Efficacy of Sodium Hypochlorite and Er,Cr:YSGG Laser Energised Irrigation Against an *Enterococcus faecalis* Biofilm

A report submitted to the University of Adelaide
in partial fulfilment of the requirements of the
Degree of Doctor of Clinical Dentistry
(Endodontics)

September 2012

Dr Jonathan Christo
BDS (Adel), BScDent (Hons) (Adel)

THE UNIVERSITY
OF ADELAIDE
AUSTRALIA

Table of Contents

Table of Figures

Table of Tables

Abstract

Aim:

Laser energised irrigation (LEI) may enhance the chemo-mechanical disinfection of the root canal system. Sodium hypochlorite (NaOCl) is the most widely used endodontic irrigant, however it is cytotoxic. LEI at high laser power settings may extrude the irrigant. In order to minimise iatrogenic injury to the patient, there is the need to minimise the concentration of sodium hypochlorite and/or laser output power whilst achieving maximum antimicrobial efficacy. The aim of this study was two-fold. Firstly, to determine an appropriate laser output power for LEI. Secondly, to establish the efficacy of various concentrations of sodium hypochlorite, with and without Er,Cr:YSGG laser energy, in extracted teeth with an *Enterococcus faecalis* biofilm.

Methodology:

One hundred and seventy six decoronated single rooted extracted human teeth (preliminary studies (Studies 1 & 2) n = 71, Study 3 n = 96) were prepared to a size 40, 0.06 taper 1 mm beyond the apex. Tooth roots were mounted within a flow cell, which was sterilised before pumping a nutrient media through the root canals. The flow cell was inoculated with *E. faecalis* (ATCC 700802) and cultivated for 4 weeks. The root ends were then sealed prior to treatment.

Preliminary studies: A range of irrigants (saline, 0.5% NaOCl, 1% NaOCl, 4% NaOCl) and laser output powers (0.1W, 0.25W, 0.5W, 1W) were compared. Following treatment, teeth were crushed and viable bacteria were quantitated by serial dilution and plating (n = 54). Scanning electron microscope images were also used to qualitatively compare groups (n = 17).

In a larger study, (Study 3), ninety six teeth were randomised to one of six groups: control (saline irrigation); 1% NaOCl standard irrigation (SI); 4% NaOCl SI; 0.5% NaOCl with laser energised irrigation (LEI); 1% NaOCl with LEI; 4% NaOCl with LEI. Following treatment, teeth were crushed and viable bacteria were quantitated by serial dilution and plating. The number of colony forming units (cfu) were compared between groups using a Kruskal-Wallis test and analysis of variance with post-hoc Wilcoxon tests. A P value of < 0.05 was considered statistically significant.

Results:

Preliminary studies: A laser output power of 0.5 W was determined to be the most appropriate power setting.

Study 3: Post-hoc analysis showed a significant difference between the control group and the 5 other treatment groups ($P < 0.001$). Significant differences were shown between 1% NaOCl (SI) and 4% NaOCl (SI) ($P = 0.036$), 1% NaOCl (SI) and 1% NaOCl LEI ($P = 0.045$) and between 1% NaOCl (SI) and 4% NaOCl LEI ($P = 0.11$). Statistical differences were not shown between the other groups.

Conclusion:

The study concluded that standard irrigation with 4% NaOCl was more effective than standard irrigation with 1% NaOCl. However lower concentrations (0.5% and 1% NaOCl) when energised with an Er,Cr:YSGG laser (at 0.5 W) were as effective as standard irrigation with 4% NaOCl.

Declaration

I, Jonathan Christo, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Declared by: Date:

Dr Jonathan Christo

Acknowledgements

There are probably very few people who complete a thesis without feeling indebted to those who have invaluably helped them.

I begin with Associate Professor Peter Cathro. Thank you for providing a positive and encouraging learning environment that set me at ease throughout the DClinDent program. Thank you for promoting evidence and analysis over dogma.

Thank you to my research supervisor, Dr Peter Zilm, for your invaluable and intimate knowledge of microbiology, and your expertise and assistance in scientific methodology and writing. Without your input, this project would not have been possible.

Thank you to Professor Geoffrey Heithersay. I have felt privileged to work under you. You have a wealth of knowledge I only aspire to. Thank you for generously sharing it.

Thank you to the staff at Adelaide Microscopy Centre, and in particular Ms Lynn Waterhouse. Thank you to Thomas Sullivan, Discipline of Public Health at the University of Adelaide, for providing the statistical analysis.

Thank you to my wife Kim. I cannot thank you enough for your love, support and patience. Thank you for reinforcing that there's a life "beyond the apex". To my beautiful daughter Annabel, I can't wait to have more time for Play School and Play-Doh.

I would like to thank my fellow DClinDent candidates Drs Aaron Seet, Mark Stenhouse and Jonathan Race for their support, direction, and friendship. I look forward to shared careers and continued lively debates.

Finally I would like to acknowledge the financial assistance from the Australian Dental Research Foundation and the Australian Society of Endodontology.

1.1 Introduction

Apical periodontitis is a remarkably widespread disease, and its magnitude is often not fully appreciated (Figdor 2002). Apical periodontitis is an inflammatory process in the peri-radicular tissues, primarily caused by bacteria within the root canal system. Bacterial invasion of the root canal system can be symptomatic or symptom free. Discomfort of varying degrees in symptomatic cases is not the only factor motivating intervention. The potential of an infection spreading along fascial planes, compromising airways, and septicaemia are examples of post root canal system infection sequella.

A fundamental concept underpinning endodontic therapy is the elimination of the causative agents, thereby providing an environment conducive to healing. Provided that bacterial numbers can be eliminated or reduced to levels undetected by paper point sampling and culture techniques, high success rates of treatment are achieved (Sjögren *et al.* 1997).

1.2 Microbiological Basis of Endodontics

Although an association between micro-organisms and apical periodontitis dates back to 1894 (Miller 1894), bacteria were not definitively established as the causative agent in apical periodontitis until the mid to late 20th century (Kakehashi *et al.* 1965; Sundqvist 1976b; Möller *et al.* 1981).

Kakehashi *et al.* (1965) conclusively established bacteria as the causal agent of pulp necrosis. The pulps of normal and germ-free rats were surgically exposed. The germ-free rats were maintained under sterile conditions, and despite the persistent trauma of food impaction into the exposed pulps, pulps remained alive and healing occurred with formation of a dentine bridge. Pulp exposures in normal (control) rats all led to pulp necrosis.

Whilst the role of bacteria in pulp necrosis was established, the role of bacteria in apical periodontitis could not be extrapolated from Kakehashi *et al.* (1965). Despite periapical chronic inflammatory tissue and abscess formation only occurring in normal rats and not in the germ-free rats, the causative agent was not established. Rather than microorganisms, the breakdown products of the necrotic pulp may have caused the periapical inflammation.

The causative agent of apical periodontitis was established by Sundqvist (1976) and Möller *et al.* (1981). Sundqvist (1976) examined 32 intact traumatised teeth that had subsequently

become necrotic or pulpless. Strict inclusion criteria and meticulous microbial sampling techniques were utilised. Bacteria were cultured from 18 of 19 teeth that had apical periodontitis. In contrast, bacteria were unable to be cultured in the 13 teeth without apical periodontitis. Although, an association between bacteria and apical periodontitis was established, a temporal relationship was not.

The temporal relationship between bacteria and apical periodontitis was established by Möller *et al.* (1981). The pulps of 78 monkey incisors without apical periodontitis were devitalized under aseptic conditions. Fifty-two teeth were infected with indigenous bacteria from the Monkey's saliva, whilst 26 were kept sterile. Six months after the devitalizing procedure, the 26 non-contaminated teeth were without clinical and radiographic signs of apical periodontitis and bacteria could not be cultured from the root canal systems. Therefore necrotic but uninfected pulp tissue did not cause apical periodontitis. In contrast, 47 of the 52 teeth that had been contaminated developed radiographic evidence of apical periodontitis. Therefore, contamination with infected saliva was responsible for the inflammatory changes in the apical region, rather than necrotic uninfected pulp tissue. Although this established bacteria as the causative agent for apical periodontitis, the method did not separate or account for the potential role of the non-microbial factors in saliva.

1.2.1 Bacterial Identification

Central to our understanding of endodontic infections are the methods used to identify bacteria. The identification of bacteria from the root canal system is a two-part process. First, a representative microbial sample must be collected from the root canal system. Following this, the bacteria must be identified by either culture or molecular techniques.

1.2.1.1 Microbial Sampling

Microbial sampling of the root canal system generally involves the absorption of the content of the canal with paper points. The aseptic techniques described by Möller (1966) have standardised the procedure and minimised contamination from the adjacent tissues and the environment. This has reduced the occurrence of false positive samples. However, given the complex anatomy of the root canal system (Vertucci 1984) and the presence of bacteria within dentinal tubules, paper point sampling is unable to provide a complete microbial sample of the root canal system. There is a high possibility of false negative results. Akpata (1976) has highlighted the limitations of paper point sampling, showing that negative cultures

produced from this technique are not an indication that the root canal system is sterile. Negative samples indicate either the presence of no micro-organisms, the presence of micro-organisms in areas inaccessible to the sampling techniques, or the inability to culture the recovered bacteria.

Alternative sampling techniques involve the collection of dentine shavings (Berber *et al.* 2006) or crushed tooth samples (Akpata 1976; Baker *et al.* 2004; Miller & Baumgartner 2010; Seet 2010). Whilst dentine shavings can be used to a limited extent *in vivo*, crushing teeth cannot. However in *in vitro* environments they are superior to paper point sampling.

1.2.1.2 Identification of Bacteria

Provided the microbial sampling technique employed has sampled viable bacteria, the sample must be maintained in an appropriate environment to prevent cell death prior to identification. Culture techniques have been widely utilised to grow and identify bacteria in microbiological investigations, and until the late 20th century it was the only method available. In order to successfully culture bacteria, the culture medium must be non-toxic and provide the required nutrients (Wade 2002). If these conditions are not met, the bacteria will not survive and a false negative result will be recorded. It is estimated that approximately only half of the oral microflora can be cultivated (Wilson *et al.* 1997). Other reasons for the inability to culture certain bacteria *in vitro* include: bacteria within the sample may produce substances inhibitory to other organisms within the sample; disruption of the bacterial cytokine network; or the separation of bacterial species that require essential synergistic effects (Wade 2002).

The sensitivity of culture techniques is highlighted by the variation in reported incidence of anaerobic bacteria. Prior to the mid 1960's, the incidence of cultured anaerobic bacteria in cases with apical periodontitis was between 24% and 31% (Brown Jr & Rudolph Jr 1957; Macdonald *et al.* 1957). Utilising the protocols established by Möller (1966), combined with the advancements in anaerobic techniques described by Moore (1966) and anaerobic glove boxes (Aranki & Freter 1972), an increase in the incidence of anaerobic bacteria isolated from endodontic samples was seen. Post-1970, 54% (Kantz & Henry 1974), 75% (Wittgow Jr & Sabiston Jr 1975) and as high as 90% (Sundqvist 1976) of species isolated from root canal systems with apical periodontitis were anaerobes. Although the methods of the previously mentioned studies, such as inclusion criterea, do vary, the striking difference in occurance of anaerobes is explained by advancements in sampling and culturing techniques.

Provided the bacteria are successfully sampled and cultured, the next challenge is identification of the bacteria to the genera and species level. Culture techniques rely on phenotypic characteristics that depend on stress, growth conditions and microbial interactions. As a result, identification can be less predictable than desired. Over the last decade there has been a rise in molecular techniques, involving polymerase chain reactions (PCR), in the study of endodontic microbiology. Polymerase chain reactions utilises a mathematical modelling technique to assess the similarity of DNA sequences of common ancestry or protein genes in a range of organisms to a known database (Wade 2002). The most widely used of these genes is the small subunit 16S ribosomal RNA gene that provides specific signature sequences which can be used for bacterial identification. Unlike culture techniques, PCR is not based on phenotypic or metabolic characteristics for bacterial identification, but utilises unchanging genetic sequences. PCR is not only more precise in bacterial identification, but due to an amplification procedure, it allows for the identification of bacteria present in relatively small numbers.

Polymerase chain reaction techniques also have their limitations. Culture techniques will detect viable bacteria, and the preparation of serial dilutions allows quantification. In contrast, the detection of bacterial DNA by PCR does not necessarily mean the bacteria were viable at the time of sampling. For example, *Enterococcus faecalis* DNA can persist for one year following cell death (Young *et al.* 2007). Therefore, the bacteria identified in the root canal system from PCR techniques may have been transient, unable to survive in the environment and not contributory to the pathogenesis of apical periodontitis. Rather than detecting DNA, reverse transcriptase PCR can identify the presence of mRNA, and therefore this PCR method will enumerate viable bacteria.

A strength of PCR is the identification of bacteria in low numbers, although this is also a potential weakness if the bacterial numbers are not quantitated. Bacteria present in very low numbers may have little pathogenic effect and may be mistaken as significant if they are not quantified. Not all PCR techniques provide quantitative data, however real time PCR is a molecular technique that can provide quantification. Real time PCR enables the detection of specific DNA sequences and quantification in either absolute numbers of copies or in relative amounts.

There are advantages and limitations of both culture and molecular techniques. Neither technique is superior, and both are required to provide a comprehensive understanding of the

root canal milieu (Sedgley *et al.* 2006b). Molecular and culture techniques have the common problem that they rely on recovery of a representative sample from the root canal system.

1.2.2 Primary Infections

1.2.2.1 Culture Based Studies

Culture studies have generally isolated up to ten species per root canal with apical periodontitis (Sundqvist 1976; Byström & Sundqvist 1981). The root canal milieu is dominated by obligate anaerobes, but facultative anaerobes can also be isolated. The bacteria within the root canal system are dynamic, with a proportional increase in obligate anaerobes with time. In an investigation in monkeys, Fabricius *et al.* (1982b) exposed the root canal system to the oral environment for seven days before sealing the teeth and taking samples at various time points. At seven days, the ratio of obligate anaerobes to aerobes was approximately equal (1.6:1), however at 180 days the ratio of obligate anaerobes to aerobes was 6.5:1 and at 1060 days, 11.3:1.

Given the environment within the root canal system, it is not surprising that anaerobic bacteria dominate. The highest occurrence of obligatory anaerobes has been reported by Sundqvist (1976). Only intact traumatised teeth, without caries or restorations, were included in this study. Whilst this is not directly applicable to teeth with caries or failing restorations, 88 different species were identified in pulpless teeth with apical periodontitis. Generally there were 6-10 species per canal, and 90% of the species were obligate anaerobes.

The most commonly cultured obligate anaerobes are *Fusobacterium*, *Prevotella* and *Peptostreptococcus* spp, whilst the most common facultative anaerobes are *Streptococcus spp*. (Sundqvist 1976; Byström & Sundqvist 1981; Baumgartner & Falkler Jr 1991; Gomes *et al.* 2004). Symptomatic cases have been associated with *Prevotella* and *Porphyromonas* (Sundqvist 1976; Sundqvist *et al.* 1979; Jacinto *et al.* 2003). Sundqvist *et al.* (1979) showed that *Bacteroides* species (now known as *Prevotella or Porphyromonas*) were required to produce non-resolving purulent infections when injected subcutaneously in guinea pigs.

1.2.2.2 Molecular Based Studies

Molecular studies have broadened our understanding of endodontic infections within the untreated root canal system. Molecular techniques have identified 400 bacterial species from

primary infected root canal systems and 45% of these were previously unidentified by culture techniques (Siqueira & Rôças 2010).

Although molecular studies have generally confirmed the findings from culture based studies, the prevalence of several species in endodontic infections has been revised based on PCR identifications. Spirochetes were seldom identified in culture studies however they have been more commonly identified with molecular techniques. The most predominant spirochete in infected root canal systems is *Treponema denticola,* it has been reported between 13% and 78% of cases (Fouad *et al.* 2002; Rôças *et al.* 2003). Similarly, although *E. faecalis* can be isolated in culture studies assessing primary infections (Baumgartner & Falkler Jr 1991), it is generally not cultured (Sundqvist 1976; Byström & Sundqvist 1981). Molecular studies have shown a higher prevalence of *E. faecalis* in primary infections compared to culture techniques. Sedgley *et al.* (2006b) compared culture and real-time quantitative PCR to detect and quantify the presence of *E. faecalis* in root canal samples. *E. faecalis* was cultured in 12.5% of infected root canal systems requiring primary endodontic treatment compared to detection by PCR in 67.5% of cases. Other studies have detected *E. faecalis* with PCR techniques in 7.5% (Siqueira Jr *et al.* 2002) and 14% (Fouad *et al.* 2002) of infected cases requiring primary endodontic treatment.

1.2.3 Infections in Root Filled Teeth

The primary cause of root canal treatment failure is generally believed to be persistent intracanal infection (Nair *et al.* 1990) or microorganism invading the root canal system via coronal leakage. Due to the methodology of prior studies, few surgical specimens had shown intra-radicular bacteria. Nair *et al.* (1990) removed root apices and periapical lesions *en bloc* and examined them using transmission electron microscopy (TEM). Of the nine cases examined, four had evidence of intra-radicular bacteria and two contained yeast in the apical root canal system. Furthermore it was noted that accessory canals, apical deltas and ramifications were densely packed with bacteria. The method employed in this research is not without flaws. The microscopic technique does not establish cellular viability but rather the topography of bacteria. Although it is unusual for dead cells to maintain normal morphology, if biofilm architecture remains following cell death bacteria may mistakenly be interpreted as viable and contributing to the disease process. On the other hand, given the complex anatomy of the root canal system, visualisation methods can underestimate the presence of bacteria. In addition to visualisation studies, both culture and molecular methods have been used to investigate the role of bacteria in root-filled teeth with apical periodontitis.

Although bacteria are primarily responsible for disease persistence in previously root canal treated teeth, there are other potential reasons. These include radicular cysts (Nair *et al.* 1996), extra-radicular infections by *Actinomyces israelii* and *Propionibacterium propionicum* (Nair & Schroeder 1984) and foreign body reactions (Nair *et al.* 1990). Alternatively healing by scar formation (Nair *et al.* 1990; Nair *et al.* 1999) can be misinterpreted as a persistent infection when viewed on radiographs.

1.2.3.1 Culture Based Studies

The quality of the initial root canal treatment appears to affect the type of microbiota associated with infections in root-filled teeth. Although providing limited information, a radiographic assessment of a root canal filling is the only method to retrospectively assess the quality of a previous root canal treatment. There is a difference in microbial flora associated with teeth judged to have had a poor quality root canal treatment compared to well-treated teeth that present with apical periodontitis. Of the 54 cases included in the study by Sundqvist *et al.* (1998) only one case was classified as having radiographically poor endodontic treatment. This case contained microbial flora similar to that of an untreated necrotic pulp. Similarly, Hancock *et al.* (2001) found that canals not filled adequately and therefore probably not debrided adequately rendered more bacteria, possibly as the environment was similar to that of an untreated infected canal. This finding has been confirmed in a study that found an association of polymicrobial infections in poorly root filled teeth compared with well filled canals (Pinheiro *et al.* 2003). It is not surprising that the flora after poor quality root canal therapy is similar to the untreated canal, particularly if there is a defective coronal restoration allowing bacteria to enter the tooth and an influx of nutrients and potentially new micro-organisms (Sundqvist & Figdor 2003).

The limitations of microbial sampling and culture techniques have previously been discussed and it is likely that culture studies have understated the presence of bacteria in root-filled teeth with apical periodontitis. Sundqvist *et al.* (1998) cultured bacteria in 24 of 54 asymptomatic previously root canal treated teeth with radiographic evidence of apical periodontitis. In comparable studies, Molander *et al.* (1998) cultured bacteria in 68 of 100 cases and Hancock *et al.* (2001) isolated bacteria in 34 of 54 canals. In addition, Molander *et al.* (1998) highlighted the effect of clinical methods on the culturing of bacteria. Bacteria were cultured in only 48% of cases in which chloroform was used to aid the removal of gutta purcha compared to 78% when chloroform was not used.

Gram positive anaerobes are dominant in root-filled teeth with apical periodontitis (Molander *et al.* 1998; Sundqvist *et al.* 1998; Hancock *et al.* 2001). Sundqvist *et al.* (1998) and Molander *et al.* (1998) found that 87% and 69% of bacteria respectively were Gram positive. *E. faecalis* was the species most commonly identified with culture techniques. Sundqvist *et al.* (1998) cultured *E. faecalis* in nine cases (of the 54) and when it was isolated it was the only microorganism present. Similarly, Molander *et al.* (1998) cultured *E. faecalis* in 32 teeth (of 100 teeth) and Hancock *et al.* (2001) reported *E. faecalis* was isolated from 30% of canals that had a positive culture (34 teeth).

In comparison to infected and untreated root canal systems, infections in root-filled teeth as assessed with culture techniques have found a higher incidence of *E. faecalis.* In addition rather than polymicrobial infections, the majority (85%) of infections in root-filled teeth, had only one or two species detected (Molander *et al.* 1998).

Other species commonly isolated in root-filled teeth with apical periodontitis include Gram positive facultative anaerobic *Streptococcus*, *Lactobacillus* and A*ctinomyces* spp; Gram negative, *Fusobacterium* and *Prevotella* spp; Gram positive anaerobic *Peptostreptococcus*; Fungi and *Candida albicans* (Molander *et al.* 1998; Sundqvist *et al.* 1998; Hancock *et al.* 2001).

There does not appear to be ethnic differences between the consortia of bacteria isolated from root-filled teeth with apical periodontitis. Investigations conducted in North America (Hancock *et al.* 2001) and Scandinavia (Molander *et al.* 1998; Sundqvist *et al.* 1998) produced comparable results.

1.2.3.2 Molecular Based Studies

Sedgley *et al.* (2006b) directly compared culture and PCR techniques in previously root canal treated teeth with apical periodontitis. Bacteria were cultured in 27 of the 48 canals whilst PCR indicated the presence of bacteria DNA in all canals (Sedgley *et al.* 2006b). Similarly Siqueria and Rôças (2004) detected bacteria DNA within all root filled teeth with periapical lesions.

Whilst Siqueria and Rôças (2004) isolated *E. faecalis* in 77% of the 22 canals with failed endodontic treatment, Sedgley *et al.* (2006b) using real-time quantitative PCR detected the

presence of *E. faecalis* in 89.6% of retreatment cases versus 67.5% of primary root canal system samples.

In this respect, molecular techniques have supported culture studies, identifying *E. faecalis* as the most commonly isolated species. Although, compared to culture techniques, molecular studies have identified bacteria more often in cases requiring root canal retreatment. In addition, molecular studies support the presence of mixed infections and question if the dominance of *E. faecalis* in culture studies may be due to limitations with culturing techniques (Siqueira & Rôças 2004). Other species that are commonly isolated in root-filled teeth with apical periodontitis include *Pseudoramibacter alactolyticus*, *Propionibacterium propionicum, Propionibacterium acnes, Fusobacterium nucleatum* and *Streptococcus* spp (Siqueira & Rôças 2004; Rôças & Siqueira 2012).

1.3 Microbial Interactions

Communities of bacteria are more likely to occur in nature than individual species. Sundqvist (1992) showed that some species will tend to pair with others and this finding has been supported by Peters *et al.* (2002a). A synergistic relationship is likely to be driven by nutrient demands. On the other hand, bacteria with similar environmental requirements may co-exist without interaction.

Certain bacteria require the presence of other species and are unable to survive axenically. The metabolic by-product of one species may be a nutrient source for another. Fabricius *et al.* (1982a) provided an example of microbial interaction within the root canal system of monkeys. Despite *Bacteroides oralis* (now classified as *Prevotella oralis*) becoming the dominant species in an experimental mixed infection, it could not survive as a mono-species infection (Fabricius *et al.* 1982a). On the other hand, bacteria such as *E. faecalis* have the ability to survive within the root canal system axenically (Fabricius *et al.* 1982a). *E. faecalis* does not rely on synergistic relationships with other bacteria for survival within the root canal system.

The concept of microbial interactions in the root canal system pre-dated the description of biofilms in endodontic infections. Given the current understanding and importance of biofilms in endodontics, it is likely that biofilms are central to microbial interactions.

1.4 Biofilms

A biofilm is a microbial community characterised by cells that are attached to a substratum, encased in an extracellular polymeric matrix, and exhibit altered growth phenotypes (Donlan & Costerton 2002).

Biofilms form when planktonic bacteria in a liquid medium are deposited on a surface containing an organic conditioning film media (Chavez de Paz 2007). There is rather limited knowledge as to the formation of biofilms in endodontic infections. Svensäter & Bergenholtz (2004) in a review article described the dynamic process of biofilm formation within the root canal system, but noted that the attachment of bacteria to the root canal walls in progressing pulp breakdown is unknown. Following initial bacterial attachment, organisms co-adhere to the surface and multiply. Some cells will detach from the biofilm over time and may go on to re-attach and seed another community (Svensäter & Bergenholtz 2004).

The formation of biofilm provides a survival advantage for the bacteria and is an adaptive response to the environment. Bacteria functioning together in biofilms are able to degrade complex nutrient molecules that could not be efficiently degraded by an individual bacterium (Svensäter & Bergenholtz 2004). The breakdown of these nutrients requires a range of peptidases, proteases and glycosidases.

1.4.1 Biofilms in Endodontics

A landmark paper by Nair in 1987 drastically changed the way root canal infection was viewed. Nair (1987) described collections of bacteria in root canals, now known as biofilms. Dense aggregates or layers of single or multiple species adhering to dentinal walls and suspended in an amorphous extracellular matrix were observed (Nair 1987).

Whilst, Nair (1987) utilised light and transmission electron microscopy, the results were later confirmed with scanning electron microscope images of extracted pulpless, infected teeth with apical periodontitis (Molven *et al.* 1991).

Bacteria in a biofilm have been observed in lower molars with apical periodontitis, immediately following instrumentation, irrigation and obturation in a single visit. Surgical specimens examined with transmission electron microscopy revealed bacteria located in 14 of

16 canals (Nair *et al.* 2005). Despite treatment, biofilms were found in isthmuses and accessory canals of the root canal system (Nair *et al.* 2005).

Furthermore, Ricucci & Siqueira (2010) used light microscopy to examine extracted, grossly carious teeth with long established infections. Biofilms were observed in 74% of previously root treated teeth and 80% of non-root treated teeth. There was a large variation between species identified and the amount of extracellular matrix (Ricucci & Siqueira 2010).

Although biofilm morphology varies from case to case, it is well established that biofilms form within the root canal system. A detailed analysis of the formation of biofilms in endodontic infections has not been conducted.

1.4.2 Increased Resistance of Bacteria in Biofilms

Bacteria within biofilms have an inherent increase in resistance to anti-microbial agents compared to planktonic bacteria. It has been reported that this increase in resistance can be as high as a 1000 fold (Gilbert *et al.* 1997; Johnson *et al.* 2002). There are several potential features of biofilms as well as environmental adaptive features of bacteria within biofilms that can explain this increase in resistance.

1.4.2.1 Physical Barrier

The biofilm structure may act as a barrier that restricts the penetration of anti-microbial agents. Biofilm structures are multilayer, providing protection for bacteria residing in the lower layers. Additionally the polymeric matrix (glycocalyx, polysaccharides and glycoproteins) acts as a diffusion barrier, physically preventing access of the antimicrobials to the bacterial cell surface (Gilbert *et al.* 1997). Positively charged agents bind to negatively charged polymers in the biofilm matrix which retards penetration (Stewart & Costerton 2001). Additionally certain antibiotics can be deactivated in biofilms, again slowing penetration of the biofilm (Stewart & Costerton 2001).

1.4.2.2 Growth Rates

Biofilms give rise to diverse physical and chemical gradients which include the concentration of nutrients, metabolic end products, oxygen, growth factors and biocides (Svensäter & Bergenholtz 2004). Within the depths of a biofilm, growth rates will generally

be suppressed relative to planktonic cells grown in the same environment (Gilbert *et al.* 1997). Antimicrobial agents have been developed and optimised for activity against fast growing organisms (Svensäter & Bergenholtz 2004). Therefore, the slower growth of bacteria in a biofilm can provide protection from antimicrobial agents that target fast growing bacteria.

The slow dividing state of bacteria in a biofilm can be considered similar to planktonic cells in the stationary phase. *E. faecalis* cells in an exponential growth phase have been shown to be highly sensitive to calcium hydroxide and sodium hydroxide, however cells in the stationary phase are more resistant (Portenier *et al.* 2005). Furthermore, it has been shown *in vitro* that *E. faecalis* cells in a starvation phase have the ability to form biofilms that maybe more resistant to 5.25% NaOCl than those of stationary cells (Liu *et al.* 2010).

1.4.2.3 Distinct Phenotype

Physiological properties of bacteria in biofilms are different from those of the same bacterium in planktonic cultures (Svensäter & Bergenholtz 2004). A change in the rate of bacterial growth is an example of a phenotype change. Phenotypic properties of the biofilm bacteria can be optimised via gene expression that alter virulence, the ability to form biofilms, incorporation of extracellular DNA and the ability to cope with environmental stress (Cvitkovitch *et al.* 2003).

1.4.2.4 Quorum Sensing

Bacterial communication allows bacteria to coordinate the gene expression and therefore the behaviour of the entire community (Miller & Bassler 2001). This cell-cell communication occurs both within and between bacterial species. Quorum sensing is a mechanism of communication between bacteria that is mediated by diffusible molecules (autoinducers) that modify gene expression in neighbouring microorganisms in response to fluctuations in the cell population density (Svensäter & Bergenholtz 2004). A number of virulence factors may be induced or it may trigger a change in biofilm growth.

1.4.2.5 Genetic Transfer

Genetic transfer of plasmids via conjugation can also increase the virulence of individual bacteria and thus the community as a whole. Such plasmids may code for molecules that enhance antibiotic resistance and virulence traits. This may provide an effective defence

against host protective mechanisms and antimicrobial agents producing a highly resistant phenotypic state (Distel *et al.* 2002). The transfer of genetic material may occur between bacteria of the same or difference species. Transfer of plasmid carrying erythromycin resistance between *S. gordonii* and *E. faecalis* in an *ex vivo* tooth model has been shown (Sedgley *et al.* 2008).

1.5 *Enterococcus faecalis*

Enterococcus species are ubiquitous and can be isolated from a wide range of habitats including water, plants, fermented food, and the gastrointestinal tract of humans and animals (Franz *et al.* 1999). Although occurring in vast quantities in the human intestinal lumen, under most circumstances enterococci do not cause harm to the host (Stuart *et al.* 2006). In addition to endodontic infections, enterococci are opportunistic bacteria that are pathogenic in urinary tract infections, abdominal infections and endocarditis. There are 23 enterococcus species within the genus (Stuart *et al.* 2006), however *E. faecalis* accounts for close to 100% of cases in which enterococci are isolated in endodontic infections (Sirén *et al.* 1997; Sedgley *et al.* 2005b).

E. faecalis is a Gram positive, non spore forming facultative anaerobe. It is a particularly resilient bacteria able to grow in temperatures ranging from 10°C to 45°C, salinity of 6.5% and high and low pH environments (Portenier *et al.* 2003).

1.5.1 *E. faecalis* in the Oral Cavity

Given that *E. faecalis* is the species most commonly recovered from infected root-filled teeth, the most obvious origin is the oral cavity. The composition of the oral microflora varies between humans, between sites in the same oral cavity and within the same sites of the same individual at different time intervals (Socransky & Manganiello 1971). *E. faecalis* is rarely isolated in oral rinse samples provided from patients without a history of endodontic treatment (Sedgley *et al.* 2004b). In contrast, *E. faecalis* has been shown to be isolated more commonly in oral rinse samples provided from endodontic patients during treatment (Sedgley *et al.* 2004b) although the occurrence rates are still less than 17% (Sedgley *et al.* 2005c). In addition, quantitative analysis revealed that *E. faecalis* comprises proportionally less than 0.005% of the bacterial load within the oral cavity (Sedgley *et al.* 2005c).

The small representation of *E. faecalis* in the oral microbiota may reflect why *E. faecalis* is more commonly isolated when PCR techniques are employed and multiple sites are sampled rather than simple oral rinse methods. Sedgley *et al.* (2006a) detected *E. faecalis* in at least one tongue site, oral rinse or gingival sulcus sample in 68% of endodontic patients. The variation in reported occurrence rates of *E. faecalis* in the oral cavity maybe explained by the sensitivity of detection method employed or alternatively, *E. faecalis* maybe transient within the oral cavity.

Enterococci are frequently found in fermented food for raw consumption, such as cheese and meat (Franz *et al.* 2003). *E. faecium* is the main species found in meat and *E. faecalis* is the main species in cheese. Although enterococci are used as components of cheese starter cultures, they mostly occur as contaminants in fermented foods. It has been speculated that food-borne enterococci may act as a source of transient oral colonisation. Razavi *et al.* (2007) could not culture *E. faecalis* from oral rinse specimens from 50 dental students with good oral hygiene. After volunteers consumed cheese with viable *E. faecalis*, enterococci were recovered in decreasing numbers over 100 minutes. One week after the cheese was consumed *E. faecalis* could not be cultured from the volunteers (Razavi *et al.* 2007). This study demonstrated that *E. faecalis* introduced from a food source might only be transient within the oral cavity and not colonise. However, as microbial samples were collected via oral rinse samples and analysed by a culture technique it is possible that low levels of undetected *E. faecalis* still remained.

Although food-borne *E. faecalis* has been shown to be transient in the oral cavity (Razavi *et al.* 2007), it may also survive in supragingival plaque (Al-Ahmad *et al.* 2010). Al-Ahmad *et al.* (2010) established that following the consumption of cheese known to contain enterococci, *E. faecalis* was isolated after two hours and five days in a biofilm on enamel slabs worn on dental splints in six healthy volunteers. Although the time period was short and the sample size was small, the results indicate that food-borne enterococci can integrate into dental oral biofilm *in vivo*.

Although it is unlikely that *E. faecalis* is a commensal organism within the oral cavity, an environmental change may lead to an ecological niche developing that suits *E. faecalis*. Transient *E. faecalis* from sources such as food, may become established and provide a potential source for endodontic infections.

1.5.2 *E. faecalis* and Root-filled Teeth with Apical Periodontitis

Biological selection drives the type and course of infection in the root canal system (Sundqvist & Figdor 2003). The environment of the untreated root canal system and the treated root canal system are vastly different. In the coronal aspect of the untreated root canal system, exogenous nutrients may include fermentable carbohydrates. The principal nutrients in the untreated root canal system are endogenous proteins and glycoproteins (Sundqvist & Figdor 2003). Peptides and amino acids will initially be supplied from the degradation of the pulp tissue. In addition, bacterial induced periapical inflammation may lead to an influx of serum-like exudate.

During and after endodontic treatment there are variations in pH, as well as short and long term antibacterial effects of medicaments or sealants (Chavez de Paz 2007). Surviving bacteria must adapt to this environment. Some argue that the bacteria that survive root canal treatment are not the most robust organisms but the ones best able to adapt to the new environmental niche (Chavez de Paz 2007).

The nutrients available in root filled canals will depend on the quality of the initial treatment and the state of the coronal restoration. Compared to an untreated canal, obturated canals have a lower oxygen tension and a reduced supply of nutrients. There are several possible explanations for the disparity between the high frequency of *E. faecalis* recovered from filled root canals with apical periodontitis compared to the low proportion recovered from untreated canals (Sundqvist & Figdor 2003). Either *E. faecalis* survived the antimicrobial treatment of the initial endodontic treatment or had entered the canals after treatment.

It is possible that *E. faecalis* is present in untreated canals but in low numbers, and as a result paper point microbial sampling and culture techniques have underestimated its presence. In some cases, the change in environment caused by endodontic treatment may favour *E. faecalis,* aiding its growth to recoverable proportions. The higher incidence of *E. faecalis* found via molecular compared with culture techniques, gives plausibility to this theory, although it is unlikely to be the sole reason.

Alternatively, it is likely that *E. faecalis* enters the canal during the process of treatment, during or between treatment sessions (Sundqvist & Figdor 2003). Sirén *et al.* (1997) found a higher proportion of canals with *E. faecalis* that had been inadequately filled, or were treated

over 10 or more appointments. It is therefore hypothesised that *E. faecalis* can infect the canal due to poor isolation of the tooth during treatment, or via defective temporary & permanent restoration margins.

Irrespective of whether *E. faecalis* enters the root canal system prior to or during endodontic treatment, in order to establish or maintain apical periodontitis, it must survive chemo-mechanical preparation and obturation of the canal. *E. faecalis* in stationary or starvation phases have the ability to form biofilms (Liu *et al.* 2010), and like other species can invade the dentinal tubules (Love 2001). This can provide protection from both mechanical preparation and the antimicrobials used in endodontic treatment.

E. faecalis has been shown to grow as a biofilm in the presence of calcium hydroxide (Distel *et al.* 2002). It can survive a pH of 11.1 due to a functioning proton pump (Evans *et al.* 2002). *In vitro* studies using bovine dentine cylinder models have shown that *E. faecalis* can survive following a ten day dressing with calcium hydroxide (Ørstavik & Haapasalo 1990).

E. faecalis can withstand long periods of starvation and it has been shown to reside and remain viable within dentinal tubules of obturated canals for at least 12 months (Sedgley *et al.* 2005a). Cells within a starvation state have been shown to be capable of recovery upon addition of serum (Figdor *et al.* 2003). Presumably, if a nutrient source is to become available, *E. faecalis* residing within dentinal tubules could flourish and induce the inflammation associated with persistent periapical infections.

1.5.2.1 Virulence Factors

There is a high inter-individual variability of *E. faecalis* isolated from root canal systems and a prevalent genotype has not been identified (Zoletti *et al.* 2011). *E. faecalis* has multiple virulence factors that can be classified as secretion factors, adhesins and surface structures such as capsular polysaccharides (Sundqvist & Figdor 2003). Virulence factors of interest include those that promote adaptation and survival in different environments.

Secretion factors associated with *E. faecalis* include cytolysin and gelatinase. Cytolysin helps prevent phagocytosis and aids the establishment and proliferation of bacteria. The prevalence of the cytolysin gene is reported in between 16–60% of isolated *E. faecalis* strains (Kayaoglu & Ørstavik 2004). Gelatinase, regulated by quorum sensing, is a proteolytic enzyme with the capacity for cleaving insulin, casein, hemoglobin, gelatin and fibrin. The

production of gelatinase may aid the binding of bacteria to dentine (Hubble *et al.* 2003) and biofilm formation (Zoletti *et al.* 2011). Zoletti *et al.* (2011) isolated the *gel*E gene in all strains of *E. faecalis* recovered from obturated root canals. However, only half of the recovered strains hydrolysed gelatin using a phenotypic test (Zoletti *et al.* 2011). Similarly, Sedgley *et al.* (2005b) examined *E. faecalis* isolated from root canals and found 70% expressed gelatinase activity.

E. faecalis adhesins include aggregation substances, enterococcus surface protein (*Esp*) and collagen adhesin (Ace) (Sundqvist & Figdor 2003). *Esp* may be involved in colonisation and persistence of *E. faecalis* during infections. Zoletti *et al.* (2011) isolated the *Esp* gene in 40% of endodontic strains of *E. faecalis*, whilst Sedgley *et al.* (2005b) isolated the *Esp* gene in 61% of strains. Ace has been shown to aid the binding of *E. faecalis* to dentine (Hubble *et al.* 2003). Sedgley *et al.* (2005b) identified Ace in all endodontic strains of *E. faecalis* examined.

1.5.2.2 Viable but Non-culturable

Although controversial, some bacteria may enter a viable but non-culturable (VBNC) state and fail to grow on routine media on which they would normally grow and develop colonies, however they are still alive (Oliver 2010). Most investigators believe it to be a survival strategy adopted by bacteria exposed to environmental stress. The VBNC state for *E. faecalis* has some support (Heim *et al.* 2002) although this is not unanimous (Bogosian *et al.* 1998). The significance of a VBNC state for *E. faecalis* and its involvement in endodontic infections is unknown.

1.5.3 *E. faecalis* is not the Sole Bacteria in Root-filled Teeth with Apical Periodontitis

Apical periodontitis is a dynamic process involving an interaction between the host and living bacteria. In order to establish persistent apical periodontitis micro-organisms must survive the anti-microbial treatment, survive the root filling and have the capacity to inflame host tissues.

Koch's postulates have formed the basis for determining a causal relationship between certain microbial species and an infectious disease. Historically, much of the earlier research into dental caries, gingivitis and periodontitis was focused on correlating a single specific

organism with the disease to satisfy these strict criteria (Chavez de Paz 2007). This gave rise to a relative small number of organisms from the oral microflora being implicated.

The "ecological plaque hypothesis", proposed by Marsh (2003) explains changes that take place within dental plaque that lead to the development of caries or periodontal disease. The "ecological plaque hypothesis" has improved the understanding of caries and periodontal disease although some would argue that endodontic research has been slow to accommodate this concept. There is no disputing the higher frequency of *E. faecalis* in root-filled teeth with apical periodontitis compared with infected cases requiring primary endodontic treatment. Erroneously, many endodontic studies refer to endodontic pathogens as bacteria isolated from a symptom-associated root canal that grows *in vitro* in a specific media (Chavez de Paz 2007). As there is not any evidence to suggest that a particular species is not involved in the pathogenesis of apical periodontitis, all bacteria found in the root canal system may be considered potential endodontic pathogens (Sundqvist & Figdor 2003).

As previously discussed it has been shown *in vitro* that *E. faecalis* has the ability to reside in the root canal system as a single species without the support of other micro-organisms (Fabricius *et al.* 1982a) and mono-cultures of *E. faecalis* have commonly been identified in root-filled teeth with apical periodontitis using culture techniques (Molander *et al.* 1998; Sundqvist *et al.* 1998). Although this may reflect the limitations of either the sampling technique (Akpata 1976) or the culture technique in which other bacteria were not potentially identified. Molecular techniques have supported culture studies, identifying *E. faecalis* as the most commonly isolated species in root-filled teeth with apical periodontitis (Molander *et al.* 1998; Sundqvist *et al.* 1998; Hancock *et al.* 2001). In contrast to culture studies, molecular studies have generally found mixed infections and therefore question if the dominance of *E. faecalis* in culture studies maybe due to limitations within culturing (Siqueira & Rôças 2004).

Given that mono-infections rarely occur in nature and the basis of the ecological approach is not individual species, but rather polymicrobial entities, the view of *E. faecalis* as the sole bacteria in root-filled teeth with apical periodontitis is simplistic and incorrect.

1.5.4 Rationale for using *E. faecalis* in *in vitro* Research

It is essential to validate the bactericidal action of different disinfection methods employed in endodontic treatment. Given the vast array of possible materials and concentrations, *in vitro* research is most often utilised. As *E. faecalis* may persist following endodontic

treatment regimes and it is prevalent in endodontic infections, it is an appropriate bacterium for *in vitro* research. However it is acknowledged that no single species will fully represent the highly variable intra-canal flora found *in vivo.*

E. faecalis is recovered from infected root canal systems requiring primary endodontic treatment (Fouad *et al.* 2002; Sedgley *et al.* 2006b) although more commonly from root-filled teeth with apical periodontitis (Molander *et al.* 1998; Sundqvist *et al.* 1998; Hancock *et al.* 2001). *E. faecalis* has the ability to reside in the root canal system as a single species without the support of other micro-organisms (Fabricius *et al.* 1982a). Mono-cultures of *E. faecalis* have commonly been identified in root-filled teeth with apical periodontitis as assessed by culture techniques (Molander *et al.* 1998; Sundqvist *et al.* 1998), although this may only highlight the limitation of the method employed.

E. faecalis can invade the dentinal tubules (Love 2001), withstand long periods of starvation (Sedgley *et al.* 2005a), and withstand high pH environments (Ørstavik & Haapasalo 1990; Distel *et al.* 2002; Evans *et al.* 2002). For these reasons, *E. faecalis* has become the most commonly used bacteria for *in vitro* endodontic research. Although maintaining the status quo is not sufficient reason to continue, until more information is available as to the polymicrobial nature of endodontic infections, and how these can be established *in vitro*, an *E. faecalis* biofilm will likely remain the most commonly used standard for *in vitro* research.

1.6 Endodontic Treatment

Endodontic treatment encompasses procedures that maintain the health of all or part of the dental pulp. When the dental pulp is diseased or injured, treatment is aimed at preserving healthy periradicular tissues. When apical periodontitis has occurred, treatment is aimed at restoring the periradicular tissues to health (European Society of Endodontology 2006).

The fundamental concept underpinning endodontic therapy is the elimination of the causative agent thereby providing an environment conducive to healing. Provided that numbers can be eliminated or reduced to undetectable numbers, high success rates of treatment are achieved (Byström *et al.* 1987; Sjögren *et al.* 1997).

Multiple approaches are aimed at the disinfection of the root canal system: instrumentation; irrigation and medication. Collectively, this is termed chemo-mechanical

preparation. In addition, obturation and a coronal restoration are important parts of root canal treatment. Technically, instrumentation and irrigation aim to remove all necrotic and vital organic tissue, as well as some hard tissue, from the root canal system. In so doing, this provides a convenience form for placement of a medicament (if deemed necessary) and a root filling material (Haapasalo *et al.* 2005).

1.6.1 Instrumentation

Mechanical instrumentation is the one of the methods for bacterial reduction of the infected root canal system (Haapasalo *et al.* 2005). Byström & Sundqvist (1981) assessed bacterial reduction in 15 single rooted teeth, instrumented over five appointments with saline irrigation. Mechanical preparation decreased bacterial numbers 100-1000 fold, however complete bacterial elimination was not achieved after one visit in any teeth. After five visits, seven of the 15 root canals still contained culturable bacteria (Byström & Sundqvist 1981).

Dalton *et al.* (1998), in a design similar to Byström & Sundqvist (1981), assessed the difference between intra-canal bacterial reduction with traditional stainless steel files with a step-back technique compared to rotary Ni-Ti instruments. Saline irrigation was used for both techniques and a difference was not found between stainless steel instruments and rotary Ni-Ti instruments. Complete eradication of bacteria was not predictable, with only 28% of canals rendered bacteria free.

Mechanical preparation without the use of an antimicrobial irrigant cannot predictably eliminate bacteria from the root canal system (Byström & Sundqvist 1981; Dalton *et al.* 1998).

1.6.1.1 Challenges to Instrumentation

1.6.1.1.1 Anatomy

The anatomical complexities of the root canal system and irregularities of the root canal wall, such as oval extensions, isthmuses, lateral canals, and apical deltas (Vertucci 1984), make complete and thorough shaping and cleaning of the root canal almost impossible. Micro-CT imaging has shown that at least 35% of the root canal surface area of a maxillary first molar remains unprepared by endodontic instrumentation techniques (Peters *et al.* 2001).

Endodontic files are designed to create preparations round in cross-section. It is common for root canals to have an oval shaped cross-section in the apical 5 mm (Wu *et al.* 2000) and

the majority of oval canals (65%) have been shown to have un-instrumented recesses when mechanically prepared (Wu & Wesselink 2001).

Given that a significant proportion of the root canal wall remains untouched by instruments, it is not surprising that mechanical preparation alone does not render canals sterile. Bacteria will remain within dentinal tubules and other parts of the root canal system, such as fins, lateral canals, accessory canals, transverse anastomoses and apical deltas.

1.6.1.1.2 Bacterial Invasion of Dentine

The dental pulp and the dentine function as a unit that is described as the pulpo-dentinal complex. Odontoblasts line the periphery of the pulp tissue and have extensions into the inner part of dentine. A dentinal tubule forms around each of the major odontoblastic processes.

Following pulp necrosis and bacterial invasion of the root canal system, the frequency of bacterial invasion of dentinal tubules varies between 50 and 90% (Haapasalo *et al.* 2003). Bacterial invasion of dentinal tubules is more common in the coronal aspect of the root canal, followed by mid and then apical regions (Harrison *et al.* 2010). The contents and architecture of dentinal tubules influences bacterial invasion (Love 2004).

Several studies have demonstrated that bacteria are able to invade the dentinal tubules in excess of 130 µm (Kouchi *et al.* 1980; Ørstavik & Haapasalo 1990; Chivatxaranukul *et al.* 2008) and *E. faecalis* has been shown to penetrate between 50–300 µm in human dentine (Safavi *et al.* 1990; Schäfer & Bossmann 2005).

Matsuo *et al.* (2003) showed bacterial invasion into dentinal tubules in 70% of teeth extracted after a diagnosis of apical periodontitis. In teeth that underwent canal enlargement, Matsuo *et al.* (2003) reported the frequency of bacteria found in the dentinal tubules was almost equally high at 65%. Thus, additional modalities are required to remove microorganisms from the areas of the root canal system that mechanical debridement does not clean.

1.6.2 Irrigation

An ideal root canal irrigant should have a broad antimicrobial spectrum, dissolve pulp tissue remnants, inactivate endotoxins and either prevent the formation of a smear layer during instrumentation, or dissolve it if one has formed (Zehnder 2006). In addition, irrigants should be non toxic and non-caustic to peri-radicular tissues (Zehnder 2006).

The most commonly used endodontic irrigant is sodium hypochlorite (NaOCl) (Haapasalo *et al.* 2005). It is an effective antibacterial agent, dissolves soft tissue, has a good shelf life, is cheap, and is readily available. Several other solutions have been, and some continue to be, used as endodontic irrigants. These include chlorhexidine, MTAD (a mixture of tetracycline isomer, acid and detergent), Tetraclean (contains doxycycline hyclate at a lower concentration than MTAD, an acid and detergent), EDTA (ethylene diamine tetra-acetic acid), hydrogen peroxide, iodine compounds, alcohol, saline and local anaesthetic solution.

The superior performance of NaOCl compared to other endodontic irrigants has been highlighted in several *in vitro* studies using various bacteria as mono-species biofilms (Spratt *et al.* 2001; Dunavant *et al.* 2006; Giardino *et al.* 2007). In addition, the tissue dissolution capacity of NaOCl has been shown to be superior to other endodontic irrigants such as 10% chlorhexidine, 3% and 30% hydrogen peroxide, 10% peracetic acid, 5% dichloroisocyanurate and 10% citric acid (Naenni *et al.* 2004).

Sodium hypochlorite is not without its drawbacks, the most important of which is its failure to remove the smear layer (Baumgartner & Mader 1987). McComb & Smith (1975) showed that standard instrumentation techniques produced a canal wall that was smeared and often packed with debris. The smear layer can be packed into dentinal tubules up to 40 μm (Mader *et al.* 1984). In addition to dentine chips, the smear layer can consist of both necrotic and viable tissue, which can include remnants of odontoblastic processes, pulp tissue and bacteria (Pashley 1984; Ørstavik & Haapasalo 1990). It has been hypothesised that the smear layer might inhibit penetration of the root canal irrigant and medicament into the dentinal tubules and affect the adhesion of the root filling cement (Pashley 1984; Ørstavik & Haapasalo 1990).

EDTA is effective at removing the smear layer (McComb & Smith 1975), however it lacks sufficient antimicrobial properties (Spratt *et al.* 2001; Dunavant *et al.* 2006; Giardino *et al.* 2007) and therefore must be combined with another anti-microbial irrigant. Several studies

have shown the combination of NaOCl (2.5-5%) and EDTA (10-17%) is particularly effective in the removal of organic and inorganic debris (Goldman *et al.* 1982; Yamada *et al.* 1983; Baumgartner & Mader 1987).

1.6.2.1 Limitations of Instrumentation and Irrigation

Chemo-mechanical instrumentation does not reliably produce sterile canals. Both microbial sampling and microscopic investigations have highlighted the limitations of chemo-mechanical preparation.

Using paper point sampling and culture techniques, mechanical instrumentation and irrigation with NaOCl has been reported to render between 50-85% of infected root canals free from bacteria at the end of the first appointment (Byström & Sundqvist 1983; Sjögren *et al.* 1991; Shuping *et al.* 2000; Peters *et al.* 2002b). Therefore between 15-50% of canals may still harbour bacteria.

The results of post chemomechanical preparation and microbial sampling have been confirmed using microscopy studies (Sjogren *et al.* 1997; Nair *et al.* 2005). Nair *et al.* (2005) resected the apical region of mesial roots of lower molars with apical periodontitis immediately following chemo-mechanical preparation and obturation in a single visit. Light and transmission electron microscopy revealed residual intracanal infections in 14 of the 16 teeth. Bacteria were present in inaccessible recesses, intracanal isthmuses and accessory canals.

Although visualisation studies do not provide information as to the viability of bacteria detected, when these findings are combined with the microbial sampling, the evidence is conclusive. To further explore the limitations of irrigation within the root canal system it is important to consider irrigation dynamics.

1.6.2.2 Irrigation Dynamics

Numerous *in vitro* studies have investigated the parameters that govern penetration of irrigating solutions. Various methods have been utilised, including radiopaque dyes (Ram 1977; Abou-Rass & Piccinino 1982; Bronnec *et al.* 2010), bio-molecular film intended to simulate a biofilm (Huang *et al.* 2008) and bacterial solutions (Sedgley *et al.* 2004a; Falk & Sedgley 2005; Sedgley *et al.* 2005d; Nguy & Sedgley 2006).

The depth of needle placement within the canal is an important parameter in determining irrigation efficacy (Abou-Rass & Piccinino 1982; Sedgley *et al.* 2005d; Huang *et al.* 2008; Bronnec *et al.* 2010). Furthermore increasing the volume of irrigant used significantly reduced bacterial load regardless of the needle depth (Abou-Rass & Piccinino 1982; Sedgley *et al.* 2005d).

The size of canal preparation is also important. Huang *et al.* (2008) found canal preparation size (20 versus 40) was significant, which was in agreement with Ram (1977). Falk and Sedgley (2005) irrigated canals with a 28 gauge safety ended endodontic needle (Max-I Probe) and found irrigation was significantly less effective in canals prepared to size 36 (with 0.04 taper) compared to canals prepared to size 60. Increasing the taper of the preparation has also been shown to improve the efficacy of irrigation (Huang *et al.* 2008; Bronnec *et al.* 2010).

Canal curvature impacts on the efficacy of irrigation. Nguy & Sedgley (2006) did not show a difference between straight canals (curvature less than 20°) prepared to a size 27 or 46 (all with 0.04 taper), however a difference in irrigation efficacy was shown in curved canals (24-28°). All irrigation was performed 1 mm from the working length, so differences were attributed to irrigation dynamics rather than the depth of needle placement. This study demonstrates that with increasing root canal curvatures, apical enlargement of the root canal system may facilitate the efficacy of endodontic irrigation. However, increasing apical preparation sizes in curved canals clinically may not be practical.

The size of the canal preparation in combination with the dimensions of the needle will determine the depth of needle tip placement. Medical stainless steel needle specifications according to the ISO stipulate that the external diameter of gauge 21 is 0.8 mm; gauge 23, 0.6 mm; gauge 25, 0.5 mm; gauge 27, 0.4 mm and gauge 30, 0.3 mm (Boutsioukis *et al.* 2007). Therefore a gauge 30 needle could be placed without binding 1 mm short of working length in a canal prepared to a size 30 or at working length in a canal prepared to a size 35. Although gauge 23 or 25 were traditionally used, smaller gauged needles (27 or 30) are now preferred (Gulabivala *et al.* 2010).

With an understanding of irrigation needle dimensions it is not surprising that Senia *et al*. (1971) did not find a difference in tissue dissolving effect between 5.25% NaOCl and saline despite the known advantages of NaOCl. Senia *et al.* (1971) prepared the mesial canals of

mandibular molars to a size 25, irrigated with a 26 gauge needle and assessed the tissue dissolving effect of the irrigants 1, 3, and 5 mm from working length. A difference in tissue dissolution was not found between saline and 5.25% NaOCl at the 1 mm and 3 mm levels, which was a reflection of preparation size and irrigation needle gauge. An *in vivo* study assessing bacterial reduction reported similar results. Despite the known bactericidal properties of NaOCl, Shuping *et al.* (2000) did not find a difference in bacteria reduction between irrigation with 1.25% NaOCl and saline during instrumentation until canal preparation sizes were increased above specific sizes.

The lack of penetration of an irrigant beyond the tip of the needle is a limitation of irrigation. Canals behave as a closed end channel which results in air entrapment at its closed end (Tay *et al.* 2010). This produces a vapour lock effect during irrigant delivery. Tay *et al.* (2010) compared an "open" and "closed" system in an *in vitro* study and found the presence of an apical vapour lock adversely affects debridement efficacy. This study only used side vented needles (30-G Max-i-Probe needle), which limits the extrapolation to other irrigation systems.

The stagnation plane beyond which the irrigant cannot pass has been observed in several clinical studies (Senia *et al.* 1971; Shuping *et al.* 2000). This phenomenon was explained with computational fluid dynamics in an inter-disciplinary review that integrated the science that underpins irrigation in endodontics (Gulabivala *et al.* 2010). The stagnation plane can be controlled by needle tip design, fluid flow rate and relative size of the needle to the canal diameter (Gulabivala *et al.* 2010).

The majority of *in vitro* research assessing irrigation dynamics has focused on the penetration to the canal terminus. The canal anatomy is complicated, irrigation must not only access fins, recesses and isthmuses but in addition, it ideally should access bacteria in dentinal tubules. The ability for irrigants to access bacteria is largely unknown. Berutti *et al.* (1997), infected maxillary central incisors with *E. faecalis* and incubated them for 20 days to ensure penetration into the dentinal tubules. Histological analysis with a Brown and Brenn stain revealed irrigation with a combination of 5% NaOCl and 10% EDTA produced a bacteria free band with an average depth of 130 μm from the canal lumen. Beyond the bacteria free zone, a band of moderate tubular infection of variable extension was evident. This further highlights the limitation of standard irrigation in accessing bacteria within dentinal tubules.

1.6.3 Medicaments

Some of the limitations of instrumentation and irrigation can be overcome by the placement of an intra-canal, inter-appointment medicament to help control the residual infection. Calcium hydroxide is the most commonly used medicament.

Calcium hydroxide dissociates into calcium and hydroxyl ions in an aqueous environment. It has a pH of approximately 12.5 and is primarily used as an endodontic medicament for its antimicrobial action and tissue dissolving capabilities.

The efficacy of calcium hydroxide as a medicament has been shown in several *in vivo* studies using paper point sampling and culture techniques. Byström *et al.* (1985) dressed 35 infected canals with calcium hydroxide for four weeks. Following the removal of the medicament and prior to microbial sampling, canals were left empty for between two and four days. Culturable bacteria were recovered in only one of the 35 canals. Calcium hydroxide dressing placed for four weeks had rendered 97% of the canal bacteria free. In a similar study, Sjögren *et al.* (1991) dressed nine infected canals with calcium hydroxide for one week. Following this treatment, no bacteria were recovered from any canals. Shuping *et al.* (2000) rendered 62% of canals bacteria free following preparation of infected canals with Ni-Ti rotary instruments and 1.25% NaOCl. Root canals were then medicated with calcium hydroxide for a minimum of one week. This rendered 92.5% of canals bacteria free which was statistically significant compared to treatment without a medicament.

Despite the success of the *in vivo* studies cited previously, several *in vitro* studies have questioned the success of calcium hydroxide. Ørstavik & Haapasalo (1990) showed the survival of *E. faecalis* after 10 days following treatment with calcium hydroxide in infected bovine dentine. *E. faecalis* has been shown to withstand a pH of 11.1 due to a functioning proton pump (Evans *et al.* 2002) and forms a biofilm in the presence of calcium hydroxide (Distel *et al.* 2002).

In line with these *in vitro* studies, Sundqvist *et al.* (1998) found that three of the nine canals in which *E. faecalis* was originally isolated still sampled positive after chemo-mechanical preparation including a calcium hydroxide dressing.

Despite a calcium hydroxide dressing further increasing the proportion of bacteria free canals (Law & Messer 2004), recent outcome studies (Trope *et al.* 1999; Weiger *et al.* 2000;

Peters & Wesselink 2002) and a meta-analysis (Sathorn *et al.* 2005), have not shown a difference between the outcome of single and multi-visit endodontic treatment. As a result, the efficacy of a calcium hydroxide dressing has been questioned.

1.6.4 Obturation

Obturation of the root canal system serves three main functions, to provide an additional barrier to the coronal ingress of bacteria, to entomb any remaining bacteria within the root canal system thereby preventing bacteria and/or their endotoxins from irritating the periapical tissues and finally to prevent ingress of fluid apically which may serve to provide nutrition for bacteria.

Although the reliability of *in vitro* leakage studies has been questioned (Wu & Wesselink 1993), current obturation methods appear incapable of providing a seal that is impenetrable by bacteria (Torabinejad *et al.* 1990; Khayat *et al.* 1993) and endotoxins (Trope *et al.* 1995). A commonly cited study that radiographically assessed the quality of root fillings and coronal restoration in relation to periapical condition concluded that the coronal restoration had a greater effect on the absence of periapical infection (Ray & Trope 1995). It is commonly misinterpreted that the quality of the obturation is not as important as the coronal restoration. When the bulk of the literature is assessed it is clear that both restoration quality and obturation quality are important for periapical health (Ray & Trope 1995; Kirkevang *et al.* 2000; Tronstad *et al.* 2000; Hommez *et al.* 2002; Dugas *et al.* 2003).

Microbial root canal sampling prior to obturation is seldom done. However root canal systems that sample negative to bacteria, assessed by paper point sampling, have been shown to have a higher success rate compared to canals positive to bacteria at the time of obturation (Sjögren *et al.* 1997). Sjögren *et al.* (1997) treated 55 single rooted teeth in a single visit. Microbial sampling confirmed the presence of bacteria in all root canal systems prior to treatment. At the completion of chemo-mechanical preparation, 22 root canals (40%) still contained recoverable bacteria. Fifty-three teeth of the included 55 teeth (96%) could be recalled. Twenty-nine of the 31 teeth with a negative sample at the time of root filling healed, a success rate of 94%. In contrast, a 68% success rate (15 of the 22 cases) from which bacteria were recovered at the time of root filling healed.

Although a higher success rate is achieved when canals sample negative to bacteria prior to obturation, obturation of infected canals may still result in periapical healing (Sjögren *et al.*

1997). In a unique study that assessed the role of obturation alone in the healing of apical periodontitis, it was found that obturation of infected canals had a significant effect on healing (Katebzadeh *et al.* 1999; Katebzadeh *et al.* 2000). It was proposed that obturation reduced the space and nutrition for multiplication of bacteria and that less bacteria will result in reduced inflammation. Although an important finding, there are limitations to these studies. The investigation was conducted in dogs and the histology or radiographic assessment of post treatment periapical condition was assessed at 6 months, which is insufficient time for complete healing.

Healing of canals that sample positive to bacteria at the time of root filling will be dependent on the quality of the root filling and/or the number and type of bacteria. A high quality root filling may entomb bacteria and/or eliminate access to nutritional material, thus leading to death of the bacteria (Figdor 2002). Alternatively the bacteria maybe eliminated by antimicrobial agents used in root filling material or sealants (Saleh *et al.* 2004).

In cases where viable bacteria remain in the root canal system, there is a constant risk that they may continue to maintain periapical inflammation. As such the goal of root canal treatment is the elimination of microorganisms.

1.7 Sodium Hypochlorite

Claude Louis Berthollet first produced a weak solution of hypochlorite, termed "Javel water" in 1789. Following the work of Dakin (1915) NaOCl was used as a wound irrigant during the First World War, and its use as an endodontic irrigant followed shortly after. Sodium hypochlorite has a broad-spectrum of antimicrobial activity. It can rapidly kill vegetative bacteria, bacterial spores, fungi, protozoa and viruses (Siqueira *et al.* 1998; McDonnel & Russell 1999). In addition NaOCl has tissue dissolving properties (Grossman & Meiman 1941), is readily available, cheap, and has a long shelf life. It is available from pharmaceutical suppliers, or as commercial household bleach. Sodium hypochlorite is the most commonly used endodontic irrigant (Haapasalo *et al.* 2005). In Australia, the most commonly used NaOCl solutions are "Miltons" and "White King" brands of domestic bleach (Clarkson *et al.* 2003). Neither solution has been approved by the TGA for endodontic use.

1.7.1 Mode of Action

In water, NaOCl ionises to produce Na^+ and a hypochlorite anion OCl^-.

$$NaOCl + H_20 \longleftrightarrow NaOH + HClO \longleftrightarrow Na^+ + OH^- + H^+ + OCl^-$$

The hypochlorite anion (OCl^-) is in equilibrium with hypochlorous acid (HClO).

$$OCl^- + H^+ \longleftrightarrow HClO$$

The equilibrium is pH dependant. Between pH 4 and 7, chlorine exists predominantly as the active moiety HClO (pKa approximately 7.5), whereas above pH 9, OCl^- predominates (McDonnel & Russell 1999). Both forms are extremely reactive oxidising agents. However, at identical levels of available chlorine, hypochlorous acid is more bactericidal than the hypochlorite anion (Bloomfield & Miles 1979). Unlike OCl^-, HClO easily penetrates a cell membrane because it is electrically neutral and because the spatial organisation of the molecule is close to that of water (Camps *et al.* 2009).

Estrela *et al.* (2002) attributes the antibacterial properties of NaOCl to two characteristics. The first is related to a chloramination reaction and the second to the high pH of NaOCl.

Hypochlorous acid (HClO) and hypochlorite anions (OCl^-) cause amino acid degradation and hydrolysis. A chloramination reaction occurs when hypochlorous acid is in contact with organic tissue. It acts as a solvent and chlorine is released. The chlorine, a strong oxidant, combines with the protein amino group and chloramines are produced. The chloramines interfere with cell metabolism and irreversibly inhibit essential bacterial enzymes (Estrela *et al.* 2002). Hypochlorous acid has been found to disrupt oxidative phosphorylation and other membrane-associated activities (Barrette *et al.* 1989).

The high pH of NaOCl interferes with the cytoplasmic membrane integrity and results in irreversible enzymatic inhibition. The high pH also causes alterations in cellular metabolism and phospholipid degradation (lipidic peroxidation) (Estrela *et al.* 2002). Other researchers have indicated that DNA synthesis may be inhibited by sodium hypochlorite (McKenna & Davies 1988). Although there is limited evidence for the concept, the antibacterial properties of sodium hypochlorite have been associated with its ability to osmotically draw fluids out of cells (Johnson & Remeikis 1993).

1.7.2 Concentration Debate

Sodium hypochlorite is clinically used in concentrations ranging from 0.5%-5.25%. The ideal concentration is a debated topic, and several factors need to be considered in its determination. Namely, the tissue dissolving and antibacterial capabilities of NaOCl need to be balanced against the potential toxicity. As the importance of bacteria in endodontic infections was not established until 1965 (Kakehashi *et al.* 1965), early research on NaOCl was centred on its tissue dissolving properties.

1.7.2.1 Tissue Dissolving Capabilities

Grossman & Meiman (1941) conducted one of the first endodontic studies to investigate the tissue dissolving properties of NaOCl. Since then, numerous *in vitro* animal models have been used, including rat epithelium and connective tissue (Hand *et al.* 1978), necrotic tissue of rat abdominal walls (Thè 1979), rabbit liver (Moorer & Wesselink 1982) and porcine pulps (Clarkson *et al.* 2006). Human umbilical cord has also been used (Johnson & Remeikis 1993). *In vitro* studies have shown a direct relationship between an increase in NaOCl concentration and tissue dissolving capabilities. Hand *et al*. (1978) measured the weight change of tissue following immersion in varying concentrations of NaOCl and found 5.25% NaOCl was superior to 2.5% NaOCl, and 1% NaOCl showed a similar weight change to saline. Although these experiments have indicated the dissolving properties of NaOCl, the test material was not dental pulp. It has been questioned if these *in vitro* studies can be extrapolated to the human pulp and the clinical setting (Clarkson *et al.* 2006).

Clarkson *et al.* (2006) confirmed that tissue dissolving of porcine pulps was proportional to the NaOCl concentration. Sodium hypochlorite solutions with added surfactants (White King 1% NaOCl; Hypochlor 1% NaOCl) were also more effective at dissolving the pulp samples compared to NaOCl formulations without surfactants (Miltons 1% NaOCl). A study by Stojicic *et al.* (2010) supported this finding. Clarkson *et al.* (2006) further concluded that little pulp dissolution could be expected clinically with Miltons. However Clarkson *et al.* (2006) did not replicate a clinical scenario, but rather placed whole porcine pulps into 25 mL of solution and measured weight change of the pulps. Mechanical instrumentation of the root canal system, as is done clinically, disrupts and increases the surface area of the pulp tissue. The surface area of the organic material has been shown to be an important factor in tissue dissolution (Moorer & Wesselink 1982). Therefore, the conclusions from Clarkson *et al.* (2006) can not be extrapolated to the clinical setting.

Moorer & Wesselink (1982) tested the interaction between NaOCl and organic matter in a homogenous system by measuring the active chlorine that is consumed in the process. It was shown that an excess of organic matter rapidly depletes the activity of the NaOCl during the first moments of the reaction. For NaOCl to be effective, it should be in excess in relation to the amount of organic material that is to be digested. Thus, considering the amount of organic matter and the complicated anatomy of the root canal system, frequent application of fresh irrigant, and/or the use of high concentrations of NaOCl are required.

Mechanical agitation has a significant influence on the tissue dissolving capabilities of NaOCl (Moorer & Wesselink 1982; Stojicic *et al.* 2010). Moorer & Wesselink (1982) postulated that fluid movement might be a more important variable than the initial concentration of NaOCl, and the amount of hypochlorite and/or its frequency of application seemed more important than the strength of NaOCl. This finding was supported by Shih *et al.* (1970). Moorer & Wesselink (1982) concluded that with better mechanical debridement and frequent changes of NaOCl, a lower concentration may be used. They recommended that a solution between 0.5 and 2% be used in clinical practice.

Several studies have utilised tooth models in an attempt to mimic the clinical scenario. Senia *et al.* (1971) used the mesial roots of lower first molars. One canal was irrigated with 5.25% NaOCl and the other with saline. Despite the high concentration of NaOCl, the results questioned the ability of NaOCl to dissolve pulp tissue in the apical region, narrow isthmus areas, or in canals with a small diameter.

Trepagnier *et al.* (1977) noted the importance of an irrigant to act in the complicated anatomical areas of a canal that are inaccessible to mechanical preparation. Using single rooted extracted human teeth, they did not find a significant difference in tissue dissolving capacity between a 5 minute treatment with 2.5% and 5% NaOCl, therefore recommending the use of a less toxic 2.5% solution. They also found, however, that 0.5% exhibited significantly less solvent activity compared to either of the higher concentrations.

Although a tooth model was utilised in both the Senia *et al.* (1971) and Trepagnier *et al.* (1977) studies, the NaOCl was not used as it would be in normal clinical scenarios. The irrigant was not renewed or agitated during the experimental time period. Thus, inadequate and clinically irrelevant volumes were used. Multiple studies have shown that contact time

and volume of NaOCl are important clinical parameters (Trepagnier *et al.* 1977; Hand *et al.* 1978; Thè 1979; Abou-Rass & Piccinino 1982).

The results from the *in vitro*, non-tooth model studies show a clear relationship between the concentration of NaOCl and the amount of tissue dissolution (Hand *et al.* 1978; Clarkson *et al.* 2006). Sodium hypochlorite formulations with surfactants have also been shown to be more effective at tissue dissolution (Clarkson *et al.* 2006; Stojicic *et al.* 2010). Despite the efficacy of high concentrations of NaOCl to dissolve tissue *in vitro*, the contact time and the volume of NaOCl are important clinical parameters that govern tissue dissolution (Trepagnier *et al.* 1977; Hand *et al.* 1978; Thè 1979; Abou-Rass & Piccinino 1982). The importance of frequent irrigant renewal, agitation of the irrigant, and the surface area of the organic material have also been highlighted (Moorer & Wesselink 1982).

1.7.2.2 Antibacterial Action

It is essential to validate the bactericidal action of the different disinfection methods employed in endodontic treatment. Contemporary research into endodontic irrigating solutions has been directed towards the antimicrobial action of the irrigant, rather than the tissue dissolving capabilities. Given the vast array of possible materials and concentrations, *in vitro* research is most often utilised. Although randomised clinical controlled studies are the gold-standard, well designed *in vitro* models have multiple advantages. The anatomy of the root canal system and bacterial inoculations can be standardised, thus allowing a direct comparison of the test antimicrobial agents. Importantly, the sample size of *in vitro* studies can be larger, enabling a stronger statistical analysis. In addition, bacterial sampling (shavings or crushing) can be performed to identify bacteria within dentinal tubules. The significance of microbial sampling techniques and identification methods has been previously discussed. The advantages and disadvantages of the various methods must be considered when comparing studies.

There are a wide range of *in vitro* models used in endodontic studies. It is essential to evaluate the method employed, to assess if it is appropriate to compare studies and whether results can be extrapolated to a clinical setting. Important clinical parameters such as the volume of irrigant used, the contact time, how the irrigant is delivered and whether or not it is replenished, need to be assessed.

The majority of research on the antimicrobial properties of NaOCl has focused on bacteria, although several studies have assessed antifungal properties (Sen *et al.* 1999; Waltimo *et al.* 1999). As bacteria form biofilms within the root canal system (Nair 1987) and biofilms have an increase in resistance to anti-microbial agents compared to planktonic bacteria (Gilbert *et al.* 1997; Johnson *et al.* 2002), an appropriate *in vitro* investigation should ideally use a bacterial biofilm containing relevant bacteria.

1.7.2.2.1 In vitro (not involving teeth)

Earlier research on NaOCl demonstrated its broad antibacterial action against a variety of Gram-positive, Gram-negative, and spore-forming microorganisms (Senia *et al.* 1975; Martin, 1975). In the laboratory setting, the antimicrobial activity of NaOCl was related to its concentration and studies have generally advocated high concentrations of NaOCl (Shih *et al.* 1970; Martin 1975; Senia *et al.* 1975; Harrison & Hand 1981; Gomes *et al.* 2001). Early microbial endodontic research *in vitro* involved the use of planktonic bacteria in solution.

Gomes *et al.* (2001) grew *E. faecalis* planktonically and ultrasonically mixed the bacteria with NaOCl (0.5%, 1%, 2.5%, 4%, 5.25%) for 10 seconds for contact times ranging from 10 seconds to 2 hours. All concentrations of NaOCl were effective in eliminating the test bacteria. However the higher concentrations required less time to inhibit bacterial growth. Less than 30 seconds was required for 5.25% NaOCl to eliminate planktonic *E. faecalis,* 2.5% required 10 minutes, 1% NaOCl required 20 minutes and 0.5% required 30 minutes. Thus NaOCl is bactericidal against planktonic bacteria and high concentrations will disinfect in a quicker time frame.

Dunavant *et al.* (2006) used a continuous flow system to establish a biofilm on porcelain coupons. The percentage kill of viable bacteria was calculated, following treatment with several endodontic irrigants. A statistical difference was not found between 1% and 6% NaOCl. It should be noted that percentage kill could be misleading in terms of remaining viable bacteria. Given a high initial bacterial count, it is possible to have a high percentage kill, yet concurrently a large number of viable bacteria may remain. Furthermore, the biofilm was established on porcelain coupons rather than within a root canal system.

Clinically relevant potential inhibitors, such as dentine (Haapasalo *et al.* 2000) need to be considered when assessing an *in vitro* model, and models that do not involve a root canal per se are ignoring the complications of anatomical confinements. In a simple test tube study

with planktonic bacteria, the tested antibacterial agent has easy access to the target bacterium. This is not the case in the root canal system. The anatomy of the root canal system involves irregularities of the root canal wall, such as fins, isthmuses, oval extensions and apical deltas. For an irrigant to have an effect in these regions it must be delivered to them. Furthermore, bacteria are not only positioned on the root canal wall, but are also found within dentinal tubules. The presence of bacteria growing as a biofilm within the tubules obviously provides a greater challenge for the antibacterial agent. Given the limitations of the methods employed in *in vitro* research with planktonic bacteria, results cannot be extrapolated to the clinical setting.

1.7.2.2.2 In Vitro (involving teeth)

Shih *et al.* (1970), having previously shown the bactericidal effect of 0.5% NaOCl against planktonic bacteria *in vitro,* were unable to replicate the results in a root canal model. The root canals of extracted teeth were inoculated with either *E. faecalis* or *Staphylococcus aureus.* The bactericidal efficiency of 5.25% NaOCl was demonstrated, whilst 0.5% NaOCl was ineffective against the test microorganisms. A proposed explanation was the complex anatomy of the canal systems reducing the contact between bacteria and irrigant. Given the results, Shih *et al.* (1970) recommended the use of 5.25% NaOCl, which is in agreement with Berber *et al.* (2006) but in contrast to Siqueira *et al.* (2000).

Siqueira *et al.* (2000) inoculated *E. faecalis* into the canals of extracted human single rooted teeth. Paper point microbial sampling of the root canal system was conducted pre and post instrumentation. Instrumentation combined with NaOCl irrigation reduced bacterial numbers 100–100,000 fold. A statistically significant difference was not established between 1%, 2.5%, 5.25% NaOCl. Siqueira *et al.* (2000) questioned the use of higher concentrations of NaOCl and suggested that frequent and copious irrigation with a dilute NaOCl solution may maintain a sufficient chlorine reserve to eliminate a significant number of bacteria.

There were two notable limitations of the method employed by Siqueira *et al.* (2000). *E. faecalis* was incubated for only 24 hours, which is insufficient for the establishment of a mature biofilm. Furthermore only paper point microbial sampling was conducted. Berber *et al.* (2006) addressed these limitations and established an *E. faecalis* biofilm with dentinal tubule penetration over 21 days. Microbial sampling was conducted with both paper point and dentine chip samples. A significant difference in antibacterial effect was not detected between 0.5%, 2.5% and 5.25% of NaOCl with paper point sampling which is in agreement

with Siqueira *et al.* (2000), however bacteria were recovered in dentine samples. Berber *et al.* (2006) suggested that 5.25% NaOCl has a greater antibacterial activity against *E. faecalis* inside the dentinal tubules. Clegg *et al.* (2006) had similar results with a polymicrobial biofilm on hemisectioned root apices, finding 6% NaOCl more effective than lower concentrations, but citing that the lower concentrations may have been more effective against the bacteria if they were replenished or given additional time. As is the case for tissue dissolution, the contact time of NaOCl has been shown to be a crucial factor in antibacterial action against bacteria in biofilms (Spratt *et al.* 2001).

Retamozo *et al.* (2010) grew an *E. faecalis* biofilm on dentine cylinders derived from bovine teeth. The biofilm was challenged with various concentrations of NaOCl (1.3%, 2.5%, 5.25%) for between 5–40 minutes. Although the method of application and volumes of the irrigant were not given, the results showed that irrigation with 5.25% NaOCl for 40 minutes was the only regime capable of completely eliminating *E. faecalis*. This finding was in agreement with Berber *et al.* (2006) and Clegg *et al.* (2006).

1.7.2.2.3 In vivo

Few clinical studies have assessed and compared the antibacterial action of different concentrations of NaOCl. Byström & Sundqvist (1983) treated 15 single rooted teeth over five appointments and established that mechanical preparation with 0.5% NaOCl irrigation was more bactericidal than mechanical preparation with saline irrigation. In a second publication (Byström & Sundqvist 1985), the antibacterial effects of 0.5% and 5% NaOCl were compared. Only single rooted teeth with necrotic pulps and periapical radiolucent lesions were included in the study. Twenty canals were mechanically prepared and irrigated with 0.5% NaOCl and another 20 with 5% NaOCl. Treatment was carried out over two visits, with paper point microbial sampling conducted following a two to four day period where the canals were empty. At the third appointment, following two chemo-mechanical preparation appointments and two to four days of the canal being empty, 12 canals (60%) irrigated with 0.5% NaOCl did not have culturable bacteria compared to 14 (70%) irrigated with 5% NaOCl. Although a statistical analysis was not undertaken, it was concluded that there was not a difference in antibacterial effect between 0.5% and 5% NaOCl. This finding was in agreement with Cvek *et al.* (1976b).

1.7.2.2.4 Conclusion

Sodium hypochlorite has a broad antibacterial action against a variety of Gram-positive, Gram-negative, and spore-forming microorganisms (Senia *et al.* 1975). Testing of planktonic cultures of *E. faecalis* has shown that a concentration between 0.5 and 5.25% NaOCl can eliminate *E. faecalis*. However, higher concentrations achieve sterilisation in a shorter time frame (Gomes *et al.* 2001).

A difference in bactericidal effect between 0.5% and 5% NaOCl has not been established in *in vivo* studies (Cvek *et al.* 1976b; Byström & Sundqvist 1985). However, given the lack of strong, well designed studies with adequate sample sizes, *in vitro* research is relied upon for the comparison of concentrations of NaOCl.

Given the heterogeneity of *in vitro* research using different test bacteria, incubation times, sampling methods and test protocols, comparison between studies is difficult. Some studies have supported the use of higher concentrations of NaOCl (Shih *et al.* 1970; Berber *et al.* (2006); Clegg *et al.* 2006; Retamozo *et al.* 2010), whilst others have not found a difference between high and low concentrations (Siqueira *et al.* 2000; Dunavant *et al.* 2006).

1.7.2.3 Toxicity

Sodium hypochlorite is nonspecific in its action and is cytotoxic to all cells except heavily keratinised epithelium (Pashley *et al.* 1985). The decision on NaOCl concentration for clinical use needs to be made on the basis of a balance between cytotoxicity and the antimicrobial and tissue dissolving effects.

Following the plethora of literature recommending higher concentrations of NaOCl for clinical use based on tissue dissolving effects, Spångberg *et al.* (1973) conducted one of the first studies which cautioned the use of 5.25% NaOCl. Based on toxicity studies in which suspensions of HeLa (immortal cell line derived from cervical cancer cells) and L cells (a cultured line of C3H mouse fibroblast cells) were subjected to prolonged (4-24 hour) contact with various concentrations of NaOCl, the authors concluded that high concentrations were highly toxic and irritating to tissue. It was reported that NaOCl uncontrollably and quickly dissolved vital as well as necrotic tissue and may cause post-operative problems. Furthermore, it was claimed that lower concentrations of NaOCl dissolved necrotic but not vital tissue, had reduced toxicity, and retained an antimicrobial effect for all bacteria

commonly present in necrotic cases. Although not having direct experimental evidence for some of these claims, the basis for a biologically acceptable therapy was shown. Later research recommended that the belief that diluted NaOCl would only affect necrotic tissue should be abandoned (Pashley *et al.* 1985).

In the clinical setting during irrigation, NaOCl may enter the periapical or periodontal tissues if a communication exists due to a wide apical foramen, iatrogenic perforation or resorptive defects. Additionally, extreme pressure during irrigation or binding of the irrigation needle tip in the root canal with no release for the irrigant coronally may result in large volumes of the irrigant contacting apical tissues (Hülsmann & Hahn 2000). The tissue dissolving properties of the irrigant can then lead to tissue necrosis. Lamers *et al.* (1980) investigated the effects of 1% NaOCl used under clinical conditions in monkeys. Although the mean scores of inflammation suggested a stronger reaction to NaOCl than to the controls irrigated with saline, statistical differences were not shown. It was concluded that when used in monkey teeth, 1% NaOCl did not add a noticeable noxious effect to the operative trauma.

Clearly methods such as that utilised by Lamers *et al.* (1980) cannot be conducted in humans. Without the option of a histological investigation, Harrison *et al.* (1978) assessed the toxicity of a 5.25% NaOCl in a clinical setting by using intra-appointment pain as a measure of periapical tissue irritation. In this study of 253 endodontic patients, 65.6% were free of inter-appointment symptoms, 27.7% had slight pain and 6.7% had pain that required palliative treatment. The incidence and degree of inter-appointment pain associated with the use of NaOCl was actually less than those associated with the use of normal saline solution as an irrigant. The results of this clinical investigation suggest that the clinical toxicity of 5.25% NaOCl is no greater than the clinical toxicity of normal saline solution when used as an endodontic irrigant. Although this study is of interest, it must be viewed in the light that clinical toxicity is not considered the sole cause of post-operative pain or as the only indicator for potential tissue destruction.

Despite being ranked low in a hierarchy of evidence, case presentations are often influential in clinical decision making. Sodium hypochlorite incidents are a good example of this. Becker *et al.* (1974) presented an early report of an incident involving NaOCl. The case involved the forceful injection of 5.25% NaOCl into the periapical tissues following binding of the needle in the canal. In what are the regular features of such NaOCl incidents, Becker *et al.* (1974) reported immediate pain for the patient, as well as oedema and ecchymosis of the overlaying soft tissues within 30 seconds. In addition patients can experience extension of

oedema over the injured half side of the face, upper lip, infraorbital region as well as profuse bleeding from the root canal (Hülsmann & Hahn 2000). Furthermore, secondary infection and reversible anaesthesia or paraesthesia are possible (Hülsmann & Hahn 2000). It has been reported that following a NaOCl incident, 66% of patients fully recover in less than one week (Kleier *et al.* 2008).

In a survey of American endodontists, 42% of respondents reported at least one accident with NaOCl (Kleier *et al.* 2008). Incidents were more common in maxillary teeth, in the molar or premolar region, and more in females than males. The use of 5.25% NaOCl (72 of 166) or a 2.63% NaOCl (1:1 dilution of 5.25% NaOCl) (66 of 166) was reported for 83% of accidents.

Several mishaps during root canal irrigation have been described in the literature, ranging from damage to the patient's clothing, splashing the irrigant into the patient or operator's eye, injection through the apical foramen, and air emphysema (Hülsmann & Hahn 2000).

Hypersensitivity reactions to NaOCl have also been reported (Kaufman & Keila 1989; Çalışkan *et al.* 1994). The symptoms described by Çalışkan *et al.* (1994) were as described for NaOCl incidents, but in addition the patient also described difficulty in breathing. In all described NaOCl incidents, the tissue response seems out of proportion to the volume of the irrigant (Sabala & Powell 1989). Sabala & Powell (1989) proposed that the response may be similar to angio-neurotic oedema, which involves a release of histamine or histamine-like substances, and vasodilation with subsequent transudation of plasma, plus haemorrhage.

Several studies have compared the cytotoxicity of various root canal irrigants, but few studies have reported on the other factors of biocompatibility. Genotoxicity tests performed on human lymphocytes, showed that NaOCl did not induce any chromosome alterations (Aubut *et al.* 2010).

It is well established that NaOCl is cytotoxic, and this is concentration dependant (Spångberg *et al.* 1973). The majority of NaOCl incidents involve higher concentrations (5.25% or a 1:1 dilution) of NaOCl (Kleier *et al.* 2008) whilst a histological investigation in a monkey model has demonstrated that irrigation with 1% NaOCl did not add a noticeable noxious effect to the operative trauma (Lamers *et al.* 1980). Although post-operative pain is multifactorial, a difference in post-operative pain following treatment with 5.25% NaOCl and

saline has not been shown (Harrison *et al.* 1978). The cytotoxicity of NaOCl needs to be considered when selecting a concentration for clinical use.

Cases at higher risk of a NaOCl incident include teeth with a wide apical foramen, iatrogenic perforations, over preparation of the canal or resorptive defects. Simple suggestions to minimise the occurrence of NaOCl incidents include marking the working length on the irrigation needle, keeping the needle loose in the canal, not using excessive force on the irrigation syringe, using a perforated needle tip, and keeping the needle in constant motion while irrigating (Kleier *et al.* 2008).

1.7.2.4 Interaction between Sodium Hypochlorite and Dentine

The interaction between an irrigant and dentine is an important consideration. Dentine is a substrate with a complex organic and inorganic structure. By weight, dentine consists of 70% hydroxyapatite (a mineral), 20% organic material and 10% water (Ten Cate 1998). The organic matrix consists mostly of type I collagen. Sodium hypochlorite may affect the mechanical properties of dentine via the degradation of the organic components (Pascon *et al.* 2009). The main causes that predispose teeth to fracture have been identified as the loss of tooth structure resulting from caries or access cavity preparation (Reeh *et al.* 1989). However, Sim *et al.* (2001) showed that compared to saline, irrigation with 5.25% NaOCl reduces the flexural strength and elastic modulus of dentine. In a review of the literature, Pascon *et al.* (2009) concluded that there was a decrease in flexural and tensile strength, elastic modulus and micro-hardness when NaOCl was used as an endodontic irrigant. The clinical significance of these changes is unknown. In addition, NaOCl may adversely affect dentine bonding with resin based materials (Yiu *et al.* 2002; Erdemir *et al.* 2004)

The potential negative effects of an irrigant on dentine are seldom considered. The majority of research that has assessed the interaction between dentine and NaOCl has been conducted with high concentrations of NaOCl. Despite a lack of evidence, it is reasonable to assume that lower concentrations of NaOCl would have less impact on the mechanical properties of dentine. Obviously further research is needed to legitimise this assumption although if shown to be true, it may justify a lower concentration of NaOCl.

1.7.3 Improving the Efficacy of NaOCl without an Increase in Concentration

1.7.3.1 Buffering NaOCl

Pure NaOCl solutions as they are used in endodontics have a pH of 12 (Frais *et al.* 2001), and thus the entire available chlorine is in the form of OCl^-. As hypochlorous acid is more bactericidal than hypochlorite one would assume that by buffering NaOCl, therefore reducing the pH and shifting the equilibrium towards hypochlorous acid, the bactericidal effect could be increased. It has also been suggested that buffered hypochlorite solution would be less toxic to vital tissues than a non-buffered counterpart (Cotter *et al.* 1985). Cotter *et al.* (1985), interested in the treatment of burns, buffered 0.5% NaOCl with bicarbonate to physiological levels and noted an increase in bactericidal effect. This however also rendered the solution unstable, with a decrease in shelf life to less than one week (Cotter *et al.* 1985). Camps *et al.* (2009) showed that a neutralised 2.5% NaOCl solution needed to be used within two hours, although the neutralised solution (2.5% NaOCl) presented a better antibacterial activity compared to an unbuffered solution (2.5% NaOCl) against *E faecalis* in an *in vitro* human root canal model.

Research comparing the tissue dissolution effect of buffered and unmodified NaOCl has had mixed findings. Camps *et al.* (2009) found neutralised 2.5% NaOCl dissolved bovine dental pulp during the first 5 minutes of contact, as efficiently as the unmodified 2.5% NaOCl solution. Whilst Aubut *et al.* (2010) showed that the dissolving action of 2.5% NaOCl (on pig palatal mucosa) is about five times higher than that of the neutralised 2.5% NaOCl. Furthermore, neutralised NaOCl used in an *in vitro* model has been shown to be more cytotoxic than an unbuffered solution (Aubut *et al.* 2010).

In a review of the literature, Rossi-Fedele *et al.* (2011) concluded that neutralising (pH 6-7.5) NaOCl may increase the antimicrobial effect of NaOCl but the tissue dissolving effect is reduced. From an endodontic irrigant view point, the limiting factor in buffering NaOCl would be the short shelf life.

An alternative option is to buffer NaOCl, but not as far as to make the solution pH neutral. Zehnder *et al.* (2002) suggested that lowering the pH from 12 to 9, according to Dakin's method, maintained but did not improve the antibacterial effects and did not render the solution less aggressive on fresh tissue. It is the amount of available chlorine, not the osmolarity, pH, or buffer capacity, which is responsible for the tissue-dissolving properties of hypochlorite solutions (Zehnder *et al.* 2002). Zehnder *et al.* (2002) concluded that a safer

irrigant with less dissolving potential if extruded may be obtained by simply diluting stock solutions of NaOCl with water rather than buffering with sodium bicarbonate.

1.7.3.2 Effect of Temperature

Heating NaOCl to increase is effectiveness was proposed as early as 1936 (Costigan 1936), and provides a possible alternative to increasing the concentration. It would be desirable to increase the tissue dissolving and antibacterial effects of NaOCl whilst not increasing cytotoxicity. It would also be desirable for the shelf life of NaOCl to be unaffected.

Multiple irrigant heating devices are available for clinical use. These devices are relatively inexpensive and irrigants are heated within a clinically acceptable time frame. Importantly, it has been shown that 1- 5.25% NaOCl, retains all of its available chlorine when heated to 37°C for four hours (Cunningham & Balekjian 1980) or up to 60°C for one hour (Sirtes *et al.* 2005). Reductions in available chlorine have been reported when solutions have been warmed for 24 hours (Cunningham & Balekjian 1980). Therefore, it is recommended that heated NaOCl be prepared immediately prior to use.

Pre-heated lower concentrations of NaOCl have been shown to dissolve tissue more effectively than higher concentrations at room temperature (Cunningham & Balekjian 1980; Abou-Rass & Oglesby 1981; Sirtes *et al.* 2005). Cunningham & Balekjian (1980) found that 2.6% NaOCl pre-heated to 37°C was as effective at dissolving bovine tendon collagen as 5.25% NaOCl at room temperature (21°C). Sirtes *et al.* (2005) weighed human pulp tissue and found that 1% NaOCl at 45°C was equally as effective as 5.25% NaOCl at 20°C, while 1% NaOCl at 60°C was significantly more effective than 5.25% NaOCl at 20°C.

Several *in vitro* studies have shown an increased antibacterial action of heated NaOCl. Cunningham & Joseph (1980) showed that 2.6% NaOCl at 37°C was more effective against *S. aureus, Streptococcus sanguis, Eschericia coli, Proteus vulgaris, and Bacillus subtilis* spores than at room temperature. Sirtes *et al.* (2005) showed a temperature raise of 25°C (45°C as compared to 20°C) increased NaOCl efficacy by a factor of 100 against planktonic *E. faecalis* cells. In contrast to these results, Raphael *et al.* (1981) infected extracted teeth with *E. faecalis*, *S. aureus, Pseudomonas aeruginosa* and did not find an increase in bactericidal efficiency between NaOCl at 21°C, 29°C, and 37°C.

Heating NaOCl provides an opportunity for a lower concentration solution to be used to achieve the desired clinical result. This would be an advantage if the heated solution were less toxic than a higher concentration at room temperature. Cunningham & Balekjian (1980) reported that when irrigants are used at room temperature (21°C), an equilibrium temperature of 31°C to 33.5°C is reached within the canal after one to two minutes. If the reverse is true, then pre-heated irrigants (above 33.5°C) may undergo a reduction in temperature within the root canal relatively quickly. Once an equilibrium temperature is established, it is possible that a heated lower concentration of NaOCl maybe less toxic than a higher concentration of room temperature NaOCl. On the other hand, once the heated solution drops to this equilibrium temperature, the increased effect achieved by heating the irrigant will have ceased. The proposed mechanism on toxicity is unsubstantiated and requires further research. At this stage it could be concluded that heating NaOCl increases the antibacterial and tissue dissolving effect, but changes in toxicity are unknown.

1.7.4 Conclusion

Sodium hypochlorite is currently the first choice of irrigant in endodontics. It is an effective antibacterial agent, dissolves tissue, has a good shelf life, is cheap, and is readily available. However, NaOCl is not without drawbacks, namely its toxicity and failure to remove the smear layer. Whilst the use of an additional irrigant, such as EDTA, can overcome sodium hypochlorite's inability to remove the smear layer, the toxicity of NaOCl cannot be avoided.

The excellent *in vitro* results of NaOCl against predominately planktonic bacteria may not be replicated in the clinical scenario, and although NaOCl has the most effective antibacterial action compared to other irrigants, it still does not render all canals bacteria free. Access of the irrigant to bacteria within the root canal system appears to be an important rate determining step, possibly more important than the concentration of the irrigant. The contact time and volume of the irrigant delivered into the canal are important parameters. It is paramount that the irrigant is constantly replenished throughout canal preparation. The irrigant needs to be delivered to the apical region, and to achieve this the canal must be suitably prepared and enlarged so that the needle tip can be positioned near the working length. Furthermore the irrigant requires access to micro-organisms within the dentinal tubules, canal fins, isthmuses and lateral canals for example. These are flaws/confinements of all irrigants, and not specific to NaOCl. There is renewed interest in energising irrigants as a

tool to improve the efficiency of endodontic irrigation. There are various ways that this can be achieved, such as sonic, ultrasonic and laser energy.

The effective concentration of NaOCl is a highly debated topic. Despite the known toxicity of higher concentrations, several popular texts have recommended the use of high concentrations of NaOCl. In particular, with the current increased acceptance of single visit endodontic treatment, using a higher concentration or heating irrigants is becoming more popular. There is no compelling evidence that higher concentrations or the heating of NaOCl can lead to a more effective clinical antimicrobial agent. There is however, conclusive evidence that higher concentrations are more toxic. If the antimicrobial effect of a lower concentration NaOCl could be improved, such as is speculated to occur by energising the solution, then this could become the preferred option.

1.8 Lasers

Laser is an acronym for "Light Amplification by Stimulated Emission of Radiation". Lasers generate light with three unique properties that differentiate the light produced from natural light. Lasers produce light of a single wavelength, known as monochromatic light. The light is coherent, meaning the photons comprising the energy beam are emitted in phase. Finally the light is collimated, which refers to the low beam divergence (Peters *et al.* 2011).

1.8.1 Laser Classification

1.8.1.1 Active Medium

There are three basic components to a laser; a pump, an active material, and a resonator (Menzel 2007). Lasers can typically only emit photons within a small, defined wavelength range and this is determined by the active medium. Lasers can be classified by the type of active medium; solid state, gas or semi-conductors (diode).

1.8.1.1.1 Solid-state Lasers

The active medium of solid state lasers are cylindrical rods of glass or single crystals, which are doped with special atoms, ions or molecules that can be optically pumped into an excited state (Demtröder 2006). Examples of solid state lasers are the ruby, neodymium, holmium and erbium lasers. The two most common erbium lasers are the erbium,

chromium:yttrium-scandium-gallium-garnet laser (Er,Cr:YSSG) and the erbium:yttrium-aluminium-garnet laser (Er:YAG).

1.8.1.1.2. Semiconductor (Diode) Lasers

The active material of semiconductor lasers is a *p-n* semiconductor diode. An electrical current through the diode transports electrons between the *n* and *p* sections. The change between energetically higher states to a lower state is accompanied by the emission of energy in the form of electromagnetic radiation (Demtröder 2006).

1.8.1.1.3. Gas Lasers

Generally the active medium of gas lasers is a gas discharge (Demtröder 2006). Argon and CO_2 lasers are examples of gas lasers. The CO_2 laser has the highest output power of all gas lasers (Demtröder 2006), although it is unlikely to gain widespread application in dentistry, as its energy cannot be applied through flexible fibres.

1.8.1.2 Mode of Operation

Lasers can also be classified by their mode of operation, i.e. continuous wave or pulsed wave. Continuous wave lasers emit laser light continuously compared to the short bursts of emissions from a pulsed laser. The pulse repetition rate is measured in hertz (Hz), where 20 Hz equals 20 pulses per second. The output power of the laser is given in watt (W). A watt measures the rate of energy conversion and is defined as one joule per second (W=J/s). The pulse energy (measured in J or mJ) of a pulsed wave laser can be calculated by dividing the laser output power (W) by the pulse repetition rate (Hz). For example an output power setting of 0.5 W at 20 Hz has pulse energy of 25 mJ (0.5/20 = 0.025 J = 25 mJ).

1.8.1.3 Electromagnetic Spectrum

Lasers can be classified according to the frequency/wavelength emission located in the visible, infrared (near infra-red, middle infra-red) and ultraviolet regions on the electromagnetic spectrum. The photon is the basic unit of electromagnetic radiation. Electromagnetic radiation is a form of energy measured in joules (J), that exhibit wave-like behaviour as it travels through space. A wave consists of successive troughs and crests. The wavelength, measured in meters (m), is the distance over which the wave's shape repeats and

is inversely proportional to frequency, measured in hertz (Hz). The energy of photons is related to the frequency of the wave.

1.8.1.4 Dental Application

Lasers in dentistry have been anecdotally classified into 'hard' and 'soft' tissue lasers (Parker 2007). 'Soft' tissue lasers are low power lasers which achieve their effect through indirect methods. Diode lasers are the most common soft tissue laser used in dental applications, and they are used for procedures such as gingivectomies and frenectomies. 'Hard' tissue lasers are high power lasers that achieve their effect through direct interactions. Erbium lasers, for example Er,Cr:YSSG and Er:YAG, are hard tissue lasers that can be used for caries removal and cavity preparation. Most hard tissue lasers can also be used for soft tissue applications.

1.8.2 Laser Interaction with Tissue

There are four processes in which incident light energy will interact with a medium that is denser than air: reflection; scattering; transmission and absorption (Parker 2007). These four concepts underpin the interaction between laser light and tissue.

Light can be reflected from the medium. Depending on the angle of incidence that photons strike a tissue surface and the surface's refractive index, a portion (4-10%) of photons will be reflected. The amount of reflection will depend on the angle of incidence of the light (Steiner *et al.* 2011).

Light can be scattered by the medium it is incident upon. The scattering behaviour of a tissue is an important parameter as it determines the volume of distribution of light intensity in the tissue (Steiner *et al.* 2011). Scattering is accompanied by a change in propagation direction without loss of energy of the photon. Scattering structures can be muscle fibres, skin layers, dentinal tubules, or individual cells or intracellular structures (Steiner *et al.* 2011).

Transmission of light occurs when the beam enters the medium, without causing interaction between the beam and the medium. The beam will emerge unchanged or partially refracted (Parker 2007).

Finally, light may be absorbed by the medium. Atoms in the ground state are at the lowest possible energy level. Electrons in atoms and molecules can change energy levels by emitting or absorbing a photon whose energy must be exactly equal to the energy difference between the two levels. Therefore, absorption is determined by matching incident energy to atoms in their electron shells (Parker 2007). Laser energy absorbed by tissue is transferred into another form and results in thermal reactions.

The main mechanism explaining the operation of lasers in dental applications is through thermal reactions. When laser photons are absorbed, the energy is transferred into heat. The physical change in the tissue caused via the heat transfer is termed photothermolysis (Parker 2007). Tissue temperatures of 60°C are known to cause denaturing of proteins, coagulation of collagens and membrane permeabilisation. At temperatures in excess of 100°C, vaporisation, tissue carbonisation and photo-ablation effects result (Steiner *et al.* 2011).

The amount of laser light absorbed is dependent on the laser incident power, the laser power density (altered by the laser fibre tip), beam movement and coolants (Parker 2007). Coolants can be endogenous, such as blood flow, or exogenous, such as water, air and endodontic irrigants (Parker 2007). Additionally, it is the components of the tissue that will determine the amount of energy absorbed. Absorbing components of tissue are water, hydroxyapatite, porphyrin, haemoglobin, melanin, flavin, retinol, nucleic acids and reduced nicotinamide adenine dinucleotide (Parker 2007; Steiner *et al.* 2011). For near infra-red and middle infra-red lasers, tissue absorption is dominated by water absorption (Steiner *et al.* 2011). The absorption coefficients of several components of tissue are shown in Figure 1. The absorption maximum of water (approximately 10^4) corresponds closely to the wavelength of erbium lasers (Er:YSGG 2.79 μm, Er:YAG 2.94 μm).

Figure 1. Absorption coefficients of haemoglobin, melanin, hydroxyapatite and water, relative to laser wavelength. From Parker (2007).

1.8.3 Endodontic Laser Applications

The first functioning laser was produced in 1960 (Maiman 1960), and applications into the medical field soon followed. Preliminary investigations of the application of lasers into dentistry focused on the effects of the ruby laser on dental hard tissues (Goldman *et al.* 1964; Stern & Sognnaes 1964). Weichman & Johnson (1971) were the first to publish an endodontic application of a laser. The aim of their *in vitro* study, which was unsuccessful, was to seal the apical foramen with a newly developed CO_2 laser.

Although not widely used in endodontics, lasers have been proposed for multiple applications. Both carbon dioxide (Elliott *et al.* 1999) and Nd-YAG lasers (Odabas *et al.* 2007) can be successfully used in pulpotomy procedures and provide an alternative to traditional formocresol pulpotomies. Laser Doppler flowmetry (Jafarzadeh 2009) has been used to measure pulp blood flow for diagnostic purposes. This is valid for pulp vitality testing, as it measures vascular rather than a nerve response (Kimura *et al.* 2000). However the technology is not presently advanced enough for routine clinical use.

The ability of lasers to remove hard tissue has applications in root canal preparation. However canal preparation with rotary instruments has been shown to be more efficient and less likely to cause procedural errors such as ledged or blocked canals (Radatti *et al.* 2006).

As a result, laser preparation of root canal systems has not gained widespread popularity. Similarly other applications have received conflicting reports such as the treatment of dentinal hypersensitivity (He *et al.* 2011), retro grade canal preparation (Karlovic *et al.* 2005; Çalışkan *et al.* 2010) and root end resections (Sullivan *et al.* 2009).

In an attempt to overcome the limitations of instrumentation and irrigation in disinfecting the root canal system, lasers have been proposed as an adjunct. It is hoped that the laser aided disinfection will reach areas inaccessible by traditional methods (for example isthmuses, lateral canals, deep fins, dentinal tubules). Three laser techniques have been proposed for disinfection of the root canal system; direct irradiation, photo-activated disinfection and laser energised irrigation.

1.8.3.1 Direct Irradiation of the Root Canal System

The majority of laser research in root canal disinfection has focused on the direct application of lasers. This involves the irradiation of a dry root canal system with laser light. The bactericidal effect of laser light was first shown in 1986 with a CO_2 laser (Zakariasen *et al.* 1986). In order for the laser light to be introduced into the root canal system, flexible fibres are attached to a handpiece and placed within the canal. The CO_2 laser has limited application in endodontics as its energy cannot be applied through flexible fibres.

Following approval from the US Food and Drug Administration (FDA) for use of the Nd:YAG laser in dentistry, the Nd:YAG laser has become the most widely used and researched laser for direct canal irradiation (Hardee *et al.* 1994; Rooney *et al.* 1994). The use of Er:YAG, Er,Cr:YSGG and diode lasers has also been proposed.

Gutknecht *et al.* (1996) were one of the first to investigate the bactericidal effect of the Nd:YAG laser in root canal systems *in vitro*. Laser irradiation produced a 99.91% reduction of *E. faecalis*, compared to the non-treated control group. Canals were treated immediately following bacterial inoculation. As such, bacteria were in a planktonic state without attachment to the canal wall, nor penetrating the dentinal tubules.

Bergmans *et al.* (2006), improved upon the method of Gutknecht *et al.* (1996), by incubating extracted human teeth with *E. faecalis* for two days. While this is still insufficient time for the establishment of a mature biofilm, the Nd:YAG laser treatment showed a

statistical reduction in bacterial counts between the laser irradiated groups and the non-treated control. Additionally, no canals were rendered sterile (Bergmans *et al.* 2006).

Although the bactericidal effect of laser light had been established (Gutknecht *et al.* 1996; Bergmans *et al.* 2006), a comparison between the antibacterial effect of laser irradiation and NaOCl irrigation was needed. Folwaczny *et al.* (2002) compared 20 seconds of Nd:YAG laser irradiation to 0.5 mL of 1% NaOCl over 10 seconds. Extracted single rooted human teeth had been inoculated with either *E. coli* or *S. aureus*, however they had only been incubated for two hours. Sodium hypochlorite treatment was more effective than laser irradiation (Folwaczny *et al.* 2002). Mehl *et al.* (1999) did not find a difference between 60 seconds of Er:YAG laser irradiation and 1.25% NaOCl (0.5 mL over 2 minutes).

The Er,Cr:YSGG laser was approved for use in endodontic treatment by the FDA in 2002. Both Yavari *et al.* (2010) and Eldeniz *et al.* (2007) investigated the bactericidal efficacy of Er,Cr:YSGG laser irradiation against *E. faecalis* in similar study designs, although utilising different laser settings. Both studies incubated straight single canals with *E. faecalis* for 48 hours and collected bacterial samples with dentine shavings. Both showed a two log bacterial reduction, however no root canal systems were rendered free of bacteria (Eldeniz *et al.* 2007; Yavari *et al.* 2010). Furthermore, both studies found treatment with NaOCl more effective than direct laser irradiation, although the irrigation times were not comparable to the laser irradiation times. Yavari *et al.* (2010) irrigated with 15 mL of 1% NaOCl over 15 minutes and Eldeniz *et al.* (2007) with 15 mL of 3% NaOCl also over 15 minutes. Laser irradiation times were 16 and 20 seconds respectively.

Using experimentally infected root canals, Wang *et al.* (2007) compared the antibacterial effect of the Er,Cr:YSGG and Nd:YAG lasers to NaOCl. Again, straight single rooted teeth were infected with *E. faecalis* and incubated for three weeks. Comparing the two laser systems, the Nd:YAG laser was a more effective laser than the Er,Cr:YSGG laser. The 2.5% NaOCl solution was the most effective treatment group, with complete bacterial elimination as assessed by paper point sampling.

1.8.3.1.1 Laser Penetration

Several studies have investigated the penetration effectiveness of laser irradiation (Klinke *et al.* 1997; Schoop *et al.* 2004; Noiri *et al.* 2008). This may be an important property of laser light. It has been well established that bacteria penetrate dentinal tubules in an infected root

canal system (Matsuo *et al.* 2003) and irrigation has a limited effect on these bacteria (Berutti *et al.* 1997).

Research into the transmission of laser light through tooth structure has been conducted with the Helium-neon laser (He:Ne), which is used in the laser Doppler probe (Vaarkamp *et al.* 1995; Odor *et al.* 1999). Light propagation in dentine is a result of scattering by the cylindrically shaped tubules (Vaarkamp *et al.* 1995; Odor *et al.* 1999; Kienle *et al.* 2003). Vaarkamp *et al.* (1995) highlighted this by showing the transmitted intensities of laser light parallel to the tubules was three times that of laser light perpendicular to the tubules. As laser light can penetrate dentine, it has the potential to have a bactericidal effect on bacteria residing within dentinal tubules.

Klinke *et al.* (1997) investigated the penetration power of an Nd:YAG laser on various dentine slices between 100-1000 μm thick. Whist there was a trend for the bactericidal effect of the Nd:YAG laser to decrease as the depth of penetration increased, a bactericidal effect was still possible at 1000 μm. The bacteria were not in a biofilm however, as discs were inoculated and immediately treated.

The Er:YAG has been shown to be more effective than a Nd:YAG, Er,Cr:YSGG and a diode laser when irradiating either *E. coli* or *E. faecalis* through a 1000 μm dentine disk (Schoop et al., 2004). Both the Nd:YAG and Er:YAG lasers showed approximately a two log fold reduction in bacteria over a four hour incubation of *E. faecalis*.

Despite the bactericidal ability of Nd:YAG and Er:YAG lasers through dentine 1000 μm thick, these *in vitro* studies can not be extrapolated to clinical models (Klinke *et al.* 1997; Schoop et al., 2004). Neither study incorporated bacterial biofilms and investigations that have assessed the bactericidal effects of laser light against a bacterial biofilm have shown the laser effect to be reduced (Bergmans *et al.* 2006; Noiri *et al.* 2008). Both studies used end firing laser fibres placed perpendicular to dentine disks. This is not clinically reproducible. The anatomy of the root canal system dictates that end firing laser fibres cannot be placed perpendicular to the dentine surface, thus reducing or possibly eliminating the penetration of the lasers.

1.8.3.1.2 Radial Firing Tips

Laser light is unidirectional, however due to the reflectance of the fiber walls the laser beam is expanded to a certain degree when leaving the end of the fiber tip (Schoop *et al.* 2009). Despite this, the majority of the laser light is propagated straight, which directs the light towards the root apex within the root canal system. Previous studies have recommended tilting the fiber tip or using spiral movements to maximise lateral emissions. Alternatively, radial-firing tips can be utilised.

Having previously investigated traditional end firing laser fibres (Schoop *et al.* 2007), Schoop *et al.* (2009) repeated their earlier study but with radial firing tips. The radial-emitting fiber tips gave a conical outline with a cone angle of 60° (Schoop *et al.* 2009). As for the 2007 study, canals were incubated for a short period with *E. faecalis*, thus a biofilm was not established, and a NaOCl control group was not included. The Er,Cr:YSGG laser reduced *E. faecalis* numbers, but the majority of samples contained detectible bacteria. Importantly, an SEM analysis revealed no signs of melting or cracking on the root canal wall. Temperature rises during irradiation were also monitored on the external root surface. The temperature rise at the root surface did not exceed 1.6°C with the settings used; therefore damage to the surrounding periodontal structures was unlikely.

Given the heterogeneity of study designs, assessing and comparing the bactericidal effect of laser light is difficult. Various power settings and times of irradiation have been used, however few studies have investigated these parameters within the same study. Gordon *et al.* (2007) evaluated the efficacy of an Er,Cr:YSGG laser with radial firing tips to disinfect dentine cylinders with two different power settings and five different time intervals. Increasing the time of irradiation led to greater bacterial reduction, which was in agreement with Mehl *et al.* (1999). Higher power outputs also produced increased bacterial reduction, which was in agreement with Folwaczny *et al.* (2002). Gordon *et al.* (2007) produced the highest bacterial reduction of 99.7%, using 240 seconds of 0.35 W at 20 Hz laser irradiation. This was comparable to the 99.2% bacterial reduction for 3.0 mL of 2.5% NaOCl (Gordon *et al.* 2007).

1.8.3.1.3 Curved Canals

It has been shown that irrigation is significantly less effective in curved canals with a small apical diameter than in curved canals with a larger apical diameter (Nguy & Sedgley 2006). Similarly, if the root canal system is to be disinfected with a laser, the laser fiber must be able to access the apical region. This will be highly influenced by canal curvature. The vast majority of the studies investigating direct laser irradiation have utilised single rooted and straight extracted human teeth. The extracted teeth have been endodontically prepared to apical sizes up to size 70. This facilitates the placement of the laser fibre to the working length.

Dewsnup *et al.* (2010 and) Yasuda *et al.* (2010) have investigated direct laser irradiation in curved root canal systems. Yasuda *et al.* (2010) compared the bactericidal effect of Nd:YAG and Er:YAG lasers in straight and curved root canals. Unfortunately, there was minimal difference between the two groups as straight was classified at 0-5° and curved 5-10°. Teeth were inoculated and incubated for two weeks with *E. faecalis*. A difference was not found between the Nd:YAG and Er:YAG lasers, however there was a trend towards the Er:YAG being more effective. The Er:YAG laser produced a higher bacterial reduction in straight canals compared to curved canals. Irrigation with 5.25% NaOCl for two minutes contact time (volume unknown) was more effective than laser irradiation, and led to the complete elimination of *E. faecalis* using paper point sampling.

Unlike Yasuda *et al.* (2010), Dewsnup *et al.* (2010) classified teeth as curved between 20° -40°. The teeth were decoronated and the canals prepared to an apical size of 40 with a 0.06 taper. Canals were inoculated with *E. faecalis,* however they were only incubated for 48 hours. Laser treatment was performed in dry canals with an Er,Cr:YSGG laser with a radial firing tip totalling two minutes of irradiation. A statistical difference was not found between curved versus straight canals. Similarly a difference was not found between 6.15% NaOCl treatment and laser irradiation. The results would suggest curved canals do not hamper laser irradiation. It is unlikely that this conclusion could be extrapolated to the clinical scenario as canals were only inoculated for 48 hours. It is therefore unlikely that a biofilm had developed within the root canal system, or that dentinal tubular penetration of bacteria had occurred in that time. Furthermore, the placement of a laser fibre to the working length was aided because teeth had been decoronated and prepared to a size 40/0.06. In the clinical setting it is unlikely that the laser fibre could be placed to working length in a curved canal as the fibres

are not flexible. The sample size in the study was small, with only 10 teeth within each treatment group and this may have influenced the statistical analysis.

1.8.3.1.4 Summary of Direct Irradiation

Various *in vitro* studies have been used to assess the use of direct laser irradiation. These have used dentine disks (Klinke *et al.* 1997; Noiri *et al.* 2008), dentine cylinders (Gordon *et al.* 2007), extracted straight teeth (Gutknecht *et al.* 1996; Folwaczny *et al.* 2002; Bergmans *et al.* 2006; Eldeniz *et al.* 2007; Schoop *et al.* 2007; Wang *et al.* 2007; Schoop *et al.* 2009; Yavari *et al.* 2010), and extracted curved roots (Dewsnup *et al.* 2010; Yasuda *et al.* 2010). Various bacterial species have been inoculated and incubated for different times ranging from zero (Gutknecht *et al.* 1996), two hours (Folwaczny *et al.* 2002), four hours (Schoop *et al.* 2007; Schoop *et al.* 2009), two days (Bergmans *et al.* 2006; Eldeniz *et al.* 2007; Dewsnup *et al.* 2010; Yavari *et al.* 2010), 7 days (Gordon *et al.* 2007), two weeks (Yasuda *et al.* 2010), to three weeks (Wang *et al.* 2007). The short incubation periods of the majority of these studies dictate that bacteria were not in a biofilm state. Additionally, various bacterial sampling techniques have been utilised, but only some (Eldeniz *et al.* 2007; Gordon *et al.* 2007; Yavari *et al.* 2010) used dentine shavings, and generally inferior methods such as paper point sampling were used (Akpata 1976).

The antibacterial effects of the Nd:YAG (Moritz *et al.* 1997; Folwaczny *et al.* 2002; Bergmans *et al.* 2006; Wang *et al.* 2007; Yasuda *et al.* 2010), Er:YAG (Mehl *et al.* 1999; Schoop *et al.* 2004; Yasuda *et al.* 2010) and Er,Cr:YSGG (Schoop *et al.* 2004; Gordon *et al.* 2007; Schoop *et al.* 2007; Dewsnup *et al.* 2010; Yavari *et al.* 2010) have been shown. It is difficult to draw conclusions due to the heterogeneity of study designs with different lasers, bacterial species, incubation times and treatment protocols. Both time of irradiation (Mehl *et al.* 1999; Gordon *et al.* 2007) and laser output power (Schoop *et al.* 2004; Gordon *et al.* 2007; Noiri *et al.* 2008) are important parameters.

At the proposed output powers for direct irradiation of the root canal system, damage to the canal wall or increase to the outer root temperature is not of concern (Schoop *et al.* 2009). Therefore, direct laser canal irradiation appears safe clinically. Generally NaOCl outperformed laser irradiation (Folwaczny *et al.* 2002; Eldeniz *et al.* 2007; Wang *et al.* 2007; Yasuda *et al.* 2010; Yavari *et al.* 2010) however this was not always the case (Mehl *et al.* 1999; Gordon *et al.* 2007; Dewsnup *et al.* 2010) as it depended on the test parameters.

Importantly the inability to position the laser tip at working length *in vivo* would likely further reduce the effectiveness of direct irradiation within the root canal system.

1.8.3.2 Photo-Activated Disinfection (PAD)

Photo activated disinfection (PAD) (Lee *et al.* 2004a; Bonsor *et al.* 2006; Bergmans *et al.* 2008; Dickers *et al.* 2009; Meire *et al.* 2009) is also referred to in the literature as 'Photodynamic therapy' (PDT) (Konopka & Goslinski 2007; Souza *et al.* 2010; Ng *et al.* 2011; Rios *et al.* 2011; Siqueira Jr & Rôças 2011) or less commonly as 'Light activated disinfection' (LAD) (Lim *et al.* 2009). The concept of PAD has been known since 1900. It has been further researched since the 1970s as a treatment for cancer, based on the concept that the nontoxic photosensitiser can be preferentially bound to malignant tissues (Dolmans *et al.* 2003). More recently dental applications of PAD have included disinfection of periodontal pockets (Sarkar & Wilson 1993), carious lesions (Williams *et al.* 2004) and root canals.

Photo activated disinfection involves three components; light, a photo-sensitiser and oxygen (Konopka & Goslinski 2007). Light of a specific wavelength activates a nontoxic photoactive dye (photosensitiser) (Siqueira Jr & Rôças 2011). This is a photo-chemical reaction and the photosensitiser molecule, originally in a low energy ground state, undergoes a transformation into an excited singlet state (Konopka & Goslinski 2007). The high-energy state can transfer its energy to an oxygen molecule (Lee *et al.* 2004a). This produces highly reactive oxygen species (singlet oxygen and free radicals) that kill microorganisms by damaging essential cellular molecules (Konopka & Goslinski 2007).

Photo activated disinfection does not involve canal enlargement, and is recommended as an additional disinfection procedure following chemo-mechanical preparation (Siqueira Jr & Rôças 2011). Most photosensitisers are activated by red light between 630 and 700 nm (Konopka & Goslinski 2007). Diode laser systems are commonly utilised as they are easy to handle, portable and cost-effective (Konopka & Goslinski 2007).

Cytotoxicity is managed by using a dye that selectively binds to bacteria and not to human cells (Lee *et al.* 2004a). This ensures that the reactive oxygen species are applied to cell membranes of bacteria and not human cells. This is an area where PAD may have an advantage over NaOCl irrigation. Xu *et al.* (2009) have shown in an *in vitro* study that PAD had only modest effects on human gingival fibroblasts and osteoblasts compared to NaOCl. Furthermore, there is only minimal heat produced during laser activation (less than 1°C) so

periodontal tissues are not affected (Dickers *et al.* 2009). Additionally, low concentrations of dyes are utilised to prevent radicular or coronal dentine discolouration and soft tissue irritation if extruded (Lee *et al.* 2004a).

1.8.3.2.1 Efficacy of PAD

Multiple publications have confirmed that bacterial species in the oral cavity are susceptible to killing by visible light provided they have been treated with an appropriate photosensitiser (Lee *et al.* 2004a). An ideal photosensitiser should be non-toxic and only display toxicity after activation by illumination (Konopka & Goslinski 2007). Several photosensitisers have been used including phenothiazine dyes (methylene blue and toluidine blue), phthalocyanines, chlorines, porphyrins, xanthenes and monoterpene (Konopka & Goslinski 2007). In the endodontic literature, methylene blue and toluidine blue are the most commonly used.

The bactericidal effect of PAD has been investigated in multiple *in vitro* studies, generally utilising extracted single rooted human teeth infected with *E. faecalis* for various time periods (Soukos *et al.* 2006; Bergmans *et al.* 2008; Meire *et al.* 2009; Souza *et al.* 2010; Rios *et al.* 2011). When the bactericidal effect of PAD has been compared to NaOCl, NaOCl has been shown to be superior (Meire *et al.* 2009; Rios *et al.* 2011). PAD is not a replacement for NaOCl but rather an adjunctive disinfection technique. It is recommended for use after chemo-mechanical preparation.

Rios *et al.* (2011) highlighted the benefit of PAD after the use of NaOCl. Similarly, several studies have investigated the effect of PAD after chemo-mechanical canal preparation. These investigations have been conducted *in vitro* (Souza *et al.* 2010; Rios *et al.* 2011), *ex vivo* (Ng *et al.* 2011) and *in vivo* (Bonsor *et al.* 2006) and mixed findings have been reported.

A qualitative *in vitro* study with SEM analysis has questioned the ability of PAD when dealing with bacteria in a biofilm (Bergmans *et al.* 2008). PAD effectively removed bacteria from the superficial layers of the biofilm, but the deep layers appeared undisturbed (Bergmans *et al.* 2008).

For PAD to be effective, it is essential that the photosensitiser is deposited on the cell wall of the bacteria. Bergmans *et al.* (2008) proposed that either the photosensitiser did not penetrate the full thickness of the biofilm, or the laser light could not be efficiently distributed

throughout the entire thickness, with greater credence given to the former (Bergmans *et al.* 2008). Thus, properties of the photoactivator such as penetration depth, diffusion/wetting capacity, amount of adsorption by the membranes, time of contact and the capacity to generate reactive oxygen species, may define the destructive potential of PAD (Bergmans *et al.* 2008). In essence PAD has similar limitations to standard irrigation as it is hampered by access.

Insufficient oxygenation due to the low oxygenated dentinal tubule microenvironment, and a rapid consumption of any available molecular oxygen have been alternatively proposed as explanations for incomplete bacterial elimination in dentinal tubules treated with PAD (Soukos *et al.* 2006; Souza *et al.* 2010; Ng *et al.* 2011). Under such conditions the bactericidal effect of PAD could be reduced or eliminated as the formation of cytotoxic oxygen derivatives may be minimised or precluded (Souza *et al.* 2010).

With only minimal *in vivo* research, and only a few studies examining the effect of PAD in supplementing bacterial elimination after chemo-mechanical preparation, it is difficult to draw any conclusions on PAD. Further research is required to refine the PAD protocol. Several variables need to be established, including the light parameters, light delivery techniques, photosensitisers and concentration of photosensitisers (Souza *et al.* 2010).

1.8.3.3 Laser Energised Irrigation

Laser irradiation within an irrigant has been termed, Laser Activated Irrigation (LAI) (George & Walsh 2008; de Groot *et al.* 2009; De Moor *et al.* 2009; De Moor *et al.* 2010). Unlike photo activated disinfection (also termed photo dynamic therapy), the laser is not activating a previously inactive irrigant. Rather, the laser is energizing or creating turbulence within an endodontic irrigant such as EDTA or NaOCl, with the goal of increasing the efficacy of the irrigant dispersal. Alternatively the term, Photon-Initiated Photoacoustic Streaming (PIPS) (Peters *et al.* 2011; DiVito *et al.* 2012; Pedullà *et al.* 2012) has been used to describe the combination of laser energy and an irrigant. Although PIPS is an accurate description of the action of the laser, it is an unnecessarily complicated term. Laser Energised Irrigation (LEI) is proposed as a simple, yet accurate term to describe the action of a laser within a root canal filled with an irrigant.

1.8.3.3.1. Mode of Action

1.8.3.3.1.1 Cavitation

The flux of molecules within a liquid takes place through two mechanisms; diffusion and convection (Macedo *et al.* 2010). Diffusion describes the random movement of molecules from a region of higher concentration to a region of lower concentration. Convection occurs when molecules are transported by motion of a fluid. During standard irrigation techniques (non-energised solutions), diffusion has been proposed to be the main mechanism of molecular transport (Macedo *et al.* 2010). However, this is a simplistic view as turbulence is created within the irrigant during delivery, as well as during instrumentation. Adjuncts to endodontic irrigation, such as sonic, ultrasonic and laser energisation, aim to increase the flux of molecules by convection.

Agitation of the irrigant can displace the apical gas entrapment from a closed system (Huang *et al.* 2008; McGill *et al.* 2008) and overcome the vapour lock effect discussed by Tay *et al.* (2010). The goal of energising irrigants is to disperse newly introduced irrigants throughout the root canal system and to dilute the resident debris by flushing it out through displacement motion (Gulabivala *et al.* 2010).

Fluid motion has been shown with LEI via a cavitation effect. Cavitation effects produce turbulence within the irrigant, and the movement of the irrigating substance to areas of complex anatomy and areas beyond the prepared root canal (Siqueira Jr & Rôças 2011).

Cavitation is the formation and then immediate implosion of small liquid-free zones or bubbles, termed cavities, within a liquid. Blanken & Verdaasdonk (2007) explained three mechanisms that can create cavitations.

- An object moving at high speed through a liquid creates an area of low pressure or vacuum behind the object.
- Pressure waves (such as with ultrasound) can create areas of low pressure, causing a liquid to boil at room temperature. The low pressure phase is generally not maintained and the bubble implodes.
- Heating a liquid to boiling temperature can cause cavitations.

Using high-speed imagery and a glass cylinder to simulate the anatomy of a root canal, Blanken & Verdaasdonk (2007) were the first to show the cavitation effect created by a laser

within a root canal model filled with liquid. A sequence of images from Matsumoto *et al.* (2011) depicting laser induced cavitation is shown in Figure 2.

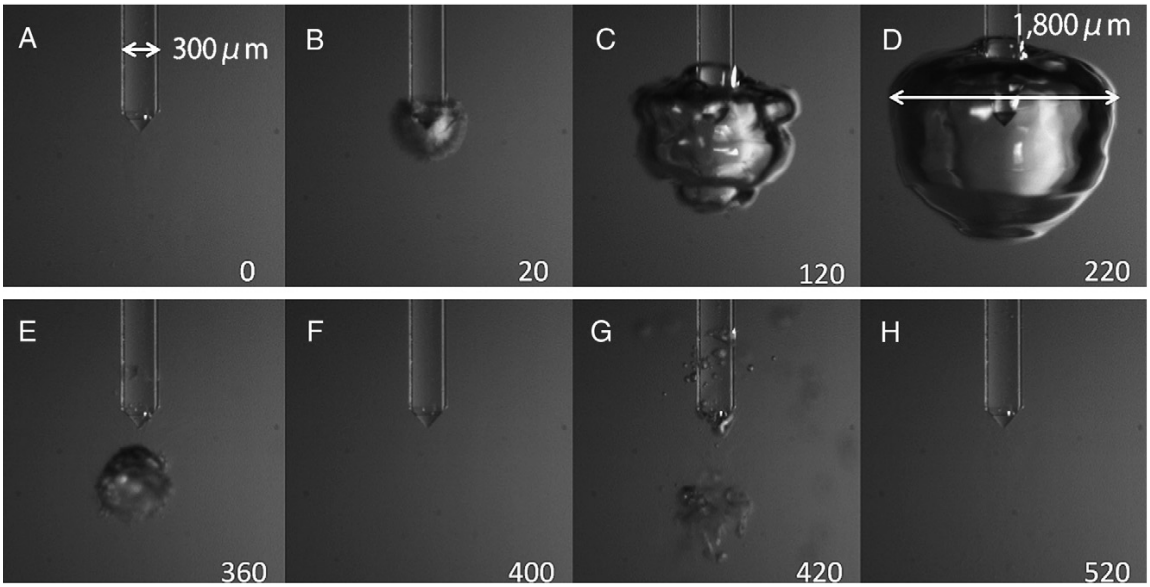


Figure 2. Images from (Matsumoto *et al.* 2011), laser induced cavitation.
Numbers show the time interval (microseconds) since onset of the laser pulse. (A) Laser fibre in water, (B) initiation of vapour bubble, (D) largest vapour bubble, (E) implosion, (G) secondary cavitation effect.

Lasers emits an energy pulse and at the beginning of the pulse (0-50 μs), the energy is absorbed in a 2 μm layer that is instantly heated to boiling temperature and turned into vapour (Blanken & Verdaasdonk 2007). This vapour expands at high speed and provides an opening in front of the fibre for the laser light (Blanken *et al.* 2009). In effect, the laser "drills" a channel through the liquid until the pulse ends. van Leeuwen *et al.* (1991) has described this as the "Moses effect in the microsecond region".

The expanding vapour bubble creates a lower pressure inside the bubble compared to the pressure in the surrounding liquid. When the laser pulse stops, the vapour bubble starts to cool and condense. This results in an implosion of the vapour bubble (Figure 2(E)). Implosion commences with the separation of the vapour bubble from the laser tip and the surrounding liquid rushes into the bubble from the region of separation with the laser fibre. This sequence is more noticeable when conducted in a large volume of liquid (e.g. a large beaker as in Figure 2), as the vapour bubble takes on a sickle shape prior to complete implosion (Blanken & Verdaasdonk 2007; Blanken *et al.* 2009; Matsumoto *et al.* 2011). The dynamics are different within the root canal model, as the restricted space prevents the vapour from expanding freely laterally, pushing the water both forward and backwards in the canal (Blanken & Verdaasdonk 2007; Blanken *et al.* 2009; Matsumoto *et al.* 2011). Matsumoto *et al.* (2011) measured the expansion of the vapour bubble and found it could reach 4500 μm.

During expansion of the vapour bubble, irrigant can be expelled from the canal and later drawn back in during implosion.

Secondary to the implosion of the vapour bubble, the formation of new bubbles can be observed (Figure 2 (G)). This is termed the 'secondary cavitation effect', and arises due to low pressure (Blanken *et al.* 2009). This cycle of cavitation (implosion followed by cavitation) can repeat itself up to ten times per laser pulse, however the size and number of cavitations decreases each time (Matsumoto *et al.* 2011). The resulting fluid turbulence induced by the laser extends up to several milliseconds beyond the time frame of the laser pulse (Blanken *et al.* 2009; Matsumoto *et al.* 2011). With the use of glass beads, Matsumoto *et al.* (2011) has shown the turbulence to be both in front of and behind the laser tip. The distance from the tip is dependent on the laser output power.

The expansion velocity of the vapour bubble during the pulse duration is in the order of 1 m/s, as measured using high-speed imagery capturing 14000 frames per second (de Groot et al 2009). The implosion of the vapour bubble occurs at a similar rate (de Groot et al 2009), although higher fluid velocities have been estimated; 21 m/s (Blanken *et al.* 2009) and 27.8 m/s (Blanken & Verdaasdonk 2007).

The cavitational effect created by lasers within the irrigant produces turbulence, and this moves the irrigant to areas of complex anatomy (Siqueira Jr & Rôças 2011). Cavitation may cause a disturbance or de-agglomeration of biofilms, or flush out bacteria from the canal (Gu *et al.* 2009). It has been proposed that cavitation may produce a transient weakening of the bacterial cell wall and cytoplasm, thus making the bacteria more susceptible to the antibacterial effects of NaOCl (Gu *et al.* 2009).

1.8.3.3.1.1.1 Laser Power Setting

The cavitation effect can be produced at low power settings. For example, Blanken *et al.* (2009) showed cavitation with a Er,Cr:YSSG laser at 12.5 mJ at 20 Hz (0.25 W) and the primary cavitation increased linearly with energy. Higher energy settings mean the vapour bubble formation starts earlier, but the onset of the implosion is later. For lower energies, the threshold for cavitation is reached later, and the energy drops below the threshold quicker. This explains why it takes longer for the vapour bubble to be produced and implosion occurs sooner (Blanken & Verdaasdonk 2007).

1.8.3.3.1.1.2 Type of Laser

Visualisation studies have not directly related cavitational effects to the type of laser used. In the middle infra-red range, wavelengths produced by both Er,Cr:YSSG (Blanken & Verdaasdonk 2007; Blanken *et al.* 2009) & Er:YAG lasers (de Groot *et al.* 2009; Matsumoto *et al.* 2011) are strongly absorbed by water, and cavitation has been demonstrated. Cavitation has also been shown with near-infrared lasers (Hmud et al 2010).

Hmud *et al.* (2010) observed cavitational effects produced by two diode laser systems, the Sirolaser (Sirona, Bensheim, Germany) system, which emits light at 980 nm, and the Ezlase (Biolase, San Clemente, CA), which emits light at 940 nm. These wavelengths are close to the absorption peak of water in the near infra-red range, however their absorption is significantly less than that of lasers in the middle infra-red range. The lowest power settings that could achieve cavitation in distilled water within 5 seconds of laser irradiation were 2.5 W/25 Hz for the Sirolaser and 4 W/10 Hz for the Ezlase. The Sirolaser generated cavitation more readily in distilled water than the Ezlase, which correlates with the known near infra-red absorption characteristics of water. As well as distilled water, this study also used hydrogen peroxide as the absorption curves of water and hydrogen peroxide are different. Although the Sirolaser produced cavitation more rapidly in water, the Ezlase was faster than the Sirolaser at producing cavitation in hydrogen peroxide. The effect with other liquids, such as EDTA and NaOCl, were not investigated.

1.8.3.3.1.1.3 Irrigant

Water and NaOCl were used in the visualisation investigations with erbium lasers (Blanken & Verdaasdonk 2007; Blanken *et al.* 2009; de Groot *et al.* 2009; Matsumoto *et al.* 2011). Blanken *et al.* (2009) reported an identical cavitational effect with Er,Cr:YSGG laser irradiation of both NaOCl and water. Alternatively, de Groot *et al.* (2009) observed different results in that the Er:YAG laser cavitation expanded further, took longer to collapse, and more secondary cavitational effects occurred in NaOCl compared to water.

1.8.3.3.1.1.4 Laser Fibres

Various laser tips have been investigated; plain fibre/end firing (Blanken & Verdaasdonk 2007; Blanken *et al.* 2009; de Groot *et al.* 2009) and radial firing (Matsumoto *et al.* 2011). Blanken & Verdaasdonk (2007) compared various laser tip diameters (200, 320 and 400 μm Biolase Z4 Endotip) within free water. The length of the initial cavitational event was larger

with the larger diameter tips at the same energy settings. but the diameter of the laser fibre within the root canal system is restricted by canal anatomy.

1.8.3.3.1.2 Effect of Heating the Irrigant

The endodontic literature has focussed on cavitation as the main mechanism of LEI. Heat generation on the outer root surface following laser irradiation has also been widely investigated, yet temperature changes within the canal have received little attention. As has been previously highlighted, increasing the temperature of NaOCl leads to an increase in tissue dissolution and antibacterial action. Macedo *et al.* (2010) showed that laser irradiation may increase the reaction kinetics of NaOCl. The only study that has investigated temperature changes within an irrigant was carried out with near infra-red diode lasers (Hmud *et al.* 2010). The root canal systems of extracted human teeth were filled with distilled water and temperatures were measured by thermo-couplings placed in the apical, middle and coronal aspects of the canal. After a 20 second irradiation period, the highest temperature increase measured of coronal water was 4.4°C. In the apical region, the temperature increase was as high as 30°C. It should be noted that the laser tip (200 μm) was placed 2 mm from the working length (apical size 350 μm, taper unknown), and therefore in close proximity to the thermo-couple in the apical region. Significant increases in irrigant temperatures were therefore recorded adjacent to the laser tip, whilst the effect some distance from the laser tip was minimal. Furthermore, cooling by irrigating with a room temperature fluid caused a return to baseline temperatures between laser exposures.

It is unknown if these results could be extrapolated to other irrigants and with other laser types. The antimicrobial action of NaOCl is increased with increased temperatures and the heating from laser irradiation may play a role in LEI. However as the heating effect is located only close to the laser tip, and baseline temperatures return after fresh irrigant is delivered, it is likely to play only a secondary role. The mechanism of cavitation has been highlighted in two studies that have assessed smear layer removal. Water does not have an ability to dissolve the smear layer, but laser energised water increased smear layer removal (George *et al.* 2008; DiVito *et al.* 2012). This suggests the effectiveness of LEI is associated with cavitation instead of the effects from heating the irrigant.

1.8.3.3.2 LEI Effectiveness

To date, there have been ten published articles that have assessed the effectiveness of LEI in endodontic treatment. All the research has been conducted on extracted human teeth and the tests have evaluated debris removal (de Groot *et al.* 2009; De Moor *et al.* 2009; De Moor *et al.* 2010), smear layer removal (George *et al.* 2008; DiVito *et al.* 2012), sealant penetration (Moon *et al.* 2012) and more recently antimicrobial effects (Peters *et al.* 2011; Sahar-Helft *et al.* 2011, Seet *et al. in press*). Irrigants used have included chlorhexidine (Sahar-Helft *et al.* 2011), NaOCl (de Groot *et al.* 2009; De Moor *et al.* 2009; De Moor *et al.* 2010), EDTA (George *et al.* 2008; DiVito *et al.* 2012), water (George *et al.* 2008; DiVito *et al.* 2012) and hydrogen peroxide (George *et al.* 2008).

1.8.3.3.2.1 Smear Layer Removal

George *et al.* (2008) created an artificial smear layer in extracted teeth by preparing the root canals with only water as an irrigant. Smear layer removal was assessed with SEM images taken 2 mm from the apex. It was found that laser irradiation improved the action of EDTAC. There was no difference between the effect of an Er,Cr:YSGG (1.25W 20Hz) and an Er:YAG (4W 20Hz) when matched for the same fibre design and same irrigant. Conical fibre ends were prepared by etching fibres with 50% hydrofluoric acid solution. The modified conical fibres removed the smear layer more effectively compared to the plain tips. As would be expected, EDTAC was more effective than water or hydrogen peroxide at smear layer removal when matched for the same laser and same fibre design.

DiVito *et al.* (2012) also investigated the effectiveness of LEI to remove the smear layer artificially created in extracted teeth. Both qualitative and quantitative assessments were undertaken with SEM images. Two minute irrigation with saline (control) was ineffective in removing the smear layer. Distilled water irradiated with an Er:YAG (20mJ 15Hz) for 20 seconds was more effective at smear layer removal compared to the control. As distilled water does not have an ability to dissolve components of the smear layer, it was assumed that the turbulence created by the laser aided the debridement of the root canal system. Laser energisation of 17% EDTA was the most effective group at removing the smear layer and 40 seconds of laser energisation was more effective than 20 seconds. Unfortunately, this study did not include 17% EDTA irrigation without laser irradiation. It did highlight however, the effective removal of the smear layer using a low laser power setting. In addition, the laser

fibre was not placed within the root canal but rather into the coronal access opening of the pulp chamber, and was kept stationary, not advancing beyond the canal orifice.

Moon *et al.* (2012) evaluated the efficacy of LEI by measuring sealer penetration into dentinal tubules. This is an indirect measure of smear layer removal, as the smear layer can prevent the penetration of sealant into dentinal tubules (Kokkas *et al.* 2004). Although this interpretation is based on the assumption that sealer will penetrate dentinal tubules when a smear layer is not present. Moon *et al.* (2012) prepared extracted root canals with more than 20° of curvature to a size #30.06 with NaOCl irrigation. Teeth underwent a final irrigation regime with either 5.25% NaOCl standard irrigation, 17% EDTA standard irrigation, laser energised 5.25% NaOCl or laser energised 17% EDTA. For LEI groups, a Nd:YAG laser (150mJ/pulse, 10Hz, 1.5W, 200 μm fibre) was used. Root canals were obturated (continuous wave of condensation technique) with gutta percha and a fluorescent labelled sealer. Penetration of sealant into dentinal tubules was assessed by confocal laser scanning microscopy. Laser energised 5.25% NaOCl resulted in a significant increase in sealant penetration compared to standard irrigation with either 5.25% NaOCl or 17% EDTA. Laser activation of 17% EDTA resulted in more penetration of sealant than standard irrigation with EDTA, however this was not statistically significant. Although this study employed an indirect measure of smear layer removal, it proposes that laser energised NaOCl may have superior smear layer removal ability compared to standard irrigation with EDTA. Further research is required to strengthen the case for energised NaOCl. If energised NaOCl is shown to effectively remove the smear layer, it may replace the need to use additional irrigants such as EDTA.

1.8.3.3.2.2 Debris Removal

Based on the experimental method published by Lee *et al.* (2004b) several studies have assessed the effectiveness of debris removal with LEI (de Groot *et al.* 2009; De Moor *et al.* 2009; De Moor *et al.* 2010). This method involves longitudinally sectioning mounted extracted teeth in a manner that allows them to be reassembled. A 4 mm groove, positioned between 2-6 mm from the apex was prepared (0.2 mm wide and 0.5 mm deep). The groove was filled with dentine debris that had been pre-soaked in 2% NaOCl which aimed to simulate an un-instrumented canal extension. There are several advantages to this methodology. The conditions immediately prior to and after any treatment are recorded. This allows a direct assessment of the effectiveness of any intervention. Also, the root canal

anatomy and the amount of debris are standardised. The method has been validated for sensitivity and reproducibility (Van Der Sluis *et al.* 2007).

De Moor *et al.* (2009) compared standard irrigation with 2.5% NaOCl, to 20 seconds of either passive ultrasonic irrigation (PUI) or laser energised 2.5% NaOCl. Laser irradiation was performed with an Er,Cr:YSGG laser (Z2 Endolase tip 200 μm fiber, four times for 5 seconds, 75 mJ, 20 Hz, stationary at 5 mm from the apical stop). Laser energised irradiation was shown to be significantly more effective at removing artificially placed dentine debris than either PUI or standard irrigation.

In a similar study to De Moor *et al.* (2009), de Groot *et al.* (2009) produced comparable results with an Er:YAG laser (280 μm 30 mm tip 80mJ 15Hz). De Moor *et al.* (2010) compared an Er,Cr:YSGG or Er:YAG laser using the same dentine debris model as previously described and did not find a difference between the two lasers. This is in agreement with George *et al.* (2008) who did not find a difference between an Er,Cr:YSGG laser and Er:YAG laser for smear layer removal.

Furthermore, De Moor *et al.* (2010) found 20 seconds of laser irradiation (with either laser) was more effective at debris removal compared to 20 seconds of PUI. This was in agreement with previous research (de Groot *et al.* 2009; De Moor *et al.* 2009). However, repeating the 20 second cycle of PUI and 2 mL irrigation with 2.5% three times, was comparable to 20 seconds of laser energised 2.5% NaOCl. Under the test conditions, if the volume of irrigation and time of ultrasonic activation was increased, a comparable result with a relatively shorter time period of LEI was achieved.

1.8.3.3.2.3 Antimicrobial Effect

While the success of endodontic treatment relies on the removal of microorganisms, the potential antibacterial effects of LEI have received attention only recently.

Hardee *et al.* (1994) combined the use of an Nd:YAG laser and NaOCl to investigate a synergistic effect. Extracted human teeth were decoronated, prepared to a size 50, and infected with *Bacillus stearothmophilus* spores, which are not found in infected root canals. Four treatments were compared. Group one had the canal filled with 0.5% NaOCl which was left for three minutes and was not replenished. Group two underwent laser irradiation with a Nd:YAG laser at 3 W 20 Hz for one minute in a dry canal. Laser energised irrigation groups

were treated as for the 0.5% NaOCl group, however following the three minute soak of NaOCl, the irrigant was energised for either one or two minutes with the Nd:YAG laser. The high laser output power lead to an increase in root temperature so that the teeth became extremely hot to touch. All treatment groups led to a bacterial spore reduction, but there were no significant differences between the groups. A synergistic effect was not detected, but given the high laser output power, it is likely that the irrigant was quickly evaporated or expelled from the canal, preventing any synergistic effect. Furthermore, a relatively low concentration of NaOCl was used, and this was not replenished after three minutes. It is also likely the available chlorine had been depleted prior to the application of the laser. The findings from this study have not been supported by more recent studies (Peters *et al.* 2011; Sahar-Helft *et al.* 2011; Seet *et al. in press*).

Seet *et al.* (*in press*) established a mature *E. faecalis* biofilm within the root canal system of extracted human teeth. The infected roots were treated with standard irrigation with 5 mL of either saline or 4% NaOCl. Additional samples underwent sonic agitation (EndoActivator, Dentsply), or laser energisation of the test irrigant. For the LEI group, following the delivery of 1.25 mL of irrigant over ten seconds the irrigant was energised with an Er,Cr:YSGG laser (0.25 W, 20 Hz) for five seconds. This process was repeated four times using a total of 5 mL of irrigant delivered over a total treatment time of one minute. Based on qualitative SEM images, the most dramatic reduction in viable *E. faecalis* was seen with laser energised 4% NaOCl. The results of Seet *et al.* (*in press*) supported the use of LEI, and is in agreement with the work of Peters *et al.* (2011).

In an *in vitro* study, Peters *et al.* (2011) assessed the disinfection of the root canal system with what they termed photon-initiated photoacoustic streaming (PIPS). A biofilm was established *in situ* in extracted human premolars. Infected root canals were treated with standard irrigation with 6% NaOCl, 6% NaOCl PUI for 30 seconds, or 6% NaOCl energised with an Er:YAG laser (50mJ 10Hz) for 30 seconds (PIPS). Laser energised irrigation of 6% NaOCl (10/20 rendered bacteria free) was significantly better than standard irrigation of 6% NaOCl (1/20) and ultrasonic energised 6% NaOCl (2/20) in achieving more bacteria-free samples. Bacteria were recovered using a modified paper point sampling technique. It should be noted however, that when assessing bacterial reduction rather than negative cultures, a significant difference was not found between LEI 6% NaOCl (99.5% bacterial reduction) and 6% NaOCl standard irrigating (96.6% reduction). This finding is in agreement with the work of Pedullà *et al.* (2012).

Pedullà *et al.* (2012) did not find a statistical difference between 5% NaOCl standard irrigation (97.13% bacterial reduction) and LEI 5% NaOCl (99.80% reduction). In contrast to Peters *et al.* (2011), Pedullà *et al.* (2012) did not find a difference between LEI and standard irrigation when assessing canals rendered bacteria-free (assessing negative cultured versus percentage of bacterial reduction). Pedullà *et al.* (2012) inoculated and established an *E. faecalis* biofilm over 15 days in extracted single rooted teeth. Irrigation with 5% NaOCl (3 mL over 30 seconds) produced 25 of 32 teeth bacteria-free, Er:YAG energised 5% NaOCl (3 mL 5% NaOCl irrigated over 30 seconds followed by LEI, (20 mJ, 15 Hz for 30 seconds)) produced 30 of 32 teeth bacteria-free. Although LEI with 5% NaOCl produced the most negative cultures and greatest reduction in bacteria, the differences were not statistically significant.

Both Seet *et al.* (*in press*) and Peters *et al.* (2011) have shown the additional benefits of laser energised NaOCl. Sahar-Helft *et al.* (2011) is currently the only study that has assessed LEI using chlorhexidine. Again the synergistic effect of LEI with an irrigant was shown. The lowest concentration (0.0001%) of chlorhexidine only had an inhibitory effect on planktonic *E. faecalis* when the solution was energised with an Er:YAG laser (3.6 – 6 W, 12 Hz, 15 seconds x 4) compared to no LEI. Although this work increased the understanding of LEI using chlorhexidine, the microbiological basis of the study was weak, a bacterial suspension of *E. faecalis* was used and a tooth model was not incorporated. Sahar-Helft *et al.* (2011), correctly cautioned extrapolation of the results to the clinical setting. Interestingly, this was the first study to investigate a range of irrigant concentrations, and demonstrated that lower concentrations of chlorhexidine could be combined with LEI, and equal or exceed the antibacterial effect of higher concentrations of chlorhexidine when non-energised. A similar experimental study has not been investigated using LEI of NaOCl.

1.8.3.3.3 Safety of LEI

1.8.3.3.3.1 Temperature

In any medical procedure patient safety is paramount. Thermal changes on the external root surface may result in injury to periodontal ligament cells and the alveolar bone. The classical study by Eriksson & Albrektsson (1983) on rabbits showed that heating bone by 10°C resulted in bone injury. Lasers used for preparation of the root canal system in a dry canal can produce significant increases in temperature. A histological investigation in dogs has shown that a Nd:YAG laser (at 3 W/25 Hz), used for preparation in a dry canal, caused ankylosis, cemental lysis and major bone remodeling (Bahcall *et al.* 1992). Lasers used for

LEI are used at sub-ablative powers however, and only minimal increases in outer root temperature have been recorded.

DiVito *et al.* (2010) measured the temperature changes on the external root surface in an *in vitro* study. An Er:YAG laser (20 mJ 15 Hz) was used to energise EDTA in the root canal of extracted human teeth. An increase of 1.2°C was recorded after 20 seconds and 1.5°C after 40 seconds. Schoop *et al.* (2009) measured temperature changes on the external root surface using a Er,Cr:YSGG laser at power settings of 2 W and 3 W and 20 Hz. Radial firing tips with a calibration factor of approximately 70% were used, meaning that approximately two-thirds of the laser energy was absorbed within the fibre. Actual laser output was 0.6 W and 0.9 W respectively. Canals were irradiated dry for 5 second cycles, followed by a 20 second break. This was repeated five times. Temperature changes were 1.3°C for the 0.6 W and 1.6°C for 0.9 W. The temperature increases were well below the 10°C threshold. It must also be noted that this investigation was conducted within a dry canal, which is likely to increase any rise in temperature. Furthermore, irrigating with a room temperature fluid between LEI causes a rapid return to baseline temperatures (Hmud *et al.* 2010).

There are several limitations to the current *in vitro* investigations. Studies by Schoop *et al.* (2009) and DiVito *et al*. (2010) were conducted at room temperature rather than body temperature. Also, the tip location of the laser fibre relative to the thermocouple is important. DiVito *et al.* (2010) used a 1.5 mm diameter thermocouple placed 5 mm from the apex. The laser fiber was placed in the coronal access opening and was not advanced into the orifice of the canal. Schoop *et al.* (2009) mounted teeth on a 10 mm by 10 mm thermocouple with a silicon heat conductive compound. The laser fiber was placed as far as possible into the canal and activated whilst withdrawing from the canal.

Despite the limitations of the *in vitro* investigations, it appears unlikely that external root surface temperature increases are of concern for LEI.

1.8.3.3.3.2 Extrusion

The cyctoxicity and other deleterious effects of extrusion of endodontic irrigants are well established (Pashley *et al.* 1985). Visualisation studies on LEI have estimated fluid velocities as high as 27.8 m/s (Blanken & Verdaasdonk 2007), although estimates from other authors have been significantly lower (de Groot et al 2009). The potential to extrude the irrigant into the periapical tissues with LEI must be considered.

A bench top investigation has shown the extrusion of dye from prepared root canals following LEI (George & Walsh 2008). Although, this study did not account for an intact periodontium, the laser power settings were high, and the apical foramina were prepared with either a size 15 or 20 file. Teeth were mounted horizontally, filled with dye, and irrigated with two needle designs as well as LEI with both an Er,Cr:YSSG (1.25 W 20 Hz) and Er:YAG (4 W 20 Hz) laser. Both bare and conical laser tips were used at 5 mm and 10 mm from the apex. The volume of extruded irrigant was measured by calculating the total area covered with extruded dye. The distance of extruded dye from the apex of the tooth was also measured. Extruded dye was recorded in all groups, apart from the side-vented needle placed 10 mm from the apex. A significant difference was not found between laser type (Er,Cr:YSSG or Er:YAG), laser fibre design (conical or bare), or fibre placement from the apex (5 or 10 mm). There was no difference between the groups for the area covered with extruded dye, however dye in the laser treatment groups was distributed up to approximately four times further from the apex. Interestingly, when matched for the same laser, tip and distance from the apex, there was a three-fold increase in dye extrusion for the size 20 apical foramen compared to the size 15. This highlights the need to preserve the apical anatomy of the tooth. Caution should therefore be used in short straight roots with open apical foramen.

The clinical relevance of the above *in vitro* study is unknown. There is no resistance from the periradicular tissues when working on extracted teeth, allowing a free flow of irrigant through the foramen. Regardless, it would seem appropriate to minimise laser output power to reduce the potential for extrusion. Furthermore, minimising the concentration of any cytotoxic endodontic irrigants would also be prudent.

1.9 Gaps in the Literature

Instrumentation and irrigation cannot reliably produce a bacteria-free root canal system (Byström & Sundqvist 1985; Shuping *et al.* 2000). Medicaments such as calcium hydroxide can potentially overcome some of the limitations of instrumentation and irrigation, however, their use is not without shortcomings and necessitates a multi-visit approach. Devices such as sonics, ultrasonics and lasers have been proposed as adjuncts to the chemo-mechanical preparation of the root canal system.

Erbium laser energisation creates cavitation effects within an irrigant and high speed photography has shown the formation and rapid implosion of vapour bubbles (de Groot *et al.*

2009; Matsumoto *et al.* 2011). It is hypothesised that laser energised irrigation facilitates the access of the irrigant to those parts inaccessible with a standard needle irrigation technique, thus improving the efficacy of the irrigant.

The effectiveness of LEI has been evaluated for debris removal (de Groot *et al.* 2009; De Moor *et al.* 2009; De Moor *et al.* 2010), smear layer removal (George *et al.* 2008; DiVito *et al.* 2012; Moon *et al.* 2012) and antimicrobial effects (Hardee *et al.* 1994; Peters *et al.* 2011; Sahar-Helft *et al.* 2011; Pedullà *et al.* 2012; Seet *et al. in press*). Apart from one of these investigations for reasons previously cited (Hardee *et al.* 1994), all have shown the advantages of LEI.

Laser output power is an important parameter. The effectiveness of LEI in removal of debris and smear layer has been shown with EDTA and Er,Cr:YSGG lasers at higher settings of 1.25 W (62.5 mJ at 20 Hz) (George *et al.* 2008) and 1.5 W (75 mJ at 20 Hz) (De Moor *et al.* 2009; De Moor *et al.* 2010). Laser energised irrigation with EDTA and an Er:YAG lasers at 1.2 W (80 mJ at 15 Hz) has shown increased debris removal (de Groot *et al.* 2009). A difference between an Er:YAG and an Er,Cr:YSGG laser has not been shown for smear layer (George *et al.* 2008) or debris removal (De Moor *et al.* 2010). More recently, lower laser powers have still been shown to be effective. DiVito *et al.* (2012) has shown smear layer removal with Er:YAG energised EDTA at 0.3 W (25 mJ and 15 Hz) and Peters *et al.* (2011) has shown an increased antibacterial effect of LEI of NaOCl with 0.5 W (50 mJ 10 Hz) using an Er:YAG laser. In agreement with Peters *et al.* (2011), Seet *et al.* (*in press*) qualitatively showed the antibacterial efficacy of Er,Cr:YSGG laser (0.25 W 20 Hz) energised 4% NaOCl.

Despite successful endodontic treatment relying on the removal of microorganisms, the potential antibacterial effect of LEI has received little attention. The three studies that have investigated the antimicrobial effect of laser energised NaOCl have used high concentrations of NaOCl, and relatively low laser power settings. Seet *et al.* (*in press*) used 4% NaOCl, Pedullà *et al.* (2012) 5% NaOCl and Peters *et al.* (2011) used 6% NaOCl. It is established that higher concentrations of NaOCl are more cytotoxic (Spångberg *et al.* 1973). Albeit with chlorhexidine and planktonic bacteria *in vitro*, LEI has been shown to increase the efficacy of lower concentrations of chlorhexidine to be equal or superior to non energised higher concentrations of chlorhexidine (Sahar-Helft *et al.* 2011). Such an investigation with NaOCl has not been undertaken.

Sodium hypochlorite is the most commonly used endodontic irrigant. It is a potent antimicrobial agent that has tissue dissolving properties. Sodium hypochlorite is nonspecific

in its action and is cytotoxic (Pashley *et al.* 1985). Sodium hypochlorite is used in concentrations ranging from 0.5-6%, however most investigations have not supported the use of higher concentrations (Zehnder 2006).

Laser energised irrigation at high laser power settings can extrude the irrigant (George & Walsh 2008). This has obvious clinical implications and research should aim to minimise the concentration of NaOCl and/or laser output power without diminishing its antimicrobial efficacy.

1.10 Aim

Overall, the aim of this study was to establish the efficacy of laser energised irrigation in endodontic treatment. Two parameters were investigated; laser output power and concentration of sodium hypochlorite. Given the vast number of possible combinations of laser output powers and sodium hypochlorite concentrations, and to ensure an adequate sample size, an *in vitro* model was required. An *E. faecalis* biofilm was established in the root canal system of extracted single rooted human teeth. The study was conducted in two parts; the preliminary studies (Study 1 and Study 2) and experimental study (Study 3).

1.10.1 Preliminary Studies (Study 1 and 2)

Given the large number of potential combinations of laser output powers and sodium hypochlorite concentrations, preliminary studies aimed to narrow the treatment groups prior to conducting the experimental study (Study 3) with an adequate sample size to allow for meaningful statistical analysis.

Therefore, the aim of the preliminary studies (Study 1 and Study 2) was to establish an effective Er,Cr:YSGG laser output power for laser energised irrigation. The aim was to determine if laser energised irrigation was more efficient at eradicating an *E. faecalis* biofilm *in vitro* and if so to determine the lowest laser output power to have an improved bactericidal effect.

1.10.2 Study 3. 'Efficacy of Sodium Hypochlorite and Er,Cr:YSGG Laser Energised Irrigation Against an *Enterococcus faecalis* Biofilm.'

The aim of 'Study 3' was to establish the efficacy of eradicating an *E. faecalis* biofilm *in vitro* using various concentrations of NaOCl with and without Er,Cr:YSGG laser energised

irrigation (at the laser power setting determined in Studies 1 and 2). The sample size used in this study allowed for statistical analysis.

The null hypothesis was that there is no difference in the antibacterial effect between laser energised irrigation and standard irrigation when using 1% and 4% NaOCl. Additionally that there was no difference in the antibacterial effect between the 1% and 4% NaOCl.

2.0 Method and Materials for Studies 1, 2 & 3

2.1 Tooth Collection, Storage and Root Preparation

Ethics approval (# H-062-2010) was granted from The University of Adelaide Human Research Ethics Committee. Teeth were obtained from the Oral Surgery Department of the Adelaide Dental Hospital. A total of 167 (Study 1, 23 teeth; Study 2, 48 teeth; Study 3, 96 teeth) extracted single rooted human teeth with completely formed apices were used in the study. The teeth were stored in 0.02% (v/v) thymol in 0.9% saline (w/v) until use. Before preparation, teeth were immersed in 4% NaOCl (Endosure, Dentalife, Ringwood, Australia) for ten minutes and lightly curetted to remove the periodontal ligament from the root surface.

The teeth were decoronated with a diamond coated disc (3M, Minnesota, USA) and the length of each root adjusted to 14 mm. A glide path was established with size 10 and 15 stainless steel K-files (SybronEndo, California, USA). The root canals were prepared in a crown down manner with FlexMaster files (VDW, Munich, Germany). Two mL of 4% NaOCl (Endosure, Dentalife, Ringwood, Australia) irrigation was used between each file. All irrigation was performed with a 3 mL syringe and a 27-gauge needle (Monojet, Kendall, Covidien, Mansfield, USA). A minimum of 16 mL of 4% NaOCl was used during instrumentation. Final instrumentation was completed with a size 40 file with a 0.06 taper, 1 mm beyond the apical foramen. This produced an apical size of 0.46 mm. An open apex was created to facilitate the flow of media through the canal to allow the establishment of a biofilm. The apical foramina were later occluded prior to treatment protocols. The smear layer was removed with 5 mL of 17% (w/v) ethylenediamine tetra-acetic acid (EDTA) for two minutes followed by a final flush with 5 mL of 4% (v/v) NaOCl.

The root canals were dried with paper points and the external surfaces of the teeth were allowed to air dry. Two coats of nail varnish (Deeply Chili 741, Revlon, New York City, USA) were applied to the entire root surface. Handling of the tooth roots was facilitated by placing two size 40 paper points into the root canal, one of the paper points extruded through

the apex to prevent occluding the apical foramen with nail varnish. When the nail varnish had dried, the paper points were removed and the roots were mounted in the flow cell.

2.2 *E. faecalis*

E. faecalis (V583 ATCC 700802) was purchased from the American Type Culture Collection (Cryosite. NSW, Australia). The organism was maintained on THB agar (Oxoid, Victoria, Australia). Frozen stock cultures were stored at -80°C in THB containing 40% (v/v) glycerol. The purity of the cultures were periodically checked by Gram staining and plating onto bile aesculin agar (Oxoid, Victoria Australia).

2.3 The Flow Cell

The study employed the use of a continuous flow cell which was designed by the University of Adelaide Endodontic research group (Seet *et al. in press*). It was designed to establish a biofilm within decoronated tooth roots.

The flow cell was constructed from Delrin poly-acetyl resin (Dupont, Wilmington, DE, USA) measuring 200 mm (W) x 200 mm (L) x 60 mm (H). The flow cell was divided into a sealed upper and lower chamber, separated by a Perspex plate (Figure 3). Holes (10 mm in diameter) were drilled into the Perspex plate to facilitate mounting of 24 tooth roots (Figure 4).

The apparatus allowed a growth medium to be pumped into the upper chamber, via a port inserted through the Perspex viewing window. A second port in the viewing window (upper outlet valve) was used to allow air to bleed from the flow cell during the initial filling of the flow cell with growth medium. The port was closed once the flow cell was filled with growth medium. The upper chamber was approximately 4 mm in depth and acted as a reservoir for the growth medium. The Perspex plate containing the tooth roots and the Perspex viewing window were both secured to the body of the flow cell with stainless steel screws (Figure 4). Two neoprene gaskets were used to ensure that the flow cell had a hermetic seal so growth medium could only pass through the tooth roots and into the lower chamber before exiting the flow cell via the outlet port (Figure 3).

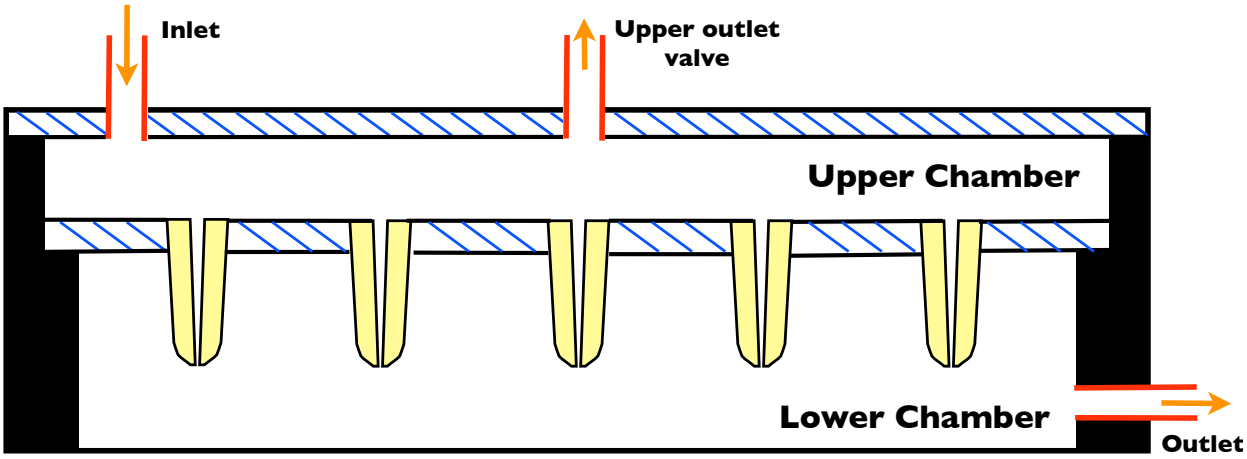


Figure 3. Schematic drawing of flow cell in cross-section.
Perspex plates are shown with blue shading. The upper Perspex plate, 'viewing window' contains the inlet valve and upper outlet valve. The upper outlet valve was only opened during initial filling of the flow cell. The Perspex plate containing the tooth roots (five roots shown in image with yellow shading) separated the flow cell into an upper and low chamber. Growth medium pumped into the upper chamber via the inlet port could only pass through the tooth roots and into the lower chamber before exiting the flow cell via the outlet port.

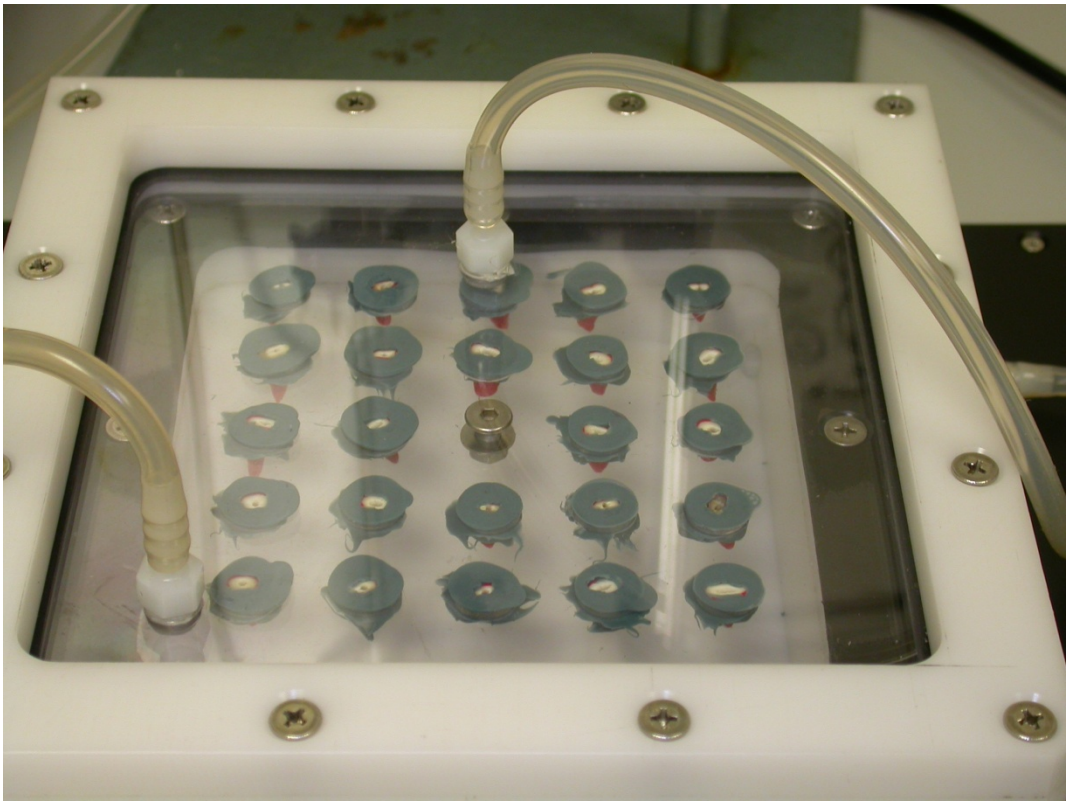


Figure 4. Photograph of the flow cell.
The Perspex viewing window with the inlet valve (lower left of image) and upper outlet valve (top of image) was attached to the flow cell with twelve screws. The Perspex plate containing 24 tooth roots is attached to the flow cell by eight screws. Holes (10 mm in diameter) were drilled into the Perspex plate to facilitate mounting of 24 tooth roots. Medium body impression material was used to mount the teeth. Neoprene gaskets were positioned between the Perspex plates and the body of the flow cell to ensure a hermetic seal.

2.4 Installation of Teeth and Sterilisation of the Flow Cell

Tooth roots were mounted onto the Perspex plate by syringing medium body polyvinyl siloxane impression material (3M ESPE, Seefeld, Germany) around the roots (Figure 4). The assembled flow cell containing the tooth roots was sterilised using ethylene oxide (Steritech Pty Ltd, Dandedong, Victoria, Australia).

2.5 Experimental Apparatus and Growth of the Biofilm

The experimental apparatus consisted of the flow cell, nutrient reservoir, silicone tubing inoculation port, peristaltic pump, waste vessel and a slide dryer which kept the flow cell at 37°C (Figure 5). The silicone tubing, nutrient reservoir containing growth medium and the waste vessel were sterilised by autoclaving at 15 psi at 121°C for 15 minutes and the system was connected aseptically.

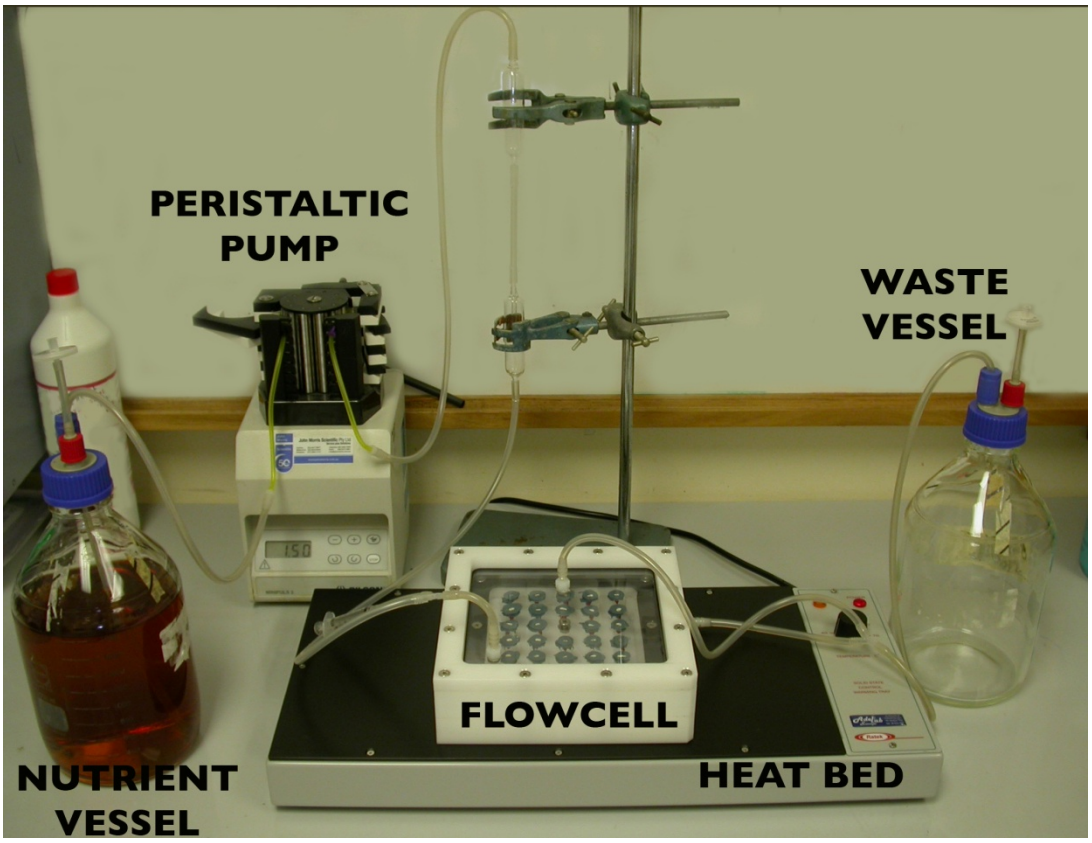


Figure 5. Photograph of the experimental apparatus.
The flow cell was seated on a slide dryer labelled 'heat bed'. The nutrient medium (lower left of image) is pumped into the flow cell via the peristaltic pump. The nutrient medium exits the flow cell and enters the waste vessel.

Sterile Todd Hewitt Broth (THB) (Oxoid, Victoria, Australia) was pumped from the nutrient reservoir into the flow cell through silicone tubing using a peristaltic pump (Gilson,

Middleton, USA). The medium line had a glass "breaker" between the peristaltic pump and the flow cell to prevent bacteria growth back into the nutrient reservoir (Figure 6).

When the flow cell was filled with THB the peristaltic pump was stopped and air bubbles were cleared from the flow cell through the second outlet in the viewing window (Figure 6). The flow cell was placed on a slide dryer, and the temperature maintained at 37°C.

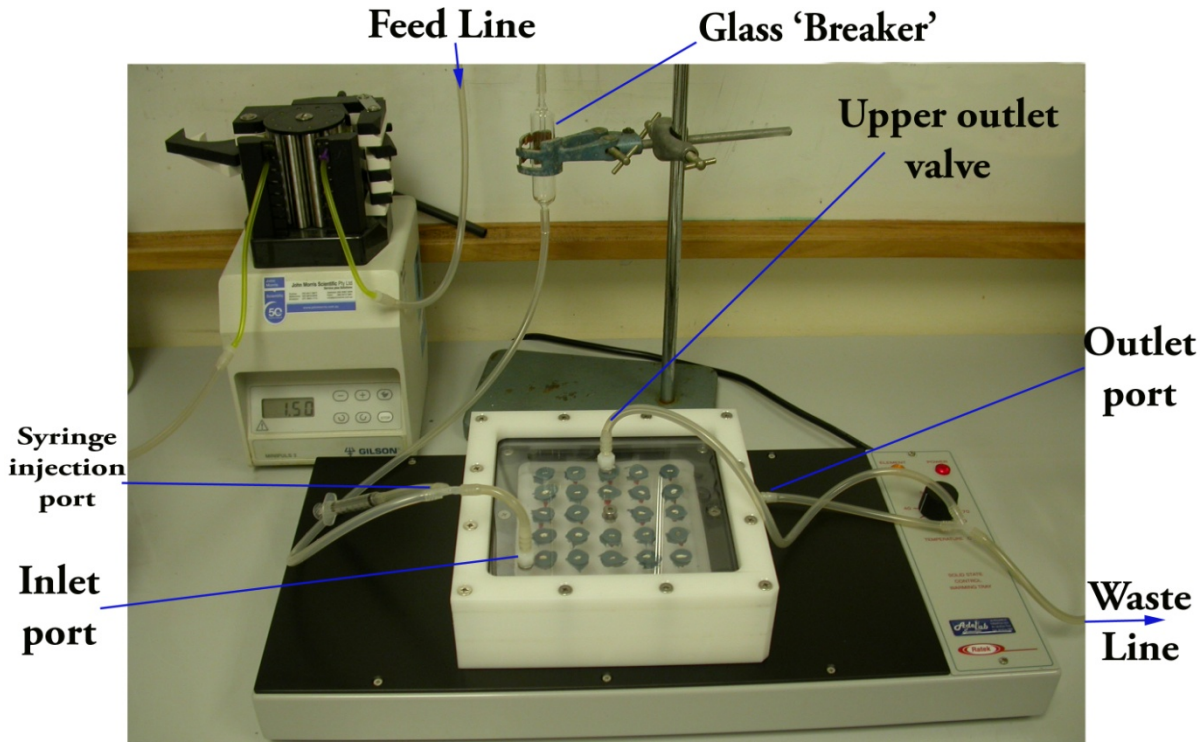


Figure 6. Photograph of the experimental apparatus.
Growth medium was pumped into the flow cell through silicon tubing labels 'Feed line'. The 'feed line' had a glass 'breaker' between the peristaltic pump and the flow cell to prevent bacteria growth back into the nutrient reservoir. Inlet and outlet ports are labelled. The 'syringe injection port' used for the inoculation of *E. faecalis,* is labelled.

To confirm sterility, the flow cell was left for 24 hours at 37°C. Following confirmation of sterility (the absence of turbidity), the flow cell was inoculated with 20 mL of an overnight culture of *E. faecalis* (V583 ATCC 700802) through a syringe injection port located in the medium feed line.

Twenty four hours after the flow cell was inoculated, the peristaltic pump was activated and THB was pumped into the flow cell at 23 mL/hr (dilution rate of 0.038 hr^{-1}) (Seet 2010). THB from the waste vessel was routinely checked by Gram staining and plating onto bile aesculin agar to confirm the presence of *E. faecalis* and absence of contamination. The flow cell was run with a continuous flow of THB for four weeks to allow a biofilm to grow in the root canal system.

After four weeks, the flow cell was drained of media and aseptically opened. The coronal openings of the 24 roots within the flow cell were sealed with Cavit (3M ESPE, Seefeld, Germany) and two coats of nail varnish. The apical foramina were sealed with cyanoacrylate (Loctite, Henkel, Düsseldorf, Germany). The flow cell was filled with 4% NaOCl (Endosure, Dentalife, Ringwood, Australia) and the teeth were immersed for one hour to remove any bacteria on the root surfaces (Plutzer 2009). Placing Cavit or cyanoacrylate prevented NaOCl from accessing the root canal system. The flow cell was then rinsed with 1 L of 5% sodium thiosulphate to deactivate the effects of NaOCl, and prevent any residual anti-microbial action.

2.6 Treatment Groups

Immediately prior to treatment, the Cavit was removed from the coronal openings with a sterile spoon excavator. The apical foramina were left sealed to simulate the clinical setting and to create a closed-end channel allowing the potential for a vapour lock effect. At all times the tooth roots were handled with sterile gauze and sterile instruments. Individual treatment groups used in Studies 1, 2 and 3 are presented in Sections 3, 4 and 5 respectively.

2.7 Crushed Tooth Samples

After treatment, roots were removed from the flow cell with sterile extraction forceps and placed into a sterile custom-made tooth crushing device (Seet 2010). This device consisted of a sterile brass cylinder and piston (Figure 7). The tooth sample was placed inside the cylinder. The piston was inserted into the cylinder, a hammer was used to strike the piston three times. The piston was removed and 2 mL of 0.9% sterile saline was added to the cylinder containing the crushed tooth fragments. The contents of the cylinder were then placed into a sterile tube.

The piston and cylinder were sterilised between samples with a 20 mL rinse of 70% ethanol, followed by 100 mL of sterile 0.9% saline.

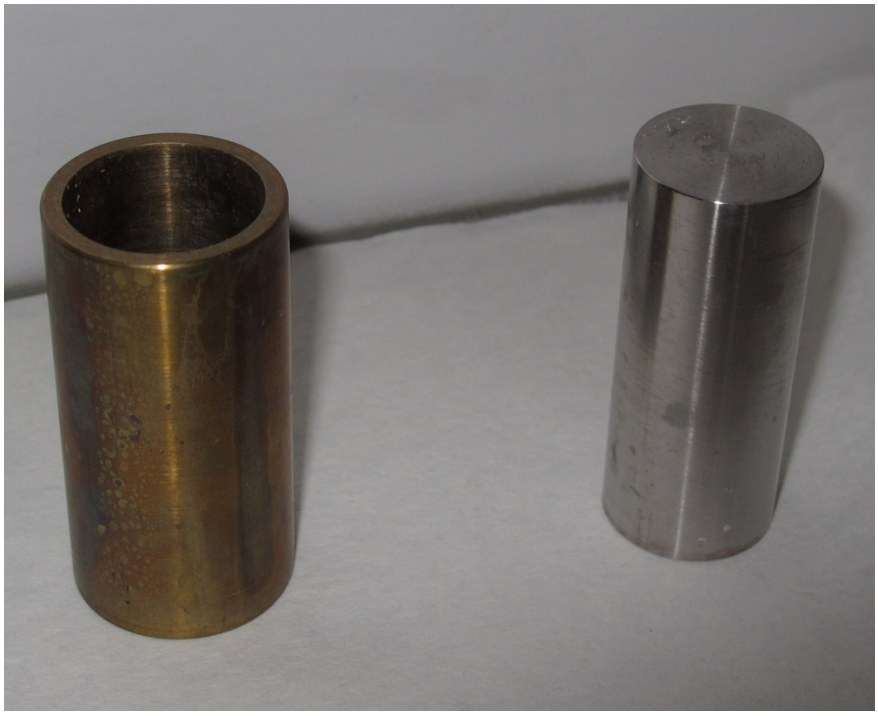


Figure 7. Photograph of the tooth crushing device.
Brass cylinder (left of images) and piston (right of image) are shown. The tooth sample was placed inside the cylinder. The piston was inserted into the cylinder, a hammer was used to strike the piston three times.

2.7.1 Cellular Viability

The tubes containing the crushed tooth samples in 2 mL of 0.9% saline were vortexed for 30 seconds. Four ten-fold serial dilutions were prepared and 100 µL aliquots of each dilution were plated in duplicate onto THB agar plates. Plates were incubated aerobically at 37°C for 24 hours, and colony forming units (cfu) per mL were calculated.

2.7.2 Protein Concentration

The amount of bacterial biofilm within the root canal system was normalised by measuring the amount of protein released after sonication of the crushed root samples. Protein was measured with a Bradford assay. A 450 µL aliquot of the crushed tooth suspension was combined with 50 µL of 1M sodium hydroxide in an Eppendorf tube and boiled for 30 minutes. A 150 µL aliquot of each sample was placed into a microtitre plate well containing an equal volume of Coomassie Plus protein assay reagent (Pierce biotechnology, Roskford, Illinois, USA). Each sample was tested in duplicate. The microtitre plate was then shaken for five minutes, and the absorbance read at 595 nm, using a microplate reader (Bio-Tek Instruments, Winooski, Vermont, USA). Standards containing bovine serum albumin were assayed in parallel with the samples. Protein calculations were measured in µg/mL.

2.8 Scanning Electron Microscope Images

Samples from each of the experimental protocols used in the preliminary studies were prepared for SEM analysis. Whole root samples were fixed (1.25% glutaraldehyde, 4% paraformaldehyde and 4% sucrose in PBS, pH 7.2) for 24 hours prior to longitudinal sectioning. The root surface was notched with a diamond-coated disc conducted in a laminar flow hood, and the tooth root was split with a sterile plastic instrument. The split roots were placed in fixative for a further 24 hours, immersed in serial dilutions of ethanol, critical point dried, mounted on SEM specimen stubs and coated with carbon and gold sputter coater. Specimens were viewed on a field emission SEM (Phillips XL30, Eindhoven, Netherlands) at 10kV accelerating voltage. Images were recorded of the main canal lumen as well as images providing a cross-sectional view of dentinal tubules (Figure 8).

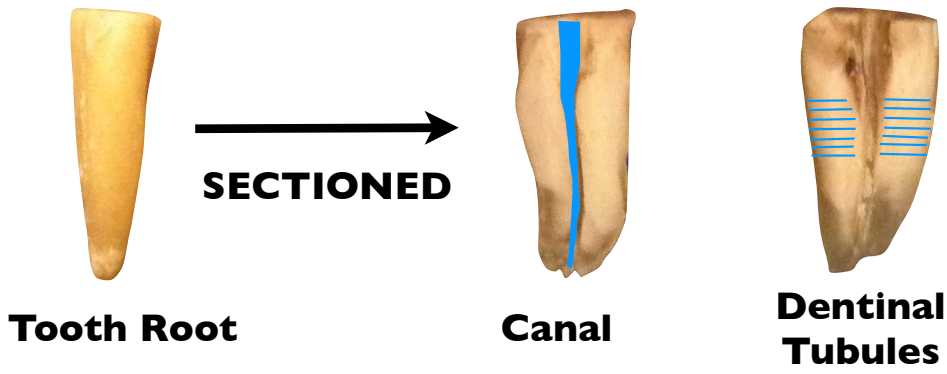


Figure 8. Photograph showing preparation of a sectioned tooth root for SEM imaging. The tooth root is shown on the left. The root is longitudinally sectioned. This allowed imaging of the main lumen surface (highlighted in blue in the middle image), as well as viewing of the dentinal tubules in cross-section (shown as blue lines on image on right).

3.0 Study 1. 'Antibacterial Efficacy of Sodium Hypochlorite and Er,Cr:YSGG Laser Energised Irrigation at 0.1 and 0.25 W. A Preliminary Study.'

3.1 Aim

The aim of the preliminary studies was to establish an appropriate Er,Cr:YSGG laser output power for laser energised irrigation. 'Study 1' aimed to determine if laser energised irrigation was more efficient at eradicating an *E. faecalis* biofilm *in vitro* and if so to determine the lowest laser output power to have an improved bactericidal effect. 'Study 1'

investigated the bactericidal effect of 0.5% and 4% NaOCl with and without laser energisation at the two lowest power settings available on the Er,Cr:YSGG laser.

3.2 Method and Materials

One flow cell was used in this preliminary study (n = 23). Details of the method and materials are explained in Section 2.0. The treatment protocols are detailed below and summarised in Table 1.

Group 1. No Treatment. (n = 4; 2 samples culture analysis and 2 samples SEM analysis).

Group 2. 0.5% NaOCl standard irrigation (SI). Standard irrigation of 5 mL of 0.5% NaOCl irrigation delivered with a 27 gauge Monoject (Kendall, Covidien, Mansfield, USA) needle. The needle tip was positioned as far apically as possible without binding. The 0.5% NaOCl was prepared by diluting one part 4% NaOCl (Endosure, Dentalife, Ringwood, Australia) with seven parts RO water. Treatment time was 1 minute (n = 3; 2 samples culture analysis and 1 sample SEM analysis).

Group 3. 4% NaOCl SI. As for group 2, but 4% NaOCl was used for irrigation (n = 1; culture).

Group 4. 0.5% NaOCl laser energised irrigation (LEI) at 0.1 W. Standard irrigation with 1.25 mL of 0.5% NaOCl for 10 seconds followed by laser energisation for 5 seconds, using an Erbium, chromium: yttrium-scandium-gallium garnet (Er,Cr:YSGG) laser (Waterlase, Biolase Technology, Irvine, USA). The laser power output was set at 0.1 W, with a pulse repetition rate of 20 Hz. The air or water function was not used. The laser tip, RFT 3 17 mm (Endolase, Biolase Technology, Irvine, USA), was positioned 5 mm from the orifice. The irrigant was not aspirated from the canal, so laser irradiation was always performed within an irrigant filled canal. The cycle was repeated four times. The total volume of irrigant was 5 mL and total time of treatment was 1 minute (n = 3; 2 samples culture analysis and 1 sample SEM analysis).

Group 5. 0.5% NaOCl LEI at 0.25 W. As for group 4, but laser output power was set at 0.25 W (n = 3; 2 samples culture analysis and 1 sample SEM analysis).

Group 6. 4% NaOCl LEI at 0.1 W. As for group 4, but 4% NaOCl was used as the irrigant (n = 3; 2 samples culture analysis and 1 sample SEM analysis).

Group 7. 4% NaOCl LEI at 0.25 W. As for group 5, but 4% NaOCl was used as the irrigant (n = 3; 2 samples culture analysis and 1 sample SEM analysis).

Group 8. Saline LEI at 0.25 W. As for group 5, but 0.9% saline was used as the irrigant (n = 3; 2 samples culture analysis and 1 sample SEM analysis). Due to insufficient numbers of roots available in the flow cell, standard irrigation and LEI at 0.1W were omitted.

	Saline	**0.5% NaOCl**	**4% NaOCl**
Standard irrigation		Group 2 (n = 3)	Group 3 (n = 1)
0.1 W LEI		Group 4 (n = 3)	Group 6 (n = 3)
0.25 W LEI	Group 8 (n = 3)	Group 5 (n = 3)	Group 7 (n = 3)

Table 1. Table summarising 'Study 1' treatment groups.
In addition, Group 1 (n = 4) received no treatment following biofilm growth over 28 days. LEI, Laser energised irrigation.

3.3 Results

3.3.1 Bacterial Viability

Results for 'Study 1' are presented in Table 2. Due to the small sample size, a statistical analysis was not conducted.

Group	**n**	**Median cfu/µg protein**
No treatment	2	8.45E+04
0.5% NaOCl SI	2	2.02E+03
4% NaOCl SI	1	1.11E+03
0.5% NaOCl + 0.1 W LEI	2	2.25E+04
0.5% NaOCl + 0.25 W LEI	2	1.49E+03
4% NaOCl + 0.1 W LEI	2	4.97E+02
4% NaOCl + 0.25 W LEI	2	8.30E+02
Saline + 0.25 W LEI	2	2.43E+04

Table 2. Viable *E. faecalis* expressed cfu/µg protein) following treatment protocols.
LEI, laser energised irrigation; SI, standard irrigation.

Irrigation with 0.5% and 4% NaOCl led to a *ca.* 10-fold reduction in cfu/µg protein compared to the group that received no treatment. Laser energised irrigation (0.1W and 0. 25

W) of 0.5% NaOCl did not reduce bacterial numbers lower than those achieved with standard irrigation of 0.5% NaOCl. Laser energised irrigation (0.1W and 0. 25 W) of 4% NaOCl led to *ca.* 10-fold reduction in cfu/µg protein compared to standard irrigation with 4% NaOCl. Laser energised saline (0.25 W) did not reduce bacterial numbers compared to the untreated group. Furthermore all groups treated with NaOCl with or with laser energisation had a greater reduction in cfu/µg protein compared to laser energised saline (0.25 W).

3.3.2 SEM Analysis

Scanning electron microscope images taken at 4 weeks revealed a confluent, dense and multilayered biofilm (Figure 9). Attachment between neighbouring cells and between cells and dentine is associated with an extra-cellular material. Fibres extending from bacteria appear to be aiding attachment to the dentine surface (Figure 10 (b)).

Bacterial penetration of dentinal tubules was present to varying depths. Bacteria were consistently visualised in the dentinal tubules 10-20 µm from the canal (Figure 11). Bacteria had also penetrated further (Figure 12). Bacterial numbers within tubules varied, some tubules were densely colonised with bacteria while bacteria were undetectable in adjacent tubules (Figure 13).

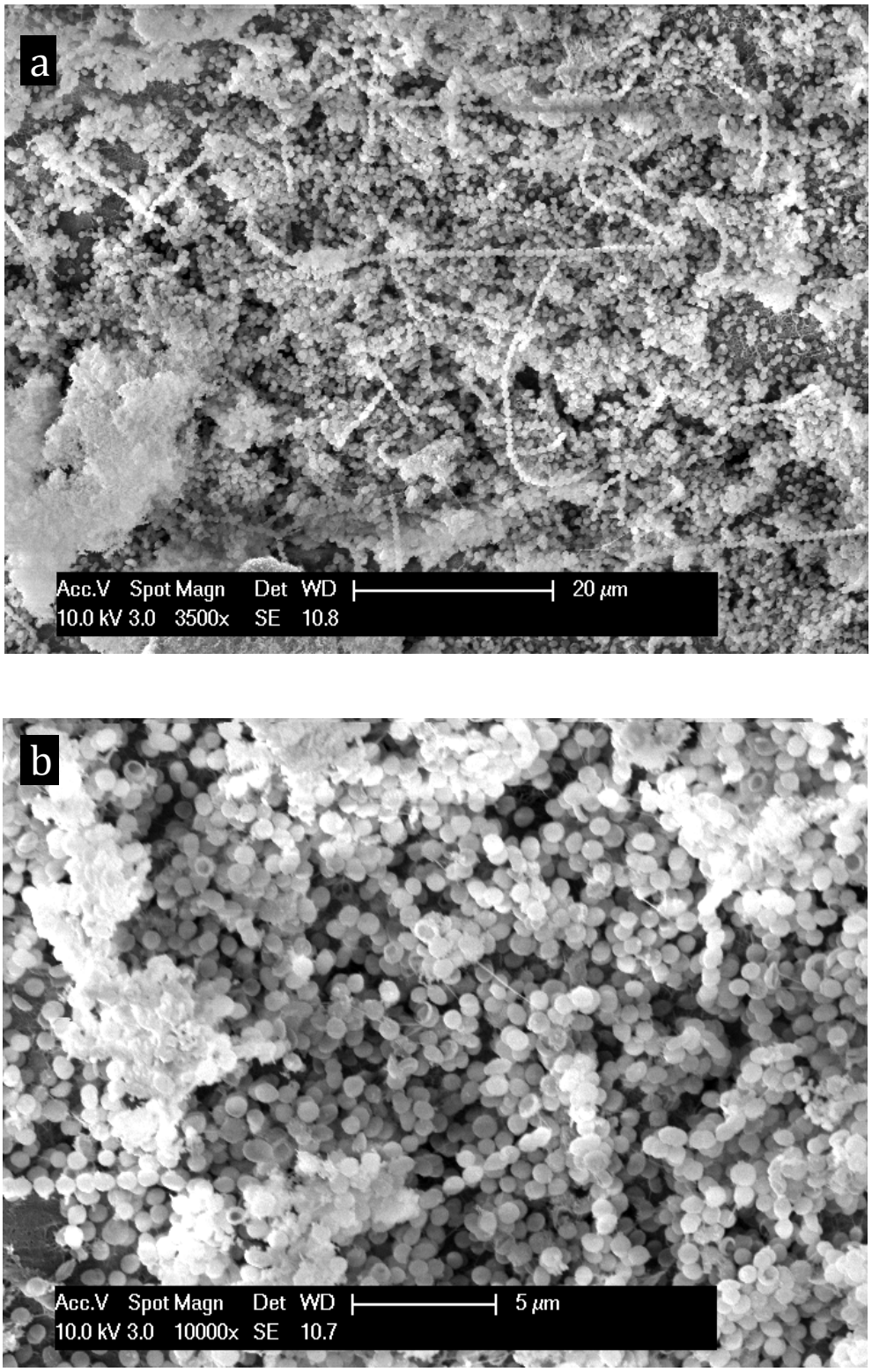


Figure 9. SEM image of 4 week biofilm (no treatment) within the root canal.
A confluent, dense and multilayered biofilm is evident. Image (a) 3 500 x magnification and (b) 10 000 x magnification.

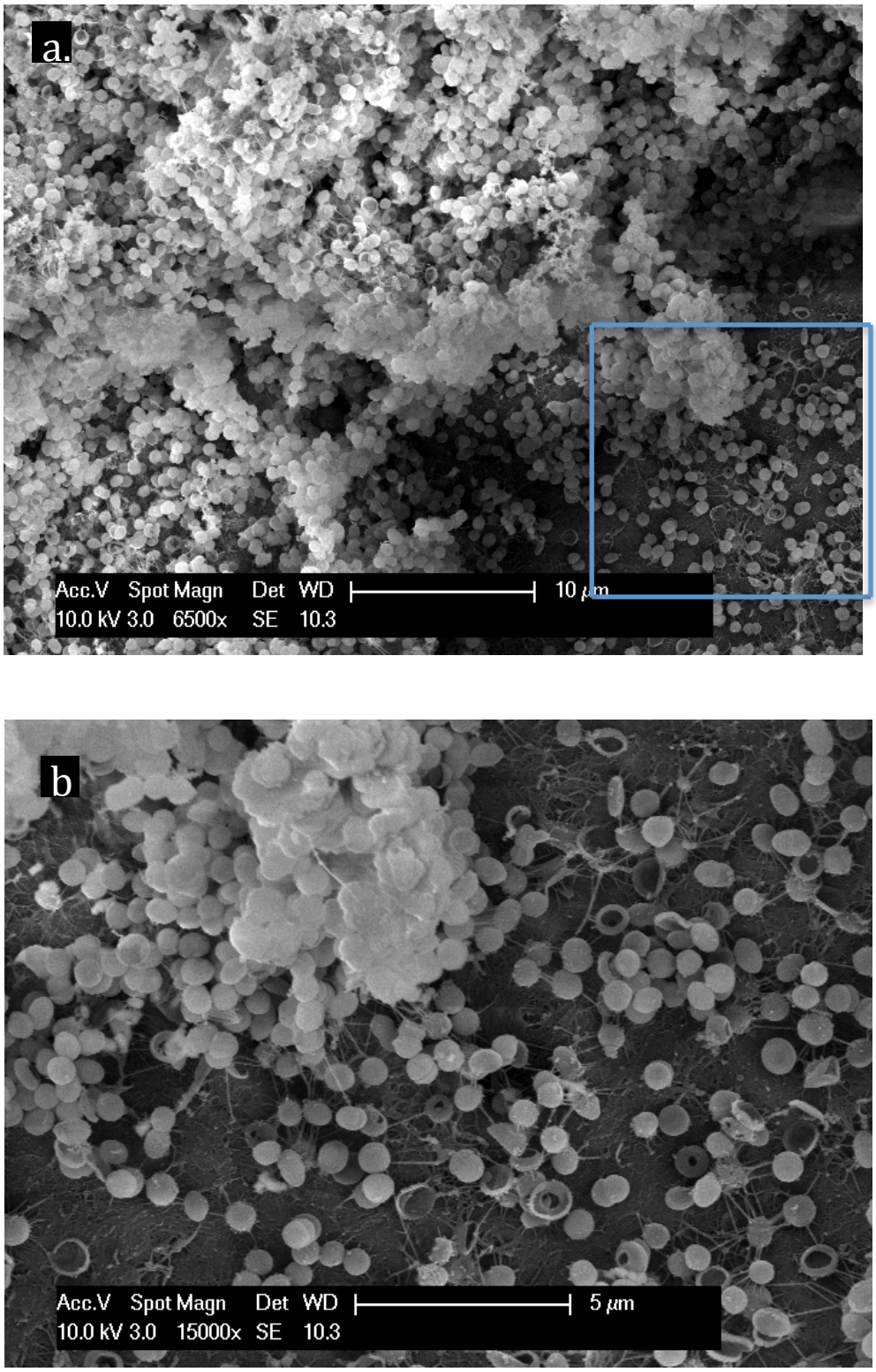


Figure 10. SEM image of 4 week biofilm (no treatment group) within the root canal. Attachment between neighbouring cells and between cells and dentine is associated with an extra-cellular material. Image (a) 6 500 x magnification, the blue rectangle is enlarged in (b) 15 000 x magnification.

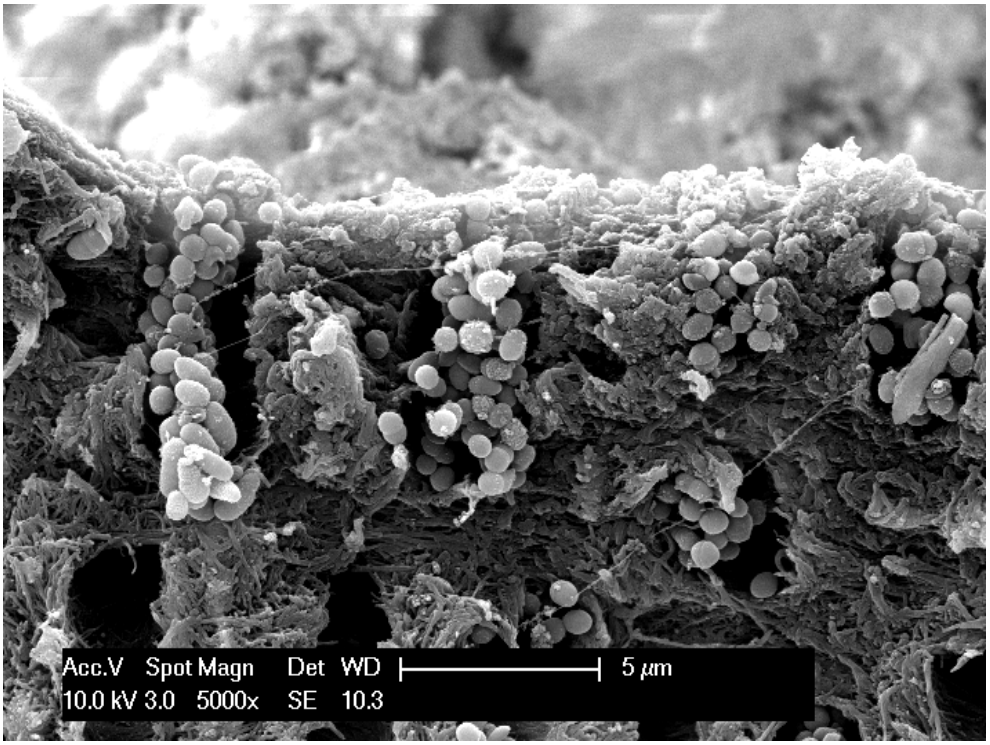


Figure 11. SEM image displaying a cross-sectional view of dentinal tubules.
Bacteria were consistently visualised in the dentinal tubules 5-10 µm from the canal (5 000 x magnification).

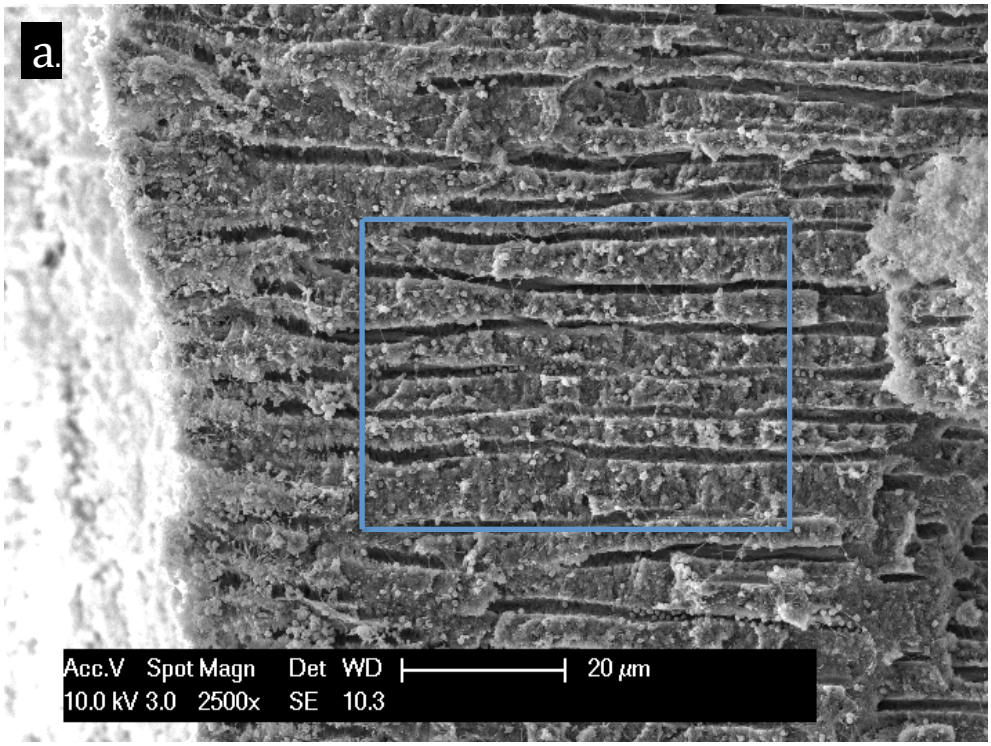
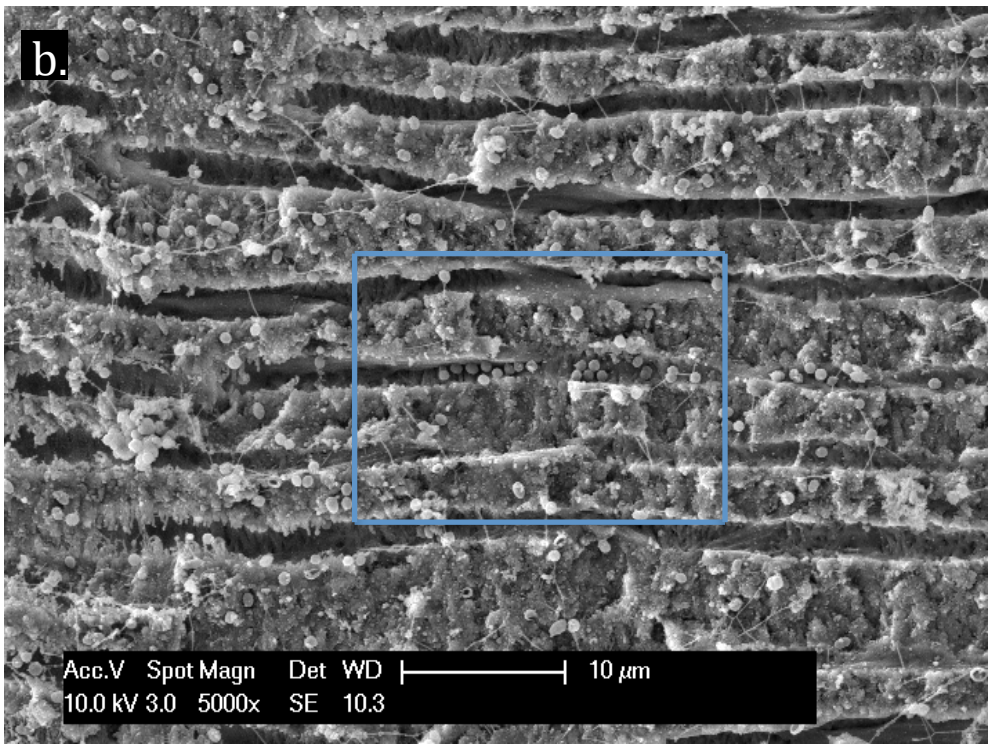


Figure 12 (a). SEM image displaying a cross-sectional view of dentinal tubules containing *E. faecalis*.
The main lumen is on the left side of the image. Image (a) 2 500 x magnification, blue box is further magnified in Figure 12 (b).



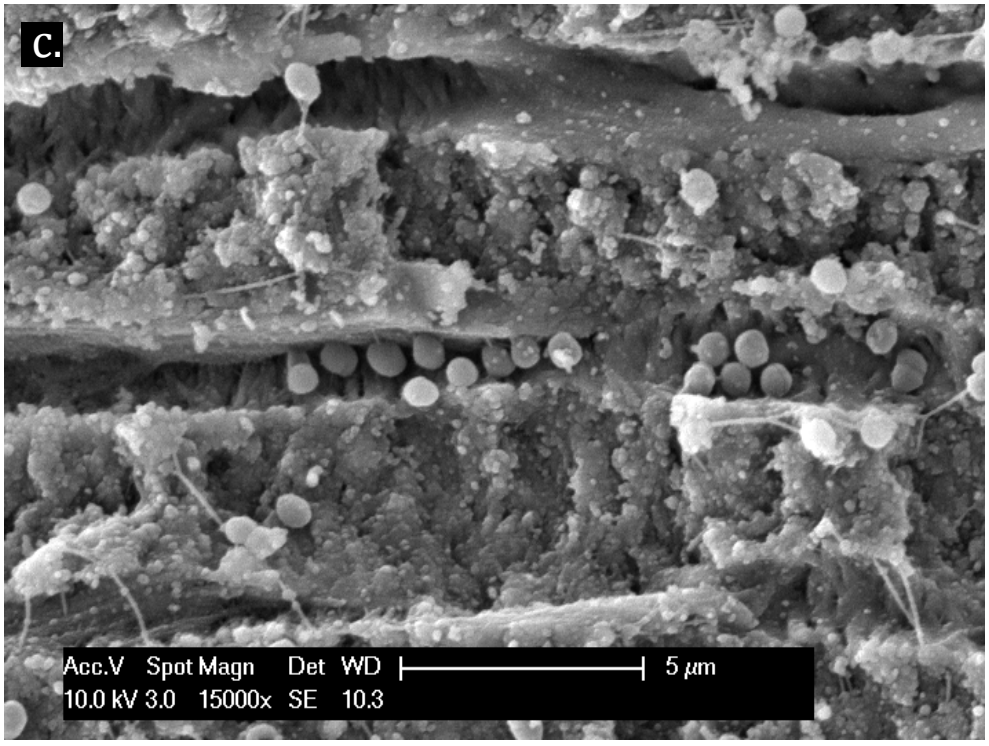


Figure 12 (b) & (c). SEM image displaying a cross-sectional view of dentinal tubules containing *E. faecalis*.
The main lumen is on the left side of the image. (b) (5 000 x magnification). The blue box is enlarged in image (c) 15 000 x magnification.

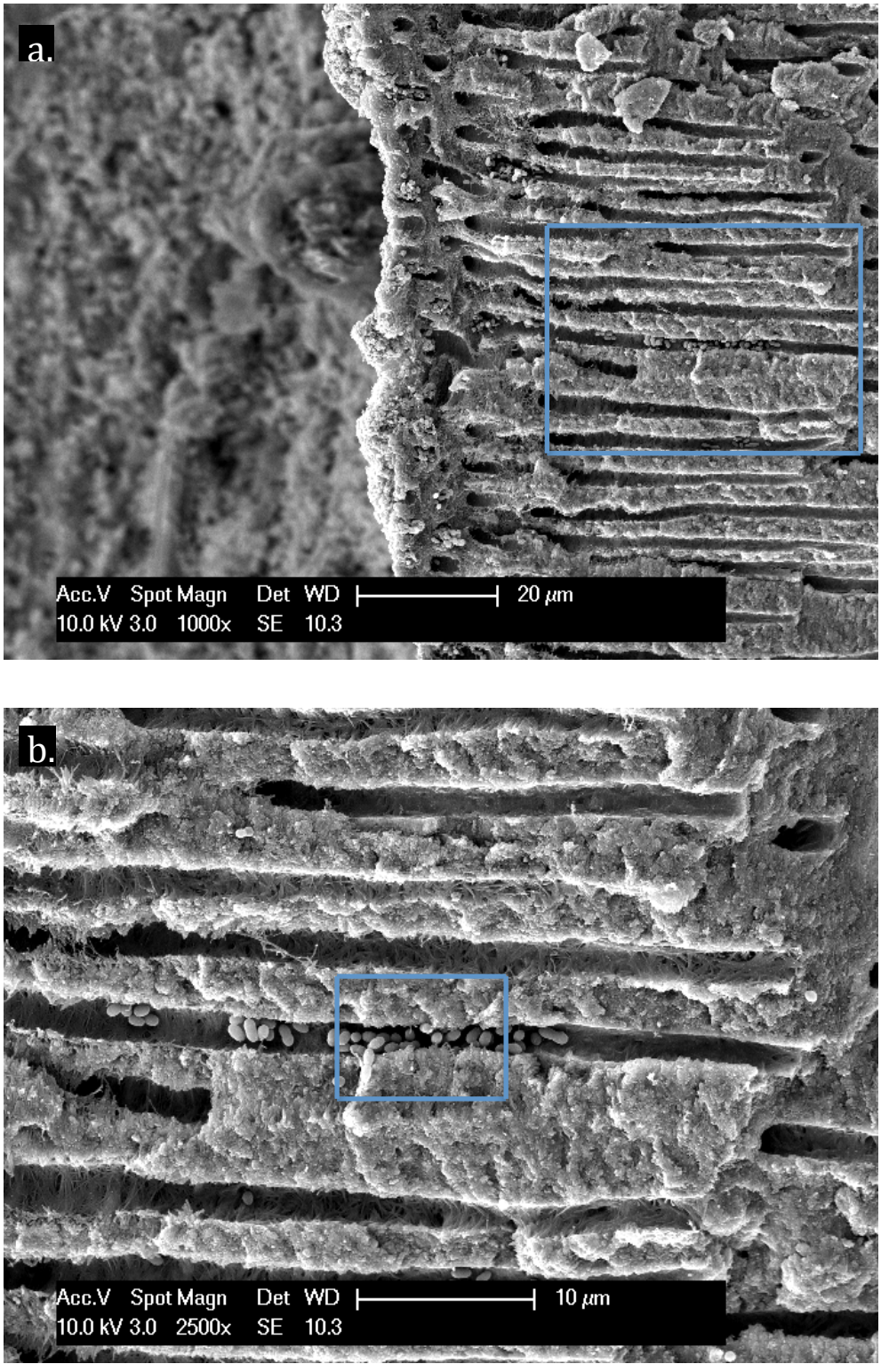


Figure 13 (a) & (b). SEM image displaying a cross-sectional view of dentinal tubules containing *E. faecalis*.
The main lumen is on the left side of the image. Image (a) 1 500 x magnification, the blue box in (a) is enlarged in (b) (2 500 x magnification).

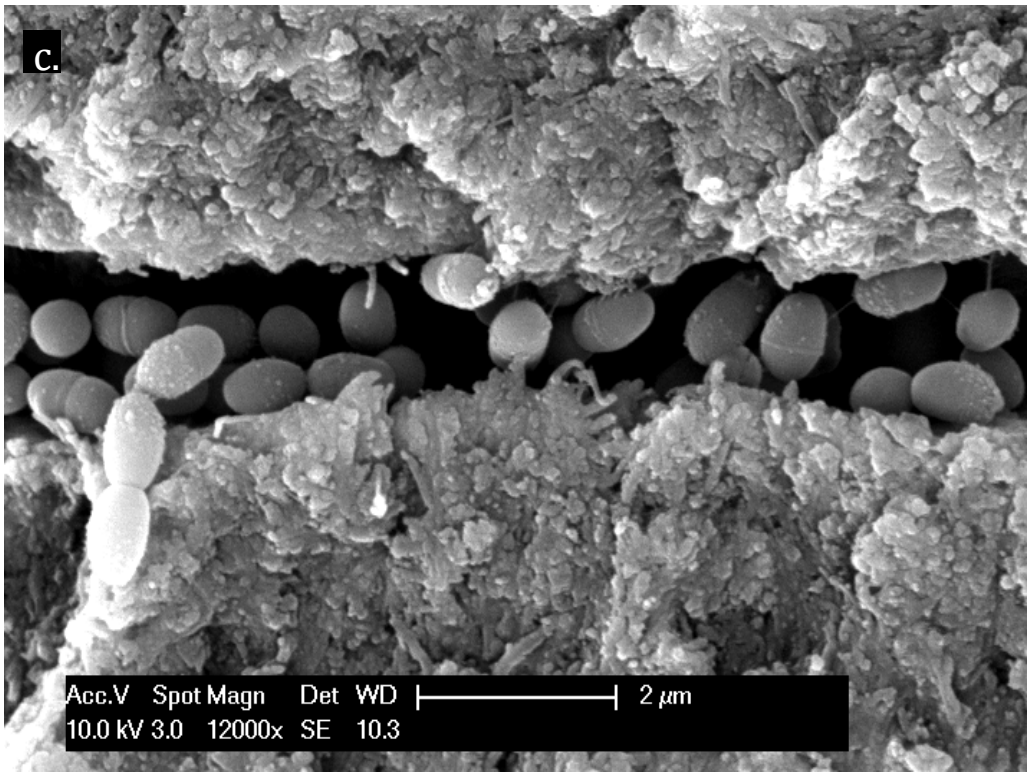


Figure 13 (c). SEM image displaying a cross-sectional view of dentinal tubules containing *E. faecalis*.
The main lumen is on the left side of the image. The blue box in Figure 13 (b) is enlarged in image (c) 12 000 x magnification.

3.4 Discussion

This study consisted of one flow cell containing 23 tooth roots. Both viable counts and SEM image analysis confirmed the establishment of an *E. faecalis* biofilm within the root canal system of the extracted teeth.

The sample size in each group was low and a statistical analysis was not conducted. It was observed that one minute of irrigation with either 0.5% or 4% NaOCl led to a *ca.* 10-fold reduction in cfu/μg protein compared to the no treatment group, however considerable numbers of *E. faecalis* (*ca.* 10^3 cfu/μg protein) still remained. This is not surprising given a confluent, dense and multilayered biofilm with penetration of dentinal tubules was established, instrumentation of the canal was not performed, and irrigation was conducted for only one minute. It was decided therefore to increase the treatment time from one minute of irrigation to two minutes for 'Study 2'.

Laser output power of 0.1 W did not appear to increase the bactericidal effect of either 0.5%, or 4% NaOCl compared to standard irrigation. To further investigate the cavitational

effects of LEI at different power settings, an *in vitro* investigation utilising a dye and an artificial root canal model was used (Section 3.5).

3.4.1 Laser Energised Saline

Although the sample size was small, laser energised saline (0.25W) did not appear to be an effective antibacterial protocol and all groups treated with NaOCl had superior bacterial reduction. This finding was in agreement with both Seet *et al.* (*in press*) and Pedullà *et al.* (2012).

Saline is not a contemporary routine endodontic irrigant as it is not an effective antibacterial agent (Byström & Sundqvist 1981; Shuping *et al.* 2000), it does not have tissue dissolution properties (Trepagnier *et al.* 1977) nor does it remove the smear layer (Baumgartner *et al.* 1984). Despite these limitations, laser energised saline has been shown to have improved smear layer removal ability (DiVito *et al.* 2012) and antibacterial effect (Pedullà *et al.* 2012; Seet *et al. in press*) compared to standard irrigation with saline. The improved performance of laser energised saline can be attributed to the turbulence and flushing action created within the irrigant via a cavitational effect.

Seet *et al.* (*in press*) has shown that laser energised irrigation of saline was superior to standard irrigation with saline. Laser energised saline disrupted the biofilm but areas of dense aggregates of *E. faecalis* were still present. Despite the improved performance of laser energised saline, standard irrigation with 4% NaOCl was a superior bactericidal agent.

In agreement with Seet *et al.* (*in press*), Pedullà *et al.* (2012) found a statistical difference in cfu counts between saline irrigation and laser energised saline. Again, standard irrigation with NaOCl was a statistically significant more effective treatment than laser energised saline.

As sodium hypochlorite irrigation is an established protocol and is superior to laser energised saline, laser energised saline is not a clinically relevant protocol. Therefore, laser energised saline was not further investigated in Study 2 or Study 3.

3.5 Study 1B. 'Fluid Movement Created by LEI within an Artificial Root Canal Model.'

3.5.1 Aim

Laser energised irrigation (LEI) creates cavitation effects within an irrigant and high speed photography has shown the formation and rapid implosion of vapour bubbles (de Groot *et al.* 2009; Matsumoto *et al.* 2011). This creates turbulence within an endodontic irrigant such as EDTA or NaOCl and it is hypothesised that LEI facilitates the access of the irrigant to those parts inaccessible with a standard needle irrigation technique, thus leading to an increased antibacterial effect. The results from Study 1 (Section 3.3) displayed no or minimal increase in bactericidal effect of LEI at 0.1 W or 0.25 W output power with an Er,Cr:YSGG laser compared to standard irrigation. The aim of this study was to investigate the ability of cavitational effects of different laser output powers to induce fluid movement within an artificial root canal model.

3.5.2 Methods and Materials

An artificial acrylic root canal was prepared to an apical size 40 with 0.06 taper. The canal was filled with sterile water. An aspiration technique was used to remove a volume of sterile water from the canal that equated to 6 mm of height as measured from the canal orifice. Crystal violet dye (Oxoid, Victoria, Australia) was placed into the canal to a depth of 6 mm (as shown in Figure 14a).

The laser tip (RFT 3 17 mm (Endolase, Biolase Technology, Irvine, USA)) of an Erbium, chromium: yttrium-scandium-gallium garnet (Er,Cr:YSGG) laser (Waterlase, Biolase Technology, Irvine, USA) was positioned 5 mm apically from the orifice. The air and water function was not used. The laser was activated for 20 seconds at 0.1 W, with a pulse repetition rate of 20 Hz.

Following this, the dye was not replenished. The laser was then activated for 20 seconds at 0.25 W, with a pulse repetition rate of 20 Hz, using the same technique as described previously.

Following 0.25 W of laser energisation, again the dye was not replenished (as it had not been displaced from the canal). The laser was then activated for 20 seconds at 0.5 W, with a pulse repetition rate of 20 Hz.

The movement of dye throughout the study was recorded in high definition video (1 920 x 1 080, 30 fps) with a Canon EOS 7D (Canon Australia, North Ryde, NSW).

3.5.3 Results

Figures 14 - 16 display images prior to and immediately after laser energy at 0.1, 0.25 and 0.5 W.

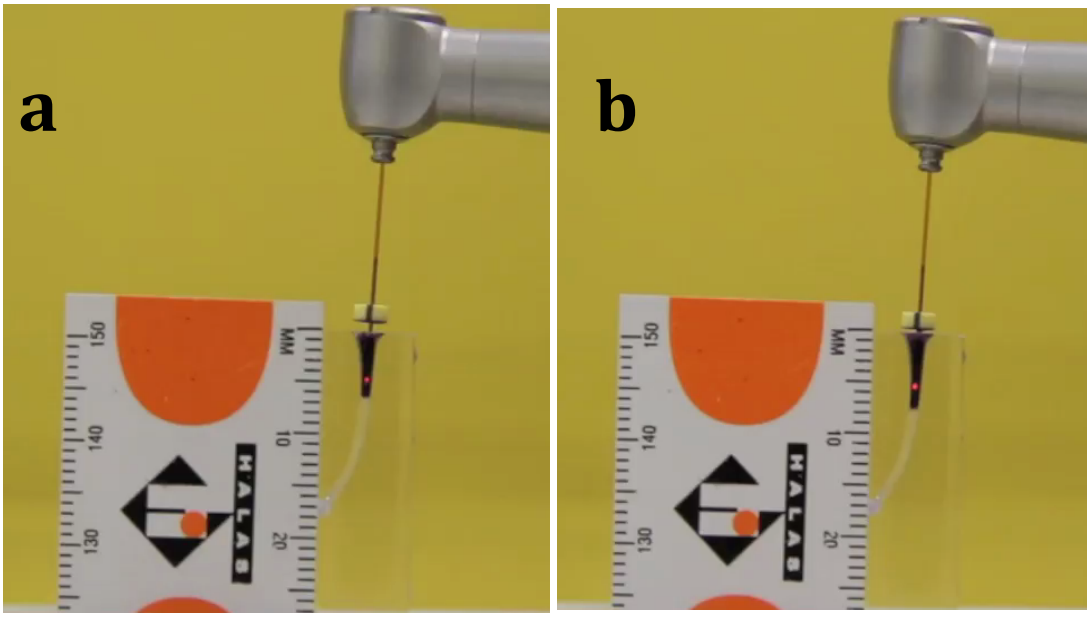


Figure 14. Dye movement following laser energisation at 0.1 W.
(a) Photograph taken prior to laser energising crystal violet dye (b) Photograph taken following 20 seconds of laser irradiation at 0.1 W.

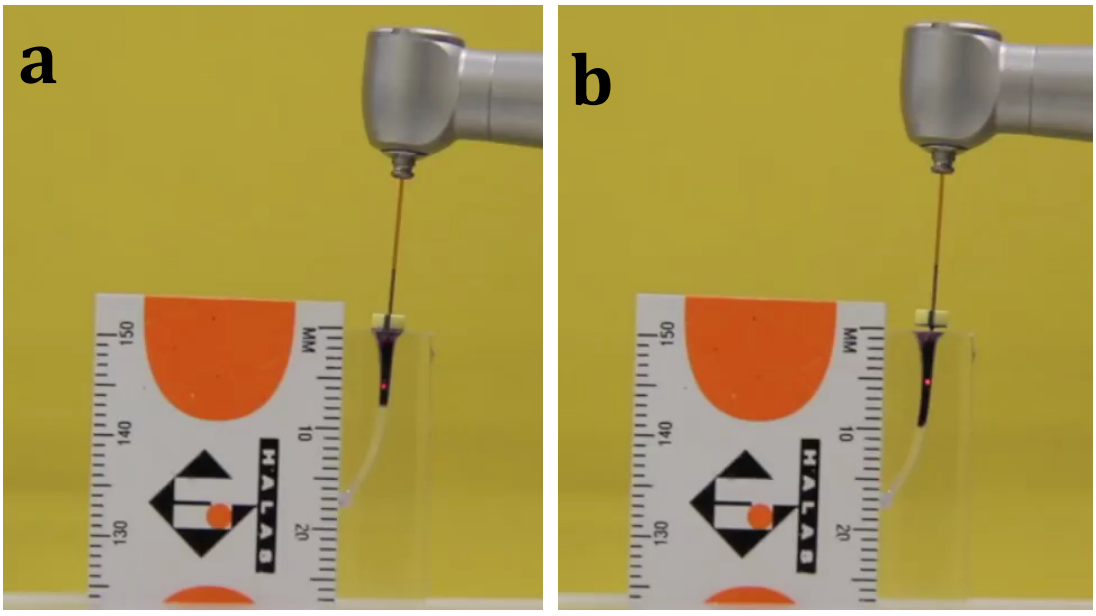


Figure 15. Dye movement following laser energisation at 0.25 W.
(a) Photograph taken following laser energisation at 0.1 W and immediately prior to laser energisation at 0.25 W. (b) Photograph taken immediately following 20 seconds of laser energisation at 0.25 W.

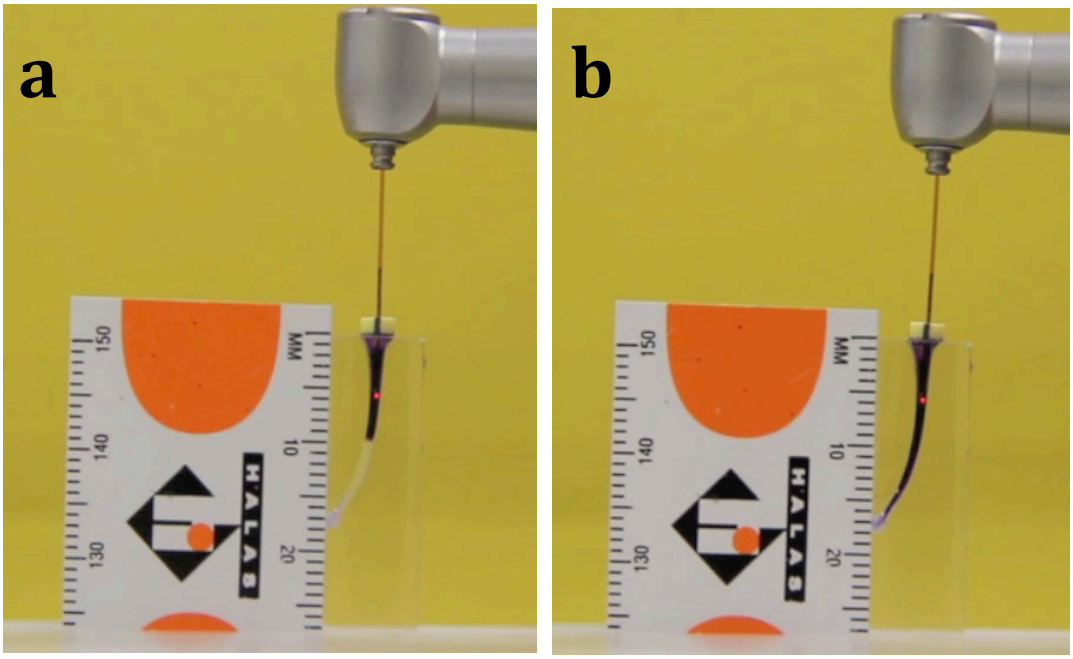


Figure 16. Dye movement following laser energisation at 0.5 W.
(a) Photograph taken following laser irradiation at 0.25 W and immediately prior to laser energisation at 0.5 W. (b) Photograph taken immediately following 20 seconds of laser energisation at 0.5 W. The dye can be seen occupying the entire root canal.

3.5.4 Discussion

This simple *in vitro* study is not without limitations. However it shows that at a laser output power of 0.1 W, minimal dye movement occurred within the artificial root canal model. Some movement of the dye was produced with a laser output power of 0.25 W, but the most dramatic effect was seen with 0.5 W. Although extrapolations should be made with caution it was anticipated that LEI at 0.1 W would be unlikely to improve the delivery of an endodontic irrigant to parts of the root canal system not accessible using a standard irrigation technique. Therefore it was decided that there was little merit in LEI at 0.1 W. Laser energised irrigation at 0.25 W, 0.5 W and 1 W was further investigated in 'Study 2'.

4.0 Study 2. 'Antibacterial Efficacy of Sodium Hypochlorite and Er,Cr:YSGG Laser Energised Irrigation at 0.25, 0.5 and 1 W. A Preliminary Study.'

4.1 Method and Materials

Two flow cells were run simultaneously (n = 48). Details of the method and materials are explained in Section 2.0. Treatment groups in 'Study 2' used a two minute treatment time instead of the one minute treatment time used in 'Study 1' as it was thought that increased exposure times could improve the antibacterial effect. The treatment protocols are detailed below and summarised in Table 3.

Group 1. No treatment. (n = 5; 4 samples culture analysis and 1 sample SEM analysis).

Group 2. 1% NaOCl standard irrigation (SI). Standard irrigation of 5 mL of 1% NaOCl irrigation delivered with a 27 gauge Monojet (Kendall, Covidien, Mansfield, USA) needle. The needle tip was placed as far apically as possible without binding. Treatment time was 2 minutes (n = 5; 4 samples culture analysis and 1 sample SEM analysis).

Group 3. 4% NaOCl SI. As for group 2, but 4% NaOCl was used for irrigation (n = 5; 4 samples culture analysis and 1 sample SEM analysis).

Group 4. 0.5% NaOCl laser energised irrigation (LEI) at 0.25 W. Standard irrigation of 1.25 mL of 0.5% NaOCl irrigation for 15 seconds followed by laser energisation for 15 seconds, using an Erbium, chromium: yttrium-scandium-gallium garnet (Er,Cr:YSGG) laser. The laser power output was set at 0.25 W, with a pulse repetition rate of 20 Hz. The air or water function was not used. The laser tip (RFT 3 17 mm), was positioned 5 mm apically from the orifice. The irrigant was not aspirated from the canal, so laser irradiation was always performed within an irrigant filled canal. The cycle was repeated four times. The total volume of irrigant was 5 mL and total time of treatment was two minutes (n = 3; 2 samples culture analysis and 1 sample SEM analysis).

Group 5. 0.5% NaOCl LEI at 0.5 W. As for group 4, but laser output power was set at 0.5 W (n = 5; 4 samples culture analysis and 1 sample SEM analysis).

Group 6. 0.5% NaOCl LEI at 1 W. As for group 4, but laser output power was set at 1 W (n = 3; 3 samples culture analysis).

Group 7. 1% NaOCl LEI at 0.25 W. As for group 4, but 1% NaOCl was used for irrigation (n = 3; 2 samples culture, 1 sample SEM analysis).

Group 8. 1% NaOCl LEI at 0.5 W. As for group 7, but laser output power was set at 0.5 W (n = 5; 4 samples culture analysis and 1 sample SEM analysis).

Group 9. 1% NaOCl LEI at 1 W. As for group 7, but laser output power was set at 1 W (n = 3; 3 samples culture analysis).

Group 10. 4% NaOCl LEI at 0.25 W. As for group 4, but 4% NaOCl was used for irrigation (n = 6; 5 samples culture analysis, 1 sample SEM analysis).

Group 11. 4% NaOCl LEI at 0.5 W. As for group 10, but laser output power was set at 0.5 W (n = 3; 2 samples culture analysis and 1 sample SEM analysis).

Group 12. 4% NaOCl LEI at 1 W. As for group 10, but laser output power was set at 1 W (n = 3; 3 samples culture analysis).

	0.5% NaOCl	**1% NaOCl**	**4% NaOCl**
Standard irrigation		Group 2 (n = 5)	Group 3 (n = 5)
0.25 W LEI	Group 4 (n = 3)	Group 7 (n = 3)	Group 10 (n = 6)
0.5 W LEI	Group 5 (n = 5)	Group 8 (n = 5)	Group 11 (n = 3)
1 W LEI	Group 6 (n = 3)	Group 9 (n = 3)	Group 12 (n = 3)

Table 3. Table summarising treatment groups.
In addition, Group 1 (n = 5) received no treatment following biofilm growth. LEI, Laser energised irrigation.

Crushed tooth samples, viability counts and protein concentrations were conducted for each tooth root. Details are provided in Section 2.7. Scanning electron microscope images were also analysed as described in Section 2.8.

4.2 Results

4.2.1 Bacterial Viability

Results are presented in Table 4. Due to the small sample size, a statistical analysis was not conducted.

Group	n	Median cfu/µg protein
No treatment	4	4.29E+04
1% NaOCl (SI)	4	6.48E+03
4% NaOCl (SI)	4	2.05E+02
0.5% NaOCl + 0.25 W LEI	2	8.59E+02
0.5% NaOCl + 0.5 W LEI	4	4.66E+02
0.5% NaOCl + 1 W LEI	3	2.77E+02
1% NaOCl + 0.25 W LEI	2	2.86E+03
1% NaOCl + 0.5 W LEI	4	6.23E+01
1% NaOCl + 1 W LEI	3	1.51E+01
4% NaOCl + 0.25 W LEI	5	2.46E+01
4% NaOCl + 0.5 W LEI	2	8.84E+01
4% NaOCl + 1 W LEI	3	2.01E+01

Table 4. Viable *E. faecalis* expressed in cfu/µg protein, following different treatment protocols.
LEI, Laser energised irrigation, SI, standard irrigation.

Irrigation with 1% NaOCl led to a *ca.* 10-fold reduction in cfu/µg protein compared to the group that received no treatment. Irrigation with 4% NaOCl led to a *ca.* 100-fold reduction in cfu/µg protein. Laser energised irrigation (0. 25 W, 0.5 W and 1 W) of 4% NaOCl was *ca.* 100-fold more effective than LEI of 0.5% NaOCl. Laser energised irrigation of 4% and 1% NaOCl produced comparable results at 0.5 W and 1 W, however at 0.25 W of energisation, 4% NaOCl reduced bacterial numbers to levels lower than those achieved with 1% NaOCl.

4.3 Comparison of Results from the Preliminary Studies (Study 1 & 2)

Combining the untreated control groups from Study 1 (n = 2) and Study 2 (n = 4), the median cell viability was 4.29×10^4 cfu/µg protein (range 3.47×10^3-1.66×10^5). This indicated that results from different flow cells were comparable.

Results for ‘Study 1’ and ‘Study 2’ are presented in Table 5. Due to the small sample size, a statistical analysis was not conducted.

	Study 1		Study 2	
Group	**n**	**median cfu/µg protein**	**n**	**median cfu/µg protein**
No treatment	2	8.45E+04	4	4.29E+04
0.5% NaOCl (SI)	2	2.02E+03		
1% NaOCl (SI)			4	6.48E+03
4% NaOCl (SI)	1	1.11E+03	4	2.05E+02
0.5% NaOCl + 0.1 W LEI	2	2.25E+04		
0.5% NaOCl + 0.25 W LEI	2	1.49E+03	2	8.59E+02
0.5% NaOCl + 0.5 W LEI			4	4.66E+02
0.5% NaOCl + 1 W LEI			3	2.77E+02
1% NaOCl + 0.25 W LEI			2	2.86E+03
1% NaOCl + 0.5 W LEI			4	6.23E+01
1% NaOCl + 1 W LEI			3	1.51E+01
4% NaOCl + 0.1 W LEI	2	4.97E+02		
4% NaOCl + 0.25 W LEI	2	8.30E+02	5	2.46E+01
4% NaOCl + 0.5 W LEI			2	8.84E+01
4% NaOCl + 1 W LEI			3	2.01E+01
saline + 0.25 W LEI	2	2.43E+04		

Table 5. Viable *E. faecalis* expressed in cfu/µg protein for ‘Study 1’ and ‘Study 2’, following different treatment protocols.
LEI, Laser energised irrigation, SI, standard irrigation.

4.3.1 Treatment Time

Following treatment protocols in ‘Study 1’ considerable numbers of viable *E. faecalis* (*ca.* 10^3 cfu/µg protein) still remained. This was not unexpected given that a confluent, dense and multilayered biofilm with penetration of dentinal tubules was established, instrumentation of the canal was not performed, and irrigation was conducted for only one minute. Treatment time was increased from one minute of irrigation in ‘Study 1’ to two minutes in ‘Study 2’, in an attempt to lead to further bacterial reduction.

Although it was not the aim of ‘Study 2’ to provide a comparison between one and two minutes of treatment time, a comparison can be made between three groups (4% NaOCl (SI),

0.5% NaOCl LEI at 0.25 W, 4% NaOCl LEI at 0.25 W). As the sample size was small, any comparison should be interpreted with caution. However, it was observed that two minutes of treatment time led to a further *ca.* 10 fold reduction in cfu/µg protein for the three treatment groups (Table 5).

4.3.2 Sodium Hypochlorite Concentration

Irrigation with either 0.5% (Study 1, 1 minute treatment time) or 1% NaOCl (Study 2, 2 minute treatment time) led to *ca.* 10-fold reduction in cfu/µg protein compared to the group that received no treatment (Figure 17). Irrigation with 4% NaOCl led to a *ca.* 100-fold reduction in cfu/µg protein (Figure 17).

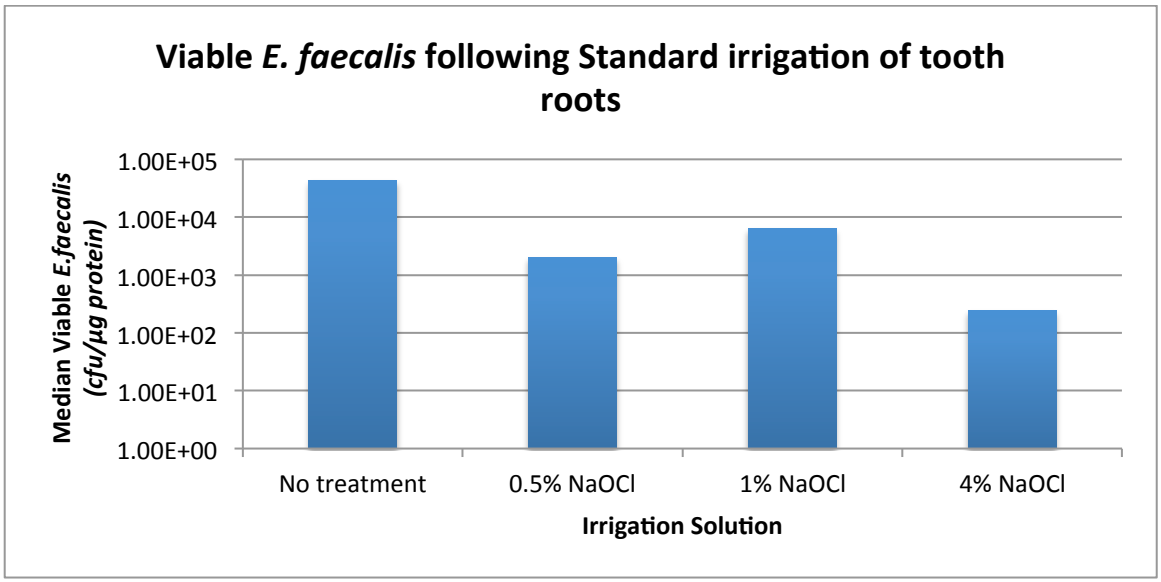


Figure 17. Viable *E. faecalis* (cfu/µg protein) in the no treatment group and following standard irrigation with 0.5% NaOCl, 1% NaOCl and 4% NaOCl.
*note 0.5% NaOCl SI, is from Study 1 and had 1 minute of treatment time. All other groups had two minutes of treatment time.

4.3.3 Laser Output Power

Laser output powers of either 0.1 W (Study 1) or 0.25 W (Study 2) did not appear to increase the bactericidal effect of either 0.5%, 1% or 4% NaOCl (Figure 18). Laser energisation of 0.5% or 1% NaOCl at a laser power of 0.5 W gave a 10-fold reduction in cfu/µg protein, compared to standard irrigation with either 0.5% or 1% NaOCl (Figure 18). Laser energisation of 4% NaOCl at 1 W lead to a 10-fold reduction in cfu/µg protein compared to standard irrigation with 4% NaOCl (Figure 18).

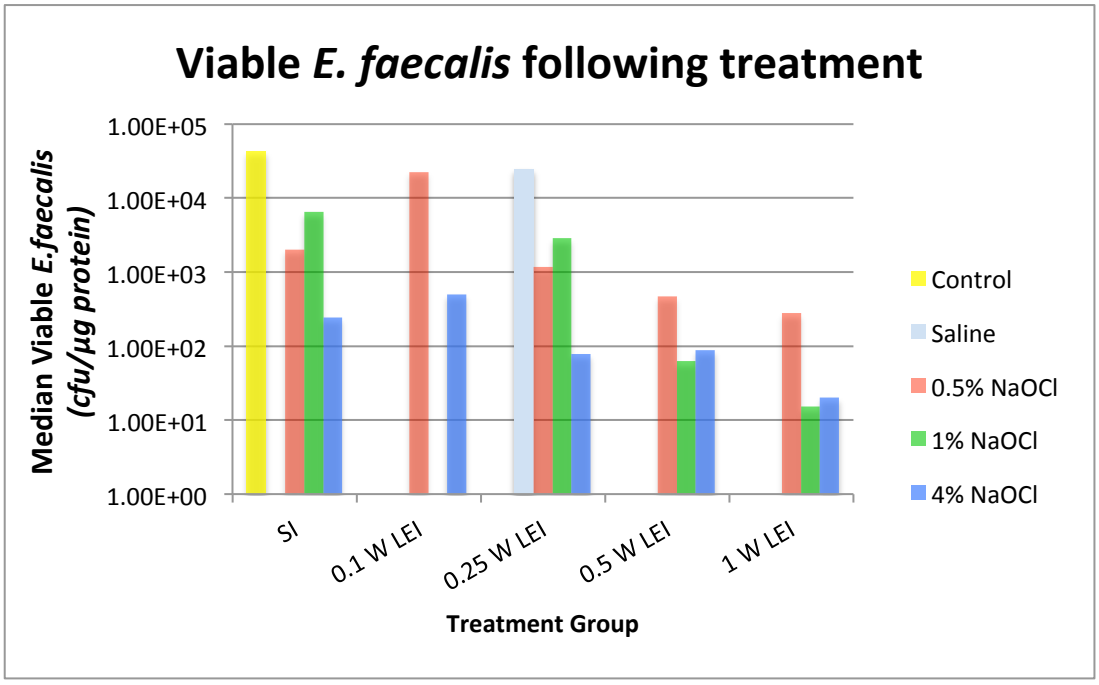


Figure 18. Viable *E. faecalis* (cfu/µg protein) following standard irrigation (SI) or laser energised irrigation (LEI) of irrigants (0.5%, 1% and 4% NaOCl) at a laser power of 0.1 W, 0.25 W, 0.5 W and 1 W.
*note 0.5% NaOCl at 0.1 W LEI, 4% LEI at 0.1W, 0.5% NaOCl SI, saline LEI at 0.25W are from Study 1 and had 1 minute of treatment time. All other groups had two minutes of treatment time.

4.4 SEM Image Analysis

4.4.1 Sodium Hypochlorite Concentration

Representative SEM images of the root canal system taken after standard irrigation with 0.5%, 1% and 4% NaOCl are shown in Figure 19. Standard irrigation with all concentrations of NaOCl led to a qualitative reduction in bacteria within the root canal system. After standard irrigation with 0.5% NaOCl, isolated *E. faecalis* biofilms remained in the root canal and within dentinal tubules (Figure 19 (a)). Following irrigation with 1% NaOCl, an *E. faecalis* biofilm could not be visualised, however, single bacterial cells remained within dentinal tubules (Figure 19 (b)). Similarly, the bacterial biofilm could not be detected following irrigation with 4% NaOCl, but again, *E. faecalis* potentially remained in dentinal tubules (Figure 19 c).

Cross-sectional views of the dentinal tubules of the no treatment group showed the presence of bacteria within 10-20 µm of the canal (Figures 11-13). Irrigation with 1% NaOCl reduced the number of bacteria identified within the tubules but some densely populated tubules remained (Figure 20). Irrigation with 4% NaOCl further reduced bacterial numbers within the tubules although isolated bacteria were still identified (Figure 21).

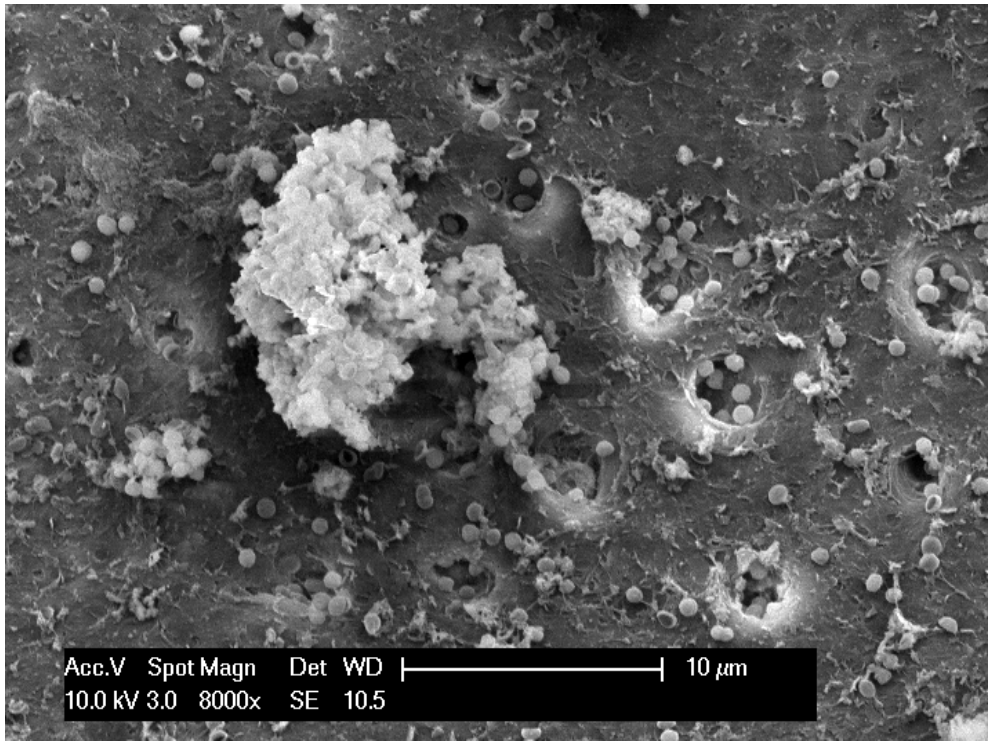


Figure 19 (a). SEM image following irrigation with 0.5% NaOCl (8,000 x magnification). Remnants of the bacterial biofilm remain as bacterial co-aggregates. In addition, *E. faecalis* cells appear within dentinal tubules.

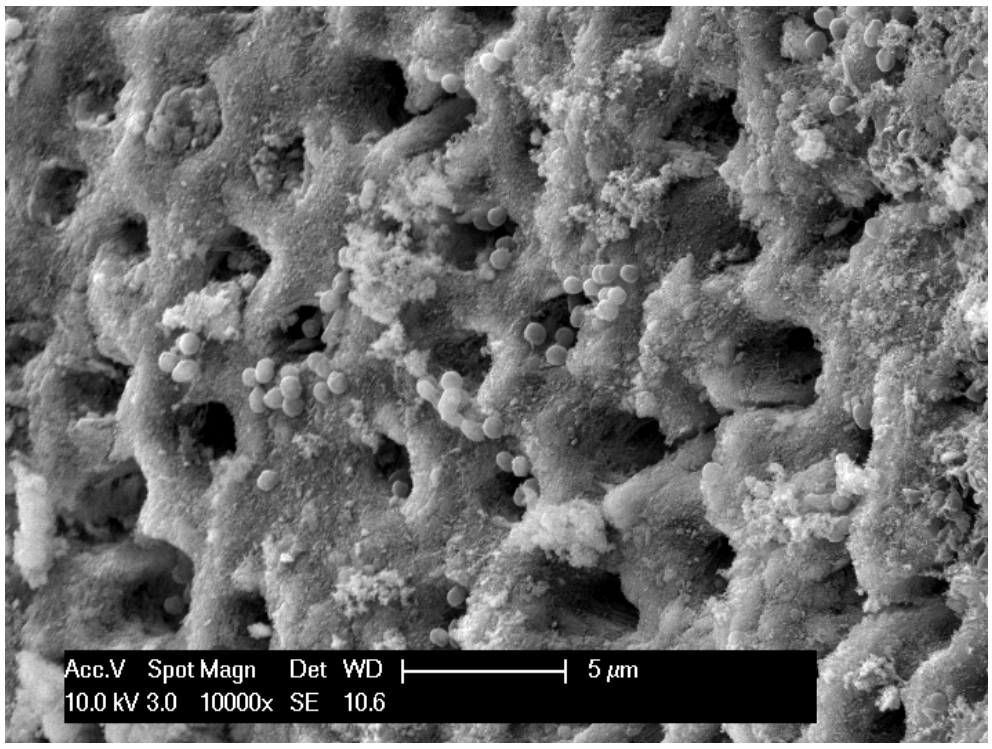


Figure 19 (b). SEM image of the root canal surface following irrigation with 1% NaOCl (10 000 x magnification).
A bacterial biofilm could not be visualised, however, single bacterial cells were clustered around dentinal tubules.

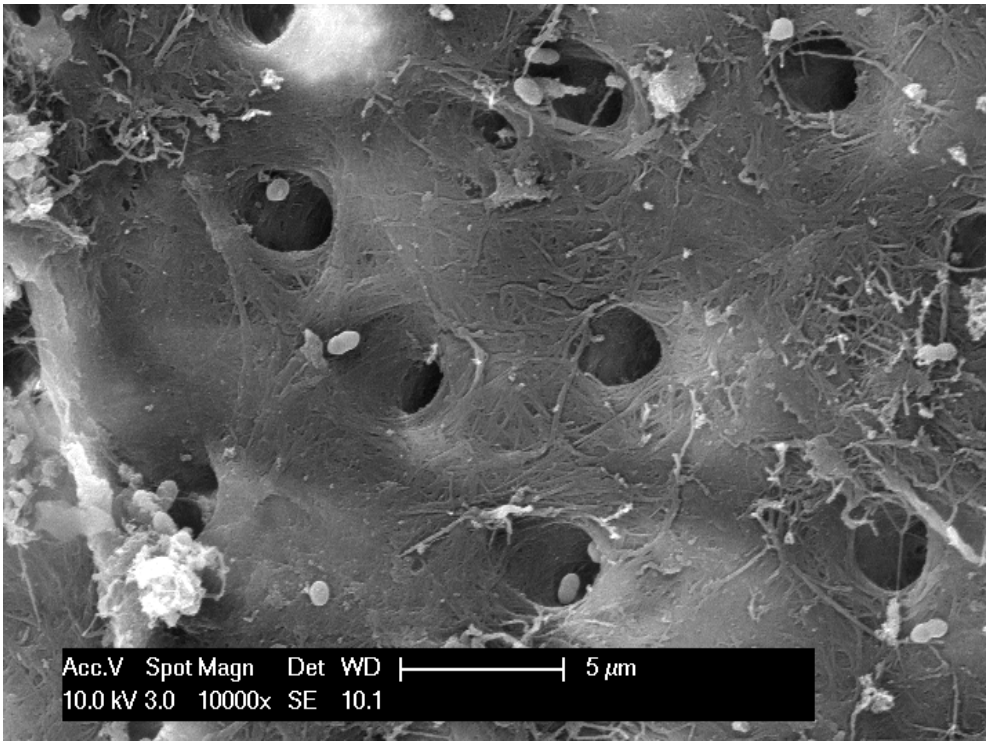


Figure 19 (c). SEM image following irrigation with 4% NaOCl (10 000 x magnification).
A bacterial biofilm could not be visualised, however, single bacterial cells were present around dentinal tubules.

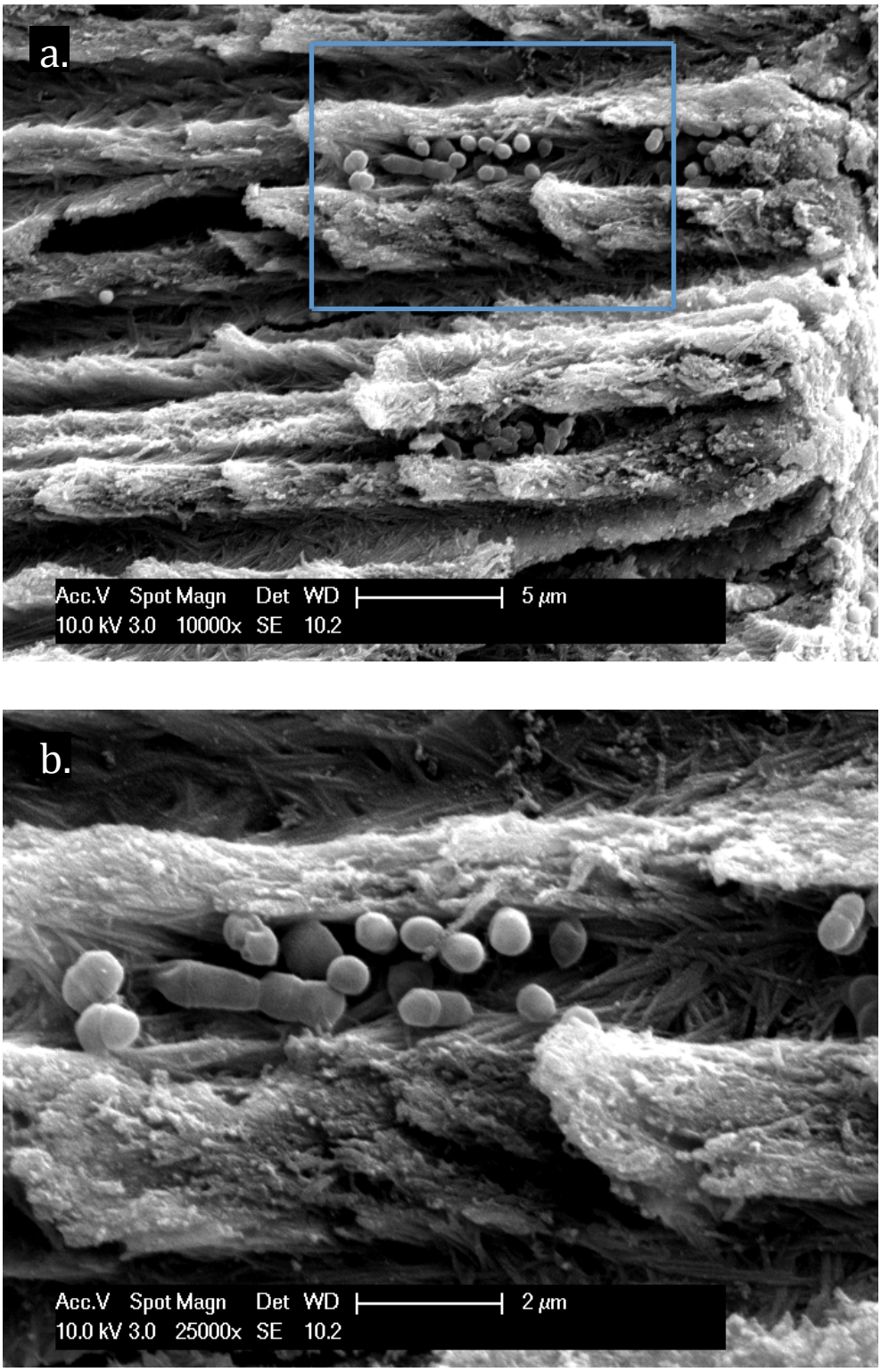


Figure 20 (a) SEM image of dentinal tubules in cross-section following irrigation with 1% NaOCl.
The canal lumen is on the right edge of the image. Irrigation with 1% NaOCl reduced the number of bacteria identified within the tubules, however some densely populated tubules were still identified within 5–10 µm of the lumen (10 000 x magnification). (b) SEM image is the area boarded in blue in (a) (25 000 x magnification).

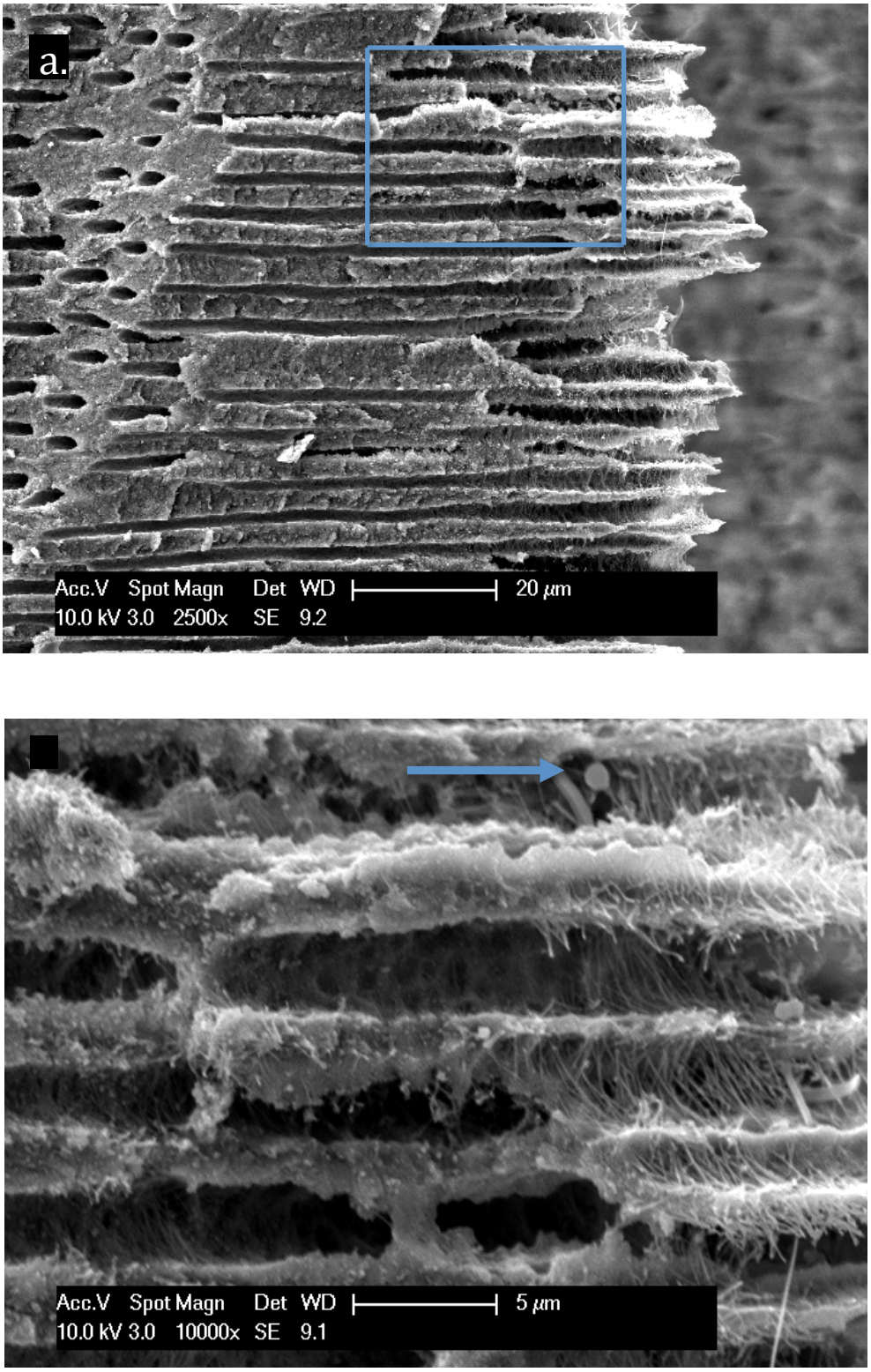


Figure 21 (a & b). SEM image of dentinal tubules in cross-section following irrigation with 4% NaOCl.
The canal lumen is on the right edge of the image. Irrigation with 4% NaOCl qualitatively reduced the number of bacteria that could be identified within the tubules, however isolated bacterial cells still remained within 5–10 µm of the canal lumen (2 500 x magnification). (b) SEM image is a magnification of the area boarded in blue in (a). An *E. faecalis* cell is shown by the arrow (10 000 x magnification).

4.4.2 Laser Output Power

A range of laser output powers were investigated (0.1 W, 0.25 W, 0.5 W and 1 W). Representative SEM images of 0.5% NaOCl treatment without laser irradiation and with laser irradiation at output powers of 0.1 W, 0.25 W and 0.5 W are shown in Figure 22. A difference was not observed between standard irrigation with 0.5% NaOCl and laser energised irrigation of 0.5% NaOCl with a power output of 0.1 W. Laser energised irrigation with output power of 0.25 W lead to a further reduction in bacterial numbers. After laser energised irrigation at 0.5 W, bacteria could not be identified within the root canal (Figure 22 (d)). At a laser power output of 0.5 W, energisation of 0.5%, 1% or 4% NaOCl, *E. faecalis* were seldom identified within dentinal tubules within close proximity to the root canal (Figure 23(a) and 24). Despite a noticeable reduction in bacterial numbers within the dentinal tubules with 0.5 W laser energisation, bacterial cells further from the root canal were still identified within dentinal tubules (Figure 23(b) and 24).

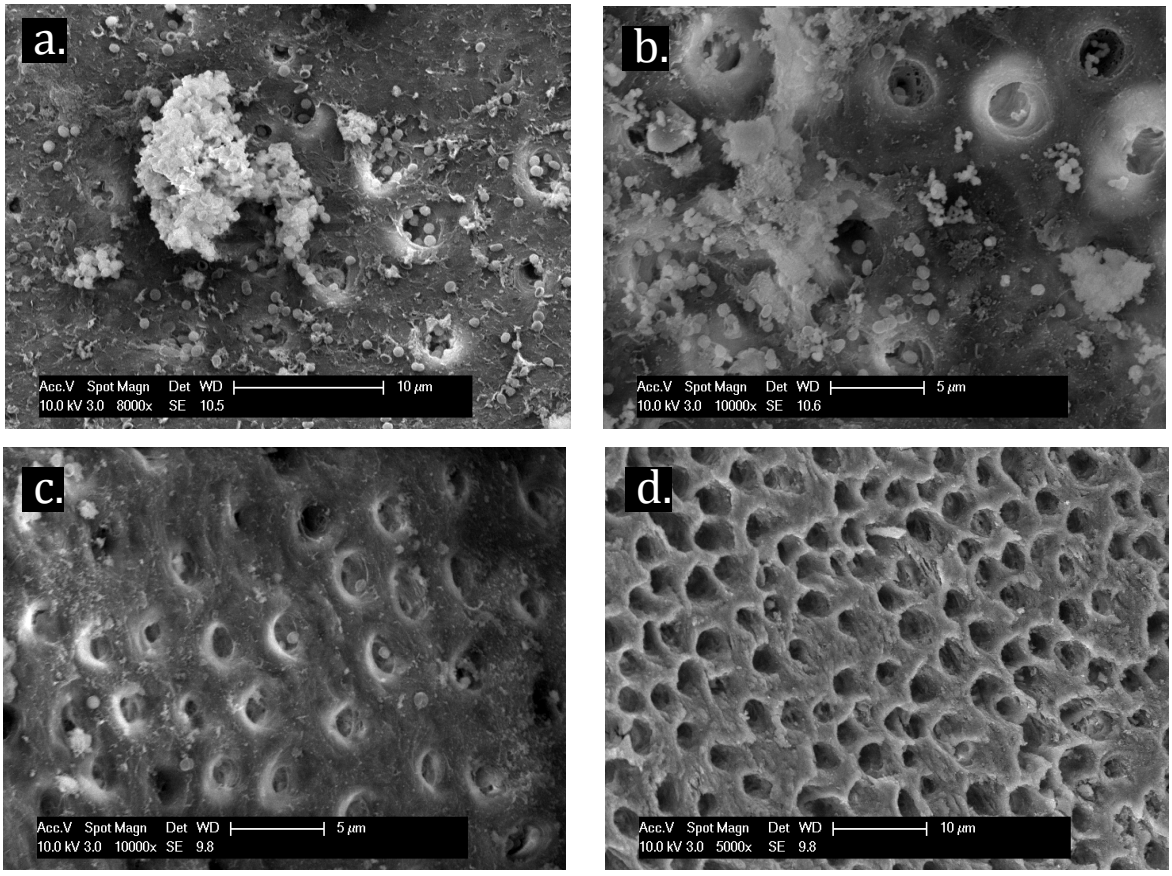


Figure 22 (a). Representative SEM images of 0.5% NaOCl treatment without laser energisation and with laser energisation at power outputs of 0.1 W, 0.25 W and 0.5 W.
(a) Standard irrigation with 0.5% NaOCl (8 000 x magnification). (b) Laser (0.1 W) energised irrigation of 0.5% NaOCl (10 000 x magnification). The image is from 'Study 1'. (c) Laser (0.25 W) energised irrigation of 0.5% NaOCl (10 000 x magnification). (d) Laser (0.5 W) energised irrigation of 0.5% NaOCl (5 000 x magnification).

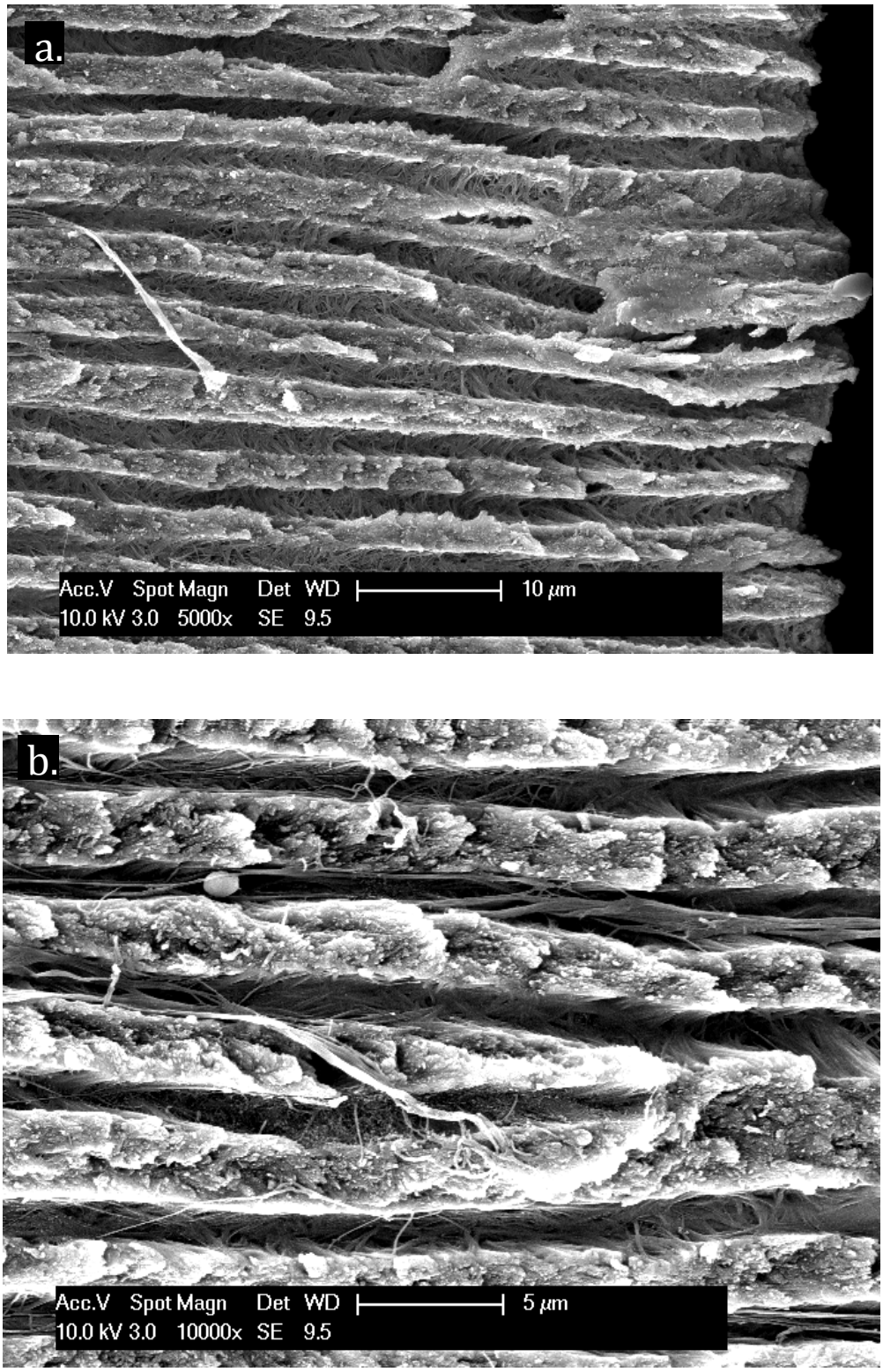


Figure 23. SEM image of dentinal tubules in cross-section, canal lumen is on the right side of image.
Following treatment with laser energised irrigation (0.5 W) of 0.5% NaOCl bacteria were not identified near the canal (a) (5000 x magnification n). Further along the dentinal tubules, bacteria could be identified (b) (10 000 x magnification).

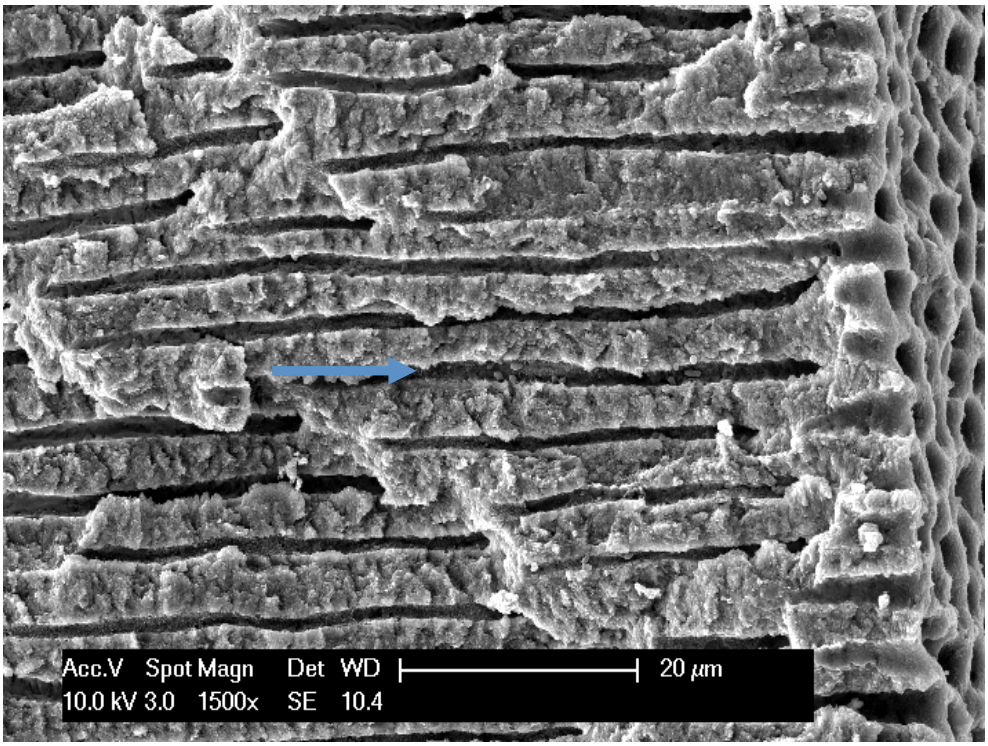


Figure 24. SEM image of dentinal tubules in cross-section, canal lumen is on the right side of image.
Following treatment with laser energised irrigation (0.5 W) of 0.5% NaOCl, the majority of dentinal tubules near the canal had no detectable bacteria. Although not all tubules were free of bacteria (arrow) (1 500 x magnification).

4.5 Discussion

The results from 'Study 1' and 'Study 2' showed that laser output powers of either 0.1 W and 0.25 W did not appear to increase the bactericidal effect of either 0.5%, 1% or 4% NaOCl (Figure 18). Laser energisation of 0.5% or 1% NaOCl at a laser power of 0.5 W gave a 10-fold reduction in cfu/µg protein, compared to standard irrigation with either 0.5% or 1% NaOCl (Figure 18). Although increasing the laser output power gave a general trend to increased antibacterial effect, the risk of irrigant extrusion is also increased. Laser energised irrigation at 0.5 W was the lowest output power to show a trend towards increased antibacterial efficacy compared to standard irrigation. Therefore, a power setting of 0.5 W was deemed most effective for laser energised irrigation. Laser energised irrigation at 0.5 W was further investigated in 'Study 3'.

5.0 Study 3. 'Efficacy of Sodium Hypochlorite and Er,Cr:YSGG Laser Energised Irrigation Against an *Enterococcus faecalis* Biofilm.'

5.1 Introduction and Aim

The aim of 'Study 3' was to establish the efficacy of eradicating an *E. faecalis* biofilm *in vitro* using several concentrations of NaOCl with and without Er,Cr:YSGG laser energised irrigation. The power setting used was 0.5 W as determined in 'Study 1 and 2". This study also used a sample size that allowed for statistical analysis of the quantitative results.

The null hypothesis was that there is no difference between a low and high concentration of NaOCl. Additionally, there is no difference between laser energised irrigation and standard irrigation.

5.2 Treatment Protocols

To this point it was unknown whether the positioning of tooth roots within the flow cell produced a significant change in the biofilm density in the root canal. To compare the effect of the positioning of the tooth roots within the flow cell and to allow a comparison between flow cells, each treatment group was equally represented from a defined zone in each flow cell. Each flow cell had the capacity for 24 tooth roots. Four flow cells were used in this study (n = 96). The study was limited to six treatment groups to allow for four samples within each flow cell and a total of 16 samples (4 samples within each flow cell, 4 flow cells). The treatment groups were determined from the results of 'Study 1' and 'Study 2' and further justification is given below.

5.2.1 Control

In 'Study 3', standard irrigation using saline (5 mL for 2 minutes) was used as a control following establishment of the biofilm within the root canal system. This protocol ensured that planktonic bacteria within the canal lumen were flushed from the root canal system whilst the biofilm adhering to the root canal system remained.

5.2.2 Laser Energised Irrigation

Results from 'Study 1' and 'Study 2' had shown that laser energised irrigation at 0.5 W was the lowest output power to show a trend towards increased antibacterial efficacy compared to standard irrigation. Laser energised irrigation (0.5 W) of a range of NaOCl concentrations (0.5%, 1% and 4% NaOCl) were investigated in 'Study 3' to determine if a lower NaOCl concentrations could be coupled with laser energy to equal or exceed the bactericidal effect of standard irrigation (non-energised) with a higher NaOCl concentrations.

5.2.3 Standard Irrigation

Laser energised 0.5%, 1% and 4% NaOCl were included as treatment groups in 'Study 3'. Ideally these would be compared with standard irrigation with 0.5%, 1% and 4% NaOCl, however this was not possible. Due to a limit of 24 roots within each flow cell and the need for an adequate sample size within each treatment group, 'Study 3' was restricted to six treatment groups. Therefore one of the standard irrigation groups (0.5%, 1% and 4% NaOCl) needed to be omitted.

Standard irrigation with 1% NaOCl was included as a treatment group as it is the most commonly used irrigant by Australian endodontists (Clarkson *et al.* 2003). Whilst concentrations of NaOCl higher than 2.5% are rarely used in Australia (Clarkson *et al.* 2003), a survey of US endodontists showed 84% were using greater than 2.5% NaOCl, and 58% were using greater than 5% NaOCl (Dutner *et al.* 2012). In Australia, 4% NaOCl is approved by the Therapeutics Goods Administration and it was therefore included as a treatment group in 'Study 3'.

In the preliminary studies, standard irrigation with 4% NaOCl led to a *ca.* 10-fold reduction in cfu/µg protein compared to standard irrigation with 1% NaOCl. Standard irrigation with 1% and 4% NaOCl were included in 'Study 3' to allow comparisons to LEI and to further investigate any potential difference in antibacterial effect for standard irrigation between the two concentrations.

Standard irrigation with 0.5% NaOCl is rarely used in Australia (Clarkson *et al.* 2003). With the need to eliminate a group it was therefore omitted.

5.3 Method and Materials

Four flow cells were used (n = 96). An *E. faecalis* biofilm was established within the root canal system of extracted single rooted teeth as described. Details of the method and materials are explained in Sections 2.1 – 2.6. Treatment groups used a two minute treatment time. Details of the treatment protocols are detailed below.

5.3.1 Tooth Positioning within Flow Cell

It was unknown whether the positioning of tooth roots within the flow cell produced a significant change in the biomass. In light of this, when removing teeth for the allocated six treatment groups, the flow cell was divided into four zones (Figure 25), which were related to proximity to the growth medium inlet. Each of the 6 treatment groups had one tooth removed from each of the four zones of the flow cell (Figure 25).

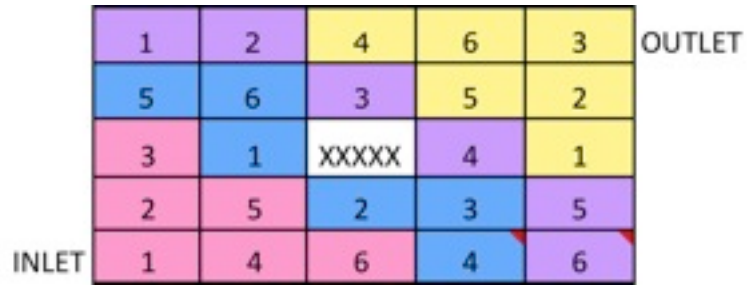


Figure 25: Schematic diagram of tooth root sample positioning.
The flow cell inlet is in the lower left and the outlet in the upper right. The flow cell was divided into 4 zones (Zone A, B, C and D, shown as pink, Blue, purple and yellow, respectively). The numbers represent each of the six treatment groups so each treatment group contained one tooth root from each zone.

5.3.2 Treatment Groups

The teeth were randomly divided into 6 treatment groups with 16 replicate teeth per group (four teeth from each treatment group within each of the four flow cells) (n = 96). Groups are detailed below and summarised in Table 6.

Group 1. Control. Standard irrigation with 5 mL of 0.9% saline delivered with a 27 gauge Monoject (Kendall, Covidien, Mansfield, USA) needle. The needle tip was positioned as far apically as possible without binding. Treatment time was 2 minutes.

Group 2. 1% NaOCl standard irrigation (SI). As for the control group, but 1% NaOCl was used as the irrigant.

Group 3. 4% NaOCl SI. As for control but 4% NaOCl was used as the irrigant.

Group 4. 0.5% NaOCl laser energised (0.5 W) irrigation (LEI). Standard irrigation of 1.25 mL of 0.5% NaOCl irrigation for 15 seconds followed by laser energisation for 15 seconds. Laser activation was performed with an Er,Cr:YSGG laser. The laser power output was set at 0.5 W, with a pulse repetition rate of 20 Hz. The air or water function was not used. The laser tip, RFT 3 17 mm (Endolase, Biolase Technology, Irvine, USA), was positioned 5 mm apically from the orifice. The irrigant was not aspirated from the canal, so laser irradiation was always performed within an irrigant filled canal. The cycle was repeated four times. The total volume of irrigant was 5 mL and total time of treatment was two minutes.

Group 5. 1% NaOCl LEI (0.5 W). As for group 4, but 1% NaOCl was used as the irrigant.

Group 6. 4% NaOCl LEI (0.5 W). As for group 4, but 4% NaOCl was used as the irrigant.

Following treatment, NaOCl was inactivated with 5 mL of 5% sodium thiosulphate for one minute. A sterile paper point was used to absorb the contents of the canal after neutralisation.

	Saline	**0.5% NaOCl**	**1% NaOCl**	**4% NaOCl**
Standard irrigation	Group 1		Group 2	Group 3
0.5 W LEI		Group 4	Group 5	Group 6

Table 6. Table summarises the six groups included in 'Study 3'.
There were 16 tooth roots within each group. LEI, Laser energised irrigation.

Crushed tooth samples, viability counts and protein concentrations were conducted for each tooth root as described in Section 2.7.

5.3.3 Statistics

All calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). Colony forming units (cfu) per mL were calculated and normalised against protein concentration (cfu/µg protein). As the data was not normally distributed, the median, range and quartile range are presented. Values were compared between groups using a Kruskal-Wallis test. In addition, comparison was made between the four experimental flow cells, and between the four zones within each flow cell. A *p*-value ≤0.05 was considered statistically significant. To further explore the differences, post-hoc Wilcoxon tests were fitted to the data.

5.4 Results

5.4.1 Analysis of variability between the Four Flow Cells

Descriptive statistics for cfu/µg protein, by flow cell, are presented in the Table 7.

Flow Cell	cfu/µg protein					
	N	Min	Q1	Median	Q3	Max
E1	24	1.3×10^0	9.8×10^0	2.39×10^1	2.28×10^2	3.16×10^4
E2	24	0.0	1.0×10^0	1.72×10^1	2.47×10^2	2.14×10^4
E3	24	0.0	1.8×10^0	7.66×10^1	5.59×10^3	4.10×10^5
E4	24	2.2×10^0	3.86×10^1	1.49×10^2	3.32×10^2	1.97×10^4

Table 7. Descriptive statistics for cfu/µg protein, by flow cell.
'Study 3' involved four flow cells (E1, E2, E3 and E4) each containing 24 teeth.

Colony forming units (cfu/mL) normalised against protein concentrations (µg/mL) were also compared between groups using a Kruskal-Wallis test. The test was not found to be statistically significant (p = 0.1546), showing that there was not a difference between the flow cells.

5.4.2 Analysis of Root Position within the Flow Cell

Descriptive statistics for cfu/µg protein, by position in the flow cell, are presented in the Table 8.

Zone	cfu/µg protein					
	N	Min	Q1	Median	Q3	Max
A	24	2×10^{-1}	3.0×10^{0}	2.00×10^{2}	3.04×10^{2}	3.19×10^{4}
B	24	3×10^{-1}	3.9×10^{0}	2.29×10^{2}	2.89×10^{2}	9.84×10^{4}
C	24	0.0	1.52×10^{1}	9.16×10^{2}	2.32×10^{2}	4.10×10^{5}
D	24	4×10^{-1}	7.7×10^{0}	8.15×10^{2}	1.28×10^{3}	2.19×10^{4}

Table 8. Descriptive statistics for cfu/µg protein, by tooth position within the flow cell. The four zones (A, B, C and D) are explained in Figure 25.

Colony forming units (cfu/mL) normalised against protein concentrations (µg/mL) were compared between groups using a Kruskal-Wallis test. The test was not found to be statistically significant ($p = 0.6045$), showing that there was not a difference between sample positioning within the flow cell.

5.4.3 Analysis by Treatment Group

Statistics for cfu/µg protein, by group, are presented in Table 9.

		cfu/µg protein					
		N	Min	Q1	Median	Q3	Max
Group	Treatment						
1	Saline (SI)	16	2.25×10^{2}	3.18×10^{3}	1.37×10^{4}	2.68×10^{4}	4.10×10^{5}
2	1% NaOCl (SI)	16	4×10^{-1}	1.51×10^{1}	1.42×10^{2}	3.46×10^{2}	1.66×10^{4}
3	4% NaOCl (SI)	16	2×10^{-1}	2.6×10^{0}	1.08×10^{1}	1.17×10^{2}	1.51×10^{3}
4	0.5% NaOCl + 0.5W LEI	16	4×10^{-1}	6.2×10^{0}	2.39×10^{1}	1.12×10^{2}	5.85×10^{2}
5	1% NaOCl + 0.5W LEI	16	0.0	3.5×10^{0}	2.00×10^{1}	1.07×10^{2}	5.03×10^{2}
6	4% NaOCl + 0.5W LEI	16	0.0	1.8×10^{0}	7.2×10^{0}	3.33×10^{1}	1.05×10^{3}

Table 9. Descriptive statistics expressed in cfu/µg protein.
SI, standard irrigation. LEI, Laser energised irrigation.

Colony forming units (cfu/mL) normalised against protein concentrations (µg/mL) were compared between groups using a Kruskal-Wallis test. The test was found to be statistically significant ($p < 0.0001$), suggesting a significant difference in the distribution of cfu/µg protein scores between the six groups. To further explore the differences, post-hoc Wilcoxon tests were fitted to the data. The results are shown in Table 10.

Comparison	Wilcoxon statistic	*P* - value
Saline vs. 1% NaOCl	371.0	0.0004 *
Saline vs. 4% NaOCl	389.0	<0.0001 *
Saline vs. 1% NaOCl + 0.5 W	390.0	<0.0001 *
Saline vs. 4% NaOCl + 0.5 W	391.0	<0.0001 *
Saline vs. 0.5% NaOCl + 0.5 W	390.0	<0.0001 *
1% NaOCl vs. 4% NaOCl	321.0	0.0413 *
1% NaOCl vs. 1% NaOCl + 0.5 W	319.0	0.0485 *
1% NaOCl vs. 4% NaOCl + 0.5 W	339.0	0.0086 *
1% NaOCl vs. 0.5% NaOCl + 0.5 W	309.0	0.1036
4% NaOCl vs. 1% NaOCl + 0.5 W	265.0	0.9851
4% NaOCl vs. 4% NaOCl + 0.5 W	282.0	0.5144
4% NaOCl vs. 0.5% NaOCl + 0.5 W	248.0	0.5633
1% NaOCl + 0.5 W vs. 4% NaOCl + 0.5 W	283.5	0.4793
1% NaOCl + 0.5 W vs. 0.5% NaOCl + 0.5 W	248.0	0.5633
4% NaOCl + 0.5 W vs. 0.5% NaOCl + 0.5 W	228.0	0.1906

Table 10. Results of post-hoc Wilcox tests comparing cfu/µg protein between groups from 'Study 3'.
A *p*-value ≤0.05 was considered statistically significant. * notes statistically significant values.

6.0 Discussion

The fundamental concept underpinning endodontic therapy is the elimination of the causative agent, thereby providing an environment conducive to healing. Provided that bacterial numbers can be eliminated or reduced to numbers undetectable by microbial sampling and culture techniques, high success rates of treatment are achieved (Sjögren *et al.* 1997).

Instrumentation and irrigation are unable to reliably produce a sterile root canal system, rendering 50-70% of root canals bacteria free when measured by culture sampling techniques (Byström & Sundqvist 1985; Shuping *et al.* 2000). Medicaments such as calcium hydroxide can potentially overcome some of the limitations of instrumentation and irrigation, however its use is not without shortcomings and necessitates a multi-visit approach. Increasing the

concentration of NaOCl, with or without the use of adjuncts such as sonics, ultrasonics and lasers, have been proposed to aid the chemo-mechanical disinfection of the root canal system.

The aim of this study was to establish the efficacy of eradicating an *E. faecalis* biofilm *in vitro,* using various concentrations of NaOCl, with and without laser energised irrigation.

6.1 Method and Materials

A flow cell adapted from Seet *et al.* (*in press*) was used in this study. This apparatus allowed the establishment of a mature, *E. faecalis* biofilm within the root canal system and dentinal tubules (Figures 9–13). Statistically analysis did not show a difference in biomass between flow cells nor between tooth positioning within the flow cell.

E. faecalis is the most frequently cultured species in previously well obturated root-filled teeth with apical periodontitis (Molander *et al.* 1998; Sundqvist *et al.* 1998). As *E. faecalis* is a highly resistant bacterium involved in endodontic infections, and it is easily grown in the laboratory, it is an appropriate bacterium to use for research *in vitro.* However it is acknowledged that no single species will fully represent the highly variable intra-canal flora found *in vivo.*

Whole roots were utilised, rather than dentine coupons or filter membranes, which has several advantages. The anatomical constraints and complex anatomy of the root canal system were accounted for, as was any potential buffering effect of dentine. *E. faecalis* has the ability to penetrate as far as 250 μm into the dentinal tubules (Schäfer & Bossmann 2005), and the presence of bacteria within dentinal tubules provides a greater challenge for the antibacterial agents.

The limitations of paper point microbial sampling are well established (Akpata 1976). To minimise the occurrence of false negative cultures due to incomplete microbial sampling, the present study used a device to crush teeth prior to bacterial sampling. This greatly improved the detection of viable bacteria residing deep within the root canal system and dentinal tubules.

6.2 Concentration of Sodium hypochlorite

There are conflicting findings regarding the most appropriate concentration of NaOCl for clinical use. Factors that need to be considered when establishing an appropriate

concentration include antibacterial action, tissue dissolution and safety. There have been relatively few clinical investigations comparing concentrations of NaOCl (Cvek *et al.* 1976a; Byström & Sundqvist 1985). Paper point microbial sampling after canal preparation has not shown a significant difference between the bactericidal effect of either 0.5% or 5% NaOCl (Cvek *et al.* 1976a; Byström & Sundqvist 1985). The majority of research comparing concentrations of NaOCl has been conducted *in vitro.*

In vitro studies involving biofilms have both supported and contraindicated the use of higher concentrations of NaOCl. Dunavant *et al.* (2006) did not find a bactericidal difference between 1% and 6% NaOCl. This is in contrast to the current study, as standard irrigation with 4% NaOCl was more bactericidal than 1% NaOCl. Whilst both studies grew *E. faecalis* biofilms, Dunavant *et al.* (2006) used porcelain coupons rather than whole roots. The irrigant would presumably have greater exposure to bacteria grown on coupons compared to within the root canal system. This may account for the lack of statistical significance between 1% and 6% NaOCl reported by Dunavant *et al.* (2006).

The present study found that standard irrigation over two minutes with 4% NaOCl was more bactericidal than 1% NaOCl. The null hypothesis that there was no difference in the antibacterial effect between the 1% and 4% NaOCl was therefore rejected. This finding is in agreement with Berber *et al*. (2006). Berber *et al.* (2006) in an *in vitro* study inoculated the root canal system and established an *E. faecalis* biofilm over 21 days. Following irrigation with a range of NaOCl concentrations, microbial samples were taken with both paper point and a dentine chip technique. Bacteria were present within the dentine chip samples, and 5.25% NaOCl was found to have a greater antibacterial activity against *E. faecalis,* compared with 0.5% and 2.5% NaOCl. This difference was not evident with paper point sampling, which is in agreement with Siqueira *et al.* (2000) who also used paper point sampling. The limitations of paper point microbial sampling have been highlighted. It is possible that this technique lacks the required sensitivity to establish a difference between various concentrations of NaOCl. Furthermore, Siqueira *et al.* (2000) inoculated canals with *E. faecalis* only 24 hours prior to experimental treatment protocols. The present study and that of Berber *et al*. (2006) both established biofilms over extended periods. Twenty four hours is insufficient time to establish a mature biofilm and this may be a further explanation why Siqueira *et al.* (2000) did not find a significant difference between 0.5%, 2.5% and 5.25% NaOCl.

6.3 Laser Energised Irrigation

The null hypothesis that there was no difference in the antibacterial effect between laser energised irrigation and standard irrigation when using 1% NaOCl was rejected. The null hypothesis that there was no difference in the antibacterial effect between laser energised irrigation and standard irrigation when using 4% NaOCl was accepted. A significant difference was not shown between standard irrigation and laser energised 4% NaOCl. Notably however, laser energised 1% NaOCl had a greater antibacterial effect compared to standard irrigation with 1% NaOCl. Therefore, the increased antibacterial effect of laser energised irrigation was established with 1% NaOCl but not replicated with 4% NaOCl. Presumably in the *in vitro* model, the increase in NaOCl concentration (4%) negated the need to laser energise the irrigant. These findings are in agreement with others (Peters *et al.* 2011; Pedullà *et al.* 2012).

Peters *et al.* (2011) has shown the increased antibacterial effect of laser energised NaOCl. A biofilm was established *in-situ* in extracted human premolars. Laser energised irrigation of 6% NaOCl was significantly more effective than either standard irrigation of 6% NaOCl or PUI of 6% NaOCl in achieving bacteria-free samples, as assessed with a modified paper point sampling technique. When bacterial reduction rather than negative cultures were assessed, a significant difference was not found between laser energised 6% NaOCl and standard irrigating techniques with 6% NaOCl (Peters *et al.* 2011). Similarly, Pedullà *et al.* (2012) did not find a statistical difference between laser energised 5% NaOCl and standard irrigation with 5% NaOCl.

The effect of different concentrations of NaOCl and laser energy has not previously been investigated. Previous studies investigating the antibacterial effect of LEI have used 4% (Seet *et al. in press*), 5% (Pedullà *et al.* 2012) or 6% (Peters *et al.* 2011) NaOCl. Sahar-Helft *et al.* (2011) has assessed different concentrations of laser energised chlorhexidine. At a concentration of 0.0001%, chlorhexidine had an inhibitory effect when the solution was energised with an Er:YAG laser whilst it had no effect when not energised. Although this work increased the understanding of laser energised chlorhexidine, the microbiological basis of the study was weak, a planktonic *E. faecalis* rather than a biofilm was used, and a tooth model was not incorporated. Sahar-Helft *et al.* (2011) highlighted that lower concentrations of an irrigant could be combined with laser energy to equal or exceed the bactericidal effect of non-energised higher concentrations. This finding has been supported in the present study.

Standard irrigation of 4% NaOCl was found to be more bactericidal than 1% NaOCl, however a statistical difference between standard irrigation with 4% NaOCl and LEI of 0.5% or 1% NaOCl was not shown. Therefore, laser energised irrigation has the potential to increase the antibacterial effect of lower concentrations of NaOCl (0.5% and 1%). The maximum antibacterial effect could be achieved with either standard irrigation with 4% NaOCl, or laser energised irrigation using 0.5%, 1% or 4% NaOCl.

6.3.1 The Efficacy of LEI Sterilisation of the Root Canal System

Laser energised irrigation did not reliably produce bacteria-free canals in this study. Bacteria were not detected in only two of the 96 roots (including the 16 controls that were irrigated with saline) following treatment. The two samples were each from a laser energised group, using 1% NaOCl and 4% NaOCl respectively. The number of canals free of detectible bacteria was lower than other studies assessing the antibacterial effect of LEI (Peters *et al.* 2011; Pedullà *et al.* 2012).

Pedullà *et al.* (2012) reported a high incidence of bacteria 'free' cultures (5% NaOCl produced 25/32 negative cultures, 5% LEI produced 30/32). However, all samples with less than 3 000 000 cfu/mL may have been classified as 'negative cultures'. Only dilutions of 10^4, 10^5 and 10^6 were examined and only plates with greater than 30 colonies were counted (30 colonies on 10^4 plate). This would account for their high incidence of bacteria free samples and explains why the results are not comparable to either the present study or those reported by Peters *et al.* (2011).

Peters *et al.* (2011) were unable to sample and culture bacteria in ten of 20 samples following treatment with laser energised 6% NaOCl. In the current study, laser energised 4% NaOCl rendered one of the 16 samples without detectible bacteria. Peters *et al.* (2011) used a modified paper point sampling technique and did not sample dentine shavings/powder. This method would be expected to underestimate the number of remaining bacteria, and is the likely explanation for the difference in results between the two studies. The sampling following tooth crushing used in the present study would be expected to increase the detection of viable bacteria remaining within dentinal tubules. The SEM images provided qualitative data that supports this theory (Figures 23 & 24). The main root canal was generally free of detectible bacteria following standard irrigation with 4% NaOCl or laser energised 0.5%, 1% or 4% NaOCl. Although following these treatments, bacteria were still identified further

along dentinal tubules. The histological findings from Peters *et al.* (2011) were in agreement with this.

The SEM images provided qualitative evidence of the presence of bacteria. The tooth roots were split in a longitudinal direction that allowed visualisation of the dentinal tubules and root canal system. It was difficult to standardise the images to allow uniformed viewing of the dentinal tubules. The notching of the roots with a diamond coated disc prior to splitting created a smear layer, and the separation of the two fragments with a plastic instrument can not control the direction of the fracture line. Thus, there is no guarantee that the crack will longitudinally follow the dentinal tubules. Furthermore SEM analysis provides evidence of the presence of bacteria, but is unable to determine the viability of bacteria.

Other studies have stained cross-sections of tooth roots using a Brown and Brenn technique and have used a light microscope to assess the presence of bacteria remaining within the canal and dentinal tubules (Harrison *et al.* 2010; Peters *et al.* 2011). Harrison *et al.* (2010) found that standard chemo-mechanical preparation with 1% NaOCl and 15% EDTA was ineffective in removing bacteria from the dentinal tubules, but an additional step, one minute ultrasonic activated irrigation with 1% NaOCl or a seven day placement of calcium hydroxide, led to a significant reduction in bacteria within tubules. Although neither treatment could reliably render the root canal sterile, nor was there a statistical difference between the two.

Peters *et al.* (2011) assessed remaining intracanal bacteria and dentinal tubule penetration in root cross-sections 1 mm and 4 mm from the apex. A consistent pattern of bacterial removal from dentinal tubules close to the root canal lumen was not observed following standard irrigation with 6% NaOCl or LEI or PUI (also with 6% NaOCl). At 1 mm from the apex, laser energised irrigation performed significantly better than both other techniques when assessing cross-sectional areas covered with bacteria.

The fate of bacteria remaining within dentinal tubules after chemo-mechanical preparation is unknown (Peters *et al.* 1995), and the concept of entombment and the potential role of bacteria remaining in dentinal tubules in persistent apical periodontitis is not clear. Given the high success rates achieved with root canal treatment, and the apparent inability to predictably eliminate all bacteria within the tubules, the impact of relatively small numbers of bacteria left within the dentinal tubules is questionable. Until established otherwise however, the goal of rendering the root canal system bacteria free still remains.

6.3.2 Laser Output Power

A range of laser output powers (0.1 W, 0.25 W, 0.5 W and 1 W) were investigated in the preliminary studies, with the aim to maximise antibacterial effect whilst minimising laser output power. Laser output powers of 0.1 W and 0.25 W did not appear to increase the bactericidal effect of either 0.5%, 1% or 4% NaOCl. Although increasing the laser output power gave a general trend to increased antibacterial effect, the risk of irrigant extrusion is also increased. The preliminary studies found LEI at 0.5 W was the lowest output power to show a trend towards increased antibacterial efficacy compared to standard irrigation. Therefore, a power setting of 0.5 W was deemed most appropriate for LEI. Laser energised irrigation at 0.5 W was further investigated in 'Study 3'.

The present study showed the increased antibacterial effect of laser energised 1% NaOCl at 0.5 W (25 mJ at 20 Hz) compared to standard irrigation with 1% NaOCl. The output power used in this study was lower than the output used in other studies that have assessed the efficacy of LEI with an Er,Cr:YSGG lasers (George *et al.* 2008; De Moor *et al.* 2009; De Moor *et al.* 2010). The efficacy of LEI in the removal of debris and smear layer has been shown with EDTA and Er,Cr:YSGG lasers at 1.25 W (62.5 mJ at 20 Hz) (George *et al.* 2008) and 1.5 W (75 mJ at 20 Hz) (De Moor *et al.* 2009; De Moor *et al.* 2010).

6.4 Clinical Considerations

Laser output power for LEI must be a balance between efficacy and minimising the potential for iatrogenic injury from heat generation and periapical irrigant extrusion. Heat generation is unlikely to be of concern if laser output power is minimised since the use of an erbium laser using similar power settings to the current study and in a dry or wet canal increased the outer root temperature by less than 2°C (Schoop *et al.* 2009; DiVito *et al.* 2012).

Laser energised irrigation at high laser power settings may extrude the irrigant which has obvious clinical implications, particularly if a cytotoxic irrigant such as NaOCl is used. In a bench top investigation, the extrusion of dye from prepared root canals following laser energised irrigation was shown (George & Walsh 2008). The study did not take into effect an intact periodontium however, and the laser power settings were relatively high (1.25 W). The potential for LEI to extrude the irrigant into the periapical tissues requires further investigation. It can be speculated that a relatively low laser power setting such as 0.5 W, and

confinement of the laser tip to the coronal third of the canal will minimise the risk of extrusion. Further research is required to support this theory.

This study found that the maximum antibacterial effect could be achieved with either standard irrigation with 4% NaOCl or laser energised 0.5%, 1% or 4% NaOCl. It is accepted that higher concentrations of NaOCl are more cytotoxic (Spångberg *et al.* 1973). A potentially safer alternative to 4% NaOCl irrigation could potentially be achieved by using LEI with 0.5% NaOCl. However, the risk of apical extrusion of the irrigant during LEI needs to be compared with the cytotoxicity of the irrigant.

The antibacterial and debris removal properties of LEI has been compared with passive ultrasonic irrigation (de Groot *et al.* 2009; De Moor *et al.* 2009; De Moor *et al.* 2010; Peters *et al.* 2011). Peters *et al.* (2011) found laser energised irrigation (6% NaOCl, Er:YAG laser 50 mJ 10 Hz, 30 seconds) was significantly superior to either irrigation alone (6% NaOCl 30 seconds) or PUI (of 6% NaOCl) in generating negative (no growth detected) samples. However the difference in bacterial reduction among the three groups did not reach the level of statistical significance.

Laser energised irrigation has been shown to be more effective at removing artificially placed dentine debris from the apical part of the root canal system than PUI when both were conducted over comparable treatment times (de Groot *et al.* 2009; De Moor *et al.* 2009; De Moor *et al.* 2010). If the volume of irrigant and time of ultrasonic activation are increased (60 seconds, 6 mL 2.5% NaOCl), PUI achieved a comparable result to a relatively shorter time period of LEI (Er,Cr:YSGG or Er:YAG laser 4 x 5 seconds 75 mJ at 20 Hz and 2 mL of 2.5% NaOCl) (De Moor *et al.* 2010).

There are current drawbacks to implementing LEI clinically. The financial cost of the laser unit must be considered. Erbium lasers are expensive, as are the laser fibres that are single use. The units are also cumbersome which may pose an additional challenge in the confines of a dental surgery. Although these are current limitations, future advancements may see improvements in these areas.

6.5 Limitations of this Study

Results from *in vitro* research cannot directly be extrapolated to the clinical setting. In the present study straight canals were used, root canal instrumentation (which would have

reduced the bacterial load considerably) was not performed, and irrigation was carried out over two minutes.

Molecular techniques are becoming increasingly popular for the identification of bacterial species, as they have improved specificity and sensitivity compared to culture techniques. A culture technique was used in the present study as it allowed both quantification of viable bacteria and assessment of their viability. The improved specificity of a molecular technique was not required as a single species (*E. faecalis*) was used in this *in vitro* model. Furthermore, the sensitivity of the bacterial sampling and culture technique was testament to the low number of samples (two of 96) with undetectable levels of bacteria.

6.6 Further Research

The ability of laser energised irrigation to increase the antibacterial efficacy of 1% NaOCl has been shown, but the same effect with 4% NaOCl (and LEI) was not shown. Presumably the higher concentration of NaOCl negates the need to energise the irrigant. However, all research that has assessed the antibacterial efficacy of LEI with NaOCl has been conducted in single rooted teeth with relatively simple anatomy. The benefits of LEI should be investigated in more complicated root canal anatomy such as curved canals or isthmuses. The benefits of laser energised irrigation in canals with more complicated anatomy form part of ongoing research in our laboratory.

Several concepts need to be addressed prior to LEI being recommended for clinical use. The safety of LEI has been insufficiently addressed, in particular the issue of extrusion of the irrigant. Furthermore, the type of laser and optimal laser settings need to be established. Both Er,Cr:YSGG (Seet *et al. in press*) and Er:YAG (Peters *et al.* 2011; Pedullà *et al.* 2012) lasers have been used for LEI investigations. As yet, the antibacterial efficacy of the two laser systems has not been directly compared. A difference in LEI efficacy has not been found between an Er,Cr:YSGG and Er:YAG laser using a dentine debris model (De Moor *et al.* 2010), nor is there a difference in smear layer removal (George *et al.* 2008). Diode laser systems have recently been shown to induce cavitation effects in water based media (Hmud *et al.* 2012), but no studies have compared the efficacy of LEI with diode lasers.

Although the present study goes some way towards establishing parameters for LEI (Er,Cr:YSGG laser at 0.5 W, 20 Hz, RFT 3 17 mm laser tip, 15 seconds irrigation followed by 15 seconds laser energisation with the cycle repeated four times), other parameters that

require investigation include optimal laser power setting (including Hertz), laser tip design (diameter and shape), laser tip positioning, and the time of irradiation. All of these parameters may have an impact on the efficacy of LEI. These parameters require further investigation with *in vitro* experiments. Once the parameters are established, the efficacy of LEI will need to be assessed in clinical trials.

7.0 Conclusion

Within the limitations of this *in vitro* study, a laser output power of 0.5 W with an Er,Cr:YSGG laser was determined to be the minimum setting for LEI. Laser energised irrigation of 1% NaOCl (Er,Cr:YSGG laser at 0.5 W) was significantly more effective than standard irrigation with 1% NaOCl. Standard irrigation using 4% NaOCl was more effective at disinfecting an *E. faecalis* biofilm compared to standard irrigation with 1% NaOCl. However, lower concentrations (0.5% and 1%) of NaOCl when energised with a Er,Cr:YSGG laser at 0.5 W were as effective as standard irrigation with 4% NaOCl.

8.0 Appendix

8.1 Study 1 Results

Sample	Treatment Group	Analysis	cfu/mL	µg protein/mL	cfu/µg protein
1	Control	SEM			
2	Control	SEM			
3	Control	Viability	2.60E+07	157.08	1.66E+05
4	Control	Viability	1.99E+05	57.29	3.47E+03
5	Saline + 0.25 W	SEM			
6	Saline + 0.25 W	Viability	1.73E+06	37.90	4.55E+04
7	Saline + 0.25 W	Viability	6.00E+04	18.87	3.18E+03
8	0.5% NaOCl	SEM			
9	0.5% NaOCl	Viability	6.60E+04	34.95	1.89E+03
10	0.5% NaOCl	Viability	7.45E+04	34.59	2.15E+03
11	0.5% NaOCl + 0.1 W	SEM			
12	0.5% NaOCl + 0.1 W	Viability	1.24E+05	22.98	5.37E+03
13	0.5% NaOCl + 0.1 W	Viability	1.36E+06	34.17	3.97E+04
14	0.5% NaOCl + 0.25 W	SEM			
15	0.5% NaOCl + 0.25 W	Viability	2.55E+04	39.30	6.49E+02
16	0.5% NaOCl + 0.25 W	Viability	5.75E+04	24.72	2.33E+03
17	4% NaOCl + 0.1 W	SEM			
18	4% NaOCl + 0.1 W	Viability	2.69E+03	34.80	7.73E+01
19	4% NaOCl + 0.1 W	Viability	2.65E+04	28.89	9.17E+02
20	4% NaOCl + 0.25 W	SEM			
21	4% NaOCl + 0.25 W	Viability	1.17E+04	16.14	7.22E+02
22	4% NaOCl + 0.25 W	Viability	1.36E+04	14.49	9.39E+02
23	4% NaOCl	Viability	3.20E+04	28.89	1.11E+03

8.2 Study 2 Results

8.2.1 Flow Cell 1 (Study 2)

Sample	Treatment Group	Analysis	cfu/mL	µg protein/mL	cfu/µg protein
1	Control	SEM			
2	Control	Viability	8.75E+06	50.36	1.74E+05
3	Control	Viability	3.15E+05	43.87	7.18E+03
4	1% NaOCl	SEM			
5	1% NaOCl	Viability	1.90E+05	24.96	7.61E+03
6	1% NaOCl	Viability	9.75E+04	30.14	3.23E+03
7	4% NaOCl	SEM			
8	4% NaOCl	Viability	7.60E+03	31.50	2.41E+02
9	4% NaOCl	Viability	9.50E+02	33.68	2.82E+01
10	0.5 NaOCl + 0.25 W	SEM			
11	0.5 NaOCl + 0.25 W	Viability	1.45E+03	34.44	4.21E+01
12	0.5 NaOCl + 0.25 W	Viability	4.60E+04	27.43	1.68E+03
13	0.5 NaOCl + 0.5 W	SEM			
14	0.5 NaOCl + 0.5 W	Viability	2.35E+04	31.56	7.45E+02
15	0.5 NaOCl + 0.5 W	Viability	4.40E+03	23.49	1.87E+02
16	1% NaOCl + 0.25 W	SEM			
17	1% NaOCl + 0.25 W	Viability	1.12E+05	32.62	3.42E+03
18	1% NaOCl + 0.25 W	Viability	6.45E+04	27.96	2.31E+03
19	1% NaOCl + 0.5 W	SEM			
20	1% NaOCl + 0.5 W	Viability	2.00E+03	25.37	7.88E+01
21	1% NaOCl + 0.5 W	Viability	4.95E+03	26.49	1.87E+02
22	4% NaOCl + 0.25 W	SEM			
23	4% NaOCl + 0.25 W	Viability	5.00E+01	15.06	3.32E+00
24	4% NaOCl + 0.25 W	Viability	4.25E+03	41.45	1.03E+02

8.2.2 Flow Cell 2 (Study 2)

Sample	Treatment Group	Analysis	cfu/mL	µg protein/mL	cfu/µg protein
1	Control	Viability	4.00E+05	23.52	1.27E+04
2	Control	Viability	1.80E+06	23.89	7.31E+04
3	1% NaOCl	Viability	1.95E+05	33.99	9.86E+03
4	1% NaOCl	Viability	1.02E+05	19.87	5.35E+03
5	4% NaOCl	Viability	1.55E+04	22.72	5.55E+02
6	4% NaOCl	Viability	3.70E+03	16.14	1.69E+02
7	0.5% NaOCl + 0.5 W	Viability	7.00E+02	14.93	3.19E+01
8	0.5% NaOCl + 0.5 W	Viability	8.25E+04	15.17	3.26E+03
9	0.5% NaOCl + 1 W	Viability	8.00E+03	16.57	3.69E+02
10	0.5% NaOCl + 1 W	Viability	7.45E+02	19.95	2.94E+01
11	0.5% NaOCl + 1 W	Viability	6.00E+03	19.22	2.77E+02
12	1% NaOCl + 0.5 W	Viability	1.00E+03	13.53	4.19E+01
13	1% NaOCl + 0.5 W	Viability	1.05E+03	19.28	4.58E+01
14	1% NaOCl + 1 W	Viability	3.00E+01	19.19	1.44E+00
15	1% NaOCl + 1 W	Viability	3.50E+02	21.04	1.51E+01
16	1% NaOCl + 1 W	Viability	9.00E+02	24.39	4.28E+01
17	4% NaOCl + 0.25 W	Viability	5.50E+02	13.34	2.46E+01
18	4% NaOCl + 0.25 W	Viability	6.50E+01	14.01	2.95E+00
19	4% NaOCl + 0.25 W	Viability	1.60E+03	19.83	7.78E+01
20	4% NaOCl + 0.5 W	Viability	4.40E+03	13.04	1.73E+02
21	4% NaOCl + 0.5 W	Viability	7.50E+01	23.90	3.59E+00
22	4% NaOCl + 1 W	Viability	4.25E+02	22.20	2.01E+01
23	4% NaOCl + 1 W	Viability	3.55E+02	20.16	1.71E+01
24	4% NaOCl + 1 W	Viability	5.10E+03	14.07	2.21E+02

8.3 Study 3 Results

8.3.1 Flow Cell 1 (Study 3)

Sample	Position	Treatment Group	cfu/mL	µg protein/mL	cfu/µg protein
1	A	Saline	1.90E+05	28.22	6.73E+03
2	B	Saline	6.40E+04	27.91	2.29E+03
3	C	Saline	8.50E+05	26.93	3.16E+04
4	D	Saline	3.50E+05	31.87	1.10E+04
5	A	1% NaOCl	6.00E+02	17.79	3.37E+01
6	B	1% NaOCl	1.25E+04	18.90	6.61E+02
7	C	1% NaOCl	2.50E+02	21.59	1.16E+01
8	D	1% NaOCl	3.00E+02	20.60	1.46E+01
9	A	4% NaOCl	4.65E+02	18.69	2.49E+01
10	B	4% NaOCl	1.35E+02	16.99	7.95E+00
11	C	4% NaOCl	4.70E+03	18.53	2.54E+02
12	D	4% NaOCl	2.65E+02	19.33	1.37E+01
13	A	1% NaOCl + 0.5 W	3.50E+02	17.67	1.98E+01
14	B	1% NaOCl + 0.5 W	5.50E+02	21.50	2.56E+01
15	C	1% NaOCl + 0.5 W	3.70E+03	18.35	2.02E+02
16	D	1% NaOCl + 0.5 W	4.00E+01	20.97	1.91E+00
17	A	4% NaOCl + 0.5 W	3.00E+01	22.73	1.32E+00
18	B	4% NaOCl + 0.5 W	3.65E+02	26.13	1.40E+01
19	C	4% NaOCl + 0.5 W	5.50E+01	17.91	3.07E+00
20	D	4% NaOCl + 0.5 W	1.40E+02	26.13	5.36E+00
21	A	0.5% NaOCl + 0.5W	1.75E+02	23.38	7.48E+00
22	B	0.5% NaOCl + 0.5W	1.35E+03	23.13	5.84E+01
23	C	0.5% NaOCl + 0.5W	5.50E+02	22.02	2.50E+01
24	D	0.5% NaOCl + 0.5W	4.05E+02	17.73	2.28E+01

8.3.2 Flow Cell 2 (Study 3)

Sample	Position	Treatment Group	cfu/mL	µg protein/mL	cfu/µg protein
1	A	Saline	6.00E+03	26.70	2.25E+02
2	B	Saline	8.15E+04	31.91	2.55E+03
3	C	Saline	5.50E+04	32.95	1.67E+03
4	D	Saline	6.20E+05	28.91	2.14E+04
5	A	1% NaOCl	4.05E+02	26.13	1.55E+01
6	B	1% NaOCl	7.00E+03	26.13	2.68E+02
7	C	1% NaOCl	1.00E+01	24.50	4.08E-01
8	D	1% NaOCl	1.75E+02	18.28	9.57E+00
9	A	4% NaOCl	5.00E+00	24.84	2.01E-01
10	B	4% NaOCl	1.50E+01	16.20	9.26E-01
11	C	4% NaOCl	1.60E+03	23.46	6.82E+01
12	D	4% NaOCl	4.00E+01	14.70	2.72E+00
13	A	1% NaOCl + 0.5 W	1.15E+04	22.76	5.03E+02
14	B	1% NaOCl + 0.5 W	1.05E+02	20.89	5.03E+00
15	C	1% NaOCl + 0.5 W	0.00E+00	15.59	0.00E+00
16	D	1% NaOCl + 0.5 W	1.05E+02	18.19	5.77E+00
17	A	4% NaOCl + 0.5 W	5.00E+00	19.33	2.59E-01
18	B	4% NaOCl + 0.5 W	1.00E+01	26.13	3.83E-01
19	C	4% NaOCl + 0.5 W	6.30E+02	21.47	2.93E+01
20	D	4% NaOCl + 0.5 W	2.60E+03	22.18	1.17E+02
21	A	0.5% NaOCl + 0.5W	4.50E+02	22.27	2.02E+01
22	B	0.5% NaOCl + 0.5W	2.00E+01	18.59	1.08E+00
23	C	0.5% NaOCl + 0.5W	3.75E+02	19.97	1.88E+01
24	D	0.5% NaOCl + 0.5W	1.21E+04	20.67	5.85E+02

8.3.3 Flow Cell 3 (Study 3)

Sample	Position	Treatment Group	cfu/mL	µg protein/mL	cfu/µg protein
1	A	Saline	7.50E+05	23.52	3.19E+04
2	B	Saline	2.35E+06	23.89	9.84E+04
3	C	Saline	1.40E+07	33.99	4.10E+05
4	D	Saline	4.35E+05	19.87	2.19E+04
5	A	1% NaOCl	2.20E+05	22.72	9.66E+03
6	B	1% NaOCl	4.15E+03	16.14	2.57E+02
7	C	1% NaOCl	1.95E+03	14.93	1.31E+02
8	D	1% NaOCl	2.52E+05	15.17	1.66E+04
9	A	4% NaOCl	4.00E+01	16.57	2.41E+00
10	B	4% NaOCl	1.00E+01	19.95	5.01E-01
11	C	4% NaOCl	6.15E+03	19.22	3.20E+02
12	D	4% NaOCl	2.04E+04	13.53	1.51E+03
13	A	1% NaOCl + 0.5 W	1.10E+02	19.28	5.71E+00
14	B	1% NaOCl + 0.5 W	5.00E+00	19.19	2.61E-01
15	C	1% NaOCl + 0.5 W	4.65E+02	21.04	2.21E+01
16	D	1% NaOCl + 0.5 W	3.00E+01	24.39	1.23E+00
17	A	4% NaOCl + 0.5 W	1.20E+02	13.34	8.99E+00
18	B	4% NaOCl + 0.5 W	4.00E+01	14.01	2.85E+00
19	C	4% NaOCl + 0.5 W	0.00E+00	19.83	0.00E+00
20	D	4% NaOCl + 0.5 W	1.37E+04	13.04	1.05E+03
21	A	0.5% NaOCl + 0.5W	1.50E+01	23.90	6.27E-01
22	B	0.5% NaOCl + 0.5W	3.10E+03	22.20	1.40E+02
23	C	0.5% NaOCl + 0.5W	1.00E+02	20.16	4.96E+00
24	D	0.5% NaOCl + 0.5W	5.00E+00	14.07	3.55E-01

8.3.4 Flow Cell 4 (Study 3)

Sample	Position	Treatment Group	cfu/mL	µg protein/mL	cfu/µg protein
1	A	Saline	6.50E+05	39.84	1.63E+04
2	B	Saline	1.40E+05	36.72	3.81E+03
3	C	Saline	2.00E+05	34.49	5.80E+03
4	D	Saline	7.50E+05	37.98	1.97E+04
5	A	1% NaOCl	1.00E+04	26.13	3.83E+02
6	B	1% NaOCl	7.50E+03	24.26	3.09E+02
7	C	1% NaOCl	3.00E+03	26.01	1.15E+02
8	D	1% NaOCl	3.50E+03	23.08	1.52E+02
9	A	4% NaOCl	8.00E+01	22.37	3.58E+00
10	B	4% NaOCl	1.35E+02	22.40	6.03E+00
11	C	4% NaOCl	3.65E+03	21.85	1.67E+02
12	D	4% NaOCl	9.50E+02	20.66	4.60E+01
13	A	1% NaOCl + 0.5 W	4.45E+03	25.67	1.73E+02
14	B	1% NaOCl + 0.5 W	4.50E+02	22.44	2.01E+01
15	C	1% NaOCl + 0.5 W	7.50E+02	18.73	4.00E+01
16	D	1% NaOCl + 0.5 W	6.15E+03	20.62	2.98E+02
17	A	4% NaOCl + 0.5 W	4.00E+01	17.88	2.24E+00
18	B	4% NaOCl + 0.5 W	2.80E+02	19.32	1.45E+01
19	C	4% NaOCl + 0.5 W	3.70E+03	17.62	2.10E+02
20	D	4% NaOCl + 0.5 W	8.00E+02	21.51	3.72E+01
21	A	0.5% NaOCl + 0.5W	1.90E+03	22.93	8.29E+01
22	B	0.5% NaOCl + 0.5W	1.25E+03	20.70	6.04E+01
23	C	0.5% NaOCl + 0.5W	3.10E+03	21.18	1.46E+02
24	D	0.5% NaOCl + 0.5W	6.80E+03	19.14	3.55E+02

9.0 Bibliography

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.

Abou-Rass M, Piccinino MV (1982) The effectiveness of four clinical irrigation methods on the removal of root canal debris. *Oral Surgery, Oral Medicine, Oral Pathology* **54,** 323-328.

Akpata ES (1976) Effect of endodontic procedures on the population of viable microorganisms in the infected root canal. *Journal of Endodontics* **2,** 369-373.

Al-Ahmad A, Maier J, Follo M *et al.* (2010) Food-borne enterococci integrate into oral biofilm: An in vivo study. *Journal of Endodontics* **36,** 1812-1819.

Aranki A, Freter R (1972) Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. *American Journal of Clinical Nutrition* **25,** 1329.

Aubut V, Pommel L, Verhille B *et al.* (2010) Biological properties of a neutralized 2.5% sodium hypochlorite solution. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **109,** e120-e125.

Bahcall J, Howard P, Miserendino L, Walia H (1992) Preliminary investigation of the histological effects of laser endodontic treatment on the periradicular tissues in dogs. *Journal of Endodontics* **18,** 47-51.

Baker NE, Liewehr FR, Buxton TB, Joyce AP (2004) Antibacterial efficacy of calcium hydroxide, iodine potassium iodide, betadine, and betadine scrub with and without surfactant against e faecalis *in vitro*. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology* **98,** 359-364.

Barrette WC, Hannum DM, Wheeler WD, Hurst JK (1989) General mechanism for the bacterial toxicity of hypochlorous acid: Abolition of ATP production. *Biochemistry* **28,** 9172-9178.

Baumgartner JC, Brown CM, Mader CL, Peters DD, Shulman JD (1984) A scanning electron microscopic evaluation of root canal debridement using saline, sodium hypochlorite, and citric acid. *Journal of Endodontics* **10,** 525-531.

Baumgartner JC, Mader CL (1987) A scanning electron microscopic evaluation of four root canal irrigation regimens. *Journal of Endodontics* **13,** 147-157.

Baumgartner J, Falkler Jr W (1991) Bacteria in the apical 5 mm of infected root canals. *Journal of Endodontics* **17,** 380.

Becker GL, Cohen S, Borer R (1974) The sequelae of accidentally injecting sodium hypochlorite beyond the root apex: Report of a case. *Oral Surgery, Oral Medicine, Oral Pathology* **38,** 633-638.

Berber VB, Gomes BPFA, Sena NT *et al.* (2006) Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing *Enterococcus faecalis* within root canals and dentinal tubules. *International Endodontic Journal* **39,** 10-17.

Bergmans L, Moisiadis P, Huybrechts B, Van Meerbeek B, Quirynen M, Lambrechts P (2008) Effect of photo-activated disinfection on endodontic pathogens *ex vivo*. *International Endodontic Journal* **41,** 227-239.

Bergmans L, Moisiadis P, Teughels W, Van Meerbeek B, Quirynen M, Lambrechts P (2006) Bactericidal effect of Nd:YAG laser irradiation on some endodontic pathogens *ex vivo*. *International Endodontic Journal* **39,** 547-557.

Berutti E, Marini R, Angeretti A (1997) Penetration ability of different irrigants into dentinal tubules. *Journal of Endodontics* **23,** 725-727.

Blanken J, Verdaasdonk R (2007) Cavitation as a working mechanism of the Er,Cr:YSGG laser in endodontics: A visualization study. *Journal of Oral Laser Applications* **7,** 97-106.

Blanken J, De Moor RJ, Meire M, Verdaasdonk R (2009) Laser induced explosive vapor and cavitation resulting in effective irrigation of the root canal. Part 1: A visualization study. *Lasers in Surgery and Medicine* **41,** 514-519.

Bloomfield SF, Miles GA (1979) The antibacterial properties of sodium dichloroisocyanurate and sodium hypochlorite formulations. *Journal of Applied Microbiology* **46,** 65-73.

Bogosian G, Morris PJL, O'Neil JP (1998) A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. *Applied and Environmental Microbiology* **64,** 1736-1742.

Bonsor SJ, Nichol R, Reid TMS, Pearson GJ (2006) Microbiological evaluation of photo-activated disinfection in endodontics (an *in vivo* study). *British Dental Journal* **200,** 337-341.

Boutsioukis C, Lambrianidis T, Vasiliadis L (2007) Clinical relevance of standardization of endodontic irrigation needle dimensions according to the ISO 9626:1991 and 9626:1991/amd 1:2001 specification. *International Endodontic Journal* **40,** 700-706.

Bronnec F, Bouillaguet S, Machtou P (2010) Ex vivo assessment of irrigant penetration and renewal during the final irrigation regimen. *International Endodontic Journal* **43,** 663-672.

Brown Jr L, Rudolph Jr C (1957) Isolation and identification of microorganisms from unexposed canals of pulp-involved teeth. *Oral Surgery, Oral Medicine, Oral Pathology* **10,** 1094.

Byström A, Sundqvist G (1981) Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *European Journal of Oral Sciences* **89,** 321-328.

Byström A, Sundqvist G (1983) Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. *Oral Surgery, Oral Medicine, Oral Pathology* **55,** 307-312.

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.

Byström A, Claesson R, Sundqvist G (1985) The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Dental Traumatology* **1,** 170-175.

Byström A, Happonen R, Sjögren U, Sundqvist G (1987) Healing of periapical lesions of pulpless teeth after endodontic treatment with controlled asepsis. *Dental Traumatology* **3,** 58-63.

Çalışkan MK, Turkun M, Alper S (1994) Allergy to sodium hypochlorite during root canal therapy: A case report. *International Endodontic Journal* **27,** 163-167.

Çalışkan M, Parlar N, Oruçoğlu H, Aydın B (2010) Apical microleakage of root-end cavities prepared by Er,Cr:YSGG laser. *Lasers in Medical Science* **25,** 145-150.

Camps J, Pommel L, Aubut V *et al.* (2009) Shelf life, dissolving action, and antibacterial activity of a neutralized 2.5% sodium hypochlorite solution. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **108,** e66-e73.

Chavez de Paz L (2007) Redefining the persistent infection in root canals: Possible role of biofilm communities. *Journal of Endodontics* **33,** 652-662.

Chivatxaranukul P, Dashper SG, Messer HH (2008) Dentinal tubule invasion and adherence by *Enterococcus faecalis*. *International Endodontic Journal* **41,** 873-882.

Chow TW (1983) Mechanical effectiveness of root canal irrigation. *Journal of Endodontics* **9,** 475-479.

Clarkson RM, Podlich HM, Savage NW, Moule AJ (2003) A survey of sodium hypochlorite use by general dental practitioners and endodontists in Australia. *Australian Dental Journal* **48,** 20-26.

Clarkson RM, Moule AJ, Podlich H *et al.* (2006) Dissolution of porcine incisor pulps in sodium hypochlorite solutions of varying compositions and concentrations. *Australian Dental Journal* **51,** 245-251.

Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR (2006) The effect of exposure to irrigant solutions on apical dentin biofilms *in vitro*. *Journal of Endodontics* **32,** 434-437.

Costigan SM (1936) Effectiveness of hot hypochlorites of low alkalinity in destroying *Mycobacterium tuberculosis*. *Journal of bacteriology* **32,** 57-63.

Cotter JL, Fader RC, Lilley C, Herndon DN (1985) Chemical parameters, antimicrobial activities, and tissue toxicity of 0.1 and 0.5% sodium hypochlorite solutions. *Antimicrobial Agents and Chemotherapy* **28,** 118-122.

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.

Cunningham WT, Joseph SW (1980) Effect of temperature on the bactericidal action of sodium hypochlorite endodontic irrigant. *Oral Surgery, Oral Medicine, Oral Pathology* **50,** 569-571.

Cvek M, Hollender L, Nord CE (1976a) Treatment of non-vital permanent incisors with calcium hydroxide. A clinical, microbiological and radiological evaluation of treatment in one sitting of teeth with mature or immature root. *Odontologisk Revy* **27,** 93-108.

Cvek M, Nord CE, Hollender L (1976b) Antimicrobial effect of root canal debridement in teeth with immature root. A clinical and microbiologic study. *Odontologisk Revy* **27,** 1-10.

Cvitkovitch D, Li Y, Ellen R (2003) Quorum sensing and biofilm formation in streptococcal infections. *Journal of Clinical Investigation* **112,** 1626-1632.

Dakin H (1915) The use of certain antiseptic substances in treatment of infected wounds. . *British Medical Journal* **2,** 318-320.

Dalton BC, Ørstavik D, Phillips C, Pettiette M, Trope M (1998) Bacterial reduction with nickel-titanium rotary instrumentation. *Journal of Endodontics* **24,** 763-767.

de Groot SD, Verhaagen B, Versluis M, Wu MK, Wesselink PR, van der Sluis LW (2009) Laser-activated irrigation within root canals: Cleaning efficacy and flow visualization. *International Endodontic Journal* **42,** 1077-1083.

De Moor RJG, Blanken J, Meire M, Verdaasdonk R (2009) Laser induced explosive vapor and cavitation resulting in effective irrigation of the root canal. Part 2: Evaluation of the efficacy. *Lasers in Surgery and Medicine* **41,** 520-523.

De Moor RJG, Meire M, Goharkhay K, Moritz A, Vanobbergen J (2010) Efficacy of ultrasonic versus laser-activated irrigation to remove artificially placed dentin debris plugs. *Journal of Endodontics* **36,** 1580-1583.

Demtröder W (2006) Lasers; atoms, molecules and photons. Springer Berlin Heidelberg: pp. 283-319

Dewsnup N, Pileggi R, Haddix J, Nair U, Walker C, Varella CH (2010) Comparison of bacterial reduction in straight and curved canals using erbium, chromium:yttrium-

scandium-gallium-garnet laser treatment versus a traditional irrigation technique with sodium hypochlorite. *Journal of Endodontics* **36,** 725-728.

Dickers B, Lamard L, Peremans A *et al.* (2009) Temperature rise during photo-activated disinfection of root canals. *Lasers in Medical Science* **24,** 81-85.

Distel J, Hatton J, Gillespie M (2002) Biofilm formation in medicated root canals. *Journal of Endodontics* **28,** 689-693.

DiVito E, Peters O, Olivi G (2012) Effectiveness of the erbium:Yag laser and new design radial and stripped tips in removing the smear layer after root canal instrumentation. *Lasers in Medical Science* **27,** 273-280.

Dolmans DEJGJ, Fukumura D, Jain RK (2003) Photodynamic therapy for cancer. *Nature Reviews Cancer* **3,** 380-387.

Donlan R, Costerton J (2002) Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* **15,** 167.

Dugas NN, Lawrence HP, Teplitsky PE, Pharoah MJ, Friedman S (2003) Periapical health and treatment quality assessment of root-filled teeth in two canadian populations. *International Endodontic Journal* **36,** 181-192.

Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL (2006) Comparative evaluation of endodontic irrigants against *Enterococcus faecalis* biofilms. *Journal of Endodontics* **32,** 527-531.

Dutner J, Mines P, Anderson A (2012) Irrigation trends among American Association of Endodontists members: A web-based survey. *Journal of Endodontics* **38,** 37-40.

Eldeniz AU, Ozer F, Hadimli HH, Erganis O (2007) Bactericidal efficacy of er,cr:Ysgg laser irradiation against *Enterococcus faecalis* compared with naocl irrigation: An *ex vivo* pilot study. *International Endodontic Journal* **40,** 112-119.

Elliott RD, Roberts MW, Burkes J, Phillips C (1999) Evaluation of the carbon dioxide laser on vital human primary pulp tissue. *Pediatric Dentistry* **21,** 327-331.

Endodontology ESo (2006) Quality guidelines for endodontic treatment: Consensus report of the european society of endodontology. *International Endodontic Journal* **39,** 921-930.

Erdemir H, Ari H, Güngüneş H, Belli S (2004) Effect of medications for root canal treatment on bonding to root canal dentin. *Journal of Endodontic* **30,**113-116.

Eriksson AR, Albrektsson T (1983) Temperature threshold levels for heat-induced bone tissue injury: A vital-microscopic study in the rabbit. *Journal of Prosthetic Dentistry* **50,** 101-117.

Estrela C, Estrela CR, Barbin EL, Spano JC, Marchesan MA, Pecora JD (2002) Mechanism of action of sodium hypochlorite. *Brazilian Dental Journal* **13,** 113-117.

Evans M, Davies J, Sundqvist G, Figdor D (2002) Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *International Endodontic Journal* **35,** 221-228.

Fabricius L, Dahlén G, Holm S, Möller A (1982a) Influence of combinations of oral bacteria on periapical tissues of monkeys. *European Journal of Oral Sciences* **90,** 200-206.

Fabricius L, Dahlén G, Möller A (1982b) Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. *European Journal of Oral Sciences* **90,** 134-144.

Falk KW, Sedgley CM (2005) The influence of preparation size on the mechanical efficacy of root canal irrigation *in vitro*. *Journal of Endodontics* **31,** 742-745.

Figdor D (2002) Apical periodontitis: A very prevalent problem. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **94,** 651-652.

Figdor D, Davies J, Sundqvist G (2003) Starvation survival, growth and recovery of *Enterococcus faecalis* in human serum. *Oral Microbiology and Immunology* **18,** 234-239.

Folwaczny M, Mehl A, Jordan C, Hickel R (2002) Antibacterial effects of pulsed Nd:YAG laser radiation at different energy settings in root canals. *Journal of Endodontics* **28,** 24-29.

Fouad A, Barry J, Caimano M *et al.* (2002) PCR-based identification of bacteria associated with endodontic infections. *Journal of Clinical Microbiology* **40,** 3223.

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.

Franz CMAP, Holzapfel WH, Stiles ME (1999) Enterococci at the crossroads of food safety? *International Journal of Food Microbiology* **47,** 1-24.

Franz CMAP, Stiles ME, Schleifer KH, Holzapfel WH (2003) Enterococci in foods - a conundrum for food safety. *International Journal of Food Microbiology* **88,** 105-122.

George R, Meyers IA, Walsh LJ (2008) Laser activation of endodontic irrigants with improved conical laser fiber tips for removing smear layer in the apical third of the root canal. *Journal of Endodontics* **34,** 1524-1527.

George R, Walsh LJ (2008) Apical extrusion of root canal irrigants when using Er:YAG and Er,Cr:YSGG lasers with optical fibers: An *in vitro* dye study. *Journal of Endodontics* **34,** 706-708.

Giardino L, Ambu E, Savoldi E, Rimondini R, Cassanelli C, Debbia EA (2007) Comparative evaluation of antimicrobial efficacy of sodium hypochlorite, MTAD, and tetraclean against *Enterococcus faecalis* biofilm. *Journal of Endodontics* **33,** 852-855.

Gilbert P, Das J, Foley I (1997) Biofilm susceptibility to antimicrobials. *Advances in Dental Research* **11,** 160-167.

Goldman L, Hornby P, Meyer R, Goldman B (1964) Impact of the laser on dental caries. *Nature* **203,** 417.

Goldman M, Goldman LB, Cavaleri R, Bogis J, Lin PS (1982) The efficacy of several endodontic irrigating solutions: A scanning electron microscopic study: Part 2. *Journal of Endodontics* **8,** 487-492.

Gomes BPFA, Ferraz CCR, M. E V, Berber VB, Teixeira FB, Souza-Filho FJ (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.

Gomes B, Pinheiro E, Gade-Neto C *et al.* (2004) Microbiological examination of infected dental root canals. *Oral Microbiology and Immunology* **19,** 71-76.

Gordon W, Atabakhsh VA, Meza F *et al.* (2007) The antimicrobial efficacy of the erbium, chromium:Yttrium-scandium-gallium-garnet laser with radial emitting tips on root canal dentin walls infected with *Enterococcus faecalis*. *Journal of the American Dental Association* **138,** 992-1002.

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

Gu L-S, Kim JR, Ling J, Choi KK, Pashley DH, Tay FR (2009) Review of contemporary irrigant agitation techniques and devices. *Journal of Endodontics* **35,** 791-804.

Gulabivala K, Ng YL, Gilbertson M, Eames I (2010) The fluid mechanics of root canal irrigation. *Physiological Measurement* **31,** R49-R84.

Gutknecht N, Moritz A, Conrads G, Sievert T, Lampert F (1996) Bactericidal effect of the Nd:YAG laser in *in vitro* root canals. *Journal of Clinical Laser Medicine and Surgery* **14,** 77-80.

Haapasalo HK, Sirén EK, Waltimo TM, Ørstavik D, Haapasalo MP (2000) Inactivation of local root canal medicaments by dentine: An *in vitro* study. *International Endodontic Journal* **33,** 126-131.

Haapasalo M, Udnæs T, Endal U (2003) Persistent, recurrent, and acquired infection of the root canal system post-treatment. *Endodontic Topics* **6,** 29-56.

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

Hancock HH, Sigurdsson A, Trope M, Moiseiwitsch JR (2001) Bacteria isolated after unsuccessful endodontic treatment in a north american population. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics* **91,** 579-586.

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.

Hardee MW, Miserendino LJ, Kos W, Walia H (1994) Evaluation of the antibacterial effects of intracanal Nd:YAG laser irradiation. *Journal of Endodontics* **20,** 377-380.

Harrison AJ, Chivatxaranukul P, Parashos P, Messer HH (2010) The effect of ultrasonically activated irrigation on reduction of *Enterococcus faecalis* in experimentally infected root canals. *International Endodontic Journal* **43,** 968-977.

Harrison JW, Svec TA, Baumgartner JC (1978) Analysis of clinical toxicity of endodontic irrigants. *Journal of Endodontics* **4,** 6-11.

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.

He S, Wang Y, Li X, Hu D (2011) Effectiveness of laser therapy and topical desensitising agents in treating dentine hypersensitivity: A systematic review. *Journal of Oral Rehabilitation* **38,** 348-358.

Heim S, Del Mar Lleo M, Bonato B, Guzman CA, Canepari P (2002) The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. *Journal of Bacteriology* **184,** 6739-6745.

Hmud R, Kahler WA, George R, Walsh LJ (2010) Cavitational effects in aqueous endodontic irrigants generated by near-infrared lasers. *Journal of Endodontics* **36,** 275-278.

Hommez GMG, Coppens CRM, De Moor RJG (2002) Periapical health related to the quality of coronal restorations and root fillings. *International Endodontic Journal* **35,** 680-689.

Huang TY, Gulabivala K, Ng YL (2008) A bio-molecular film ex-vivo model to evaluate the influence of canal dimensions and irrigation variables on the efficacy of irrigation. *International Endodontic Journal* **41,** 60-71.

Hubble T, Hatton J, Nallapareddy S, Murray B, Gillespie M (2003) Influence of *Enterococcus faecalis* proteases and the collagen binding protein, ace, on adhesion to dentin. *Oral Microbiology and Immunology* **18,** 121-126.

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

Jacinto R, Gomes B, Ferraz C, Zaia A, Souza Filho F (2003) Microbiological analysis of infected root canals from symptomatic and asymptomatic teeth with periapical periodontitis and the antimicrobial susceptibility of some isolated anaerobic bacteria. *Oral Microbiology and Immunology* **18,** 285-292.

Jafarzadeh H (2009) Laser doppler flowmetry in endodontics: A review. *International Endodontic Journal* **42,** 476-490.

Johnson BR, Remeikis NA (1993) Effective shelf-life of prepared sodium hypochlorite solution. *Journal of Endodontics* **19,** 40-43.

Johnson SA, Goddard PA, Iliffe C *et al.* (2002) Comparative susceptibility of resident and transient hand bacteria to para-chloro-meta-xylenol and triclosan. *Journal of Applied Microbiology* **93,** 336-344.

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.

Kantz W, Henry C (1974) Isolation and classification of anaerobic bacteria from intact pulp chambers of non-vital teeth in man. *Archives of Oral Biology* **19,** 91-96.

Karlovic Z, Pezelj-Ribaric S, Miletic I, Jukic S, Grgurevic J, Anic I (2005) Erbium:YAG laser versus ultrasonic in preparation of root-end cavities. *Journal of Endodontics* **31,** 821-823.

Katebzadeh N, Hupp J, Trope M (1999) Histological periapical repair after obturation of infected root canals in dogs. *Journal of Endodontics* **25,** 364-368.

Katebzadeh N, Sigurdsson A, Trope M (2000) Radiographic evaluation of periapical healing after obturation of infected root canals: An in vivo study. *International Endodontic Journal* **33,** 60-65.

Kaufman AY, Keila S (1989) Hypersensitivity to sodium hypochlorite. *Journal of Endodontics* **15,** 224-226.

Kayaoglu G, Ørstavik D (2004) Virulence factors of *Enterococcus faecalis*: Relationship to endodontic disease. *Critical Reviews in Oral Biology and Medicine* **15,** 308-320.

Khayat A, Lee SJ, Torabinejad M (1993) Human saliva penetration of coronally unsealed obturated root canals. *Journal of Endodontics* **19,** 458-461.

Kienle A, Forster FK, Diebolder R, Hibst R (2003) Light propagation in dentin: Influence of microstructure on anisotropy. *Physics in Medicine and Biology* **48,** N7-14.

Kimura Y, Wilder-Smith P, Matsumoto K (2000) Lasers in endodontics: A review. *International Endodontic Journal* **33,** 173-185.

Kirkevang LL, Ørstavik D, Hörsted-Bindslev P, Wenzel A (2000) Periapical status and quality of root fillings and coronal restorations in a danish population. *International Endodontic Journal* **33,** 509-515.

Kleier DJ, Averbach RE, Mehdipour O (2008) The sodium hypochlorite accident: Experience of diplomates of the American Board of Endodontics. *Journal of Endodontics* **34,** 1346-1350.

Klinke T, Klimm W, Gutknecht N (1997) Antibacterial effects of Nd:YAG laser irradiation within root canal dentin. *Journal of Clinical Laser Medicine and Surgery* **15,** 29-31.

Kokkas AB, Boutsioukis A, Vassiliadis LP, Stavrianos CK (2004) The influence of the smear layer on dentinal tubule penetration depth by three different root canal sealers: An *in vitro* study. *Journal of Endodontics* **30,** 100-102.

Konopka K, Goslinski T (2007) Photodynamic therapy in dentistry. *Journal of Dental Research* **86,** 694-707.

Kouchi Y, Ninomiya J, Yasuda H, Fukui K, Moriyama T, Okamoto H (1980) Location of *Streptococcus mutans* in the dentinal tubules of open infected root canals. *Journal of Dental Research* **59,** 2038-2046.

Lamers AC, van Mullem PJ, Simon M (1980) Tissue reactions to sodium hypochlorite and iodine potassium iodide under clinical conditions in monkey teeth. *Journal of Endodontics* **6,** 788-792.

Law A, Messer H (2004) An evidence-based analysis of the antibacterial effectiveness of intracanal medicaments. *Journal of Endodontics* **30,** 689-694.

Lee MT, Bird PS, Walsh LJ (2004a) Photo-activated disinfection of the root canal: A new role for lasers in endodontics. *Australian Endodontic Journal* **30,** 93-98.

Lee SJ, Wu MK, Wesselink PR (2004b) The effectiveness of syringe irrigation and ultrasonics to remove debris from simulated irregularities within prepared root canal walls. *International Endodontic Journal* **37,** 672-678.

Lim Z, Cheng JL, Lim TW *et al.* (2009) Light activated disinfection: An alternative endodontic disinfection strategy. *Australian Dental Journal* **54,** 108-114.

Liu H, Wei X, Ling J, Wang W, Huang X (2010) Biofilm formation capability of *Enterococcus faecalis* cells in starvation phase and its susceptibility to sodium hypochlorite. *Journal of Endodontics* **36,** 630-635.

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

Love RM (2004) Invasion of dentinal tubules by root canal bacteria. *Endodontic Topics* **9,** 52-65.

Macdonald J, Hare G, Wood A (1957) The bacteriologic status of the pulp chambers in intact teeth found to be nonvital following trauma. *Oral Surgery, Oral Medicine, Oral Pathology* **10,** 318-322.

Macedo RG, Wesselink PR, Zaccheo F, Fanali D, van der Sluis LWM (2010) Reaction rate of NaOCl in contact with bovine dentine: Effect of activation, exposure time, concentration and pH. *International Endodontic Journal* **43,** 1108-1115.

Mader CL, Baumgartner JC, Peters DD (1984) Scanning electron microscopic investigation of the smeared layer on root canal walls. *Journal of Endodontics* **10,** 477-483.

Maiman TH (1960) Stimulated optical radiation in ruby. *Nature* **187,** 493-494.

Marsh PD (2003) Are dental diseases examples of ecological catastrophes? *Microbiology* **149,** 279-294.

Martin H (1975) Quantitative bactericidal effectiveness of an old and a new endodontic irrigant. *Journal of Endodontics* **1,** 164-167.

Matsumoto H, Yoshimine Y, Akamine A (2011) Visualization of irrigant flow and cavitation induced by Er:YAG laser within a root canal model. *Journal of Endodontics* **37,** 839-843.

Matsuo T, Shirakami T, Ozaki K, Nakanishi T, Yumoto H, Ebisu S (2003) An immunohistological study of the localization of bacteria invading root pulpal walls of teeth with periapical lesions. *Journal of Endodontics* **29,** 194-200.

McComb D, Smith DC (1975) A preliminary scanning electron microscopic study of root canals after endodontic procedures. *Journal of Endodontics* **1,** 238-242.

McDonnel G, Russell AD (1999) Antiseptics and disinfectants : Activity, action, and resistance. *Clinical Microbiology Reviews* **12,** 147-179.

McGill S, Gulabivala K, Mordan N, Ng YL (2008) The efficacy of dynamic irrigation using a commercially available system (Rinsendo®) determined by removal of a collagen 'bio-molecular film' from an ex vivo model. *International Endodontic Journal* **41,** 602-608.

McKenna SM, Davies KJ (1988) The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. *The Biochemical Journal* **254,** 685-692.

Mehl A, Folwaczny M, Haffner C, Hickel R (1999) Bactericidal effects of 2.94 microns Er:YAG laser radiation in dental root canals. *Journal of Endodontics* **25,** 490-493.

Meire MA, De Prijck K, Coenye T, Nelis HJ, De Moor RJG (2009) Effectiveness of different laser systems to kill *Enterococcus faecalis* in aqueous suspension and in an infected tooth model. *International Endodontic Journal* **42,** 351-359.

Menzel R (2007) Lasers, photonics. pp. 359-531: Springer Berlin Heidelberg.

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. *Annual Review of Microbiology* **55,** 165-199.

Miller TA, Baumgartner JC (2010) Comparison of the antimicrobial efficacy of irrigation using the endovac to endodontic needle delivery. *Journal of Endodontics* **36,** 509-511.

Miller W (1894) An introduction to the study of the bacteriopathology of the dental pulp. *Dental Cosmos* **36,** 505-528.

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

Möller AJR (1966) Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies. *Odontologisk Tidskrift* **74**.

Möller AJR, Fabricius L, Dahlén G (1981) Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *Scandinavian Journal of Dental Research* **89,** 475-484.

Molven O, Olsen I, Kerekes K (1991) Scanning electron microscopy of bacteria in the apical part of root canals in permanent teeth with periapical lesions. *Dental Traumatology* **7,** 226-229.

Moon Y-M, Kim H-C, Bae K-S, Baek S-H, Shon W-J, Lee W (2012) Effect of laser-activated irrigation of 1320-nanometer Nd:YAG laser on sealer penetration in curved root canals. *Journal of Endodontics* **38,** 531-535.

Moore W (1966) Techniques for routine culture of fastidious anaerobes. *International Journal of Systematic and Evolutionary Microbiology* **16,** 173.

Moorer WR, Wesselink PR (1982) Factors promoting the tissue dissolving capability of sodium hypochlorite. *International Endodontic Journal* **15,** 187-196.

Moritz A, Doertbudak O, Gutknecht N, Goharkhay K, Schoop U, Sperr W (1997) Nd:YAG laser irradiation of infected root canals in combination with microbiological examinations. *Journal of the American Dental Association* **128,** 1525-1530.

Naenni N, Thoma K, Zehnder M (2004) Soft tissue dissolution capacity of currently used and potential endodontic irrigants. *Journal of Endodontics* **30,** 785-787.

Nair PNR, Schroeder H (1984) Periapical actinomycosis. *Journal of Endodontics* **10,** 567-570.

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

Nair PNR, Sjögren U, Krey G, Kahnberg K, Sundqvist G (1990) Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: A long-term light and electron microscopic follow-up study. *Journal of Endodontics* **16,** 580-588.

Nair PNR, Pajarola G, Schroeder HE (1996) Types and incidence of human periapical lesions obtained with extracted teeth. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **81,** 93-102.

Nair PNR, Sjögren U, Figdor D, Sundqvist G (1999) Persistent periapical radiolucencies of root-filled human teeth, failed endodontic treatments, and periapical scars. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **87,** 617-627.

Nair PNR, Henry S, Cano V, Vera J (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **99,** 231-252.

Ng R, Singh F, Papamanou DA *et al.* (2011) Endodontic photodynamic therapy ex vivo. *Journal of Endodontics* **37,** 217-222.

Nguy D, Sedgley C (2006) The influence of canal curvature on the mechanical efficacy of root canal irrigation *in vitro* using real-time imaging of bioluminescent bacteria. *Journal of Endodontics* **32,** 1077-1080.

Noiri Y, Katsumoto T, Azakami H, Ebisu S (2008) Effects of Er:YAG laser irradiation on biofilm-forming bacteria associated with endodontic pathogens *in vitro*. *Journal of Endodontics* **34,** 826-829.

Odabas ME, Bodur H, Bariotas E, Demir C (2007) Clinical, radiographic, and histopathologic evaluation of Nd:YAG laser pulpotomy on human primary teeth. *Journal of Endodontics* **33,** 415-421.

Odor TM, Chandler NP, Watson TF, Ford TRP, McDonald F (1999) Laser light transmission in teeth: A study of the patterns in different species. *International Endodontic Journal* **32,** 296-302.

Oliver JD (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews* **34,** 415-425.

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

Parker S (2007) Verifiable CPD paper: Laser-tissue interaction. *British Dental Journal* **202,** 73-81.

Pascon FM, Kantovitz KR, Sacramento PA, Nobre-dos-Santos M, Puppin-Rontani RM (2009) Effect of sodium hypochlorite on dentine mechanical properties. A review. *Journal of Dentistry* **37,** 903-908.

Pashley DH (1984) Smear layer: Physiological considerations. *Operative Dentistry.* Supplement **3,** 13-29.

Pashley EL, Birdsong NL, Bowman K, Pashley DH (1985) Cytotoxic effects of NaOCl on vital tissue. *Journal of Endodontics* **11,** 525-528.

Pedullà E, Genovese C, Campagna E, Tempera G, Rapisarda E (2012) Decontamination efficacy of photon-initiated photoacoustic streaming (PIPS) of irrigants using low-energy laser settings: An ex vivo study. *International Endodontic Journal*. in press.

Peters LB, Wesselink PR, Moorer WR (1995) The fate and the role of bacteria left in root dentinal tubules. *International Endodontic Journal* **28,** 95-99.

Peters LB, Wesselink P (2002) Periapical healing of endodontically treated teeth in one and two visits obturated in the presence or absence of detectable microorganisms. *International Endodontic Journal* **35,** 660-667.

Peters LB, Wesselink P, Van Winkelhoff A (2002a) Combinations of bacterial species in endodontic infections. *International Endodontic Journal* **35,** 698-702.

Peters LB, van Winkelhoff AJ, Buijs JF, Wesselink PR (2002b) Effects of instrumentation, irrigation and dressing with calcium hydroxide on infection in pulpless teeth with periapical bone lesions. *International Endodontic Journal* **35,** 13-21.

Peters OA, Schönenberger K, Laib A (2001) Effects of four NiTi preparation techniques on root canal geometry assessed by micro computed tomography. *International Endodontic Journal* **34,** 221-230.

Peters OA, Bardsley S, Fong J, Pandher G, Divito E (2011) Disinfection of root canals with photon-initiated photoacoustic streaming. *Journal of Endodontics* **37,** 1008-1012.

Pinheiro ET, Gomes BPFA, Ferraz CCR, Sousa ELR, Teixeira FB, Souza-Filho FJ (2003) Microorganisms from canals of root-filled teeth with periapical lesions. *International Endodontic Journal* **36,** 1-11.

Plutzer B (2009) Comparative efficacy of endodontic medicaments against *Enterococcus faecalis* biofilms. *[D.Clin.Dent thesis] The University of Adelaide, Australia*.

Portenier I, Waltimo T, Haapasalo M (2003) *Enterococcus faecalis*: The root canal survivor and star in post treatment disease. *Endodontic Topics* **6,** 135-159.

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.

Radatti DA, Baumgartner JC, Marshall JG (2006) A comparison of the efficacy of Er,Cr:YSGG laser and rotary instrumention in root canal debridement. *Journal of the American Dental Association* **137,** 1261-1266.

Ram Z (1977) Effectiveness of root canal irrigation. *Oral Surgery, Oral Medicine, Oral Pathology* **44,** 306-312.

Raphael D, Wong TA, Moodnik R, Borden BG (1981) The effect of temperature on the bactericidal efficiency of sodium hypochlorite. *Journal of Endodontics* **7,** 330-334.

Ray HA, Trope M (1995) Periapical status of endodontically treated teeth in relation to the technical quality of the root filling and the coronal restoration. *International Endodontic Journal* **28,** 12-18.

Razavi A, Gmür R, Imfeld T, Zehnder M (2007) Recovery of *Enterococcus faecalis* from cheese in the oral cavity of healthy subjects. *Oral Microbiology and Immunology* **22,** 248-251.

Reeh ES, Messer HH, Douglas WH (1989) Reduction in tooth stiffness as a result of endodontic and restorative procedures. *Journal of Endodontics* **15,** 512-516.

Retamozo B, Shabahang S, Johnson N, Aprecio RM, Torabinejad M (2010) Minimum contact time and concentration of sodium hypochlorite required to eliminate *Enterococcus faecalis*. *Journal of Endodontics* **36,** 520-523.

Ricucci D, Siqueira J (2010) Biofilms and apical periodontitis: Study of prevalence and association with clinical and histopathologic findings. *Journal of Endodontics* **36,** 1277-1288.

Rios A, He J, Glickman GN, Spears R, Schneiderman ED, Honeyman AL (2011) Evaluation of photodynamic therapy using a light-emitting diode lamp against *Enterococcus faecalis* in extracted human teeth. *Journal of Endodontics* **37,** 856-859.

Rôças IN, Siqueira JF, Andrade AFB, Uzeda M (2003) Oral treponemes in primary root canal infections as detected by nested PCR. *International Endodontic Journal* **36,** 20-26.

Rôças IN, Siqueira JF (2012) Characterization of microbiota of root canal-treated teeth with posttreatment disease. *Journal of Clinical Microbiology* **50,** 1721-1724.

Rooney J, Midda M, Leeming J (1994) A laboratory investigation of the bactericidal effect of a Nd:YAG laser. *British Dental Journal* **176,** 61-64.

Rossi-Fedele G, Guastalli AR, Doğramacı EJ, Steier L, De Figueiredo JAP (2011) Influence of pH changes on chlorine-containing endodontic irrigating solutions. *International Endodontic Journal* **44,** 792-799.

Sabala CL, Powell SE (1989) Sodium hypochlorite injection into periapical tissues. *Journal of Endodontics* **15,** 490-492.

Safavi KE, Spångberg LS, Langeland K (1990) Root canal dentinal tubule disinfection. *Journal of Endodontics* **16,** 207-210.

Sahar-Helft S, Slutzky-Goldberg I, Moshonov J *et al.* (2011) Synergistic effect of Er:YAG laser irradiation in combination with chlorhexidine on the viability of *Enterococcus faecalis*: An *in vitro* study. *Photomedicine and Laser Surgery* **29,** 753-758.

Saleh IM, Ruyter IE, Haapasalo M, Ørstavik D (2004) Survival of *Enterococcus faecalis* in infected dentinal tubules after root canal filling with different root canal sealers *in vitro*. *International Endodontic Journal* **37,** 193-198.

Sarkar S, Wilson M (1993) Lethal photosensitization of bacteria in subgingival plaque from patients with chronic periodontitis. *Journal of Periodontal Research* **28,** 204-210.

Sathorn C, Parashos P, Messer H (2005) Effectiveness of single versus multiple visit endodontic treatment of teeth with apical periodontitis: A systematic review and meta analysis. *International Endodontic Journal* **38,** 347-355.

Schäfer E, Bossmann K (2005) Antimicrobial efficacy of chlorhexidine and two calcium hydroxide formulations against *Enterococcus faecalis*. *Journal of Endodontics* **31,** 53-56.

Schoop U, Kluger W, Moritz A, Nedjelik N, Georgopoulos A, Sperr W (2004) Bactericidal effect of different laser systems in the deep layers of dentin. *Lasers in Surgery and Medicine* **35,** 111-116.

Schoop U, Goharkhay K, Klimscha J *et al.* (2007) The use of the erbium, chromium:yttrium-scandium-gallium-garnet laser in endodontic treatment: The results of an *in vitro* study. *Journal of the American Dental Association* **138,** 949-955.

Schoop U, Barylyak A, Goharkhay K *et al.* (2009) The impact of an erbium, chromium:yttrium-scandium-gallium-garnet laser with radial-firing tips on endodontic treatment. *Lasers in Medical Science* **24,** 59-65.

Sedgley C, Applegate B, Nagel A, Hall D (2004a) Real-time imaging and quantification of bioluminescent bacteria in root canals *in vitro*. *Journal of Endodontics* **30,** 893-898.

Sedgley C, Lennan S, Clewell D (2004b) Prevalence, phenotype and genotype of oral enterococci. *Oral Microbiology and Immunology* **19,** 95-101.

Sedgley C, Lennan S, Appelbe O (2005a) Survival of *Enterococcus faecalis* in root canals ex vivo. *International Endodontic Journal* **38,** 735-742.

Sedgley C, Molander A, Flannagan S *et al.* (2005b) Virulence, phenotype and genotype characteristics of endodontic *Enterococcus spp*. *Oral Microbiology and Immunology* **20,** 10-19.

Sedgley C, Nagel A, Shelburne C, Clewell D, Appelbe O, Molander A (2005c) Quantitative real-time PCR detection of oral *Enterococcus faecalis* in humans. *Archives of Oral Biology* **50,** 575-583.

Sedgley CM, Nagel AC, Hall D, Applegate B (2005d) Influence of irrigant needle depth in removing bioluminescent bacteria inoculated into instrumented root canals using real-time imaging *in vitro*. *International Endodontic Journal* **38,** 97-104.

Sedgley C, Buck G, Appelbe O (2006a) Prevalence of *Enterococcus faecalis* at multiple oral sites in endodontic patients using culture and PCR. *Journal of Endodontics* **32,** 104-109.

Sedgley C, Nagel A, Dahlen G, Reit C, Molander A (2006b) Real-time quantitative polymerase chain reaction and culture analyses of *Enterococcus faecalis* in root canals. *Journal of Endodontics* **32,** 173-177.

Sedgley CM, Lee EH, Martin MJ, Flannagan SE (2008) Antibiotic resistance gene transfer between streptococcus gordonii and *Enterococcus faecalis* in root canals of teeth ex vivo. *Journal of Endodontics* **34,** 570-574.

Seet A (2010) An *in vitro* evaluation of the effectiveness of endodontic irrigants, with and without sonic and laser activation, in the eradication of *Enterococcus faecalis* biofilm. *[D.Clin.Dent thesis] The University of Adelaide, Australia*.

Seet A, Zilm P, Gully N, Cathro P. A qualitative comparison of sonic or laser energisation of 4% sodium hyochlorite on an *Enterococcus faecalis* biofilm grown *in vitro*. *Australian Endodontic Journal*. (*in press*).

Sen BH, Safavi KE, Spångberg LS (1999) Antifungal effects of sodium hypochlorite and chlorhexidine in root canals. *Journal of Endodontics* **25,** 235-238.

Senia ES, Marshall FJ, Rosen S (1971) The solvent action of sodium hypochlorite on pulp tissue of extracted teeth. *Oral Surgery, Oral Medicine, Oral Pathology* **31,** 96-103.

Senia ES, Marraro RV, Mitchell JL, Lewis AG, Thomas L (1975) Rapid sterilization of gutta-percha cones with 5.25% sodium hypochlorite. *Journal of Endodontics* **1,** 136-140.

Shih M, Marshall FJ, Rosen S (1970) The bactericidal efficiency of sodium hypochlorite as an endodontic irrigant. *Oral Surgery, Oral Medicine, Oral Pathology* **29,** 613-619.

Shuping GB, Ørstavik D, Sigurdsson A, Trope M (2000) Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. *Journal of Endodontics* **26,** 751-755.

Sim TP, Knowles JC, Ng YL, Shelton J, Gulabivala K (2001) Effect of sodium hypochlorite on mechanical properties of dentine and tooth surface strain. *International Endodontic Journal* **34,** 120-132.

Siqueira JF, Batista MM, Fraga RC, de Uzeda M (1998) Antibacterial effects of endodontic irrigants on black-pigmented gram-negative anaerobes and facultative bacteria. *Journal of Endodontics* **24,** 414-416.

Siqueira JF, 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.

Siqueira JF, Rôças IN, Souto R, de Uzeda M, Colombo AP (2002) *Actinomyces species, Streptococci*, and *Enterococcus faecalis* in primary root canal infections. *Journal of Endodontics* **28,** 168-172.

Siqueira JF, Rôças I (2004) Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **97,** 85-94.

Siqueira JF, Rôças I (2010) Microbiology and treatment of endodontic infections. In: Hargreaves K, Cohen S, eds. *Pathways of the Pulp, 10th edition*, Mosby: pp. 559-601.

Siqueira JF, Rôças IN (2011) Optimising single-visit disinfection with supplementary approaches: A quest for predictability. *Australian Endodontic Journal* **37,** 92-98.

Sirén E, Haapasalo M, Ranta K, Salmi P, Kerosuo E (1997) Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. *International Endodontic Journal* **30,** 91-95.

Sirtes G, Waltimo T, Schaetzle M, Zehnder M (2005) The effects of temperature on sodium hypochlorite short-term stability, pulp dissolution capacity, and antimicrobial efficacy. *Journal of Endodontics* **31,** 669-671.

Sjögren U, Figdor D, Spångberg L, Sundqvist G (1991) The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. *International Endodontic Journal* **24,** 119-125.

Sjögren U, Figdor D, Persson S, Sundqvist G (1997) Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *International Endodontic Journal* **30,** 297-306.

Socransky SS, Manganiello SD (1971) The oral microbiota of man from birth to senility. *Journal of Periodontology* **42,** 485-496.

Soukos NS, Chen PS, Morris JT *et al.* (2006) Photodynamic therapy for endodontic disinfection. *Journal of Endodontics* **32,** 979-984.

Souza LC, Brito PR, de Oliveira JC *et al.* (2010) Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of *Enterococcus faecalis*. *Journal of Endodontics* **36,** 292-296.

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,** 856-871.

Spratt DA, Pratten J, Wilson M, Gulabivala K (2001) An *in vitro* evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. *International Endodontic Journal* **34,** 300-307.

Steiner R, Raulin C, Karsai S (2011) Laser-tissue interactions. In: Laser and IPL Technology in Dermatology and Aesthetic Medicine. Springer Berlin Heidelberg: pp. 23-36.

Stern RH, Sognnaes RF (1964) Laser beam effect on dental hard tissues (abstract) *Journal of Dental Research* **43,** 873.

Stewart P, Costerton J (2001) Antibiotic resistance of bacteria in biofilms. *The Lancet* **358,** 135-138.

Stojicic S, Zivkovic S, Qian W, Zhang H, Haapasalo M (2010) Tissue dissolution by sodium hypochlorite: Effect of concentration, temperature, agitation, and surfactant. *Journal of Endodontics* **36,** 1558-1562.

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.

Sullivan J, Pileggi R, Varella CH (2009) Evaluation of root-end resections performed by Er,Cr:YSGG laser with and without placement of a root-end filling material. *International Journal of Dentistry,* 1-6.

Sundqvist G (1976) *Bacteriological studies of necrotic dental pulps*: Thesis. Dept. of Oral Microbiology, University of Umeå.

Sundqvist G, Eckerbom M, Larsson A, Sjögren U (1979) Capacity of anaerobic bacteria from necrotic dental pulps to induce purulent infections. *Infection and Immunity* **25,** 685.

Sundqvist G (1992) Ecology of the root canal flora. *Journal of Endodontics* **18,** 427-430.

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 Endodontology* **85,** 86-93.

Sundqvist G, Figdor D (2003) Life as an endodontic pathogen. *Endodontic Topics* **6,** 3-28.

Svensäter G, Bergenholtz G (2004) Biofilms in endodontic infections. *Endodontic Topics* **9,** 27-36.

Tay FR, Gu L-S, Schoeffel GJ *et al.* (2010) Effect of vapor lock on root canal debridement by using a side-vented needle for positive-pressure irrigant delivery. *Journal of Endodontics* **36,** 745-750.

Ten Cate A (1998) Oral Histology. Development, Structure and Function. *5th edn St Louis, Missouri, USA: Mosby,* 128-149.

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

Thè SD, Maltha JC, Plasschaert AJM (1980) Reactions of guinea pig subcutaneous connective tissue following exposure to sodium hypochlorite. *Oral Surgery, Oral Medicine, Oral Pathology* **49,** 460-466.

Torabinejad M, Ung B, Kettering JD (1990) *In vitro* bacterial penetration of coronally unsealed endodontically treated teeth. *Journal of Endodontics* **16,** 566-569.

Trepagnier CM, Madden RM, Lazzari EP (1977) Quantitative study of sodium hypochlorite as an *in vitro* endodontic irrigant. *Journal of Endodontics* **3,** 194-196.

Tronstad L, Asbjørnsen K, Døving L, Pedersen I, Eriksen HM (2000) Influence of coronal restorations on the periapical health of endodontically treated teeth. *Dental Traumatology* **16,** 218-221.

Trope M, Chow E, Nissan R (1995) *In vitro* endotoxin penetration of coronally unsealed endodontically treated teeth. *Dental Traumatology* **11,** 90-94.

Trope M, Delano E, Ørstavik D (1999) Endodontic treatment of teeth with apical periodontitis: Single vs. Multivisit treatment. *Journal of Endodontics* **25,** 345.

Vaarkamp J, ten Bosch JJ, Verdonschot EH (1995) Propagation of light through human dental enamel and dentine. *Caries Research* **29,** 8-13.

Van Der Sluis LWM, Wu MK, Wesselink PR (2007) The evaluation of removal of calcium hydroxide paste from an artificial standardized groove in the apical root canal using different irrigation methodologies. *International Endodontic Journal* **40,** 52-57.

van Leeuwen TG, van der Veen MJ, Verdaasdonk RM, Borst C (1991) Noncontact tissue ablation by Holmium:YSGG laser pulses in blood. *Lasers in Surgery and Medicine* **11,** 26-34.

Vertucci F (1984) Root canal anatomy of the human permanent teeth. *Oral Surgery, Oral Medicine, Oral Pathology* **58,** 589-599.

Wade W (2002) Unculturable bacteria - the uncharacterized organisms that cause oral infections. *Journal of the Royal Society of Medicine* **95,** 81-83.

Waltimo TM, Ørstavik D, Sirén EK, Haapasalo MP (1999) *In vitro* susceptibility of *Candida albicans* to four disinfectants and their combinations. *International Endodontic Journal* **32,** 421-429.

Wang QQ, Zhang CF, Yin XZ (2007) Evaluation of the bactericidal effect of Er,Cr:YSGG, and Nd:YAG lasers in experimentally infected root canals. *Journal of Endodontics* **33,** 830-832.

Weichman JA, Johnson FA (1971) Laser use in endodontics. A preliminary investigation. *Oral Surgery* **31,** 416.

Weiger R, Rosendahl R, Löst C (2000) Influence of calcium hydroxide intracanal dressings on the prognosis of teeth with endodontically induced periapical lesions. *International Endodontic Journal* **33,** 219-226.

Williams JA, Pearson GJ, Colles MJ, Wilson M (2004) The photo-activated antibacterial action of toluidine blue in a collagen matrix and in carious dentine. *Caries Research* **38,** 530-536.

Wilson MJ, Weightman AJ, Wade WG (1997) Application of molecular ecology in the characterization of uncultured micro-organisms associated with human disease. *Reviews in Medical Microbiology* **8,** 91-101.

Wittgow Jr W, Sabiston Jr C (1975) Microorganisms from pulpal chambers of intact teeth with necrotic pulps. *Journal of Endodontics* **1,** 168-171.

Wu MK, Wesselink PR (1993) Endodontic leakage studies reconsidered. Part i. Methodology, application and relevance. *International Endodontic Journal* **26,** 37-43.

Wu MK, R'oris A, Barkis D, Wesselink P (2000) Prevalence and extent of long oval canals in the apical third. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **89,** 739-743.

Wu MK, Wesselink PR (2001) A primary observation on the preparation and obturation of oval canals. *International Endodontic Journal* **34,** 137-141.

Xu Y, Young MJ, Battaglino RA *et al.* (2009) Endodontic antimicrobial photodynamic therapy: Safety assessment in mammalian cell cultures. *Journal of Endodontics* **35,** 1567-1572.

Yamada RS, Armas A, Goldman M, Lin PS (1983) A scanning electron microscopic comparison of a high volume final flush with several irrigating solutions: Part 3. *Journal of Endodontics* **9,** 137-142.

Yasuda Y, Kawamorita T, Yamaguchi H, Saito T (2010) Bactericidal effect of nd:Yag and er:Yag lasers in experimentally infected curved root canals. *Photomedicine and Laser Surgery* **28,** S-75-S-78.

Yavari HR, Rahimi S, Shahi S *et al.* (2010) Effect of Er,Cr:YSGG laser irradiation on *Enterococcus faecalis* in infected root canals. *Photomedicine and Laser Surgery* **28,** S-91-S-96.

Yiu CK, Garcia-Godoy F, Tay FR *et al.* (2002) A nanoleakage perspective on bonding to oxidized dentin. *Journal of Dental Research* **81,** 628-632.

Young G, Turner S, Davies JK, Sundqvist G, Figdor D (2007) Bacterial DNA persists for extended periods after cell death. *Journal of Endodontics* **33,** 1417-1420.

Zakariasen KL, Dederich DN, Tulip J, DeCoste S, Jensen SE, Pickard MA (1986) Bactericidal action of carbon dioxide laser radiation in experimental dental root canals. *Canadian Journal of Microbiology* **32,** 942-946.

Zehnder M, Kosicki D, Luder H, Sener B, Waltimo T (2002) Tissue-dissolving capacity and antibacterial effect of buffered and unbuffered hypochlorite solutions. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **94,** 756-762.

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

Zoletti GO, Pereira EM, Schuenck RP, Teixeira LcM, Siqueira Jr JF, dos Santos KTRN (2011) Characterization of virulence factors and clonal diversity of *Enterococcus faecalis* isolates from treated dental root canals. *Research in Microbiology* **162,** 151-158.