmat['temperature60:experimental.time10m',
'temperature60:experimental.time10m']+2*covmat['temperature60',
'NaOCl5%:temperature60']+2*covmat['temperature60',
'temperature60:experimental.time10m']+2*covmat['temperature60:experimental.time10m',
'NaOCl5%:temperature60']
> print(varD)
[1] 0.0009805479
```

Therefore, to test whether $D$ is 0, the t-test statistic is

$$T = \frac{\hat{D}}{\sqrt{\widehat{Var}(D)}} = \frac{0.0255}{\sqrt{0.0009805479}} = 0.8143.$$

From the R output on page 12 of the report, we know that the degrees of freedom for the residuals is 23.

The hypotheses of the two-sided test are:

**Null hypothesis** $H_0$**:** $D = 0$;

**Alternative hypothesis** $H_1$**:** $D \neq 0$.

The p-value for testing the significance of $D$ is

```
> 2*(1-pt(0.8143,df=23)).
[1] 0.4238244
```

Since the p-value is much greater than 0.05, we cannot reject the hypothesis that $D$ is 0.

If you have prior knowledge that high temperature may be more effective in killing the bacteria than low temperature, one-sided hypothesis test is more relevant. The hypotheses are

**Null hypothesis** $H_0$: $D \leq 0$;

**Alternative hypothesis** $H_1$: $D > 0$.

The p-value for testing the significance of $D$ under one-tailed setting is

```
> 1-pt(0.8143,df=23).
[1] 0.2119122
```

Again the p-value is much greater than 0.05, we cannot reject the null hypothesis. Hence, our conclusion is that there is no significant difference in the response between high and low temperatures when the concentration of NaOCl is 5.25% and the experiment time is 10 minutes.

Tests under other combination of treatment factors can be done following the same procedure.

The results for testing whether the expected percentage of the bacteria killed given high temperature is equal to that given low temperature are summarized in Table 1. The results show that when the concentration is 5.25% and the experiment time is 10 minutes, the effect of temperature is not significant. Otherwise, high temperature is more effective in killing bacteria than low temperature.

| NaOCl | Experiment time | $D$ | Estimate of $D$ | S.D. of $D$ | t statistic | Two-sided p-value | Significance at 5% level |
|---|---|---|---|---|---|---|---|
| 5.25% | 10 | $\beta_2 + \beta_{12} + \beta_{21}$ | 0.0255 | 0.0313137 | 0.8143 | 0.424 | No |
| 5.25% | 3 | $\beta_2 + \beta_{12}$ | 0.16561 | 0.0313137 | 5.2887 | 2.286e-05 | Yes |
| 2% | 10 | $\beta_2 + \beta_{21}$ | 0.16846 | 0.0313137 | 5.3798 | 1.827e-05 | Yes |
| 2% | 3 | $\beta_2$ | 0.30852 | 0.0313137 | 9.8526 | 1.061e-09 | Yes |

Table 1: The results for testing whether the expected percentage of the bacterial given high temperature is equal to that given low temperature.

The results for testing whether the expected percentage of the bacteria killed given high concentration of NaOCl is equal to that given low concentration of NaOCl are summarized in Table 2. The results show that when the temperature is 60 degrees and the experiment time is 10 minutes, the effect of concentration is not significant. Otherwise, high concentration is more effective in killing bacteria than low concentration.

| Temperature | Experiment time | $D$ | Estimate of $D$ | S.D. of $D$ | t statistic | Two-sided p-value | Significance at 5% level |
|---|---|---|---|---|---|---|---|
| 60 | 10 | $\beta_1 + \beta_{12} + \beta_{13}$ | -0.05548 | 0.0313137 | -1.7717 | 0.0837 | No |
| 60 | 3 | $\beta_1 + \beta_{12}$ | 0.11145 | 0.0313137 | 3.5591 | 0.00167 | Yes |
| 20 | 10 | $\beta_1 + \beta_{13}$ | 0.08743 | 0.0313137 | 2.7921 | 0.0104 | Yes |
| 20 | 3 | $\beta_1$ | 0.25436 | 0.0313137 | 8.1230 | 3.298e-08 | Yes |

Table 2: The results for testing whether the expected percentage of the bacterial given high concentration of NaOCl is equal to that given low concentration of NaOCl.

The results for testing whether the expected percentage of the bacteria killed given long experiment time is equal to that given short experiment time are summarized in Table 3. The results show that when the concentration of NaOCl is 5.25% and the temperature is 60 degrees, short experiment time is more effective in killing bacteria. When the concentration of NaOCl is 5.25% and the temperature is 20 degrees, the effect of experiment time is not significant. Otherwise, long experiment time is more effective in killing bacteria than short experiment time.

, high concentration is more effective in killing bacteria than low concentration.

| NaOCl | Temperature | $D$ | Estimate of $D$ | S.D. of $D$ | t statistic | Two-sided p-value | Significance at 5% level |
|---|---|---|---|---|---|---|---|
| 5.25% | 60 | $\beta_1 + \beta_{13} + \beta_{23}$ | -0.08312 | 0.0313137 | -2.6544 | 0.0142 | Yes |
| 5.25% | 20 | $\beta_1 + \beta_{13}$ | 0.05694 | 0.0313137 | 1.8184 | 0.0821 | No |
| 2% | 60 | $\beta_1 + \beta_{23}$ | 0.08381 | 0.0313137 | 2.6765 | 0.0135 | Yes |
| 2% | 20 | $\beta_1$ | 0.22387 | 0.0313137 | 7.1493 | 2.719e-07 | Yes |

Table 3: The results for testing whether the expected percentage of the bacterial given long experiment time is equal to that given short experiment time.

**Appendix B – Mean Dead Bacteria (%) of Every Experimental Group using 2 % NaOCl**

| | NaOCl concentration | Temperature (°C) | Exposure time (min) | *E. faecalis* strain | Biofilm age | Dead Bacteria (%) |
|---|---|---|---|---|---|---|
| 1 | 2 % | 20 | 3’ | Gel 31 | 3d | 30.24 % |
| 2 | 2 % | 20 | 3’ | VP3-181 | 3d | 30.06 % |
| 3 | 2 % | 20 | 10’ | Gel 31 | 3d | 59.10 % |
| 4 | 2 % | 20 | 10’ | VP3-181 | 3d | 60.17 % |
| 5 | 2 % | 60 | 3’ | Gel 31 | 3d | 63.56 % |
| 6 | 2 % | 60 | 3’ | VP3-181 | 3d | 72.65 % |
| 7 | 2 % | 60 | 10’ | Gel 31 | 3d | 63.42 % |
| 8 | 2 % | 60 | 10’ | VP3-181 | 3d | 71.46 % |
| 9 | 2 % | 20 | 3’ | Gel 31 | 3w | 29.74 % |
| 10 | 2 % | 20 | 3’ | VP3-181 | 3w | 29.08 % |
| 11 | 2 % | 20 | 10’ | Gel 31 | 3w | 60.97 % |
| 12 | 2 % | 20 | 10’ | VP3-181 | 3w | 57.23 % |
| 13 | 2 % | 60 | 3’ | Gel 31 | 3w | 65.49 % |
| 14 | 2 % | 60 | 3’ | VP3-181 | 3w | 69.64 % |
| 15 | 2 % | 60 | 10’ | Gel 31 | 3w | 66.00 % |
| 16 | 2 % | 60 | 10’ | VP3-181 | 3w | 75.17 % |

**Appendix C – Mean Dead Bacteria (%) of Every Experimental Group Using**

| | NaOCl concentration | Temperature (°C) | Exposure time (min) | *E. faecalis* strain | Biofilm age | Dead Bacteria (%) |
|---|---|---|---|---|---|---|
| 1 | 5.25 % | 20 | 3’ | Gel 31 | 3d | 61.06 % |
| 2 | 5.25 % | 20 | 3’ | VP3-181 | 3d | 66.89 % |
| 3 | 5.25 % | 20 | 10’ | Gel 31 | 3d | 60.36 % |
| 4 | 5.25 % | 20 | 10’ | VP3-181 | 3d | 64.35 % |
| 5 | 5.25 % | 60 | 3’ | Gel 31 | 3d | 66.28 % |
| 6 | 5.25 % | 60 | 3’ | VP3-181 | 3d | 73.26 % |
| 7 | 5.25 % | 60 | 10’ | Gel 31 | 3d | 65.54 % |
| 8 | 5.25 % | 60 | 10’ | VP3-181 | 3d | 74.03 % |
| 9 | 5.25 % | 20 | 3’ | Gel 31 | 3w | 60.47 % |
| 10 | 5.25 % | 20 | 3’ | VP3-181 | 3w | 61.25% |
| 11 | 5.25 % | 20 | 10’ | Gel 31 | 3w | 61.24 % |
| 12 | 5.25 % | 20 | 10’ | VP3-181 | 3w | 57.70 % |
| 13 | 5.25 % | 60 | 3’ | Gel 31 | 3w | 72.79 % |
| 14 | 5.25 % | 60 | 3’ | VP3-181 | 3w | 74.78 % |
| 15 | 5.25 % | 60 | 10’ | Gel 31 | 3w | 70.41 % |
| | 5.25 % | 60 | 10’ | VP3-181 | 3w | 72.69 % |

**5.25 % NaOCl**