Longitudinal studies into the effect of fluoridated milk on artificial caries lesion mineral change using an *in-situ* model

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Author's Declaration

This thesis is the original work of the author.

Ailsa J. Nicol

Summary

It has been known since the early part of the twentieth century that fluoride has a beneficial effect on dental caries. It is now recognised this occurs through the action of preventing demineralisation and encouraging remineralisation, with a minor effect of inhibition of the plaque bacteria involved in the caries process. Fluoride can be delivered to the oral cavity in a number of ways, and is commonly found in toothpastes used by a large proportion of the population in developed countries. Since the mid-1970s, the routine use of fluoride toothpastes in such countries has reduced the caries rate significantly (Haugejorden *et al.*, 1997; Newbrun, 1999). However, for children from disadvantaged areas who may have no access to toothbrushes or toothpastes and whose diet is highly cariogenic, additional sources of fluoride may be beneficial. It has been proved clinically, and confirmed in a systematic review of the literature, that increased exposure to fluoride enhances its caries preventive effect (Marinho *et al.*, 2004b).

Fluoridated milk has the benefit that it is possible to target children who would benefit most from it. Milk has excellent nutritional value; after all, it has been recommended by the Scientific Advisory Committee on Nutrition (SACN), which advises the UK Department of Health, as the sole food for infants up to the age of six months.

There are a number of research questions still unanswered regarding the use of fluoridated milk. The topic was reviewed on behalf of the World Health Organisation by Stephen *et al.*(1996). They stated that further research was required to determine, for example: the optimum frequency of intake of fluoridated milk; the optimum concentration of fluoride to be added to milk; the optimum age of the child to whom the milk should be given and the optimum time for which fluoridated milk should be provided. Two recent systematic reviews of the literature pertaining to fluoridated milk stated that the evidence regarding the efficacy of fluoridated milk was impossible to determine because of a lack of suitably designed studies (Holm, 2002; Yeung *et al.*, 2005). Finally, much of the research regarding the efficacy of fluoridated milk as a delivery system was obtained prior to the routine use of fluoridated dentifrices. Thus, the evidence for benefits from the concomitant use of fluoridated milk with a fluoridated dentifrice is lacking.

One of the main aims of the work reported in this thesis was to use an *in situ* model to investigate the effect of milk, with or without the addition of fluoride, on the remineralisation and/or further demineralisation of artificial carious lesions. The design of the study also included investigation of the effect of using a fluoridated dentifrice slurry to simulate toothbrushing twice-daily, in addition to the milk intake.

The change in mineral content of artificial enamel lesions, created in human tooth blocks was measured using two techniques, namely QLF and TMR. A specific study determined the repeatability of the new QLF technique.

Using a complete denture *in situ* model, it was possible to place multiple caries lesions at different sites within each subject's oral cavity. The final aim of this work was to compare the response of caries lesions at different sites to exposure to the experimental protocols, and to determine whether the results supported previous work relating to the site-specificity of caries.

In the studies described in this thesis, repeatability estimates for the image capture and image analysis parts of the QLF technique were classed as being 'substantial' by the criteria suggested by Shrout (1998). It was demonstrated that the operator (AJN) could achieve levels of consistency similar to operators described as "experienced" in other studies examining the repeatability of QLF.

Subjects who used the fluoridated dentifrice slurry to simulate toothbrushing twice daily tended to have a greater increase in the mineral content between pre- and post-protocol measurements than those who did not use the slurry. This finding applied to both QLF and TMR evaluations, and was statistically significant for some, but not all of the measured parameters. These findings demonstrated that, in this study, the positive effect, on lesion mineral content, of simulating toothbrushing twice daily, was greater than that of the experimental protocols involving fluoridated milk consumption alone. The effect of the dentifrice slurry concurs with literature published in recent years which suggests that fluoridated toothpaste is very effective in the prevention of dental caries (Stephen *et al.*, 1988; Marinho *et al.*, 2004b).

Looking at the effect of the experimental protocols, there was overall net mean remineralisation of the artificial lesions in the tooth blocks used in the study. However, it is unlikely that this was as a result of the experimental protocols alone, because the 'no

beverage' control showed similar amounts of change to that found in the milk and fluoridated milk protocols.

There were differences in the remineralisation achieved at different sites within the mouth. Lesions at the labial position in the upper denture were notable, because in several instances, they achieved the least amount of remineralisation. This result concurs with previous work suggesting that there is a reduced salivary film velocity at this site, thereby reducing salivary clearance and increasing the likelihood of demineralisation.

In summary, this work has supported the benefit of regular use of fluoridated dentifrice. With regard to the effect of fluoridated milk, this work appears to suggest limited value in the use of fluoridated milk as a sole source of fluoride, or as a source of fluoride in addition to the regular use of dentifrices in the caries-preventive process.

List of Abbreviations

<	less than
%	percentage
%Vol mineral.μm	percentage volume of mineral times micrometre – unit of IML
°C	degrees Centigrade, temperature
ΔF	Delta F, also known as QLF average fluorescence loss
ΔQ	Delta Q, average change in fluorescence loss multiplied by area
ΔZ	Delta Z, also known as Integrated Mineral Loss
®	registered trademark
μm	micrometre, 10^{-6} m
ANCOVA	Analysis of covariance, statistical test
ATP	Adenosine Tri-phosphate
BSE	Bovine spongiform encephalopathy
CaF_2	calcium fluoride
$Ca_{10}(PO_4)_6(OH)_2$	hydroxyapatite
CCD	Charge coupled device
CI	confidence interval
Cu(Kα)	Copper (K alpha)
dfs	decayed, filled surfaces (deciduous dentition)

DFS	decayed, filled surfaces (permanent dentition)
dmfs	decayed, missing, filled surfaces (deciduous dentition)
DMFS	decayed, missing, filled surfaces (permanent dentition)
dmft	decayed, missing, filled teeth (deciduous dentition)
DMFT	decayed, missing, filled teeth (permanent dentition)
et al.	and others
F	fluorine
F^-	fluoride ion
FAB	Fastidious Anaerobe Broth
FOTI	Fibre optic Transillumination
g	gram
GLM	General Linear Modelling, statistical test
HF	hydrogen fluoride
ICC	Intra-class correlation, statistical test
IML	Integrated mineral loss
in situ	in situation, e.g. in the oral cavity
in vitro	in a laboratory environment
in vivo	in life (i.e. observations of patients/subjects)
Ip	Iodine permeability

kg	kilogram
KI	Potassium iodide
KSp	solubility product
kV	kilovolts
LD	Lesion depth
LMR	Longitudinal Microradiography
M	Molar
mA	milliamps
mg	milligram
min	minute
mL	millilitres
mm	millimetre, 10^{-3} m
mV	millivolts
n	number
NaF	sodium fluoride
Na_2FPO_3	sodium monofluorophosphate
nm	nanometer, 10^{-9} m
(OH^-)	hydroxyl ion
p	p-value of statistical probability

PBS	Phosphate buffered saline
(PO_4^{3-})	phosphate ion
ppm	parts per million
QLF	Quantitative light fluorescence
QLF^{AREA}	QLF parameter area
QLF^{AVER}	QLF parameter average % fluorescence loss
QLF^{MAX}	QLF parameter maximum % fluorescence loss
r	statistical symbol of correlation coefficient
RCT	randomised controlled trial
SMFP	sodium monofluorophosphate
SnF_2	Tin fluoride
Sp.	Species
St. Dev.	standard deviation
T	Treatment group
TD	Treatment plus dentifrice slurry group
TMR	Transverse Microradiography
TSE	Transmissible spongiform encephalopathy
UHT	ultra-heat treated
vCJD	variant Creutzfeldt - Jakob disease

WHO	World Health Organisation
WIM	Wavelength induced microradiography

1. Introduction and review of literature

1.1 Introduction

This chapter describes the reasons why the studies in this thesis were designed and gives a critical review of the relevant literature.

The dental caries process will be discussed first, followed by the effect of fluoride and then milk on the caries process. The methods of delivering fluoride to the oral cavity will then be reviewed. Thereafter, the effect of fluoridated milk on the caries process will be discussed, followed by the methods and designs used to study this process. The methods available to measure caries in experimental studies will then be described.

A summary of the literature review and the aims of the work described in this thesis will conclude this chapter.

1.2 Dental caries

1.2.2 Definition of dental caries

Dental caries is a multi-factorial disease process. Key factors related to the process include a tooth coated with cariogenic bacteria, exposed to a fermentable carbohydrate, over time. Dental caries has been defined in many ways, and these definitions have been further refined as knowledge of the disease process has increased. In 1985, Nikiforuk stated that, "*Dental caries is a peculiarly local disease which involves destruction of the hard tissues of the teeth by metabolites produced by oral microorganisms*"(Nikiforuk, 1985a). Increased knowledge about the influence of saliva and fluoride on the caries process has modified this further. In 1996, Featherstone defined dental caries as "*A plaque-related disease, dependent on the presence of simple sugars in the diet, driven by frequency of eating simple carbohydrates, modified by fluoride, salivary-flow and composition of saliva.*"(Featherstone, 1996).

However, neither of these definitions reflects the fact that dental caries occurs over a length of time, and the clinical appearance of caries is a reflection of the accumulated events which have occurred in the past. In 1997, Fejerskov stated it seemed most appropriate to use the term "dental caries" to refer to the recorded mineral loss that

presents itself in the clinic at any particular time. He went on to define the "carious process" as being the dynamic de- and remineralizing processes resulting from microbial metabolism on the tooth surface which, over time, may result in a net loss of mineral, and possibly, but not always, leading to cavitation (Fejerskov, 1997).

Differentiation between the caries process and the lesion itself can cause confusion. This is multiplied when the term "dental caries" is used synonymously to describe both the caries lesion and the caries process. Such is the importance of defining the terminology associated with dental caries accurately, that consensus statements were developed during an International Consensus Workshop on Caries Clinical Trials, in 2002, to define the 'Caries Process' and the 'Caries Lesion'. These statements by Kidd and Fejerskov (2004) were:

(a) '*The Caries Process occurs as an interaction between the (plaque) biofilm and the tooth surface and sub-surface*'; and

(b) '*The Caries Lesion is the manifestation of the stage of the process at one point in time.*' The term 'biofilm' in the above statement being the communities of microorganisms attached to a surface. Dental plaque is an example of a microbial biofilm (Marsh and Bradshaw, 1995).

Therefore, in simplistic terms, the caries lesion is a 'snapshot' of the caries process. To understand the caries lesion, one should have an understanding of the mechanisms of de- and remineralisation which occur as a result of the caries process.

1.2.2 Clinical manifestation of the caries process

The manifestations of the caries process occur both within the different mineralised structures of the tooth, and at different sites around the tooth. The process may manifest as an enamel caries lesion with an intact surface and progress to frank cavitation and dentine involvement, which may encroach on the dental pulp. The process is site-specific and can occur at different parts of the tooth, i.e. on the smooth surfaces of enamel, or in the occlusal fissures or pits of teeth. The caries process results in loss of tooth mineral due to an imbalance in the mineral dynamics between the tooth surface and the aqueous environment of the oral cavity, a prevailing overall loss of mineral leading ultimately, to cavitation of the tooth surface. The lesion is relatively easy to detect when there is obvious

breakdown of the tooth structure. However, once caries has reached this stage in enamel, active restorative treatment will be required (Kidd and Fejerskov, 2003). The challenge in dentistry is to detect the presence of the disease at an early stage while it is an incipient, non-cavitated enamel lesion, and then modify the oral environment to encourage arrest or reversal of the process.

Similar criteria can be applied to root surface caries, where there is also subsurface demineralisation and the surface may appear softened early in lesion development. However, the carious lesions here are seldom more than 0.5 to 1.0mm deep and are amenable to preventive therapies such as plaque control and fluoride therapy, thereby arresting the lesions, which may not require restoration (Kidd and Fejerskov, 2004).

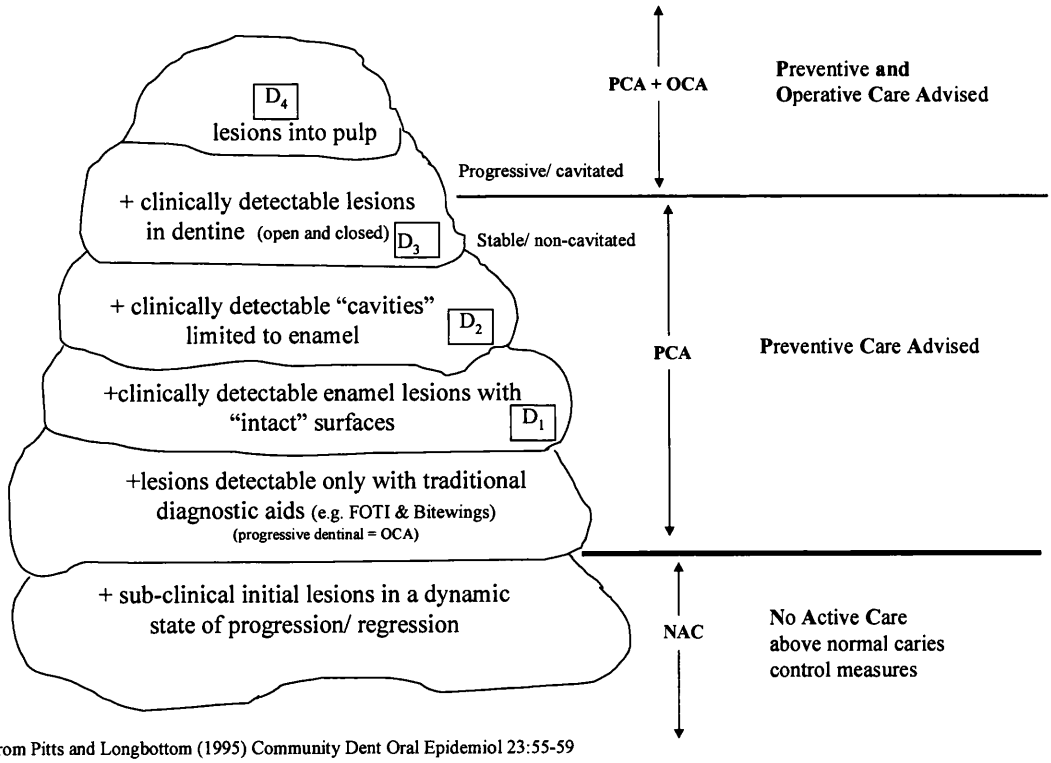
The earliest clinical sign of dental caries is the white spot (incipient caries) lesion, which is most visible when the tooth is clean and dry. A roughness may be detected when a dental probe is run over the lesion surface, but there is no clinical evidence of cavitation at this stage. The clinical white spot is a result of prevailing, sub-surface mineral loss producing subsurface porosity within the mineral structure of the tooth. This porosity fills with fluid and air, which has a different refractive index from sound tooth structure. Light scatters differently within the lesion compared with sound tooth structure, and this difference is visually interpreted by the human eye as a white spot (ten Bosch, 1996). Prior to breakdown of the tooth surface, it is possible for mineral exchange between tooth and the oral environment to be reversed: a process known as re-mineralisation. Indeed, studies of incipient lesions have shown that, even within the same lesion, certain areas can be remineralising while others are demineralising (Zero, 1999). It is thought that a net transfer of mineral back into the lesion may also result in take-up of extrinsic brown stain. The lesion may even take on a brown appearance and be known as a brown spot lesion. Such a lesion is often arrested or reversed by remineralisation due to the instigation of an effective preventive programme (Nikiforuk, 1985b).

If cavitation does occur, the biofilm then occupies a protected niche, inaccessible to measures designed to arrest or reverse the caries process (Marsh and Nyvad, 2003), and the rate of lesion progression increases clinically (von der Fehr and Haugejorden, 1997). The observation of these signs of dental caries is important, however the decision about how to manage the process in a clinical manner, is also required. There is consensus that when macroscopic cavitation occurs, then operative intervention is required. However, when

cavitation is not evident or is not able to be observed directly, the decision-making process is more difficult. Current concepts, indicating whether preventative treatment or both preventative and operative treatment are required, can be illustrated using the "iceberg of dental caries" (Figure 1) and contemporary treatment need/advice (Pitts and Longbottom, 1995).

The detection of dental caries in enamel that has been exposed to fluoride (as discussed in Section 1.3), particularly during development, is more difficult than that of enamel that has not been exposed to fluoride (Pitts and Deery, 1994). This is because fluoridated apatite may have a whiter, more opaque colouring than non-fluoridated apatite. The situation is also complicated by the fact that cavitation of more highly fluoridated apatite occurs later than less fluoridated apatite (Pitts and Deery, 1994). This can, on occasion reveal a significant amount of dentinal caries that may not be amenable to simple restorative techniques. In this situation, caries may go undetected on the enamel surface and following microscopic enamel cavitation, dentinal caries progresses undetected; leaving little more than an enamel shell, with extensive dentinal caries that is close to, if not involving the dental pulp (Weerheijm *et al.*, 1992a; Weerheijm *et al.*, 1992b; Pitts and Deery, 1994).

Figure 1 – The "iceberg of dental caries"



From Pitts and Longbottom (1995) Community Dent Oral Epidemiol 23:55-59

1.2.3 Demineralisation/remineralisation. The mechanism of the caries process in enamel

As stated previously, the caries process is multi-factorial, occurring over time and alternating between periods of demineralisation and remineralisation.

This section will consider the formation of dental plaque, the enamel structure and the chemical changes which drive the caries process.

1.2.3.1 The formation of dental plaque

The growth and metabolic activity of the oral microflora is maintained and regulated by saliva, which buffers the normal oral pH to values between 6.75 and 7.25 as a result of several buffering systems (Edgar and Higham, 1996). These include the carbonic acid/bicarbonate system and the inorganic orthophosphate system.

Saliva contains glycoproteins and proteins that act as the primary carbohydrate, peptide and amino acid source for the growth of the oral microflora. Saliva can sustain the growth of oral microflora which degrade the oligosaccharide side-chains of salivary glycoproteins such as mucins (Marsh and Nyvad, 2003).

The communities of microorganisms which form the biofilm of plaque adhere to the tooth surface via the acquired pellicle. The pellicle forms quickly as a film, <1 μm thick, on the tooth surface within two hours of thorough tooth cleaning. It is derived from salivary proteins, glycoproteins, lipids and glycolipids, as well as extracellular molecules from bacteria. Oral microorganisms are transported via saliva, and attracted to the acquired pellicle by physico-chemical interactions and short-range specific stereo-chemical molecular interactions with the primary colonising bacteria. Following this, secondary colonising bacteria adhere to the primary colonisers, and horizontal and vertical stratification develops leading, within two weeks, to a climax community of dental plaque (Marsh and Bradshaw, 1995).

As plaque develops, bacteria, which metabolise simple sugars, synthesize extracellular polysaccharides and organic acids. These extracellular polysaccharides were initially

thought to improve the adhesion of the plaque but, it is now known, they facilitate penetration of sugars deeper into the plaque and inhibit the effect of the buffering capacity of saliva at the plaque-enamel interface (Marsh and Bradshaw, 1995). The organic acids (lactate, acetate, formate, propionate and butyrate) produced by plaque lead to a reduction in the pH at the plaque-enamel interface and within the plaque fluid (Rugg-Gunn, 1993). Where there is a carbohydrate-rich diet, the growth-rate of oral bacteria increases and there is a change in the composition of the microflora towards more aciduric species, which thrive in a lower pH environment (Marsh and Nyvad, 2003).

1.2.3.2 Enamel structure

Although the caries process occurs in all of the dental hard tissues, it is the demineralisation and remineralisation process which occurs in enamel which is the principal interest of this thesis. The caries process affecting the crown of the tooth begins most often in enamel, which is the most highly mineralised tissue in the human body. The mineral is in the form of calcium phosphate crystals. A repeating pattern of calcium, phosphate and hydroxyl ions are arranged in a crystal lattice structure resembling hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. The hydroxyapatite lattice is contaminated with many other elements and compounds including carbonate, sodium, fluoride, lead and magnesium. The hydroxyapatite crystals are long and thin, approximately 50nm wide in cross-section and more than 100 μm long (ten Cate *et al.*, 2003), and are tightly packed into enamel prisms. Organic matter and water are found between the crystals in the intercrystaline and interprismatic spaces. The approximate composition of enamel and dentine are listed in Table 1. It is the susceptibility of these hydroxyapatite crystals to dissolution by acid that forms the chemical basis of dental caries (Eisenmann, 1989).

Table 1 – Approximate composition of enamel and dentine by % weight and by % volume

Tissue Component	Enamel		Dentine	
	% by weight	% by volume	% by weight	% by volume
Mineral = Carbonated hydroxyapatite	96	85	70	47
Protein / Lipid	1	3	20	33
Water	3	12	10	20

Taken from Featherstone JDB: Prevention and reversal of dental caries: role of low level fluoride. Community Dent Oral Epidemiol, 1999;27:31-40

1.2.3.3 Chemical changes which drive the caries process

Normally, saliva and other oral fluids that bathe the teeth are supersaturated with respect to several calcium phosphate compounds, collectively known as apatites. The most commonly occurring enamel apatite is hydroxyapatite, and substitution of carbonate and fluoride into the enamel apatite changes its solubility product (KSp). Carbonate substitution increases the solubility of the hydroxyapatite lattice, whereas solubility is decreased with fluoride substitution (ten Cate *et al.*, 2003). The supersaturated apatite concentration of oral fluids prevents these apatites, and similar minerals, dissolving from the teeth into saliva. However, when the pH of saliva and the fluids within the oral cavity drops, the solubility of the tooth apatite increases, resulting in dissolution along a concentration gradient between the low-volume/ high-concentration intercrystalline fluids and relatively-larger-volume/ lower-concentration fluids of plaque and saliva. In general, for each decrease in pH unit, the solubility of the apatites increases by a factor of 10. Dissolution of apatite generally occurs in the pH range 4.0-5.5 (ten Cate *et al.*, 2003).

When phosphate ions (PO_4^{3-}), hydroxyl ions (OH^-) and calcium ions accumulate in solution, it becomes saturated and dissolution of the hydroxyapatite slows. However,

further exposure to acids as a by-product of sugar metabolism by the oral microorganisms again changes the proportion of ions in solution which may further drive the dissolution process (ten Cate *et al.*, 2003). Fluoridated apatite and hydroxyapatite are soluble at different pH levels (Lagerlöf, 1983). It is thought that the critical pH, which is the level at which the caries process develops and net mineral loss occurs from the tooth, is when the saliva and plaque fluid are supersaturated with respect to fluoridated apatite and under-saturated with respect to hydroxyapatite. As a result, subsurface hydroxyapatite leaches from the dental hard tissues into plaque fluid and saliva, while fluoridated apatite forms in the surface layer of the developing caries lesion. It is this concurrent super-saturation with fluoridated apatite that appears to be responsible for the formation of the surface layer of the non-cavitated white-spot lesion, which may be 20-50 μm thick (ten Cate *et al.*, 2003). This surface layer reduces the demineralisation effect in the body of the caries lesion when a drop in pH occurs.

When demineralisation occurs initially, there is loss of interprismatic mineral. There is no surface layer for a period of about a month (Øgaard et al., 1996). At this stage, the initial demineralisation may be known as 'surface-softened enamel' rather than an early caries lesion, which exists when the surface layer is present (Arends and Christoffersen, 1986). The method of development of this surface layer is subject to debate. *In vitro* it can be created artificially with the use of various acidified gels, though an unsaturated calcium phosphate solution may also be used to create lesions (Arends and Christoffersen, 1986). *In vivo*, numerous methods have been proposed, but it is thought to form due to the presence of an inhibitor of mineral loss. Fluoride and proteins are suggested examples, but whether they are both required, or only one of them individually, is not clear (Arends and Christoffersen, 1986).

Experiments have shown that the surface morphology of the early caries lesion is different from that of sound enamel. The surface enamel of an early caries lesion is both porous and mineral-rich, and the intact enamel surface layer gives it potential for repair. Below the surface layer is the body of the lesion which , at 10-70 %vol is lower in mineral than the surface zone (Arends and Christoffersen, 1986). This is where the majority of the loss of mineral occurs, hence the term, sub-surface demineralisation.

Remineralisation of the dental tissues occurs when partially demineralised apatite crystals are exposed to supersaturated apatite solutions (ten Cate *et al.*, 2003). Partially

demineralised crystals are present in carious lesions. The surface layer of the lesion appears to be the most responsive to the remineralisation process. This is because of the small pores present in the surface lesion, which slow down the diffusion process of the supersaturated apatite solution into the main body. As a result of this, the lesion body fails to remineralise completely, leaving a white scar which is visible through the remineralised surface layer. Fluoride has a most important role in the remineralisation process, and this will be discussed in more detail later.

Once the lesion has cavitated, the intact surface layer is no longer present and the body of the lesion is subject to the pH fluctuations of the oral cavity. Although there is free access for salivary calcium, phosphate and fluoride ions into the body of the lesion, there is also free access for the cariogenic acids, provision of a non-accessible niche for ever-expanding quantities of plaque, and an inevitable increase in the rate of clinical lesion progression (von der Fehr *et al.*, 1970).

1.3 Effect of fluoride on caries process

1.3.1 Discovery of the effect of fluoride on the caries process

Fluoride has long been thought to work in one of two ways: "systemically", if the fluoride is ingested by a foetus/ child, then incorporated directly into the pre-calcified tooth tissues as they form; and "topically", where fluoride is adsorbed on to the outer, and pulpal surfaces of the formed tooth, and particularly when the tooth has early decalcification present, making such enamel more resistant to decay.

It was originally thought that the main effect of fluoride on the caries process was when it was incorporated "systemically" into the developing dental tissues. This theory originated from studies in the United States of America undertaken in the early part of the 20th century by Black and McKay (Black, 1916; McKay, 1916a; McKay, 1916b; McKay, 1916c; McKay, 1916d). Black and McKay investigated 'Colorado brown stain' which caused mottling of enamel in the Colorado area. McKay arrived in Colorado Springs in 1901 and soon noticed that residents who had lived in the area all their lives had a permanent brown stain on their teeth. By 1909, McKay had not been able to find any literature describing this brown stain, so he contacted Black, a revered expert from the Northwest Dental School of Chicago, USA. They studied 2,945 children native to the town and determined that the prevalence of this 'brown stain' in native children was 87.5% and that the stain was a malformation of tooth enamel. However, although these teeth were

malformed they did not appear to have an increased amount of tooth decay, compared with children who were not affected. In 1912, McKay also noticed that the same condition had been described in Italian immigrants from certain areas around Naples (Eager, 1902). It was at this point that McKay established mottled enamel occurred in a high proportion of children who had been born and lived all their lives in definite geographic areas. Children who moved to the affected areas when they were two or three years of age were not affected. The environment of the children, and whether they were from an affluent background, did not influence the condition. This eliminated diet as an aetiological factor.

In 1916, McKay was informed that prior to a change in water supply in 1898, citizens in Britton, South Dakota had no mottling on their teeth, whereas children born since this change had mottled teeth. It was at this point that McKay suspected that the water supply could be responsible. A similar situation occurred in Bauxite, Arizona, where increased mottling was caused by a change in water supply, due to expansion of ALCOA (Aluminium Company of America) activity. However it was not until 1931, that Churchill, an ALCOA analytical chemist ordered testing of the drinking water to be undertaken. The water was apparently normal but further testing for trace elements found that, in this part of Arizona, the water contained 13 ppm of F^- (Churchill, 1931). Thereafter, McKay re-contacted areas where mottled enamel had been reported previously, and arranged for testing of these waters to be undertaken. They were all found to have high, varying levels of fluoride.

Further investigation was then undertaken by H Trendly Dean (1931), who worked within the US Public Health Service. He was assigned to investigate the relationship between fluoride concentration in drinking water, tooth/enamel mottling and dental caries. He reported there were 97 localities within the USA where mottled enamel was said to occur. This mottling of enamel was Dental Fluorosis and was of no public health significance. His aim was to find the "minimum threshold" of fluoride, i.e. the level at which it began to blemish teeth visibly. He developed a mottling classification in order to record, objectively, the severity of mottling in each area (Dean, 1934). Dean then went on to relate the concentration of fluoride in the drinking water with fluorosis-severity and dental caries. As a result, he determined that the optimum non-mottling level of fluoride for drinking water was around 1 ppm F^- (Dean, 1934; Dean and Elvove, 1936). Furthermore, he demonstrated, conclusively, that the severity of mottling increased with increasing fluoride concentration in the drinking water. A number of areas with previously high levels of F in

the water supply, were changed to a level < 1ppm F^-. This resulted in a halt in the mottling of enamel for those teeth formed after the water F^- concentration-change.

However, it was a British investigator, Ainsworth in 1925, who determined in Maldon, Essex that the mottled enamel was more resistant to dental caries than non-mottled. He found an inverse relationship between water F^- levels consumed and increased caries incidence (Medical Research Council, 1925; Ainsworth, 1928).

In 1939, Dean, having read Ainsworth's work, studied four Illinois cities, two with fluoride concentrations of 1.7 ppm and 1.8 ppm and two with the lower fluoride concentration of 0.2 ppm. Dean determined that the caries experience in the fluoridated cities was half that of the cities with the lower fluoride concentration. In 1942, another of his studies showed that near maximal reduction in caries experience, with little or no visible mottling occurred with a concentration of 1ppm F^- in the drinking water (Dean *et al.*, 1942). Thereafter, a significant amount of dental research concentrated on the systemic effect of fluoride by increasing the concentration of fluoride incorporated into the developing dental tissues.

As a result of these studies, the first attempt to fluoridate drinking water artificially was planned for Grand Rapids, Michigan, with Muskegon, Michigan acting as a control. Following baseline data collection, fluoridation began in 1945 (Dean *et al.*, 1942), with the first results being collected in 1951 (Arnold *et al.*, 1953). It was shown that, for those born and raised in fluoridated Grand Rapids, a near 50% caries-reduction now existed compared with the Muskegon control. For many years, comment on data for older children was virtually ignored, although it was evident that even for those aged 4-10 years at outset, caries reductions – which could only be due to topical fluoride action – were substantial.

In spite of the early, and sustained, bias towards a systemic-only mode of action for F^-, the suggestion that fluoride may have a topical effect as well as a systemic effect was first suggested when epidemiologists reported that some of the caries inhibition occurred in teeth only exposed to fluoride post-eruptively. Deatherage (1943a; 1943b) noted that the DMFT of males who received water containing 1mg F^- /litre from the age of 8 years onwards, was lower than the DMFT of men who had lived continuously in low-fluoride areas, although higher than from those who had received fluoridated water from birth. Similarly, Weaver (1944), observed an effect of fluoride on 11-14 year old children who

moved into a water-fluoridated area in a study involving children from South Shields, Sunderland and Jarrow in the UK.

In parallel with the projects above, several workers pursued studies to investigate the possible effects of topically - delivered fluoride. Thus, as early as 1955, Muhler *et al.* (1955a; 1955b) reported on the successful reduction of caries via fluoride-containing dentifrices. Wellock *et al.* (1963), formulated caries - inhibiting 12,300 ppm F^- Acidulated Phosphate Fluoride (APF) topical gels. In 1965, Torrell and Erikson demonstrated benefits which could be derived from rinsing regularly with various fluoride solutions, while substantial caries reductions were obtained by Aasenden & Peebles (1974) and Stephen & Campbell (1978), via fluoride supplements. In the latter case, these were only dispensed daily at school, with instructions that they should be allowed to dissolve slowly within the mouth. A resulting 81% caries reduction was obtained by Stephen and Campbell (1978). The topical-only benefits of fluoride, delivered daily via school milk, were demonstrated by Stephen *et al.* (1984), after a 5 year study. Also in the UK, the post-eruptive effect of water-fluoridation was shown clearly in a study by Hardwick *et al.*, (1982). Thus, it was in 1983, that scientists agreed, at an international caries congress in Zurich, that the main caries-beneficial effects of fluoride on teeth, were indeed topical (Fejerskov, 1984). Nonetheless, there are still a few advocates of a possible, albeit minor, systemic role (Kalsbeek et al., 1992).

1.3.2 Presence of fluoride in the oral cavity

Fluoride is present in the oral cavity in teeth, saliva, oral mucosa and crevicular fluid. It is also stored in plaque and calculus.

1.3.2.1 Teeth

The content of fluoride in tooth tissues is related to the amount of fluoride present at the time of tooth formation (systemic effect) and also the amount of fluoride in the oral fluids bathing the tooth during and after eruption (topical effect). The fluoride content of teeth is therefore variable, depending on fluoride intake during tooth formation and, in the outer surface of the tooth, on the fluoride content of the fluid that bathes it. Fluoride content increases in the outer 100 micrometres of enamel during the months following tooth-eruption, due to inward diffusion of fluoride from the oral cavity. The concentration of fluoride may reach levels between 1000 - 2000 ppm at the enamel surface (Robinson *et al.*, 1996), the subsurface enamel typically containing 20-100 ppm of fluoride. However, for

almost half a century, it has been known that even higher concentrations of fluoride are found within incipient caries lesions at the enamel surface (Dowse and Jenkins, 1957). This increase in concentration was not quantified for many years until Robinson and co-workers measured up to an 800% increase in the fluoride concentration within the mineral at the surface of a remineralised white spot lesion, compared with the adjacent unaffected enamel surfaces (Robinson *et al.*, 1983).

When fluoride is applied topically to the surface of enamel, a calcium fluoride-like material (CaF_2) is formed. It is understood this occurs as a result of a chemical reaction between apatite and soluble fluoride, described as a "double decomposition" reaction (Gerould, 1945). Small globules (<1μm) of CaF_2 can be visualised on the surface of the enamel when viewed using scanning electron microscopy, which may act as a fluoride reservoir. The solubility of these calcium fluoride globules increases when there is a pH drop, as occurs during a cariogenic challenge. This is believed to be a major cariostatic effect of topical fluoride (Øgaard, 2001).

1.3.2.2 Saliva

Salivary fluoride concentration is influenced by topical applications of fluoride such as fluoridated dentifrice, fluoridated mouth-rinse and also (when available) fluoridated milk and water. The speed of excretion of fluoride from saliva is dependent on salivary flow-rates, and is described as the 'salivary fluoride clearance' (Dawes, 1983; Weatherell *et al.*, 1984; Dawes and Weatherell, 1990).

As discussed previously, saliva "at rest" is supersaturated with respect to hydroxyapatite and fluoridated apatite, and contains a source of calcium and phosphate ions, essential for repair of early enamel lesions. The additional presence of fluoride in low concentrations in saliva is important in tipping the demineralisation / remineralisation balance towards remineralisation. The method by which this occurs will be discussed in more detail in Section 1.3.3.

1.3.2.3 Oral mucosa

Human oral mucosa can absorb fluoride and retain it within the tissue. Therefore, the oral mucosa may act as a reservoir of fluoride that is replenished when the fluoride concentration in saliva is high and is depleted as the concentration falls. Zero *et al.* (1990) compared the fluoride retention of topically applied agents in dentate and edentulous

individuals. They found that the retention of fluoride in the oral mucosa of edentulous subjects using fluoride gel containing 5000ppm fluoride was significantly greater than that of dentate patients. In this study, no difference was found between these subjects when lower concentrations of fluoride were used, either in a fluoridated dentifrice (1100 ppm F^-) or a fluoride rinse (226 ppm F^-). Jacobsen *et al.* (1992) also demonstrated fluoride absorption and retention into the oral mucosa following rinsing with a 0.2% NaF (900 ppm F^-) solution and demonstrated its subsequent release from the oral tissues over a period of two hours. Whether this release was into saliva or plasma, is not known (Jacobsen *et al.*, 1992). It was noted, however, that mucosal fluoride levels remained elevated for considerably longer than those of saliva, suggesting the oral mucosa acts as a major intra-oral reservoir of fluoride (Jacobsen, 1995).

1.3.2.4 Dental plaque

Dental plaque contains fluoride in both ionic and bound forms (Kashket and Bunick, 1978), which correlate generally with salivary fluoride levels, although other sources of plaque fluoride include the diet and crevicular fluid (MacFadyen *et al.*, 1979). Plaque fluoride concentrations vary, depending on the site within the mouth and are higher than salivary fluoride concentrations. This may be due to slower elimination of the ion from dental plaque, thickness of the salivary film or fluoride being released from other sources (Fejerskov *et al.*, 1996).

Fluoride is known to accumulate in dental plaque and may rise to levels 100-200 times that present in whole saliva (Larsen and Bruun, 1994). Fluoride in plaque fluid is present in plaque lying adjacent to the tooth and may therefore provide a fluoride source at the time of acid challenge resulting from plaque metabolism. It is thus desirable to have a constant source of salivary fluoride to replenish plaque fluid levels (ten Cate and Featherstone, 1996).

1.3.3 Mechanistic action of fluoride on the caries process

In the past twenty years, research has been concentrated on determining the mechanism of the topical effect of fluoride on the caries process. As a result, current opinion is that there are three major mechanisms of fluoride action on the caries process. These mechanisms are: fluoride (i) inhibition of demineralisation at the crystal surfaces; (ii) enhanced

subsurface remineralisation, resulting in arrestment or reversal of caries and (iii) inhibition of plaque bacterial metabolism (Featherstone, 1999).

1.3.3.1 Inhibition of demineralisation by fluoride

Fluoride present in the aqueous phase at the apatite crystal surface, may inhibit enamel demineralisation. When the local pH falls to approximately pH 4.5-5.5, hydroxyapatite will dissolve, but fluorapatite will simultaneously be forming in the demineralised area due to a super-saturation of fluorapatite within the oral fluids. Featherstone *et al.* (1990) showed, in an *in vitro* study, that fluoride at concentrations of 0.1-50ppm within a buffer in an acidic solution, inhibited demineralisation. They also demonstrated that the carbonated apatite dissolution rate was equivalent to that of hydroxyapatite when as little as 1ppm fluoride was added to an acid solution. The change in dissolution rate was in proportion to the logarithm of the fluoride concentration (Featherstone *et al.*, 1990). Elevations in fluoride concentration levels, even below 1ppm, resulted in significant reductions in the mineral loss caused by plaque acids (Larsen and Bruun, 1994).

The rate of caries lesion formation is also modified by fluoride. The rate reduces if fluoride is added to an artificial caries-forming solution (ten Cate and Featherstone, 1996). Fluoride present during the acid challenge to a tooth will combine with hydrogen ions to form HF. The hydrogen fluoride will then be transported rapidly into the enamel before dissociating in the fluid between enamel crystallites thereby completing the equilibrium reaction:

$$HF \rightleftharpoons H^+ + F^-$$

The fluoride ion can then be absorbed strongly on to the surface of carbonated apatite, protecting the crystal surface from dissolving. Featherstone (1999) suggested that low levels of fluoride present during an acid challenge from the bacteria, allowed fluoride to travel with the acid into the sub-surface of the tooth, and to be absorbed on to the crystal surface, thus preventing it from being dissolved.

1.3.3.2 Fluoride enhances remineralisation

It is known that fluoride levels as low as 0.1ppm may be sufficient to enhance growth of enamel crystals, and hence promote remineralisation (Brown, 1974). Therefore, the presence of fluoride in the aqueous environment of the teeth, at relatively low physiological levels, will have a stabilising effect on the dental minerals. For mineral to go back into the tooth, the saliva is required to be supersaturated with respect to calcium and phosphate. Partially-dissolved crystals in enamel act as nucleators for remineralisation and fluoride is absorbed on to such surfaces, attracts calcium ions (and therefore phosphate ions) and excludes carbonate. This produces a much less soluble surface coating for crystallites, with a solubility which is less than hydroxyapatite, but greater than fluorapatite. This partially remineralised enamel requires stronger acid challenges of longer duration to dissolve it. Therefore, fluoride present in solution from topical sources enhances remineralisation by speeding-up the growth of a new surface on the partially demineralised sub-surface crystals in the carious lesion. *In vivo* work has suggested that fluoride is more effective in inhibiting demineralisation of enamel than increasing remineralisation of lesions (Jeansonne and Feagin, 1979).

1.3.3.3 Fluoride inhibits plaque bacteria

Fluoride in its ionised form (F^-) cannot cross the bacterial cell wall/ membrane. As cariogenic bacteria produce acid, the pH in the surrounding area drops, creating hydrogen ions (via dissociation from the bacterially produced acid) which bind with fluoride to form HF (as described by the equilibrium equation described above). The HF diffuses rapidly across the cell wall and into the cell, where it dissociates into H^+ and F^-, creating a concentration gradient which encourages further diffusion of HF into the cell. The fluoride ion within the cell inhibits the glycolytic enzyme enolase of the plaque bacteria (Featherstone, 1999), which is responsible for converting 2-P-glycerate to P-enolpyruvate within the cell. The reduced output of the P-enolpyruvate inhibits sugar transport (Hamilton, 1990). The hydrogen ion within the cell causes acidification of the cell cytoplasm, which also inhibits the proton-pumping H +/ATP-ase (Marquis, 1990). Thus, all of these mechanisms inhibit plaque bacterial activity which, in turn, will influence the rate of fermentable carbohydrate catabolism to extracellular acids. By so doing, they will prevent proliferation of cariogenic bacteria suited to a low pH environment.

To summarise the mechanistic action of fluoride: the main effect is inhibition of demineralisation, followed by enhanced remineralisation. The inhibition of plaque bacteria by fluoride is known to occur, but probably has a limited effect on the overall process.

1.3.3.4 Summary of mechanistic action of fluoride on the caries process

There is strong evidence today that fluorides work mainly topically by impeding demineralisation and promoting remineralisation and of the tooth hard tissues.

Epidemiological studies confirm that good oral hygiene combined with fluoride application interferes with caries progression. However, Bjarnason and Finnbogason (1991) controversially stated that fluoride levels in dentifrices have little effect on the progression of radiographically detectable enamel lesions, though a higher concentration of fluoride (1000 ppm F^-) caused a less pronounced caries progression than a lower concentration (250 ppm F^-). They concluded that the benefit of topical fluoride application in the form of a dentifrice is mostly in retarding the initiation of new lesions, while the reduction is far less in the progression of already established caries. Conversely, Lawrence *et al.* (1997) demonstrated that lifetime residents of a fluoridated area demonstrated a significantly lower progression of enamel lesions compared to children resident in a non-fluoridated area. The general consensus is that fluoride has a maximum benefit when it is available constantly (or if that is not possible, frequently) in low levels within the oral cavity.

Fluoride does reduce caries prevalence. Over the last 25 – 30 years, caries prevalence in the USA and most European countries has dramatically declined. Although it is difficult to give a straightforward reason for the present lower caries prevalence in these populations, consensus exists on the important role of fluoride, particularly widespread home use of fluoride toothpaste and the increasing use of professionally applied topical fluorides (Haugejorden *et al.*, 1997; Newbrun, 1999).

So is the use of fluoridated toothpaste enough? Is there an additional benefit from using other forms of topical fluorides? These can be in the form of fluoridated water, salt, milk, tablets, mouthrinses, gels and varnishes, and are discussed in more detail in Section 1.5. A recent systematic review (Cochrane) examined the additional use of a topical fluoride along with fluoridated toothpaste and found that there was a further modest caries reducing benefit compared to toothpaste use alone (Marinho *et al.*, 2004c).

1.4 Effect of milk on the caries process

Milk is regarded as one of the most nutritionally complete foods, containing fat, protein, essential vitamins e.g. A & D and minerals such as calcium and phosphate. The UK Department of Health recommends that infants are fed on milk alone (preferably human) until they are weaned on to solid foods at the age of six months. Whether children are fed on cow's milk, formula feed or human milk, milk is regarded as one of the ideal sources of nutrition. Furthermore, the amount of nutrients supplied by milk is high in relation to its calorie content.

Milk (along with water) has also been suggested as a drink that is not harmful to teeth, and is therefore thought a suitable alternative to sugary or fizzy drinks for both children and adults. It is promoted strongly by both the dental and dairy industry as an ideal beverage.

There has been extensive debate over the years, regarding the cariogenic potential of milk. While milk is known to contain sugar in the form of lactose (4-5% in cow's milk; 7% in human milk (Darke, 1976)), in the great majority of situations it is thought to be anti-cariogenic (McDougall, 1977; Bowen *et al.*, 1991; Bowen and Pearson, 1993; Erickson and Mazahari, 1999). However, in a very few cases, human milk has been shown to cause "nursing caries" (a form of early childhood caries) in children who have been fed "on demand", throughout the night when sleeping alongside their mothers (Dilley *et al.*, 1980; Derkson and Ponti, 1982; Roberts, 1982).

As well as the above mentioned constituents, milk contains a range of antibacterial substances: lysosyme, peroxidase and lactoferrin (Kosikowski, 1970), which could affect the microflora of the oral cavity. However, as liquids are swallowed quickly, any components are less available to intra-oral bacteria as compared to sticky, more retentive foods.

In studies using enamel slabs *in vitro*, milk has been shown to cause less enamel solubility than lactose or sucrose solutions (Jenkins and Ferguson, 1966; McDougall, 1977). Milk is also known to contain caries-protective factors, such as calcium, phosphate, casein and lipids. Moynihan *et al.* (2003) report that calcium and phosphate are present in cow's milk in high concentrations (125mg and 96mg/100g respectively), but less-so in human milk

(34mg and 15mg/100g respectively). It was these high concentrations of calcium and phosphate that were initially thought to be the reason why milk was anti-cariogenic. However, when milk was compared with a water-based solution containing equivalent calcium and phosphate concentrations, enamel was found to be less soluble with milk than the water-based solution (Jenkins and Ferguson, 1966). These workers concluded that the effect of milk was more complicated than can be accounted for by its calcium and phosphate content alone, and that some other constituent must also have an effect on enamel solubility. Later studies showed that casein may protect against demineralisation of enamel, but unfortunately this is unpalatable to humans (Reynolds and Black, 1987; Reynolds *et al.*, 1995). More recent studies (Grenby *et al.*, 2001) tried to determine the caries-protective agents of milk *in vitro*. Their main findings were that when fat, lactose, casein and other proteins were removed from milk, it was still caries-protective; i.e. it seems calcium and phosphate plays a part in inhibiting caries. However, they stated that milk contains other protective factors more powerful than these, and further investigations suggested that proteose-peptones may be involved.

Milk has been shown to reduce the cariogenic potential of sugar-containing foods (Jenkins and Ferguson, 1966; Thompson *et al.*, 1984). However, an increase in plaque-acid production was noted in an intra-oral plaque study with 5% sucrose in milk (Thompson et al., 1984), though another study found no such effect, albeit demineralisation was produced with 5% sucrose in water (Mor and McDougall, 1977). Hence, apparent contradictions exist.

1.5 Fluoride delivery systems

1.5.1 Ingestion, absorption, storage and excretion of fluoride

Fluorine is the most electronegative of all chemical elements, is seventeenth in the order of elemental abundance in the Earth's crust (Fleischer, 1953) and, in its ionic form fluoride (F^-), is ingested via water, foods and drinks. The main source of ingested fluoride is from fluoridated dentifrices, other sources include fluoridated mouthwashes and varnishes (Fomon and Ekstrand, 1996). Fluoridated water may also have a significant effect in a naturally or artificially fluoridated area (in temperate climates, the 'ideal concentration' is 1 ppm F^-). In addition, small amounts may be acquired from the use of fluoridated milk, Teflon-coated cooking vessels, airborne origins and fluoride-containing drugs. Of the ingested fluoride, the major portion is absorbed from the stomach and duodenum (75-90%), 1% is absorbed through the oral mucosa, and 10-25% passes through the body to be excreted in faeces (Murray *et al.*, 1991).

When fluoride is absorbed via the stomach and duodenum, it is carried around the body within blood plasma. Concentrations of fluoride in ductal saliva and gingival crevicular fluid, as well as urine and bile, are related to plasma levels. Any change in the fluoride concentration of plasma creates a simultaneous and proportional change in the fluoride levels of these body fluids (Murray *et al.*, 1991). Approximately 99% of fluoride absorbed following ingestion is stored by the body in the calcified tissues, where it is strongly bound to apatite and other calcium phosphate compounds (WHO expert Committee on Oral Health Status and Fluoride Use, 1994). Any absorbed fluoride not stored, is excreted in urine (Murray *et al.*, 1991).

Fluoride has an affinity for apatite tissue (found in tooth and bone). The fluoride content of bone reflects plasma fluoride concentration at the time of calcification and during remodelling. Therefore, it changes in relation to the levels of fluoride ingested. As discussed earlier, fluoride may substitute for hydroxyl groups in hydroxyapatite to form a fluoridated hydroxyapatite.

Research has shown that fluoride is most effective in dental caries protection when a low level of fluoride is constantly maintained in the oral cavity. The World Health Organisation Oral Health Strategy, encourages the use of '*community-based public health programmes*

to implement the most appropriate method of maintaining a constant low level of fluoride in as many mouths as possible'(World Health Organisation, 2005). There is clear evidence that long-term exposure to an optimum level of fluoride results in diminishing levels of dental caries in both children and adults. This can be achieved using one or more of the methods described below.

1.5.2 Fluoridated water

As was detailed in Section 1.3, water was the first vehicle whereby the beneficial caries-preventive action of fluoride was observed (Ainsworth, 1928). Fluoridation of the drinking water, either naturally occurring or as a therapeutic additive, is still the preferred method of administering fluoride to a population, as the water is drunk both by consumers and is also used for cooking. Furthermore, its consumption requires no active compliance of the individual. Thus the frequency of exposure of teeth to fluoride is fairly constant and guaranteed.

Artificial fluoridation adjusts the fluoride level in the water supply to 1.0 ppm F^- in temperate climates, 1.2 ppm F^- in arctic regions and 0.6 ppm F^- in tropical regions. A recent UK systematic review commissioned by the Chief Medical Officer of the Department of Health published in 2000, examined 214 studies further to an electronic search of 25 databases, with no language restrictions and hand search of appropriate journals. One of the aims of the review was to determine the evidence regarding the effect of water fluoridation on dental caries. Twenty six studies were included in this part of the review; a large number of studies were excluded because they were cross-sectional studies and did not satisfy the inclusion criteria. It concluded that the best available evidence suggests that fluoridation of drinking water supplies does reduce caries prevalence, both as measured by the proportion of children who are caries-free, and by the mean change in dmft/DMFT score. The range of the mean difference in the proportion (%) of caries-free children is -5% to 64%, with a median of 14.6%.Therefore, it was not possible to determine, the degree to which dental caries was reduced from the data available (McDonagh et al., 2000).

Unfortunately, domestic water within and surrounding Glasgow has a fluoride concentration of less than 0.03 ppm F^-.

1.5.3 Fluoridated milk

As will be described in greater detail, in section 1.6, fluoridated milk was considered over 40 years ago in areas with non-fluoridated water supplies. Milk was recommended as a vehicle because of its additional benefit as a good food for infants and children. Numerous studies involving fluoridated milk have been undertaken in the USA (Rusoff *et al.*, 1962), Switzerland (World Health Organisation, 1970), Scotland (Stephen *et al.*, 1981; Stephen *et al.*, 1984), Hungary (Bánóczy *et al.*, 1983; Bánóczy *et al.*, 1985), Israel (Zahlaka *et al.*, 1987), North West England (Ketley *et al.*, 2003), and most recently in Russia (Maslak *et al.*, 2004).

While generally positive benefits were reported, the methodologies employed were often far from ideal. In addition, one of the difficulties with using fluoridated milk is the logistics of producing sterilised fluoridated milk, delivering it to those who need it, and then ensuring its compliant consumption.

1.5.4 Fluoridated salt

Fluoridated salt, like fluoridated milk was pioneered in Switzerland, where it was first introduced in 1955. It is currently used by approximately 80% of the population. The fluoride concentration is currently 250 mg F/kg salt, with the average ingestion of 7-10g per day, 3-5g of which is via domestic salt. It is also used in Hungary, Columbia, France, Spain and Germany. Studies in Switzerland (De Crousaz *et al.*, 1985) and Hungary (Toth, 1976) have indicated that fluoridation of salt results in substantial dental caries prevention. For example, in the Hungarian study, there was a decrease in the DMFT of 58%, after 8 years of salt fluoridation in children aged 7-11 years. As a community-based means of fluoride delivery, fluoridated salt also uses relatively simple technology and is cheap to produce and purchase. In addition, as it requires little positive effort, it has good acceptability with the public. However, with public health messages encouraging the reduction of salt in the diet, as a method of reducing hypertension, it may be that the promotion of fluoridated salt has limited use in the 21st century.

1.5.5 Fluoridated dentifrice

Millions of people worldwide use fluoridated toothpaste. It is one of the World Health Organisation's policies to support the widespread use of affordable fluoridated toothpaste in developing countries, particularly as a result of the changing diet and nutritional status in these countries (World Health Organisation, 2005).

Many changes have taken place with respect to formulation of toothpaste over time as in the 1940s, the fluoride salt (sodium fluoride) was found to bind to the abrasive and become clinically inactive. The formulations of mono-fluorophosphate and stannous fluoride were developed, both of which were compatible with calcium-containing abrasives. Then, in the 1980s, new abrasives were developed e.g. hydrated silica, which was compatible with sodium fluoride. Amines can also be used, which in conjunction with the stannous ion, may act as F^- counter-ions thereby having additional benefits of being antimicrobial. Today, the most common sources of fluoride in toothpastes are sodium fluoride (NaF) and sodium monofluorophosphate (Na_2FPO_3, often abbreviated to SMFP). Although NaF is acknowledged, since 1974, as being marginally more effective (Stamm, 1995), SMFP is often used because it is compatible with a wider range of ingredients, particularly those which are less expensive.

Toothpastes are designed to act in a topical manner (i.e. act locally within the mouth, on the surfaces of erupted teeth) rather than act following ingestion (systemic effect). As high doses of fluoride can cause fluorosis and be toxic, the maximum fluoride level allowed in Europe for "over the counter sales" is 1450ppm F (0.32% NaF, 1.14% SMFP), and in USA 1100ppm F (0.22% NaF, 0.76% SMFP). The efficacy is known to increase by 6% per 500 ppm F^- increase in relative concentration (Stephen *et al.*, 1988). The British Society of Paediatric Dentistry has recommended that children under the age of six use 600 ppm F^- toothpaste, unless they are of higher caries risk, when 1000 ppm F^- toothpaste should be used, with only a small pea-sized amount (Rock, 1994). Children over the age of six should use 1000 or 1450 ppm F^- toothpaste (Holt et al., 1996). Recent studies using fluoridated toothpastes with a fluoride content of 2800 ppm F^- demonstrated an increase of 20% in its protective effect (Biesbrock *et al.*, 2001) and such a formulation " Colgate Duraphat® 2800 Fluoride toothpaste" (Colgate-Palmolive (UK) Ltd, Guildford, UK) is available on prescription in the UK.

A recent systematic review investigated the effectiveness of fluoridated toothpastes in the prevention of dental caries in children and adolescents. It found clear evidence that fluoride toothpastes are efficacious in preventing dental caries. The effect of fluoride toothpaste increased when the baseline levels of D(M)FS were high; when a higher fluoride concentration was used; with higher frequency of use; and with supervised brushing. This effect was not altered by exposure to water fluoridation. On average, fluoride toothpastes reduced the DMFS 3-year increment by 24% (Marinho et al., 2004b).

Furthermore, increased efficacy, over-and-above that of increased brushing frequency has been proven when post-brushing rinsing with water is eliminated, as the fluoride is then not removed from the mouth and is thus available to exert a greater topical benefit (Sjogren *et al.*, 1995; O'Mullane *et al.*, 1997; Chestnutt *et al.*, 1998).

1.5.6 Fluoride supplements

Where water fluoridation is not possible, other vehicles of fluoride delivery must be considered. One of the first suggestions for enhancing fluoride exposure was fluoride tablets. The first use of this form was attributed to Erharde (1874) in the form of potassium fluoride tablets, though the first trial was not recorded until 1945 by Beaudet and reported by Bibby *et al.*(1955) using calcium fluoride tablets. Arnold *et al.* (1960) reported on 121 subjects who had started to take 0.5- or 1-mg F^- (sodium fluoride) tablets daily, between birth and 6 years of age, over periods ranging from 1 to 12 years and published dmft and DMFT results which were comparable with similarly aged subjects in naturally fluoridated Aurora and artificially fluoridated Grand Rapids, Newburgh and Brantford.

The daily dosage of fluoride in tablet/drop form was calculated from estimates of fluoride ingestion from water. The main drawback of this method is compliance, especially where tablets are given for home use (relying on parental and child compliance) rather than in supervised school programmes. Current British opinion, based on the majority view and current evidence (British Society of Paediatric Dentistry) is that children who live in areas containing less than 0.3 ppm F^-, and who are considered to be at high risk of developing dental caries, should take a fluoride supplement of 0.25 mg F^- per day for those aged 6 months up to 3 years; 0.5 mg F^- per day for those aged 3 up to 6 years and 1.00 mg F^- per day for those aged 6 years and over (Holt et al., 1996), albeit the efficacy of such a regimen has still never been tested clinically. When fluoride is given as tablets, these should be allowed to dissolve slowly in the mouth in order to give a maximal topical effect (McCall *et al.*, 1981) as well as any parallel post-ingestion systemic benefit.

1.5.7 Fluoridated mouthwash

Fluoridated mouthwashes (also known as mouthrinses) act topically within the mouth. They usually contain 0.05% sodium fluoride (225 ppm F) if used as a daily rinse or 0.1-0.75 % sodium fluoride (450-3000 ppm F) if used weekly. Over 30 trials in 14 countries have shown that daily mouthrinsing helps to prevent dental caries. A systematic review

(Cochrane) completed in 2004, determining the efficacy of fluoride mouthrinses showed they reduce the DMFS 3-year increment by 26% (Marinho *et al.*, 2004a). Sodium fluoride is the preferred formulation (Stephen, 1994), and increased frequency is more efficacious than increased concentration (Heifetz *et al.*, 1982).

1.5.8 Fluoride varnish

Fluoride varnish application is a topical method of delivering fluoride to the erupted surface of the tooth. The most common UK formulation is Duraphat® varnish (Colgate Oral Pharmaceuticals, Canton, MA, USA) which contains 50mg sodium fluoride in 1 ml, equivalent to 22.6 mg (22,600 ppm F^-) of fluoride in an alcoholic solution of resins. Topical fluoride varnishes are of proven benefit in preventing caries and in helping to arrest caries in children with early childhood caries (previously known as "nursing bottle caries") and cervical decalcification. These are highly concentrated vehicles for fluoride and the recommended dose should not be exceeded (Ekstrand *et al.*, 1981; Shaw, 1997).

A recent systematic (Cochrane) review investigating the effectiveness of fluoride varnishes in the prevention of dental caries in children (Marinho *et al.*, 2005b), examined nine studies involving 2709 subjects. They concluded there was a substantial caries-inhibiting effect of fluoride varnish in both permanent and deciduous dentitions, based largely on trials with "no treatment" controls. However, they were not able to estimate the D(M)FS reduction as a result of the relatively poor quality of most of the included studies and the wide confidence intervals around the estimates of effect.

1.5.9 Fluoride gel

The use of a thixotropic gel has also been employed as a topical method of delivering fluoride to the mouth. Current British recommendations (British Society of Paediatric Dentistry) state that "*....professionally applied fluoride gels (1.23 % acidulated phosphate fluoride APF) and solutions (8% stannous fluoride) are recommended by some authorities, but have been shown to be of poor cost-benefit, although clinically beneficial. Children at high risk should be considered for application of topical fluorides twice yearly*" (Shaw, 1997).

A recent systematic review investigated the effectiveness of fluoride gels in the prevention of dental caries in children (Marinho et al., 2005a) and included 25 studies involving 7747 youngsters. They concluded there was clear evidence of the caries-inhibiting effect of

fluoride gel, the best estimate of this being a 21% reduction (95% CI, 14 to 28%) in D(M)FS. However, inadvertent ingestion can achieve toxic F^- levels unless delivery and swallowing of the F^- gel is well controlled (Ekstrand *et al.*, 1981; McCall *et al.*, 1983).

1.5.10 Fluoridated foodstuffs

The fluoride content of fresh food generally ranges from 0.01 to 1.0 ppm F^- (Whitford, 1996). Fish, such as sardines, may contribute to a higher dietary intake if the bones are eaten. Brewed teas may also contain fluoride concentrations of 1 ppm to 6 ppm F^- depending on the amount of dry tea used, where the tea leaves were grown, the local water fluoride concentration, and the brewing time (Whitford, 1996).

Chewing gum has also been used as a method of delivering fluoride to the oral cavity. Brunn and Givskov (1978) reported on salivary fluoride levels following the chewing of gum containing 0.25 mg F^-. However, with only 6% of the gum fluoride remaining after 10 minutes of chewing, the salivary fluoride content was low at 3.9 ppm F^-. They later admitted that the fluoride-containing gum might be more readily accepted by children than other methods, but this creates a potential problem of excessive intake via unsupervised usage (Bruun and Givskov, 1979). Furthermore, there have been no controlled clinical caries-inhibiting studies using this delivery vehicle.

1.5.11 Slow release fluoride devices

More recently, another method of fluoride delivery has been developed: the slow release fluoride device. The aim of this device is to provide a constant source of fluoride to the oral cavity and overcome problems of poor subject compliance. Initial work on slow release fluoride was completed using co-polymer membranes retained on teeth in orthodontic brackets (Mirth *et al.*, 1982; Mirth *et al.*, 1983). Further work has been completed using glass beads, which contain trace elements in the form of inorganic radicals and it has been possible to place fluoride as the trace element in such a device (Curzon and Toumba, 2004). Slow releasing glass fluoride devices containing 13.3% fluoride, placed on the buccal aspect of maxillary first permanent molar teeth have been shown to increase long-term salivary fluoride concentration (Curzon and Toumba, 2004). Fluoride delivered in this manner has been shown to be effective as a caries-preventive device *in vivo* in schoolchildren from a low socio-economic background (Toumba and Curzon, 2005).

1.5.12 Summary

Fluoride is without question a most powerful caries preventive agent, and is probably the only one for which substantial efficacy has been shown beyond doubt. It is also a therapeutic agent which is by-and-large safe for use, as shown in many long-term studies. Currently, the WHO Global Health Programme is undertaking demonstration projects in Africa, Asia and Europe to assess the relevance of affordable fluoridated toothpaste, water fluoridation and salt fluoridation in these areas (World Health Organisation, 2005).

Fluoride's topical effectiveness regarding dental caries prevention, has been firmly established via the body of evidence from randomized controlled trials (Marinho *et al.*, 2005c). Fluoride toothpastes in comparison with mouthrinses or gels appear to have a similar degree of effectiveness for the prevention of dental caries in children (Marinho *et al.*, 2005d). However, acceptance is likely to be greater for fluoride toothpaste. Additionally, the use of another topical fluoride e.g. mouthrinse, gel, or varnish as well as a fluoridated toothpaste, achieved only a modest caries reduction benefit compared to that of toothpaste alone (Marinho *et al.*, 2004c).

1.6 Fluoridated milk as a fluoride delivery system

The use of milk as a vehicle for delivering fluoride has been mentioned previously. This section will discuss fluoridated milk in more detail.

The use of fluoridated milk as a possible dental caries-preventive medium was first proposed by the Swiss paediatrician Ziegler, in 1953. The method he suggested was described in a review (Zeigler, 1956), whereby he added 1mL of 0.22% NaF (1000 ppm F^-) solution to 1 litre of fresh milk. He stated that "*...the addition of fluoride in dosage of 1 mg F^- per litre (1 ppm F^-) to milk, in cases where the fluoridation of drinking water is not possible, is justified on the grounds of physiological and toxicological considerations. The advantages of this mode of administration as compared with fluoride prophylaxis with tablets, salt or even with water, seem to be significant.*"

1.6.1 Possible interactions between milk and fluoride

One of the common misconceptions about the addition of fluoride to milk is that the ionic constituents of the milk will bind with the fluoride and prevent any therapeutic benefit. There is a certain element of truth in this statement, but it is an issue only with high doses of fluoride, or where it is left for a number of hours before drinking. Konikoff (1974) and Duff (1981) noted that significant binding of milk to fluoride occurred after a period of four or five hours. Cutress *et al.* (1995) studied the effects of deposition of fluoride in ovine enamel from fluoridated milk at concentrations of 300 and 750 ppm F^-, and found that only 30% and 20% of fluoride respectively was chemically available within the milk. In the 2-5 ppm F^- concentration range, which is commonly used in milk, these interactions have a relatively small effect on the bioavailability of fluoride (Phillips, 1991; Edgar *et al.*, 1992). In the most commonly used pasteurized milk, virtually all added fluoride remains available throughout its shelf-life, extending over several days when stored below 6°C. However, ultra-heat-treated (UHT) fluoridated milk does suffer some loss of fluoride availability during processing and subsequent storage at ambient temperatures over the six month shelf-life (Stephen *et al.*, 1996).

1.6.2 Compounds used to fluoridate milk

Compounds which have been used to fluoridate milk include sodium fluoride, calcium fluoride, disodium monofluorophosphate and disodium silicofluoride. All of these compounds have been used successfully in clinical and laboratory trials (Stephen *et al.*, 1984; Bánóczy *et al.*, 1985; Villa *et al.*, 1989; Stösser *et al.*, 1993). Of these compounds,

sodium fluoride is by far the most commonly used agent for large scale production of fluoridated milk and has been used in large community schemes in Bulgaria, China, Russia and the United Kingdom. In the Chilean studies, (Stephen *et al.*, 1996) disodium monofluorophosphate was used, albeit via powdered milk. Calcium fluoride has not been used for large scale production because of its low aqueous solubility.

Sodium fluoride is usually added to the milk in the form of a concentrated aqueous solution, using a fixed volume ratio to obtain the required concentration.

1.6.3 Milk fluoride concentration and volume

To calculate the appropriate milk fluoride concentration, it is necessary to consider the volume of fluoridated milk consumed daily by a child. The volume consumed varies with location; e.g. in the UK, a child would typically receive 1/3 pint (189 mL) of school milk per day, whereas in China, kindergarten children each receive 250 mL (Stephen *et al.*, 1996). In order to deliver a dose of approximately 0.5 mg fluoride per day, to children in both areas, the fluoride concentration in the milk would need to be set at 2.65 ppm F^- and 2 ppm F^- respectively. In Bulgaria, where 200 mL per day is the typical volume consumed and the fluoride requirement is 1mg per day, the concentration of fluoride in milk is set at 5 ppm F^-.

1.6.4 Methods of measuring fluoride concentration in milk

The ionizable fluoride concentration of milk can be conveniently measured using a fluoride ion selective electrode (Orion Research of Boston, USA), in conjunction with a reference electrode, coupled to an ion meter. The use of an appropriate buffer (TISAB II, Total Ionic Strength Adjustment Buffer) allows measurement of fluoridated milk concentrations of between 1 and 10 ppm F^- via a direct concentration readout, or a millivolt output from which concentration may be computed (Stephen *et al.*, 1996).

1.6.5 Mode of action of fluoridated milk on caries process

The action of milk on the caries process has been discussed previously in Section 1.4, where the evidence for milk acting as a caries-preventive substance was shown. Stösser *et al.* (1995) demonstrated there was no evidence to suggest that the fat content of milk (skimmed, semi-skimmed or whole) nor the method in which the milk was sterilised (raw, pasteurized, UHT) had any effect on the caries-protective effect of milk. However,

Konikoff (1974) noted that for the consumption of fluoridated fresh milk to be effective, it had to be consumed within four hours of preparation.

The addition of fluoride to milk has additional benefit, as it may accumulate in the dental plaque; Kertész *et al.* (1992) noted this after eight weeks of fluoridated milk consumption. It may also accumulate within the enamel. Toth *et al.* (1987) took acid-etch enamel biopsies of children (aged 8-10 years) who had been involved in a closed community fluoridated milk study and showed a significant increase ($p<0.01$) in the fluoride content of the enamel biopsy samples after consuming fluoridated milk (200 mL milk containing 0.75mg F^-) for one year. Given that the children in the Toth *et al.* (1987) study were aged eight to ten years, it would be reasonable to assume that the increase in fluoride content of the enamel was as a result of a post-eruptive (topical) effect of fluoride.

1.6.6 Clinical/Community trials with fluoridated milk

The first clinical trial was in Yokohama, Japan (Imamura, 1959) and reported a 36% caries reduction in the permanent teeth from the fluoride group, which consisted of 167 11-year olds who consumed 2-2.5 mg NaF in milk, or soup, added to school meals over 150-180 days a year.

In the USA, Rusoff *et al.* (1962) enrolled 65 school children, aged six to nine years at the outset and provided milk (3.5 ppm F^-) to them daily at school. They achieved an overall caries reduction of 35% in the six year-old children, and a 78% difference between the test and control groups.

In Switzerland, Zeigler (1956) and Wirz (1964), published reports on a large scale experiment in Winterthur, Switzerland. They gave one ppm F^- to 749 test children (553 controls) who were aged between nine and 44 months at the beginning of the trial, and after six years, the caries reduction was between 14.8% and 31.5% in primary teeth and from 64.2% to 65.2% in permanent molars.

In Scotland, in the early 1980s, a carefully designed double-blind study with substantial pre-trial stratification, was run over five years. Milk was given to 4½ to 5½ year-old school children by providing those in the test group with 200 mL of milk with 1.5 mg F^- added (approx. 7 ppm F^-) while those in the control group were given 200 mL of milk. Both groups were given milk for 200 days per year. There were 187 subjects recruited, 94 were

in the test group and 93 in the control group. The children had annual clinical and radiographic examinations. The clinical examinations and milk distribution were on a double-blind basis and the radiographs were also read blind. Compliance was assessed by laboratory-based urine analysis, at the end of the second year of the trial. The fluoridated milk solution was prepared by adding 300 mL of sterile sodium fluoride solution to five gallons of milk. At the end of five years there were 50 subjects remaining in the test group and 56 in the control group. There was a 31.2% difference in the DMFT scores between the fluoridated and non-fluoridated milk scores, and a 43.1% difference in the DMFS scores (Stephen *et al.*, 1984).

In Hungary, in 1979, a milk fluoridation study was implemented. Here each child consumed 200 mL of milk or cocoa-milk daily, 0.4 mg F^- was added to kindergarten children and 0.75 mg F^- was added to the milk of primary children. Fluoride aliquots were prepared by the pharmacy and these were added to the milk and stirred for 10 minutes, ensuring that the milk was consumed within 30 minutes. Urinary fluoride excretion was analysed initially, weekly then monthly. Clinical examinations were carried out each year, DMFS, dmfs, DMFT and dmft indices were calculated without radiographic examination. Data were analysed at three, five and ten years. The overall mean caries increment was calculated between the test and control groups, there was a 36.8% DMFT, and a 40.0% DMFS reduction favouring subjects in the fluoridated milk group (Bánóczy *et al.*, 1985; Gyurkovics *et al.*, 1992).

In the United States, a school-based study was designed to investigate the effect of using fluoridated chocolate-flavoured milk on caries incidence in elementary school children over two and three years (Legett *et al.*, 1987). They suggested that chocolate flavoured, sweetened milk is strongly preferred and this may increase the compliance of milk ingestion. However, although a control group is described in the study, it was not described whether this was fluoridated and/or sweetened or not. They describe compliance issues and a large interruption in the delivery of milk to the schools following dairy refurbishment as well as a high attrition rate which led them to recruit additional subjects to analyse over a two-year period. Overall, they describe a 77% caries reduction in their two-year study when children ingested chocolate-flavoured fluoridated milk. Because of these procedural difficulties, these results should be interpreted with caution.

A study in Israel (Zahlaka *et al.*, 1987) investigated the caries-reducing effect of reconstituted powdered cow's milk (100 mL) supplemented with 1 mg F^- as NaF. This was provided to children aged between four and seven years at the start of the study for a period of three years at school. There were 120 subjects in each of the test and the control groups. The control group had no beverage; a more suitable control would have been to have the reconstituted powdered cow's milk with no fluoride supplement. It is known that milk may exert a cariostatic effect, therefore, it is difficult to determine whether the results of this study are as a result of effect of the fluoride or the combined effect of the milk and fluoride.

Community based studies have been undertaken in Bulgaria, where Pakhomov *et al.* (1995) instigated a school-based fluoridated milk study involving 3-10 year olds ingesting 200 mL of milk containing 1 mg of F^- (equivalent to 5ppm F^- as NaF) in a town, Asenovgrad. Their control group were children in a nearby town, Panaguriche, who received milk from a different dairy. Cross-sectional random samples of 100 children from each town were examined at age six and a half, and again three years later. The fluoridated milk cohort showed a decrease in dmft of 40% and in the mean DMFT of 89% compared to baseline. However, they discussed that these benefits may not have been as a result of fluoridated milk alone, and there may also have been additional effects of improved oral hygiene and improved dietary habits.

A large school-based milk fluoridation study was undertaken in North West England by Ketley *et al.* (2003). This study involved children, initially aged 3-5 years. Those in the fluoridated school milk group were given 189mL of milk containing 0.5mg of F^- (equivalent of 2.65 ppm F^-), those in the non-fluoridated milk group had the same volume, and both groups drank with a straw for approximately 180 days per year. Blinded, clinical visual examination was undertaken also examination using Fibre-optic Transillumination (FOTI) for DMFT, DFS, dmft and dfs, at baseline and four years later (7-9 years). The fluoridated-milk group (n=318) had DMFT 0.40 (st.dev.= 0.85), DFS 0.45 (st.dev.=1.12), dmft 2.28 (st.dev.=2.06) and dfs 4.49 (st.dev.=4.91). The non-fluoridated milk group (n=233) had DMFT 0.40 (st.dev=0.87), DFS 0.55 (st.dev.= 1.35), dmft 1.96 (st.dev.= 2.18) and dfs 4.12 (st.dev.= 4.85). Therefore, this group found no caries reduction in the primary dentition and very minimal impact on the permanent dentition. They discussed a number of potential explanations for their findings differing from those of previously published work, including: age of entry into the study; the numbers of days the children consumed the milk;

the DMFT in the non-fluoridated milk control group (which was five times less than that in the Stephen *et al.* (1984) study); diet; fluoride toothpaste and use of fissure sealants. Many of the earlier fluoridated milk studies were conducted before fluoridated dentifrices were readily available or fluoridated dentifrices were not used by test subjects. However, in the UK (and elsewhere), fluoridated toothpaste is very readily available and was used at least twice a day in 63-66% of subjects in this study.

Most recently, in Volvograd in Russia, Maslak *et al.(2004)* investigated the effect of fluoridated milk in kindergarten children. One hundred and sixty-six children who were caries-free aged three, were randomly assigned into two groups. Group 1 (n= 75) consumed 180-200 mL of fluoridated milk when at school, group 2 (n=91) consumed non-fluoridated milk for four years. Preliminary three year results showed that caries prevalence was 69.3% in Group 1 and 82.4% in Group 2, ($p< 0.05$), dmft was 2.5 (st.dev.=0.26) for Group 1 and 3.64 (st.dev.=0.26) for Group 2, ($p< 0.05$) at detection threshold D3, D4. They concluded that their milk fluoridation project was effective in reducing caries in children when given from three years of age; however, no mention was made of the concentration of fluoridated milk used in the study or whether the children were using additional fluoride vehicles possibly in the form of fluoridated toothpaste.

1.6.7 Summary of fluoridated milk as a fluoride delivery system

There is "bioavailability" evidence that the availability of fluoride is not reduced by milk in the concentrations currently used (<15 ppm F^-). The main effect of the fluoride within fluoridated milk appears to be a post-eruptive topical one, as it is still as effective in older children whose teeth have erupted prior to them being recruited into fluoridated milk studies (Toth *et al.*, 1987). However, Pakhamov *et al.* (1995) suggested that the greatest benefit of fluoridated milk occurs when it is available earlier in the child's life and in the first two to three years of the programme.

Fluoride concentrations of 5-15 ppm F^- as CaF_2, NaF, Na_2-monofluorophosphate or Na_2-silicofluoride have been shown to result in a significant reduction in caries of 40-50% and did not depend on the compound of fluoride used. The Ketley *et al.* (2003) study used fluoride concentration of 2.65 ppm F^- and observed minimal caries reduction with this concentration in the permanent dentition only.

There is some debate about the quality of the evidence supporting milk fluoridation. In a review of the literature (Mariño, 1995) concluded that "*recent evidence suggests that milk fluoridation may be regarded as a valid alternative in areas where water fluoridation cannot be used to provide the desired benefits*". More recently, a systematic review of the effectiveness of fluoride tablets, fluoride in salt and fluoride in milk with regard to prevention of caries was unable to draw conclusions due to insufficient evidence (Holm, 2002).

In conclusion, where compliance can be assessed, fluoridated milk appears to maintain a prolonged low level of ionized fluoride available within the oral cavity, and this can be capable of promoting remineralisation (Stephen *et al.*, 1996). However, currently, there is insufficient evidence to determine the extent of the caries preventive effect of fluoridated milk over-and-above that of fluoridated dentifrice. Further studies are required to investigate this.

1.7 Caries investigative models

In this section, the design and methods used for caries *in situ* and *in vitro* trial models, and the reasoning behind the methods chosen for the studies described later in this thesis, are discussed.

Ideally, to study the effect of potential caries preventive factors on de- or re-mineralisation, a randomised-controlled-clinical-trial (RCT) model would be used, involving longitudinal studies and measurement of caries increment, e.g. van Rijkom *et al.*(2004). However, there are significant difficulties with this approach, such as the difficulty of achieving ethical approval; potential bias must be avoided by the use of a randomised blind study design; and such studies tend to be time-consuming for both patients and examiners. In combination, these factors make clinical *in vivo* randomised-controlled-trials very expensive to run. Other models have therefore been developed such as *in vitro* and *in situ* models, which can simulate an *in vivo* situation to a greater or lesser extent.

1.7.1 Introduction

As discussed previously (Section 1.2) dental caries is a multi-factorial process. *In vitro* and *in situ* models are useful to investigate the caries process because they allow multiple variables to be held constant in order to facilitate the measurement of a single variable (Mellberg, 1992). This situation is virtually impossible to replicate in a clinical longitudinal randomised controlled trial. Also, dental caries may progress slowly over a long time-period making randomised controlled trials *in vivo* expensive to run. This is in addition to the inevitable subject attrition that occurs over time. Models have the advantage that the caries process can be accelerated, or designed in such a way that smaller changes in mineral content can be more accurately measured in a non-invasive and ethical manner, often using fewer subjects.

The disadvantages of models are that because relatively small numbers of subjects are used, the sample population is not necessarily comparable with the general population. Also, the studies require a certain amount of compliance from the subject and can be difficult to ascertain if this has been sufficient. Any lack of compliance could have a significant effect on experimental outcome.

Given the multi-factorial nature of dental caries, these models should include: a tooth substrate (either enamel or dentine), the formation or presence of dental plaque with

cariogenic potential, a carbohydrate challenge (provided by the subject's normal diet or experimentally controlled), with all of these factors occurring over time (Zero, 1995). The method in which the formation or presence of plaque and carbohydrate challenge are delivered to the model is dependent on the protocol used to induce the change and the place in which the study will take place i.e. *in vitro, in situ* or *in vivo*. The method used to observe and measure the change is also important and will be discussed in more detail in Section 1.8.

Intra-oral model systems provide an essential intermediate step between test procedures in animals and *in vitro* investigations on the one-hand, and clinical and field trials on the other (Manning and Edgar, 1992).

1.7.2 In vitro models

These are models which are used, out of the oral cavity, in a highly controlled laboratory situation. Such laboratory-based studies are useful for initial investigations into the effects of various parameters on de- and re-mineralisation. They allow the use of untested products which have yet to be proven as non-detrimental to human health. They are frequently quicker to perform, cheaper and can be undertaken at times convenient to the investigators (Manning and Edgar, 1992). However, there are limitations to this type of study as it can be difficult to extrapolate information determined in this manner, directly to a clinical situation.

1.7.3 In situ models

"*In situ models involve the use of appliances or other devices which create defined conditions in the human mouth that simulate the process of dental caries" (Zero, 1995).*

In situ models use a hard-tissue substrate placed within the oral cavity for the purpose of studying changes in the substrate due to a treatment or modification of the oral environment (Mellberg, 1992). Numerous model systems designed to measure the loss or gain in mineral by tooth tissues over short periods have been developed. These provide direct evidence of the process of caries development, without incurring some of the ethical and practical problems involved in clinical trials (Manning and Edgar, 1992).

In situ models tend to use hard tissue substrates in the form of tooth slabs or single sections cut from extracted teeth (Øgaard and Rølla, 1991). Enamel blocks containing pre-prepared

artificial caries lesions are usually employed to evaluate the effects of products or conditions on remineralisation, though they are also used to determine demineralisation and fluoride uptake (Mellberg, 1992). The study of naturally occurring carious lesions is the most clinically relevant method of analysing remineralisation and further demineralisation in caries trials. However, many uncertainties occur when using such lesions, namely, the extent of the caries lesion, whether it has arrested or has the potential to become active, and the site of the dental caries. It is known that remineralisation occurs more slowly in an *in situ,* as compared with an *in vitro* situation, as a result of the dynamic de- and re-mineralising situation that occurs within saliva *in situ* (Manning and Edgar, 1992).

Prepared tooth blocks or sections need to be mounted in an intra-oral device to retain them within the oral cavity. This technique was first developed by Koulourides and Volker(1964). Demineralisation or remineralisation regimes can be delivered to the tooth block or section in two ways. First, by removal of the intra-oral appliance and applying the fluoride/ dietary item directly to the tooth, and second by having the subject wearing the intra-oral appliance and taking the fluoride/dietary item directly into the mouth, as naturally as possible with the appliance in place. The second method is preferable since it permits normal oral clearance, salivary interactions and other physiological interactions to occur (Manning and Edgar, 1992). There are a number of methods used to deliver the substrate to the oral cavity, the enamel slabs can be mounted in removable appliances (e.g. partial dentures, complete dentures) or in fixed appliances (e.g. temporary crowns or orthodontic bands).

The partial denture model was developed first by Koulourides and Volker (1964) and expanded by Koulourides (1974) where foreign hard tissue was mounted in posterior flanges of lower acrylic dental prostheses. This design required that teeth be missing from the arch but has been used successfully in many studies (Dijkman *et al.*, 1986; ten Cate and Rempt, 1986). Appliances have been designed to wear as palatal plates containing a number of enamel blocks (Brudevold *et al.*, 1984; Zero *et al.*, 1992). Also, single section models have been used to carry tooth sections rather than tooth blocks in intra-oral removable appliances (Creanor *et al.*, 1986; Wefel *et al.*, 1987; Strang *et al.*, 1988; Macpherson *et al.*, 1990).

Fixed appliances have been used to carry tooth sections or blocks. Wefel *et al.* (1987; 1992) used an intra-oral gold shell crown model. This involved mounting single sections of enamel containing white spot lesions, within a rectangular slot constructed within a cast gold shell crown. Øgaard *et al.* (1992) developed a model using tooth blocks mounted in a removable appliance. These blocks were carried beneath orthodontic bands which had 0.8mm wire posts welded to the inside of the band allowing plaque to accumulate between the tooth specimen and the band.

To enable the use of multiple treatment protocols to be applied to subjects involved in *in situ* studies, it is essential for there to be regular 'washout' periods between protocols to eliminate any effect of one protocol (possibly containing fluoride) on another (possibly not containing fluoride). Previous studies have shown that fluoride is not generally retained in the oral cavity for longer than two weeks after its last usage (Schafer, 1989). However, in a study undertaken by Stephen *et al.* (1992), they noted an unexpectedly high remineralisation rate in the subjects who were using non-fluoridated dentifrice, who had used 2500 ppm F^- prior to the two week washout period. They suggested that a washout period of four weeks should be recommended, to minimise potential problems.

1.7.4 In vivo models

In vivo studies investigating the effect of a treatment on a condition will always be the method of choice. However, ethically, only a treatment which shows no detrimental effect can be used in such a study. In order to conduct a laboratory investigation to examine natural early enamel caries, which may be reversible, it would be unreasonable to extract a natural human tooth. Some other tooth pathology or clinical indication would need to be present to justify extraction of a tooth for this type of study. Furthermore, there are disadvantages of undertaking an *in vivo* study involving lesion microanalyses unless the teeth are pre-scheduled for extraction. These disadvantages include difficulties in standardising the effect of the previous oral environment and the tooth type.

Intra-oral models based on vital unextracted teeth should also be considered as *in vivo* models (Øgaard and Rølla, 1991). Caries lesions can be developed on such teeth using metal plates (Nygaard-Östby *et al.*, 1957), beneath orthodontic bands (Hals and Simonsen, 1972; Øgaard and Rølla, 1992), or beneath gauze (Ostrom *et al.*, 1977; Gallacher and Pearce, 1979). Many of these models have been used in children when teeth were to be extracted later for orthodontic purposes e.g. Øgaard *et al.* (1988). This model is thought to

be the most accurate in relation to extrapolation of findings to the *in vivo* situation, however, it also introduces further ethical dilemmas.

1.7.5 Enamel in caries investigative models

Early caries of the tooth crown tends to occur in enamel. This is observed, clinically, as a white opaque spot that is slightly softer than the surrounding sound enamel, and which increases in whiteness when dried with air (Arends and Christoffersen, 1986). Thus, much of the research on early caries lesions has examined enamel caries. Primary dentine caries may occur as root caries, after gingival recession. Dentine caries also occurs in the coronal portion of the tooth, deep to enamel caries, with or without cavitation of the overlying enamel lesion (Kidd and Fejerskov, 2003).

One might expect that natural, unaltered human enamel would always be the best choice of substrate in experiments relating to the caries process but, depending on the objectives of a study, this may not always be so. Sound surfaces are necessary to observe caries formation. However, to be able to examine the effect of fluoridated substances on the reversal of the caries process, it is important to start experiments with demineralised enamel, thus allowing measurement of remineralisation as well as potential further demineralisation.

Historically, *in situ* and *in vitro* studies have used a number of different enamel substrates in caries trials. These include human, bovine, ovine, canine and porcine enamel. Although the majority of the characteristics of mammalian animal enamel are generally similar to that of humans, there are some minor differences (Featherstone and Mellberg, 1981). If conclusions from *in situ* and *in vivo* trials are to be extrapolated and applied to clinical situations, as many of these differences as possible should be eliminated. Most importantly, the substrate should reflect the effects of an agent on a natural human caries lesion (Mellberg, 1992). Bovine enamel was easily obtainable in large quantities and it has the advantage of having large, relatively flat surfaces. However, it is more porous than human enamel which results in more rapid diffusion rates and lesion formation (Flim and Arends, 1977; Featherstone and Mellberg, 1981; Edmunds *et al.*, 1988). Comparing all of the animal substrates available, bovine enamel produces lesions most like those in human teeth (Edmunds *et al.*, 1988). However, the advent of bovine spongiform encephalitis (BSE) in the UK has made this substance more difficult to obtain and unsuitable for use in *in situ* trials.

Human enamel is usually considered to be the substrate of choice, for *in situ* studies. However, a major problem can be accessing a sufficient quantity of extracted teeth.

Whilst dental caries occurs naturally *in vivo* on smooth surfaces, and in pits and fissures, the smooth surfaces of teeth have fewer anatomical variations than the occlusal surfaces. Dental caries is therefore easier to measure consistently on smooth surfaces. Also, more methods are available to measure early enamel caries on smooth surfaces.

Human enamel may contain defects, either from previous natural caries challenges or other causes, and is likely to be of variable age and source which gives it a variable composition, leading to variations in test response (Mellberg, 1992). As has been described in Section 1.3, fluoride can be taken into the outer layer of enamel post-eruptively. Significant variation occurs in the amount of fluoride present in the outer layer depending on the exposure to fluoride following tooth eruption into the oral cavity. To eliminate this discrepancy, the outer 500 micrometres of enamel can be removed (as described in Chapter 2) and the enamel polished, resulting in more consistent *in vitro* or *in situ* artificial caries lesion formation (Arends and Gelhard, 1983). The resultant flattened tooth surface as a result of the removal of the outer enamel also facilitates microdensitometric analysis of radiographs of lesions, by reducing the surface curvature of the tooth. However, it should be recognised, that subsurface enamel exposed by previous grinding and polishing will demineralise more readily than the original surface enamel when exposed to a cariogenic challenge (Theuns *et al.*, 1986). Nevertheless, if all of the enamel surfaces used in a study are abraded, the lesions created subsequently should all behave in a more similar manner than if they were created on non-abraded enamel surfaces.

Enamel blocks with lesions formed *in vitro* are the most widely used substrate in *in situ* studies. They allow for greater control of the demineralisation created and such early caries lesions are capable of further de- and re-mineralisation. While it is still not possible to create lesions with identical degrees of demineralisation, this methodology is more controllable than using natural lesions.

Artificial lesions can be created in tooth blocks/slabs *in situ* by placing the sound enamel substrate within the mouth and covering it (e.g. with gauze) to facilitate plaque accumulation, as described in Section 1.7.3. However, these techniques can be time-consuming and it is quicker to develop lesions *in vitro,* then place them *in situ. In vitro*

artificial lesions can also be created in thin tooth sections, rather than tooth blocks. However, care should be taken when using thin tooth sections, because these sections are not robust enough to withstand more than the most careful handling.

1.7.6 Methods of artificial caries lesion creation in enamel

A number of methods have been used to create artificial enamel caries lesions. Each method can result in lesions with different characteristics which may cause them to respond differently to an anti-caries treatment or a caries challenge. These lesions can be classified into several types. Arends and ten Cate (1981) classified them as being: surface-etched; surface-softened, or subsurface lesions. The simplest method is to etch the enamel with orthophosphoric acid, creating a surface-etched lesion which is more representative of erosion rather that caries (Gängler and Hoyer, 1984). Surface-softened lesions are intermediate between subsurface lesions and surface-etched lesions, but can be difficult to quantify as it may be problematic to locate the original specimen surface, which can be lost during handling. However, their use can be justified on the basis that surface-softening occurs as a preliminary stage of natural white spot lesion formation (Arends and Christoffersen, 1986; Øgaard *et al.*, 1986). Naturally- formed white spot lesions are subsurface lesions with well-formed surface layers which form partially as a result of recurrent intra-oral demineralisation and remineralisation episodes. Artificial subsurface lesions are often developed with a surface layer by using a surface protective agent, such as fluoride or other chemicals which alters the transport of tooth mineral ions in and out of the tooth (Mellberg, 1992).

In an attempt to mimic the surface zone of a naturally-created caries lesion, White (1987b) created an lactic acid based demineralising solution containing a polymer called "Carbopol" which enhances the surface zone formation of an artificially-created caries lesion. This method has been used successfully for creation of artificial caries lesions within the Hard Tissue Laboratory at the University of Glasgow Dental School as well as other research units involved in this type of work (Stookey, 1992).

1.7.7 Site of in situ blocks

Enamel blocks can be located in numerous areas of the mouth. Their location relative to salivary flow patterns and facility for plaque accumulation may be important in determination of the severity of the caries challenge, or the amount of demineralisation or remineralisation which occurs subsequently (Mellberg, 1992). The average total salivary

volume produced per day is approximately 570 mL, though this is dependent on the length of time spent awake, eating and sleeping (Dawes, 1987; Watanabe and Dawes, 1988). The average unstimulated whole saliva flow rate is about 0.32 mL/min. It has been calculated that the average salivary film is only 0.1 mm or less in thickness (Lagerlöf and Dawes, 1984). This figure has been used to calculate the salivary film velocity at various sites of the mouth (Dawes *et al.*, 1989). Unstimulated salivary film velocity has been estimated to be greatest at the lower anterior lingual site (7.6 mL/min), with the upper posterior lingual region having a velocity of 6.8 mL/min. Lower velocities were noted at the lower anterior buccal site, (1.0 mL/min), with the upper anterior buccal site (0.8 mL/min), having the lowest salivary film velocity recorded. It has been suggested that a slow velocity of flow of the salivary film over different tooth surfaces will slow the clearance of plaque acids, thereby prolonging the Stephan curve (Lecomte and Dawes, 1987).

With respect to variations in mineral exchange of artificial enamel lesions as a result of the thickness of plaque in *in situ* models, it has been shown that more demineralisation occurs under a thick layer of plaque rather than a thin layer (Essig *et al.*, 1987; Mellberg *et al.*, 1990). Thus it is important to consider with respect to specimen location within the mouth whether plaque presence is likely, and how thick it will be (Mellberg, 1992). Ultimate plaque bulk is also dependent on whether the substrate is mounted proud of the appliance, recessed within the appliance (hence creating a trough for plaque accumulation), or flush with the appliance surface, thereby resulting in a thinner layer of surface plaque (Creanor *et al.*, 1996).

1.8 Measurement of mineral change of the smooth surface carious lesion *in vitro* and *in situ*

In *in situ* studies, it is important to determine how much mineral has been lost or gained from a caries lesion within a tooth specimen and where within the lesion the mineral change has occurred. The techniques detailed below (Section 1.8.1-1.8.6) have been used to measure overall mineral loss from a caries lesion as well as the distribution of mineral throughout the lesion. Furthermore, repeated evaluations with the techniques may enable monitoring of lesion behaviour over time. It is essential to measure these parameters to determine whether de- or re-mineralisation has occurred within a study. It can also be helpful to determine where the mineral loss or gain has occurred within a lesion; for example; from the surface zone or deeper within the body of the lesion.

The ideal method of measurement would be: (a) easy to use; (b) non-technique sensitive; (c) non-destructive to the lesion being measured (therefore allowing longitudinal assessment) and (d) permit, by direct measurement, the mineral loss or gain in a quantitative manner (by percentage volume or weight of mineral).

Several methods have been developed; each method of measurement will be described and discussed below.

1.8.1 Microhardness

Microhardness indentation measurements were used in the first *in situ* studies (Koulourides, 1966). A Knoop or Vickers diamond is positioned on the surface of a caries lesion. A given load is applied to the diamond for a specific time. The diamond indentation-length left on the specimen is measured microscopically in μm. This measurement gives an indirect value of mineral content. If indentation length values increase, then there has been continued loss of mineral; if indentation length values decrease, then there has been a net gain in mineral. These measurements are only applicable for use with enamel. In dentine the change in hardness measurement do not relate to the changes in mineral (Herkströter *et al.*, 1989). Clearly, this method involves some degree of destruction as a different part of the lesion must be measured subsequently.

There are two types of microhardness measurement:

Surface microhardness (SMH), where the indenter load is perpendicular to the polished tissue surface, and Cross-sectional microhardness (CSMH), where the indenter load is parallel to a cut surface through the lesion.

Surface microhardness needs a flat surface, which can be difficult to obtain on a curved tooth, and provides only qualitative information. However, lesion shape, mineral distribution within the lesion, and protein uptake *in situ,* may influence indentation length values. Furthermore, the linear relationship between indentation length and lesion depth is valid only in a limited range of lesion depth values (Arends *et al.*, 1980). White (1987a) reported that for early carious lesions of shallow depth (between 25 and 50 μm), the net remineralisation measured by surface microhardness was highly correlated with remineralisation measured with microradiography ($r = 0.94$; $p < 0.01$).

Cross-sectional microhardness has the advantage that indirectly, the mineral content can be determined quantitatively. In 1983, Featherstone *et al.*, showed that the volume percentage of mineral as determined by microradiography, was directly proportional to the Knoop indentation length (μm), with a correlation coefficient of 0.92 (Featherstone *et al.*, 1983). This allows the mineral profile (volume % of mineral as a function of the distance from the outer surface) to be obtained. The disadvantage is that it cannot measure the 25 μm nearest the edge of the section (Arends and ten Bosch, 1992).

To determine the actual mineral loss or gain, the measurements obtained must be calibrated against a quantitative technique, commonly Transverse Microradiography (TMR) (Featherstone *et al.*, 1983). This technique is relatively straight-forward to perform and, as a result, it is used regularly in research, both within academic institutions and in commercial laboratories (White *et al.*, 1992).

1.8.2 Iodine tests

1.8.2.1 Iodine absorptiometry

This methodology was introduced as a quantitative method for longitudinal study of enamel demineralisation *in vitro.* Iodine (^{125}I) is used to irradiate longitudinal tooth sections, in a similar arrangement to that of LMR (see section 1.8.4.2). The incident and transmitted radiation are measured with a scintillation counter. The amount of absorbed photon radiation is a measure of the mineral per unit area ($kg.m^{-2}$). The change in photon radiation due to a dentine sample placed in the beam is linearly correlated ($r=0.83$) with the

amount of calcium ion lost *in vitro,* as determined by chemical analysis (Almqvist *et al.*, 1988).

This technique provides quantitative mineral loss and gain data, though it is destructive and requires the tooth specimen to be cut into sections prior to analysis. It can also be used to assess both enamel and dentine samples.

Given that the main study described in this thesis will be an *in situ* study, this technique will not be discussed further.

1.8.2.2 Iodine permeability

Iodine permeability (Ip) measurements can give sensitive estimates of initial stages of de- and re-mineralisation in relation to enamel pore volume. Here, tooth specimens are covered with 2M KI solution for 3 minutes, then the KI is wiped off. The window created by the KI solution on the enamel specimen is then covered with water for 40 seconds to allow back-diffusion of iodide. The water is quantitatively removed with an absorbent disc, the iodine content of which is measured with an iodine-specific electrode and is a measure of Ip. This technique was first described by (Bakhos *et al.*, 1977). In 1991, ten Bosch and Angmar-Månsson showed a moderate correlation coefficient (0.55) between the Ip change and the calcium ion change.

However, this technique does not measure mineral loss or change directly and is sensitive to enamel pore blockage. Thus, it is not suitable for use in *in vivo* or *in situ* studies (Zero *et al.*, 1990). Nonetheless, it is a non-destructive technique and therefore can be used to measure on a longitudinal basis. This technique has not been used in recently published studies.

1.8.3 Chemical analysis

For this technique, tooth tissue sampling for analysis of the calcium and phosphate content is undertaken. Microsamples are obtained by dissection, abrasion or micro-drilling of the tooth substance. These microsamples are then dissolved in acid, and the solutions analysed for calcium and/or phosphate content. Calcium is usually determined via atomic absorption spectroscopy, whereas phosphate content is estimated by the formation of a coloured

complex with molybdate. It is, in principle, a good method to quantify de- and re-mineralisation of dental tissues (Arends and ten Bosch, 1992).

Again, this is a single measurement technique (as it is also destructive) and only flat samples can be used. It has been cited as a 'gold standard' technique for determining the mineral dissolved from samples in *in vitro* studies (ten Bosch and Angmar-Månsson, 1991; Huysmans and Longbottom, 2004). However, the disadvantages of the technique are: that only a large gain or loss of mineral is measurable; curved samples cannot be used; and mineral distributions are not measurable in practice (Arends and ten Bosch, 1992). Given these disadvantages, and that many *in vitro* studies are of short duration, a more accurate estimate of the measurement of mineral change is desirable. Therefore, this technique is not routinely used.

1.8.4 Microradiography

It was Thewlis (1940) who first wrote about the technique of mineral quantification by means of X-ray absorption. Since then microradiography has developed into one of the 'gold standards' for measuring mineral change in tissues, with three different microradiographical techniques having been described: transverse microradiography (TMR); longitudinal microradiography (LMR) and wavelength independent microradiography (WIM).

1.8.4.1 Transverse microradiography (TMR)

Transverse Microradiography involves cutting the tooth specimen into thin (80 – 140 μm) plano-parallel slices or sections, perpendicular to the tooth surface. The sections are placed on an unexposed film along with an aluminium step-wedge and irradiated with monochromatic X-rays. The absorption of the X-rays is proportional with the optical density of the film (described in more detail in Chapter 2, Section 2.3.2.2.). Micro-densitometry or image-analysis is then used to calculate the mineral content using Angmar's formula. Mineral content is expressed in volume% or $kg.m^{-3}$ (Angmar *et al.*, 1963b; Mallon and Mellberg, 1985). The two parameters commonly measured are: Integrated Mineral Loss (IML) and Lesion Depth (LD). IML is the integrated difference between the microradiograph of the sample with mineral loss, and that of the sound sample. LD values are determined from the mineral distribution in the microradiograph as

the distance from the outer surface of the specimen to the position where the mineral content is 95% of that in sound tissue (Dijkman *et al.*, 1986).

If a lesion remineralises, both LD and IML decrease, whereas if it demineralises, then both LD and IML values increase (Arends and ten Bosch, 1992). The advantages of this technique are that mineral change can be measured in a quantitative, accurate manner, and that mineral distribution can also be determined. The main disadvantage is that the sample is destroyed by cutting into sections, thus not permitting longitudinal measurement, unless a single section model is used (Creanor *et al.*, 1986; Mellberg *et al.*, 1986; Wefel *et al.*, 1987; Strang *et al.*, 1987). When measuring the outer 10 μm of the sample, edge effects as a result of sectioning, often results in inaccurate values. Furthermore, if there have been any heavy metal ions (e.g. from SnF_2 containing dentifrices) absorbed into the enamel surface, these may distort the image analysis of the microradiograph. Image analysis may be further prejudiced if the thin tooth section is not plano-parallel.

This technique is regularly used to indirectly and quantitatively measure the mineral content of caries lesions and is recognised as a 'gold standard' method (Huysmans and Longbottom, 2004).

1.8.4.2 Longitudinal microradiography (LMR)

To overcome the difficulties of tooth sample destruction, longitudinal microradiography was developed. In this technique, longitudinal tooth sections are prepared parallel to the anatomical tooth surface (approximately 0.3-0.5mm thick). The sections are placed on to unexposed photographic film along with an aluminium step-wedge and the processed films are analysed using image analysis. By so doing, the absolute amount of mineral per unit area can be calculated (de Josselin de Jong *et al.*, 1987). The advantage of this technique is that the mineral content in enamel and dentine can be determined repeatedly, allowing consecutive quantitative changes to be monitored (Zuidgeest *et al.*, 1990). This technique has been used to measure mineral loss through dental caries (de Josselin de Jong *et al.*, 1988) and erosion (Hall *et al.*, 1997). The technique is routinely used to measure change in mineral content.

1.8.4.3 Wavelength independent microradiography (WIM)

The most recent development in microradiography is wavelength independent microradiography (WIM) and was first described by (Herkströter *et al.*, 1990). The method uses high-energy X-rays (≤60Kv) for non-destructive determination of mineral content in whole teeth, and uses a step-wedge with a wavelength-independent ratio to the mass attenuation coefficients of enamel and dentine. It is possible to measure enamel and dentine from 0.3 to 6.0mm in thickness, with or without natural curved surfaces (Arends and ten Bosch, 1992). This is a relatively recently developed technique that has not been used frequently.

1.8.5 Polarised light microscopy

Polarised light microscopic (PLM) analysis shows changes in hard tissue sections by measuring birefringence. Tooth sections are created with a thickness of 80μm. A beam of light shone onto the section, splits into two plane-polarised rays, called ordinary and extra-ordinary rays, respectively. The tooth section is placed between a polariser and an analyser and the mineral crystals exhibit birefringence (ten Bosch and Angmar-Månsson, 1991). The difference between the ordinary ray refractive index and the extra-ordinary ray refractive index is calculated. Polarised light microscopy can be used to determine whether the difference between the refractive indices of the two rays become smaller or larger. If the difference between the ordinary and extra-ordinary ray refractive indices becomes smaller, this indicates that the pore size in the body of the lesion has become smaller indicating remineralisation and vice versa. However, polarised light experiments are difficult to interpret quantitatively (Arends and ten Bosch, 1992). It should be noted that prism shape and orientation, carbonate content, water content and organic content may all have an effect on the total birefringence measured by this technique. It is also a single-use technique as the tooth specimen needs to be sectioned prior to analysis and the use of imbibing agents such as quinoline have an undetermined effect on the mineral changes when such sections are analysed repeatedly at intervals throughout the demineralising or remineralising protocol. It is a relatively common technique.

1.8.6 Optical quantification techniques

For many years, the loss of mineral from enamel and dentine has been known to alter the optical properties or visual appearance of teeth e.g. the 'white spot'. The following techniques are based on this property.

1.8.6.1 Light-scattering

The light-scattering technique is based on the white appearance of a carious lesion and the scattering of light by enamel crystallites in relation to their environment. The light emerging (flux) from specimens as a result of light-scattering is expected to be related to the size and number of crystallites within a unit volume. Ten Bosch *et al.*(1984) and Angmar-Månsson and ten Bosch (1987) used two narrow beams of white light which illuminated a white spot lesion of 0.5 mm diameter, to develop a device called the optical caries monitor. The light emerging from the lesion was collected and measured. Linear relationships were found between the collected light flux and the mineral loss measured by microradiography. The correlation coefficient was 0.9 for lesions created on flat surfaces of enamel.

As this method is non-destructive, it can be used for measuring re- and de-mineralisation on intact enamel samples. However, it is only useful for smooth surface caries lesions. This technique has now been superseded by techniques such as Quantitative Light Fluorescence.

1.8.6.2 Quantitative light fluorescence (QLF)

It has been known for many years that, when a light is shone on a tooth, the tooth will auto-fluoresce (Benedict, 1928; Armstrong, 1963; Spitzer and ten Bosch, 1976). It was subsequently noted that laser fluorescence facilitated detection of early carious lesions on both smooth and fissured tooth surfaces (Bjelkhagen *et al.*, 1982). Further work developed quantification of mineral loss in natural, early caries lesions on smooth surfaces of extracted teeth (de Josselin de Jong *et al.*, 1995). Later, the original argon laser light source was changed to a white (Xenon) light source which was filtered to a wavelength band of around 520 nm. The system using filtered white light was subsequently validated by Al-Khateeb *et al.* (1997).

The basic methodology of the technique (described in more detail in Chapter 2, Section 2.3.1), is that white light is filtered to allow blue light to shine on a tooth containing a lesion. The tooth autofluoresces and the image of this autofluorescence is captured with a camera using a yellow barrier filter in front of the lens. The image is stored and analysed using QLF software which is calibrated to give the area (mm^2) of the white spot lesion as well as, maximum loss of fluorescence (%), and average loss of fluorescence (%), of the lesion when compared with the sound tooth substance which surrounds it. QLF has been validated by comparing it with TMR and LMR (Hafström-Björkman *et al.*, 1992; Emami *et al.*, 1996; Lagerweij *et al.*, 1999). However, concerns have been raised (ten Bosch, 2000) that comparisons should not be made between microradiography and QLF, because microradiography analyses a section of a lesion and QLF analyses the whole lesion, therefore they are not directly comparable.

QLF's advantages are that it is relatively quick to perform and non-destructive; therefore, it can measure a change in caries lesions over time. Following training, it is relatively quick and easy to use, and, once the necessary hardware has been purchased, it is relatively in-expensive to use. Its disadvantages are an inability to determine changes throughout the depth of the lesion, and that data obtained by inexperienced users' may not be reproducible (Pretty *et al.*, 2002). This technique is commercially available for purchase within the United States of America (Clin-QLF, Inspektor Research BV, Amsterdam, The Netherlands).

1.8.7 Summary of methods to measure mineral content over time

The different methods described previously can be summarised in Table 2. This demonstrates that Transverse Microradiography is the only method available that can directly measure mineral content, mineral change and also determine mineral distribution throughout the smooth surface enamel lesion. However, the only way to assess these values repeatedly over time is by using the single section model (as mentioned in Section 1.7.4), and these thin sections are difficult to handle as a result of their delicate nature. In the conventional enamel block method of TMR, the specimen is destroyed during the cutting process, therefore repeated measurements of the blocks are not possible. Therefore measurement of mineral change over time can only be achieved by Quantitative Light Fluorescence (QLF); Longitudinal Microradiography (LMR); Wavelength Induced Microradiograhy (WIM) and Iodine Absorptiometry.

For studies undertaken in this thesis, LMR could have been chosen as a longitudinal method, but evidence suggests that the samples would not have withstood the *in situ* environment (de Josselin de Jong *et al.*, 1987). In addition, WIM is a relatively expensive technique, with limited evidence supporting its use. Another possibility, Iodine Absorptiometry, required specialised radiographic equipment that was not available, whereas QLF hardware and software were available. QLF therefore became the method of measurement of mineral change within the study, with TMR as a 'gold standard' technique of direct measurement of mineral content for comparison (Huysmans and Longbottom, 2004).

Table 2 – Techniques to assess de- and re-mineralisation of smooth surface enamel lesions

Technique		Determine Mineral Content	Determine Mineral Change	Determine Mineral Distribution	Multiple Measurement Over Time Possible
Microhardness	Surface	No	No	Minor	No
	Cross-sectional	Yes (Indirect)	Quantitative (Indirect)	Yes	No
Polarised Light Microscopy		No	Qualitative only	No	No
Chemical analysis		Yes (Direct)	Quantitative (Direct)	No	No
Microradiography	Transverse (TMR)	Yes (Direct)	Quantitative (Integration)	Yes	No (Yes, in single section model)
	Longitudinal (LMR)	No	Quantitative (Direct)	No	Yes
	Wavelength Induced (WIM)	No	Quantitative (Direct)	No	Yes
Iodine	Absorptiometry	No	Quantitative (Direct)	No	No
	Permeability	No	Qualitative only	No	Yes
Optical	Light Scattering	No	Quantitative (Indirect)	No	Yes
	Quantitative Light Fluorescence (QLF)	No	Quantitative (Direct)	No	Yes

This table is modified from (Arends and ten Bosch, 1992)

1.9 Summary and aims

1.9.1 Summary

It has been known since the early part of the twentieth century that fluoride has a beneficial effect on dental caries. It is now recognised this occurs through the action of preventing demineralisation and encouraging remineralisation, with a minor effect of inhibition of the plaque bacteria involved in the caries process. Fluoride can be delivered to the oral cavity in a number of ways and these have been discussed in detail in Section 1.5. Fluoride is commonly found in toothpastes used by a large proportion of the population in developed countries. Since the mid-1970s, the routine use of fluoride toothpastes in such countries has reduced the caries rate significantly (Haugejorden *et al.*, 1997; Newbrun, 1999). However, for children from disadvantaged areas who may have no access to toothbrushes or toothpastes and whose diet is highly cariogenic, additional sources of fluoride may be beneficial. It has been proved clinically, and confirmed in a systematic review of the literature, that increased exposure to fluoride enhances its caries preventive effect (Marinho *et al.*, 2004b).

Ideally, in areas with high caries levels, fluoride should be delivered to the population in the water supply. However, this is not possible in areas that do not have access to a piped domestic water supply or where political pressures prevent this measure from being introduced. When fluoridated water is not available, alternatives must be sought.

One alternative source of fluoride is fluoridated salt. However, its introduction in the UK would be difficult at present, with health professionals campaigning to reduce salt intake as a way of managing hypertension and reducing risk factors for cardiac disease. Fluoridated milk has also been suggested as an alternative, and it has the benefit that it is possible to target children who would benefit most from it. Milk has excellent nutritional value; after all, it has been recommended by the Scientific Advisory Committee on Nutrition (SACN), which advises the UK Department of Health, as the sole food for infants up to the age of six months.

There are a number of research questions regarding the use of fluoridated milk. The topic was reviewed on behalf of the World Health Organisation by Stephen *et al.* (1996). They stated that further research was required to determine, for example: the optimum frequency

of intake of fluoridated milk; the optimum concentration of fluoride to be added to milk; the optimum age of the child to whom the milk should be given and the optimum time for which fluoridated milk should be provided. A recent systematic review of the literature pertaining to fluoridated milk stated that the evidence regarding the efficacy of fluoridated milk was impossible to determine because of a lack of suitably designed studies (Holm, 2002). Finally, much of the research regarding the efficacy of fluoridated milk as a delivery system was obtained prior to the routine use of fluoridated dentifrices. Thus, the evidence for benefits from the concomitant use of fluoridated milk with a fluoridated dentifrice is lacking.

1.9.2 Aims

One of the main aims of the work reported in this thesis was to use an *in situ* model to investigate the effect of milk, with or without the addition of fluoride, on the remineralisation and demineralisation of artificial carious lesions. The design of the study also included investigation of the effect of using fluoridated dentifrice to simulate toothbrushing twice-daily, in addition to the milk intake.

A further aim of the study was to compare the effect of fluoridated milk consumption with a low concentration of fluoride, three times per day, to that found with use of a higher concentration of fluoride in milk consumed only once per day; both milk regimes having the same overall fluoride content. The effect of increasing frequency of fluoridated milk intake had not been studied previously, and was one of the deficiencies in the evidence highlighted in the fluoridated milk monograph by Stephen *et al. (1996),* as described above.

To determine whether the fluoride was effective in preventing further demineralisation and promoting remineralisation, changes in mineral content of the artificial lesions were measured. The relatively new technique of QLF is non-invasive and can be used to measure mineral change longitudinally, by taking repeated images of the carious lesion at regular intervals. The study aimed to determine the feasibility of using QLF with a novel *in situ* complete denture model. Furthermore, a specific study addressed the question of repeatability when using the QLF technique. Having established the suitability of QLF to measure mineral change in a repeatable manner, interpretation of the results obtained using

the QLF method were compared with the interpretation of results obtained with TMR, which has been recognised previously as a 'gold standard' technique.

Using an *in situ* appliance, it is possible to place multiple caries lesions at different sites within the subjects' oral cavity. The final aim of this work was to compare the response of caries lesions at different sites to exposure to the experimental protocols, and to determine whether the results supported previous work relating to the site-specificity of caries.

Therefore, in summary, the following research questions were developed:

- Could a given operator both obtain and analyse an image in a repeatable manner with the available QLF machine?

- Was there an effect of using fluoridated dentifrice on the mineral content of enamel lesions?

- Were there any effects of consumption of fluoridated milk on mineral content of enamel lesions a) with or b) without the effect of fluoridated dentifrice?

- Were there any differences in the change in the mineral content of enamel lesions at different intra-oral sites?

2.0 Materials and Methods

2.1 Introduction

This chapter describes the methods involved in selecting suitable teeth for use in the study, preparing tooth blocks and creating artificial lesions in enamel. It also describes the techniques used to monitor and measure the mineral content of the artificially-created enamel lesions. The specific methods relating to the investigation of repeatability of QLF measurements and the main *in situ* study are detailed in Chapters 3 and 4, respectively.

All experiments reported in this thesis were completed using blocks of human enamel with underlying dentine and, for the reasons detailed in Section 1.8, were measured using Quantitative Light Fluorescence (QLF). In addition, for the main study described in Chapter 4, Transverse Microradiography (TMR) was also used.

2.2 Tooth preparation

2.2.1 Tooth selection

Extracted human teeth were collected from general dental practices around Scotland and from the Oral Surgery Department of Glasgow Dental Hospital & School (fluoride level in domestic water supply <0.04ppm F^-). One thousand suitable molar teeth were selected from those collected and stored in 0.12% saturated solution of Thymol prior to use. Suitable molar teeth were defined as those with no caries or minimal restorations, devoid of obvious cracks or other defects and which were of a suitable shape to cut out a tooth block of enamel.

2.2.2 Preparation of tooth blocks

Initially, soft tissue debris was removed from each tooth using a discoid excavator (Dentsply Ash Instruments, Devon, UK). The teeth were then cleaned with a slurry of pumice and water, using a rubber cup in a slow-speed dental handpiece. The buccal and lingual surfaces of the molar teeth were abraded to remove the fluoride-rich outer 300-500 micrometres of enamel thereby exposing a more reactive surface for lesion creation (Weatherell *et al.*, 1973; Weatherell *et al.*, 1977; Stookey, 1992). This was achieved using a high-speed diamond veneer bur (LVS-2, Set 4151, Laminate Veneer System, Komet Medical, Konstanz, Germany) to create depth grooves of 300 micrometres in the natural enamel surface. The grooves were then smoothed using a high-speed diamond chamfer bur to create a flat smooth surface (LVS-4, Set 4151, Laminate Veneer System, Komet

Medical, Konstanz, Germany). The enamel surface was polished with Sof-lex ™ Pop-on polishing discs (3M ESPE, St Paul, MN, USA) of decreasing abrasiveness.

The teeth were then cut into blocks, using the Labcut 1010 machine (Agar Scientific Ltd, Cambridge, UK). Each tooth was fixed to a chuck at the desired orientation with sticky wax which was allowed to harden. The chuck was attached to the Labcut 1010 machine and the machine switched on. The specimen was gently brought down on to the blade until it started to cut, and then allowed to continue until the cut was complete. The cuspal enamel was removed first, followed by the roots above the amelo-cemental junction (Figure 2). The abraded buccal and lingual surfaces were then cut from the tooth, creating a block, approximately 2-3 mm in depth, with dentine to support the overlying enamel. The shorter mesial and distal surfaces of the tooth were cut-off to construct enamel slabs approximately 3-4 mm long x 3-4 mm wide x 3 mm deep. These were reserved and used to collect plaque samples for microbiological analysis (for a concurrent microbiological study described later).

Figure 2 – Diagram of how the molar teeth were cut into blocks

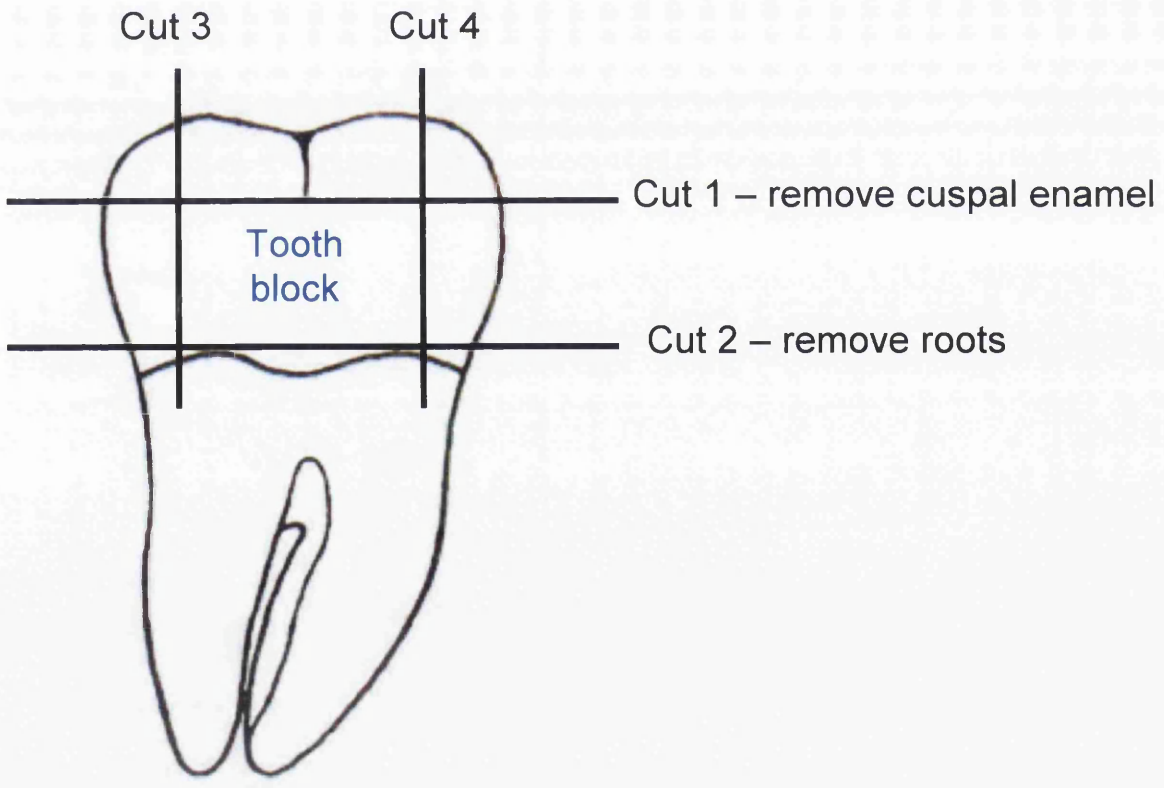
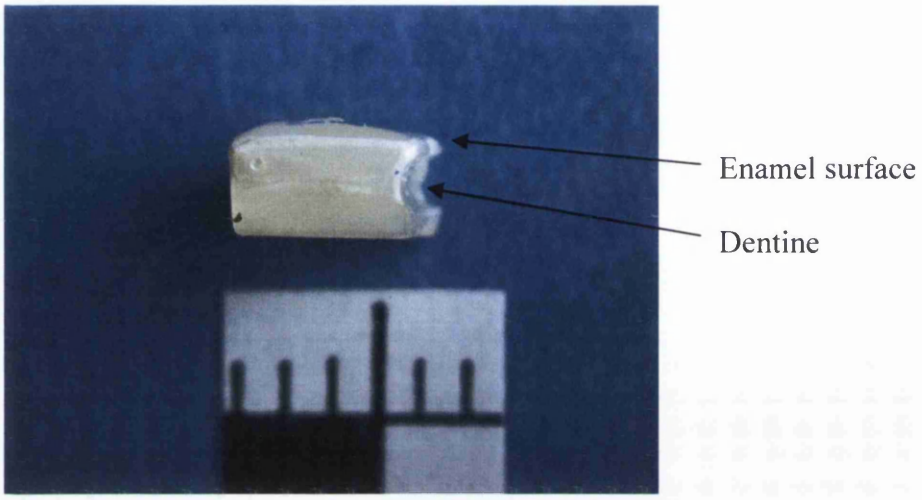


Diagram modified from Wheeler's Dental Anatomy, Physiology and Occlusion, p324, 8th Ed. Saunders

The buccal and lingual surfaces were then tidied up on a grinding wheel to produce tooth blocks approximately 6 mm long x 4 mm wide x 3 mm deep. To orientate the blocks, a groove was cut in the upper edge of the slab using a diamond bur in a high-speed handpiece. This groove denoted the top surface of the block and was used for orientation (Figure 3) when the studies described in this thesis were carried out and during subsequent microradiography and image analysis.

Figure 3 – Photograph of a prepared tooth block with a notch for orientation. (Scale in mm)



2.2.3 Disinfection of the tooth blocks

Prior to use in the mouth, all tooth blocks were disinfected using a 'prion disinfection protocol'. This protocol was provided by Professor William Wade, Professor of Oral Microbiology, King's College Dental Institute, University of London, UK. The purpose of the protocol was to try and eliminate any risk of prion transfer, or the transmission of the Transmissible Spongiform Encephalopathies (TSEs) to subjects taking part in the studies described later in this thesis. In particular, concern was expressed over the possible transmission of Bovine Spongiform Encephalopathy (BSE) which may cause variant Creutzfeldt - Jakob disease (vCJD) in humans (Bruce *et al.*, 1997).

The protocol used was a proven anti-Scrapie protocol. Scrapie is another prion disease, similar to BSE, but occuring in sheep. At the time the studies in this thesis were conceived, this was the most progressive prion disinfection protocol available. The prion disinfection protocol involved:

a) storage of the prepared tooth blocks in 10% Formalin for 7 days,

b) washing in de-ionised water,

c) storage in 5% Sodium Dodecyl Sulphate for 24 hours and,

d) washing three times in phosphate-buffered saline (PBS).

As no data were available at the time to determine whether this treatment would alter the potential for artificially-created caries lesions to demineralise and remineralise, a pilot study was undertaken. This study will not be discussed in detail in this thesis. However, a summary of the work was presented to the British Society of Dental Research in 2001 and can be found in Appendix 1.

2.2.4 Varnishing and fissure sealing

Prior to the creation of artificial caries lesions, prepared tooth blocks were covered with either acid-resistant varnish or fissure sealant, leaving exposed a 4 x 3 mm window on the abraded and polished enamel surface. Initially, acid-resistant varnish was used as this had been successfully employed in previous *in situ* studies at the University of Glasgow Dental School. However, it was later discovered that the use of acid-resistant varnish was not always sufficiently robust to survive a six-week intra-oral period, in this particular *in situ* model; therefore an alternative methodology was developed. Both methods are described below.

In the first method, on removal from the prion disinfection protocol, two coats of clear acid-resistant varnish (Max Factor Diamond Hard Nail Lacquer, Crystal Clear 101, Procter and Gamble UK, Weybridge, UK) were applied free-hand on all surfaces of the tooth block leaving an exposed window 3 x 4 mm on the abraded and polished enamel surface.

For the method involving fissure sealant, following prion disinfection, blocks were dried and a 4 x 3 mm piece of adhesive tape (Letraline 1/32 x 650 blue flex tape, Letraset, USA) was placed in the centre of the abraded and polished enamel surface. A 37% orthophosphoric acid gel (Scotchbond etchant, 3M ESPE dental products, St Paul, MN 55144, USA) was applied to the exposed enamel around the edges of the tape for 30 - 40 seconds prior to thorough washing and rinsing with tap water and drying with compressed air. The etched enamel was then coated with opaque fissure sealant (Estiseal® LC, Kulzer,

Germany) and cured with a conventional halogen light (QHL75™, Dentsply Detrey GmbH, Konstanz, Germany) for 40 seconds. The adhesive tape was removed along with any traces of adhesive. Traces of adhesive were removed with a microbrush® (Dungarvan, Co. Waterford, Ireland) soaked in 95% ethanol and the task was performed with the aid of a dissecting microscope. After the abraded and polished enamel surface was coated with fissure sealant, the remaining sides of the prepared tooth block were covered with acid-resistant varnish as described previously.

2.2.5 Artificial caries lesion creation

The varnished and fissure sealed tooth blocks had artificial caries lesions created in the exposed enamel window by placing the lesions in a lactic acid-based demineralising solution containing a polymer (Carbopol, B & F Goodrich and Company Chemical Group, Avon Lake, Ohio, USA) to ensure the preservation of an intact surface. The demineralisation solution was manufactured following the protocol described in Appendix 2 (White, 1987b). For every one square millimetre of exposed enamel, two millilitres of demineralising solution was used to create the lesion. In this way the ratio of exposed enamel area to volume of demineralisation solution was kept constant. The lesion size was approximately 4mm x 3mm, giving a lesion area of 12 mm^2. Therefore, approximately 24 mL of solution was required for each tooth block.

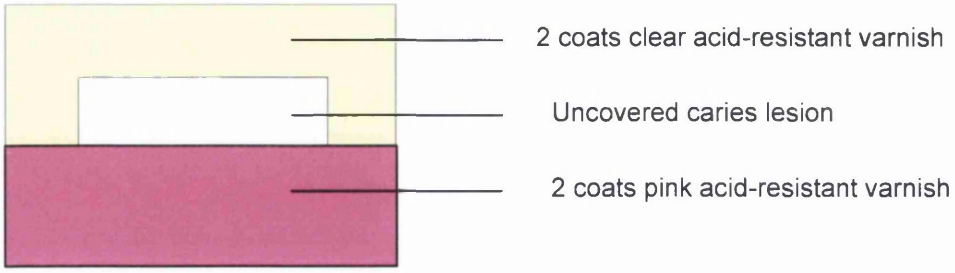
Each tooth block was placed in a numbered tube, containing the demineralisation solution, for seven to fourteen days. The tubes were incubated at 37°C and lesion development checked every 24-48 hours. Once significant demineralisation, without surface cavitation, was evident on the clean, dry tooth surface, verification was made via Quantitative Light Fluorescence (discussed in Section 2.3.1). A minimum Average Fluorescence Loss of -13% was used to define adequate demineralisation. This value was chosen as, potentially, both further demineralisation and remineralisation could be demonstrated by QLF from this degree of demineralisation. No maximum Average Fluorescence Loss was defined, though in practice, values greater than -30% were seldom achieved after 7-14 days of demineralisation.

2.2.6 Creation of lesion controls

Once an artificial caries lesion had been created (as described in Section 2.2.5), one half of the lesion was then covered with two coats of pink, acid-resistant varnish (Sugar Pink,

Proctor and Gamble UK, Weybridge, UK), while the other half was left exposed (Figure 4). The covered (varnished) area of each tooth block thus acted as an *in situ* lesion control.

Figure 4 - Diagram of tooth specimen covered with acid-resistant varnish



For blocks coated with fissure sealant, following lesion creation, the lower half of the created lesion and the adjacent fissure sealant was covered with more fissure sealant and cured (Figure 5) using the method described in Section 2.2.4 above.

Figure 5 - Photograph of tooth block containing a caries lesion with the lower half of the tooth block covered with fissure sealant and the upper half containing the exposed caries lesion surrounded with fissure sealant prior to use in the study. (Scale in mm)

2.3 Mineral change assessment - detailed methodology

Two methods were chosen to measure changes in mineral content: 1) Quantitative Light Fluorescence (QLF) and 2) Transverse Microradiography with Image analysis (TMR).

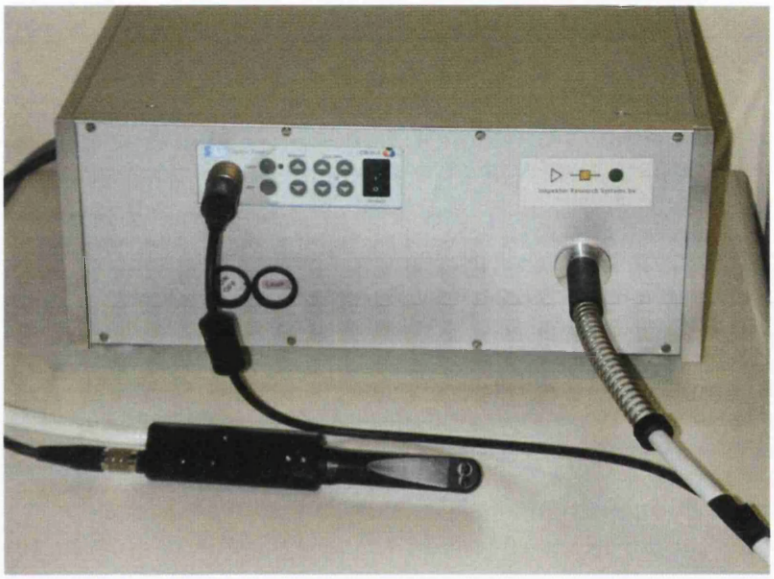
QLF was used in all of the experiments described in this thesis, while TMR was used only in the main study, described in Chapter 4.

2.3.1 Quantitative light fluorescence (QLF)

Quantitative Light Fluorescence is a non-invasive technique that may be used to detect and monitor loss and gain of mineral in caries lesions.

The QLF system comprises hardware (Figure 6) that captures an image of the tooth, and software that allows manipulation and analysis of that image.

Figure 6 - Photograph of QLF hardware. The black coloured handpiece in the front of the picture contains the light source, CCD micro-video camera and prism.



The hardware consists of a white light from a Xenon arc lamp which is filtered to produce blue light with a peak wavelength of 370 nm. This was used to illuminate the tooth surface, which then auto-fluoresces. In the studies described in this thesis (when the tooth blocks were not present in the test dentures), the tooth blocks containing the lesions were mounted in Blu-Tack (Blu-Tack, Bostick-Findley Ltd., Stafford, UK) on a lab-stand. The lab-stand was moved up and down in a vertical direction to focus the lesion image on the computer screen. The focal length of the Glasgow Clin-QLF camera was 14 mm. A colour charge-coupled device (CCD) micro-video camera (Panasonic WV-KS 152), with a yellow-high pass filter of wavelength 520 nm placed in front of the camera, was used to capture the fluorescent image. Any light with a wavelength of less than 520 nm was thereby excluded.

This also eliminated any reflection from the tooth (Al-Khateeb *et al.*, 1997). The captured image was viewed on a computer monitor for manipulation and analysis. When viewing the captured image, sound tooth structure appeared yellow/green in colour, while the artificial caries induced light scattering to produce a darker area in the centre of the tooth block. After analysis, the images and analysis results were stored on a computer.

The software (QLF version 1.97i, Inspektor Research Systems BV, Amsterdam, Netherlands) allowed storage, processing and analysis of the image. Three parameters were measured: lesion area - QLF^{AREA} (mm^2); maximum fluorescence loss - QLF^{MAX} (% Fluorescence loss); and average fluorescence loss - QLF^{AVER} (% Fluorescence loss). The fluorescence loss of the caries lesion was calculated by comparing it to the surrounding sound enamel, as described by de Josselin de Jong *et al.*(1995). The fluorescence radiance of surrounding sound enamel was taken to be 100% and the QLF software simulated reconstruction of sound enamel fluorescence radiance values at the lesion site through two-dimensional linear interpolation of sound enamel values around the lesion. The percentage change in fluorescence was then calculated by comparing the difference between the actual and reconstructed fluorescence images, with caries being defined as a difference larger than 10% with respect to the reconstructed values of sound enamel (de Josselin de Jong *et al.*, 1995).

QLF^{AREA} describes the two-dimensional size of the lesion over the surface of the tooth block. It was determined by counting the total number of pixels which the QLF program had defined as being dental caries. In turn, the number of pixels was used to calculate the lesion area measured in square millimetres (mm^2). QLF^{MAX} describes the maximum reduction in pixel intensity compared with the fluorescence of sound enamel. It was described as a percentage loss in fluorescence. It is "the largest difference between the actual and reconstructed radiance that is found in the lesion". QLF^{AVER} describes the mean reduction in intensity across the lesion and was also measured as a percentage (% loss in fluorescence). QLF^{AVER} is "the mean of all of the differences between the actual and reconstructed radiance values that are found within the lesion area" (de Josselin de Jong *et al.*, 1995).

The comparison of the caries lesion with the surrounding sound enamel is undertaken by placing a rectangular analysis patch over the image of the lesion. The edges of the patch are placed over sound enamel, close to the border with the lesion. Given that the patch is

rectangular in shape, it therefore had four borders. These borders can be moved or "stretched" into a position so they are placed over adjacent sound enamel. If a border cannot be placed on sound enamel, e.g. if a caries lesion was adjacent to a gingival margin, then this border is "turned off". In fact, with the QLF software (QLF version 1.97i), any combination of the four borders can be switched off, as long as one border of the rectangular patch is "switched on" and placed over sound enamel. For most studies described in this thesis, where QLF was used, three borders out of the four in the rectangular analysis patch were "switched on". The reason for this was one half of each lesion was covered with either acid-resistant varnish or fissure sealant and the border of the rectangular analysis patch adjacent to this had to be switched off, as it was not overlying sound enamel.

Once QLF values for the each of the three parameters: lesion area; maximum fluorescence loss, and average fluorescence loss, were calculated, they were exported from the programme and imported to a spreadsheet computer file (Excel, Microsoft® Corporation, USA). Data were exported in this manner then downloaded to a database (Access, Microsoft® Corporation, USA) for storage, prior to statistical analysis.

2.3.2 Transverse microradiography (TMR) and image analysis

This technique has been described as one of the "gold standards" for measuring mineral content of tooth substance, and has been used extensively in measuring de- and re-mineralisation in dental caries research (ten Bosch and Angmar-Mansson, 1991; Huysmans and Longbottom, 2004). The two parameters measured using TMR during the study were: integrated mineral loss (IML), which is the integral of the change in mineral content over a known distance from the surface of the tooth (vol%mineral.μm), (ten Bosch and Angmar-Mansson, 1991); and lesion depth (LD), which was measured in micrometres (μm).

Microradiography was used at the end of each experimental protocol described in the main study (Chapter 4).

2.3.2.1 Tooth preparation prior to microradiography

Microradiography was performed on very thin tooth sections, approximately 120-140 μm thick, cut from the tooth blocks. Blocks of teeth were embedded in Epoxy resin (Epofix, Struers, Glasgow, UK) prior to sectioning. Sections were cut using the Accutom-50 machine (Figure 7) to an approximate thickness of 200 μm.

Figure 7 - Accutom-50 machine used to cut the tooth blocks into 200 µm sections

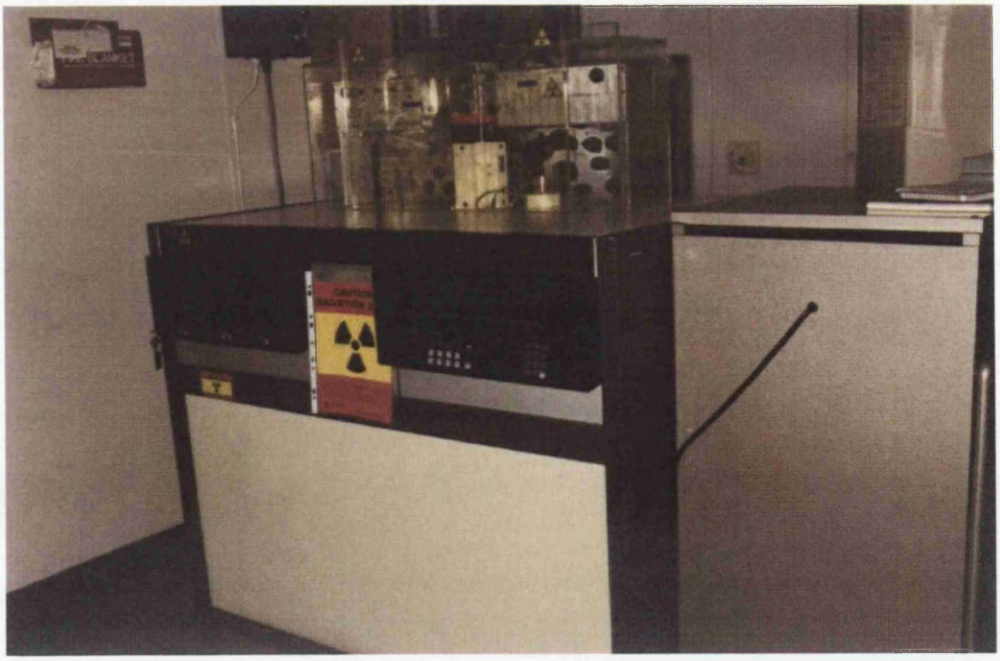
Each section was examined on a glass slide under a microscope at x30 magnification. When a lesion was seen clearly as a dark rectangle at the enamel surface, the section was set aside for further preparation. From each tooth block, two such sections were chosen to be ground and microradiographed. These sections were ground and polished from a thickness of 200 µm to 120-140 µm prior to microradiography. This was achieved by rubbing the sections on a round glass slab using a fine abrasive slurry (1200 grade, White Bauxlite Honing Abrasive [Al_2O_3], Raymond Lamb, London, England). Any ridges present on the tooth section from the cutting process, were thus removed. The thickness of a section was confirmed by measuring a number of points along the edge of the enamel surface at regular intervals, using a digital micrometer (Mitutoyo, Tokyo, Japan). These measurements also gave an estimation of the planoparallelity of the section. If between 120-140 µm, the section was labelled using a soft pencil and stored in a 0.12% Thymol solution. One of the tooth sections was then randomly chosen and prepared for microradiography and image analysis.

In preparation for microradiography, the tooth sections were washed in cold running water, mounted in cling-film and labelled before being placed in a microradiograph cassette. The cassettes were loaded in a dark room, with the clingfilm-mounted sections, unexposed high resolution film (Holographic film SO-181, Eastman Kodak Company, Rochester, New York, USA) and an aluminium step-wedge. The step-wedge was comprised 25 μm-thick sheets of Aluminium foil, making a stepwedge with 12 increments, from 0 to 275 μm. The cassettes were sealed with light-excluding tape.

2.3.2.2 Microradiography and film development

Cassettes were placed at a source to specimen distance of 300mm from a Cu(Kα) Ni-filtered X-ray source (Diffractus 582, Enraf Nonius, Delf, Holland) operating at 30mV and 20 kV for 20 minutes (Figure 8). Following exposure to X-rays, the cassettes were opened in a dark room and the exposed film was processed under standard conditions. These standard conditions were: all solutions at 21°C; 1 minute in clearing solution to remove the silver halide film backing; 2.5 minutes in the developing solution; 1 minute wash in water; 5 minutes in the fixing solution and then 10 minutes under running cold water. The developed films were then placed in a drying cabinet at 37 °C for 24 hours prior to analysis.

Figure 8 - Photograph of the Cu(Kα) x-ray Diffractus 582 equipment



Once the microradiographs were developed, the tooth sections were removed from the cling film and stored in a 0.12% saturated solution of thymol. Any microradiographs

deemed to be unsatisfactory as a result of a processing fault, were retaken. Microradiographs containing lesions with surface zones that were no longer intact were re-taken using another section that had been cut from that particular tooth specimen, if one was available. These re-taken microradiographs were analysed and the values recorded were substituted in place of those from the damaged tooth specimens.

2.2.2.3 Image analysis

The hardware used to analyse the images consisted of a CCD camera attached to a Leitz transmission microscope (Leitz-Wetzlar, Germany) with a stabilised power source to ensure constant illumination. The microscope was linked to a computer (Figure 9). Images were analysed at x 40 magnification in transmitted light.

Figure 9 - Photograph of image analysis hardware used to measure the mineral content of the microradiographs in TMR

Image analysis involved the assessment of the absorption of monochromatic X-rays by the tooth sections. X-ray absorption of the tooth sections was proportional to the resultant optical density of the film and was calculated using the known properties of X-ray absorption of aluminium from the aluminium step-wedge described previously. Mineral loss was calculated using the TMR analysis software version 1.25e (Inspektor Research Systems BV, Amsterdam, The Netherlands), using Angmar's formula (Angmar *et al.*, 1963a). This provided a quantitative measurement of mineral content at specific points

within the section. In addition, information about the mineral distribution within the lesion from the surface towards the amelodentinal junction was also determined.

The camera used a greyscale with 256 increments (0-255). As described above, the aluminium step-wedge had 12 steps of 25 μm increments from 0 - 275 μm. The correct illumination for each film was set using the thickest step on the step-wedge (275 μm). This was determined by adjusting the lamp current until illumination was deemed satisfactory by the computer software. This was indicated by a green light on the computer screen and was in the region of 2152 mA current supplied to the light source. The camera was then calibrated using the remaining steps of the aluminium step-wedge image. Each step was scanned to record an average grey value. The average grey values were plotted against step-wedge thickness. A fourth order polynomial curve was fitted to the data. A good step-wedge produced a curve with a linear central portion with flattened regions at the thinner and thicker parts of the step-wedge. It was then possible to equate any grey level on the film to a known thickness of aluminium. Angmar's Equation (Angmar *et al.*, 1963a) converts the grey levels on the film of a known thickness of aluminium to a known volume % of hydroxyapatite. An image of a stepwedge curve and a plot of numerical loss against depth from the TMR programme is given in Figure 10 below.

Once calibration using the step-wedge had taken place, the images on each film were scanned and then analysed with the TMR software in the following manner:

a. The image of the lesion surface was aligned parallel to the y-axis of the computer screen. This ensured any calculations of mineral content were made perpendicular to the surface of the lesion;

b. The region of interest was scanned, ensuring that the area was as broad as possible and that its depth extended from the surface of the section into sound tooth enamel;

c. A plot was then constructed of average volume % mineral across the breadth of the scan (y-axis) against depth from the surface of the lesion (x-axis);

d. Zero% mineral content was defined on the y-axis using a moveable cursor on the screen;

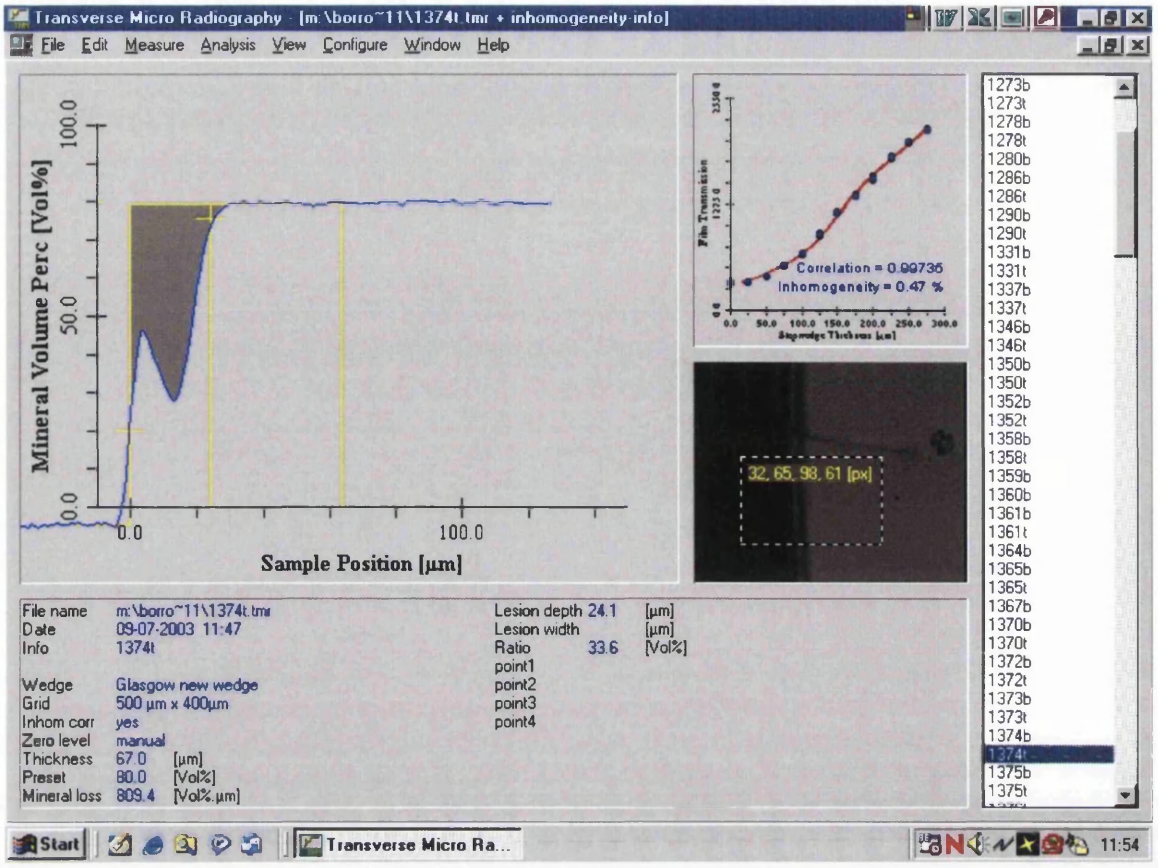
e. 100% mineral content of sound enamel was defined on the y-axis using a moveable cursor on the screen;

f. The start of the lesion was suggested by the computer programme as that point on the x-axis where the mineral content was 20% by volume mineral on the y axis;

g. The depth of the lesion was defined as a point on the x-axis where the mineral content reached 95% of sound enamel (Mallon and Mellberg, 1985). This was indicated by the computer programme and the x-axis co-ordinate determined by the operator (95% of sound enamel tissue, when sound enamel is defined as 80% mineral by volume);

h. For the purposes of calculation of mineral loss it was assumed that sound enamel contained 80% by volume mineral. The y-axis was adjusted accordingly and the TMR software calculated 'Integrated Mineral Loss' and 'Lesion Depth'. Integrated mineral loss was the difference between sound and carious mineral by integration. An image was produced on the computer screen as shown in Figure 10.

Figure 10 – Image of TMR software output, showing step-wedge curve (top right), TMR image (bottom right) and TMR graph (top left) with the IML highlighted in dark grey.



The data for each individual tooth section following analysis were then exported into a spreadsheet computer programme (Excel, Microsoft® Corporation, USA). These data were then imported into a database (Access, Microsoft® Corporation, USA) for storage and manipulation prior to statistical analyses.

3.0 The repeatability of QLF

3.1 Introduction and aims

Quantitative Light Fluorescence (QLF) is a relatively new caries diagnostic method which can be used to measure quantitatively the change in mineral content of teeth. It is non-invasive and is employed to evaluate the difference in scattering of fluorescent light between sound and demineralised tooth structure. This method has been validated both *in-vitro* and *in-vivo* (Chapter 1, Section 1.8.6). However, it was essential to examine whether a given QLF operator (i.e. AJN), who would be capturing and analysing images for studies such as those described in this thesis, could use the method repeatedly.

To allow comparison of QLF findings, both within and between different studies and machines, the QLF technique must be reliable, repeatable and reproducible. Reliability, is the noun used to describe the ability of something to be relied upon (Concise Oxford English Dictionary, 2002) and is often used interchangeably in statistics to mean repeatability or reproducibility. Repeatability can be defined statistically as "*the extent to which repeated measurements by the same observer in identical conditions agree*" (Petrie and Sabin, 2000). Reproducibility, however, can be defined as "*the extent to which the same results can be obtained in different circumstances, e.g. by two methods of measurement, or by two observers*" (Petrie and Sabin, 2000).

Despite the abundance of reliability studies published to date, there is still no agreement in the levels of reliability required for a method or instrument to be deemed acceptable. Landis and Koch (1977) proposed a series of cut-off levels to describe levels of reliability. However, these values are not based on any objective criteria and are often criticized (Dunn, 1989; Shrout, 1998). Shrout (1998) published modified, more stringent, cut-off levels to those proposed by Landis and Koch (1977) and used the following adjectives for describing levels of repeatability or reproducibility:

(0.00 – 0.10) – virtually none;

(0.11 – 0.40) – slight;

(0.41 – 0.60) – fair;

(0.60 – 0.80) – moderate;

(0.81 – 1.0) – substantial.

The QLF machine available in Glasgow was a prototype with a white-light Xenon arc lamp, rather than a laser, as the light source (for methodology see Section 2.3.1). It was thus necessary to confirm the repeatability of use of this QLF machine, particularly as the main study in this thesis relied on taking repeated images of the same lesion, by the same observer, on a longitudinal basis.

Reproducibility and repeatability of Quantitative Light Fluorescence has been studied previously (Tranaeus *et al.*, 2002; Pretty *et al.*, 2002), and has included both intra-examiner and inter-examiner assessment. The statistical tests used to determine repeatability and reproducibility were t-tests, ANOVA and intra-class correlation coefficient statistics (ICC).

Tranaeus *et al.* (2002) investigated *in vivo* repeatability and reproducibility of the QLF method with respect to three variables; QLF^{AREA}, QLF^{MAX} and QLF^{AVER}. They examined both the image-capturing stage and the analytical stage using two different light sources, the Xenon arc lamp (as described in Chapter 2) and the former light source, the argon ion laser system. For this stage, intra-examiner repeatability was not investigated formally. However, for the image capture stage, inter-examiner reproducibility amongst the three analysts in the study produced intra-class correlation coefficients (ICC) of between 0.95 and 0.98 for the three QLF parameters. Of the three analysts, two were described as "experienced" and the other as a "novice" and it was noted there was a statistically significant difference between the "novice" and one of the "experienced" examiners in repeatability of image capture. However, there was no evidence of consideration of the effect of potential bias between the different image capture occasions, though the results obtained were "substantial" when described by the adjectives proposed by Shrout (1998).

For the analytical stage, Tranaeus *et al.* (2002) showed intra-examiner repeatability values (ICC) of between 0.93 and 0.99, and inter-examiner reproducibility of between 0.95 and 0.99; again these results could be described as "substantial". However, Pretty *et al.* (2002) suggested that repeatability and reproducibility appear to be dependent on the level of examiner experience. They investigated the repeatability and reproducibility of QLF image analysis amongst 10 examiners of varying experience, and reported inter-examiner reproducibility to be significantly different between novice and experienced examiners; the latter being superior.

Both the Tranaeus *et al.* (2002) and Pretty *et al.* (2002) studies demonstrated the importance of ensuring that an examiner is trained in the Quantitative Light Fluorescence method of image capture and analysis, to ensure any differences detected are attributable solely to the mineral change within the lesion. They also both noted a significant difference between novice and experienced examiners with regard to intra-examiner repeatability. The novice examiners had difficulty in consistently repeating their QLF analysis, and there were statistically significant differences determined for their image capture and analysis when compared with the more experienced examiners.

A study was designed, therefore, to assess the repeatability of QLF measurements made by an operator (AJN) who would be capturing and analysing the images in the studies reported within this thesis. Statistical advice was sought with regards to study protocol and statistical analysis. The study was designed to answer the following questions:

- Could the operator (AJN) obtain a repeatable image with the QLF machine?

- Could the operator (AJN) analyse the same image in a repeatable way?

3.2 Materials and methods

3.2.1 Tooth specimen preparation

Extracted caries-free human premolar teeth, 10 in total, were sectioned longitudinally through their mesial and distal surfaces using a water-cooled rotating diamond wheel (Labcut 1010, Agar Scientific Ltd, Cambridge, UK) to produce 20 specimens. These were then cleaned, abraded and polished as described in Section 2.2.2.

Each tooth specimen was lettered (a-t) and covered with two layers of acid-resistant varnish (Max Factor Diamond Hard Nail Lacquer, 549 Passion Red, Proctor and Gamble, Weybridge, UK) leaving an exposed area of approximately 4 mm x 3 mm in the middle of the buccal or lingual surface. All specimens were then placed into a lactic acid-based Carbopol demineralisation gel (White, 1987b), (Appendix 2) for 48 hours, to create an artificial caries lesion within the exposed area. The acid-resistant varnish was then removed with acetone and alcohol, to leave an artificially-created caries lesion surrounded by intact enamel on either the buccal or lingual tooth surface.

All tooth specimens were stored in a 0.12% saturated thymol solution, prior to and during the study, to prevent desiccation of the artificial caries lesions. Before image capture, each specimen was dried for five seconds with compressed air and left to air-dry for 30 minutes. This was to reduce the possibility of a change in QLF values as a result of lesion desiccation, as previously noted by several authors (Al-Khateeb *et al.*, 1998; van der Veen *et al.*, 2003).

3.2.2 Image capture and analysis

The QLF image capture and analysis were accomplished using hardware described previously (Chapter 2, Section 2.3.1), and QLF software (version 1.97i, Inspektor Research Systems BV, Amsterdam, The Netherlands).

Image capture

(a) To answer the question: "*Could the operator (AJN) obtain a repeatable image with the Glasgow QLF machine?*"

For this purpose, the 20 tooth specimens containing artificially-created caries lesions were labelled "a-t" and had images captured three times, approximately one week apart, in a random order. The randomisation was achieved in a simplistic manner, by pulling the corresponding letter out of an envelope. The 60 images were then analysed once by the same examiner, using the Glasgow QLF software, in a similarly generated random order, with values recorded for the three QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER}.

Image analysis

(b) To answer the question: *"Could the operator (AJN) analyse the same image in a repeatable manner?"*

Here one image was captured of each of the 20 tooth specimens containing an artificially-created caries lesion. Each image was then analysed on three separate days (20 images, analysed x3), in random order (as described above), by the same examiner using the Glasgow QLF software, with values recorded for the three QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER}. All analyses were completed within a two-week time period.

Statistical analysis

The statistical analysis used to determine the repeatability of the QLF technique was chosen in order to take into consideration the potential effect of systematic bias, which it is

important to be aware of, as it is possible that there could be bias between the image capture occasions or days.

Repeatability was determined using the "components of variance" technique, with the components being estimated using, "repeated measures analysis of variance", for each of the QLF parameters. These components were: the order; the image capture or day and the tooth specimen. The effect of the 'image capture' or 'day' was investigated to examine for any systematic bias. Data were plotted and components of variance estimated, and the repeatability-estimate derived from these, for each of the three QLF parameters. These estimates were compared with the levels proposed by Shrout (1998).

3.3 Results and analysis of repeatability of QLF

Image capture

The results of image capture for these 20 tooth specimens for the three QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER} are displayed in Figure 11, Figure 12 and Figure 13 respectively. Each coloured line represents one of the twenty tooth specimens for each of the three image capture days. In the ideal situation, where every image of a given tooth specimen is the same, parallel, straight lines would be displayed.

Examining the plots in Figure 11, Figure 12 and Figure 13, it would appear from the increased number of almost straight lines, that QLF^{MAX} (Figure 12) is the most repeatable of the QLF parameters captured for these 20 tooth specimens.

Figure 11 - Plot of QLF^{AREA} versus day for three separate images captured

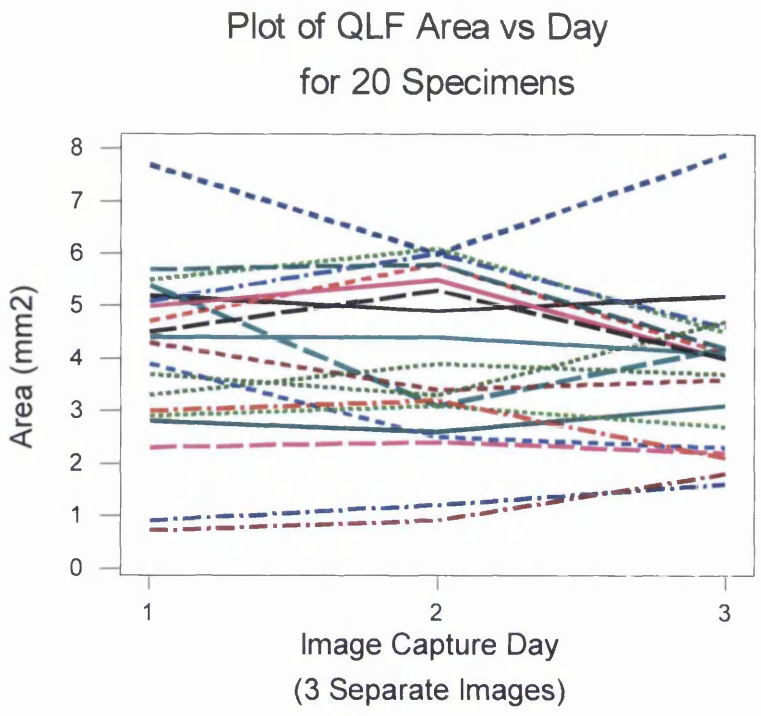


Figure 12 - Plot of QLF^{MAX} versus day for three separate images captured

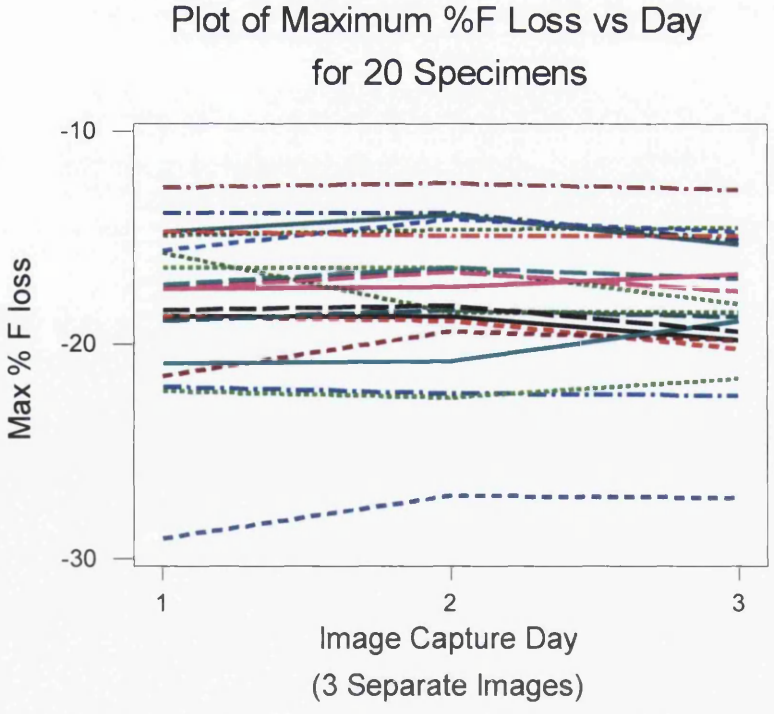
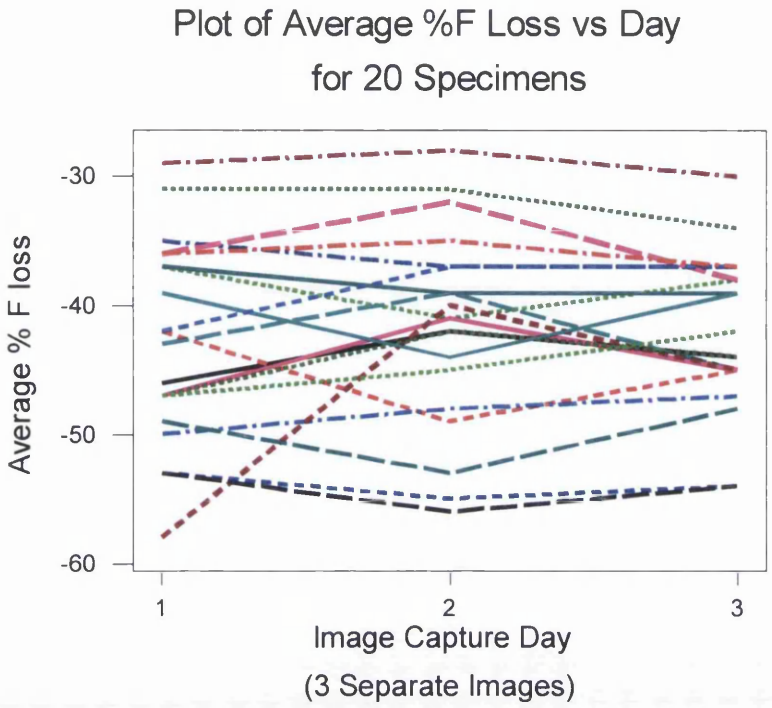


Figure 13 - Plot of QLF^{AVER} versus day for three separate images captured



The descriptive statistics for the twenty specimens from the image captures, on three different days, are given in Table 3 for the QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER}. Data in Table 3 demonstrate that the QLF values obtained over the three days have similar means, standard deviations and ranges for each of QLF^{AREA}, QLF^{MAX} and QLF^{AVER}.

Table 3 – Descriptive statistics for image capture of 20 specimens over three separate days

QLF Parameter	Number of specimens	Day 1		Day 2		Day 3	
		Mean (St. Dev.)	Range	Mean (St. Dev.)	Range	Mean (St. Dev.)	Range
QLF^{AREA} (mm^2)	20	4.05 (1.7)	(0.7, 7.7)	3.97 (1.6)	(0.9, 6.1)	3.73 (1.4)	(1.6, 7.9)
QLF^{MAX} (%F Loss)	20	-42.8 (7.8)	(-58, -29)	-41.7 (7.7)	(-56, -28)	-42.1 (6.2)	(-54, -30)
QLF^{AVER} (%F Loss)	20	-18.0 (3.8)	(-29, -13)	-17.8 (3.6)	(-27, -12)	-18.1 (3.3)	(-27, -13)

From the statistical models, for all three QLF parameters, the effects of order and day were not statistically significant (p-values for these effects were > 0.1, for each of the QLF parameters), whilst the random specimen effect was highly significant ($p < 0.001$) in each of the three models. This significant effect of specimen was expected and indicated that all the tooth specimens were unique. From the simple models involving just the random effect of specimen, the components of variance were estimated, and the repeatability-estimate derived from these, for each of the three QLF parameters. Approximate 95% confidence intervals for the repeatability-estimates were also calculated, and are displayed in Table 4.

Table 4 - Repeatability of QLF for image capture on three separate days

QLF Parameter	Estimate of Repeatability	Approx. 95% CI for Repeatability
QLF^{AREA} (mm^2)	83%	(68, 92)%
QLF^{MAX} (%F Loss)	96%	(91, 98)%
QLF^{AVER} (%F Loss)	83%	(68, 92)%

Data in Table 4 confirm that QLF^{MAX} was the most repeatable QLF parameter for QLF capture, as was suggested in Figure 11, Figure 12 and Figure 13. The estimate of repeatability was 96%, with a narrow 95% confidence interval of between 91% - 98%. QLF^{AREA} and QLF^{AVER} had lower estimates of repeatability (83%), with wider 95% confidence intervals of between 68% - 92%.

Image analysis

The results of the image analysis experiment, where each tooth specimen had one image captured and this image was then analysed on three different days, are displayed for the three QLF parameters in Figure 14, Figure 15 and Figure 16, for QLF^{AREA}, QLF^{MAX} and QLF^{AVER} respectively.

Figure 14 - Plot of QLF^{AREA} versus day for one image analysed three times

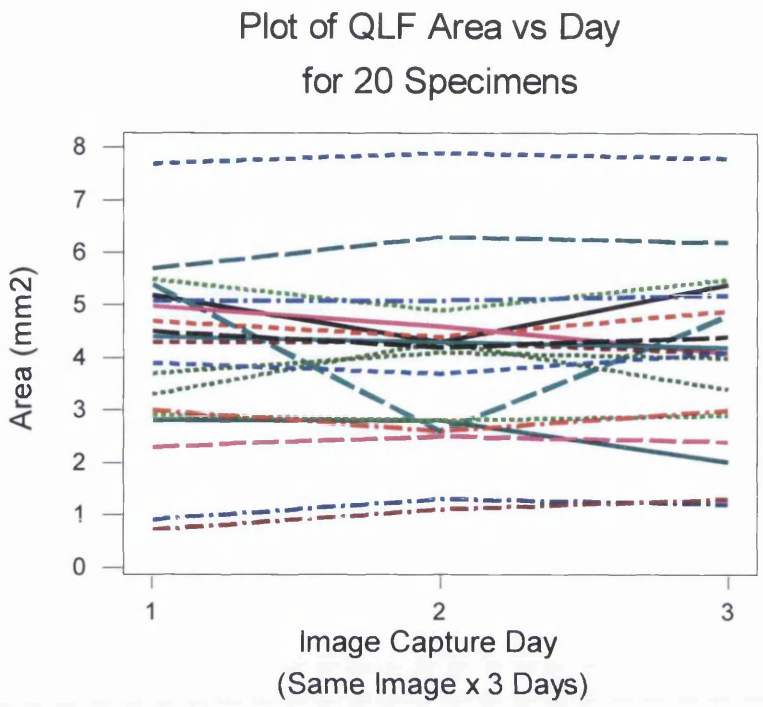


Figure 15 - Plot of QLF^{MAX} versus day for one image analysed three times

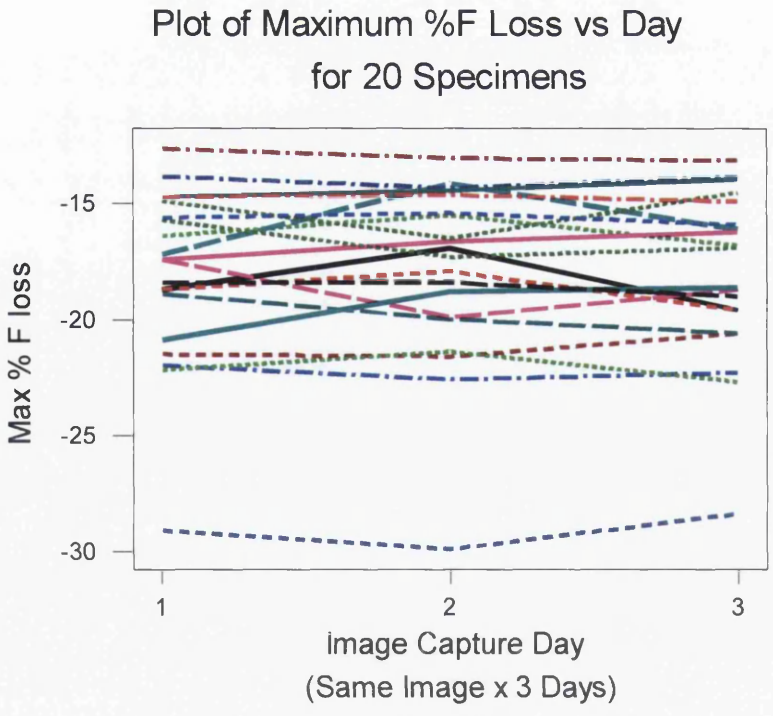
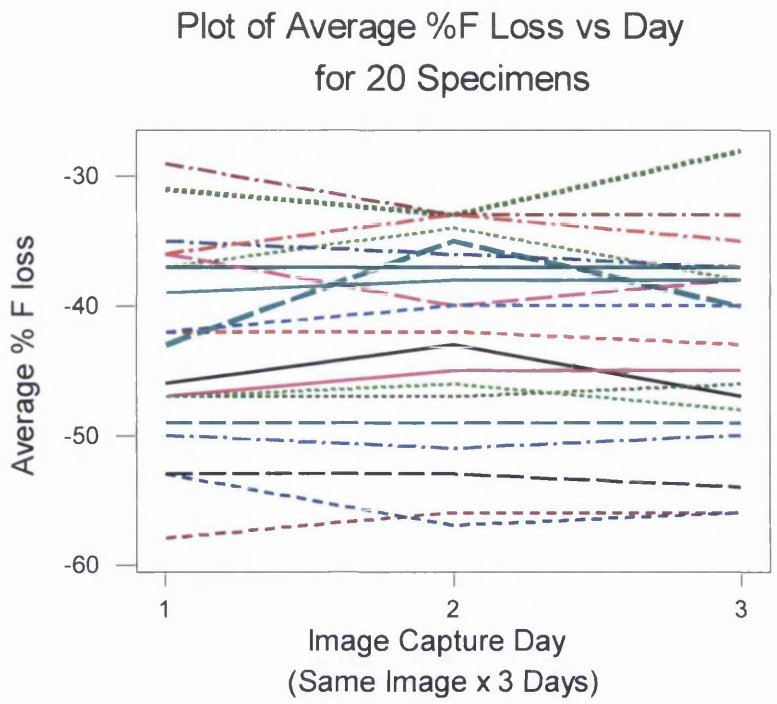


Figure 16 - Plot of QLF^{AVER} versus day for one image analysed three times



On examining the plots in Figure 14, Figure 15 and Figure 16, it would appear from the increased number of almost straight lines, that QLF^{MAX} and QLF^{AVER} were slightly more repeatable than QLF^{AREA} for image analysis for these 20 tooth specimens.

The descriptive statistics for analysis of the images of twenty specimens, three times on different days are described in Table 5 for the QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER}.

Data in Table 5 demonstrate that the QLF values obtained over the three days had similar means, standard deviations and ranges for QLF^{AREA}, QLF^{MAX} and QLF^{AVER}. This would suggest that there was little evidence of any systematic difference (bias) between the three days.

Table 5 – Descriptive statistics for image analysis of 20 specimens over three separate days

QLF Parameter	Number of specimens	Day 1		Day 2		Day 3	
		Mean (St. Dev.)	Range	Mean (St. Dev.)	Range	Mean (St. Dev.)	Range
QLF^{AREA} (mm^2)	20	4.05 (1.7)	(0.7, 7.7)	3.90 (1.6)	(1.1, 7.9)	4.04 (1.6)	(1.2, 7.8)
QLF^{MAX} (%F Loss)	20	-42.8 (7.8)	(-58, -29)	-42.4 (7.8)	(-57, -33)	-42.9 (7.8)	(-56, -28)
QLF^{AVER} (%F Loss)	20	-18.0 (3.8)	(-29, -13)	-18.0 (3.9)	(-30, -13)	-18.1 (3.7)	(-28, -13)

Statistical analysis of these results was conducted using the "repeated measures analysis of variance" technique, where for each of the outcomes (QLF parameters), the day and the order were modelled as fixed effects, and the tooth specimen was modelled as a random effect.

For all three QLF parameters, the effects of order and day were not statistically significant (p-values for both fixed effects, being > 0.1, for each of the QLF parameters). The random specimen effect was highly significant ($p<0.001$) in each of the three models. This specimen effect was to be expected and confirmed that each of the tooth specimens was unique. From the simple models involving just the random effect of specimen, the components of variance were estimated and the repeatability-estimate derived from these, for each of the QLF parameters. Approximate 95% confidence intervals for the repeatability-estimates were calculated. The repeatability estimates, together with the corresponding confidence intervals, are contained in Table 6.

Table 6 - Repeatability of QLF for image analysis of one image on three separate days

QLF Parameter	Estimate of Repeatability	Approx. 95% CI for Repeatability
QLF^{AREA} (mm^2)	93%	(86, 97)%
QLF^{MAX} (%F Loss)	95%	(91, 98)%
QLF^{AVER} (%F Loss)	96%	(91, 98)%

Data shown in Table 6 confirm the evidence from Figure 14, Figure 15 and Figure 16, which shows that QLF^{MAX} and QLF^{AVER} were slightly more repeatable QLF parameters for QLF analysis, with estimates of repeatability of 95% and 96% respectively. Both had narrow 95% confidence intervals of between 91% - 98%. QLF^{AREA} had a slightly lower estimate of repeatability (93%), with a wider 95% confidence interval of between 86% - 97%.

3.4 Discussion of repeatability of QLF

It was essential to determine the repeatability of the operator (AJN) in this study to validate the QLF results of the studies described in this thesis. Previous work by Pretty *et al.* (2002) and Tranaeus *et al.* (2002) have demonstrated that an inexperienced QLF examiner is less repeatable than an experienced one. Therefore, repeatability measurements were useful to help define the operator's level of QLF experience, i.e. "*the extent to which repeated measurements by the same observer in identical conditions agree*" (Petrie and Sabin, 2000).

To this end, the study was carefully designed by a statistician from the University of Glasgow. The methodology used was standard to the QLF technique. Images were captured and analysed in the manner described earlier, and identical to the technique used within the main study described in this thesis (Chapter 4). The statistical analysis used was the "components of variance" technique, with the components being estimated using "repeated measures analysis of variance". The method of analysis chosen in this study allowed for the examination of potential systematic bias, as well as the calculation of

repeatability estimates. Previous work on repeatability of QLF has not stated whether the potential effect of bias were considered, and instead have just reported the repeatability estimates, typically using intra-class correlation coefficients. Whilst the statistical methods are not identical, results obtained from studies utilising ICCs can be compared to the results reported here, by multiplying the ICC by 100 (i.e. converting into a percentage).

Repeatability of image capture

For the outcome measures of QLF^{AREA} and QLF^{AVER}, the estimates of repeatability were approximately 83%, with corresponding 95% confidence intervals of similar width, running from 68% - 92% (Table 4). Therefore, using the definitions from Shrout (1998), the repeatability of image capture, using the parameters QLF^{AREA} and QLF^{AVER}, is described as "substantial". However, if the lower point of the confidence interval is examined (i.e. "worst case scenario"), then there could be some cause for concern with these endpoints being "moderate". The most repeatable outcome was QLF^{MAX}, with an estimate of 96% repeatability and corresponding confidence interval from 91% - 98%. This confidence interval was much narrower than the other two, providing further evidence that it would seem easier for this operator to measure, consistently, QLF^{MAX} of the same specimen from different images. It is of interest to note that when the Clin-QLF software calculates the QLF parameters, it uses the maximum "difference between the surrounding healthy enamel and the enamel within the area for analysis of one pixel" to calculate the QLF^{MAX} value. Therefore, it would be reasonable to assume that this would be one of the most constant parameters when examining multiple images of the same tooth specimen and would explain the high average repeatability and narrower 95% confidence interval obtained.

The results obtained in this study were comparable with those obtained for image capture by the Swedish group (Tranaeus *et al.*, 2002), although, the latter study used an Argon ion laser as the light source for the image capture rather than light from a Xenon arc lamp. However, they found that their most repeatable parameter was QLF^{AREA}. They obtained ICC's of 0.97 for QLF^{AREA}, 0.88 for QLF^{AVER} and 0.94 for QLF^{MAX}.

Repeatability of image analysis

For all three of the QLF parameters, the estimate of repeatability was over 90%, suggesting that the operator could consistently measure each of the QLF outcomes, for each specimen, over a number of days. The least reproducible outcome appeared to be QLF^{AREA} (93%), with QLF^{MAX} and QLF^{AVER} having similar repeatability-estimates of 95% and 96%. This

was further emphasised by the confidence intervals for the repeatability estimates, the intervals for QLF^{AVER} and QLF^{MAX} being of similar width. In addition, both were narrower than the interval for QLF^{AREA}, suggesting more variability in consistently measuring QLF^{AREA} of a given specimen. All of these repeatability-estimates are described as "substantial" (Shrout, 1998).

The Tranaeus *et al.* (2002) study also found the image analysis stage to be more consistent than image capture. They achieved ICCs of between 0.93 and 0.98 for QLF^{AREA}; between 0.96 and 0.99 for QLF^{AVER} and between 0.96 and 0.99 for QLF^{MAX}. These results were obtained for three different examiners, with the lower values being obtained for the "novice" examiner. Similarly to the findings in this study, Tranaeus *et al.* also found QLF^{AREA} to be the least repeatable parameter, with QLF^{MAX} and QLF^{AVER} the more repeatable parameters for QLF image analysis.

Summary

Overall, it appeared that the image capture stage was not as repeatable as the image analysis stage. One of the reasons for this may be the change in magnification of the image depending on how close the lesion is to the hand-piece. Not all images of the lesions were captured at exactly the same distance because it was dependent on focus. It is likely that this difference in magnification could account for the differences in lesion area (QLF^{AREA}). To eliminate this potential error, it is now possible to use a depth guide attached to the camera, to ensure that the lesion image is captured at optimum focal length. Another method of eliminating image capture error is to use the image realignment software that has recently been developed within the QLF software (de Josselin de Jong and van der Veen, 2000). The software allows alignment of multiple images captured longitudinally of the same lesion. A 'patch' can be saved when the first image is analysed and this can be superimposed on the image when capturing subsequent images. After necessary adjustment by horizontal, vertical or rotational images the analysis can then be performed. Unfortunately, this software was not available at the beginning of the studies in this thesis.

From a statistical point of view, one of the weaknesses in this study was that the lesion sizes showed a lack of range. For repeatability studies, a wide range of lesion size is desirable. Despite the lesions in the current study ranging between approximately 0.5 and 8.0 mm^2, this was narrower than that of comparable ranges in other studies. A larger variety of lesion size and degree of demineralisation was achieved in the Tranaeus study,

and the slightly higher repeatability-estimates may reflect this greater variety of lesions (though as mentioned before, the authors do not describe any examination of potential systematic bias). However, in the current study, one of the intentions was to create artificial caries lesions which were of a similar size and degree of demineralisation, to allow additional confirmation of the method chosen to create the lesions for the main study, the technique of which is described in Chapter 1, Section 1.7 and in Chapter 4.

Although the design of this study was planned so that the statistical method applied considered the potential effect of systematic bias, it was not considered important to determine bias as a result of operator error. This would have needed to involve many other operators of varying experience to measure potential systematic bias between operators i.e. if one QLF operator was to consistently measure QLF parameters lower or higher on each occasion, in comparison with a different operator. In the studies completed in this thesis, there was one operator who captured and analysed all of the images (AJN), so although it was not possible to determine reproducibility, it is possible to determine the repeatability of the operator. Future work could include comparison with other QLF operators, of varying experience.

The operator (AJN) had approximately six months of QLF image capture and analysis experience which was gained prior to the beginning of the experiments described in this thesis. In addition, this repeatability study was undertaken prior to the main study described in Chapter 4. The results from this study show that the operator in this thesis was able to capture and analyse images at a "substantially" repeatable level. The repeatability estimates obtained would suggest that the operator could achieve levels of consistency similar to operators described as "experienced" in other studies examining the repeatability of QLF.

The results also demonstrated that the QLF image analysis stage was more repeatable than the QLF image capture stage for all QLF parameters, with the exception of QLF^{MAX}, which was highly repeatable in both stages.

4.0 The effect of fluoridated and non-fluoridated milk on longitudinal mineral content changes in artificial, caries enamel lesions

4.1 Introduction and aims

This chapter describes a study designed to evaluate the effect of fluoridated milk, on change in mineral content of artificial enamel lesions, using a complete denture model *in situ*. The study protocol was designed to investigate a number of research questions in relation to the efficacy of fluoridated milk usage. In addition, little is known about the additional cariostatic benefit obtained when fluoridated milk is taken in conjunction with the normally recommended twice-daily use of a fluoridated dentifrice. Hence, the study protocol was designed to address the question of fluoridated milk ingestion frequency, with and without the simultaneous use of a fluoridated dentifrice. Any potential site differences in caries mineral dynamics that occur within the oral cavity were also studied.

The study used an enamel caries *in situ* appliance model to investigate the following questions:

- Was there an effect of using fluoridated dentifrice on the mineral content of enamel lesions?
- Were there any effects of consumption of fluoridated milk on mineral content of enamel lesions a) with or b) without the effect of fluoridated dentifrice?
- Were there any differences in the change in the mineral content of enamel lesions at different intra-oral sites?

4.2 Methods

4.2.1 Subject selection

Ethical approval for the study was obtained from the Area Dental Ethics Committee of North Glasgow University Hospitals NHS Trust.

Subjects were recruited to allow comparisons both in terms of the effects of fluoridated/ non-fluoridated milk and frequency of milk ingestion, and also to allow for the investigation of any carry-over effects between experiments. The details about the study protocol, the number of subjects to be recruited and the subject allocation are described in Sections 4.2.5 and 4.2.7.

Subjects were recruited for selection from three sources: patients of colleagues in general dental practice; a list of patients who had previously attended for treatment in the undergraduate Prosthodontic clinic of Glasgow Dental Hospital and School; and by opportunistic recruitment of individuals known to the study researchers.

In total, 71 potential subjects were approached (Appendix 3), with 50 agreeing to attend for further screening and assessment. Selection criteria were determined and are listed below:

Selection criteria

It was judged that subjects suitable for possible inclusion in the study should:

(i) be able to attend on a regular basis and, where relevant, have a good attendance history.

(ii) be mobile i.e. not requiring ambulance transport services.

(iii) be edentulous.

(iv) consider themselves to be a good denture wearer, who did not require complex treatment.

(v) have a "normal" diet with neither excessive nor insufficient refined carbohydrate.

(vi) have "sufficient" resting whole salivary flow-rate i.e. >0.1 mL per minute.

(vii) have *Lactobacillus sp.* intra-orally.

(viii) ideally be free of oral candidiasis.

(ix) have no evidence of Sjögren's syndrome.

(x) not be on long-term antibiotics, or use chlorhexidine gluconate mouthwash/gel.

(xi) not be taking medication such as antihypertensives, anticholinergics, tricyclic antidepressants/sedatives/tranquillisers, diuretics or antihistamines that might reduce the salivary flow-rate.

Potential subjects were identified for selection and the above criteria verified as follows. Subjects were invited to attend an assessment appointment. They were given further information about the study (Appendix 4) and informed they would be provided with two new sets of dentures; one of which would carry intra-oral test specimens. In addition, it was explained that a financial reward would also be provided to compensate for their time and travelling expenses. If the subject agreed to participate, a consent form was signed (Appendix 5). Subjects were then asked to provide a completed three-day diet diary form sent with the introductory letter, which included the request that one of the days should record what they ate and drank at a weekend. This allowed subjective evaluation as to whether the diet contained excessive or insufficient refined carbohydrate, such as might modify their potential caries experience. A full medical and dental history and oral examination was completed for each consenting participant and an assessment made of the alveolar ridge-form to determine the level of difficulty of denture construction. An unstimulated whole saliva sample was collected over two minutes by asking subjects to drool into a graduated tube. An adequate unstimulated salivary flow-rate was defined as being greater than 0.1 mL per minute (Dawes, 1996). Following this, an intra-oral rinse of 10mL of 0.01M Phosphate Buffered Saline (PBS) at pH 7.4 (Sigma Chemical Co., St Louis, MO, USA) was undertaken for one minute with the subject's current dentures in place. The rinse and saliva sample were then cultured for microbiological assessment to detect whether *Streptococcus mutans* and *Lactobacillus sp.* were present in the volunteer's oral microflora. A requirement of a microbiology Ph.D study involving the same subjects, and running in parallel with this project, was that the participants had *Lactobacillus sp.*, and that the intra-oral presence or absence of *Streptococcus mutans* was known at the selection stage.

4.2.2 Complete denture model

As the subjects were edentulous, the intra-oral appliance design consisted of modifying a set of complete upper (maxillary) and complete lower (mandibular) dentures to carry human tooth blocks with artificially-created caries lesions therein. Rather than merely

altering the existing dentures, it was decided that new complete dentures should be constructed. These "test" dentures were to be worn during each six-week experiment. The time between experiments was decreed a "washout period", details of which are described later. During washout periods, subjects were to be required to leave their "test" dentures out of their mouths. Hence, two sets of complete dentures were constructed, one to wear as the intra-oral appliance during each experiment, and one to wear between experiments. In total, 32 subjects each had two sets of dentures provided for them (64). The two sets of dentures were essentially duplicates with only the test dentures modified to carry tooth blocks.

The complete dentures were constructed with a variation from the normal practice. Primary impressions were taken in red impression compound (Kerr UK Ltd, Peterborough, UK) to enable construction of individual impression trays in light-cured acrylic. Master impressions were taken of the patients using individual trays modified with green impression compound (Kerr UK Ltd, Peterborough, UK), and Permlastic, polysulphide impression material (Kerr UK Ltd, Peterborough, UK). Master casts were constructed in dental stone and duplicated to create a second set of master casts. Wax record blocks were made, jaw registration taken and acrylic teeth (Senator, Wright Health Group Ltd, Dundee, UK) selected for trial dentures. Once both clinician (AJN) and subject were happy with the appearance and registration, the dentures were processed in heat-cured acrylic resin, and finished. Templates and impressions were made of these prostheses and a duplicate set of upper and lower complete dentures constructed on the duplicate master casts. Subjects were then asked to wear the dentures and attend for adjustment and review until they were able to wear both sets comfortably. Thereafter, volunteers were required to choose which set of dentures they preferred to wear as their "test" dentures, as the experimental protocol required the subjects to use these dentures continuously (day and night) for several six-week periods. A washout period followed each of the six-week test periods during which the alternate dentures were worn for four weeks. To prevent any intra-oral discomfort or infection, instruction was given on denture cleaning (Appendix 6). Subjects were advised to brush only the fitting surface of each denture with a soft brush, and informed that any food debris could be removed by simply holding the dentures under running water. Throughout the study periods, regular examination of the subject's oral tissues was undertaken, though no subject developed any signs or symptoms from wearing the dentures continuously.

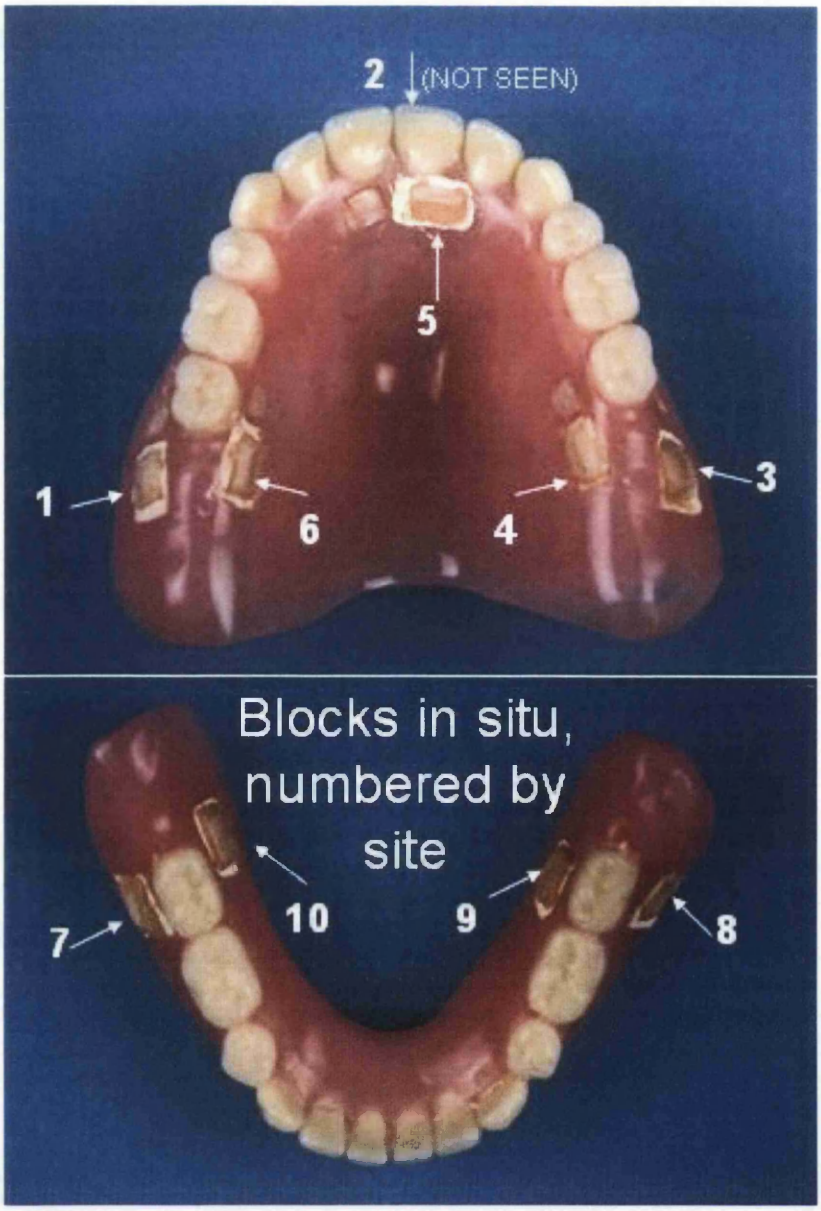
4.2.3 Tooth blocks containing artificial caries lesions

As described in Chapter 2, Section 2.2.2, blocks of abraded human enamel with underlying dentine were created with approximate dimensions of 8 x 6 x 3 mm. Artificial caries lesions were then prepared using a lactic acid-based demineralisation gel containing a polymer (Carbopol, B & F Goodrich and Company Chemical Group, Avon Lake, Ohio, USA) to facilitate the formation of subsurface demineralisation with an intact surface zone. Within 1-2 weeks, adequate demineralisation was observed in the tooth blocks by visual examination, and then confirmed with Quantitative Light Fluorescence (QLF), with the lesions having an Average Fluorescence Loss (QLF^{AVER}) of greater than 13%, as described in Chapter 2, Section 2.3.1, being suitable for inclusion in the study.

4.2.4 Site selection for artificial caries lesions

The complete dentures used in the experimental phases of the study were modified to create test sites by drilling rectangular depressions in the buccal and palatal/lingual aspects of each set of dentures, to allow cementation of the enamel blocks flush with the polished surface of the dentures. Within the appliance model, 10 test locations were chosen to represent intra-oral sites with differing salivary-film velocities (Dawes and Macpherson, 1993). These were six sites in the upper denture and four sites in the lower denture (Figure 17). The six chosen sites in the upper intra-oral appliance were: right buccal (site 1); midline labial (site 2); left buccal (site 3); left palatal (site 4); midline palatal (site 5) and right palatal (site 6). The four sites in the lower intra-oral appliance were: right buccal (site 7); left buccal (site 8); left lingual (site 9) and right lingual (site 10).

Figure 17 - Photograph of test dentures containing tooth blocks in 10 test locations

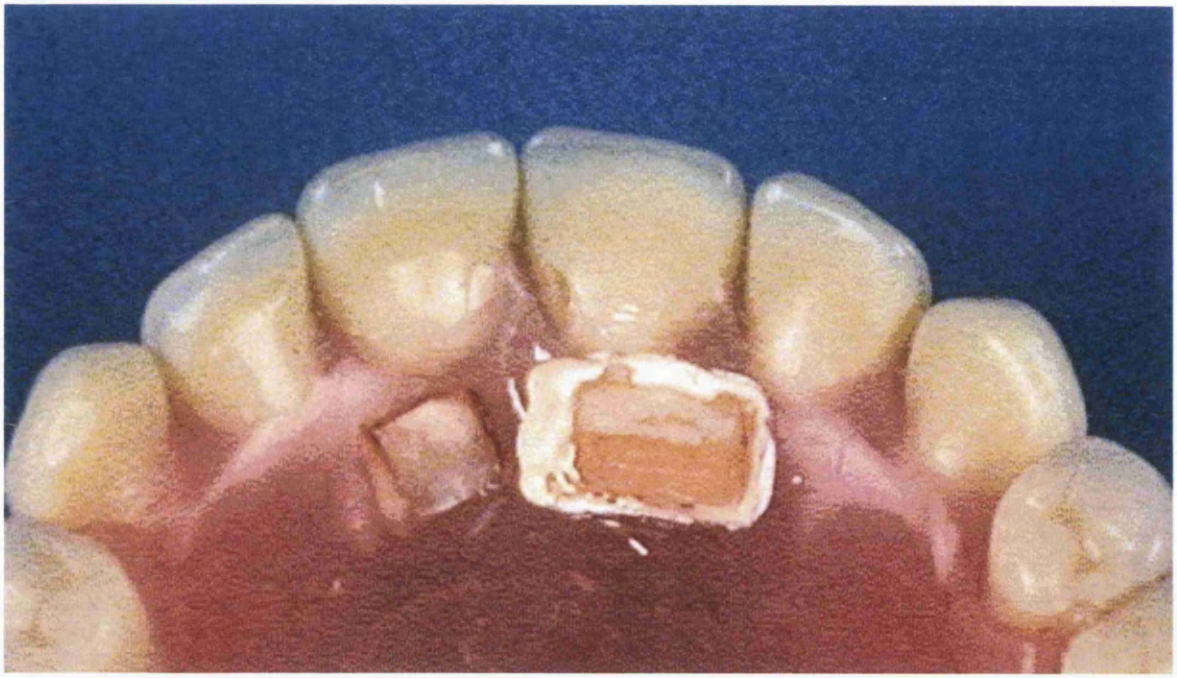


The sites in the upper denture each retained two enamel blocks, whereas there was only one block per site within the lower denture. The additional block at each site in the upper denture was for plaque collection relating to a concurrent Ph.D study described briefly in Section 4.2.1. This additional block was smaller than that described above, with approximate dimensions of 4 x 4 x 3 mm and did not contain an artificial caries lesion.

The smaller non-demineralised blocks, used solely for plaque collection, were fixed into the six sites in the upper intra-oral appliance for each of the experimental protocols, using a combination of self-cure acrylic (Simplex Rapid, Austenal Dental Products Ltd, Harrow, UK) and cyanoacrylate adhesive (Tufloc, TILP adhesives Ltd, Grantham, UK). The large blocks of tooth, containing a demineralised lesion, were allocated randomly to test sites

and cemented into the appliances using Tempbond NE™ (Kerr UK Ltd, Peterborough, UK). This allowed relatively easy removal of the tooth blocks from the test dentures on completion of each of the experimental protocols. The typical appearances of both large and small blocks, *in situ* are shown in Figure 18.

Figure 18 - Photograph of small and large tooth block in upper test denture



4.2.5 Study protocol

Selected subjects were allocated randomly to one of two groups: treatment only (T), or treatment plus dentifrice (TD); further details are provided in Section 4.2.7. Those following the TD protocol conformed to an industry-accepted method of simulating twice daily toothbrushing of the teeth (Manly, 1943). A dentifrice (1100 ppm F) slurry diluted 1:4 in water was prepared and checked in the laboratory, prior to distribution with a small marked measuring cup. The subjects involved measured 4 mL of the slurry into the measuring cup, took this to the mouth and swilled it around the oral cavity, once in the morning and once in the evening, for two minutes before voiding. The subjects were told not to rinse their mouth out following use of this dentifrice slurry.

For both groups, there were five experimental protocols. Each subject was allocated (in the manner described in Section 4.2.7) to follow all five of the experimental protocols in a random order. Each protocol was of six weeks' duration and was followed by a minimum "washout period" of four weeks (Stephen *et al.*, 1992). The five protocols were:

A. 0.5 mg F in 200 mL milk, three times per day (equivalent to 2.5 ppm F)

B. 1.5 mg F in 200 mL milk, once per day (equivalent to 7.5 ppm F)

C. 200 mL milk, once per day

D. 200 mL milk, three times per day

E. No beverage

Each protocol was followed in addition to the subject's usual diet, which had been assessed previously (Section 4.2.1). The subject was asked at the beginning of each experiment and during each experiment if there had been any changes to their medical history or medication taken, which could have excluded them from the study according to the selection criteria (Section 4.2.1).

4.2.6 Fluoridated milk preparations

To prepare the fluoridated milk, solutions of 0.5 mg F and 1.5 mg F were prepared in the laboratory, along with deionised, distilled water for use as a placebo. The Pharmacy Production Department of the Western Infirmary, Glasgow, filter-sterilised all of the solutions, and 2 mL volumes were placed into sterile, labelled, plastic bijou bottles (Figure 19).

Figure 19 - Photograph of the three bijou bottles used in the study. The three solutions 1.5 mg F, 0.5 mg F and distilled, deionised water were randomly allocated A, B and C.

Prior to each experiment, the Research Assistant gave each subject verbal and written instructions on how to follow the experimental protocol and explained the function and duration of the four week "washout period" between each study phase (Appendix 7 contains the details of the instructions given to subjects for each experiment for the two groups, T and TD). The Research Assistant also arranged for delivery of the UHT milk with fluoride, and the fluoride slurry, where applicable, to the subject's home, if required. The milk was skimmed, semi-skimmed or whole, depending on the subject's preference. The required numbers of bijou containing either a sodium fluoride solution or placebo distilled water, were provided (if required) along with sufficient quantities of UHT milk appropriate for any one complete protocol run. Only the Research Assistant knew which solutions corresponded with the bijou labels A, B and C. Subjects were instructed to measure 200 mL of milk into the measuring cup provided, and then empty the contents of a bijou bottle into the milk, and stir prior to ingesting the beverage. The concentration of fluoride in the milk was confirmed with a fluoride meter, in the laboratory, prior to the experimental process.

The processes relating to patient allocation, milk usage, and milk delivery were undertaken by the Research Assistant, to ensure the clinical investigator was blind to the actual experimental protocol which each subject was following.

On the last day of each six-week experiment, subjects were asked to return all bijou bottles they had been given for the duration of that experiment, to the Research Assistant. Those who had been allocated to the TD group were also asked to return the large bottle which had contained the dentifrice slurry solution in order to evaluate compliance. Compliance was also assessed via a questionnaire distributed to each volunteer following completion of their total involvement i.e. at the end of all five experiments (Appendix 8).

4.2.7 Subject allocation and number of subjects

As mentioned before, subjects were each to undertake 5 different experiments (A. 0.5mg F in 200 mL milk x 3/day, B. 1.5mg F in 200 mL milk x 1/day, C. 200 mL milk x 1/day, D. 200 mL milk x 3/day and E. no beverage). With respect to the subject randomisation process, two elements were involved: firstly, subjects were allocated randomly to one of the two groups (T or TD); secondly, the order of experiments (A - E) had to be determined for each subject.

Randomisation of subject to a group

To allocate subjects to either the T or TD group, a randomisation list was prepared by the research statistician, using the random permutated blocks method (Pocock, 1983), to ensure approximately equal numbers of subjects were allocated to each group following recruitment. This process was set up prior to the commencement of subject recruitment.

This randomisation list was then transcribed into a log-book, which contained sequential subject study numbers, and against each study number, the allocated group. When a subject was recruited into the study, the Research Assistant assigned the subject the next available subject study number, from which the allocated group was deduced and assigned.

Randomisation of order of experiments for a subject

Prior to study commencement, the statistician created experimental schedules, these providing the order in which the five experiments were to be allocated to each subject. The experimental orders were determined using a Balanced Latin Squares Design (Pocock, 1983), to ensure that within each group, each sequential pair of experiments was to be given to at least two subjects, assuming every subject completed all five experiments. This design was used to enable the potential effect of experimental order to be examined, assuming all participants completed all experiments.

The Balanced Latin Squares Design indicated that ten subjects would be required in each group, thus a total of twenty subjects was needed. However, for the design to be balanced, it was necessary that all twenty subjects completed all five experiments. Given the target population, it was decided to aim to recruit a minimum of thirty persons, to try to ensure that all the required sequential experimental orders were undertaken by at least two subjects. It was also decided that, if any subjects dropped-out of the study, without completing all five experiments, within the first year of the study commencing, the experimental schedule for such a subject would be re-started with a new volunteer. After this first year, once the first ten experimental schedules for each group had been allocated, further schedules were created for any remaining recruited subjects. By so doing, it was hoped to minimise the effect of any subjects dropping-out before completion of all five experiments, in the latter phases of the study.

4.2.8 Transverse microradiography and image analysis (TMR)

On completion of the experimental protocols, and after all images were captured with QLF, the tooth specimens were cut into sections, hand-lapped then microradiographed as described in Chapter 2, Section 2.3.2.1. As many sections as possible were prepared from each block. Initially a single section from the centre of the block was used for TMR analysis. Image analysis was undertaken using the TMR software, version 1.25e, (Inspektor Research Systems BV, Amsterdam, The Netherlands). Image analysis was undertaken on both the covered (control) area of the lesion and also on the exposed area of each tooth section.

Tooth blocks were included for analysis only if they had an exposed and covered reading constituting a "pair". Therefore, if there was no reading from either the covered "control" portion or exposed portion of the tooth specimen, it was excluded from statistical analysis. This occurred if there was loss of the intact surface zone of the caries lesion, believed to occur, on occasion, both during the *in situ* period and during preparation of the tooth section for microradiographical analysis. In such cases another section from the same block was used. No TMR data could be recorded from a block if it was either lost during the experiment, or no intact sections could be retrieved from that block. Obviously, use of TMR only at the end of an experiment makes it is impossible to obtain a true baseline value. Therefore the TMR data calculated for the covered part of the lesions represented "proxy" control values. This aspect will be discussed in more detail later.

4.2.9 Quantitative Light Fluorescence (QLF) image numbering and analysis

A maximum of nine QLF images were captured of each tooth specimen during the study. They were numbered "Image 1 - 9" according to the stage of image capture and are illustrated schematically in Figures 20, 21 and 24.

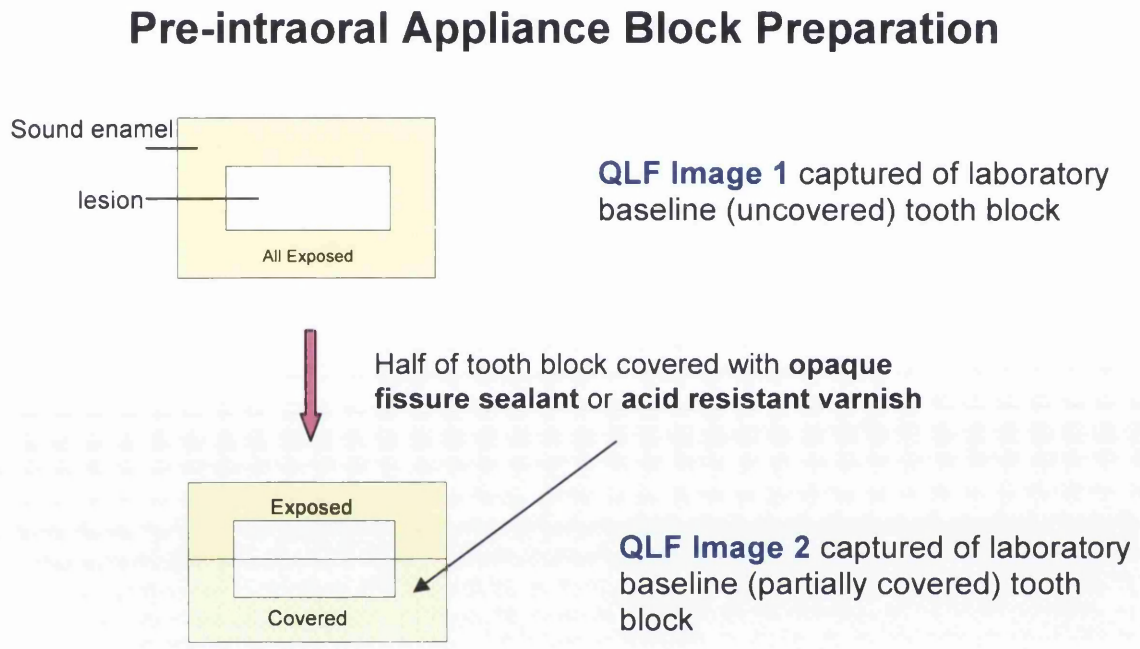


Figure 20 - Diagram summarising block preparation prior to placement in the intra-oral appliance

Figure 20 summarises block preparation prior to placement within the intra-oral appliance. Once the artificial caries lesion was created on the tooth block, it was examined visually to determine whether there was an evenly demineralised "white spot lesion" on the enamel surface. This was verified by taking a QLF image of the block (named QLF Image 1) and analysing it to confirm that the lesion demonstrated an Average Fluorescence Loss (QLF^{AVER}) of 13% or greater. Tooth blocks which satisfied this criterion were then partially covered by placing either acid-resistant varnish or fissure sealant over the lower half of the tooth block. Another QLF Image was captured at this stage (named QLF Image 2 or "baseline image"), this was to confirm that the exposed lesion still demonstrated an

Average Fluorescence Loss (QLF^{AVER}) of 13% or greater, i.e. the demineralisation of the lesion was fairly even between the covered (control) and exposed areas. This image also acted as a baseline image, for later comparisons.

Figure 21 - Diagram summarising the order of the QLF image capture

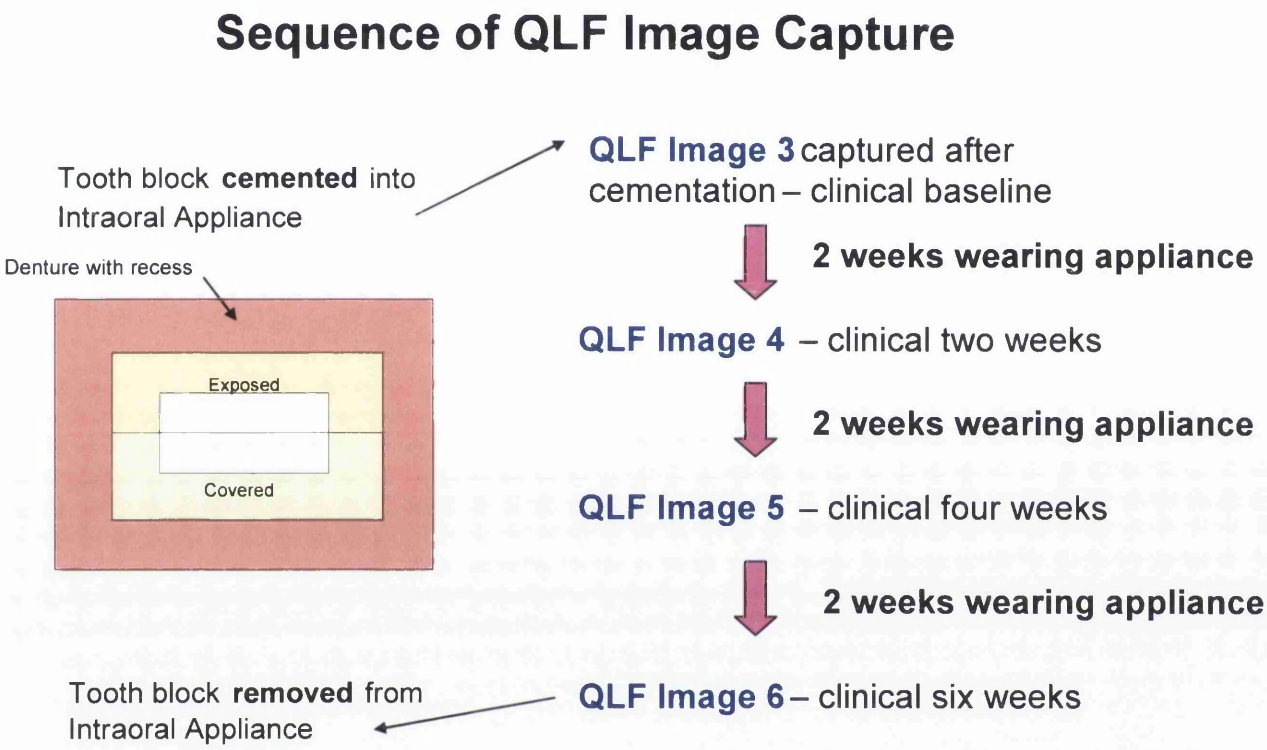


Figure 21 outlines the sequence of QLF image capture associated with the clinical aspect of the study i.e. with the block in the appliance. When subjects attended for the first visit of the six-week experiment, blocks were allocated to the appliance in a random manner. The tooth blocks were cemented into the appliance using temporary dental cement, Tempbond NE™ (Kerr UK Ltd, Peterborough, UK) and a clinical baseline image was captured using QLF (named "QLF Image 3"). Examples of QLF images of tooth blocks are shown in Figures 22 and 23.

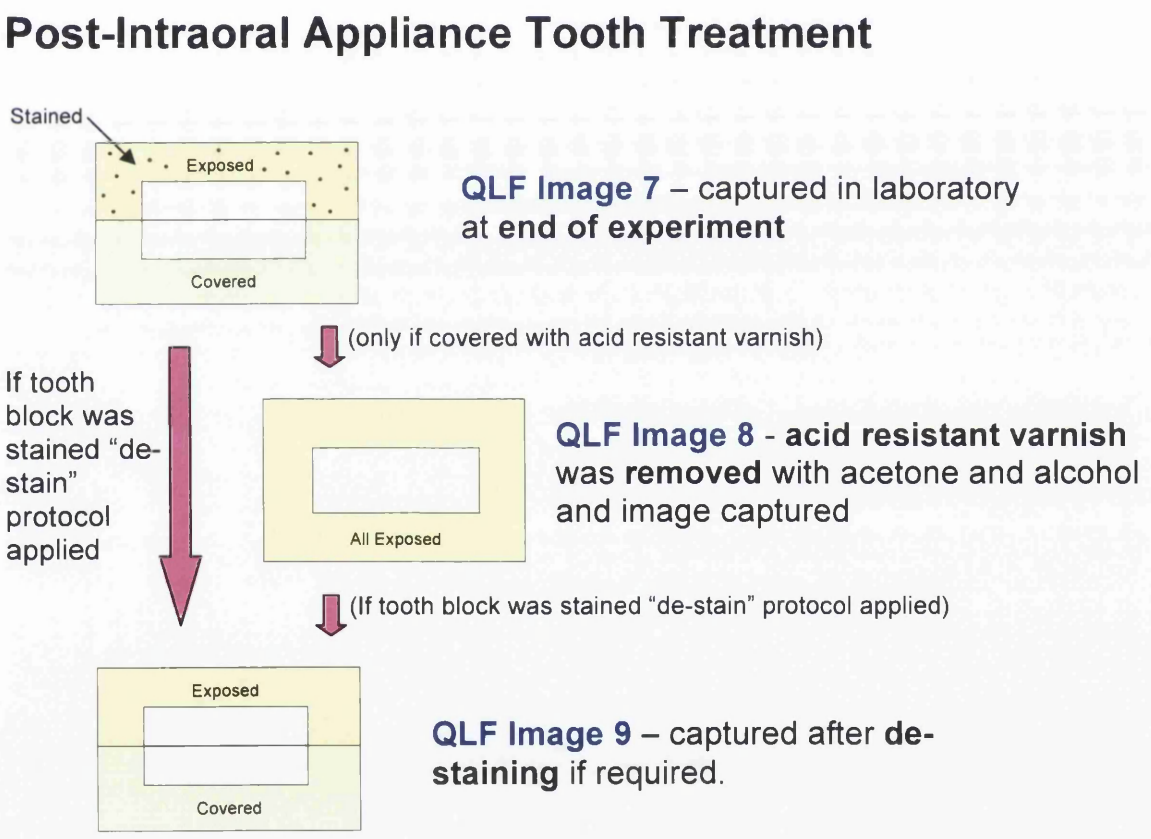
Figure 22 - Image of computer screen showing a QLF image 3 for a tooth block with the lower half covered with acid-resistant varnish

Figure 23 - Image of computer screen showing a QLF image 3 for a tooth block with the lower half covered with fissure sealant

Images were then captured of the tooth block at every subsequent attendance of the subject, which was at two-week intervals. Therefore "QLF Image 4" was captured two weeks into the experiment, "QLF Image 5" at four weeks and "QLF Image 6" at the sixth week, and end-point of the protocol. The tooth block was then removed from the intra-oral appliance by gentle elevation from the rectangular depression.

In Figure 24, the QLF image capture of a tooth block on removal from the intra-oral appliance is outlined. Immediately on removal, the tooth blocks had an image captured (named "QLF Image 7"). The tooth blocks which had been partially covered with acid-resistant varnish had the varnish removed using acetone and alcohol and a QLF Image was captured (named "QLF Image 8"). QLF Image 8 analysed values were not used in the overall statistical analysis.

Figure 24 - Diagram of treatment of tooth block on removal from intra-oral appliance



In some subjects there was evidence of staining present on the tooth block when removed from the intra-oral appliance and it is known that the presence of stain modifies the

specificity of QLF as stain may also fluoresce and absorb light. Thus a false reading, indicating greater demineralisation than was actually present, could arise. Hence, if visible stain was present on any tooth block, it was placed into a de-stain protocol (place block in 30% Hydrogen Peroxide for four hours, rinse thoroughly in running water, store in water for 24 hours, rinse block and container, then store in saturated solution of Thymol 0.12%). An abstract describing this technique, was presented at PEF in 2002, Appendix 11. Following this, where applicable, a final QLF Image was captured, "QLF Image 9".

To allow ease of manipulation of the data for statistical analysis, a "proxy" final image was created and named "Image 10". This was not a newly captured image, and was the data of image 9, or if no stain had been present, the data of image 7. This proxy "Image 10" was taken as the final result.

QLF image analysis

The captured QLF Images were analysed using the software Clin-QLF version 1.25e (Inspektor Research Systems BV, Amsterdam, The Netherlands) as described in Chapter 2, Section 2.3.1. Because of the shape of the artificial caries lesion used in this study, a maximum of three borders could be placed on sound tooth substance surrounding the lesion prior to analysis. This was because the 4th and last remaining border would have to be placed at the division between the covered and uncovered lesion, and hence would not have been on sound enamel. This is illustrated in Figures 25 and 26. Each lesion was analysed at a threshold of "10". Therefore, when the mean intensity of the pixels selected for sound enamel was compared with the intensity of any pixels within a prescribed area enclosing the lesion, those pixels with an intensity reduction of 10% or greater were categorised as dental caries. In this way the total number of pixels counted as carious could be used to calculate the lesion area (QLF^{AREA} mm^2), the average reduction in pixel intensity compared with sound tooth enamel (QLF^{AVER} % Fluorescence Loss), as well as the maximum reduction in pixel intensity (QLF^{MAX} % Fluorescence Loss).

Figure 25 - Image of computer screen showing a QLF image of a tooth block covered with acid-resistant varnish with the analysis patch superimposed.

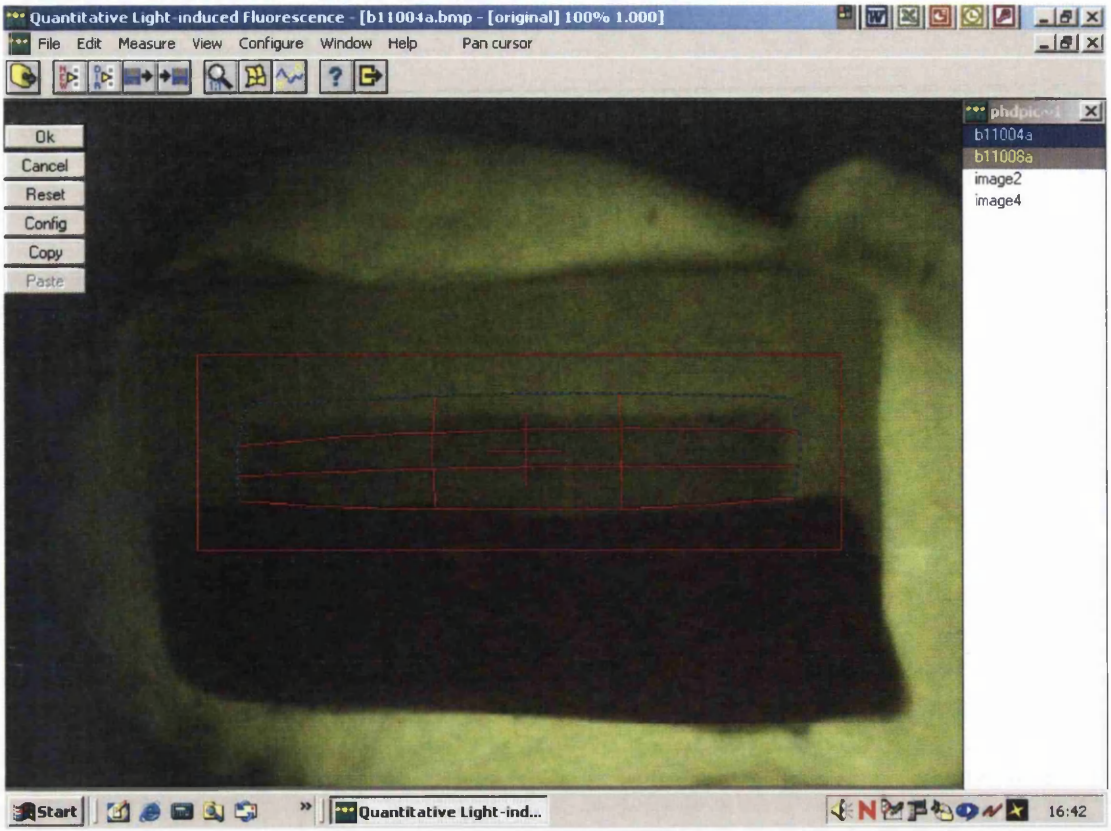
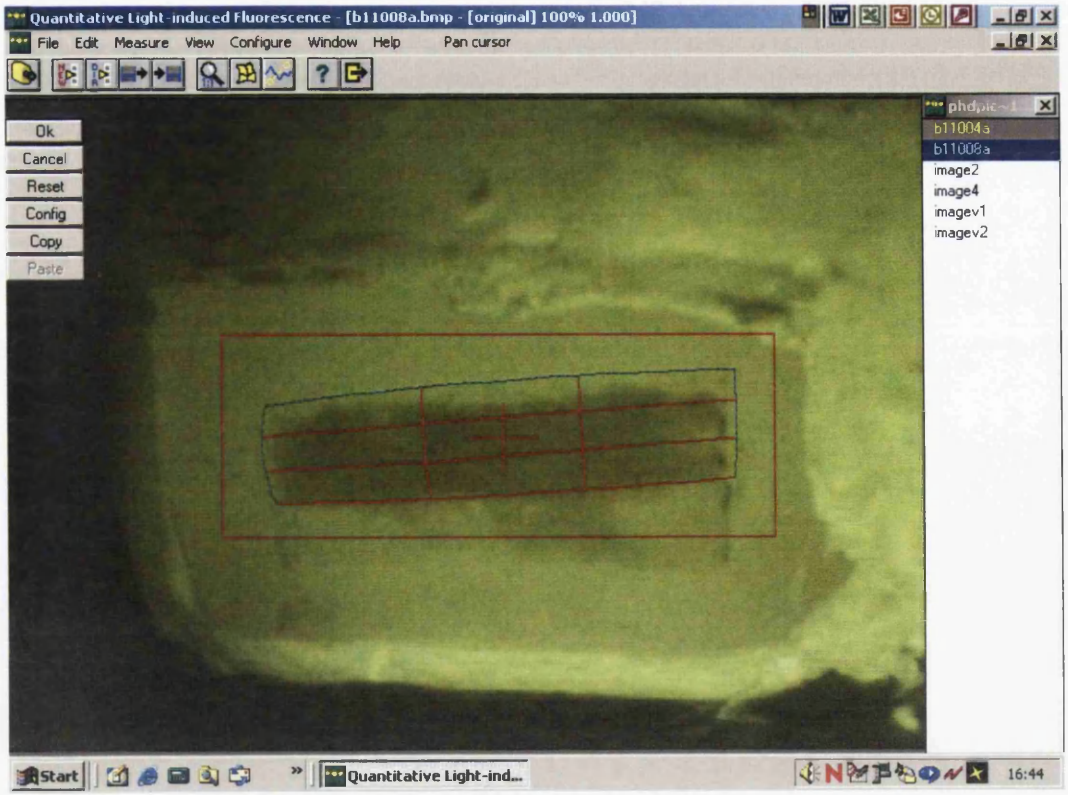
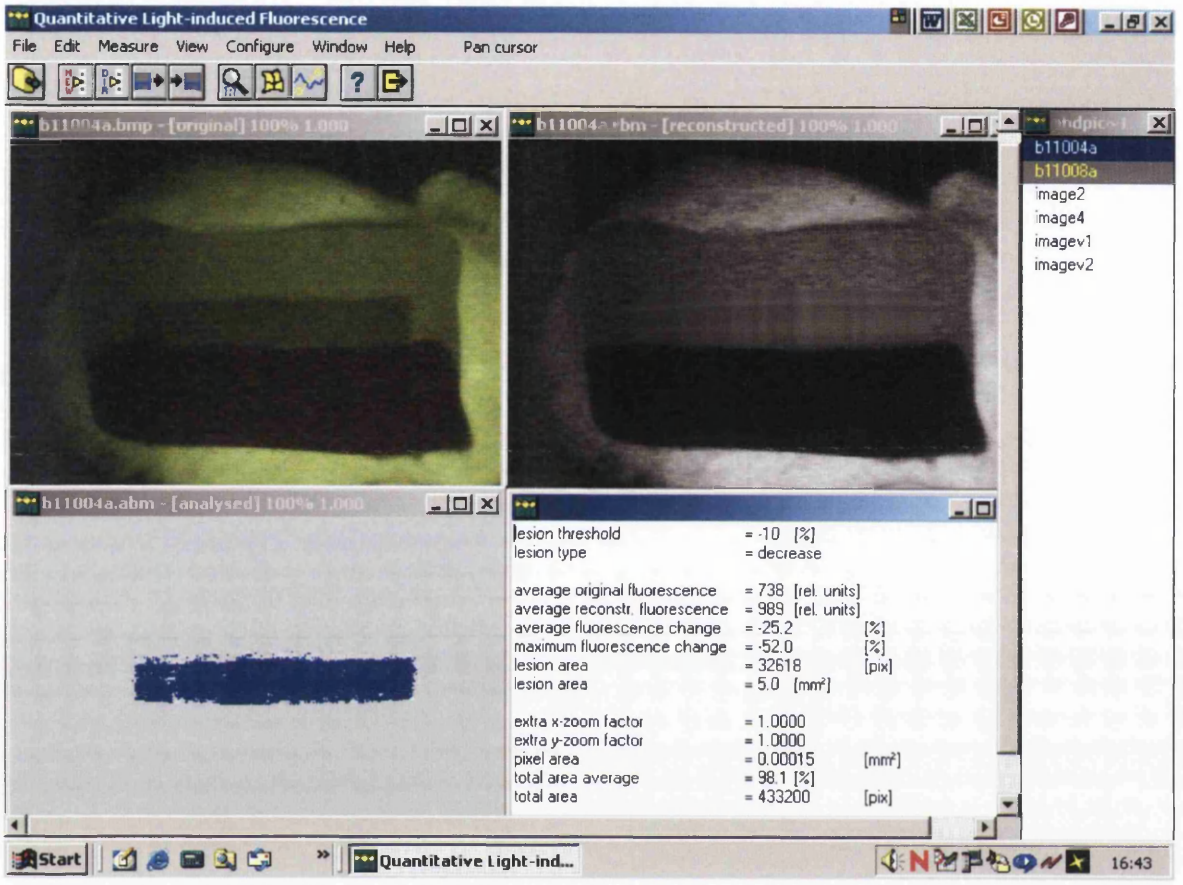


Figure 26 - Image of computer screen showing a QLF image of a tooth block covered with fissure sealant with the analysis patch superimposed.



Thus the three QLF parameters recorded were: Area (QLF^{AREA}); Average % Fluorescence Change (QLF^{AVER}) and Maximum % Fluorescence Change (QLF^{MAX}). Examples of the analysis given from the Clin-QLF software are shown in Figures 27 and 28.

Figure 27 - Image of the computer screen following analysis (with the Clin-QLF programme) of the varnished tooth block illustrated above



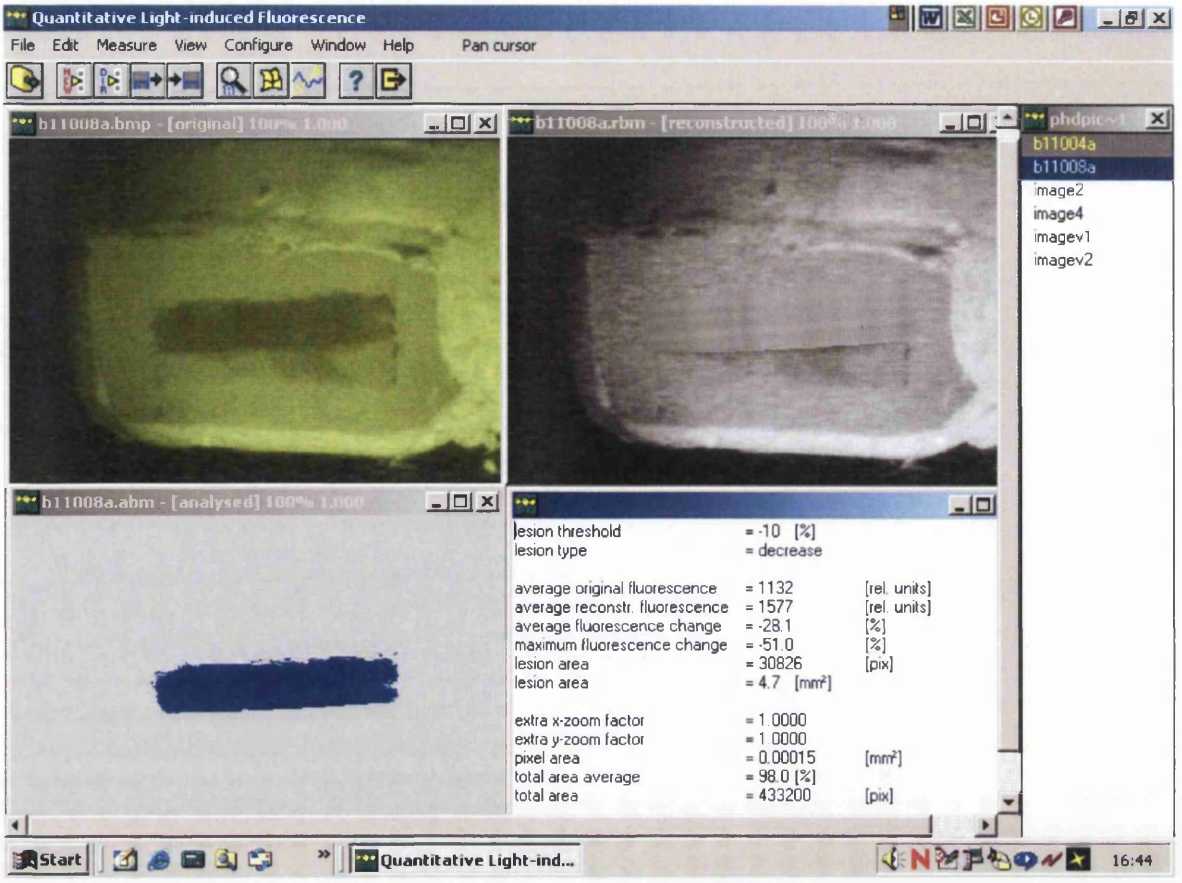
The QLF results for the tooth block illustrated in Figure 27 were:

QLF^{AREA}: 5.0 mm²

QLF^{MAX}: -52 % fluorescence loss

QLF^{AVER}:-25.2 % fluorescence loss

Figure 28 - Image of the computer screen following analysis (with the Clin-QLF programme) of the fissure sealed tooth block illustrated above



4.2.10 *Data handling and statistical methodology*

All data collected were entered into a custom-designed database (Microsoft Access®, Microsoft ® Corporation, USA). This was to permit storage and manipulation of the large amount of data from the image analysis using both TMR and QLF. Storage of these data in the custom database allowed subsequent manipulation so they could be imported into the statistical computer package (Minitab, Minitab Inc, PA, USA).

The main objective of the statistical analysis was to examine systematically whether the difference between the control lesions and the lesions exposed to the protocols were related to any of the factors of interest (i.e. group, experiment and site). Descriptive statistics, i.e. mean, standard deviation and range, were calculated for integrated mineral loss and lesion depth, for TMR, and QLF^{AREA}, QLF^{MAX} and QLF^{AVER}. Data were plotted to examine their distributions. All of the data were approximately normally distributed; therefore, parametric statistical tests were applied.

In the descriptive analyses of both the QLF and TMR outcomes, the data were summarised across all appropriate specimens. The decision was taken to present the data in this manner, rather than determining summary statistics on a subject basis (i.e. across all sites per subject), as it was felt that, at a descriptive level, the large between-specimen variability was of interest. However, the formal statistical analyses using the general linear models (detailed further below), took into account the multiple observations from each subject.

For both the TMR and QLF 'baseline' control data, statistical analysis was used to examine whether the 'baseline' control lesions were of similar magnitude with regards to group and experiment. Because of the composition of the data, this was undertaken slightly differently for TMR and QLF. Thus the statistical methodology will be described separately for TMR and QLF data analysis. However, for all statistical tests, p-values of less than 0.05 were taken to be statistically significant.

4.2.10.1 Statistical methodology for TMR

Throughout this section, the objective was to examine the mineral content (integrated mineral loss and lesion depth), as measured by TMR, and to investigate potential differences between groups, experiments and sites. The following data were examined:

- all specimens
- subdivided by group - Treatment only (T)
  Treatment and Dentifrice (TD)
- subdivided by experiment - 0.5mg Fluoride in milk, three times per day
  1.5mg Fluoride in milk, once per day
  200ml milk, once per day
  200ml milk, three times per day
  no beverage (negative control)
- subdivided by site - 1 - upper posterior buccal, right
  2 - upper midline buccal
  3 - upper posterior buccal, left
  4 - upper posterior palatal, left

5 - upper midline palatal

6 - upper posterior palatal, right

7 - lower posterior buccal, right

8 - lower posterior buccal, left

9 - lower posterior lingual, left

10 - lower posterior lingual, right

The TMR parameters of integrated mineral loss and lesion depth were examined in the following way:

- Covered area of the lesion (proxy 'baseline' control)
- Area of the lesion exposed to the experimental protocol
- Difference between the control and exposed lesion.

When considering the covered (control) lesion data, the mean mineral content of the lesions allocated to the two independent treatment groups (T and TD) were compared using a two sample t-test and corresponding 95% confidence interval. The mean mineral content data for the covered lesions, by the five experiments to which the specimens were allocated, were compared using one-way ANOVA. Where required, follow-up Bonferroni-corrected multiple comparisons were used to identify which of the five experiments differed significantly.

To determine which of the factors of interest detailed above had a significant effect on change in mineral content, the difference of covered lesion data minus exposed lesion data, was calculated for each block. A general linear model procedure was then used to determine which of the factors: experiment, group and site, together with potential interactions between these factors, had a significant effect on the difference, for integrated mineral loss and lesion depth separately.

Additionally, it was decided to examine the tooth specimens from sites where it was anticipated there would be the greatest amount of remineralisation in further detail. The four tooth blocks that were placed in Sites 1, 3, 9 and 10 (upper posterior buccal sites and lower posterior lingual sites) were likely to be exposed to the greatest amount of salivary

flow. Thus the greatest amount of remineralisation was anticipated in these blocks. Further analysis was undertaken of this group of blocks, in a manner similar to that listed above.

4.2.10.2 Statistical methodology for QLF

The objective was to examine the area (QLF^{AREA}) and change in the fluorescence as an average (QLF^{AVER}) and a maximum (QLF^{MAX}), as measured by QLF and to again look for potential differences in terms of the factors: group, experiment and site; as defined in the list in Section 4.2.10.1 above.

The QLF parameters of QLF^{AREA}, QLF^{AVER} and QLF^{MAX} were examined for:

- baseline QLF (Image 2) data
- difference between the baseline (Image 2) and post-experimental protocol QLF data (Image 10).

When considering the baseline (Image 2) data, the means of the lesions allocated to the two independent treatment groups (T and TD) were compared using a two sample t-test and corresponding 95% confidence interval. The mean baseline (Image 2) data for the lesions, by the five experiments to which the specimens were allocated, were compared using one-way ANOVA. Where required, follow-up Bonferroni-corrected multiple comparisons were used to identify which of the five experiments differed significantly.

To determine which of the factors of interest detailed above had a significant effect on change in mineral content, the difference of baseline (Image 2) data and final (Image 10) data, was calculated for each block. For QLF^{AVER} and QLF^{MAX} the difference was taken as final image (Image 10) minus baseline (Image 2) image, because negative numbers were being dealt with, as explained in Section 4.3.2.2. The exception to this was QLF^{AREA} which was taken as Image 2 (baseline) - Image 10 (final image), with a positive number signifying further demineralisation.

A general linear model procedure was then used to determine which of the factors: experiment, group and site, together with potential interactions between these factors, had a significant effect on the difference for QLF^{AREA}, QLF^{MAX} and QLF^{AVER} separately.

For the reasons described in Section 4.2.10.1 above, it was decided to examine the tooth blocks from sites where it was anticipated there would be the greatest amount of remineralisation. Therefore, the four tooth blocks placed in Sites 1, 3, 9 and 10 (upper posterior buccal sites and lower posterior lingual sites) were examined in further detail. The statistical analysis undertaken for this group of blocks was in a manner similar to that detailed above.

4.3 Results

Of the 50 subjects who agreed to attend for screening, 32 fulfilled the selection criteria and were recruited. Of the remaining 18, two failed to attend for screening, nine declined the recruitment invitation, and seven were unsuitable either because of inadequate salivary flow or an unsuitable intra-oral flora.

A total of 32 volunteers were recruited in case some failed to complete the study. Three volunteers failed to start the experimental protocols due to illness. Hence, 29 volunteers started at least one of the five experiments within the study (mean age 68 years, SD 9 years). There were 15 male volunteers (mean age 70 years, SD 9 years) and 14 female volunteers (mean age 67 years, SD 9 years). The age range of the volunteers is shown in Table 7.

Table 7 - Age range of volunteers at start of study

Age Range (years)	59 or less	60 - 69	70 -79	80 or over
Number of volunteers (29)	5	11	9	4

Of these 29 volunteers, 13 were randomly allocated to the T group and 16 were randomly allocated to the TD group. Of the 29 subjects, 25 volunteers completed all five experimental protocols (12 were in the T group and 13 were in the TD group). Details relating to those who failed to do so are listed as follows:

Subject 1, (TD group) had an unexpected illness and died after completing two experimental protocols.

Subject 18, (TD group) withdrew after completing two experimental protocols.

Subject 20, (TD group) completed three experimental protocols then failed to return because of chronic illness.

Subject 17, (T group) completed four experimental protocols, had an accident during the final protocol and was too immobile to return and repeat the final experiment.

Therefore of the 145 possible experiments (i.e. 29 subjects x 5 experiments), results were obtained for 136 (25 x 5; 2 x 2; 1 x 3; 1 x 4). In total, 1360 (136 x 10) tooth blocks were used in these experiments.

The study design and randomisation were planned to allow analysis of the effect of order of experiments. However, because an insufficient number of the subjects completed all five of the experimental protocols, it was not possible to incorporate this factor into the statistical models.

Compliance with the experimental protocols was assessed at the end of all five experimental protocols with a postal questionnaire (Appendix 8). The results of this are displayed in Section 4.3.4.

4.3.1 Results of transverse microradiography and image analysis (TMR) evaluation

4.3.1.1 Survival of specimens suitable for TMR evaluation

Of the 1360 tooth blocks used in the experimental protocols, there were 939 blocks at the end of the study from which a section could be cut with a matched "pair" of a covered (control) and exposed lesion suitable for analysis by transverse microradiography. The 421 tooth blocks without TMR results were because of either a loss of the tooth block during the experimental protocol or a mix up of tooth blocks on retrieval from the intraoral appliance (four) or failure to cut a section with a matched covered and exposed lesion suitable for TMR evaluation.

4.3.1.2 Analysis of covered (control) TMR data

Summary statistics of TMR integrated mineral loss (IML) and lesion depth (LD) values for the covered (control) lesions from the tooth blocks were calculated to determine if there

were differences in the covered data when analysed by group and by experiment. These IML and LD values were considered to be proxy values for baseline lesion size (control), as they were calculated on completion of the experiment.

Covered lesions

A. Integrated mineral loss

Table 8 contains the summary statistics for IML data for all the covered TMR lesions. A wide range of values was seen and the standard deviation was almost half that of the mean (mean = 2214, St. Dev. = 1053).

Table 8 - Summary statistics of integrated mineral loss (IML) of all covered TMR lesions

N*	Mean	Standard Deviation	Range
939	2214	1053	(217, 7369)

Integrated mineral loss is measured in %Vol mineral.µm
N* = number of lesions available for analysis

B. Lesion depth

Table 9 contains the summary statistics of lesion depth (LD) for all of the covered TMR lesions. The standard deviation was again relatively high when compared with the mean. The range of values was wide, with the deepest lesion being almost three times that of the mean.

The summary statistics in Tables 8 and 9 demonstrate the large variability in terms of the proxy control lesions, suggesting that the covered lesions were not all of similar size with regard to either integrated mineral loss or lesion depth.

Table 9 - Summary statistics of lesion depth (LD) of all covered TMR lesions

N*	Mean	Standard Deviation	Range
939	75.9	28.4	(10.4, 212.4)

Lesion depth is measured in µm
N* = number of lesions available for analysis

Covered lesions by group

Tables 10 and 11 show the results of division of the TMR data into the two treatment groups, Treatment (T) and Treatment plus dentifrice (TD). The T group was smaller than the TD group by 73 tooth specimens.

A. Integrated mineral loss

Table 10 demonstrates a difference in the IML values of the covered lesions between the T and TD groups. The mean IML of the T group was greater than that of the TD group. This was of the order of approximately 12%. The standard deviations of both groups were similar and the ranges were very large. Use of a two sample t-test showed a statistically significant difference in the proxy control values between the two groups, with the T group having greater IML than the TD group. The 95% confidence interval for the mean difference in IML was 155 to 425 %Vol mineral.μm.

Table 10 - Summary statistics of IML of all covered TMR lesions by treatment group

Group	N*	Mean (St. Dev.)	Range	2 sample t-test	95% CI (T - TD)
T	433	2371 (1091)	237 - 7369	**p < 0.001**	(155, 425)
TD	506	2081 (1002)	217 - 5791		

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of lesions available for analysis

B. Lesion depth

The summary statistics of lesion depth, shown in Table 11, demonstrate the differences in the proxy control LD data between the two groups, T and TD. Again, the standard deviations and ranges were large. A two sample t-test demonstrated a statistically significant difference between T and TD, with the mean lesion depth in the T group being greater than that in the TD group. The 95% confidence interval for the mean difference was 7.4 to 14.6 μm.

Table 11- Summary statistics of LD of all covered TMR lesions by treatment group

Group	N*	Mean (St. Dev.)	Range	2 sample t-test	95% CI (T - TD)
T	433	81.8 (29.6)	10.4 - 212.4	**p < 0.001**	(7.4, 14.6)
TD	506	70.8 (26.4)	12.4 - 168.6		

Lesion depth is measured in μm
N* = number of lesions available for analysis

Covered lesions by experiment

The summary statistics of the covered lesions following random allocation into the five experimental protocols are shown in Tables 12 and 13. These tables show that equal numbers of lesions were not evaluated for each of the five experiments. The lowest numbers of tooth lesions were evaluated for the no beverage experiment, where there were 33 fewer specimens than in the experiment with the largest number of lesions (the 1.5mg F x 1/day).

A. Integrated mineral loss

Table 12 shows the mean, standard deviation and range of the proxy control IML data for the tooth lesions allocated to the five experimental protocols. The mean data demonstrate the relatively large lesion sizes. The 1.5mg F x 1/day protocol had the highest mean lesion value and the lowest mean IML value occurred in the specimens allocated to the 0.5mg F x 3/day protocol. However, the standard deviations and ranges were also large, demonstrating the large variability across the lesions within those allocated to each experiment. A one-way analysis of variance, used to compare the control IML areas across the five independent experiment groups, resulted in a p-value of 0.043. This suggests that there was a statistically significant difference between the mean IML values across the five groups. However, follow-up Bonferroni-corrected multiple comparisons indicated that the only difference which reached close to statistical significance (p=0.054) was between the 0.5 mg F x 3/day and 1.5 mg F x 1/day protocols.

Table 12 - Summary statistics of IML of covered TMR lesions by experiment

Experiment	N*	Mean (St. Dev)	Range
0.5mg F x 3/day	179	2080 (950)	(351, 5682)
1.5mg F x 1/day	203	2380 (1198)	(237, 7369)
200mL milk x1/day	192	2167 (1083)	(355, 5395)
200mL milk x3/day	195	2150 (807)	(602, 4807)
No beverage	170	2283 (1162)	(216, 6343)

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of lesions available for analysis

B. Lesion depth

Summary statistics for covered TMR lesions by experiment for LD are shown in Table 13. These findings are similar to the IML data. The lesions from the 1.5mg F x 1/day and the 0.5mg F x 3/day protocols had the greatest and least mean lesion depths, respectively. The standard deviations and the ranges were large and again, the narrowest standard deviation and range occurred in the 200mL milk x 3/day group, indicating that the depths of the lesions randomly allocated to this group were the most consistent. When formal analysis using a one way analysis of variance was applied, there was no evidence of a statistically significant difference between the mean LD values of the proxy control areas across the five groups ($p = 0.126$).

Table 13 - Summary statistics of LD of covered TMR lesions by experiment

Experiment	N*	Mean (St. Dev.)	Range
0.5mg F in 200 mL milk x 3/day	179	73.3 (26.7)	(17.5, 161.7)
1.5mg F in 200 mL milk x 1/day	203	80.4 (30.2)	(10.4, 212.4)
200mL milk x1/day	192	74.8 (29.3)	(14.2, 169.8)
200mL milk x3/day	195	75.4 (22.0)	(25.6, 153.3)
No beverage	170	74.8 (32.9)	(12.4, 168.6)

Lesion depth is measured in µm.
N* = number of lesions available for analysis

4.3.1.3 Comparison of the difference between covered (control) and exposed lesions measured by TMR

Descriptive statistics of the differences between the covered (control) and the exposed lesions for IML and LD are shown in Tables 14 - 21. These measurements represented the changes in lesions over the experimental periods.

The calculation was covered minus exposed (cov-exp). If remineralisation occurred this resulted in a positive IML or LD difference but if further demineralisation occurred then this resulted in a negative IML or LD difference. This can be illustrated as follows:

Section from tooth specimen 1630, IML

- (cov-exp) = 2018 - 453 = 1565 %Vol mineral.µm
- this is a positive value and indicates *remineralisation.*

Section from tooth specimen 1342, IML

- (cov - exp) = 1379 - 2585 = -1206 %Vol mineral.μm

- this is a negative value and indicates further *demineralisation.*

All lesions

A. Integrated mineral loss

The descriptive statistics for covered and exposed IML, as well as the difference (covered - exposed), are shown in Table 14.

Table 14 - Summary statistics of IML, covered, exposed and the differences between covered and exposed

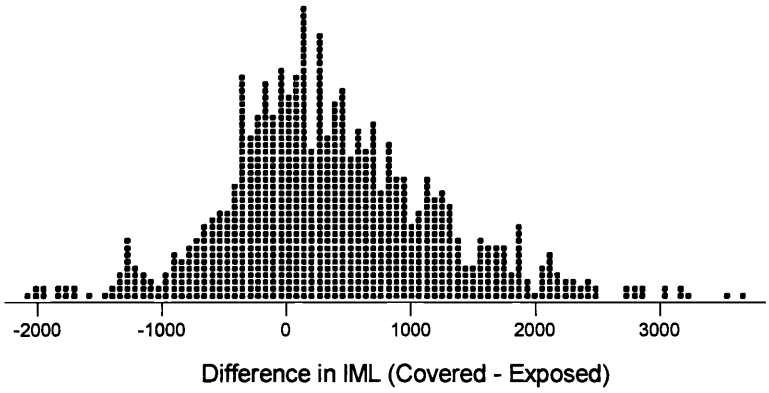
	N*	Mean (St. Dev.)	Range
Covered	939	2214 (1053)	(217, 7369)
Exposed	939	1848 (928)	(146, 5790)
Difference (Cov-Exp)	939	366 (863)	(-2059, 3639)

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of lesions available for analysis

There was on average a mineral gain of 366 %Vol mineral.μm, indicating remineralisation of the tooth specimens. However, it should be noted that the standard deviation of the differences were high. The range for the difference between covered and exposed lesions was very wide and indicated that not all of the tooth specimens behaved in a similar manner during the experimental protocols.

When plotted, the difference in IML (covered - exposed), has an approximately normal distribution (Figure 29).

Figure 29 - Dot plot of differences in IML (covered - exposed) TMR lesions



Integrated mineral loss is measured in %Vol mineral.µm (n=939)

B. Lesion depth

The descriptive statistics for covered and exposed LD and the difference (covered - exposed) are shown in Table 15.

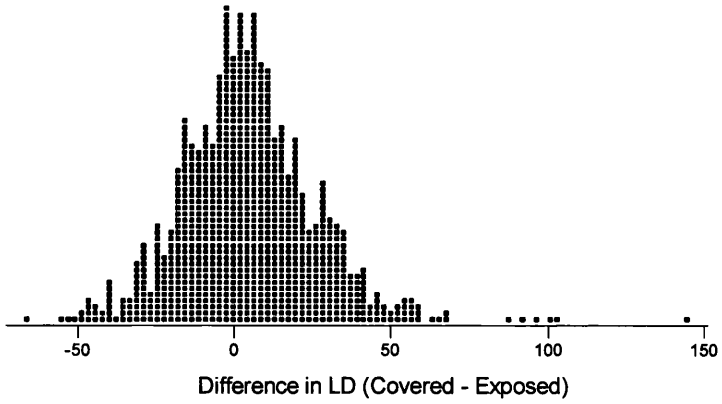
Table 15 - Summary statistics of LD, covered, exposed and the differences between covered and exposed lesions

	N*	Mean (St. Dev.)	Range
Covered	939	75.9 (28.4)	(10.4, 212.4)
Exposed	939	71.2 (25.5)	(6.6, 180.4)
Difference (Cov-Exp)	939	4.6 (21.8)	(-65.3, 143.6)

Lesion depth is measured in µm
N* = number of lesions available for analysis

The mean lesion depths of the covered and exposed lesions were similar, making the difference between them small. However, the standard deviation of the mean differences (covered - exposed) were large. This indicates that the lesions were not all behaving in a similar manner. This was confirmed from the extent of the range, indicating that some lesions demineralised further and others remineralised. When plotted, the difference between lesion depths (covered - exposed) has an approximately normal distribution (Figure 30).

Figure 30 - Dot plot of differences in LD (covered - exposed) all lesions



Lesion depth is measured in µm (n=939)

Lesions by group

When dividing the tooth specimens into the different treatment groups and experiments, only the summary statistics for the difference between the covered and exposed tooth specimens (covered - exposed) are displayed.

A. **Integrated mineral loss**

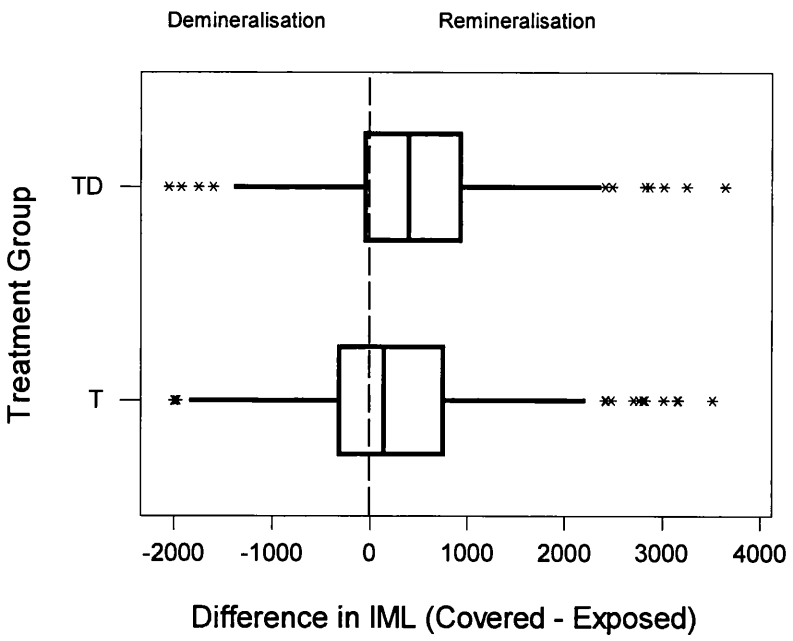
Table 16 - Summary statistics for the IML differences (covered - exposed) by treatment group

Group	N*	Mean IML difference (St. Dev.)	Range
T	433	257 (903)	(-2004, 3515)
TD	506	459 (817)	(-2059, 3639)

Integrated mineral loss is measured in %Vol mineral.µm
N* = number of lesions available for analysis

As discussed in Section 4.3.1.2 above, there were not equal numbers of tooth specimens available from the two treatment groups T and TD. However, Table 16 demonstrates an IML difference for the TD group which is just over 200 %Vol mineral.µm, greater than the IML difference of the T group. In other words, both groups showed a net remineralisation but there was more remineralisation with the TD group. The standard deviations of these IML differences were very large, and were approximately two to three times the size of the mean. The ranges of both groups were extremely wide and include values that were both negative and positive, indicating that some of the lesions demineralised and others remineralised (Figure 31).

Figure 31 - Box plot of the IML differences (covered - exposed) by treatment group



Integrated mineral loss is measured in %Vol mineral.µm

B. **Lesion depth**

Table 17 - Summary statistics for the LD differences (covered - exposed) by treatment group

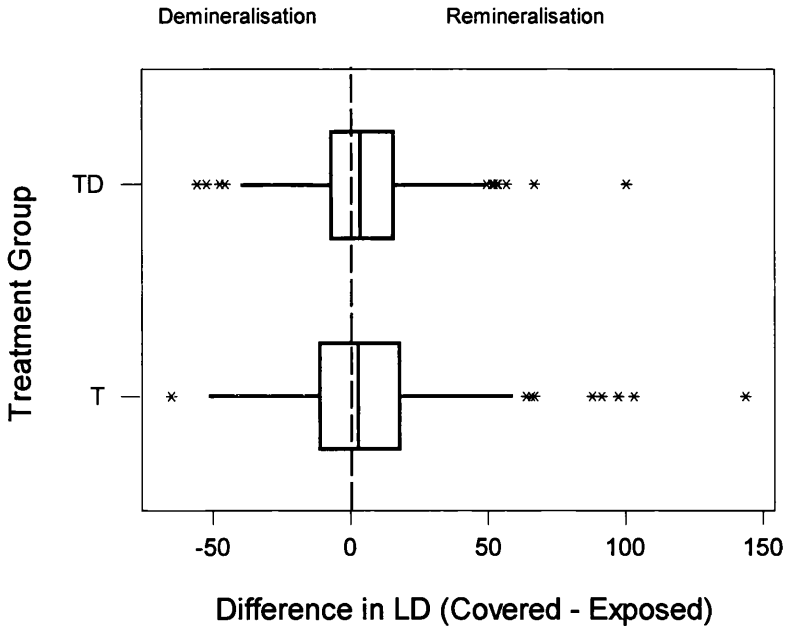
Group	N*	Mean LD difference (St. Dev.)	Range
T	433	4.7 (24.9)	(-65, 143.6)
TD	506	4.6 (18.8)	(-56, 100.5)

Lesion depth is measured in µm
N* = number of lesions available for analysis

Table 17 demonstrated that there was very little difference in the mean LD difference between the two groups. The TD group had a very slightly smaller mean lesion depth difference. The standard deviations were large and were four to five times the size of the

mean. The ranges were wide and included negative as well as positive values, indicating that there had been further lesion demineralisation as well as remineralisation (Figure 32).

Figure 32 - Box plot of the LD differences (covered - exposed) by treatment group



Lesion depth is measured in µm

Lesions by experiment

The summary statistics of the IML- and LD-difference (covered - exposed) lesion data by experiment are shown below.

A. Integrated mineral loss

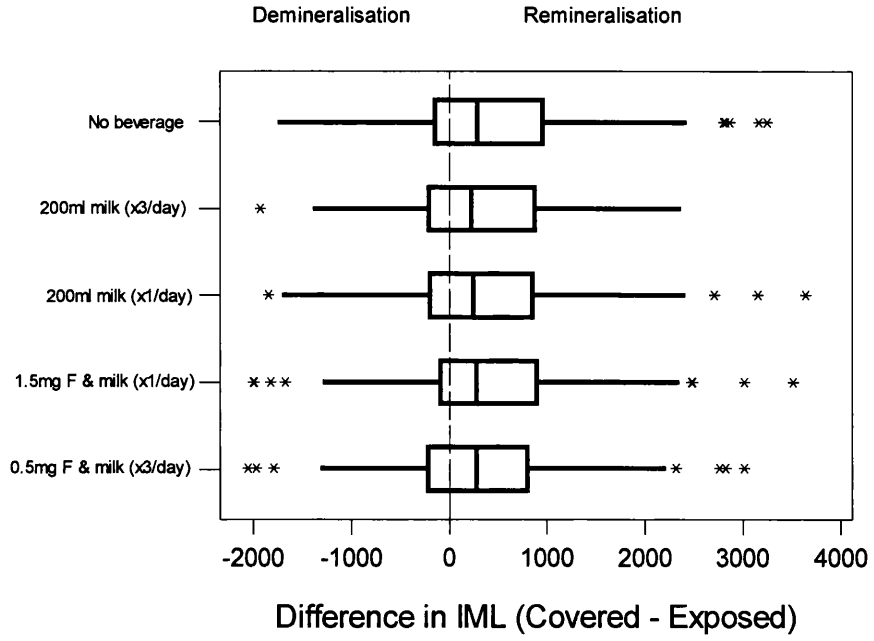
Table 18 - Summary statistics for the IML differences (covered - exposed) by experiment

Experiment	N*	Mean IML difference (St. Dev.)	Range
0.5mg F in 200 mL milk x 3/day	179	331 (855)	(-2059, 3015)
1.5mg F in 200 mL milk x 1/day	203	411 (890)	(-2004, 3515)
200mL milk x1/day	192	345 (882)	(-1852, 3639)
200mL milk x3/day	195	313 (800)	(-1935, 2371)
No beverage	170	435 (892)	(-1752, 3253)

Integrated mineral loss is measured in %Vol mineral.µm
N* = number of specimens available for analysis

Table 18 contains the summary statistics for the IML differences (covered - exposed) for the five experimental groups. All IML mean differences were positive indicating remineralisation (Figure 33). The smallest IML difference occurred in the 200mL milk x 3/day group and the greatest IML difference was in the no beverage group, which had approximately a third greater mean IML difference than the smallest mean difference, 200mL milk x 3/day.

Figure 33 - Box plot of the IML differences (covered - exposed) by experiment



Integrated mineral loss is measured in %Vol mineral.µm

B. Lesion depth

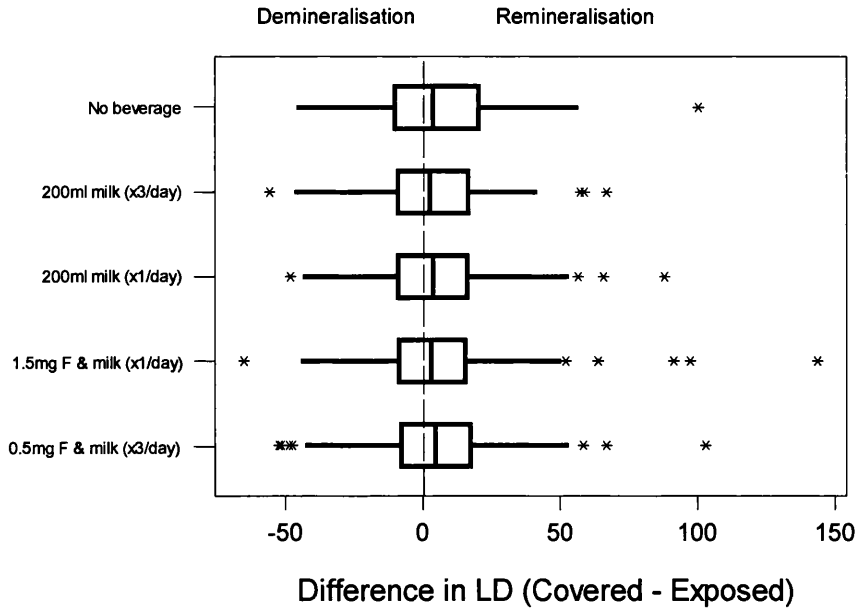
Table 19 - Summary statistics for the LD differences (covered - exposed) by experiment

Experiment	N*	Mean LD difference (St. Dev.)	Range
0.5mg F x 3/day	179	5.0 (22.6)	(-52.3, 102.9)
1.5mg F x 1/day	203	5.3 (23.4)	(-65.3, 143.6)
200mL milk x1/day	192	4.8 (21.0)	(-48.1, 87.9)
200mL milk x3/day	195	2.7 (20.5)	(-55.9, 66.7)
No beverage	170	5.6 (21.4)	(-45.9, 100.5)

Lesion depth is measured in µm
N* = number of lesions available for analysis

From the data in Table 19 it can be observed that all mean LD differences were positive indicating on average there was remineralisation through a reduction in lesion depth. The smallest LD difference occurred in the 200mL milk x 3/day group. The largest LD difference occurred in the no beverage group. The standard deviations were large and the ranges were very wide (Figure 34). The ranges included negative and positive values, demonstrating that the lesions in the five groups were behaving in an inconsistent manner.

Figure 34 - Box plot of the LD differences (covered - exposed) by experiment



Lesion depth is measured in µm

Lesions by site

The summary statistics of the IML- and LD-difference (covered-exposed) lesion data by site are shown below.

A. Integrated mineral loss

Table 20 - Summary statistics for the IML differences (covered - exposed) by site

Site	N*	Mean IML difference (St. Dev.)	Range
1 – upper right buccal	121	423	(-1852, 2347)
2 – upper mid-labial	117	208	(-2004, 3160)
3 – upper left buccal	120	344	(-1793, 3016)
4 – upper left palatal	48	344	(-849, 3152)
5 – upper mid-palatal	49	364	(-1313, 3254)
6 – upper right palatal	54	399	(-1334, 2412)
7 – lower right buccal	111	396	(-1334, 2412)
8 – lower left buccal	102	543	(-2001, 3639)
9 – lower left lingual	108	350	(-1683, 2763)
10 – lower right lingual	109	311	(-1709, 3515)

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of specimens available for analysis

From the data in Table 20 it can be observed that there were positive values in all sites indicating remineralisation. The greatest mean mineral gain occurred at site 8 and the least remineralisation occurred at site 2. The mean standard deviations of the differences were high and the ranges were very wide, and included negative and positive values, demonstrating that the lesions in the different sites were behaving in an inconsistent manner.

Table 21 - Summary statistics for the LD differences (covered - exposed) by site

Site	N*	Mean LD difference (St. Dev.)	Range
1 – upper right buccal	121	4.6	(-51.6, 58.8)
2 – upper mid-labial	117	1.7	(-55.9, 55.1)
3 – upper left buccal	120	3.4	(-46.9, 100.5)
4 – upper left palatal	48	0.71	(-43.0, 65.8)
5 – upper mid-palatal	49	3.1	(-45.8, 52.3)
6 – upper right palatal	54	7.1	(-31.1, 56.8)
7 – lower right buccal	111	5.7	(-47.4, 63.7)
8 – lower left buccal	102	9.6	(-65.3, 91.2)
9 – lower left lingual	108	4.8	(-52.3, 102.9)
10 – lower right lingual	109	4.5	(-32.7, 143.6)

Lesion depth is measured in µm
N* = number of lesions available for analysis

From the data in Table 21 it can be observed that there were positive values in all sites indicating remineralisation. The greatest mean mineral gain occurred at site 8 and the least remineralisation occurred at site 4. The mean standard deviations of the differences were high and the ranges were very wide, and included negative and positive values, again demonstrating that the lesions in the different sites were behaving in an inconsistent manner.

Formal analysis using a general linear model procedure

Formal analysis of the data used a general linear model (GLM) procedure to determine which of the three factors, experiment, group and site, together with a random subject effect, had a significant influence on the difference (covered - exposed) data for both IML and LD. Furthermore, the GLM procedure was able to examine potential interactions between these factors.

A. Integrated mineral loss

When the difference (covered - exposed) in IML was modelled on experiment, group and site, together with a random subject effect, there was evidence of a statistically significant interaction effect of 'group and site' ($p = 0.002$), together with a significant subject effect ($p = 0.001$). The subject effect was expected and confirms that the subjects were all different in terms of their 'response'. There was insufficient evidence to suggest that the experiment factor had a statistically significant effect on the outcome ($p = 0.445$).

Given the evidence of the combined effect of 'group and site', the outcome of difference in IML was modelled on experiment and site for the two groups separately. The summary statistics of the IML differences by site, for T and TD separately, are shown in Tables 22 and 23, respectively. Data from sites 4, 5 and 6 should be interpreted with caution because of the smaller number of specimens retained in the intra-oral appliances. There was insufficient evidence of an effect of experiment for either the TD group ($p = 0.717$) or the T group ($p = 0.139$).

In the T group (Table 22) there was variation in the mean IML differences with site 8 having the largest positive mean IML differences (remineralisation) of +979 %Vol mineral.μm and site 3 having the smallest mean IML difference (remineralisation) of +3 %Vol mineral.μm. The standard deviations were consistently large, as were the ranges. The GLM procedure determined that there was insufficient evidence to suggest a statistically significant site effect for the T group ($p = 0.214$).

Table 22 - Summary statistics for the IML differences (covered - exposed) by site for the T group

Site	N*	Mean IML difference (St. Dev.)	Range
1 – upper right buccal	55	149 (823)	(-1852, 2092)
2 – upper mid-labial	53	378 (948)	(-2004, 3160)
3 – upper left buccal	57	3 (865)	(-1793, 2047)
4 – upper left palatal	17	287 (964)	(-1793, 2047)
5 – upper mid-palatal	23	204 (806)	(-1313, 2095)
6 – upper right palatal	29	327 (875)	(-1333, 2414)
7 – lower right buccal	50	404 (965)	(-1981, 2825)
8 – lower left buccal	45	979 (1062)	(-2001, 3021)
9 – lower left lingual	50	244 (886)	(-1683, 2793)
10 – lower right lingual	54	184 (886)	(-1709, 3515)

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of lesions available for analysis

In the TD group (Table 23), the smallest mean IML difference occurred in site 2 with +67 %Vol mineral.μm and the largest mean IML difference occurred in sites 1 and 3 with +652 %Vol mineral.μm. Again the standard deviations and range were consistently large. The GLM procedure determined that there was a statistically significant effect of site for the TD group ($p = 0.002$).

Table 23 - Summary statistics for the IML differences (covered - exposed) by site for the TD group

Site	N*	Mean IML difference (St. Dev.)	Range
1 – upper right buccal	66	652 (710)	(-530, 2347)
2 – upper mid-labial	64	67 (849)	(-1935, 2485)
3 – upper left buccal	63	652 (912)	(-1606, 3016)
4 – upper left palatal	31	375 (675)	(-849, 2172)
5 – upper mid-palatal	26	505 (821)	(-657, 3254)
6 – upper right palatal	25	482 (558)	(-1143, 1284)
7 – lower right buccal	61	390 (806)	(-2059, 2831)
8 – lower left buccal	57	592 (931)	(-1752, 3639)
9 – lower left lingual	58	442 (768)	(-1322, 2080)
10 – lower right lingual	55	435 (773)	(-1264, 2323)

Mineral Loss is measured in %Vol mineral.µm
N* = number of lesions available for analysis

B. Lesion depth

When the LD difference (covered - exposed) was modelled on experiment, group and site, together with a random subject effect, there was again evidence of a statistically significant interaction effect of 'group and site' ($p = 0.043$), together with the expected significant subject effect ($p = 0.004$). There was insufficient evidence to suggest that the experiment factor had a statistically significant effect on the outcome ($p = 0.276$). There was also insufficient evidence of an experiment effect for the TD group ($p = 0.583$) or the T group ($p = 0.675$).

Given the significant interaction effect of 'group and site', the summary statistics for the LD difference (covered - exposed) by site, for T and TD separately, are shown in Tables 24 and 25, respectively. As discussed previously, data from sites 4, 5 and 6 should be interpreted with caution because of the smaller number of specimens retained in the intra-oral appliances.

In the T group (Table 24), there was much variation in the mean LD differences, with site 8 having the largest positive change in the mean LD difference of +11.3 μm (indicating remineralisation) while site 3 had the largest negative change in the mean LD difference of -3.3 μm, indicating further demineralisation through an increase in lesion depth at this site. A similar, but smaller, result difference was noted for site 4. The standard deviations were consistently large, as were the ranges, particularly in sites 9 and 10. The GLM procedure determined that there was insufficient evidence to suggest a significant site effect for the T group ($p = 0.295$).

Table 24 - Summary statistics for the LD differences (covered - exposed) for site, for the T group

Site	N*	Mean LD difference (St. Dev.)	Range
1 – upper right buccal	55	3.1 (23.2)	(-51.6, 58.8)
2 – upper mid-labial	53	4.6 (21.9)	(-48.8, 55.1)
3 – upper left buccal	57	-3.3 (22.5)	(-46.9, 44.2)
4 – upper left palatal	17	-0.2 (25.5)	(-43.0, 65.8)
5 – upper mid-palatal	23	0.6 (18.4)	(-24.7, 34.9)
6 – upper right palatal	29	7.8 (21.7)	(-31.1, 53.4)
7 – lower right buccal	50	6.7 (23.8)	(-45.9, 63.7)
8 – lower left buccal	45	11.3 (24.3)	(-65.3, 91.2)
9 – lower left lingual	50	7.4 (29.7)	(-38.8, 102.9)
10 – lower right lingual	54	6.7 (30.5)	(-29.8, 143.6)

Lesion depth is measured in μm
N* = number of lesions available for analysis

In the TD group (Table 25), the largest negative change in the mean LD difference occurred at site 2 (-0.71 μm) and the largest positive change in the mean LD difference occurred at site 3 (+9.41 μm). Again, the standard deviations and range were large. When the various factors were examined with relation to the outcome for the two groups separately, there was some evidence of a site effect for the TD group ($p = 0.088$), although this was not statistically significant at the 5% level.

Table 25 - Summary statistics for the LD differences (covered - exposed) for site, for the TD group

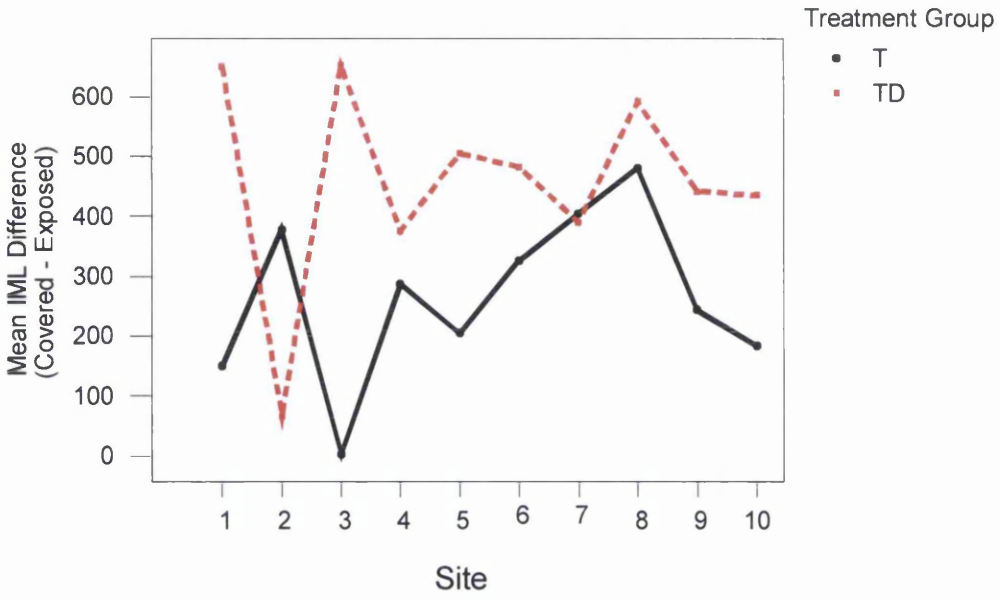
Site	N*	Mean LD difference (St. Dev.)	Range
1 – upper right buccal	66	5.9 (16.1)	(-31.2, 48.8)
2 – upper mid-labial	64	-0.7 (19.1)	(-55.9, 53.7)
3 – upper left buccal	63	9.4 (22.2)	(-31.8, 100.5)
4 – upper left palatal	31	1.2 (15.4)	(-16.2, 49.7)
5 – upper mid-palatal	26	5.4 (19.2)	(-45.8, 52.3)
6 – upper right palatal	25	6.4 (17.4)	(-25.1, 56.8)
7 – lower right buccal	61	4.9 (17.5)	(-47.4, 40.4)
8 – lower left buccal	57	8.3 (21.5)	(-39.8, 56.8)
9 – lower left lingual	58	2.5 (17.7)	(-52.3, 38.0)
10 – lower right lingual	55	2.4 (17.9)	(-32.7, 51.4)

Lesion depth is measured in μm
N* = number of lesions available for analysis

Summary

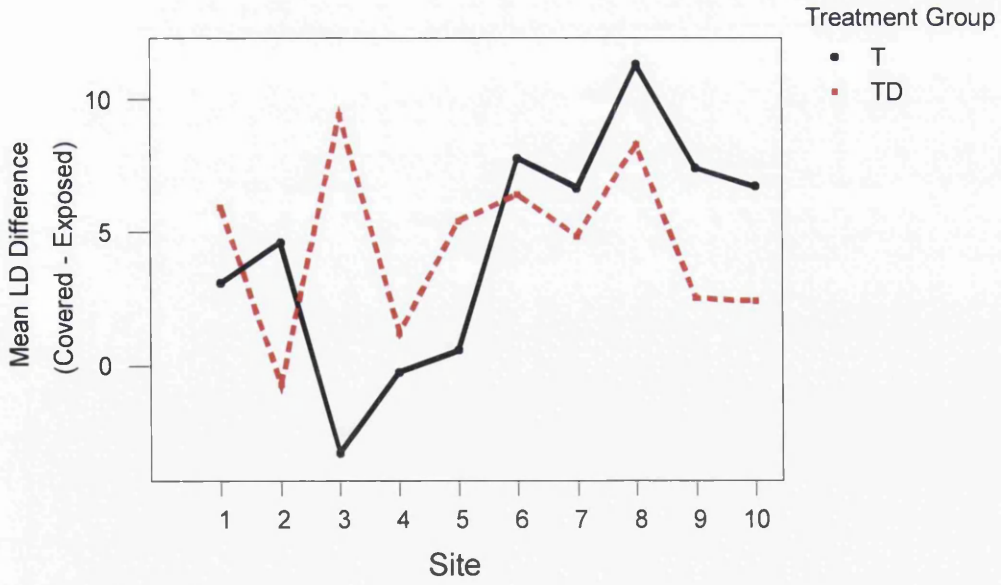
It is apparent from the results of the analysis of the IML and LD difference data that there was variability in the way that the lesions behaved in the different treatment groups at different sites. This is illustrated further in the following interaction plots of the IML and LD mean difference data, charted by site and group in Figures 35 and 36.

Figure 35 - Interaction plot of the mean IML differences by site and group



Mineral Loss is measured in %Vol mineral.µm

Figure 36 - Interaction plot of the mean LD differences by site and group



Lesion depth is measured in µm

These interaction plots indicate that there is much variability between the different sites. In order to try and clarify what was happening, it was decided that particular sites should be analysed in more detail. Specifically, sites were chosen where evidence from the literature suggested that there was an increased salivary film velocity and therefore greater remineralisation potential. The sites chosen for more detailed examination were: sites 1 and 3 (upper posterior buccal sites closest to the parotid major salivary gland duct exit into the oral cavity); and sites 9 and 10 (lower posterior lingual and were closest to the sublingual papilla, the exit site of the saliva from the submandibular and sublingual major salivary glands). These sites became known as the 'selected sites'.

4.3.1.4 Analysis of covered (control) TMR data for selected sites

Prior to presenting the results of the analysis of the difference data for the lesions allocated to the selected sites 1, 3, 9 and 10, the summary statistics for the covered (control) data will be presented (Tables 26 and 27). These sites contain similar numbers of tooth lesions, with sites 1 and 3 containing 12 more lesions than sites 9 and 10.

Tables containing summary statistics for selected sites covered (control) data by group and experiment are displayed in Appendix 9. The values displayed in these tables are very similar to those of all of the covered lesions shown in Section 4.3.1.2.

Selected lesions by site

A. Integrated mineral loss

The mean IML of the covered lesions from the selected sites were similar although the means at sites 1 and 3 were slightly larger than at sites 9 and 10 (Table 26). The standard deviations were consistently large, as were the ranges. There were no significant differences between the selected sites at baseline.

Table 26 - Summary statistics for IML covered (control) selected sites

Site	N*	Mean (St. Dev.)	Range
1 - upper right buccal	121	2297 (1012)	(501, 7369)
3 – upper left buccal	120	2229 (1064)	(217, 5123)
9 – lower left lingual	108	2115 (1032)	(537, 6251)
10 – lower right lingual	109	2158 (1011)	(237, 6385)

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of specimens available for analysis

B. Lesion depth

The mean lesion depths of the covered tooth specimens from the selected sites were also similar (Table 27). The standard deviations and ranges were consistently large. There were no significant differences between the selected sites at baseline.

Table 27 - Summary statistics for LD covered (control) selected sites

Site	N*	Mean (St. Dev.)	Range
1 - upper right buccal	121	78.7 (26.7)	(18.0, 159.4)
3 – upper left buccal	120	74.9 (30.5)	(12.4, 168.6)
9 – lower left lingual	108	74.3 (28.1)	(20.5, 161.7)
10 – lower right lingual	109	75.7 (32.2)	(10.4, 212.4)

Lesion depth is measured in um
N* = number of lesions available for analysis

4.3.1.5 Comparison of the difference between covered (control) and exposed areas measured by TMR for selected sites

Descriptive statistics of the IML and LD differences between the covered (control) and the exposed lesions in the same sections cut from the tooth specimens for selected sites 1, 3, 9 and 10, are shown below. Summary statistics of the IML and LD differences are presented by group and experiment prior to the results of formal analysis using General Linear Model (GLM) Analysis.

All selected site sections

The summary statistics for the covered and exposed IML and LD data as well as the IML and LD differences (covered - exposed) for selected site sections are shown in Tables 28 and 29. There were 458 sections available for TMR analysis from the selected sites 1, 3, 9 and 10.

A. Integrated mineral loss

Table 28 contains the IML summary statistics for all sections from the selected sites 1, 3, 9 and 10. A mean IML difference between covered and exposed values of +358 %Vol mineral.μm was observed. This is a reduction in IML and indicated a net remineralisation of the lesions. However, the standard deviation of the differences was large and the range included specimens with negative and positive values, indicating that some specimens remineralised and others demineralised further.

Table 28 - Summary statistics for IML data from selected sites

	N*	Mean (St. Dev.)	Range
Covered	458	2203 (1029)	(217, 7369)
Exposed	458	1845 (952)	(161, 5791)
Difference (Cov - Exp)	458	358 (853)	(-1851, 3515)

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of sections available for analysis

B. Lesion depth

Table 29 contains summary statistics for the LD data from the selected sites 1, 3, 9 and 10. The mean LD difference between covered and exposed tooth specimens was +4.3 μm. The standard deviation was very large and was over five times greater than the mean. The range was wide, and contained negative and positive values, indicating that not all of the specimens behaved in the same way.

Table 29 - Summary statistics for LD data from selected sites

	N*	Mean (St. Dev.)	Range
Covered	458	76.0 (29.3)	(10.4, 212.4)
Exposed	458	71.6 (26.0)	(6.6, 180.4)
Difference (Cov - Exp)	458	4.3 (22.8)	(-52.3, 143.6)

Lesion depth is μm
N* = number of sections available for analysis

Selected site sections by group

Summary statistics for the IML and LD differences (covered - exposed) for lesions from tooth specimens allocated to the two treatment groups T and TD are shown in Tables 30 and 31. There was a difference in the number of sections available for analysis between the two treatment groups, with the TD group containing 26 specimens more than the T group.

A. Integrated mineral loss

Table 30 contains summary statistics for the difference IML data from the selected sites 1, 3, 9 and 10. The mean IML difference between T and TD was 411 %Vol mineral.μm, with TD having the greater mean IML difference. The standard deviations were large and greater than the means, particularly with the T group which had a standard deviation of 863 %Vol mineral.μm. The ranges were wide, and contained negative and positive values, indicating that not all of the specimens behaved in the same way.

Table 30 - Summary statistics for the IML differences (covered - exposed) for selected sites by treatment group

Group	N*	Mean IML difference (St. Dev.)	Range
T	216	141 (863)	(-1852, 3515)
TD	242	552 (797)	(-1606, 3016)

Integrated mineral loss is measured in %Vol mineral.µm
N* = number of sections available for analysis

B. Lesion depth

The summary statistics for the LD differences for the selected sites by group are shown in Table 31. The mean LD difference between the T and TD groups was 1.9 µm, with the mean in the TD group being the greater. The standard deviations were large, being at least three times the value of the mean. The ranges were wide, and contained negative and positive values, indicating that not all of the specimens behaved in the same way.

Table 31 - Summary statistics for the LD differences (covered - exposed) for selected sites by treatment group

Group	N*	Mean LD difference (St. Dev.)	Range
T	216	3.3 (26.7)	(-51.6, 143.6)
TD	242	5.2 (18.7)	(-52.3, 100.5)

Lesion depth is measured in µm
N* = number of sections available for analysis

Selected site sections by experiment

Summary statistics for the IML and LD differences (covered - exposed) for tooth sections from the selected sites divided according to allocated experiment, are shown in Tables 32 and 33. The greatest difference in number of tooth sections available for analysis between any of the groups is 21; with the 200ml milk x 3/day containing 100 specimens and the no beverage group containing 79. The other groups all contained data from approximately 90 tooth sections.

A. Integrated mineral loss

Table 32 shows the summarised IML difference data. All mean IML differences were positive indicating remineralisation. The largest positive mean IML difference occurred in the 1.5mg F x 1/day group (+475 %Vol mineral.μm). The smallest positive mean IML difference occurred in the 200ml milk x 1/day group (+231 %Vol mineral.μm). The standard deviations were consistently high and the ranges wide, all incorporating negative and positive numbers, indicating that not all specimens behaved in a similar way.

Table 32 - Summary statistics for the IML differences (covered - exposed) for selected sites by experiment

Experiment	N*	Mean IML differences (St. Dev.)	Range
0.5mg F x 3/day	90	435 (916)	(-1793, 3016)
1.5mg F x 1/day	93	475 (883)	(-1683, 3515)
200ml milk x 1/day	96	231 (880)	(-1852, 2220)
200ml milk x 3/day	100	336 (781)	(-1274, 2292)
No beverage	79	318 (787)	(-1194, 2871)

Integrated mineral loss is measured in %Vol mineral.μm
N* = number of sections available for analysis

B. Lesion depth

Table 33 shows the summarised LD difference data. All mean LD differences were positive indicating remineralisation. The largest positive mean LD difference was in the 0.5 mg F x 3/day group (+6.3 %Vol mineral.μm); this was followed by the 1.5mg F x 1/day group (+6.1 %Vol mineral.μm). The smallest positive mean LD difference occurred in the 200ml milk x 1/day group (+2.4 %Vol mineral.μm). The standard deviations were consistently large, as were the ranges, which again included positive and negative numbers, indicating inconsistency in lesion behaviour.

Table 33 - Summary statistics for the LD differences (covered - exposed) for selected sites by experiment

Experiment	N*	Mean LD difference (St. Dev.)	Range
0.5mg F x 3/day	90	6.3 (25.6)	(-52.3, 102.9)
1.5mg F x 1/day	93	6.1 (24.6)	(-39.6, 143.6)
200ml milk x 1/day	96	2.4 (21.2)	(-48.1, 87.9)
200ml milk x 3/day	100	3.9 (21.2)	(-46.9, 66.7)
No beverage	79	2.8 (21.5)	(-35.3, 100.5)

Lesion depth is measured in μm
N* = number of sections available for analysis

Formal analysis of data from selected sites using a general linear model (GLM) procedure

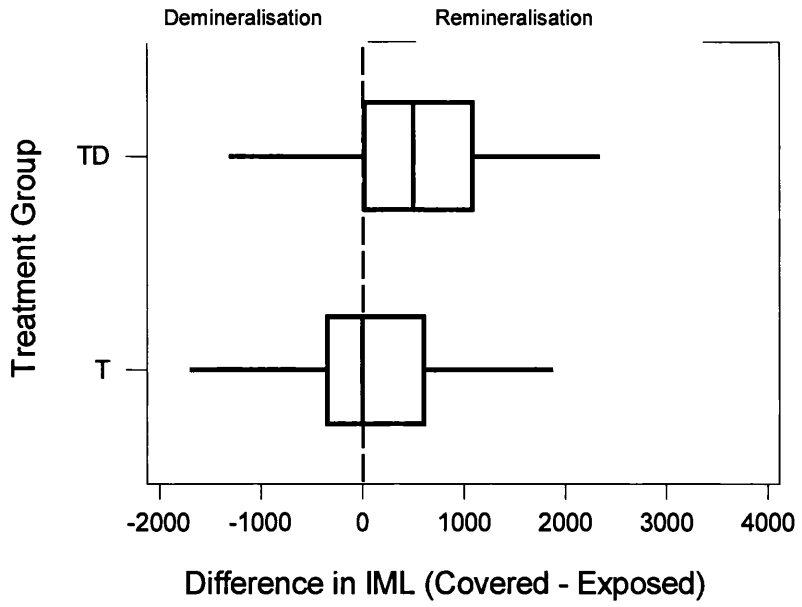
Formal analysis using a GLM procedure determined whether any of the three factors, experiment, group and site, together with the random subject effect, had a significant influence on the IML and LD difference data from sections cut from tooth specimens at the four selected sites 1, 3, 9 and 10. In addition, the GLM procedure could also examine potential interactions of these factors.

A. Integrated mineral loss at selected sites

The model for IML differences at the selected sites, indicated that there was a statistically significant effect of treatment group ($p = 0.001$) and also subject ($p = 0.021$). The subject effect was expected and confirms that the subjects were all different in terms of their 'response'. There was insufficient evidence to suggest that either the experiment ($p = 0.386$) or the site ($p = 0.843$) had a statistically significant effect on the outcome.

Figure 37 illustrates the significant influence of group. A two sample t-test was performed on the IML difference data ($p < 0.0001$, with the 95% confidence interval for the difference being -564 to -258 %Vol mineral.μm). Thus, the mean IML difference was significantly greater in the TD group than in the T group.

Figure 37 - Box plot of the selected site IML differences by group (covered - exposed)



Integrated mineral loss is measured in %Vol mineral.μm

When the factors of experiment and site were examined with relation to the outcome for the two groups separately, there was insufficient evidence that either of these factors of interest were significantly influencing the outcome (experiment - TD: $p = 0.863$, T: $p = 0.429$; site - TD: $p = 0.231$, T: $p = 0.347$).

B. Lesion depth at selected sites

The model for LD differences at the selected sites demonstrated a statistical significant interaction effect of 'group and site' ($p=0.005$) and a significant subject effect ($p=0.012$). As before, the significant subject effect was expected and demonstrated that the subjects were all different in terms of their 'response'. There was insufficient evidence to suggest that the experiment had a statistically significant effect on the outcome ($p=0.787$).

The summary statistics for the LD differences for the combined effects of 'group and site' are shown in Tables 34 and 35. All mean differences were positive indicating

remineralisation, with the exception of site 3 in the T group. The standard deviations were high and ranges were wide, all incorporating negative and positive numbers, indicating that not all specimens behaved in a similar way. There was evidence that the effect of site was different for the two treatment groups. Figure 38 illustrates this difference.

Table 34 - Summary statistics for LD differences, selected sites for the TD group

Site	N*	Mean LD difference (St. Dev.)	Range
1 - upper right buccal	66	5.9 (16.1)	(-31.2, 48.8)
3 – upper left buccal	63	9.4 (22.2)	(-31.8, 100.5)
9 – lower left lingual	58	2.5 (17.7)	(-52.3, 38.0)
10 – lower right lingual	55	2.4 (17.9)	(-32.7, 51.4)

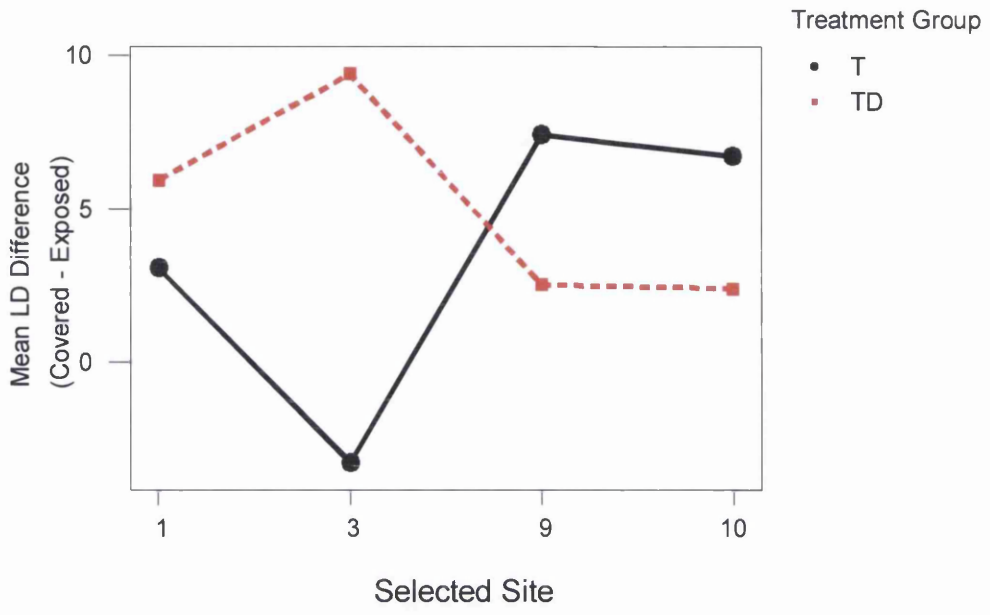
Lesion depth is measured in µm.
N* = number of sections available for analysis

Table 35 - Summary statistics for LD differences, selected sites for the T group

Site	N*	Mean LD difference (St. Dev.)	Range
1 - upper right buccal	55	3.1 (23.2)	(-51.6, 58.8)
3 – upper left buccal	57	-3.3 (22.5)	(-46.9, 44.2)
9 – lower left lingual	50	7.4 (29.7)	(-38.8, 102.9)
10 – lower right lingual	54	6.7 (30.5)	(-29.8, 143.6)

Lesion depth is measured in µm
N* = number of sections available for analysis

Figure 38 - Difference in selected site LD (covered - exposed) by group and site



When the various factors were examined in relation to the outcome for the two treatment groups separately, there was some evidence, though not statistically significant at the 5% level, of a site effect for both the T group (p=0.094) and the TD group (p=0.104). For both groups there was insufficient evidence of an experiment effect (TD: p=0.971, T: p=0.354).

4.3.2 Quantitative light fluorescence (QLF) studies

In total, 1360 tooth blocks were used in the experimental protocols. Images were captured of these 1360 tooth blocks by QLF as described in section 4.2.9 and illustrated in Figures 20, 21 and 24. A baseline image was captured (Image 2) just prior to placement in the intra-oral appliance. The final image (Image 10) was of the tooth block following removal from the intraoral appliance and following the de-stain protocol if this was required (as discussed in section 4.2.8).

The results for the parameters QLF^{AREA}, QLF^{MAX} and QLF^{AVER} (see Chapter 2) are described below.

4.3.2.1 Analysis of baseline QLF (Image 2) data prior to inclusion in the intra-oral appliance

The mean QLF^{AREA} was 4.3 mm^2 at baseline (Table 36). The standard deviation was 1.3 and the range was between 1.2 and 8.5 mm^2. The mean QLF^{MAX} was -51% fluorescence change. The standard deviation was 7 and the range was between -77 and -32% fluorescence change. The mean QLF^{AVER} was -21.0% fluorescence change. The standard deviation was 4.1, and the range was between -38 and -14% fluorescence change.

Table 36 - Summary statistics of baseline data for all blocks for the three QLF parameters

QLF Parameter	Number of Blocks	Mean (St. Dev.)	Range
QLF^{AREA} (mm^2)	1360	4.3 (1.3)	(1.2, 8.5)
QLF^{MAX} (% fluorescence loss)	1360	-51 (7)	(-77, -32)
QLF^{AVER} (% fluorescence loss)	1360	-21.0 (4.1)	(-38.3, -13.6)

The summary statistics of baseline QLF data by group and experiment are shown in Appendix 10, in a layout similar to the summary of the covered (control) TMR data in 4.3.1.4. The data in Appendix 10 show that there were some statistically significant differences detected at baseline for the QLF parameters by group and experiment. There were statistically significant differences detected at baseline between the T and TD group for QLF^{AREA}, but none detected for QLF^{MAX} and QLF^{AVER}. There were statistically significant differences detected between experimental protocols at baseline for QLF parameters QLF^{MAX} and QLF^{AVER}. For QLF^{MAX} there were significant differences between no beverage and 200mL milk x 1/day and the other three protocols. For QLF^{AVER} there were statistically significant differences determined between no beverage and the other four experimental protocols.

4.3.2.2 Comparison of baseline with post-experimental protocol QLF data

Descriptive statistics of the baseline (QLF Image 2) and the post-experimental protocol final image (QLF Image 10) for the three QLF parameters are shown below. Summary statistics of the differences between baseline and final image were calculated for all specimens, then for specimens by group and then by experiment.

The difference between baseline and post-experimental QLF data was calculated as follows: QLF^{AREA} was baseline (QLF Image 2) - final image (QLF Image 10) mm^2. Therefore, a positive number indicates that the QLF image area became smaller, suggesting that remineralisation had occurred. QLF^{MAX} and QLF^{AVER} were final image (QLF Image 10) - baseline (QLF Image 2) % fluorescence loss. Therefore a positive number indicates that there has been an overall gain in %fluorescence loss, again suggesting that remineralisation has occurred. This term is more accurately described as "% fluorescence change".

All available specimens

There were 284 tooth specimens lost during the experimental protocols; therefore there were 1041 tooth specimens available for statistical analysis. These losses were mainly as a result of failure of the temporary cement used to retain the tooth specimens within the intra-oral appliance. In the sites which were on the palatal surface of the upper denture (sites four, five and six) there were significant forces of swallowing applied during eating and drinking, therefore, loss of the tooth sections was higher in these sites.

A. QLF^{AREA}

The mean difference in QLF^{AREA} between baseline and final image was 2.0 mm^2 (Table 37). The standard deviation was large and of similar size to the difference. The range included both negative and positive numbers indicating that not all of the tooth blocks were behaving in a similar way.

Table 37 - Summary statistics of baseline, final image and difference between them for QLF^{AREA}

Image	Number of Blocks	Mean (St. Dev.)	Range
Baseline Image	1041	4.2 (1.3)	(1.2, 8.5)
Final Image	1041	2.2 (1.9)	(0.0, 9.8)
Difference between baseline and final image	1041	2.0 (2.1)	(-5.9, 7.8)

QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

The mean difference in QLF^{MAX} between final image and baseline image was 12% (Table 38). This indicated that on average remineralisation had occurred. The standard deviation, however, was large and was of similar size to the difference. The range included both negative and positive values, indicating that not all of the lesions were behaving in the same way.

Table 38 - Summary statistics of baseline, final image and difference between them for QLF^{MAX}

Image	Number of Blocks	Mean (St. Dev.)	Range
Baseline image	1041	-50 (7)	(-77, -32)
Final image	1041	-39 (12)	(-75, -11)
Difference between final image and baseline image	1041	12 (13)	(-31, 47)

QLF^{MAX} is measured in % change in fluorescence

C. QLF^{AVER}

The mean difference in QLF^{AVER} between final image and image 2 was 5.3% (Table 39). This indicates that on average the lesions had remineralised. The standard deviation was

large and of similar size to the difference. The range included both negative and positive values, indicating that not all of the lesions behaved in the same way.

Table 39 - Summary statistics of baseline, final image and difference between them for QLF^{AVER}

Image	Number of Blocks	Mean (St. Dev.)	Range
Baseline image	1041	-20.9 (4.1)	(-37.0, -13.7)
Final image	1041	-15.6 (3.7)	(-34.0, -10.2)
Difference between final image and baseline image	1041	5.3 (5.1)	(-15.4, 22.1)

QLF^{AVER} is measured in % change in fluorescence

All available tooth blocks by group

During the experimental protocols, there were 137 tooth specimens lost from the T group and 147 specimens lost from the TD group. This left 494 tooth specimens in the T group and 547 tooth specimens in the TD group.

A. QLF^{AREA}

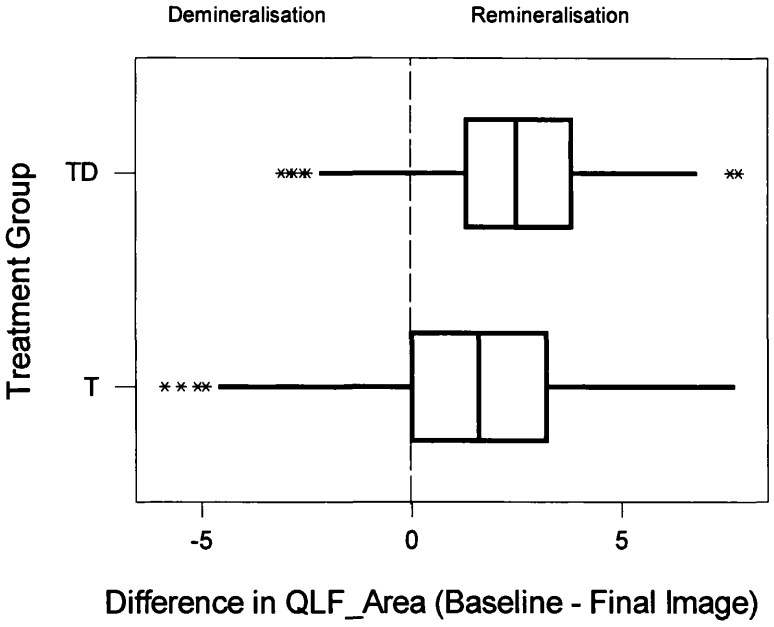
The mean difference between baseline and final image was greater for the TD group by 1.0 mm^2 than the T group for QLF^{AREA} (Table 40). This indicated that on average the tooth specimens in the TD group remineralised more than the specimens in the T group. The standard deviations were large, though smaller in the TD group. The ranges were wide and included both positive and negative numbers, indicating that not all of the lesions behaved in the same way. These data are illustrated in Figure 39.

Table 40 - Summary statistics of differences (baseline-final image) for QLF^{AREA} by group

Group	Number of Blocks	Mean Difference (St. Dev.)	Range
T	494	1.5 (2.2)	(-5.9, 7.7)
TD	547	2.5 (1.9)	(-3.1, 7.8)

QLF^{AREA} is measured in mm^2

Figure 39 - Box plot of difference (baseline-final image) for QLF^{AREA} by group



QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

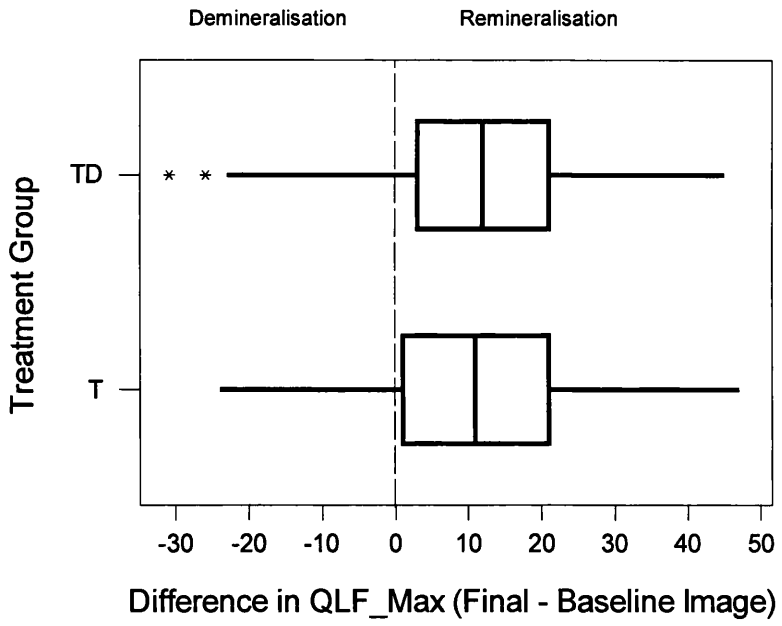
The mean difference between final image and baseline was greater in the TD group by 1% change in fluorescence when compared with the T group for QLF^{MAX} (Table 41). This indicated that slightly greater mean remineralisation occurred in the TD group than the T group. The standard deviations were large and were of similar size to the mean differences. The ranges included negative and positive numbers indicating that not all of the lesions behaved in a similar way. The differences (final image-baseline) for QLF^{MAX} are summarised in Figure 40.

Table 41 - Summary statistics of differences (final image-baseline) for QLF^{MAX} by group

Group	Number of Blocks	Mean (St. Dev.)	Range
T	494	11 (13)	(-24, 47)
TD	547	12 (13)	(-31, 45)

QLF^{MAX} is measured in % change in fluorescence

Figure 40 - Box plot of difference (final image-baseline) for QLF^{MAX} by group



QLF^{MAX} is measured in % change in fluorescence

C. QLF^{AVER}

The mean difference of final minus baseline image was greater in the TD group by 1.1% change in fluorescence (Table 42). This indicated that the lesions in the TD group, on average, remineralised more than in the T group. The standard deviations were large. The ranges were wide and included both negative and positive numbers, indicating that not all

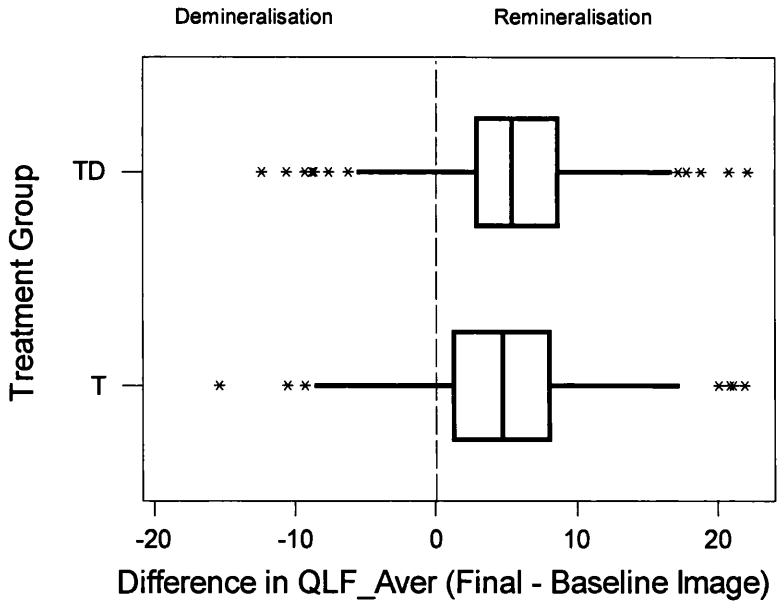
of the tooth specimens behaved in a similar way. A box plot of the differences (final image-baseline) of QLF^{AVER} by group is shown in Figure 41.

Table 42 - Summary statistics of differences (final image-baseline) for QLF^{AVER} by group

Group	Number of Blocks	Mean (St. Dev.)	Range
T	494	4.7 (5.5)	(-15.4, 21.9)
TD	547	5.8 (4.8)	(-12.4, 22.1)

QLF^{AVER} is measured in % change in fluorescence

Figure 41 - Box plot of difference (final image-baseline) for QLF^{AVER} by group



QLF^{AVER} is measured in % change in fluorescence

Blocks by experiment

There were between 54 and 61 tooth blocks lost from each experimental group: 54 blocks were lost from the no beverage and 200 mL milk x 1/day groups; 57 blocks were lost from the 200 mL x 3/day group; 58 blocks were lost from the 1.5mg F x 1/day group and 61 tooth blocks were lost from the 0.5mg F x 3/day group.

A. QLF^{AREA}

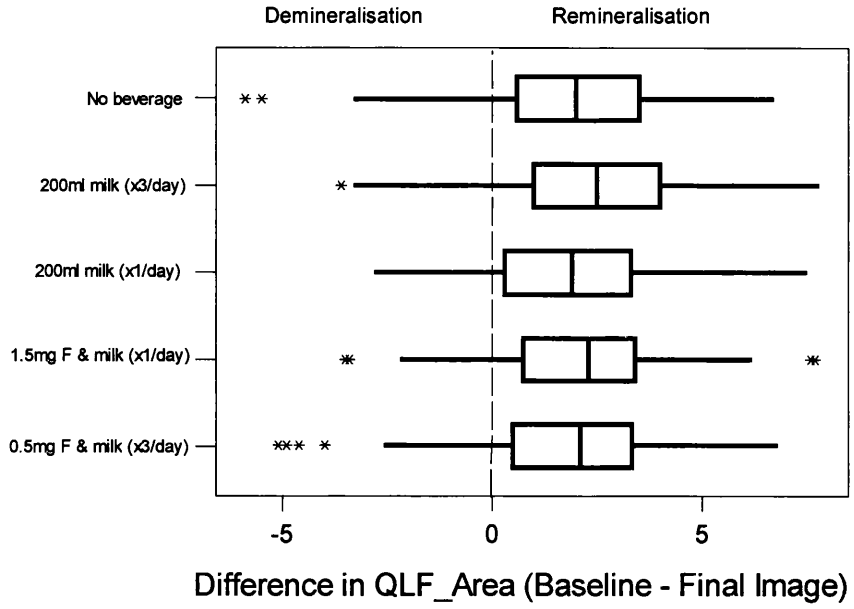
The greatest mean difference for baseline - final image for QLF^{AREA} was 2.4 mm^2 and occurred in the 200 mL milk x 3/day group (Table 43). The least difference was 1.9 mm^2 and occurred in the 0.5mg F x 3/day, 200 mL milk x 1/day and no beverage groups. The standard deviations were consistently high. The ranges all included negative and positive numbers, indicating that not all of the specimens behaved in a similar manner. This is shown diagrammatically in Figure 42.

Table 43 - Summary statistics of differences (baseline-final image) for QLF^{AREA} by experiment

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	194	1.9 (2.3)	(-5.1, 6.8)
1.5mg F x 1/day	221	2.1 (2.0)	(-3.5, 7.7)
200mL milk x 1/day	207	1.9 (2.0)	(-2.8, 7.5)
200mL milk x 3/day	218	2.4 (2.2)	(-3.6, 7.8)
No beverage	201	1.9 (2.2)	(-5.9, 6.7)

QLF^{AREA} is measured in mm^2

Figure 42 - Box plot of differences (baseline-final image) for QLF^{AREA} by experiment



QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

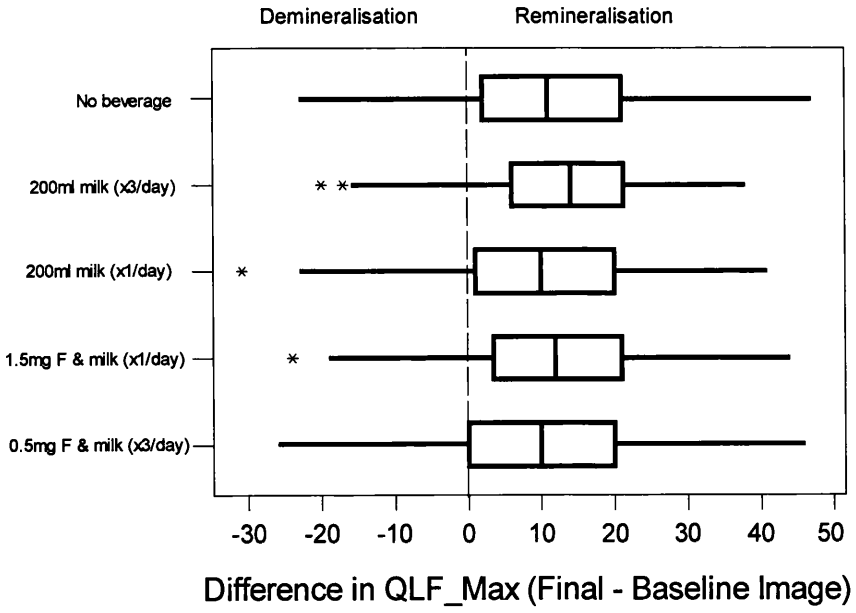
The greatest mean difference for final minus baseline image for QLF^{MAX} was 13% and again occurred in the 200mL milk x 3/day group (Table 44). The smallest mean difference was 10% and occurred in the 200 mL milk x 1/day group. The standard deviations were consistently large. The ranges included negative and positive numbers indicating that not all of the tooth blocks behaved in a similar manner. This is shown diagrammatically in Figure 43.

Table 44 - Summary statistics of differences (final image-baseline) for QLF^{MAX} by experiment

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	194	11 (14)	(-26, 46)
1.5mg F x 1/day	221	12 (12)	(-24, 44)
200mL milk x 1/day	207	10 (13)	(-31, 41)
200mL milk x 3/day	218	13 (12)	(-20, 38)
No beverage	201	12 (15)	(-23, 47)

QLF^{MAX} is measured in % change in fluorescence

Figure 43 - Box plot of differences (final image-baseline) for QLF^{MAX} by experiment



QLF^{MAX} is measured in % change in fluorescence

C. QLF^{AVER}

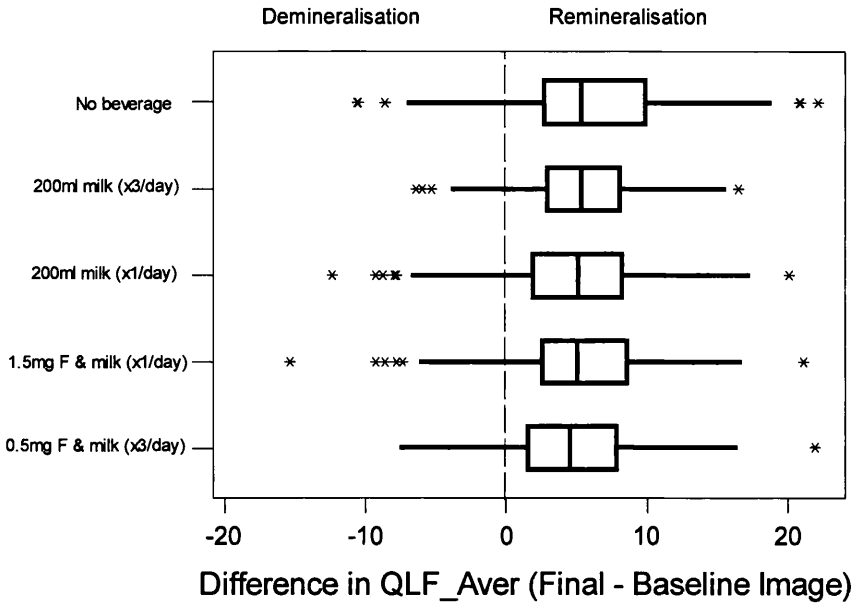
The greatest mean difference for final minus baseline image for QLF^{AVER} by experiment occurred in the no beverage group with a mean 6.2% change in fluorescence (Table 45). The smallest mean difference occurred in the 0.5mg F x 3/day with 4.8% mean change in fluorescence. The standard deviations were consistently high. The ranges were wide and included both negative and positive numbers indicating that not all of the lesions behaved in a similar way. A box plot illustrating these differences by experiment is shown in Figure 44.

Table 45 - Summary statistics of differences (final image-baseline) for QLF^{AVER} by experiment

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	194	4.8 (5.0)	(-7.6, 21.9)
1.5mg F x 1/day	221	5.1 (5.2)	(-15.4, 21.1)
200mL milk x 1/day	207	4.9 (5.2)	(-12.4, 20.1)
200mL milk x 3/day	218	5.4 (4.3)	(-6.4, 16.5)
No beverage	201	6.2 (5.9)	(-10.6, 22.1)

QLF^{AVER} is measured in % change in fluorescence

Figure 44 – Box plot of differences (final image-baseline) for QLF^{AVER} by experiment



QLF^{AVER} is measured in % change in fluorescence

Formal analysis using a general linear model procedure

Formal statistical analysis utilised a general linear model (GLM) procedure to determine which of the three factors, experiment, group and site, together with the random subject effect and the potential interactions of these factors, had a statistically significant influence on the difference between baseline and final image for the tooth specimens, for the three QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER}.

QLF^{AREA}

The final model for difference in QLF^{AREA} indicated that there was a statistically significant effect of group ($p=0.006$), experiment ($p=0.040$) and site ($p<0.001$) together with a significant subject effect ($p<0.001$). This subject effect was expected and confirmed that the subjects were all different in terms of their 'response'. There was also a statistically significant combined effect of experiment and group ($p=0.047$).

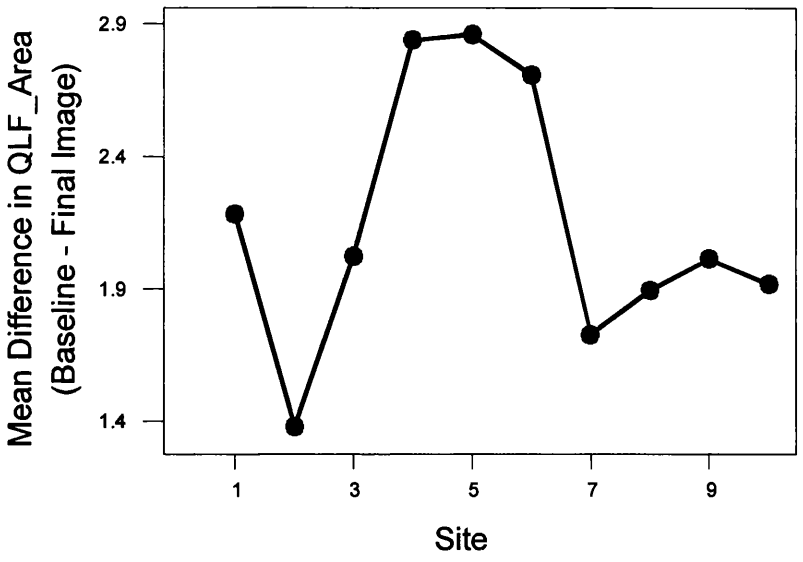
The summary statistics for the effect of site are shown below in Table 46. The significant effect of site is also illustrated (Figure 45).

Table 46 - Summary statistics for differences (baseline-final image) for QLF^{AREA} by site

Site	Number of Blocks	Mean (St. Dev.)	Range
1 – upper right buccal	133	2.2 (1.9)	(-3.1, 6.7)
2 – upper mid-labial	134	1.4 (2.0)	(-4.6, 6.2)
3 – upper left buccal	126	2.0 (2.2)	(-5.9, 7.6)
4 – upper left palatal	56	2.8 (2.1)	(-3.3, 7.5)
5 – upper mid-palatal	59	2.9 (1.9)	(-2.2, 6.8)
6 – upper right palatal	57	2.7 (2.1)	(-3.4, 6.2)
7 – lower right buccal	119	1.7 (2.1)	(-4.0, 6.3)
8 – lower left buccal	116	1.9 (2.3)	(-5.5, 7.7)
9 – lower left lingual	122	2.0 (2.1)	(-3.6, 7.8)
10 – lower right lingual	119	1.9 (2.2)	(-5.1, 6.6)

QLF^{AREA} is measured in mm^2

Figure 45 - Plot of mean difference (baseline-final image) for QLF^{AREA} by site



QLF^{AREA} is measured in mm^2

There was also a statistically significant combined effect of group and experiment. The summary statistics of this combined effect are shown in Tables 47 and 48. This significant combined effect of group and site is illustrated (Figure 46).

Table 47 - Summary statistics of differences (baseline-final image) for QLF^{AREA} by experiment for the T group

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	92	1.0 (2.3)	(-5.1, 6.1)
1.5mg F x 1/day	100	1.7 (2.2)	(-3.5, 7.7)
200mL milk x 1/day	106	1.5 (2.0)	(-2.8, 7.5)
200mL milk x 3/day	99	1.9 (2.4)	(-3.6, 6.7)
No beverage	97	1.5 (2.2)	(-5.9, 6.3)

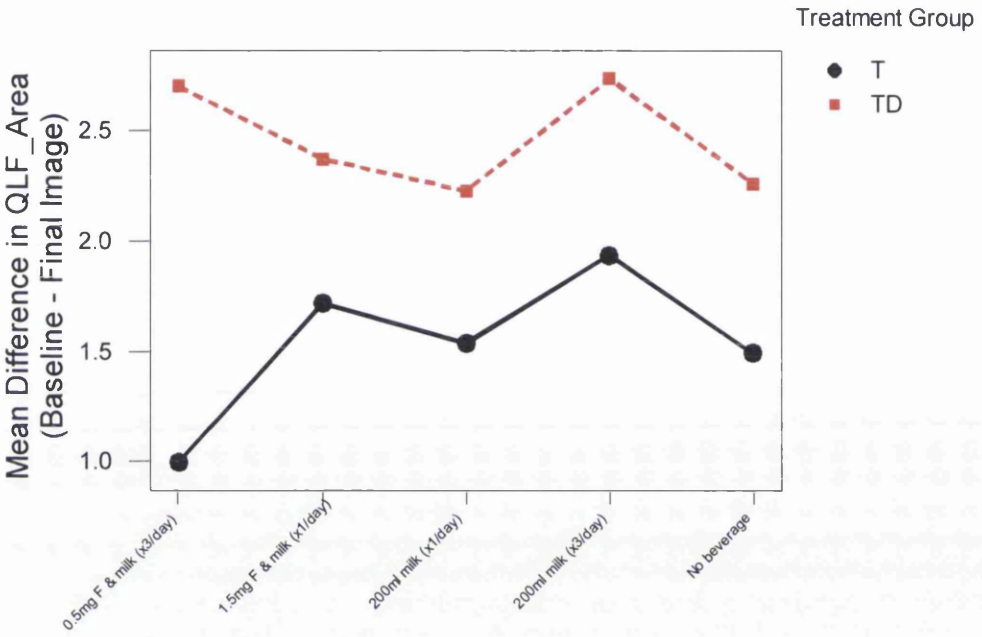
QLF^{AREA} is measured in mm^2

Table 48 - Summary statistics of differences (baseline-final image) for QLF^{AREA} by experiment for the TD group

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	102	2.7 (1.9)	(-2.6, 6.8)
1.5mg F x 1/day	121	2.4 (1.7)	(-2.2, 7.6)
200mL milk x 1/day	101	2.2 (1.9)	(-2.5, 6.6)
200mL milk x 3/day	119	2.7 (2.0)	(-3.1, 7.8)
No beverage	104	2.3 (2.1)	(-3.1, 6.7)

QLF^{AREA} is measured in mm^2

Figure 46 - Plot of mean differences (baseline-final image) for QLF^{AREA} by group and experiment



QLF^{AREA} is measured in mm^2

QLF^{MAX}

The final model for difference in QLF^{MAX} indicated that there was a statistically significant effect of site ($p=0.047$) together with a significant subject effect ($p<0.001$). This subject effect is expected and confirms that the subjects were all different in terms of their "response". There was insufficient evidence to suggest that the experiment had a statistically significant effect on the outcome ($p=0.182$) or the group ($p=0.549$).

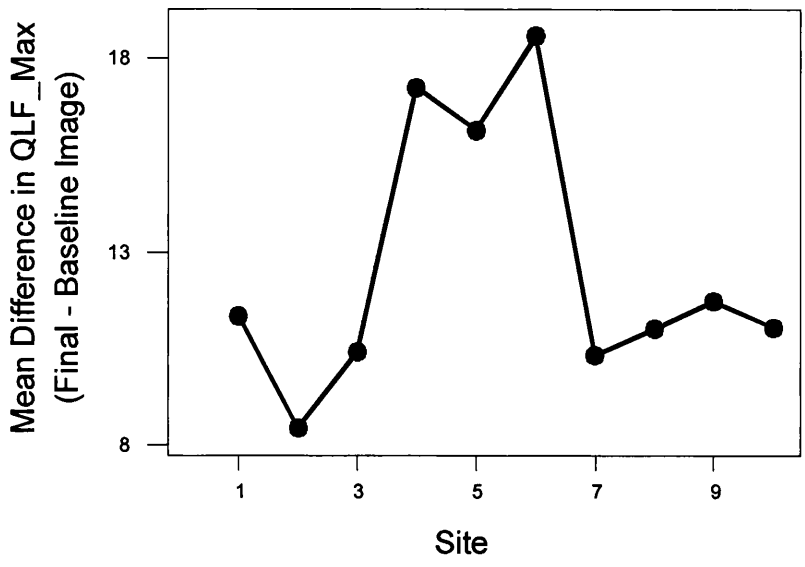
Thus there was evidence that the only factor of interest to have a significant influence on the outcome was site. The summary statistics for the difference in QLF^{MAX} by site are shown in Table 49 and the mean differences are illustrated in the summary figure below (Figure 47).

Table 49 - Summary statistics of differences (final image-baseline) for QLF^{MAX} by site

Site	Number of Experiments	Mean (St. Dev.)	Range
1 – upper right buccal	133	11 (11)	(-15, 45)
2 – upper mid-labial	134	8 (10)	(-18, 30)
3 – upper left buccal	126	10 (11)	(-12, 37)
4 – upper left palatal	56	17 (16)	(-21, 47)
5 – upper mid-palatal	59	16 (14)	(-12, 37)
6 – upper right palatal	57	19 (14)	(-18, 44)
7 – lower right buccal	119	10 (15)	(-31, 43)
8 – lower left buccal	116	11 (14)	(-20, 44)
9 – lower left lingual	122	12 (14)	(-23, 46)
10 – lower right lingual	119	11 (14)	(-19, 40)

QLF^{MAX} is measured in % change in fluorescence

Figure 47 - Plot of mean difference (final image-baseline) for QLF^{MAX} by site



QLF^{MAX} is measured in % change in fluorescence

QLF^{AVER}

The final model for difference in QLF^{AVER} indicated that there was a statistically significant effect of experiment ($p=0.022$), site ($p=0.006$) and subject ($p<0.001$). The subject effect is expected and confirms that the subjects were all different in terms of their "response". There was insufficient evidence to suggest that group had a statistically significant effect on outcome ($p=0.116$).

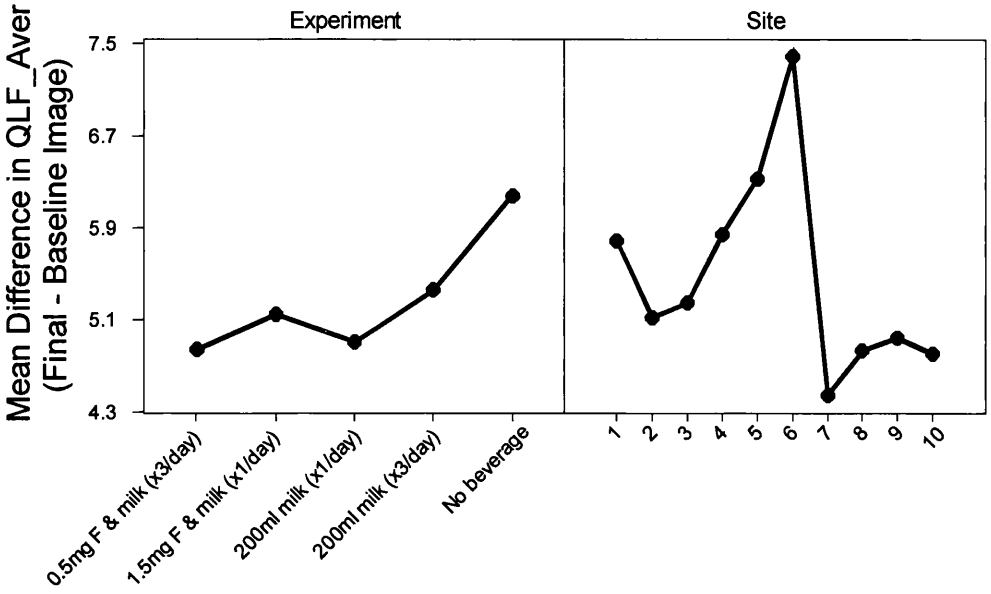
The summary statistics of the differences for QLF^{AVER} by site are shown in Table 50. The main significant effects of experiment and site are plotted (Figure 48). (Note: the summary statistics of the baseline data for experiment are shown in Appendix 10.)

Table 50 - Summary statistics of differences (final image-baseline) for QLF^{AVER} by site

Site	Number of Blocks	Mean (St. Dev.)	Range
1 – upper right buccal	133	5.8 (4.9)	(-8.8, 18.8)
2 – upper mid-labial	134	5.1 (4.7)	(-3.3, 21.1)
3 – upper left buccal	126	5.2 (4.0)	(-6.2, 16.8)
4 – upper left palatal	56	5.8 (6.3)	(-10.5, 22.1)
5 – upper mid-palatal	59	6.3 (4.8)	(-8.6, 15.3)
6 – upper right palatal	57	7.4 (4.5)	(-1.4, 20.1)
7 – lower right buccal	119	4.4 (5.6)	(-12.4, 22.1)
8 – lower left buccal	116	4.8 (5.3)	(-7.4, 18.8)
9 – lower left lingual	122	4.9 (5.8)	(-8.0, 21.9)
10 – lower right lingual	119	4.8 (5.8)	(-15.4, 16.0)

QLF^{AVER} is measured in % change in fluorescence

Figure 48 - Plot of mean differences (final image-baseline) by experiment and site forQLF^{AVER}



QLF^{AVER} is measured in % change in fluorescence

4.3.2.3 Analysis of baseline QLF data for selected sites

In a similar manner to the TMR data, the QLF data were further analysed concentrating on the sites where it was anticipated there would be the greatest potential to remineralise. These were the selected sites 1, 3, 9 and 10.

In total there were 541 tooth specimens cemented into the intra-oral appliance in the four selected sites 1, 3, 9 and 10. The summary statistics of the baseline data for these selected sites for the three QLF parameters are presented below. All of the four selected sites contained 135 tooth sections, with the exception of site 3 which contained 136 tooth sections.

A. QLF^{AREA}

The selected sites had similar mean QLF^{AREA} values at baseline (Table 51). The standard deviations and ranges were similar too, indicating that on average the lesions from the selected sites had a similar spread of QLF^{AREA} values.

Table 51 - Summary statistics of baseline data from selected sites for QLF^{AREA}

Selected site	Number of Blocks	Mean (St. Dev.)	Range
1 - upper right buccal	135	4.4 (1.4)	(1.8, 7.6)
3 – upper left buccal	136	4.2 (1.3)	(1.6, 7.9)
9 – lower left lingual	135	4.2 (1.2)	(1.5, 8.5)
10 – lower right lingual	135	4.2 (1.2)	(1.2, 7.6)

QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

The lesions from the four selected sites at baseline had similar mean values for QLF^{MAX} (Table 52). The standard deviations and ranges were also similar, indicating that there was a similar spread across the four sites in terms of the QLF^{MAX} of the artificial caries lesions.

Table 52 - Summary statistics of baseline data from selected sites for QLF^{MAX}

Selected site	Number of Blocks	Mean (St. Dev.)	Range
1 - upper right buccal	135	-50 (8)	(-73, -34)
3 – upper left buccal	136	-51 (8)	(-74, -35)
9 – lower left lingual	135	-51(7)	(-72, -32)
10 – lower right lingual	135	-50 (8)	(-77, -33)

QLF^{MAX} is measured in % fluorescence loss

C. QLF^{AVER}

The selected sites had similar mean QLF^{AVER} (Table 53). The standard deviations and ranges were also similar indicating that the spread in terms of QLF^{AVER} was similar across the four selected sites.

Table 53 - Summary statistics of baseline data from selected sites for QLF^{AVER}

Selected site	Number of Blocks	Mean (St. Dev.)	Range
1 - upper right buccal	135	-21.1 (4.2)	(-31.9, -13.7)
3 – upper left buccal	136	-20.5 (3.7)	(-31.6, -15.3)
9 – lower left lingual	135	-20.9 (4.2)	(-35.2, -14.0)
10 – lower right lingual	135	-20.8 (4.3)	(-35.0, -13.8)

QLF^{AVER} is measured in % fluorescence loss

In summary, for all three of the QLF parameters, on average there were similar sized lesions in the tooth blocks allocated to the four selected sites.

4.3.2.4 Comparison of baseline with post-experiment QLF data for selected sites

Selected sites

The summary statistics of the selected tooth blocks at baseline, for the three QLF parameters, are presented below (Table 54).

Table 54 - Summary statistics of selected sites at baseline, for the three QLF parameters

QLF Parameter	Number of blocks	Mean (St. Dev.)	Range
QLF^{AREA}	541	4.3 (1.3)	(1.2, 8.5)
QLF^{MAX}	541	-50 (7)	(-77, -32)
QLF^{AVER}	541	-20.9 (4.1)	(-35.2, -13.7)

QLF^{AREA} is measured in mm^2. QLF^{MAX} and QLF^{AVER} are measured in % fluorescence loss

There were 41 fewer blocks at the end of the experiments compared with at the beginning. These missing tooth blocks were lost from the intra-oral appliances during the experimental protocols, often when the subject was eating or drinking. The summary

statistics of the selected blocks minus the 41 lost tooth blocks at baseline, final image and the difference between them, for the three QLF parameters, are presented in Tables 55, 56 and 57.

A. QLF^{AREA}

There was a mean difference of 2.0 mm^2 between baseline and final image for QLF^{AREA} indicating that the area of the caries lesions on average became smaller (Table 55). However, the standard deviation was of similar size to the difference and the range included negative and positive values, indicating that not all of the lesions behaved in a similar way.

Table 55 - Summary statistics of selected sites at baseline, final image and difference between them for QLF^{AREA}

Image	Number of Blocks	Mean (St. Dev.)	Range
Baseline	500	4.3 (1.3)	(1.2, 8.5)
Final image	500	2.2 (1.8)	(0.0, 9.8)
Difference between baseline and final image	500	2.0 (2.1)	(-5.9, 7.8)

QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

There was a mean 11% change in fluorescence between baseline and final image for QLF^{MAX} (Table 56). The standard deviation was of a similar size to the mean difference. The range included negative and positive values, indicating that not all of the lesions behaved in the same manner.

Table 56 - Summary statistics of selected sites at baseline, final image and the difference between them for QLF^{MAX}

Image	Number of Blocks	Mean (St. Dev.)	Range
Baseline	500	-50 (7)	(-77, -32)
Final image	500	-39 (11)	(-64, -14)
Difference between final image and baseline	500	11 (11)	(-23, 46)

QLF^{MAX} is measured in % fluorescence change

C. QLF^{AVER}

There was a mean 5.2% fluorescence change between baseline and final image for QLF^{AVER} (Table 57). The standard deviation was of a similar size to the mean difference in QLF^{AVER}. The range included positive and negative values indicating that not all of the lesions behaved in a similar manner.

Table 57 - Summary statistics of selected sites at baseline, final image and difference between them for QLF^{AVER}

Image	Number of Blocks	Mean (St. Dev.)	Range
Baseline	500	-20.9 (4.1)	(-35.2, -13.7)
Final image	500	-15.7 (3.5)	(-30.6, -10.5)
Difference between final image and baseline	500	5.2 (5.0)	(-15.4, 21.9)

QLF^{AVER} is measured in % fluorescence change

Selected tooth blocks by group

There was a difference in the number of tooth blocks in the two groups, with the TD group containing 24 more specimens than the T group.

A. QLF^{AREA}

The mean change in QLF^{AREA} between the baseline and final images was 1.2 mm^2 greater for the TD group compared with the T group (Table 58). The standard deviations were similar for the two groups. The ranges were wide and included positive and negative numbers, indicating that the lesions behaved in different ways.

Table 58 - Summary statistics of selected sites differences (baseline-final image) for QLF^{AREA} by group

Group	Number of Blocks	Mean (St. Dev.)	Range
T	238	1.4 (2.1)	(-5.9, 6.2)
TD	262	2.6 (1.9)	(-3.1, 7.8)

QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

The mean change in QLF^{MAX} between the final and baseline images was 2 units of % fluorescence change greater in the TD group compared to the T group (Table 59). The standard deviations were of similar size to the mean differences. The ranges were wide and included both negative and positive numbers, indicating that not all of the blocks behaved in a similar way.

Table 59 - Summary statistics of selected sites differences (final image-baseline) for QLF^{MAX} by group

Group	Number of Blocks	Mean (St. Dev.)	Range
T	238	10 (13)	(-23, 46)
TD	262	12 (12)	(-17, 45)

QLF^{MAX} is measured in % fluorescence change

C. QLF^{AVER}

The mean change in QLF^{AVER} between the final and baseline images was 1.2 units of % fluorescence change greater in the TD group compared to the T group (Table 60). The standard deviations were large and were of a similar size as the mean differences. The ranges were wide and included negative and positive numbers, indicating that not all lesions behaved in a similar manner.

Table 60 - Summary statistics of selected sites differences (final image-baseline) for QLF^{AVER} by group

Group	Number of Blocks	Mean (St. Dev.)	Range
T	238	4.6 (5.4)	(-15.4, 21.9)
TD	262	5.8 (4.5)	(-9.3, 18.8)

QLF^{AVER} is measured in % fluorescence change

Selected tooth blocks by experiment

The summary statistics of the differences between baseline and final image by experiment for the selected sites are shown below. The numbers of tooth specimens in the five experimental groups were all between 95 and 105.

A. QLF^{AREA}

There was no great difference between the mean area differences of the experimental groups (Table 61). The standard deviations were all similar to each other and to the mean differences. The ranges all included positive and negative numbers, indicating that not all of the lesions in each group behaved in the same way.

Table 61 - Summary statistics of selected sites differences (baseline-final image) for QLF^{AREA} by experiment

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	97	1.9 (2.1)	(-5.1, 5.9)
1.5mg F x 1/day	103	2.1 (1.9)	(-2.2, 7.6)
200mL milk x 1/day	100	1.9 (2.0)	(-2.5, 6.6)
200mL milk x 3/day	105	2.2 (2.3)	(-3.6, 7.8)
No beverage	95	2.0 (2.2)	(-5.9, 6.7)

QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

The mean differences ranged from 12% to 10% change in fluorescence (Table 62). The standard deviations were of similar size to those of the mean differences. The ranges included positive and negative values, indicating that not all of the lesions behaved in the same way.

Table 62 - Summary statistics of selected sites differences (final image-baseline) for QLF^{MAX} by experiment

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	97	10 (13)	(-16, 46)
1.5mg F x 1/day	103	11 (11)	(-19, 40)
200mL milk x 1/day	100	10 (12)	(-23, 41)
200mL milk x 3/day	105	12 (12)	(-20, 37)
No beverage	95	12 (13)	(-17, 45)

QLF^{MAX} is measured in % change in fluorescence

C. QLF^{AVER}

The greatest mean difference between the final image and baseline image in the selected sites for QLF^{AVER} occurred in the no beverage group, with 6.4%, and the smallest difference occurred in the 0.5mg F x 3/day group, with 4.6% change in fluorescence (Table 63). The standard deviations were of similar size to the mean differences. The ranges included positive and negative values, indicating that not all of the lesions behaved in the same way.

Table 63 - Summary statistics of selected sites differences (final image-baseline) for QLF^{AVER} by experiment

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	97	4.6 (5.1)	(-7.3, 21.9)
1.5mg F x 1/day	103	4.8 (5.4)	(-15.4, 16.7)
200mL milk x 1/day	100	5.2 (5.0)	(-9.3, 16.8)
200mL milk x 3/day	105	5.1 (4.4)	(-6.4, 16.5)
No beverage	95	6.4 (5.1)	(-4.9, 18.8)

QLF^{AVER} is measured in % change in fluorescence

Selected tooth blocks by site

Following the experimental protocols, a number of blocks were lost from each site: 2 from site 1; 10 from site 3; 13 from site 9 and 16 from site 10.

A. QLF^{AREA}

The mean differences in QLF^{AREA} values between baseline and final images were of a similar magnitude across the four selected sites (Table 64). The standard deviations were of a similar size to the mean differences. The ranges included negative and positive values, indicating that the lesions in each site behaved in different ways.

Table 64 - Summary statistics of selected sites differences (baseline image - final image) for QLF^{AREA} by site

Site	Number of Blocks	Mean (St. Dev.)	Range
1 - upper right buccal	133	2.2 (1.9)	(-3.1, 6.7)
3 – upper left buccal	126	2.0 (2.2)	(-5.9, 7.6)
9 – lower left lingual	122	2.0 (2.1)	(-3.6, 7.8)
10 – lower right lingual	119	1.9 (2.1)	(-5.1, 6.6)

QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

The mean differences in QLF^{MAX} between final and baseline images were of a similar magnitude across the four selected sites (Table 65). The standard deviations were of similar size to the mean differences. The range included negative and positive values, indicating that not all of the lesions behaved in a similar manner.

Table 65 - Summary statistics of selected sites differences (final image-baseline image) for QLF^{MAX} by site

Site	Number of Blocks	Mean (St. Dev.)	Range
1 - upper right buccal	133	11 (11)	(-15, 45)
3 – upper left buccal	126	10 (11)	(-12, 37)
9 – lower left lingual	122	12 (14)	(-23, 46)
10 – lower right lingual	119	11 (14)	(-19, 40)

QLF^{MAX} is measured in % change in fluorescence

C. QLF^{AVER}

The mean differences in QLF^{AVER} values between final and baseline images were different across the four selected sites (Table 66), with the least mean difference (4.8%) in site 10 (lower, right lingual) and the greatest mean difference (5.8%) in site 1 (upper, right buccal). The standard deviations were large and of similar size to the mean differences.

The ranges were wide and included positive and negative numbers, indicating that not all of the lesions behaved in a similar manner.

Table 66 - Summary statistics of selected sites differences (final image-baseline image) for QLF^{AVER} by site

Site	Number of Blocks	Mean (St. Dev.)	Range
1 - upper right buccal	133	5.8 (4.9)	(-8.8, 18.8)
3 – upper left buccal	126	5.2 (4.0)	(-6.2, 16.8)
9 – lower left lingual	122	4.9 (5.3)	(-8.0, 21.9)
10 – lower right lingual	119	4.8 (5.8)	(-15.4, 16.0)

QLF^{AVER} is measured in % change in fluorescence

Formal analysis of selected sites using a general linear model procedure

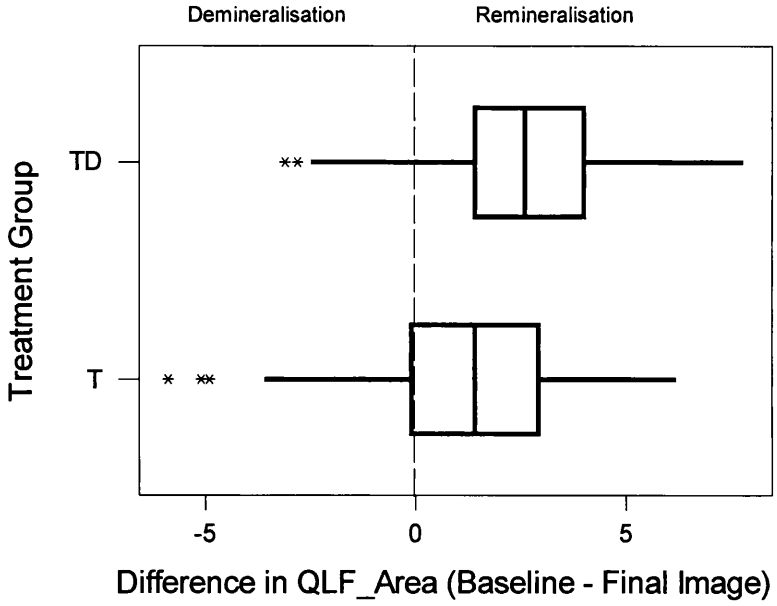
Formal analysis via a general linear model (GLM) procedure was used to determine whether any of the three factors, experiment, group and site, together with the random effect of subject and the potential interactions of these factors, had a significant influence on the difference between baseline and final image, for each of the three QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER}, for the four selected sites.

A. QLF^{AREA}

The final model for difference in QLF^{AREA}, for the selected sites, indicated that there was a statistically significant effect of group ($p=0.001$), together with a significant subject effect ($p<0.001$). This subject effect is expected and confirms that the subjects were all different in terms of their 'response'. There was insufficient evidence to suggest that the experiment had a statistically significant effect on the outcome ($p=0.640$) or the site ($p=0.730$).

Thus there was evidence that the only factor to have a statistically significant influence was group. This is illustrated in Figure 49. A two-sample t-test applied to the differences in QLF^{AREA} resulted in a p-value of <0.001 and a 95% confidence interval for (TD - T) of 0.9 to 1.6 mm^2. Thus the difference in QLF^{AREA} was significantly greater in the TD group than in the T group.

Figure 49 - Box plot for selected sites illustrating the difference in QLF^{AREA} by group



QLF^{AREA} is measured in mm^2

When the factors of site and experiment were examined in relation to the results for the two groups separately, there was insufficient evidence that either of the factors of interest were significantly influencing the difference in QLF^{AREA} (experiment -TD: p=0.749, T: p=0.630; site - TD: p=0.998, T: p=0.406).

B. QLF^{MAX}

The final model for the difference in QLF^{MAX} indicated that with the exception of a significant subject effect (p=0.012), there was no evidence of any of the other factors having a statistically significant effect (experiment: p=0.592, group: p=0.596, site: p=0.833). The subject effect was expected as this confirms that the subjects were all different in terms of their response.

C. QLF^{AVER}

The final model for the difference in QLF^{AVER}, for the selected sites, indicated that there was some evidence of an interaction between the factors of experiment and group

(p=0.054) and an effect of experiment alone (p=0.050). There was insufficient evidence of a site effect (p=0.373).

When the various factors were examined with relation to the outcome for the two groups separately, there was no evidence of a site effect for either the T group (p=0.405) or the TD group (p=0.715). For both groups there was some (although not statistically significant) evidence of an experiment effect (TD: p=0.055, T: p=0.057).

To investigate further the effect of experiment, the summary statistics for the differences in QLF^{AVER}, by experiment, for the two groups separately, are given below (Tables 67 and 68).

Table 67 shows that 200mL milk x 3/day had the lowest mean difference between final image and baseline image, and the no beverage group had the greatest mean difference for the T group. The ranges were wide and included positive and negative numbers, indicating that not all of the lesions behaved in the same way.

Table 67 - Summary statistics of selected sites differences (final image-baseline image) for QLF^{AVER} by experiment for the T group

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	45	4.1 (5.9)	(-7.3, 21.9)
1.5mg F x 1/day	47	4.7 (5.7)	(-15.4, 16.7)
200mL milk x 1/day	51	4.3 (5.3)	(-9.3, 13.2)
200mL milk x 3/day	48	3.3 (4.6)	(-6.4, 15.6)
No beverage	47	6.4 (5.3)	(-3.2, 17.3)

QLF^{AVER} is measured in % change in fluorescence

Table 68 shows similar statistics for the differences between final image and baseline image for the TD group. Here, the lowest mean difference occurred in the 1.5mg F x1/day group and the greatest difference occurred in the 200 mL milk x 3/day. Again the ranges

were wide and included positive and negative numbers, indicating that not all of the lesions behaved in a similar manner.

Table 68 - Summary statistics of selected sites differences (final image-baseline) for QLF^{AVER} by experiment for the TD group

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	52	5.0 (4.3)	(-2.6, 15.0)
1.5mg F x 1/day	56	4.8 (5.1)	(-9.3, 15.2)
200mL milk x 1/day	49	5.9 (4.6)	(-8.8, 16.8)
200mL milk x 3/day	57	6.7 (3.5)	(-1.0, 16.5)
No beverage	48	6.4 (4.8)	(-4.9, 18.8)

QLF^{AVER} is measured in % change in fluorescence

In the T group, using Bonferroni-corrected multiple comparisons, there was some evidence (although not statistically significant) of differences between no beverage and 0.5mg F x 3/day, no beverage and 200 mL milk x 1 day and no beverage and 200 mL milk x 3/day. The corrected confidence intervals for these three comparisons are given in Tables 69.

Table 69 – Bonferroni-corrected confidence intervals for selected comparisons of experiments for QLF^{AVER} for the T group

Differences between experimental protocols		Corrected CI for difference
No beverage -	0.5mg F x 3/day	(-0.5, 5.6)
No beverage -	200 mL milk x 1/day	(-0.8, 5.1)
No beverage -	200 mL milk x 3/day	(-0.02, 6.0)

QLF^{AVER} is measured in % change in fluorescence

In the TD group, using Bonferroni-corrected multiple comparisons, there was some evidence (although not statistically significant) of differences between 200 mL milk x 3/day and 0.5mg F x 3/day and 200 mL milk x 3/day and 1.5mg F x 1/day. The corrected confidence intervals for these two comparisons are given (Table 70).

Table 70 – Bonferroni-corrected confidence intervals for selected comparisons of experiments for QLF^{AVER} for the TD group

Differences between experimental protocols		Corrected CI for difference
200 mL milk x 3/day -	0.5 mg F x 3/day	(-0.4, 4.6)
200 mL milk x 3/day -	1.5 mg F x 1/day	(-0.2, 4.6)

QLF^{AVER} is measured in % change in fluorescence

4.3.3 Overall summary of results for TMR and QLF

All data

In summary, the effects of group, site and experiment and any statistically significant interaction effects on the differences in the two TMR parameters, IML and LD, for all sites are given in Table 71 below.

Table 71 - Summary of effects of group, experiment and site on the differences in the TMR parameters for data from all sites

<table>
<tr><th>TMR parameter</th><th>Group</th><th>Site</th><th>Experiment</th></tr>
<tr><td rowspan="2">IML</td><td>No (p=0.059)</td><td>No (p=0.436)</td><td rowspan="2">No (p = 0.445)</td></tr>
<tr><td colspan="2">Combined effect of group and site

Yes (p=0.002)</td></tr>
<tr><td rowspan="2">LD</td><td>No (p=0.917)</td><td>No (p=0.276)</td><td rowspan="2">No (p = 0.606)</td></tr>
<tr><td colspan="2">Combined effect of group and site

Yes (p = 0.043)</td></tr>
</table>

p-values calculated via a general linear model procedure

In summary, the effects of group, experiment and site and any statistically significant interaction effects on the three QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER} for all sites are given in Table 72 below.

Table 72 - Summary of effects of group, experiment and site on the QLF parameters for all sites

QLF parameter	Group	Experiment	Site
QLF^{AREA}	Yes (p=0.006)	Yes (p=0.040)	Yes (p<0.001)
	Combined effect of group and experiment Yes (p=0.047)		
QLF^{MAX}	No (p=0.549)	No (p=0.182)	Yes (p=0.047)
QLF^{AVER}	No (p=0.116)	Yes (p=0.022)	Yes (p=0.006)

p-values calculated via a general linear model procedure

Selected sites

The effects of group, site and experiment and any statistically significant interaction effects on the differences in the two TMR parameters, IML and LD, for the selected sites 1, 3, 9 and 10, are summarised in Table 73 below.

Table 73 - Summary of effects of group, experiment and site on the differences in the TMR parameters for the selected sites

TMR parameter	Group	Site	Experiment
IML	Yes (p=0.001)	No (p=0.843)	No (p=0.386)
LD	No (p=0.726)	No (p=0.934)	No (p=0.787)
	Combined effect of group and site (p=0.005)		

p-values calculated via a general linear model procedure

In summary, the effects of group, experiment and site and any statistically significant interaction effects on the differences for each of the three QLF parameters: QLF^{AREA}; QLF^{MAX} and QLF^{AVER} for the selected sites 1, 3, 9 and 10 are given in Table 74 below.

Table 74 - Summary of effects of group, experiment and site on the differences in the QLF parameters for the selected sites

<table>
<tr><th>QLF parameter</th><th>Group</th><th>Experiment</th><th>Site</th></tr>
<tr><td>QLF^{AREA}</td><td>Yes (p=0.001)</td><td>No (p=0.640)</td><td>No (p=0.730)</td></tr>
<tr><td>QLF^{MAX}</td><td>No (p=0.596)</td><td>No (p=0.592)</td><td>No (p=0.833)</td></tr>
<tr><td rowspan="2">QLF^{AVER}</td><td>No (p=0.147)</td><td>Yes (p=0.050)</td><td rowspan="2">No (p=0.373)</td></tr>
<tr><td colspan="2">Combined effect of group and experiment

No (p=0.054)</td></tr>
</table>

p-values calculated via a general linear model procedure

4.3.4 Results of post-experimental compliance questionnaire

A questionnaire designed to assess compliance of the volunteers with the experimental protocols was constructed. Two versions were designed; one to give to the volunteers allocated to the treatment group (T), the other, with two additional questions, was given to those allocated to the treatment plus dentifrice group (TD). These are shown in Appendix 8.

The questionnaire was distributed to the volunteers that completed all five of the experimental protocols (25) and also the individual that completed four experiments but was unable to return because of ill health. Due to loss of contact, the questionnaires were not distributed to the other three subjects. In total, 26 questionnaires were sent by post to the volunteers on completion of the study, 25 questionnaires were returned completed. Of these, seventeen subjects declared that they were fully compliant with all aspects of the experimental protocols. Eight of the subjects were not fully compliant; all eight did not wear their dentures overnight all of the time (mainly they declared that they removed the lower denture, some of the time, overnight). Of these eight, three of them did not take the UHT milk all of the time: one volunteer did not take it for three days because he had "flu";

another subject moved house and as a result did not take all of the milk during one week but claimed to take extra the next week; the third, said that he had diarrhoea when he added the fluoride to the milk (no mention of this was made during the experiments to either the research assistant or the clinical researcher).

4.4 Discussion

The following sections will discuss the methodology used and the results of the experimental studies described earlier in this chapter, and interpret them in relation to the methodology and results of previously published work.

4.4.1 Discussion of methodology

This sub-section will discuss the reasoning behind the methodology chosen in the study, in relation to the review of the literature (Chapter 1).

4.4.1.1 Experimental substrate chosen

There are several advantages to using bovine enamel in preference to human enamel. These include the fact that bovine enamel is easier to obtain in large quantities and, of all the non-human substrates, it facilitates the production of artificial caries lesions most like those in human teeth (Edmunds *et al.*, 1988). However, it was decided that bovine enamel would not be used because of increased incidence of Bovine Spongiform Encephalitis (BSE) being detected in cattle during the time of the selection and recruitment stages of the study.

Human enamel was therefore chosen to be used as the hard tissue substrate in this study, even though significant amounts were required i.e. approximately 1000 extracted permanent molar teeth with intact, caries free buccal and lingual surfaces. To obtain these teeth, substantial effort was put into liaising with general dental practitioners and oral surgeons within Glasgow and the surrounding area, requesting that they collect teeth in bottles containing 0.12% aqueous thymol following extraction.

4.4.1.2 Substrate disinfection

As discussed in Chapter 2, Section 2.2.3, all tooth blocks were disinfected using a prion decontamination protocol. This protocol had been suggested by a British Professor of Microbiology to reduce the potential risk of contracting variant Creutzfeld Jacob Disease (vCJD) from extracted teeth. This 'prion-decontamination' protocol was applied to all of

the tooth blocks used in the study and this methodology was accepted by the local ethics committee as an appropriate procedure at the time. A pilot study was designed to determine whether the prion decontamination protocol affected the potential for artificially-created caries lesions to demineralise. This was measured by both QLF and TMR. The results of this pilot study, which were presented at the British Society for Dental Research Conference in 2001, are described in Appendix 1. In summary, they demonstrated that little change occurred in the lesions, as measured by TMR and QLF, when the protocol was applied to the tooth prior to being sectioned into blocks or after it had been sectioned into blocks. However, if the protocol was applied twice to the tooth, before and after it was cut into the blocks, then differences were detected in demineralisation when compared to the control group.

The recommended protocol was subsequently found to be ineffective for prion decontamination. Current protocols for general prion decontamination therefore revert back to those outlined by Taylor (1999) and suggest placing items in 2M NaOH for 1 hour, or a combination of autoclaving (121°C) with NaOH. Although it is not known for certain what effect these latter protocols may have on any artificially created lesions or on the actual tooth structure, it can be assumed they would be very destructive. At present, there is, therefore, no known method of decontaminating tooth blocks containing artificial caries lesions that is effective against prion proteins and has been shown not to affect the structure of the block.

The current 'gold standard' for disinfection of tooth substrate for use in *in situ* studies is gamma-irradiation (Amaechi *et al.*, 1998; Amaechi *et al.*, 1999). However, prion proteins are known to be resistant to ionizing, ultraviolet and microwave radiations (Taylor, 1999). This raises significant concerns about whether *in situ* studies using human teeth should be undertaken at all, at present. Preliminary work in the disinfection of tooth substrate has been undertaken by Watson *et al.* (2004) using hypochlorite-treated and untreated autoclaved enamel surfaces. However, the effect this would have on the mineral dynamics of an artificially-created caries lesion is not clear. Further work is required in this area to determine the appropriate manner of eliminating the possible transmission of prion proteins (and other infective agents) during *in situ* studies. The prescribed protocol was considered effective for conventional microbial control prior to placement of specimens in the mouth although the sterility of the specimens was not specifically tested.

4.4.1.3 Creation of artificial caries lesions

In the present study, it was important to commence experiments with caries lesions which had the potential to either remineralise or demineralise further. For reasons outlined in the literature review (Section 1.2.3.2), it was decided that smooth surface enamel caries would be investigated as these lesions are easier to create in a standard manner than fissure caries. In addition, measurement is simplified with this lesion type, as smooth surfaces permit alignment of the measurement device perpendicular to the surface being assessed. Hence, a greater number of measurement techniques may be employed. Artificially-created enamel lesions were chosen for the study, since it is virtually impossible to identify early, natural caries lesions capable of remineralising which have all demineralised to a similar degree (Ekstrand *et al.*, 2005).

Early enamel caries can be created experimentally *in vivo* by fixing modified orthodontic bands or gauze onto a natural tooth to allow plaque accumulation (Nygaard-Östby *et al.*, 1957; Hals and Simonsen, 1972; Ostrom *et al.*, 1977; Gallacher and Pearce, 1979; Øgaard and Rølla, 1992). However, as discussed in Section 1.7.4, unless these teeth are scheduled for extraction, it is almost impossible to perform exacting lesion microanalysis. By creating artificial caries lesions *in vitro* for later use within an *in situ* model, more control of the lesion development is possible, and ideally, lesions can be developed so that they have a similar degree of baseline demineralisation. *In situ* appliances allow the placement of relatively standardised caries lesions into a more realistic environment than would be achieved in a laboratory, while permitting the testing of specific hypotheses.

In lesion development, it is hypothetically easier to standardise the creation and behaviour of these lesions if the surface enamel is abraded to remove the outer surface. This removes the "history" of the outer surface of enamel with respect to intra-oral fluoride exposure and caries experience (Arends and Gelhard, 1983). Therefore, it was decided that abraded human enamel was to be used in the studies described in this thesis, in an attempt to have consistency of enamel lesion development and response. Following examination of the literature and pilot studies within the hard tissue laboratory of the University of Glasgow Dental School, the Carbopol method of artificial caries lesion development (White, 1987b) was chosen for: (a) its ability to recreate a surface zone, (b) the capacity to investigate both remineralisation and demineralisation and (c) its potential for consistency in artificial lesion creations. Thus to conclude, the decision was made to use abraded, human enamel

as the substrate, containing artificially-created smooth surface caries lesions, in an *in situ* appliance.

4.4.1.4 *In situ* model

To resolve any ethical considerations and to permit the placement of many tooth slabs (10) in the mouth, it was decided that modified complete dentures would be used as the intra-oral model for the main *in situ* enamel caries study described in this thesis. This model allowed for the potential testing of cariogenic substrates (as had been planned initially) and also eliminated any ethical considerations involved in asking subjects to refrain from using other fluoridated substances, such as fluoridated dentifrices, for the six-week duration of each of the experimental protocols (domestic water within and surrounding Glasgow has a fluoride concentration of less than 0.04 ppm F^-).

4.4.1.5 Denture model

The sites chosen to place the tooth slabs within the denture were determined partially with reference to previous studies in this area investigating salivary film velocity and its effect on the clearance of plaque acids from areas of the mouth (Lecomte and Dawes, 1987; Dawes *et al.*, 1989) and partially by the ability to physically site the tooth blocks within the dentures. The thickness of the tooth blocks was approximately 3 mm, as it was necessary to provide support for the enamel from the underlying dentine. The tooth blocks were symmetrically placed on the right and left sides, buccally and palatally in the posterior of the upper and lower dentures (eight blocks), with additional blocks placed in the midline, buccal and palatal aspects of the upper denture (two blocks). It was anticipated that sites adjacent to major salivary gland orifices would have greater remineralisation potential than those placed, for example, on the upper buccal anterior (labial) aspect of the dentures.

One of the disadvantages of this model is that once dentures are worn, the microflora may change, with a potential increase in the colonisation by yeasts such as *Candida albicans,* as well as higher proportions of lactobacilli and staphylococci (Marsh and Martin, 1999). In particular, it is known that specific caries organisms, such as streptococci, significantly reduce in numbers when the patient is edentulous. Prior to the experimental protocols, subjects had small enamel blocks placed in the test dentures and were asked to wear these for three days before the caries-containing enamel blocks were sited. Plaque samples were collected after the three-day period and placed in 1mL of Fastidious Anaerobe Broth (FAB), prior to transfer to the microbiology laboratory for immediate processing. It is

known that the manner in which a carious lesion responds to a caries challenge is dependent, in part, on the oral microflora present. Examination of this oral microflora did take place and is described in detail in the Ph.D thesis of Elizabeth Dickson, University of Glasgow (2003). In summary, following the placement of enamel and dentine blocks, *Lactobacilli sp.* counts increased in the majority of subjects, with a minority (~30%) being recolonised by *Streptococcus mutans*.

Another potential disadvantage of the complete denture model is a possible reduction in the salivary flow rate, particularly in subjects who are elderly. This potential disadvantage was carefully managed and subjects were selected who had fulfilled stringent inclusion criteria. This topic is discussed in further detail later in Section 4.4.2.1. Subject compliance is an issue in a study involving removable appliances, and this will also be discussed in Section 4.4.2.8. In other aspects, this model acts in a similar manner to other *in situ* models.

The advantages of the complete denture model are numerous. Given that the subject no longer has their own teeth, the treatment being tested cannot cause any detrimental damage to their dental hard tissues. This allows testing of treatments that may have evidence of being caries-inducing, thereby overcoming any ethical dilemmas and constraints. At the same time, other advantages of an *in situ* model still apply, such as the ability to place caries lesions of a pre-determined size into the oral cavity, which is a much more relevant location than a test-tube or "artificial mouth". In addition, most subjects rendered edentulous would have become so as a result of their caries or periodontal disease experience and it could be argued that they are thus relevant subjects, given their disease experience.

4.4.1.6 Experimental protocols

Historically, the fluoride concentration used in milk has been between 5 and 15 ppm F^- as CaF_2, NaF, Na_2-monofluorophosphate or Na_2-silicofluoride, and has been shown to produce a significant reduction in caries of 40-50% (Stephen *et al.*, 1996). Previous studies completed in the Glasgow area (Stephen *et al.*, 1981; Stephen *et al.*, 1984) used 200 mL of milk with 1.5 mg F^- added (approx. 7.5 ppm F^-) and obtained a 43% difference in average DMFS scores between subjects exposed to fluoridated and non-fluoridated milk. However, it should be recognised that all of the children in the Stephen *et al.* (1984) study were advised to brush with a non-fluoridated toothpaste. The majority of early fluoridated milk

studies (discussed further in Chapter 1, Section 1.6.6) were undertaken without the use of fluoridated toothpastes, at a time when no fluoride was routinely available to the general population. The use of fluoridated dentifrices has become widespread over the last 25 - 30 years, and as a result of this (and the use of additional fluoride sources) the caries rate in Europe and USA has dropped significantly (Haugejorden *et al.*, 1997; Newbrun, 1999). Fluoridated toothpaste is now regarded as the main source of fluoride in areas without water fluoridation.

The consensus view is that frequent exposure to fluoride at an appropriate concentration is important to maintain the low levels of fluoride in saliva and plaque. There is uncertainty about the potential benefits of consumption of fluoridated milk, in populations who routinely use fluoridated toothpaste. Additionally, the influence of frequency of exposure to fluoridated milk has been highlighted as an area requiring investigation (Stephen *et al.*, 1996).

Therefore, the main study described in this thesis (Chapter 4) was designed to investigate the effect on enamel mineral content of exposure to milk, with and without added fluoride and to determine whether any benefit of fluoridated milk use was apparent over-and-above that associated with exposure to a fluoridated toothpaste slurry. The effect of frequency of intake of fluoridated milk was also investigated. It was decided to use the same daily dose to that used previously by Stephen *et al.* (Stephen *et al.*, 1981; Stephen *et al.*, 1984), i.e. 200 mL of milk with 1.5mg F^- (7.5 ppm F^-), and to compare it to that of 200 mL of milk containing 0.5 mg F^- (approx 2.5 ppm F^-) in three daily doses. The control was milk alone; 200 mL of milk once a day, and 200mL of milk three times per day, with an additional negative control of "no beverage". It was important to determine the effect of both a control (milk) and a negative control (no beverage) because previous work has suggested that there is a caries-preventive effect from consuming milk alone (discussed in more detail in Section 1.4).

When investigating the effect of increasing the fluoride delivery to three times daily, it was decided to divide the daily dosage of fluoride by three, thereby reducing the concentration of the fluoridated milk given to the subjects. The main reason for this was, although the subjects involved in the study were edentulous adults, fluoridated milk is generally given to young children. It was suggested therefore that the dosage of fluoride in milk given to the subjects in three daily doses should approximate with that suggested as the appropriate

daily dose, which has been calculated previously to be equivalent to the optimum fluoridated water concentration, which is 1 ppm F^-.

4.4.1.7 Study design and number of subjects

To eliminate any carry-over effect of fluoride between the six-week experimental protocols, the study design incorporated a four-week "washout" period, as research undertaken by Stephen *et al.* (1992) suggested a "washout" period of two weeks (Schäfer, 1989) was insufficient. The study design also tried to balance any potential cumulative effect of the different protocols. It was mainly a statistical design process (detailed in Section 4.2.7) and involved randomisation of the order of the experimental protocols, for each subject, after the subject had been randomly allocated to one of the two groups. This process was provided by a statistician from the University of Glasgow and administered by a research assistant, with the main operator (AJN) not party to this information for the duration of the study.

The number of subjects was determined by the design of the study, rather than following on from a more formal statistical sample size calculation, given the limited resources of time and funding available. The design of the study was described in detail in Section 4.2.7 and required a minimum of 20 subjects (10 in each group), each completing all five experiments, in order to be able to examine all the effects of interest. Therefore, as previously described, a target of 30 subjects was set. Ideally, the number of subjects would have been greater, but 30 subjects, involved the production of 1500 human tooth blocks, each containing a carious lesion. Producing these lesions was, in itself, an enormous undertaking, not including the exacting analysis of the specimens on removal from the test dentures. Previous studies in this field, involving protocols of similar duration have used smaller numbers of subjects; Sjogren *et al.* (2002) in an *in situ* study involving six four-week protocols had 15 subjects, Engstrom *et al.* (2004) used one four-week protocol and had 20 subjects.

4.4.1.8 Measurement of caries

Measurement of the caries lesions over time was undertaken using QLF. This technique became available following the loan of a prototype machine to the University of Glasgow by Inspektor Research Systems BV, Amsterdam, The Netherlands. The QLF technique has many advantages; it is non-invasive and repeated measurements can be made relatively easily. The QLF product "Clin-QLF" has been developed extensively over the last 10-12

years. Initially, the light source used to illuminate the tooth was an Argon laser (de Josselin de Jong *et al.*, 1995). However, because of health and safety regulations surrounding the use of lasers, which limit their locale and portability, a white Xenon light source was later developed (Al-Khateeb *et al.*, 1997). The prototype machine used in this study was one of the first light source prototypes developed. In addition, the manner in which the CCD (Charge Coupled Device) camera and light source are mounted in the hand-piece has been modified in recent years. The prototype machine used for this study had the light source, with a prism adjacent to a CCD camera, mounted in a hand-piece. However, the more recent Clin-QLF equipment has the light source and CCD camera mounted co-axially and this, anecdotally, appears to improve the amount of light and fluorescence available to the camera (though no evidence is available to date to substantiate this claim). Also, it should be recognised that technology advances continually and that the resolution of the CCD cameras available has improved over time. This should be remembered when making direct comparisons of QLF results obtained with the prototype machine used in this thesis and those obtained using QLF machines currently available on today's market. In addition to the advancements in the hardware, there have been developments in the software used; in particular, the image alignment software, which now allows multiple images to be captured over time at similar angulations and orientation (de Josselin de Jong and van der Veen, 2000). This is particularly valuable in the *in vivo* situation when imaging a natural curved tooth surface and allows longitudinal monitoring of a demineralised area. Unfortunately, this software was developed only in 2000 and thus was not available at the beginning of the studies described in this thesis.

A value of -13 % Fluorescence Loss for QLF^{AVER} was chosen as the minimal threshold of demineralisation for the tooth block to be included in the study. This was chosen because it was found to correspond with evidence of a white spot lesion when the tooth block was viewed dry, with the naked eye, in natural light. In addition, it was surmised that potentially, both further demineralisation and remineralisation could be demonstrated by QLF from this degree of demineralisation. There was no maximum threshold defined for inclusion in the study, though in practice, values greater than -30% were seldom achieved after 7-14 days of demineralisation. It could be suggested that a maximum threshold should have been defined, thereby reducing the variability of the specimens at baseline.

TMR was also used because it is currently recognised as a "gold standard" in the measurement of mineral content. It was considered necessary to use an accepted method to

measure the tooth mineral change in addition to the relatively new QLF technique. Initially, it was intended to compare directly the results obtained with QLF and TMR. However, this was not as straight-forward as had been anticipated. TMR examines a section or slice taken from a lesion, whereas QLF measures the mineral content of the whole lesion. Whilst some researchers have attempted to correlate results derived from the two methods (Hafström-Björkman *et al.*, 1992; Emami *et al.*, 1996; Lagerweij *et al.*, 1999), others have raised questions about the ability to compare values obtained via QLF with those obtained via microradiography (ten Bosch, 2000). Therefore, in the present study, results obtained with QLF and TMR were not directly compared, although comparisons of the conclusions of the factors influencing the differences obtained between end-point and baseline values for the various parameters of the two methods will be discussed later (Section 4.4.2.7).

4.4.1.9 Removal of stain from caries lesions

One of the difficulties, which became apparent early in the study, was the development of staining on some of the tooth blocks and/or the carious lesion situated within the blocks. Stain is known to influence the fluorescence properties of a caries lesion, which in turn can affect the ability of the QLF method to accurately measure the degree of de- or re-mineralisation. Therefore, a pilot study was undertaken to investigate the ability of various protocols to "de-stain" the caries lesion, taking into consideration the effect that each protocol may have on the ability of the lesion to de- or re-mineralise further and the effect that they may have on the lesion's surface characteristics. This protocol and the results obtained are described in more detail in Appendix 11. This work was presented at the Pan European Society for Dental Research conference in 2002. The results obtained in this pilot study demonstrated that 30% hydrogen peroxide and zircate polishing paste were both effective at removing the stain. The "de-stain" protocol used in the main study described in this thesis was therefore, 4 hours immersion in 30% hydrogen peroxide followed by thorough washing and soaking in water for 24 hours. This protocol was only applied to tooth blocks at the end of the experimental protocols where stain was observed visually. If the lesion did undergo "de-staining", an image was captured prior to this procedure (QLF Image 7) and further to the stain protocol being applied (QLF Image 9). The methodology of this protocol was described in more detail in Section 4.2.9.

4.4.1.10 Statistical analysis

Statistical analysis was undertaken as described in Section 4.2.10. It was decided to summarise the data by tooth block rather than by subject. This was because there were numerous factors involved in the design of the study; the five experimental protocols, the different sites within the mouth and whether the subject was using a dentifrice slurry to simulate tooth-brushing twice daily. Given that there were 29 subjects in the study, after consideration of these factors, there would have been small numbers in the sub-groups, potentially making it more difficult to interpret the summarised data, particularly given the high variability across tooth blocks. This is in contrast to the number of specimens available for analysis, which was 939 for TMR and 1041 for QLF.

As mentioned previously, descriptive statistics were initially used to describe the results achieved with QLF and TMR. Blocks were randomly allocated when situating them within the test dentures. However, there was a difference determined between the two groups (T and TD), in terms of the baseline measurements, as measured by both QLF and TMR, which occurred by chance. The formal statistical analyses, using a general linear model procedure, for both the QLF data and TMR data, were based on modelling the appropriate difference between baseline and final lesion size, on the various factors of group, experiment, site and subject. However, it is acknowledged that by using the differences, the baseline size of the lesion could be very important, particularly if the lesions were not all comparable to start with: lesions which were bigger prior to any experimental exposure would have more potential for remineralisation.

An alternative statistical method for analysing the data would be to model the final lesion size on the effects of group, experiment, site and subject, after adjusting for the baseline lesion size (as a covariate within the model), using analysis of covariance (ANCOVA). It is recognised that analysis of covariance (ANCOVA) would have been an alternative statistical method, however, it was impractical to report and include the analyses presented previously as well as the more complicated ANCOVA method of analysis and this alternative method was deemed to be beyond the realms of this thesis. For the purposes of this "clinical" thesis it was considered sufficient to report the findings in terms of modelling the differences in lesion size only.

4.4.2 Interpretation of results

4.4.2.1 Subjects

Given that the mean age of the participants at recruitment to the study was 72 years, and the duration of the study, it is remarkable that so many of the subjects managed to complete all five experimental protocols. Following recruitment to the study, the subjects all had two sets of complete dentures constructed and were reviewed until both sets were comfortable to wear both day and night. In addition to the time taken to achieve this, the time required to complete the five experimental protocols, with washout and vacation periods, meant the subject's commitment to the study was between two and a half and three years duration. This is significant, particularly as the subjects were often required to attend at two-weekly intervals.

To enable an analysis to be undertaken of the effect of order of the experimental protocols, it was necessary that at least 10 subjects, in each group, completed all of the protocols in the pre-determined orders. Unfortunately, this did not happen and thus, it was not possible to examine for any effect of the order in which the experiments were completed within the statistical modelling. It is thought that this inability to examine for the effect of order of the experimental protocols will have a minimal effect on the conclusions of the study, as there was a relatively lengthy four-week washout period between each protocol, which should eliminate any carryover effect from the previous protocol.

Whilst this elderly cohort of subjects may have had an age-related reduced salivary flow, all participants had resting whole salivary flow rates greater than 0.1 mL per minute at selection (Dawes, 1996). Any medication that could reduce salivary flow rates was a contraindication and excluded the subject from the study. Additionally, subjects were repeatedly asked throughout the study whether there had been any change to their medical history or medication taken which could have affected their ability to fulfil the initial selection criteria described in Section 4.2.1. No participants were found to have had any change that would have affected their salivary flow rate and therefore their suitability to remain as a participant in the study.

A number of advantages were associated with use of an elderly population group. Many of the subjects were no longer working in paid employment and therefore were more able to attend appointments. Participants made every effort to attend appointments that had been arranged, and there were only a few appointments throughout the whole study where

patients did not attend. These were usually as a result of unforeseen circumstances, such as a fall or sudden illness. The disadvantages of using a more elderly cohort of subjects are that the findings obtained might not be directly extrapolated to younger cohorts e.g. they may have a different diet and intra-oral microflora (as discussed in Section 4.4.1.5) and demographically they are more likely to develop morbidity which may prevent them from completing their agreed participation in the study.

4.4.2.2 Survival of specimens suitable for evaluation

Of the 1360 tooth blocks used in the experimental protocol for analysis by TMR, there were 939 blocks (69%) available at the end of the study with a matched "pair" of a covered (control) and exposed lesions. Therefore, a total of 31% of the blocks were not available because they had either been lost during the study (21%), did not have a matched pair of lesions at the end of the study (9.7%), or were mixed up on retrieval from the study (0.3%). Many of the blocks were lost from sites 4, 5 and 6 of the dentures, i.e. the palatal sites on the upper denture. The subjects mentioned that many of these were lost during eating. This may have been related to positioning of the blocks within the denture, or the choice of cement used to place the blocks within the denture. Blocks were placed in depressions cut in the acrylic dentures that were as near to a friction-fit as possible. The dental cement used was one designed to temporarily hold crowns and bridges onto teeth rather than hold pieces of tooth in an acrylic denture. In the choice of cement, a balance was required between selecting a material with the ability to hold the pieces of tooth securely within the denture and at the same time allowing easy removal of specimens at the conclusion of each experimental protocol. Whilst it could be perceived that the cement chosen was not an appropriate material to use, it was surmised that temporary crowns are sometimes constructed in cold-cure acrylic resin and, therefore, a reasonable tooth/acrylic bond should exist (Sjogren *et al.*, 2002).

Prior to TMR evaluation, approximately nine thin (~200 μm) sections were cut through each lesion. One section was chosen for TMR analysis, usually from the middle of the lesion. If any section was found not to have an intact surface zone, within both the covered and exposed parts of the lesion, then that section was discarded and another section chosen. The lack of a matched pair of intact exposed and covered lesions on sections at the end of the study was frequently as a result of cavitation of the lesion surface, either during section preparation at the end of the study or during the *in situ* protocol.

Of the 1360 tooth blocks initially placed in the denture, 1041 blocks were available for analysis with QLF at the end of the study. Two hundred and eighty-four blocks (21%) were lost from the dentures *in situ* because of failure of the cement used to fix the blocks in place during function (swallowing, eating and drinking).

4.4.2.3 Analysis of baseline data

For the purposes of this study, the intention was for the lesions to have a relatively similar size at baseline. The criteria for lesion selection involved capturing images of the lesions and analysing them with QLF. Lesions were prepared and then visually examined to confirm the presence of a white spot lesion. Inclusion was based on a minimum QLF^{AVER} reading of -13% Fluorescence Loss. However, there was no maximum QLF^{AVER} value suggested, the maximum demineralisation criterion being that the lesion surface should be intact and have not cavitated.

Looking at the QLF (Image 2) data as a whole, it is evident that the baseline images were of a relatively standard size and degree of demineralisation. This is demonstrated by the similar mean values and narrow standard deviations of the lesions at baseline. Additional examination of the baseline QLF data, divided by group and experiment, is shown in Appendix 10 and, in general, confirms this statement. One exception, however, is the QLF parameter QLF^{AREA} where statistical analysis of baseline data by group (Appendix 10) did demonstrate a significant difference between the T and TD groups, with the latter group having a statistically significantly greater mean lesion area. The mean difference was 0.2 mm^2. The other exception was the analysis of baseline QLF data by experiment. This found statistically significant differences between experimental protocols for QLF^{MAX} and for QLF^{AVER}. While the effect of these differences in QLF baseline values on experimental outcomes is unknown, it is felt that the magnitude of the mean differences occurring between the groups or experimental protocols at baseline is unlikely to have clinical significance.

Looking at the covered (control) TMR data as a whole, it was apparent that although the mean IML and LD values indicated highly demineralised but shallow TMR lesions, there was a wide range of lesion sizes amongst the specimens. It was impossible to measure the degree of lesion demineralisation with TMR at baseline, as the specimens would have been destroyed in the process. Unlike QLF, where the whole of the lesion is measured both at baseline and post-experimentally, with TMR, adjacent parts of the original lesion are

measured at the end of the experiment, one part having been covered with varnish representing the proxy baseline or control measurement, and the other uncovered area representing the post-experimental measurement. It is possible that the varnished/fissure sealed, covered (control) part of the lesion may not have always completely protected the control part of the lesion from further remineralisation or demineralisation as, on occasion, saliva may have been able to leach/seep under the covered area. It has to be noted, therefore, that whilst TMR is a 'gold standard' for measurement of enamel mineral content, there are some limitations associated with the use of covered proxy baseline measurements.

Although the enamel blocks with created lesions were allocated randomly to test subjects and sites within dentures, when the covered TMR data were examined with respect to treatment group (T and TD), there were differences between the T and TD groups for both mean IML and LD. This difference between groups indicated there was greater demineralisation of the covered specimens in the T group, by approximately 12%, and there was consistency in the direction of this change for both IML and LD. These differences were statistically significant ($p<0.001$ for both IML and LD). It could be argued that as the T group lesions were on average larger than those of the TD group, there was more potential for remineralisation in the former group. There could, therefore, have been a tendency for the difference between the 'baseline' covered and final values to have been greater in the T group. However, in general, the results found that more remineralisation occurred in the TD group, and it is therefore possible that the use of the dentifrice slurry may have shown an even greater effect, had the baseline values been more consistent, as discussed in Section 4.4.1.10.

4.4.2.4 Comparison of differences between 'baseline' and final lesions

This sub-section will interpret the results obtained when comparing the differences between the covered and exposed lesions for TMR, and baseline and final lesions for QLF.

TMR

For the parameters IML and LD, examining the mean values of the differences between covered 'baseline' and final lesion sizes indicated that there was overall remineralisation, although there was significant variation in lesion response.

The three main factors being investigated in the study were: 1] 'group' i.e. with and without the use of a dentifrice slurry to simulate brushing with a fluoridated dentifrice, 2] 'site' in the mouth, and 3] 'experimental protocol' i.e. various milk protocols with and without added fluoride and the 'no beverage' control.

Whilst it could have been anticipated from the literature that the use of a dentifrice slurry would have clearly increased the amount of remineralisation, and the descriptive statistics for IML showed on average more remineralisation in the TD than T group, formal statistical analysis using a general linear modelling procedure (GLM) found that the main effect of 'group' was not statistically significant for either IML ($p=0.059$) or LD ($p=0.917$). Although the reasons for this are unclear, possible explanations could relate to the difference in baseline values between the groups as discussed previously and/or the inclusion of other fluoride protocols, which could have reduced the effect of the dentifrice slurry. The analysis did, however, show a statistically significant interaction effect of 'group and site'.

Since the site pattern of change in mineral content of lesions was different in the two groups, the factor of site was examined for each group separately. Within the TD group, in relation to the IML data, a statistically significant site effect was seen and the pattern found concurs with previous literature investigating the site-specificity of caries (Lecomte and Dawes, 1987; Dawes *et al.*, 1989), with more remineralisation occurring in the upper buccal posterior regions (with high salivary film velocity) and less in the upper anterior labial region (with low salivary film velocity). A similar pattern was seen for LD, but the site effect did not reach statistical significance ($p=0.088$). Within the T group, a different pattern was seen, but the within-group site effect did not reach statistical significance for either IML or LD outcome measures, suggesting that the differences between the two groups were not consistent at all ten sites. It is difficult to understand why the different patterns across the sites occurred for the two 'groups', i.e. with the expected site pattern of remineralisation occurring in the group who used the dentifrice slurry, and exposure to relatively high concentrations of fluoride on a regular basis, but not with the treatment-only group.

With regard to the third factor under investigation, namely 'experiment', although some variation in mean change of mineral content occurred between the different experiments (with descriptive statistics surprisingly showing most remineralisation in the 'no beverage'

group), using the GLM analysis, the effect of 'experiment' was not statistically significant for either IML or LD data, after adjusting for the other significant factors. With the TD group, it could have been expected that the exposure to the toothpaste slurry may have 'swamped' the effect of the additional exposure to lower concentrations of fluoride in some of the milk experimental protocols. It could, therefore, perhaps have been anticipated that a 'group and experiment' interaction may have been seen, with more pronounced differences being present between experiments in the T group than in the TD group. However, this did not occur. Although efforts were made to facilitate subject compliance, e.g. with home delivery of products and clear instruction sheets, the potential effect of compliance on study outcomes is uncertain, particularly in relation to the effect of experiment, where it could be surmised the greatest variation from the protocol may have occurred. The potential influence of subject compliance is discussed in detail in Section 4.4.2.8.

Overall, the mean IML difference between covered and exposed lesions was 366 %Vol mineral.μm. For LD, the mean difference was very small (overall mean change = 4.6 μm). This brings into question the preciseness with which changes in lesion depth can be measured. It has been suggested that the accuracy of TMR in relation to enamel lesion depth measurement is about 5 μm and for IML 200 %Vol mineral.μm (Arends and ten Bosch, 1992). This suggests that the parameter IML may be a more appropriate measure of changes in mineral content of artificially created caries lesions, as used in this *in situ* model. This would appear to agree with work published by White *et al.* (1992), which suggested that the primary measure of mineral loss or gain should be IML (also known as ΔZ), with the secondary measure being lesion depth. However, the findings of the present study showed, in general, agreement between the IML and LD results. In both cases the individual effects of 'group', 'site' and 'experiment' were not found to be statistically significant, whilst for both outcomes an interaction between 'group and site' occurred. Furthermore, on exploring this interaction, the same overall pattern of remineralisation across sites was seen within the TD group for both IML and LD, whilst for the T group both the two outcomes showed different patterns across the ten sites.

QLF

The comparison of baseline data (Image 2) with post-experimental protocol data (Image 10) for all specimens for each QLF parameter, demonstrated mean positive differences, indicating remineralisation occurred overall. QLF^{AREA} demonstrated the greatest positive

difference of approximately 50%, this was followed by QLF^{AVER} with a 25% change towards mineralisation, then QLF^{MAX} with a positive change of approximately 20%.

For QLF^{AREA}, formal statistical analysis using a general linear modelling procedure (GLM) demonstrated statistically significant effects of 'group', 'experiment' and 'site'. By group, there was more remineralisation in the TD than T group, indicating that the dentifrice slurry was more effective than the experimental protocols alone. By experiment, the greatest mean difference for QLF^{AREA} was in the 200 mL of milk x 3/day protocol, followed by the 1.5 mg F in milk x 1/day. The finding with regard to the latter protocol is understandable, although its use could have been expected to result in most remineralisation. Whilst milk alone has been shown to be non-cariogenic (McDougall, 1977; Bowen *et al.*, 1991; Bowen and Pearson, 1993; Erickson and Mazahari, 1999), it is somewhat surprising that on average more remineralisation occurred with intake of milk three times daily than with either of the fluoride-containing milk protocols.

In relation to the QLF^{AREA} data, a statistically significant interaction effect occurred between group and experiment. Although, as expected, the average changes in mineral content were lower in the T group, the greatest difference between experimental protocols in the T group, in terms of the difference between baseline and final measurements ,was larger than with the TD group (0.9 and 0.4 mm^2, respectively). Although, as discussed previously in relation to TMR, it could be predicted that a wider range of values would be associated with the absence of dentifrice slurry use, it is surprising that in the T group, the smallest amount of remineralisation occurred, on average, with use of 0.5 mg F x 3/day. It was anticipated that the fluoride-containing milk protocols would have been seen to have more of an effect than the non-fluoride protocols, without the swamping effect of the dentifrice slurry being present.

Examining the results by site, on average, there was greatest remineralisation at sites 4, 5 and 6 (upper, palatal sites). However, there were approximately 50% fewer tooth blocks retrieved from these areas. Nevertheless, this finding is understandable as, with tongue movement, there will be good movement of saliva and little plaque accumulation at these sites. By contrast, the least amount of remineralisation occurred in site 2. The results achieved at this site would concur with previous evidence suggesting that less remineralisation occurs at areas of reduced salivary flow (Lecomte and Dawes, 1987; Dawes *et al.*, 1989).

For QLF^{MAX}, formal statistical analysis using the GLM procedure indicated that there was a statistically significant effect of site. The smallest mean positive difference between the baseline and post-experimental measurements occurred at site 2, indicating that the least remineralisation occurred at this site. As mentioned previously, this could be explained by the low salivary film velocity that has been proven to occur in this area (Lecomte and Dawes, 1987). The greatest mean differences occurred at sites 4, 5 and 6, where the difference achieved was double that which occurred at site 2. The findings in relation to these palatal sites are thus similar to those seen with the QLF^{AREA} parameter, and the potential reasons for this have been described previously.

For the QLF parameter QLF^{AVER}, formal analysis with GLM demonstrated statistically significant effects of experiment and site, but after adjusting for these effects, there was no statistically significant group effect. For experiment, the smallest mean difference occurred in the 0.5 mg F x 3/day protocol and the greatest mean difference occurred in the 'no beverage' protocol. The ranges included negative values suggesting that there was inconsistency in lesion behaviour. It was surprising to see that the negative control, i.e. 'no beverage', had the greatest positive effect on average fluorescence change. It is not understood why this should be the case, although the percentage differences in the average fluorescence obtained for these protocols were small (4.8% for the 0.5 mg F x 3/day protocol and 6.2% for the no beverage protocol). For site, the greatest mean differences between baseline and post-experimental protocol data occurred in sites 4, 5 and 6. This concurs with results obtained with the other QLF parameters. The smallest mean changes occurred at sites 7 to 10, i.e. those situated on the lower denture. This pattern is different to that seen with the other QLF parameters and is difficult to explain, as both the buccal and lingual aspects of the lower arch are exposed to a relatively high salivary film velocity compared, for example, to the upper labial site (Lecomte and Dawes, 1987; Dawes *et al.*, 1989).

In summary therefore, for QLF, the most consistent statistically significant effect on all three QLF parameters was that of the site of the tooth blocks within the test dentures. The greatest amount of remineralisation occurred on the palatal aspects of the upper denture. Many blocks were lost from these sites during the experiments, presumably due to movement of the tongue and the effect of eating. It is probably for these reasons that most remineralisation occurred here, with good salivary movement and little chance for appreciable plaque accumulation at these sites. The finding would tend to agree with

clinical observations, with caries seen relatively rarely at these locations. For both QLF^{AREA} and QLF^{AVER}, the smallest change between baseline and post-experimental measurements occurred at site 2. As discussed previously, this finding agrees with the published literature, indicating a very low salivary film velocity at this site (Lecomte and Dawes, 1987). The experimental protocol used also demonstrated a statistically significant effect on both QLF^{AREA} and QLF^{AVER}. These results varied and are difficult to interpret, with the fluoride-containing milk resulting, on average, in less mineral change than some other experimental protocols. There was also a clear effect of group, but only with regard to QLF^{AREA}. This demonstrated, for this parameter, that the remineralising effect of the additional fluoride content of the dentifrice slurry was greater than that achieved with the fluoridated milk alone.

Summary

Overall, on examining the TMR and QLF findings, the outcomes are difficult to interpret. Although the summary statistics showed, on a consistent basis, more remineralisation with the TD than T group, it was only with one parameter, QLF^{AREA}, that the group effect (as a main effect) was statistically significant, after adjusting for any other significant effects. The effect of baseline lesion size may have had some influence on these findings. There was no consistent effect of experimental protocol. Two of the QLF parameters showed 'experiment' to have a statistically significant effect, but the ordering of the experiments with regard to magnitude of change in mineral content varied and the protocols with fluoridated milk were not associated, on average, with more remineralisation than the fluoride-free experiments. As discussed earlier, the influence of subject compliance is unknown. With regard to 'site', in many cases the smallest mean change in mineral content occurred at site 2 (associated with low salivary film velocity), but again this pattern was not consistent across all parameters. A site effect was seen for all three QLF parameters and a combined effect of group and site occurred in relation to both of TMR parameters.

As the two main factors being investigated in this study were the effects of experiment and group, and as a statistically significant site effect was seen for all three QLF parameters and the combination of site and group was statistically significant for both TMR parameters, it was decided to repeat the analysis using a smaller number of sites, which it was hoped would behave in a more consistent manner. The four sites chosen were sites 1 and 3 (upper posterior buccal) and 9 and 10 (lower posterior lingual). All of these sites are close to major salivary ducts. Evidence suggests that there is a relatively high salivary film

velocity in these areas (Dawes *et al.*, 1989) and therefore greater remineralisation potential. Although, according to the QLF results, the palatal sites of the upper arch were associated with most remineralisation, many of the blocks were lost from these locations and the findings from these sites differed from others around the mouth.

4.4.2.5 Analysis of baseline data for selected sites

No statistically significant differences were found in mean 'baseline' lesion TMR or QLF values between the four selected sites. The means, standard deviations and ranges seen across the four sites were relatively similar, particularly in relation to QLF. The potential reasons why more variation may have occurred among sites measured with TMR have been discussed previously in Section 4.4.2.3. The findings suggest that lesions of similar size were allocated to the four selected sites and it could therefore be expected that lesions at these four different sites would behave in a relatively similar manner.

4.4.2.6 Comparison of differences between 'baseline' and final lesions for selected sites

TMR

The results of the selected sites TMR data are described in detail in Section 4.3.1.5 and summarised in Section 4.3.3.

For TMR, both parameters, i.e. IML and LD, demonstrated an overall positive mean difference between 'baseline' and post-experimental values, indicating remineralisation. This was more pronounced with IML. The mean difference for LD was small (overall mean change = 4.3 μm), and as discussed previously in Section 4.4.2.4, the magnitude of the change brings into question how precisely the small differences obtained can be measured.

For IML, formal statistical analysis was performed using a GLM procedure and for the selected sites, unlike for the all-sites data, a statistically significant effect of treatment group was demonstrated on changes in mineral content, with more remineralisation occurring in the lesions exposed to the dentifrice slurry. This outcome is not surprising given the fact that the dentifrice contained 1100 ppm fluoride and was used twice daily. With regard to the 'experiment' factor, the descriptive data showed some variation between experimental protocols, with a different pattern emerging to that seen when all sites were included. For the selected sites data, on average most remineralisation occurred

with use once daily of milk containing 1.5 mg of fluoride and the smallest mean change in mineral content was seen with use of fluoride-free milk once daily. Whilst this result is closer to that which would have been anticipated, compared to the findings seen with inclusion of all the sites (where 'no beverage' showed on average most remineralisation), even with use of the selected sites data, the effect of 'experiment' again failed to reach statistical significance, after adjusting for the significant effect of group. The fact that 'site' had no significant effect is expected; with the four sites having been selected in the expectation that they would behave in a similar manner. Thus for IML, the 'group' effect was strongest, suggesting that the fluoridated dentifrice slurry has the most significant effect on change in lesion mineral content.

For LD, although descriptive data showed similar patterns in relation to 'group' and 'experiment' to that seen with the IML data, analysis by GLM demonstrated insufficient evidence to suggest statistically significant main effects of 'group', 'experiment' or 'site' on the outcome. The analysis did, however, show a statistically significant interaction effect of 'group and site', suggesting that for LD, it is the combined effects of the dentifrice slurry and the position of the block in the mouth (i.e. site) that has the greatest effect on change in this parameter, rather than the experimental protocol. The finding of a 'group and site' interaction effect was to some extent unexpected, as it was anticipated that the use of the selected sites would have resulted in lesions behaving in a similar manner at the different sites and so any differences between the two groups would be similar at the four sites.

Examining the effect of site separately for the two groups, there was some evidence, though not significant at the 5% level, of an effect of site for both the T group and the TD group. The lingual surface of the lower posterior region behaved in a similar manner for both the T and TD groups on the left and right sides of the mouth (i.e. sites 9 and 10), although there was less of a difference between the covered and exposed lesions in the TD group. It was anticipated that sites 1 and 3 would also behave in a similar manner, given that they were both placed in the upper posterior buccal area of the dentures. However, this did not happen in the T group with, on average, demineralisation occurring at site 3. Whilst both the failure to see an overall 'group' effect and the detected 'group and site' interaction were unexpected and differ from the results obtained with IML, given the size of the mean changes in lesion depth occurring and the previously mentioned associated limitations of this measure with regard to the ability to measure consistently and accurately such small

changes in LD, the findings are perhaps understandable. More weight should, therefore, perhaps be attached to the IML findings, as suggested by White *et al.* (1992).

When comparing the TMR results obtained for all data with those for the selected sites, the interpretation of factors important on the outcomes was clearer with the selected sites data. Here, only the effect of 'group' had a statistically significant effect on mean change in IML, with significantly greater remineralisation occurring in the TD group. Therefore, for the selected sites IML data, the dentifrice slurry had the greatest beneficial effect on the lesions. For LD, the only statistically significant effect noted was the combined effect of 'group and site'; this was seen both when all of the data were considered, as well as with the selected site data.

QLF

For all QLF parameters, the comparison of baseline and post-experimental data for selected sites demonstrated a mean positive difference, indicating remineralisation occurred overall. QLF^{AREA} demonstrated the greatest positive difference of approximately 50%, this was followed by QLF^{AVER} with an approximate 25% change towards mineralisation, then QLF^{MAX} with a positive change of approximately 20%. These values are almost identical to those found when all sites were included in the analysis.

Formal analysis of the change in QLF^{AREA} using the GLM procedure, found a statistically significant effect of group, with more remineralisation occurring in the lesions exposed to the dentifrice slurry. Unlike the findings when all sites were analysed (when all three factors of interest had a statistically significant effect), this was the only statistically significant effect on outcome, suggesting that the fluoride within the dentifrice had a greater effect than the experimental protocols. It is not surprising that the use of the selected sites resulted in the disappearance of the site effect. It could be concluded, therefore, that for QLF^{AREA}, the most significant effect on outcome was that of group.

The GLM model indicated that for QLF^{MAX}, none of the factors examined showed evidence of a statistically significant effect on the outcome. The only factor that had shown a significant effect when all sites were included in the analysis was 'site', and as discussed above, the loss of this effect with use of selected sites data is not unexpected.

The GLM model for QLF^{AVER} indicated that there was a combined effect of 'group and experiment' on change in mineral content. This suggests that the dentifrice slurry and the

experimental protocol used in combination had an effect, that was not quite statistically significant (p=0.054), with differences between experiments not being the same for the two groups. When the various factors were investigated for the two groups separately, there was some evidence (not statistically significant at the 5% threshold) of an effect of experiment. In the T group the greatest mean difference (remineralisation) occurred in the 'no beverage' protocol and the least mean difference was in the 200 mL milk x 3/day protocol. In the TD group, however, the greatest mean difference was in the 200 mL milk x 3/day protocol and the least mean difference occurred in the 1.5 mg F x 1/day protocol. In both the T and TD groups, the ranges were wide, indicating inconsistency in lesion behaviour. Given the inconsistency in the ranking of the experimental protocols, it is difficult to draw conclusions from this data.

4.4.2.7 Comparison and interpretation of outcome of results obtained with TMR and QLF data

Although it would be interesting to have been able to correlate data obtained with QLF and TMR, the results obtained for these parameters within this study do not lend themselves to direct comparison. Lagerweij *et al.* (1999) attempted to correlate results obtained with QLF (QLF^{AVER} only), using a white arc lamp, and TMR and achieved a correlation coefficient of 0.63, which is classed as 'moderate' according to Shrout's descriptors (1998), detailed in Chapter 3. Al-Khateeb (1997) performed similar work comparing QLF (QLF^{AVER} and QLF^{AREA}), again, using a light source with TMR, and obtained a 'substantial' correlation coefficient of 0.84 for both parameters. From the results obtained within this thesis, the two techniques appear to be measuring lesion mineral content in different ways. This is not surprising, as TMR is measuring a section of a carious lesion from the surface downwards, into the depth of the lesion, while QLF is measuring the scattering of fluorescence of the surface of the carious lesion, in comparison to the sound enamel around it. This concurs with a previous suggestion by ten Bosch that the two measurement techniques are not directly comparable (ten Bosch, 2000).

However, it is possible to make indirect comparisons of the results of the statistical models obtained from the QLF and TMR data, as follows:

For the TMR data, the most useful parameter was IML, due to the minimal differences attained in lesion depth. However, analysis involving all sites showed similar results for both IML and LD. In both cases, none of the individual factors i.e. group, experiment or

site, demonstrated statistically significant effects, although for both parameters, a combined effect of 'group and site' was seen. For QLF parameters, the effects of group, experiment and site showed different results to those obtained with TMR. From the data, it is difficult to determine the most useful QLF parameter for measuring change in mineral content. When modelling all the data, for all three QLF parameters there was a statistically significant site effect with, in general, the least remineralisation occurring in the upper labial region, and most remineralisation being seen at the palatal sites. For QLF^{AREA}, a statistically significant group effect was also observed, with more remineralisation associated with exposure of lesions to the dentifrice slurry. This parameter, together with QLF^{AVER}, also showed a significant experiment effect. However, the ranking of the experiments by mean changes in mineral content were not as one would have predicted, i.e. the fluoride-containing milk protocols did not show the most remineralisation.

Statistical modelling involving use of data from the four selected sites attempted to allow the effects of group and experiment to be investigated more clearly, without the additional effect of variation in lesion response due to the positioning of the enamel blocks within the oral cavity. On the whole, this approach was successful, with none of the TMR or QLF parameters demonstrating an individual statistical significant site effect, although a combined 'group and site' effect was seen in relation to the lesion depth TMR parameter. Both QLF^{AREA} and the IML component of TMR showed a statistically significant group effect, with more remineralisation occurring with use of the dentifrice slurry containing 1100 ppm fluoride. Neither of the TMR parameters demonstrated a significant 'experiment' effect, although QLF^{AVER} parameter demonstrated a statistically significant effect of experiment (with no beverage demonstrating the greatest amount of remineralisation) and QLF^{AVER} showed a statistically significant combined effect of group and experiment. The selected site data therefore indicated that the use of the dentifrice slurry was the main factor influencing the outcome, with the use of the different experimental protocols having no additional statistically significant effect on most of the outcomes.

In summary, although it was not deemed appropriate to make direct comparisons between the QLF and TMR data because they appear to be measuring different properties, it could be suggested that the IML component of TMR and QLF^{AREA} are able to draw similar conclusions. However, before this claim could be substantiated, further work would need to be undertaken in this area. To date, there are no *in situ* studies in the literature using

QLF as a method of measuring mineral content and, therefore, no *in situ* studies have been published comparing the results obtained using both QLF and TMR.

Attempts to compare the QLF results achieved in this study with those achieved in other investigations are also complicated by the use of different parameters in other studies. Commonly, the parameters ΔF and ΔQ are now used. ΔF is equivalent to QLF^{AVER} and describes the average change in fluorescence, as a percentage, when comparing the area within the highlighted area to the sound enamel surrounding it. ΔQ is the average change in fluorescence as a percentage (ΔF) multiplied by the area, measured in mm^2, i.e. with units of mm^2.%. These changes in outcome measures have been introduced following updates to the Clin-QLF hardware and software and the majority of very recent publications reflect these changes. This makes comparison of the QLF data obtained from this and other studies difficult. As stated above, to date, no *in situ* studies have been published using QLF as a method of measuring de- or remineralisation, although a methodology has recently been published (Higham *et al.*, 2005).

4.4.2.8 Subject compliance

With any *in situ* study, it is essential to make an assessment of the level of subject compliance with the study protocols. This is particularly the case when the subjects are wearing intra-oral appliances that they are able to remove themselves. Every effort was made by those involved in the investigation to enable the subjects to fulfil all of the study requirements with minimum inconvenience. This included delivering the milk to the individuals' homes (although this was not undertaken by the main investigator to ensure blinding). In an attempt to stress the importance of compliance, the Research Assistant asked the subjects on a regular basis about their adherence to the experimental protocols and at the end of each experiment the individuals were asked to return any fluoride containers (including those which had contained the dentifrice slurry), so that ongoing assessment of compliance could be made. It is acknowledged that the positive responses received from the subjects and the return of the containers does not prove that the subjects ingested the fluoride as requested.

The evidence obtained from the study suggests that the dentifrice slurry had an effect on the caries lesions. This suggests that compliance with dentifrice slurry use was satisfactory. The protocols relating to use of the milk protocols were a little more demanding and it is likely that, on occasion, subjects would not have complied fully with requests about the

milk/fluoridated milk intake. In addition, when the subject was involved in one of the fluoridated milk protocols, it is possible that the subject forgot or neglected to add the fluoride solution to the milk, or indeed, they forgot to mix the milk and fluoride solutions together. It is unlikely that the subjects used the wrong bijou of fluoride in their experiments as the subjects were asked to return empty and unused containers to the Research Assistant at the end of each protocol. Additionally, there should not have been a problem with carry-over of one six-week protocol to the next, with a washout period of at least four weeks occurring between each experiment.

Ideally, the subjects would have been subjected to regular urine analysis to confirm that they had followed the protocols correctly. However, given the extensive commitment required of the subjects and their age, it was felt that this would have been too invasive and would have discouraged the subjects from participating. Urinary analysis is often impracticable in a field study, and for this investigation it was determined that, logistically, urinary analysis was not possible and it was therefore not undertaken.

At the end of the study, the 26 subjects who had completed four or five experiments were asked to fill in a compliance questionnaire (the results of which are described in detail in Section 4.3.4). Of the 25 subjects who replied, 17 subjects claimed to be fully compliant with the study instructions. Eight (32%) of those who replied were not fully compliant, and admitted to removing their lower denture, some of the time, overnight. This could have changed the lesion response by reducing the amount of time that the lesion had to remineralise overnight (in the absence of food and drink, bathed in saliva) and also change the manner in which the lesion responded following a period of dehydration, or immersion in water. In addition, of these eight persons, three also admitted to not taking all of the milk, for various reasons such as illness. These issues are common problems with *in situ* and other study-types involving human subjects.

4.4.2.9 Comparison of current results with the fluoridated milk literature

As outlined in Chapter 1, the use of milk as a delivery vehicle for fluoride could have a number of advantages. In addition to the potential caries-preventive benefits, there is evidence that the use of milk itself reduces general health inequalities (Smith, 1997). Thus, milk fluoridation programmes operate in a number of countries including China, The Russian Federation, Chile, Bulgaria and the United Kingdom. However, the evidence base

in relation to the effectiveness of fluoridated milk as a community-based method of caries prevention is not strong. A very recent systematic review of the topic (Yeung *et al.*, 2005) used selection criteria that included the need for a randomised or quasi-randomised control trial design and study duration of at least three years. Only two studies met the inclusion criteria. One study, involving children in Russia from three years of age at baseline, used a fluoride concentration in milk of 2.5 mg/L (or 0.5 mg/200mL), and found significant differences in caries increment for both the primary and permanent dentition between test and control groups after a study duration of three years (Maslak *et al.*, 2004). The fluoride milk concentration used corresponded to that of one of the experiments used in the present study, i.e. the 0.5 mg F in 200 mL milk x 3/day. However, limited study information is available as, to date, the work has only been published in abstract format. The second study included in the review was that of Stephen *et al.* (1984). This school-based Glasgow study involved children from five years of age at baseline. The fluoride level used was 1.5 mg / 200 mL milk, i.e. three times that used in the Russian study. Stephen *et al.* (1984) instructed use of a fluoride-free dentifrice during the study. The study design therefore most closely relates to the experimental protocol of the present study involving 1.5 mg F in 200 mL milk x 1/day in the T group. The results of the Glasgow-based RCT showed no significant difference in caries increment between test and control groups for the primary dentition at the three annual examinations, and for the permanent dentition, again no significant differences were found for the first three years. However, by years four and five, significant differences in DMFT values occurred between the two groups, with a lower caries increment in the test group. It is interesting to note that the results are not consistent between the two studies included within the review and that the differences between test and control groups were greater in the study involving the lower concentration of fluoride. However, as mentioned above, limited information regarding study design is available in relation to the Russian study and it is therefore difficult to compare study findings in a meaningful way.

The two clinical studies described above are obviously very different to the *in situ* study described in this thesis. The clinical studies measure the incidence of new caries lesions developing over a number of years, whilst the *in situ* study measured the change in mineral content of artificially created enamel lesions over a six-week period. The methods of detection / measurement of lesions were also very different. Although *in situ* studies are designed to be able to produce results in a much shorter time than clinical studies and they have been shown in the past to be a useful method for providing information relating to the

effectiveness of different concentrations of fluoride in dentifrices (Schafer, 1989; Mellberg *et al.*, 1991; Stephen *et al.*, 1992), it is nevertheless difficult to compare directly the results of the clinical and *in situ* studies. With inconsistent results being found between the clinical studies with regard to the effectiveness of fluoridated milk as a caries-preventive agent, and with no significant differences in caries increment becoming apparent in one study until the four-year time point, it is perhaps not surprising that the *in situ* study failed to show an obvious effect of the experiment protocols for either of the treatment groups. The systematic review concluded that there was some evidence, from the two studies that met the inclusion criteria, that fluoridated milk was beneficial to the permanent teeth of school children, but that at present there are an insufficient number of high quality studies available to provide a strong evidence base in relation to the effects of fluoridated milk in preventing caries in children.

In this *in situ* study the effect of group was statistically significant for some outcome parameters with, on average, more remineralisation occurring in the lesions exposed to the toothpaste slurry containing 1100 ppm F. It is perhaps surprising that the effect of group was not statistically significant for all of the outcome measures, as the literature provides strong evidence of the effectiveness of fluoridated toothpaste in the prevention of caries (Stephen *et al.*, 1988; Marinho *et al.*, 2004b).

5.0 Conclusions and further work

This chapter contains general conclusions from the preceding work, discusses the limitations of the project and suggests some recommendations for further work.

The work in this thesis evaluated the ability of an operator to both take images and analyse them in a repeatable manner using the QLF technique. Once the repeatability of the operator using this technique had been established, QLF was used, together with the established TMR methodology, to evaluate the effect of consumption of milk, with or without additional fluoride, on the change in mineral content of artificial caries lesions placed at different sites within the oral cavity. This work posed a number of specific research questions which are discussed within the remainder of this chapter.

5.1 Conclusions

The aim of the study described in Chapter 3 was to investigate the repeatability of the QLF process and answer the research question listed below:

- **Could a given operator both obtain and analyse an image in a repeatable manner with the available QLF machine?**

From the results obtained in this study, it can be concluded that the image analysis stage was more repeatable than the image capture stage of the QLF technique. For the image capture stage, of the three QLF parameters measured, QLF^{MAX} was the most repeatable, for this given operator, when using this prototype Clin-QLF machine, with a repeatability estimate of 96%. QLF^{AREA} and QLF^{AVER} had lower repeatability estimates of 83%, however, these were still classed as being 'substantial' by the criteria suggested by Shrout (1998). For the image analysis stage, the most repeatable parameters were QLF^{MAX} and QLF^{AVER} (96% and 95%, respectively), although QLF^{AREA} still had a repeatability estimate of 93%. However, it should be noted that one of the disadvantages of this study was the limited variability in the degree of demineralisation of the tooth specimens (n=20). In the main study described in Chapter 4, statistical analysis of baseline measurements of the degree of demineralisation, determined using both QLF and TMR, demonstrated greater variability between the tooth specimens (n=1360). Repeatability work described by Tranaeus *et al.* (2002) demonstrated greater repeatability and higher correlation values in a study with a greater variability in the degree of demineralisation of the tooth specimens

analysed. Therefore, it could be that the repeatability of QLF in the main study described in this thesis, is likely to be greater than that achieved in the study (Chapter 3).

In summary, QLF is a highly repeatable technique, for this given operator, for both the image analysis and image capture stage. However, it is important that the operator is experienced because the repeatability of the technique decreases significantly with inexperienced operators (Pretty *et al.*, 2002). As stated in Chapter 3, the repeatability estimates obtained would suggest that the operator in the studies described in this thesis, can achieve levels of consistency similar to operators described as "experienced" in other studies examining the repeatability of QLF. Recent developments in image re-positioning software (van der Veen and de Josselin de Jong, 2000) are likely to further improve the repeatability of image capture and subsequent analysis. QLF has significant benefits over other mineral measurement techniques in that it is possible to take multiple images over time, it is non-invasive and it is relatively straight-forward to perform. In addition, when compared with TMR, the QLF measurement obtained at baseline is a true measurement, rather than a proxy measurement, as obtained by TMR. Therefore, QLF is a useful and repeatable measurement technique for use in *in situ* and *in vivo* studies.

Having established the operator repeatability in the use of QLF to evaluate mineral loss in a non-invasive manner, QLF was applied to an *in situ* model to study the effects of fluoridated milk consumption. The aims of the study described in Chapter 4 were to answer the following research questions:

- **Was there an effect of using fluoridated dentifrice on the mineral content of enamel lesions?**

- **Were there any effects of consumption of fluoridated milk on mineral content of enamel lesions a) with or b) without the effect of fluoridated dentifrice?**

- **Were there any differences in the change in the mineral content of enamel lesions at different intra-oral sites?**

With regard to the methodology used in this study, an *in situ* model involving human enamel blocks with artificial caries lesions was selected. The complete denture model used was advantageous in that it allowed the placement of multiple tooth blocks into the mouth

of the subject and potentially allowed for cariogenic substances to be tested. However, a suitable method for disinfecting tooth specimens used in *in situ* caries models needs to be determined via further research. All of these issues have been discussed further in Chapter 4, Section 4.4.

Was there an effect of using fluoridated dentifrice on the mineral content of enamel lesions?

The design of the study included the investigation of the effect of use of fluoridated dentifrice to simulate toothbrushing twice-daily. Subjects who used the dentifrice slurry to simulate toothbrushing twice daily tended to have a much greater mean positive difference between pre- and post-protocol measurements than those who did not use the slurry. This applied to both QLF and TMR evaluations; although statistically significantly greater remineralisation was only evident for QLF^{AREA} and IML evaluations, whilst for LD and QLF^{AVER} evaluations the combined effect of group and site was statistically significant. This demonstrated that, in this study, the positive effect of toothbrushing twice daily on lesion remineralisation was greater than that of the experimental protocols involving fluoridated milk consumption alone. This was probably because of the difference in concentration of fluoride available. The maximum amount of fluoride available with the fluoridated milk was no greater than 7.5 ppm F^-, whereas the concentration of fluoride in the dentifrice was 1100 ppm F, although diluted to a slurry. Therefore, overall, greater levels of lesion remineralisation were observed with subjects using the dentifrice slurry and this was statistically significant for some but not all of the measured parameters. The effect of the dentifrice slurry concurs with literature published in recent years which suggests that fluoridated toothpaste is very effective in the prevention of dental caries (Stephen *et al.*, 1988; Marinho *et al.*, 2004b).

Were there any effects of consumption of fluoridated milk on mineral content of enamel lesions a) with or b) without the effect of fluoridated dentifrice?

Looking at the effect of the five experimental protocols, there was overall net mean remineralisation of the artificial lesions in the tooth blocks used in the study. However, it is unlikely that this was as a result of the experimental protocols alone, because the 'no beverage' protocol showed similar amounts of change to that found in the milk and fluoridated milk protocols. There were no statistically significant effects of experimental

protocol as measured by TMR. For QLF, for the parameter QLF^{AREA} there was a combined effect of 'group and experiment' determined, which was not apparent when the selected sites data were examined. There was no significant effect of experimental protocol for QLF^{MAX}. For QLF^{AVER} there was a statistically significant effect of experiment but the protocol 'no beverage' demonstrated the greatest remineralisation. Therefore, unfortunately, there was no consistent pattern seen.

Examining the effect of fluoridated milk in addition to the fluoridated dentifrice slurry, the only parameter to show a significant effect was, as stated above, QLF^{AREA}, which was a combined effect of 'group and experiment'.

It was anticipated that there might have been greater discrimination noted between experiments in the T group, which did not have the 'flooding effect' of the dentifrice slurry to contend with. Again, however, there was no consistent pattern seen with any of the parameters.

A further aim of the study was to compare the effect of consumption of fluoridated milk with a low concentration of fluoride three times per day, to that found with the consumption of milk with a higher concentration of fluoride in milk only once per day; both milk regimes having the same overall fluoride content. This was, in effect, comparing the experimental protocols: 0.5 mg F^- in 200 mL milk x 3/day with 1.5mg F^- in 200 mL milk x 1/day. The statistical analysis concluded that there was no evidence of an experimental effect, after adjusting for any other statistically significant effects. However, it was apparent that there were consistently greater mean differences observed, indicating greater remineralisation, for the 1.5mg F^- in 200 mL milk x 1/day protocol. Therefore, from the studies performed in this thesis and for this dosage of fluoride, there were no positive effects to the subject of increasing the frequency of fluoridated milk consumption greater than once per day. It may be that the fluoride in the 0.5 mg F x 3/day protocol group was too low to have a beneficial effect, though this contrasts with research which suggests that a low sustained concentration of fluoride within the oral cavity is preferred for lesion remineralisation (Larsen and Bruun, 1994; ten Cate and Featherstone, 1996). In addition, the fluoride concentrations chosen were similar to those used in successful clinical trials (Stephen *et al.*, 1984; Maslak *et al.*, 2004). However, it may be that a certain threshold of fluoride is required to elevate the fluoride retained in the oral cavity to a therapeutic level. It could be suggested, that if a higher concentration of fluoride had been

used three times per day, further remineralisation could have been obtained. However, further research is required in this area to substantiate this claim.

Were there any differences in the change in the mineral content of enamel lesions at different intra-oral sites?

The final aim of this work was to compare the response of caries lesions exposed to the same experimental protocols but at different sites within the oral cavity, and determine whether the results supported previous work relating to the site-specificity of caries (Lecomte and Dawes, 1987; Dawes *et al.*, 1989; Macpherson and Dawes, 1994). In this study, there were differences in the remineralisation achieved at different sites within the mouth. Lesions at site 2 were notable, because in several instances, they achieved the least amount of remineralisation. This site was the labial position in the upper denture. This result concurs with previous work suggesting that there is a reduced salivary film velocity at this site, thereby reducing salivary clearance and increasing the likelihood of demineralisation. There was no consistency in the ordering/ranking of other sites, in terms of the amount of change in mineral content.

In an attempt to tease out the significant results and to attempt to eliminate the variability in lesion behaviour dependent on site, specific sites were analysed in further detail (sites 1, 3, 9 and 10). These sites were chosen because it was anticipated that they would behave in a similar manner. As expected, lesions at these selected sites behaved in a more similar manner and no statistically significant differences were detected in lesion behaviour between sites for either QLF or TMR evaluations. This supports previous work relating to site specificity of caries (Lecomte and Dawes, 1987; Dawes *et al.*, 1989; Macpherson and Dawes, 1994). The only exception to this was the TMR parameter LD, which demonstrated a statistically significant combined effect of 'group and site'. However, the differences in LD obtained, in general, are small enough to question the 'usefulness' of this parameter. The results of the statistical models obtained for the selected sites data were 'clearer', once the variability of site was removed.

Summary

Clinically, this work has confirmed the benefit of regular use of fluoridated dentifrice. This work has also to some extent supported previous evidence related to the site-specificity of dental caries. With regard to the effect of fluoridated milk, this work appears to suggest

limited value in the use of fluoridated milk as a sole source of fluoride or as a source of fluoride in addition to the regular use of dentifrices in the caries-preventive process.

5.2 Limitations of the project

Subject compliance will always be a limiting factor in an *in situ* study. It was not possible to determine accurately how compliant the subjects were in fulfilling the requirements of the experimental protocols. However, given the number of appointments they were expected to attend and their excellent compliance with these appointments, the majority of subjects were compliant with at least some of the instructions provided. In addition, the post-experimental compliance questionnaire was anonymous, so one would hope their answers would be honest and true.

One method of assessing subject compliance with such experimental protocols is to measure the fluoride concentration in urine samples obtained from the subjects. Whilst considered a limitation of the study, it is accepted that urinary analysis cannot be performed in field studies, for which the numbers of subjects here, were not dissimilar to studies of that type. Inclusion of this analysis was considered prior to the commencement of studies, but it was decided that logistically, it would not be possible to perform urinary analysis on the subjects.

It is acknowledged that the multiple phases in the study, along with the required 'washout periods' meant that the duration of the study was long and arduous for the subjects. However, the Research Assistant endeavoured to limit the inconvenience and regularly delivered milk to the subjects at home. It was fortunate that of those who agreed to participate in the study, most of them completed it. In the majority of subjects who failed to complete, the reasons were due to medical problems developing, either in themselves or with their spouse. The duration of the study and the commitment required therefore limited the number of subjects recruited to and completing the study.

Clinically, most studies and programmes involving fluoridated milk have involved children. In this *in situ* study, edentulous adults were used as subjects. It is acknowledged that the oral microflora, salivary flow and diet of children will be different to that found in edentulous adults, and this may limit the ability to extrapolate the findings from these studies to an *in vivo* situation. However, the study was investigating the topical effect of

fluoride; therefore, there is no reason why the use of fluoridated milk should be restricted to children.

It is also acknowledged that the design of the study was complex. A number of parameters were being evaluated at the same time, i.e. the use of two fluoridated milk regimes, the concurrent fluoridated dentifrice use and the effect of site specificity. It was essential to study the effect of fluoridated milk with the use of fluoridated dentifrice given that the latter is considered normal practice for all dentate individuals in developed countries and as much of the key literature in the past has studied the effect of fluoridated milk alone. The statistical method of analysis, the general linear modelling procedure, took these factors and potential interactions between the different factors into consideration. With regard to the site specificity effect, it is acknowledged that published literature in this area has used dentate individuals. In the main study described in this thesis, edentulous individuals were subjects. It is possible that the flanges present on the modified complete dentures altered the salivary flow rate and the site specificity of the caries lesions.

The QLF hardware was a protocol device, loaned by Inspektor Research Systems BV, (Amsterdam, The Netherlands) and the illumination provided to the handpiece was (anecdotally) inferior to that found on newer systems. In addition, developments in the CCD cameras over time have improved with advances in technology. Also, the QLF software has continually been developed and additional features, such as the image repositioning software, are now available, which should improve the repeatability of the image capture process (de Josselin de Jong and van der Veen, 2000). Together, these advances in QLF are likely to improve both the repeatability and reproducibility of the technique. Therefore, if the studies performed in this thesis were repeated today, there may have been improvements in ease of use and some improvements in precision, but not enough to affect the conclusions. It should be remembered that this was a long-term study, which took a long time to run and analyse.

Initial work was published in the 1990s which attempted to make comparisons between lesions measured with TMR, LMR and QLF (Hafström-Björkman *et al.*, 1992; Emami *et al.*, 1996; Lagerweij *et al.*, 1999). Therefore, it was anticipated that a direct comparison between QLF and TMR could be made within this study. However, more recent work has suggested that this is not possible, as it is acknowledged that these tools are measuring different lesion properties and are, therefore, not directly comparable (ten Bosch, 2000).

Nevertheless, an attempt was made to make comparisons between the results obtained from the statistical models for the parameters from the two methods and this is described in Chapter 4, Section 4.4.2.7. Good agreement was obtained for the IML component of TMR and QLF^{AREA} and it could be suggested that these parameters could possibly be comparable. Further work would be required to substantiate this claim.

5.3 Recommendations for future work

5.3.1 General future work

One of the pilot studies undertaken prior to the main study described in this thesis (Chapter 4), was to determine whether a new prion-decontamination protocol had any effect on the behaviour of artificially created caries lesions to demineralise further and remineralise. This research was presented as an abstract at the British Society of Dental Research in 2001 (Appendix 1). This protocol was used throughout the main study in an attempt to decontaminate tooth specimens against potential prion proteins that could be present, in addition to storage of the tooth blocks in a thymol solution. Subsequently, it was demonstrated through the use of a bio-assay that 2M NaOH at 60°C for 1 hour, was the only effective protocol against prion disease. The current "gold standard" technique for disinfecting teeth prior to use in the oral cavity is gamma irradiation. However, prion proteins are known to be resistant to ionising, ultraviolet and microwave radiations (Taylor, 1999). Currently, there is no known method of sterilising tooth blocks containing artificial caries lesions without irreversibly damaging the lesions and tooth blocks. Further work is required in this area to determine an effective protocol which will eliminate the possible transmission of prion proteins (and other infective agents) during *in situ* studies and will maintain the characteristics and behaviour properties of the artificially created caries lesions, allowing them to behave in their accustomed manner, by demineralising further or remineralising when exposed to an intra-oral environment.

5.3.2 Future work related to this study

The QLF data were collected at fortnightly intervals (i.e. at baseline, 2, 4, and 6 weeks). Ideally, it would have been interesting to observe the patterns that developed from these measurements over time. However, this was out with the capabilities of this study and will be investigated in future work.

In addition, it would be interesting to determine any effect of using the new image alignment software, along with newer QLF hardware, to determine whether the measurements obtained would be more repeatable and precise.

The relationship between QLF and TMR is complex. As stated previously, it would be interesting to undertake further work to determine whether there is a relationship between the data obtained by the two measurement methods.

An alternative method of statistical analysis would be to use the ANCOVA method (analysis of covariance), rather than the GLM method (general linear modelling). The analysis undertaken in this thesis (GLM) takes into consideration the baseline lesion size; however, ANCOVA is a more powerful analysis and may be worthy of further investigation.

In addition, as mentioned previously, it would be of interest to determine if there would be an effect of increasing the frequency of fluoridated milk consumption, to three times per day, and comparing it to that used once per day, both with a similar concentration of fluoride e.g. 1.5 mg F^- in 200 mL milk x 3/day compared with 1.5 mg F^- in 200 mL milk x 1/day.

5.4 Dissemination of results

Results from the work discussed in this thesis have been presented at a number of scientific research conferences. It is the intention of the author to disseminate the results further by the production of a number of research papers and publications in peer-reviewed research journals.

Appendices

Appendix 1 - BSDR 2001 (Prion decontamination), poster text

UNIVERSITY
of
GLASGOW

Effect of a Prion-Decontamination Protocol on Artificial Caries Development

A Hannah*, L Oxford, C Smith, A Hall, L Macpherson, R Foye
(Glasgow Dental Hospital and School, Scotland)

ABSTRACT

Safety of laboratory personnel is of utmost importance. A protocol has been suggested to reduce the potential risk of contracting variant Creutzfeld Jacob Disease (vCJD) from extracted teeth. This study aimed to determine whether this protocol affects the development of artificially created caries lesions in such teeth. The protocol comprises storage in 10% Formalin for 7 days, washing in deionised water, storage in 5% Sodium Dodecyl Sulphate for 24 h and washing 3 times in Phosphate Buffered Saline (PBS). Ground and polished blocks were cut from 160 extracted teeth and divided into 4 equal groups. The groups were treated as follows. Group 1, the tooth and block were exposed to the prion-decontamination protocol. In Group 2, only the tooth was exposed to prion-decontamination protocol. In Group 3, only the block was exposed to the prion-decontamination protocol. Group 4 was a control group where neither the tooth nor the block, were exposed to the prion-decontamination protocol. Blocks were varnished and placed in lactic acid demineralisation solution for 96 hours. Quantitative Light Fluorescence (QLF) readings were taken at 0 and 96 hours. Half the blocks in each group were sectioned prior to Transverse Microradiography (TMR). QLF readings demonstrated significant demineralisation that was also measured by TMR. A one-way ANOVA demonstrated significant differences between groups for some QLF measurements ($p = 0.1$, $p = 0.04$ and $p = 0.005$ for QLF area, QLF Maximum % Fluorescence and QLF Mean % Fluorescence, respectively). No such differences were apparent for TMR measurements. From this data prion-decontamination protocols may affect the degree of demineralisation of artificial lesions but further work is required to determine the nature of this effect.

HYPOTHESIS

There is no difference between groups of artificial caries lesions created in extracted human teeth previously exposed to different prion decontamination protocols when measured by the QLF machine and this demineralisation can be confirmed by TMR (transverse microradiography).

QLF (Quantitative Light Fluorescence)

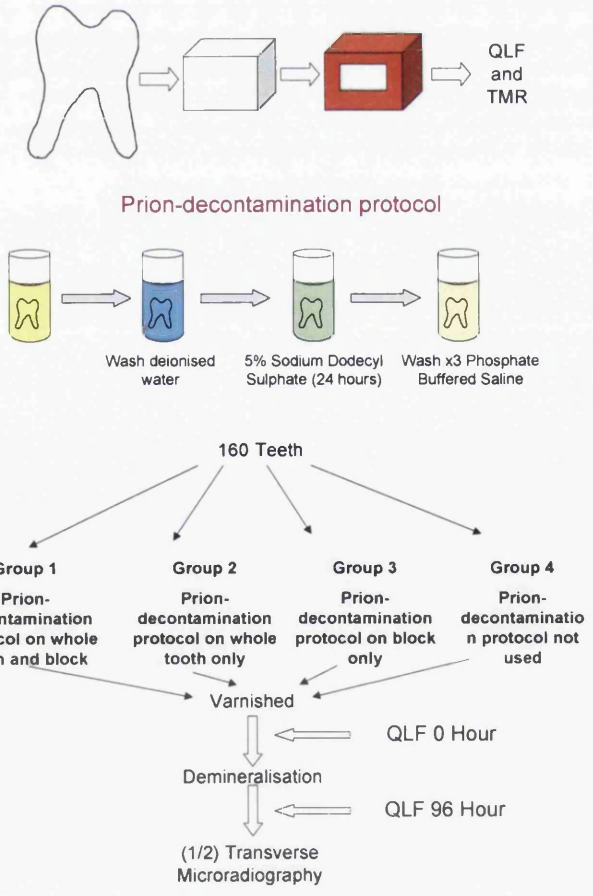
A method of measuring the scattering of fluorescent light to determine the amount of mineral loss through dental caries. Mineral loss is expressed as three different parameters:

Area of mineral loss in mm^2 (AREA)

Mean % loss of fluorescence radiance (MEAN)

Maximum % loss of fluorescence radiance (MAX)

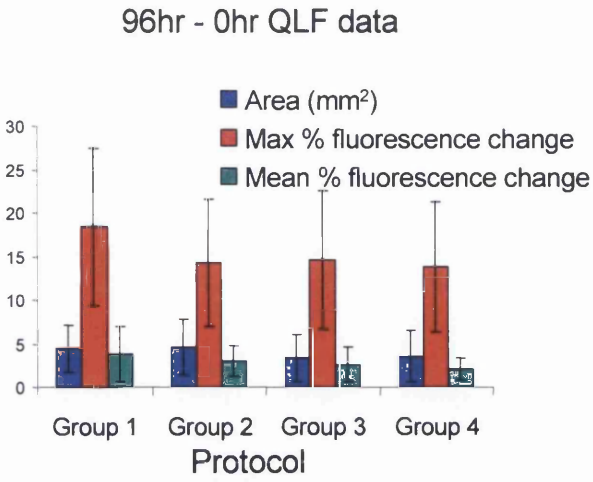
METHOD

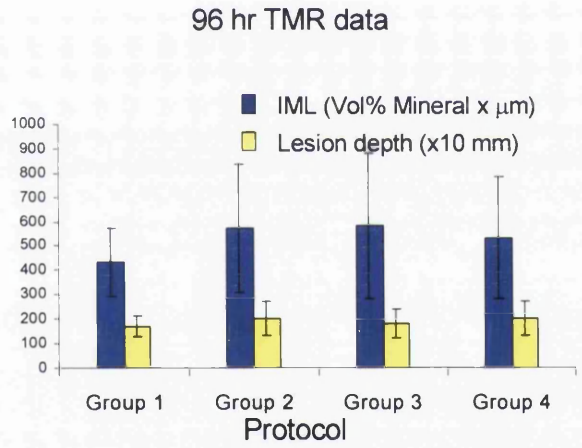


STATISTICAL ANALYSIS

One way ANOVA's were used to compare the parameters

RESULTS





PARAMETER	ONE WAY ANOVA
QLF AREA	p = 0.1
QLF MAX	p = 0.043
QLF MEAN	p = 0.005
TMR IML	p = 0.219
TMR DEPTH	p = 0.3

CONCLUSION

From this data, prion-decontamination protocols may affect the degree of demineralisation of artificial lesions created in extracted human teeth but further work is required to determine the nature of this effect.

Appendix 2 - Carbopol demineralisation solution (White, 1987b)

Carbopol / Lactic Acid gels were prepared from stock solutions of 1% polyacrylic acid (Carbopol C907, MW = 450,000 daltons) and 1.0M Lactic Acid, respectively.

1% Polyacrylic Acid

Add 10g of C907 to a 1L beaker and add approximately 600mL of distilled water. The resulting solution was stirred with a magnetic stirrer at room temperature while 10M/l NaOH solution was added to adjust the pH to 4.0. The Carbopol resin typically takes about 2-4 hours to dissolve. The resulting solution was transferred to a 1000mL volumetric flask and diluted to the mark.

1M Lactic Acid Solution

The stock lactic acid solution was prepared from reagent grade Lactic Acid and was steam distilled prior to use, (the lactic acid was heated at 90°C for 8 hours and allowed to cool to room temperature before use), after which 74mL was made up to a litre with distilled water.

Carbopol / Lactate gels

These are prepared by adding 100mL of polyacrylic acid and 100mL of 1M lactic acid to 500mL of distilled water and making up to 1000mL with distilled water, giving a solution of 0.1M lactic acid in 0.1% Carbopol. The pH of this solution was adjusted to 5.0 using 1M NaOH solution.

Saturated Carbopol / Lactic Acid gel

This is prepared by adding 2g of synthetic calcium hydroxyapatite to 1000mL of carbopol / lactate gel at pH 5.0. The pH of the resulting solution was readjusted to pH 5.0 by the drop-wise addition of 2M HCl until the solution reaches equilibrium (~4-8 h, pH drift <0.1 unit/hr). This solution was then filtered through Whatman # 42 paper and the supernatant collected. This is saturated carbopol / lactic acid gel.

Carbopol Demineralising Solution

This is prepared by adding equal volumes of carbopol / lactate gel and saturated carbopol / lactate gel. This solution was adjusted to pH 5.0 by the addition 1M NaOH before use.

The enamel specimens were demineralised in 25 mL of solution (2mL/mm^2) at 37°C for the required time period.

Appendix 3 - Patient introduction letter

Ailsa J. Nicol, BDS, FDS RCS (Edin)
Clinical Lecturer in Restorative Dentistry
Tel : 0141 – 211 9861
E-mail : a.nicol@dental.gla.ac.uk

THE EFFECT OF MILK ON ARTIFICIAL TOOTH DECAY

Hello,

My name is Ailsa Nicol and I am a Dentist who lectures at Glasgow Dental Hospital and School.

I am looking for enthusiastic ladies and gentlemen with no teeth, and with time on their hands to assist me with my research. In return you will be given free of charge, two sets of carefully made complete dentures and a financial gift to compensate you for your time and travelling expenses.

The study in which you will be asked to take part is to look at the effects of drinking milk, with and without fluoride, on little pieces of artificial tooth decay hidden in the dentures. After the dentures have been constructed, you will be required to attend the Dental School for a short visit on a fortnightly basis during each experiment.

So, if you wear complete dentures, have time on your hands and don't mind drinking milk, I would be delighted to hear from you!

If you are interested, please fill in the slip below and return it in the stamped addressed envelope. In return you will receive an appointment to attend for an introduction and assessment, further information will be given to you then. There is no obligation to participate in the study and you are able to withdraw at any time. If you have any questions please do not hesitate to contact me at the above address.

Looking forward to meeting you!

Miss Ailsa Nicol, BDS, FDS RCS(Edin)
Clinical Lecturer in Restorative Dentistry

I am interested in the above and would like an appointment for further information and assessment.

NAME: .....
ADDRESS:
.....

.....
POSTCODE:
.....
TELEPHONE NO:
.....
DATE OF BIRTH:
.....

Please return in the stamped addressed envelope. Thank you!

Appendix 4 - Patient information

Ailsa J. Nicol, BDS, FDS RCS (Edin)
Clinical Lecturer in Restorative Dentistry
Tel : 0141 – 211 9861
E-mail : a.nicol@dental.gla.ac.uk

PATIENT INFORMATION

THE EFFECT OF MILK ON ARTIFICIAL TOOTH DECAY

The project involves looking at the effects of milk containing fluoride on early tooth decay. This is important as it may aid prevention of tooth decay, particularly in children's teeth.

The study involves carefully constructing full upper and lower dentures for ladies and gentlemen with no teeth, who are judged to have a normal diet. These dentures are then copied to make another set of dentures which will contain small pieces of human enamel with artificial decay hidden in them. These are the experiment dentures which will be worn full-time for each of the five, six-week-long experiments.

As mentioned in the introductory letter, following construction of the dentures you will be required to attend the Dental School for a short visit on a fortnightly basis during each of the experiments. You will also be encouraged to follow a particular, straight-forward daily denture cleaning routine. There will be a three-week break between each of the experiments. The experiments will involve you supplementing your normal diet with one of the following:

1. nothing (control)
2. 200 mL of milk (less than half a pint, once a day)
3. 200 mL of milk (less than half a pint, three times a day)
4. 1.5mg Fluoride in 200 mL of milk, i.e. 7.5ppm F(once a day)
5. 0.5mg Fluoride in 200 mL of milk, i.e. 2.5ppm F (three times a day)

A financial gift will be given at the end of each experiment, with a lump sum available at the end of the series of experiments to cover your time and travelling expenses. In total you could receive up to £300 for completion of all the work required in addition to the two new sets of dentures specially constructed for you.

You can, of course, withdraw from the study at any time. If you have any further questions or request any further information, please do not hesitate to contact me at the above address.

Ailsa Nicol,
BDS, FDS RCS (Edin)

Appendix 5 - Consent form

Ailsa J. Nicol, BDS, FDS RCS(Edin)
Clinical Lecturer in Restorative Dentistry
Tel : 0141 – 211 9861
E-mail : a.nicol@dental.gla.ac.uk

CONSENT FORM

THE EFFECT OF MILK ON ARTIFICIAL TOOTH DECAY

Please initial box

I confirm that I have read and understood the patient information sheet for the above study and have had the opportunity to ask questions ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason. ☐

I agree to take part in the above study. ☐

_____ _____

Name of Patient Date Signature

_____ _____

Name of Researcher Date Signature

Patient Identification number: _____

1 copy to patient , 1 copy to researcher.

Appendix 6 - Denture cleaning advice

GENERAL CLEANING INSTRUCTIONS.

Dentures should be removed twice daily (morning and evening) in order to clean the fitting surface, i.e. the area in contact with your gums.

You are asked to brush this area with a toothbrush and water each time but to make sure not to touch the pieces of tooth that have been inserted into the dentures.

If necessary, you can rinse the dentures gently under running water to remove particles of food etc.

Appendix 7 - Patient information for experiments for T and TD groups

FLUORIDATED MILK STUDY

INSTRUCTIONS TO PATIENTS

GROUP 'T'

EXPERIMENT 1 : NO BEVERAGE

- Wear dentures continuously (including throughout the night) for 6 weeks.

- During the 6 week experiment, follow the ***general cleaning instructions*** (provided on a separate sheet).

- Continue with your normal diet

On the mornings of your appointments at the Dental Hospital, we would ask you
(1) not to drink any tea.
(2) not to eat for at least two hours before your appointment time, if possible.

You will be given appointments at the start of the experiment, at 2 weeks, 4 weeks and at 6 weeks (i.e. the end of the experiment).

FLUORIDATED MILK STUDY

INSTRUCTIONS TO PATIENTS

GROUP 'T'

EXPERIMENT 2/4: 200mL MILK (ONCE EACH DAY)

- Wear dentures continuously (including throughout the night) for 6 weeks.

- During the 6 week experiment, follow the ***general cleaning instructions*** (provided on a separate sheet).

- We also ask you to stir one of the small bottles of liquid which are supplied with the milk into 200 mLs of the study milk, and drink this once each day. The milk solution should be drunk all at once rather than sipped over a long period of time.

  (The milk, a plastic measuring cup and bottles of liquid will be provided by the study organisers).

- Continue with your normal diet.

On the mornings of your appointments at the Dental Hospital, we would ask you
(1) not to drink any tea.
(2) not to eat for at least two hours before your appointment time, if possible.
(3) not to drink the test milk that morning.

You will be given an appointment at the beginning of the experiment, at 2 weeks, 4 weeks and at 6 weeks (i.e. the end of the experiment).

FLUORIDATED MILK STUDY

INSTRUCTIONS TO PATIENTS

GROUP 'T'

EXPERIMENT 3/5: 200 mL MILK (THREE TIMES EACH DAY)

- Wear dentures continuously (including throughout the night) for 6 weeks.

- During the 6 week experiment, follow the ***general cleaning instructions*** (provided on a separate sheet).

- Three times a day, we also ask you to stir one of the small bottles of liquid (which are attached to the milk carton) into 200 mLs of the study milk, and drink this milk solution. On each occasion, the milk solution should be drunk all at once rather than being sipped over a long period of time. Please leave at least 2 hours in between each of these 200mL drinks.
  (The milk, a plastic measuring cup and the bottles of liquid will be provided by the study organisers).

- Continue with your normal diet.

On the mornings of your appointments at the Dental Hospital, we would ask you
(1) not to drink any tea.
(2) not to eat for at least two hours before your appointment time, if possible.
(3) not to drink the test milk that morning.

You will be given an appointment at the beginning of the experiment, at 2

weeks, 4 weeks and at 6 weeks (i.e. the end of the experiment).

FLUORIDATED MILK STUDY

INSTRUCTIONS TO PATIENTS

GROUP 'TD'

Experiment 1 : No beverage

- Wear dentures continuously (including throughout the night) for 6 weeks.

- During the 6 week experiment, follow the ***general cleaning instructions*** (provided on a separate sheet).

- We also ask you to rinse the dentures in your mouth twice each day (once in the morning and once in the evening), with the toothpaste mixture provided. The amount to use is shown by a black mark on the medicine cup attached to the bottle of toothpaste mixture.

  (The toothpaste mixture should be shaken well and stirred before use and **swirled around all parts of the mouth for 2 minutes each time**). After swirling, spit out.

- Continue with your normal diet.

On the mornings of your appointments at the Dental Hospital, we would ask you
(1) not to drink any tea.
(2) not to eat for at least two hours before your appointment time, if possible.
(3) not to swirl with the toothpaste mixture that morning.

You will be given appointments at the start of the experiment, at 2 weeks, 4 weeks and at 6 weeks (i.e. the end of the experiment).

FLUORIDATED MILK STUDY

INSTRUCTIONS TO PATIENTS

GROUP 'TD'

Experiment 2/4: 200mL milk (Once a day).

- Wear dentures continuously (including throughout the night) for 6 weeks.

- During the 6 week experiment, follow the ***general cleaning instructions*** (provided on a separate sheet).

STEP 1

- We also ask you to rinse the dentures in your mouth twice each day (once in the morning and once in the evening), with the toothpaste mixture provided. The amount to use is shown by a black mark on the medicine cup attached to the bottle of toothpaste mixture.

  (The toothpaste mixture should be shaken well and stirred before use and **swirled around all parts of the mouth for 2 minutes each time**). After swirling, spit out.

STEP 2

- The experiment also involves stirring one of the small bottles of liquid (attached to the milk carton) into 200 mLs of the study milk, and drinking this once each day. The milk solution should be drunk all at once rather than sipped over a long period of time. It should also be taken at least 2 hours after the morning toothpaste rinse and at least 2 hours before the evening toothpaste rinse.

  (The milk, plastic measuring cup and bottles of liquid will be provided by the study organisers).

- Continue with your normal diet.

On the mornings of your appointments at the Dental Hospital, we would ask you
(1) not to drink any tea.
(2) not to eat for at least two hours before your appointment time, if possible.
(3) not to drink the test milk or swirl with the toothpaste mixture that morning.

You will be given appointments at the start of the experiment, at 2 weeks, 4 weeks and at 6 weeks (i.e. the end of the experiment).

FLUORIDATED MILK STUDY

INSTRUCTIONS TO PATIENTS

GROUP 'TD'

Experiment 3/5: 200 mL milk (Three times each day).

- Wear dentures continuously (including throughout the night) for 6 weeks.

- During the 6 week experiment, follow the ***general cleaning instructions*** (provided on a separate sheet).

STEP 1

- We also ask you to rinse the dentures in your mouth twice each day (once in the morning and once in the evening), with the toothpaste mixture, which is provided by the study organisers. The amount to use is shown by a black mark on the medicine cup attached to the bottle of toothpaste mixture.

  (The toothpaste mixture should be shaken well and stirred before use and **swirled around all parts of the mouth for 2 minutes each time**). After swirling, spit out.

STEP 2

- Three times a day, we also ask you to stir one of the small bottles of liquid (which are attached to the milk cartons) into 200 mLs of the study milk and drink this milk solution. On each occasion, the milk solution should be drunk all at once rather than being sipped over a long period of time. The first 200 mL drink should be taken at least 2 hours after the morning toothpaste rinse and the third 200 mL drink at least 2 hours before the evening toothpaste rinse.

  (The milk, plastic measuring cup and bottles of liquid will be provided by the study organisers).

- Continue with your normal diet.

On the mornings of your appointments at the Dental Hospital, we would ask you
(1) not to drink any tea.
(2) not to eat for at least two hours before your appointment time, if possible.
(3) not to drink the test milk or swirl with the toothpaste mixture that morning.

You will be given appointments at the start of the experiment, at 2 weeks, 4 weeks and at 6 weeks (i.e. the end of the experiment).

Appendix 8 – Post-experimental compliance questionnaire

Post-experimental compliance questionnaire for TD group

FLUORIDATED MILK STUDY

Patient Allocation No._____ Treatment No._____

1. **Did you complete all five experiments?** YES [ ] NO [ ]

If NO, How many did you complete? _____

Please give a reason for not completing all five experiments _____

_____

2. **Did you have any problems taking the milk?** YES [ ] NO [ ]

If YES, please give details_____

_____

2A. **Were there occasions when you did not manage to take the milk?** (Please tick one).

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details_____

_____

3. **Did you have any problems adding the fluoride solution to the milk?**

YES [ ] NO [ ]

If YES, please give details_____

3A. **Were there occasions when you did not manage to add the fluoride solution to the milk?** (Please tick one)

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details_____

_____

4. **Did you have any problems swirling the toothpaste mixture in your mouth?**

YES [ ] NO [ ]

If YES, please give details_____

_____

4A. **Were there occasions when you did not manage to swirl the toothpaste mixture?** (Please tick one)

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details_____

_____

5. **Were you able to fast for 2 hours before each appointment?** (Please tick one)

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details _____

_____

6. **Did you manage to wear the dentures throughout the night during the experiments?**

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details_____

_____

7. **Did you have any problems wearing the dentures made for you?** (Please tick one)

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details _____

_____

7A. **Were those problems rectified?** [ ] Completely [ ] Partially

[ ] Not at all [ ] Does not apply

WE WOULD BE GRATEFUL IF YOU COULD RETURN THIS QUESTIONNAIRE IN THE PREPAID ENVELOPE PROVIDED.

THANK YOU AGAIN FOR TAKING PART IN THE STUDY.

Post-experimental compliance questionnaire for T group

FLUORIDATED MILK STUDY

Patient No._____ Treatment No._____

1. **Did you complete all five experiments?** YES [ ] NO [ ]

If NO, How many did you complete? _____

Please give a reason for not completing all five experiments _____

_____

2. **Did you have any problems taking the milk?** YES [ ] NO [ ]

If YES, please give details_____

_____

2A. **Were there occasions when you did not manage to take the milk?** (Please tick one).

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details_____

_____

3. **Did you have any problems adding the fluoride solution to the milk?**

YES [ ] NO [ ]

If YES, please give details_____

_____

3A. **Were there occasions when you did not manage to add the fluoride solution to the milk?** (Please tick one)

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details_____

_____

4. **Were you able to fast for 2 hours before each appointment?** (Please tick one)

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details _____

_____

6. **Did you manage to wear the dentures throughout the night during the experiments?**

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details_____

_____

7. **Did you have any problems wearing the dentures made for you?** (Please tick one)

[ ] All of the time [ ] Most of the time [ ] Some of the time [ ] Not at all

Please give details _____

_____

7A. **Were those problems rectified?** [ ] Completely [ ] Partially

[ ] Not at all [ ] Does not apply

WE WOULD BE GRATEFUL IF YOU COULD RETURN THIS QUESTIONNAIRE IN THE PREPAID ENVELOPE PROVIDED.

THANK YOU AGAIN FOR TAKING PART IN THE STUDY.

Appendix 9 – TMR results not described in Chapter 4

Covered (control) TMR data for selected sites by group and experiment

Table 75 - Summary statistics for IML data of covered (control) selected sites by group

Group	N*	Mean (St. Dev.)	Range
T	216	2345 (1070)	(237, 7369)
TD	242	2076 (977)	(217, 5791)

Integrated Mineral Loss is measured in %Vol mineral.µm
N* = number of lesions available for analysis

Table 76 - Summary statistics for LD data of covered (control) selected sites by group

Group	N*	Mean (St. Dev.)	Range
T	216	82.8 (30.0)	(10.4, 212)
TD	242	69.8 (27.4)	(12.4, 168.6)

Lesion Depth is measured in µm
N* = number of lesions available for analysis

Table 77 - Summary statistics of IML data for covered (control) selected sites by experiment

Experiment	N*	Mean (St.Dev.)	Range
0.5mg F x 3/day	90	2171 (911)	(429, 5044)
1.5mg F x 1/day	93	2424 (1214)	(237, 7369)
200mL milk x 1/day	96	2114 (1066)	(428, 5123)
200mL milk x 3/day	100	2137 (753)	(677, 4663)
No beverage	79	2170 (1159)	(217, 6252)

Integrated Mineral Loss is measured in %Vol mineral.µm
N* = number of lesions available for analysis

Table 78 - Summary statistics of LD data for covered (control) selected sites by experiment

Experiment	N*	Mean (St.Dev.)	Range
0.5mg F x 3/day	90	75.1 (29.1)	(17.5, 162)
1.5mg F x 1/day	93	90.0 (31.9)	(10.4, 212)
200mL milk x 1/day	96	73.7 (27.5)	(17.8, 153)
200mL milk x 3/day	100	76.2 (22.8)	(25.6, 153)
No beverage	79	73.4 (35.3)	(12.4, 169)

Lesion Depth is measured in μm.
N* = number of lesions available for analysis

Appendix 10 – QLF results not described in Chapter 4

Baseline QLF data by group

A. QLF^{AREA}

Table 79 - Summary statistics of baseline QLF data by treatment group for QLF^{AREA}

Group	Number of Blocks	Mean (St. Dev.)	Range	Two-sample T-Test	95% CI (T-TD)
T	640	4.1 (1.3)	(1.2, 8.0)	**p=0.001**	**(-0.4, -0.15)**
TD	720	4.3 (1.3)	(1.2, 8.5)		

QLF^{AREA} is measured in mm^2

B. QLF^{MAX}

Table 80 - Summary statistics of baseline QLF data by treatment group for QLF^{MAX}

Group	Number of Blocks	Mean (St. Dev.)	Range	Two-sample T-Test	95% CI (T-TD)
T	640	-51.0 (7.9)	(-77, -32)	**p=0.098**	**(-1.5, 0.13)**
TD	720	-50.3 (7.1)	(-77, -34)		

QLF^{MAX} is measured in % fluorescence loss

C. QLF^{AVER}

Table 81 - Summary statistics of baseline QLF data by treatment group for QLF^{AVER}

Group	Number of Blocks	Mean (St. Dev.)	Range	Two-sample T-Test	95% CI (T-TD)
T	640	-21.0 (4.2)	(-35, -14)	**p=0.852**	**(-0.4, 0.5)**
TD	720	-21.0 (4.0)	(-38, -13.6)		

QLF^{AVER} is measured in % fluorescence loss

Baseline QLF data by experiment

A. QLF^{AREA}

Table 82 - Summary statistics of baseline QLF data by experiment for QLF^{AREA}

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day	260	4.1 (1.3)	(1.2, 7.7)
1.5mg F x 1/day	290	4.2 (1.3)	(1.5, 7.9)
200mL milk x 1/day	270	4.3 (1.3)	(1.6, 8.0)
200mL milk x 3/day	280	4.3 (1.4)	(1.2, 8.5)
No beverage	260	4.3 (1.2)	(1.4, 7.6)

QLF^{AREA} is measured in mm^2
One-way ANOVA of QLF^{AREA} vs experiment - p=0.336

B. QLF^{MAX}

Table 83 - Summary statistics of baseline QLF data by experiment for QLF^{MAX}

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day [a]	260	-49.5 (7.6)	(-77, -32)
1.5mg F x 1/day [ab]	290	-50.6 (7.7)	(-75, -34)
200mL milk x 1/day [ab]	270	-50.8 (7.4)	(-72, -33)
200mL milk x 3/day [a]	280	-50.0 (7.2)	(-72, -32)
No beverage [b]	260	-52 (7.5)	(-77, -35)

QLF^{MAX} is measured in % fluorescence loss
One-way ANOVA of QLF^{MAX} vs experiment - p=0.001
Common symbol means no statistically significant difference between experimental groups

C. QLF^{AVER}

Table 84 - Summary statistics of baseline QLF data by experiment for QLF^{AVER}

Experiment	Number of Blocks	Mean (St. Dev.)	Range
0.5mg F x 3/day [a]	260	-20.3 (3.8)	(-35, -14)
1.5mg F x 1/day [a]	290	-20.9 (4.1)	(-34, -14)
200ml milk x 1/day [a]	270	-21.1 (4.3)	(-35, -14)
200ml milk x 3/day [a]	280	-20.4 (3.7)	(-35, -14)
No beverage [b]	260	-22 (4.5)	(-38, -14)

QLF^{AVER} is measured in % fluorescence loss
One-way ANOVA of QLF^{AVER} vs experiment - p= 0.001
Common symbol means no statistically significant difference between experimental groups

Appendix 11 – PEF 2002, text of poster presentation

Quantitative Light Fluorescence measurement of stain removal from artificial caries

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Abstract

Artificial caries lesions used for in situ studies may acquire extrinsic stain. This affects the ability of optical caries detection methods such as Quantitative Light Fluorescence (QLF) to measure mineral change during the study. The aim of this experiment was to investigate the effect of two different stain removal protocols on artificially created caries lesions. Sixty human premolar teeth with 4x2mm artificial caries lesions were created. Lesions were allocated to six equal-sized groups with matched mean and standard deviation values based on initial QLF assessment. Three groups of ten lesions were stained with a tea solution for 20 hours and measured again. Groups of ten teeth were subjected to the following protocol : Group 1, stained then immersed in 30% Hydrogen Peroxide for four hours; Group 2, stained then polished with Zircate Polishing Paste (Dentsply) in a rubber cup; Group 3, stained only; Group 4, Zircate Polishing Paste only; Group 5, Hydrogen Peroxide for four hours only; Group 6, no treatment. Post-treatment QLF measurements were taken. Statistical analysis of the difference between baseline QLF measurements and post-tea stain QLF measurements using a paired t-test demonstrated a significant increase in QLF values ($p < 0.001$). To compare the effect of different stain removal protocols, the difference between baseline and post-treatment QLF measurements was calculated. Using this data, a one way ANOVA and pairwise analysis using a Tukey test demonstrated the effective removal of tea stain by both Hydrogen Peroxide and Zircate Polishing Paste ($p<0.001$). In conclusion both Hydrogen Peroxide and Zircate Polishing Paste remove stain effectively from artificial caries lesions when measured by QLF.

Null Hypothesis

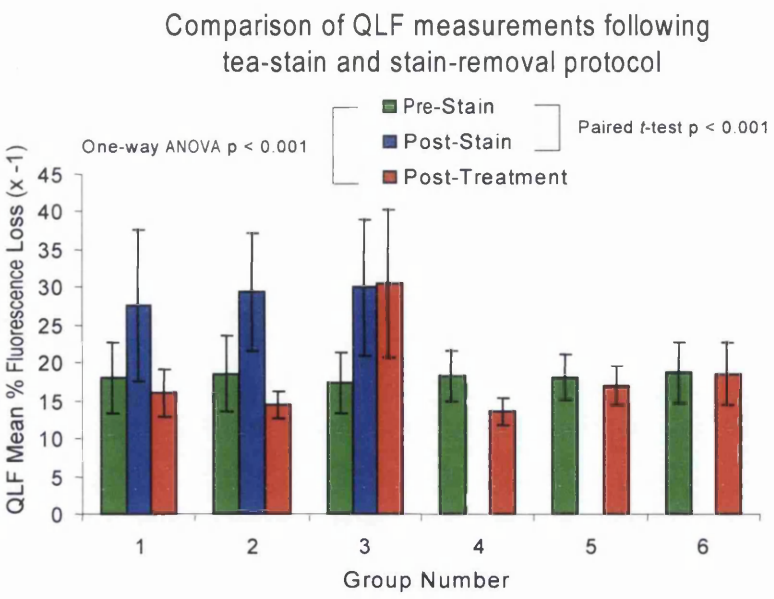
There are no differences in QLF measurements following exposure of artificial caries lesions to a tea solution for 20 hours.

Hydrogen Peroxide and Zircate Polishing Paste are not effective at removing stain from artificial caries lesions.

Method

Sixty human caries-free premolar teeth were abraded and polished to remove the outer 300 micrometres of enamel. They were varnished to leave a window of 4x2mm on the buccal surface and placed in a demineralisation solution for 72 hours to create the artificial caries lesions. Varnish was removed with acetone and alcohol. Measurements were made using QLF which is a method of measuring the scattering of fluorescent light that has been used to determine the amount of mineral loss through dental caries. Preliminary QLF measurements were made (Pre-Stain) and the lesions divided randomly into six equal sized groups. Three groups of 10 lesions were stained with a tea solution for 20 hours and measured by QLF again (Post-Stain). Groups of 10 teeth were subjected to the following protocol: Group 1, stained then immersed in 30% Hydrogen Peroxide for four hours; Group 2, stained then polished with Zircate Polishing Paste (Dentsply) in a rubber cup; Group 3, stained only; Group 4, Zircate Polishing Paste only; Group 5, Hydrogen Peroxide for four hours only; Group 6, no treatment. Further QLF measurements were made post-treatment (Post-treatment). A paired t-test was used to compare baseline QLF measurements with post-tea stain measurements. A one way ANOVA and pairwise analysis using a Tukey test were used to compare QLF measurements following stain removal.

Results



Confidence intervals from a Tukey Pairwise analysis of the difference between Pre-stain and Post-treatment QLF measurements

Group	1	2	3	4	5
2	-11.82-4.65				
3	5.33-21.80	8.92-25.39			
4	-12.46-4.01	-8.88-7.60	-26.03- -9.56		
5	-8.78-7.69	-5.19-11.28	-22.35- -5.87	-4.55-11.92	
6	-7.91-8.56	-4.33-12.14	-21.48- -5.01	-3.69-12.78	-7.37-9.10

(Note the red values denote significant differences between groups)

Conclusion

In this study exposure to a tea solution affected QLF measurements significantly. Both Hydrogen Peroxide and Zircate Polishing Paste were effective methods to remove stain from artificial caries lesions when measured using QLF.

The authors wish to acknowledge the support of Inspektor Research Systems B.V., Amsterdam for their help with the QLF measurements.

Appendix 12 – ORCA 2005, abstracts

Use of TMR in an in-situ study to measure the effect of a fluoridated dentifrice

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The aim was to determine if TMR could be used to observe mineral change in caries lesions *in-situ* exposed to a fluoridated dentifrice. Human tooth blocks (n=1450) were used in a randomised, cross-over design, utilising 29 subjects, analysis was blinded. Each block contained an artificial carious lesion, half of which was covered with acid-resistant varnish. Blocks were placed in modified complete dentures and 13 subjects followed each of five, six-week protocols: no beverage, 200mL milk x1/day; 200mL milk x3/day; 1.5mg F in 200mL milk x1/day and 0.5mg F in 200mL milk x3/day. This group of subjects followed a treatment only protocol (T). The remaining 16 subjects followed the same treatment protocols but also used a dentifrice slurry (1100ppm F) diluted 1: 4, 4mL x2/day. These subjects followed a treatment plus dentifrice protocol (TD). Integrated Mineral Loss (IML) and Lesion Depth (LD) TMR values were determined for the covered and uncovered parts of each carious lesion.

Results – T group (433 blocks in 13 subjects): mean IML= 268 %vol.mineral.μm (SD 261); mean LD= 4.9μm (SD 6.6). TD group (506 blocks in 16 subjects): mean IML= 451 %vol.mineral.μm (SD 165); mean LD= 4.8 μm (SD 3.1). After averaging across teeth and protocol for each subject, a two-sample t-test of the effect of group revealed a statistically significant difference in IML between the TD & T groups (p=0.041) with 95% CI for difference (TD-T) = (8.4, 357.3) %Vol mineral.μm, but no difference between groups in terms of Lesion Depth (p=0.980).

In conclusion, TMR was unable to conclusively demonstrate increased remineralisation when using fluoridated dentifrice twice a day, in addition to ingestion of fluoridated milk.

Using QLF to evaluate the effect of a fluoride dentifrice in an in-situ trial

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The aim of this study was to determine if QLF could be used to observe mineral change in artificial caries lesions in situ exposed to a fluoridated dentifrice slurry (TMR results in Abstract No.49). Twenty-nine edentulous subjects were recruited and provided with complete dentures containing 10 lesions per subject. Subjects were randomly allocated to one of two broad protocols: a five-treatment protocol (T) and a five-treatment plus dentifrice protocol (T+D). Each set of complete dentures contained 10 lesions which were changed after each treatment which lasted for six weeks. The dentifrice exposure comprised twice daily swilling for 2 min with 4 mL of a dentifrice slurry made from 1100ppm NaF dentifrice diluted 1:4 with deionised water. QLF measurements were made at baseline and after six weeks. Data were obtained for 494 lesions from 13 subjects and 547 lesions from 16 subjects for the T and T+D groups, respectively. Averaging across teeth and treatment for each subject, the mean (sd) differences for baseline minus week-six values were 4.8 (1.8) and 5.7 (1.1) for Average Fluorescence loss for T and T+D protocols, respectively. Maximum Fluorescence loss differences were 11.2 (4.4) and 12.1 (12.7) and the Area differences were 1.5 (0.7) and 2.4 (0.7) for T and T+D, respectively. Simple two sample t-tests were performed and demonstrated a statistically significant difference between groups for Area ($p = 0.004$) but not for Average Fluorescence Loss ($p = 0.136$) or Maximum Fluorescence loss ($p = 0.525$). In conclusion, QLF may be useful to evaluate the effect of fluoride dentifrice use using this in situ model but further work is required to establish which QLF parameters are most helpful.

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