

The Effect of Varying the Amount of Fluoride in Milk and The Frequency of Its Application on Demineralisation/Remineralisation of Bovine Dental Enamel In Vitro

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Submitted in accordance with the requirements for the degree of Doctor of
Clinical Dentistry

The University of Leeds
Leeds Dental Institute
Division of Child Dental Health

August, 2017

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Dedicated to my mom and dad

ACKNOWLEDGMENTS

A few words on paper cannot express the deep appreciation and gratitude I hold for every person that has stood by my side through this exciting experience. Completing a Post Graduate degree has always been a dream, and I can honestly say that I have been truly fortunate and blessed to be able to live my dream here at The University of Leeds. This experience all started with the kind help of Dr. N. Al-Ibrahim who encouraged me to become part of the Leeds family, and for that, I cannot thank her enough.

It has been a great privilege to be supervised by Professor K.J. Toumba, one of the most influential role models in the Paediatric Dentistry field and whom I have been fortunate enough to work with. I can truly say that It has been an absolute pleasure working on a topic that he continues to be passionate about and which I have also grown to appreciate more each year. I offer my deepest gratitude and sincere thanks to him for his continued guidance, support, and motivation which allowed me to achieve my full potential both academically and clinically. I must also thank both Professor K.J. Toumba and Professor M. Duggal for accepting me into this programme and for allowing me to advance and flourish in the field of Paediatric Dentistry.

I was fortunate to have been guided and assisted by both Dr. M. Malinowski and Mr. S. Strafford who spent a lot of their time directing and helping me carry out the 60-day laboratory experiment, and for that I am grateful. I would also like to express my appreciation to Dr. S. Keat for helping in the provision of all the required laboratory materials and to Mr. C. Sullivan for his photography skills. A special acknowledgement goes to the lovely Dent. Cru research team Mrs. Ashna Chavda and Mrs. Gillian Dukanovic for their continuous help and patience throughout.

Furthermore, I want to express my warmest appreciation and gratitude to Dr. J. Tahmassebi for her support, fairness, advice and understanding. Her consistent encouragement has truly inspired me throughout both the good times but also more importantly through those stressful situations. I am genuinely thankful for all the time and effort she kindly provided during my four years of training.

I carry the utmost respect and appreciation for both Dr. R. Bryan and Mr. S. Fayle for helping me during the course of this journey. I would like to sincerely thank them for being very understanding and allowing me the time to carry out my laboratory work between clinical duties. In addition, I want to extend my deepest gratitude to Dr. P. Kandiah and Dr. J. Monteiro for being great role models during their training years and for helping me believe in myself and thus allowing me to advance my clinical knowledge and skills. I am also very thankful

for the help and support provided by Dr. H. Nazzal and Dr. K. Kenny. I would also like to extend my gratitude to all the wonderful nurses at the Paediatric Dentistry Department for their wonderful support.

To my dearest friends and colleagues, those long days and nights at the university have finally paid off! Thank you to all the people that have been there for me through thick and thin. I wish you all the best, and I look forward to what the future has in store for us! A special thank you to Dr. W. AlMutairi for being such a good and supportive friend from the start of this programme.

Last but of course not least, my biggest acknowledgement goes to my devoted parents, Hanan and Mahmoud, for believing in me since day one. I am forever grateful for their love, nurturing, support and understanding. What was once a dream, has now become a reality I am living because of their sacrifices and unconditional giving. Thank you, mom and dad, for reminding me that I am stronger than I give myself credit. To my sister Mona, my brothers Nabil, Ahmad and Omar, their lovely wives, Dania, Sawsan and Reema, and of course my nephews and nieces, Mohammad, Abdullah, Jana, Lama and Leen, thank you for cheering me on and for keeping me updated with everything. I love you all very much!

ABSTRACT

Aim: To investigate the remineralisation and/or demineralisation of artificially created white spot lesions (WSL) on bovine enamel following a daily or intermittent exposure to 0.5 mg F or 1.0 mg F added to bovine milk using a pH-cycling in vitro model and whether the higher dose would result in a better preventive effect. **Methods:** Bovine teeth were collected and sectioned for the creation of artificial carious lesions while ensuring that there was a margin of sound enamel surrounding the white spot lesion (n=151). The prepared enamel slabs were randomly allocated to one of 6 groups: **Group 1:** Dipped into 0.0 mg F in milk, in an interrupted frequency manner. **Group 2:** Dipped into 0.5 mg F in milk, in an interrupted frequency manner. **Group 3:** Dipped into 1.0 mg F in milk, in an interrupted frequency manner. **Group 4:** Dipped into 0.0 mg F in milk, in a daily frequency manner. **Group 5:** Dipped into 0.5 mg F in milk, in a daily frequency manner. **Group 6:** Dipped into 1.0 mg F in milk, in a daily frequency manner. The enamel slabs underwent a 60 day pH-cycling regimen where the slabs were subjected to fluoridated milk twice daily (before and after pH-cycling). The quantitative light fluorescence (QLF) machine was used as the method of analysis as it measures the percentage change of demineralised enamel fluorescence in relation to the surrounding sound enamel. Images were obtained at baseline and on completion of the study. **Statistical Analysis:** Shapiro-Wilk test was used to test the normality of the data. Analysis of variance (one way ANOVA) was used to compare the different concentrations of fluoride in milk while the paired sample T-Test was used to compare between the two frequencies (Interrupted and Daily) as well as between baseline and final

readings. **Results:** Significant improvement ($p < 0.05$) between baseline and final readings was obvious within all QLF parameters: ΔF (average loss of fluorescence), Area of the lesion and ΔQ (lesion volume) for all the fluoride concentration levels including the control (0.0 ppm F). When considering the frequencies separately ΔF and ΔQ showed a significant difference ($p < 0.05$) between the control and 1.0 ppm F for the interrupted frequency while a significant difference for the daily frequency was found when comparing the highest fluoride concentration to the control and 0.5 ppm F. As for the comparison between both frequencies, it was apparent that although there was a general improvement in lesion remineralisation for all groups, ΔQ was only statistically significantly different ($p < 0.05$) at the 0.5 ppm F level in favour of the interrupted frequency. **Conclusions:** Fluoridated milk was shown to be effective in remineralisation of artificially created sub-surface enamel lesions in bovine enamel with the most improvement demonstrated at 0.5 mg F used in an interrupted frequency manner. The results need to be interpreted with caution and there is still a need for more randomised clinical studies to confirm these findings.

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	II
ABSTRACT	IV
TABLE OF CONTENTS	VI
LIST OF TABLES.....	IX
LIST OF FIGURES & GRAPHS.....	XI
1.0 LITERATURE REVIEW	1
1.1 DENTAL CARIES	2
1.1.1 <i>White spot lesions (WSL)</i>	4
1.1.2 <i>Creation of artificial WSL</i>	6
1.2 FLUORIDE AND CARIES PREVENTION	7
1.2.1 <i>Mechanisms of action of fluoride</i>	8
1.2.2 <i>Role of saliva</i>	9
1.2.3 <i>Fluoride in saliva and oral clearance</i>	10
1.2.4 <i>Fluoride absorption, distribution, and elimination from the body</i>	11
1.2.5 <i>Optimal fluoride exposure</i>	12
1.2.6 <i>Fluorosis and fluoride toxicity</i>	13
1.2.7 <i>Methods of fluoride delivery</i>	15
1.3 BOVINE MILK AND DENTAL HEALTH.....	18
1.3.1 <i>Bioavailability of fluoride in milk</i>	19
1.3.2 <i>Caries-preventive effect of milk fluoridation</i>	20
1.4 THE IN VITRO DEMINERALISATION/REMINERALISATION MODEL	26
1.5 TYPES OF ENAMEL USED IN CARIES MODELS.....	28
1.6 METHODS OF EVALUATING DEMINERALISATION AND REMINERALISATION OF DENTAL TISSUES.....	30
1.6.1 <i>Transverse Microradiography (TMR) & Polarised Light Microscopy (PLM)</i>	31
1.6.2 <i>Quantitative light-induced fluorescence (QLF)</i>	32
1.6.3 <i>Microhardness test</i>	36
2.0 AIM, OBJECTIVE & NULL HYPOTHESIS.....	39
2.1 AIM	39
2.2 OBJECTIVE	39
2.3 NULL HYPOTHESIS	39
3.0 MATERIALS AND METHODS	40
3.1 SAMPLE SIZE CALCULATION	41
3.2 RANDOMISATION AND BLINDNESS.....	41
3.3 EXPERIMENTAL MATERIALS (TEST AND CONTROL PRODUCTS):.....	41
3.4 THE STUDY GROUPS (EXPLAINED IN DETAIL IN SECTION 6.0)	42

3.5 TEETH SELECTION AND CLEANING	43
3.6 ENAMEL SLAB PREPARATION FOR TESTING WITH QLF & CREATION OF WHITE SPOT LESIONS	43
3.7 QUANTITATIVE LIGHT-INDUCED FLUORESCENCE (QLF) ANALYSIS.....	49
3.8 DATA HANDLING	54
3.9 INCLUDED ARTIFICIAL LESIONS DEPENDING ON THE ΔF RANGES.....	55
3.10 TRAINING AND CALIBRATION.....	57
3.11 INTRA-EXAMINER REPRODUCIBILITY	58
3.12 STUDY GROUPS.....	58
3.13 FREQUENCIES USED (DAILY AND INTERMITTENT).....	59
3.13.1 <i>Daily frequency of consumption during 60 days.</i>	59
3.13.2 <i>Interrupted frequency of consumption during 60 days</i>	60
3.14 THE PH CYCLING REGIME IN THE STUDY.....	63
3.15 PREPARATION OF SOLUTIONS USED FOR PH CYCLING.....	69
3.15.1 <i>Preparation of fluoridated milk with different concentrations</i>	69
3.15.2 <i>Preparation of the milk/ saliva slurry</i>	69
3.15.3 <i>Artificial saliva</i>	69
3.15.3.1 Artificial day-time saliva solution.....	70
3.15.3.2. Artificial night-time saliva solution.....	71
3.15.4 <i>Preparation of acetic acid solution</i>	72
3.16 STATISTICAL ANALYSIS	74
4.0 RESULTS	75
4.1 QUANTITATIVE LIGHT INDUCED FLUORESCENCE RESULTS (QLF).....	75
4.2 DESCRIPTIVE STATISTICS AND NORMALITY TESTS	75
4.2.1 <i>Interrupted frequency</i>	75
4.2.1.1 Normality tests for Interrupted Frequency:.....	75
4.2.1.2 Descriptive statistics and mean comparisons.....	78
4.2.2 <i>Daily frequency</i>	78
4.2.2.1 Normality test for the Daily frequency:	78
4.2.2.2 Descriptive statistics and means comparisons	81
4.2.3 <i>Difference between baseline values</i>	81
4.3 TESTING THE DIFFERENCES WITHIN EACH GROUP (BASELINE & AFTER TREATMENT)...	82
4.3.1 <i>Difference in ΔF within each fluoride concentration (Interrupted & Daily Frequency)</i>	82
4.3.2 <i>Difference in ΔQ within each fluoride concentration (Interrupted and Daily frequency)</i>	85
4.3.3 <i>Difference in Area within each fluoride concentration (interrupted and daily frequency)</i>	88
4.4 COMPARING THE FLUORIDE CONCENTRATION LEVELS IN MILK FOR EACH FREQUENCY (INTERRUPTED & DAILY).....	91
4.4.1 <i>The Interrupted frequency:</i>	91
4.4.2 <i>For the Daily frequency</i>	95
4.5 COMPARING THE FLUORIDE CONCENTRATION LEVELS IN MILK BETWEEN BOTH FREQUENCIES (INTERRUPTED VS. DAILY)	98

4.5.1 Difference in ΔF (ΔF diff) values at baseline and after treatment between the interrupted and daily frequencies.....	98
4.5.2 Difference in ΔQ (ΔQ diff) values at baseline and after treatment between the interrupted and daily frequencies.....	101
4.5.3 Difference in lesion Area (Area diff) values at baseline and after treatment between the interrupted and daily frequencies.....	105
4.6 INTRA-EXAMINER REPRODUCIBILITY.....	110
4.6.1 Intra-examiner reproducibility for ΔF	110
4.6.2 Intra-Examiner Reproducibility for ΔQ	112
4.6.3 Intra- Examiner Reproducibility for Area.....	114
4.7 SUMMARY OF THE RESULTS.....	116
5.0 DISCUSSION.....	121
5.1 THE IN VITRO MODEL.....	122
5.2 DESIGN OF THE STUDY.....	123
5.2.1 The bovine teeth caries model.....	124
5.2.2 Preparation and storage of enamel slabs.....	125
5.2.3 Creation of artificial carious lesions.....	126
5.2.4 The pH cycling experimental model.....	128
5.2.5 Study materials used.....	130
5.3 METHOD OF ANALYSIS: QUANTITATIVE LIGHT INDUCED FLUORESCENCE (QLF).....	131
5.3.1 QLF results.....	136
5.4 THE EFFECT OF FLUORIDATED MILK ON ENAMEL SUBSURFACE LESION	
REMINERALISATION.....	136
5.4.1 Interrupted vs. Daily frequency patterns.....	147
6.0 CONCLUSION.....	150
7.0 RECOMMENDATIONS AND FUTURE RESEARCH.....	152
8.0 REFERENCES.....	154
9.0 APPENDICES.....	XVI

LIST OF TABLES

TABLE 1-1 INTERNATIONAL MILK FLUORIDATION SCHEMES	24
TABLE 3-1: COMPOSITION OF SYNTHETIC DAY-TIME SALIVA SOLUTION	70
TABLE 6-2: COMPOSITION OF SYNTHETIC NIGHT-TIME SALIVA SOLUTION	72
TABLE 3-3: ACETIC ACID SOLUTION COMPONENTS	73
TABLE 4-1: NORMALITY TEST (SHAPIRO-WILK) P-VALUES FOR THE INTERRUPTED FREQUENCY	76
TABLE 4-2 DESCRIPTIVE STATISTICS FOR ΔF DIFF, ΔQ DIFF AND AREA DIFF FOR THE INTERRUPTED FREQUENCY	78
TABLE 4-3: NORMALITY TEST (SHAPIRO-WILK) P-VALUES FOR THE DAILY FREQUENCY	79
TABLE 4-4: DESCRIPTIVE STATISTICS FOR ΔF DIFF, ΔQ DIFF AND AREA DIFF FOR THE DAILY FREQUENCY	81
TABLE 4-5: THE MEAN VALUES OF ΔF AT BASELINE AND AFTER TREATMENT FOR ALL F CONCENTRATION LEVELS	83
TABLE 4-6: PAIRED SAMPLED T-TEST RESULTS FOR ΔF VALUES AT BASELINE AND AFTER TREATMENT	84
TABLE 4-7: THE MEAN VALUES OF ΔQ AT BASELINE AND AFTER TREATMENT FOR ALL F CONCENTRATION LEVELS	86
TABLE 4-8: PAIRED SAMPLED T-TEST RESULTS FOR ΔQ VALUES AT BASELINE AND AFTER TREATMENT	87
TABLE 4-9: WILCOXON SIGNED-RANK TEST FOR ΔQ VALUES AT BASELINE AND AFTER TREATMENT	87
TABLE 4-10 THE MEAN VALUES OF AREA AT BASELINE AND AFTER TREATMENT FOR ALL F CONCENTRATION LEVELS	89
TABLE 4-11 PAIRED SAMPLED T-TEST RESULTS FOR AREA VALUES AT BASELINE AND AFTER TREATMENT	90
TABLE 4-12 WILCOXON SIGNED-RANK TEST FOR AREA VALUES AT BASELINE AND AFTER TREATMENT	90
TABLE 4-13: ONE-WAY ANOVA RESULTS BETWEEN FLUORIDE CONCENTRATION LEVELS FOR THE DIFFERENCE IN ALL QLF PARAMETERS AT BASELINE AND AFTER TREATMENT	93

TABLE 4-14: MULTIPLE COMPARISONS OF THE DIFFERENCE IN ΔF AND ΔQ AT BASELINE AND AFTER TREATMENT BETWEEN ALL LEVELS OF FLUORIDE CONCENTRATIONS	94
TABLE 4-15: ONE-WAY ANOVA RESULTS BETWEEN FLUORIDE CONCENTRATION LEVELS FOR THE DIFFERENCE IN ΔF AT BASELINE AND AFTER TREATMENT	96
TABLE 4-16: MULTIPLE COMPARISONS OF THE DIFFERENCE IN ΔF AT BASELINE AND AFTER TREATMENT BETWEEN ALL LEVELS OF FLUORIDE CONCENTRATIONS..	96
TABLE 4-17: KRUSKAL- WALLIS TEST RESULTS BETWEEN FLUORIDE CONCENTRATION LEVELS FOR THE DIFFERENCE IN ΔQ AND AREA AT BASELINE AND AFTER TREATMENT	97
TABLE 4-18: PAIRWISE COMPARISON TEST OF THE DIFFERENCE IN ΔQ AT BASELINE AND AFTER TREATMENT BETWEEN ALL LEVELS OF FLUORIDE CONCENTRATIONS	97
TABLE 4-19: DESCRIPTIVE STATISTICS FOR THE DIFFERENCE IN ΔF (ΔF DIFF) AT BASELINE AND AFTER TREATMENT FOR ALL FLUORIDE CONCENTRATION LEVELS..	99
TABLE 4-20: INDEPENDENT TWO-SAMPLE T-TEST RESULTS FOR THE DIFFERENCE IN ΔF (ΔF DIFF)	101
TABLE 4-21: DESCRIPTIVE STATISTICS FOR THE DIFFERENCE IN ΔQ (ΔQ DIFF) AT BASELINE AND AFTER TREATMENT FOR ALL FLUORIDE CONCENTRATION LEVELS	102
TABLE 4-22: INDEPENDENT TWO-SAMPLE T-TEST RESULTS FOR THE DIFFERENCE IN ΔQ (ΔQ DIFF) FOR FLUORIDE CONCENTRATION LEVEL 1.0 PPM	105
TABLE 4-23: MANN-WHITNEY U TEST FOR THE DIFFERENCE IN ΔQ (ΔQ DIFF) FOR FLUORIDE CONCENTRATION LEVELS 0.0 PPM AND 0.5 PPM	105
TABLE 4-24: DESCRIPTIVE STATISTICS FOR THE DIFFERENCE IN LESION AREA (AREA DIFF) AT BASELINE AND AFTER TREATMENT FOR ALL FLUORIDE CONCENTRATION LEVELS	106
TABLE 4-25: MANN-WHITNEY U TEST FOR THE DIFFERENCE IN LESION AREA (AREA DIFF)	109
TABLE 4-26: MANN-WHITNEY U TEST FOR THE DIFFERENCE IN LESION AREA (AREA DIFF) (WITHOUT THE EXTREME VALUES)	109
TABLE 4-27: PAIRED SAMPLED TEST FOR ΔF MEASURES	110
TABLE 4-28: INTRA-CLASS CORRELATION COEFFICIENT FOR ΔF MEASURES	111
TABLE 4-29: PAIRED SAMPLES TEST FOR ΔQ MEASURES	112
TABLE 4-30: INTRA-CLASS CORRELATION COEFFICIENT FOR ΔQ MEASURES	112
TABLE 4-31: PAIRED SAMPLES TEST FOR AREA MEASURES	114
TABLE 4-32: INTRA-CLASS CORRELATION COEFFICIENT FOR AREA MEASURES	114

LIST OF FIGURES & GRAPHS

FIGURE 3-1: TEST AND CONTROL MILKS USED	42
FIGURE 3-2: WELL DIAMOND WIRE SAW CUTTING MACHINE (WELL® WALTER EBNER, CH-2400 LE LOCHE).....	45
FIGURE 3-3: ENAMEL SLABS ATTACHED TO UNIVERSAL (STERILIN®, UK) TYPE TUBE USING YELLOW DENTAL WAX “STICKY WAX”	46
FIGURE 3-4: APPLICATION OF NAIL VARNISH COATING WITH EXPOSED ENAMEL WINDOW.....	47
FIGURE 3-5: PREPARATION OF THE DEMINERALISING GEL MIXTURE	48
FIGURE 3-6: ENAMEL SLABS SUBMERGED IN THE DEMINERALISING GEL	49
FIGURE 3-7: QLF MACHINE. SLR CAMERA MOUNTED ON THE STAND WITH A FIXED DISTANCE FROM THE ENAMEL SLAB	50
FIGURE 3-8: DARKENED ROOM SETTING.....	50
FIGURE 3-9: CANON CAMERA USED WITH ATTACHED LENS AND BILUMINATOR™ APPARATUS.....	51
FIGURE 3-10: CAMERA STAND AND JACK SET AT A STANDARDISED POSITION	52
FIGURE 3-11: CREATION OF SILICONE MOULD	53
FIGURE 3-12: QLF IMAGE (WITH BLUE-LIGHT) DURING ANALYSIS, SHOWS THE DARKENED DEMINERALISED LESION SURROUNDED BY SOUND ENAMEL. THE IMAGE ALSO SHOWS THE PATCH ENCIRCLING THE LESION.....	54
FIGURE 3-13: AN EXAMPLE OF THE ANALYSIS SOFTWARE DISPLAYING QLF PARAMETER VALUES (IMAGE CAPTURED WITH BLUE-LIGHT FEATURE).....	55
FIGURE 3-14: SHOWING AN ENAMEL SLAB FROM GROUP 4 (0.0 PPM F; DAILY FREQUENCY).....	56
FIGURE 3-15: SHOWING AN ENAMEL FROM GROUP 5 (0.5 PPM F; DAILY FREQUENCY).....	57
FIGURE 3-16: SHOWING AN ENAMEL SLAB FROM GROUP 6 (1.0 PPM F; DAILY FREQUENCY).....	57
FIGURE 3-17: AN EXAMPLE OF MARKING THE CALENDAR WITH LABELS TO HIGHLIGHT WHICH DAYS THE INTERRUPTED FREQUENCY GROUPS SHOULD BE DIPPED IN MILK	63
FIGURE 3-18: EACH GROUP HAD SEPARATE LABELLED CONTAINERS. EACH STEP OF THE CYCLE REQUIRED THE USE OF A DESIGNATED CONTAINER TO PREVENT CONTAMINATION.....	64

FIGURE 3-19: DIPPING THE SLABS IN FLUORIDATED MILK FOLLOWED BY THE MILK/SALIVA SLURRY.....	65
FIGURE 3-20: DAY TIME SALIVA CONTAINERS AND INCUBATOR	66
FIGURE 3-21: ALL GROUPS UNDERGOING THE ACETIC ACID CHALLENGE	67
FIGURE 3-23: DAILY REGIME FOLLOWED FOR 60 DAYS:	68
FIGURE 3-24: PREPARATION OF ARTIFICIAL SALIVA.....	71
FIGURE 3-25: ORION PH METER	73
FIGURE 4-1: BOX-PLOTS FOR ΔF DIFF, ΔQ DIFF, AND AREA DIFF (INTERRUPTED FREQUENCY)	77
FIGURE 4-2: BOX-PLOTS FOR ΔF DIFF, ΔQ DIFF, AND AREA DIFF (DAILY FREQUENCY)	80
FIGURE 4-3: BOXPLOTS FOR MEAN OF ΔF DIFF BY FREQUENCY AND LEVEL OF FLUORIDE CONCENTRATION	100
FIGURE 4-4: BOXPLOTS FOR MEAN OF ΔQ DIFF BY FREQUENCY AND LEVEL OF FLUORIDE CONCENTRATION	104
FIGURE 4-5: BOXPLOTS FOR MEAN OF LESION AREA DIFF BY FREQUENCY AND LEVEL OF FLUORIDE CONCENTRATION	108
FIGURE 4-6: BLAND-ALTMAN PLOT FOR REPRODUCIBILITY OF ΔF MEASURES AT THE BEGINNING OF THE EXPERIMENT.....	111
FIGURE 4-7 BLAND-ALTMAN PLOT FOR REPRODUCIBILITY OF ΔQ MEASURES AT THE BEGINNING OF THE EXPERIMENT.....	113
FIGURE 4-8 BLAND-ALTMAN PLOT FOR REPRODUCIBILITY OF AREA MEASURES AT THE BEGINNING OF THE EXPERIMENT.....	115
GRAPH 4-1: THE MEAN PERCENTAGE CHANGE IN ΔF	85
GRAPH 4-2: THE MEAN PERCENTAGE CHANGE IN ΔQ	88
GRAPH 4-3: THE MEAN PERCENTAGE CHANGE IN AREA	91
GRAPH 4-4: MEAN OF THE DIFFERENCE IN ΔF (ΔF DIFF) BY FREQUENCY AND LEVEL OF CONCENTRATION	99
GRAPH 4-5: MEAN OF THE DIFFERENCE IN ΔQ (ΔQ DIFF) BY FREQUENCY AND LEVEL OF FLUORIDE CONCENTRATION	103
GRAPH 4-6 MEAN OF THE DIFFERENCE IN LESION AREA (AREA DIFF) BY FREQUENCY AND LEVEL OF FLUORIDE CONCENTRATION.....	107

LIST OF ABBREVIATIONS

%	Percentage
≥	Greater than or equal to
% F	Percentage change in fluorescence
% Q	Percentage change ΔQ
ΔF	Average fluorescence loss
ΔQ	Multiplication of ΔF and area
μg	Microgram
μm	Micrometre
am	Morning
ANOVA	Analysis of Variance
BSPD	British Society of Paediatric Dentistry
Ca	Calcium
Conc.	Concentration
CPP-ACP	Casein phosphopeptide-amorphous calcium phosphate
CSMH	Cross Sectional Microhardness
d	Day
DEJ	Dentine- enamel junction
diff	Difference (change)= final reading-baseline reading
dmfs	Decayed, Missing and Filled Surfaces (primary teeth)
DMFS	Decayed, Missing and Filled Surfaces (Permanent teeth)
dmft	Decayed, Missing and Filled Teeth (for primary teeth)
DMFT	Decayed, Missing and Filled Teeth (Permanent teeth)
e.g.	Example
EAPD	European Academy of Paediatric Dentistry
F	Fluoride
g	Gram
HA	Hydroxyapatite
HCL	Hydrochloric acid
ICDAS	International Caries Detection and Assessment System
ICC	Intra-class Correlation Coefficient
Kg	Kilogram
KOH	Potassium hydroxide

L	Litre
mg	Milligram
min	Minutes
ml or mL	Millilitre
mm	Millimetre
mol	Mole
N or n	Number
Na	Sodium
NaOH	Sodium hydroxide
nm	Nanometre
°C	Degree of Celsius
OR	Odd Ratio
p	p-value
pm	Evening
pH	Acidity
px	Pixels
PO ₄	Phosphate
ppm	Part per million
QLF	Quantitative Light-Induced Fluorescence
RCT	Randomised control trials
s	Second
SD	Standard deviation
SE	Standard error
SEM	Scanning Electron Microscopy
Sig	Statistical level
SMH	Surface Microhardness
SPSS	Statistical Package for the Social Sciences
TMR	Transverse Microradiography
vs	Versus
w/v	Weight per volume
w/w	Weight per weight
WSL	White spot lesion
wt	Weight

1.0 LITERATURE REVIEW

This process began by initially attending a library course on literature searching and how to use Endnote referencing when writing a literature review. A number of search engines were explored including: Pubmed, Embase, Ovid, google scholar and the Cochrane Library for systematic reviews. The University of Leeds Library data base was also used for obtaining additional literature. There were no restrictions made on the types of studies to be reviewed although the language was restricted to English. The terms used involved milk, fluoride, caries, demineralisation, remineralisation, children, artificial, young, adults, teenagers, bovine, human, teeth, enamel, pH-cycling, concentration, toxicity, dose, frequency, metabolism and excretion. These were searched separately and in combination or in comparison. The systematic reviews related to milk fluoridation were read and their references were searched to obtain further articles (Cagetti et al., 2013; Yueng et al. 2015). The abstracts of papers were examined first and if found relevant, were read throughout. The Borrow Foundation was also contacted and they helped provide a milk fluoridation data base that included all the scientific articles, abstracts, books, lectures, non-scientific articles and editorials related to milk fluoridation (Version 6.8, Dispatched January 2014).

Recent advances in the understanding of the dental caries process have led to moving away from the restorative approach to the more conservative biological approaches combined with the provision of ongoing prevention as per the available evidence-based guidelines (Meyer-Luckel et al. 2013). Having said that, dental caries is still considered to be one of the main common diseases,

causing significant burdens on individual and community levels as well as its major impacts on the health systems (Benzian, 2013).

1.1 Dental caries

It is well reported in the literature that dental caries is a multifactorial dynamic process, affected by a number of factors pushing the dynamic equilibrium in favour of either remineralisation or demineralisation of the tooth minerals which determines if the process starts, advances, discontinues or reverses. This activity normally takes place at the interface of the intricate biofilm that overlays the tooth surface consisting of the pellicle and plaque microflora (Pretty and Ellwood, 2013). It is therefore considered reversible and can be stopped with the elimination of the plaque (Gomez, 2015).

The caries process is caused by a low pH level for a prolonged period of time within the plaque (critical pH for human and bovine enamel is 5.5) resulting in enamel dissolution (Costa et al., 2015). The clinical causes of caries include: accumulation of plaque containing bacteria and/or acid producing bacteria of which more than 700 bacterial species have been identified in plaque samples where almost 40 of these species have been linked to dental caries alone and varying between individuals, sites within the oral cavity, diet and other factors. The frequent consumption of fermentable carbohydrates, low salivary flow or reduced buffering capacity of saliva in addition to the genetic makeup of the host also renders them to be more susceptible to caries (Salli and Ouwehand, 2015).

Dental caries continues to be considered a serious public health problem; it affects 60-90% of school children as well as adults on a global level, particularly among underprivileged groups of both developed and developing countries (Petersen, 2003). Dental Public Health England produced two oral health surveys reporting the prevalence and severity of dental decay in three and five-year-old children. There is reportedly 12% of three-year-old children with obvious dental decay of which one or more have reached dentinal level, extracted or even restored due to decay with an average dmft of 0.36 per child. While about 88% of this cohort of children were free from visually noticeable tooth decay (Oral Health Survey of three-year-old children 2013, PHE). As for the 5-year-old cohort of children in the UK, the percentage of children who had experienced dental decay reached up to 24.7% with an average dmft of 0.8 per child. This meant that around 75.2% of 5-year-old children were free from visually apparent dental decay (Oral Health Survey of five-year-old children 2015.PHE). The Royal College of Surgeons of England reported that around 46,500 children and young adults had been admitted to hospitals for a general anaesthetic due to dental caries between 2013 and 2014 (RCS Eng. 2015). These reports have stated that dental caries remains the most common causative factor for children between five and nine years of age to be admitted to hospital (30.9% of all GA's) in both England and Scotland and that these numbers are increasing each year (NHS Dental Statistics for England 2013–2014, Deery et al., 2015).

There are many factors that play a role in the development of dental caries, and diet is one of the major factors. Therefore, in order to successfully control dental caries, a combination of good oral hygiene, fluoride use, and dietary

control must be implemented. Other factors include: general health, patient's behaviour, total food intake, intake pattern, rate of salivary secretion, plaque deposition, fluoride use and socio-economic status (SIGN 138). Communities of lower socio-economic status have higher levels of dental caries compared to non-deprived communities which might have resulted in significant oral health inequalities (Pretty and Ellwood, 2013). The most important predictor of future caries risk is considered to be the individual's past caries experience. The clinician should be aware that patients' risk status fluctuates continuously to either improvement or deterioration and this could be monitored by adhering to guidelines, carrying out structured interviews or by using computer-based forms (Twetman et al., 2013).

1.1.1 White spot lesions (WSL)

Defined as the presence of a subsurface lesion with most of the mineral loss underneath a reasonably intact enamel surface layer (Toumba, 2005). It appears whiter when dried with air and softer than adjacent healthy enamel. This white opaque, chalky appearance of early enamel lesions is due to loss of minerals of surface or subsurface enamel creating an optical phenomenon by altered light properties (Sudjalim et al., 2006; Mayne et al., 2011; Tüfekçi et al., 2014). Enamel caries can, therefore, be classified as either damage of the enamel surface or a white spot lesion that extends from the outer surface of enamel up to the enamel-dentinal junction without any evidence of cavitation or discolouration beneath the enamel-dentinal junction (Ashley et al., 1998).

Teeth are formed from hydroxyapatite which is a calcium phosphate mineral. The enamel structure is built from a number of well-oriented prism-like

structures, consisting of millions of hydroxyapatite crystals that form a potentially mechanically strong but otherwise chemically vulnerable structure. This vulnerability can be attributed to the existing porosities present between the enamel prisms and hydroxyapatite crystals, allowing the organic acids formed in the plaque to penetrate into the enamel instead of dissolving the enamel layer by layer. This results in the tooth maintaining an intact surface layer as the demineralisation process continues with multiple exposures to acid challenges where the acid dissolution occurs over extended depths (ten Cate, 2013).

From a histopathological point of view, the early enamel lesion is characterised by four zones (Silverstone, 1973; Silverstone et al., 1988a; Silverstone et al., 1988b; Roopa et al., 2015):

- 1- The surface zone with 1 to <5% pore volume. This zone forms the intact surface on top of the lesion and is produced by remineralisation and redeposition of calcium and phosphate ions from saliva as the pH level returns to neutral (pH 7).
- 2- The body of the lesion consisting of >5 to 25% pore volume. This zone represents the bulk of the lesion with around 50% of its minerals lost and is located approximately 15-30 μm beneath the surface layer.
- 3- The dark zone with around 2 to 4% pore volume and is situated between the translucent zone and body of the lesion.
- 4- The translucent zone with 1% pore volume representing the advancing front of the lesion. It consists of a soluble mineral phase lacking calcium hydroxyapatite.

If the process of demineralisation continues, the acids will penetrate into greater depths, and the tooth structure will become weaker resulting in obvious

cavitation. However, there is a chance for new enamel to form in white spot lesions by deposition of calcium and phosphate ions in the oral cavity due to the neutralising effect of saliva which results in remineralisation. In the presence of fluoride ions in plaque during remineralisation, these ions become incorporated with the hydroxyapatites to form a fluor-hydroxyapatite mixed crystals structure that favours reprecipitation of the dissolved minerals, has lower solubility, and more resistance to future acid attacks (ten Cate, 2013; Roopa et al., 2015). Due to the deposition of fluorapatite and calcium phosphate precipitation on the surface layer, the surface pores become blocked, restricting diffusion into the lesion and leading to incomplete lesion remineralisation which is why this enamel retains its demineralised appearance (Dirks, 1966; Manton and Reynolds, 2016).

1.1.2 Creation of artificial WSL

The concept of creating artificial carious lesions has been investigated in early research (ten Cate, 1996; Toth et al., 1997; Amaechi et al., 1998; Lippert et al., 2011). It is important to note that there are structural differences between the natural caries process and artificial enamel caries-like lesions. The naturally occurring alternating demineralisation and remineralisation cycles present in the oral cavity are believed to be the reason why natural enamel caries lesions are more nonuniform with increased thickness and mineral content compared to artificial lesions (Phark et al., 2009; Joshi and Joshi, 2013). This dynamic process of demineralisation and remineralisation renders the enamel in natural WSL more resistant to an acid attack because these intraoral conditions result in a reduction in the size of WSL micropores compared to artificial enamel

carious lesions where more penetration of chemical substances (e.g resins or fluorides) occurs (Kidd et al., 2004; Jeffries, 2014; Li et al., 2014). An advantage of using artificially created white spot lesions is that they allow testing of different demineralisation mechanisms and investigating different parameters of the caries process one at a time under controlled conditions (Arends and Christoffersen, 1986). It is impossible to mirror the caries process that occurs in the human oral cavity due to variations in caries models used, types of teeth, the time scale of the experiment in vitro which is why the results from such experiments need to be carefully interpreted prior to extrapolation (Robinson et al., 2017).

1.2 Fluoride and caries prevention

The fluoride ion comes from one of the halogen group elements known as fluorine. It is widely available in nature and has great affinity towards mineralised tissues (Ullah and Zafar, 2015). Dental tissues are high in minerals, mainly apatite. In the presence of F^- (fluorine), it combines with the apatite to form fluorapatite. F^- is a naturally occurring element that exists in both natural and commercial forms, its concentration has usually been shown as the number of parts per million (ppm).

F^- was first added to drinking water in Grand Rapids Michigan, 1945, and that is where its therapeutic effect was initially demonstrated. The caries incidence in around 30,000 school children (measured as decayed, missing or filled permanent teeth) was found to have decreased by more than 60 percent after implementation of water fluoridation systems (Arnold et al., 1962). The improved understanding of fluoride's preventative effect against caries started

as early as the 1970's when fluoride toothpastes were first introduced and it was established that fluorides played the most important caries preventive role through its topical effect on dental tissues (WHO, 1994; Fejerskov et al., 2004; Buzalaf et al., 2011; Sket et al., 2017).

Fluoride has since been acknowledged as the most valuable method used in caries prevention as the introduction of fluoridated toothpastes resulted in less dental caries prevalence over the past decades (Twetman, 2009). Current guidelines also highlight the importance of using fluoridated toothpastes as the basic regimen in the majority of European countries (Marino et al., 2003; EAPD, 2009; Walsh et al., 2010).

1.2.1 Mechanisms of action of fluoride

A number of mechanisms of action of fluoride have been explained to demonstrate its protective effects. Fluoride can also inhibit the production of extracellular polysaccharides which affects plaque bacterial growth and glycolysis by inhibiting the enzyme enolase in the glycolytic pathway. Fluoride strongly inhibits the enzyme in the presence of inorganic phosphate in which the inhibitory species is the fluorophosphate ion that binds to magnesium and forms a complex with enolase and inactivates the enzyme (Mikesh and Bruns, 2009). In addition to that, fluoride plays another important role as it inhibits demineralisation and enhances remineralisation when combining with calcium and phosphate to form fluorapatite crystals which are more stable than hydroxyapatites and can withstand acidic attacks without dissolving the crystallites. This effect is highly dependent on the bioavailability of calcium and

phosphate ions in saliva (ten Cate, 1999; Edgar et al., 2004; Featherstone, 2006; Reynolds, 2008).

Understanding that the post-eruptive (topical) effect of fluoride is the primary mode of action in caries prevention, emphasises the importance of maintaining a frequent, low concentration exposure of fluoride in the oral cavity (Fejerskov et al., 1996).

1.2.2 Role of saliva

Saliva has a protective role in reversing and/or arresting the carious process. It helps reduce the amount of plaque retention on the tooth surface by means of mechanical cleansing (Mandel and Zeng, 1973; Mandel, 1974; Dodds et al., 2004; Stookey, 2008; Felix et al., 2012).

Dental biofilms and saliva provide a near neutral pH when under physiological conditions. The oral fluids containing calcium and phosphates in ionic form along with the intra-oral pH level will determine whether this results in super-saturation or under-saturation. This would have influence if a mineral precipitates or forms onto teeth (remineralisation) or dissolves (demineralisation) due to acids produced by cariogenic bacteria (ten Cate, 2013).

Calcium and phosphate present in saliva remain in a supersaturated state due to the actions of salivary proteins, especially statherin. This protein binds the calcium ions, making them readily available in solution (Lamkin and Oppenheim, 1993). Therefore, conditions where salivary function or flow are impaired could lead to the development of rampant caries due to the

insufficiency of calcium and phosphate needed for the remineralisation process (Featherstone, 2008).

1.2.3 Fluoride in saliva and oral clearance

Early research carried out by Baker Dirks (1966) provided evidence of the importance of remineralisation. They reported that around 50% of white spot lesions in young individuals would disappear with time due to the potential remineralising effect of saliva. There seems to be a direct relationship between the oral fluoride level and the fluoride dose applied from toothpastes where the mean salivary fluoride concentration seems to significantly increase as the fluoride concentration increases (Duckworth and Morgan, 1991; Duckworth and Stewart, 1994). In addition, oral fluid levels are also affected by the time of application of the fluoride agent and the method of delivery (Zero et al., 1992). Duckworth and Morgan demonstrated in their randomised clinical trial on different concentrations of fluoridated toothpaste that the levels of fluoride in saliva and plaque had reflected the dentifrice that had been used even 18 hours after the last brushing. Saliva fluoride levels had increased from 0.01 to 0.02 ppm while plaque showed an increase from 1.5 to 2.4 ng F/mg wet weight for the 0 and 2500 ppm F dentifrices respectively. This observation suggests fluoride reservoirs are filled when a fluoride product is being used where the fluoride would slowly be released later on (Duckworth and Morgan, 1991).

A study by Paul et al. (1993) stated that there was substantial retention of fluoride in saliva following the use of low concentration fluoride toothpaste in 7-9-year-old children where salivary fluoride levels were back to normal 30 minutes after tooth brushing. Another randomised cross-over experimental

study provided 6-8-year-old children with four types of drinking regimes including fluoridated milk and water containing 1 mg F. The unstimulated whole saliva was examined at baseline, after 15 minutes and 120 minutes while plaque samples were taken at baseline and after 2 hours only. The baseline values were between 0.01 and 0.02 mg F/ Litre for saliva and between 10.4-14.2 mg F/ Litre for plaque. Their results indicated that a statistically significant increase ($p < 0.05$) in fluoride was found in saliva 15 minutes following the consumption of fluoridated milk and water. The salivary fluoride concentration had eventually returned to baseline values 2 hours later. As for plaque fluoride levels, there was a statistically significant 2-fold increase ($p < 0.01$) in fluoride levels 2 hours after consumption of both fluoridated milk and water. These findings highlighted the protective benefits of both fluoridated milk and water in the cariostatic process as well as the fluoride storing potential of saliva and plaque (Petersson et al., 2002).

As a result of salivary secretions and swallowing, the fluoride levels in the oral cavity decline considerably. This would subsequently limit the effect of fluoride on oral bacteria as bacterial metabolism and growth are altered by fluoride concentrations of up to 10 ppm and more. Considering that fluoride provides the most preventive effect topically following tooth eruption, it is therefore important to emphasise that fluoride would be most effective when it is present in the oral cavity and not after it is swallowed (ten Cate, 2013).

1.2.4 Fluoride absorption, distribution, and elimination from the body

Approximately 90% of fluoride is absorbed in the gastrointestinal tract after it is consumed while the remaining 10% is excreted in faeces. It is then

distributed through the blood stream to the plasma and subsequently the body organs where the mean time of its peak concentration is 20-60 minutes after it is consumed. The concentration of fluoride in the plasma is usually around 0.01 ppm F and does not commonly exceed 0.06 ppm F and depends on the intake of fluoride over the day, earlier fluoride exposure and degree of fluoride accumulation in the bone (Ekstrand et al., 1984, Spak et al., 1986). Children and adults both retain some of the fluoride (50% and 36% respectively) of which most is contained in mineralised tissues, mainly bones and teeth. As for the residual part of the absorbed fluoride, it is excreted from the kidneys into the urine as the kidneys are considered the vital organ responsible for maintaining the fluoride concentration in human bodies. The excretion of fluoride through sweat and saliva is considered to be minimal (Fawell et al., 2006; Buzalaf and Whitford, 2011; Kanduti et al., 2016).

1.2.5 Optimal fluoride exposure

It is of importance to consider the multiple sources of fluoride available, including dental products as well as other available natural sources. It is also important to take into account the impact of fluoride on different age groups living in fluoridated versus non-fluoridated areas.

Exposure to fluoride is exhibited in milligrams per kilogram body weight. During the 1940's, appropriate exposure was estimated at 1.0 to 1.5 milligrams per day, which is equivalent to 0.05 mg F/kg body weight per day. More recently, optimal exposure to fluoride was considered to be at 0.05 to 0.07 milligrams per kilogram body weight per day for children younger than 12 years old and should not exceed 0.1 mg F/kg body weight for infants. Lower levels of 0.03 to 0.04

have also been proposed. It is necessary to monitor fluoride exposure in young children, because of its caries preventive role when used appropriately. (Whelton et al., 2004; Buzalaf and Levy, 2011; Buzalaf, 2017). However, fluoride intake in excessive amounts may lead to dental fluorosis in children (Fejerskov et al., 1996). Knowing that Fluoride is a toxic substance, it is important to make sure that it is used appropriately to add to patients' health while minimising the risk of causing harmful effects.

1.2.6 Fluorosis and fluoride toxicity

Enamel fluorosis is a type of hypomineralisation of the surface and subsurface enamel distinguished by the presence of more porosities than the surrounding normal enamel due to higher fluoride intake during the first six years of life which is a critical time for the immature enamel (Burt and Ekland, 1992; Whelton et al., 2004). Both the secretory and early maturation phases of amelogenesis are considered to be the timings where enamel is considered to be more prone to developing fluorosis as Evans and Stamm (1991). Reported this critical window to be as long as four months in duration starting at an age of 22 months after birth (Browne et al., 2004). Levy et al. (2010) mentioned that an excess fluoride intake throughout a child's first three years of life is directly related to the severity and prevalence of dental fluorosis in the permanent incisors. The calcification of permanent incisor crowns generally initiates at 3-4 months after birth to be completed by 4-5 years. In addition to the amount of fluoride ingested, the fluoride dose, timing, and duration of intake are all considered factors that influence the severity of fluorosis (Levy et al., 1995; Wong et al., 2010).

The clinical presentation of fluorosis can range from quantitative defects of narrow white horizontal lines with patches of yellowish to brown porous enamel to a more variable qualitative loss of enamel evident as mottling and/or pitting (Denbesten and Li, 2011).

On a population basis, studies have suggested that fluorosis can be used as a biomarker to assess the amount of exposure to fluoride in both fluoridated and non-fluoridated communities where enamel formation is taking place (Levy et al., 2010; O'Mullane et al., 2016). In such cases, where studies include groups of people rather than individuals, the daily urinary fluoride excretion can be used for the evaluation of fluoride intake to avoid unwanted risks. The urinary fluoride excretion tests are able to detect fluoride changes around six years earlier than when fluorosis is evident clinically and the WHO provided a detailed manual outlining the methodological measures needed to carry out a fluoride excretion analysis (WHO, 2014).

Population-based programmes such as the administration of water, milk or salt fluoridation as well as the use of fluoridated toothpastes are considered important caries prevention methods, as long as there is a balance between the risks and benefits. This highlights the importance of adhering to guideline recommendations when using fluoride containing products (EAPD, 2009, SDCEP, 2010; DOH, 2017).

The European Food Safety Authority (EFSA) has defined the lowest observed adverse effect level (LOAEL) of fluoridated milk causing enamel fluorosis in children from birth to the age of eight years to be 0.1 milligrams per kilogram body weight per day. Therefore, baby milk formulas have a maximum concentration of fluoride ranging from 0.6 to 0.7 milligrams per litre. However,

powdered infant milk formulas are likely to be exceeded if water containing more than 0.7 milligrams per litre fluoride is used for its preparation (Nohno et al., 2011).

Acute toxicity arises when excessive amounts of fluoride are ingested within a short period of time which could become poisonous. The signs and symptoms include nausea, hyper salivation, abdominal pain, bloody vomiting, and diarrhoea after being exposed to low doses. In higher doses, convulsions, cardiac arrhythmias, cardiac and respiratory failures and eventually death. Chronic toxicity, on the other hand, is a consequence of frequent prolonged exposure to fluoride resulting in variable expressions ranging from dental fluorosis, skeletal fluorosis to kidney damage (Heifetz and Horowitz, 1986).

The probable toxic dose (PTD) was defined by Whitford (1990) as the minimal amount of ingested fluoride that requires action and was set at 5 mg/kg body weight. For example, the PTD for a 5-year-old child with an average weight of 20 kg can result from ingesting 100 mg of fluoride. The safely tolerated dose (STD) was defined at 8-16 mg/kg body weight while the certainly lethal dose was at 32-64 mg/kg body weight (Hodge and Smith, 1965; Heifetz and Horowitz, 1986; Whitford, 1990; Whitford, 2011).

1.2.7 Methods of fluoride delivery

The use of fluorides in various vehicles for the prevention of dental caries has always been shown to be effective. The ease and different ways of fluoride administration are considered leading reasons for its success. In order for fluoride to be beneficial, it should continuously be available whether through topical application e.g. toothpastes (Walsh et al., 2010), mouth rinses (Marinho

et al., 2016), gels (Marinho et al., 2015), varnishes (Marinho et al., 2013) and slow releasing fluoride devices (Chong et al., 2014). There is an abundance of literature highlighting the efficacy of these methods on caries prevention.

Seventy-five randomised controlled trials (RCT) evaluating toothpastes with the outcome measure reported about 23% DMFS prevented fraction for the fluoridated toothpastes of concentrations equal or above 1000 ppm F. The recent Cochrane review by Marinho et al. (2016) evaluating the evidence on the caries preventive efficacy of mouth rinses for children and adolescents reported that out of 35 trials, there was on average a 27% reduction in DMFS (95% CI, 23%-30%) in permanent teeth compared to placebo. The data reported from 28 RCT's on fluoride gels revealed that there was a prevented fraction of 28% reduction in DMFS (95% CI, 19-36%) and 20% (CI 1%-38%) for the primary dentition. As for fluoride varnishes, the results reported by another Cochrane review done by Marinho et al. (2013), of the 22 RCT were assessed there seemed to be a DMFS reduction of 43% (95%CI, 30%-57%) and a dmfs of 37% (95% CI, 24%-51%). Chong et al. (2014) conducted a Cochrane review evaluating the literature on slow-release fluoride devices. Their review only found one RCT where only 48% of the children retained their beads and were available for analysis (n=174) with a lower caries increment in the intervention group (mean difference 0.72 (95% CI)) but the evidence was of very low quality.

Fluoride can also be delivered systemically via the saliva e.g. water fluoridation, supplements (tablets and drops), fluoridated milk, salt fluoridation and other dietary sources (Espelid, 2009).

Water fluoridation is considered a major breakthrough in preventive dentistry. A Cochrane review by Iheozor-Ejiofor et al. (2015) assessed the data

of 107 RCT's showing a reduction in dmft of 1.18 (95% CI, 1.31-2.31) and DMFT of 1.16 (95% CI, 0.72-1.61) which equates to 35% and 26% reduction in dmft and DMFT respectively. The authors also reported their estimation of possible dental fluorosis related to water fluoridation at 0.7 ppm to be of aesthetic concern in approximately 12% of the participants. However, about 95% of the evaluated studies were at high risk of bias with numerous variations between the studies. Hence, water fluoridation remains a safe and effective public health method for the prevention of dental caries. The effectiveness of fluoride supplements on caries prevention was discussed in a Cochrane review by Tubert-Jeannin et al. (2011), where 11 RCT's were assessed and showed an average of 24% reduction in DMFS (95% CI, 16%-33%).

Milk fluoridation programmes are currently in progress worldwide since milk is considered an important part of a child's diet in addition to being another feasible vehicle for the delivery of fluoride. (Twetman, 2005). Although the available literature suggests a beneficial caries preventive effect from milk fluoridation, the evidence is still lacking in high quality RCT to confirm those findings (Yueng et al., 2005). The updated Cochrane review by Yeung et al. (2015) highlighted the lack of good quality evidence examining the benefits and harms of fluoridated milk in the prevention of dental caries. Following the authors' revision of their protocols, they included one unpublished RCT based on the published abstract (Maslak et al., 2004) while excluding the 5 year double blind RCT study by Stephen et al. (1984) due to lack of adequate randomisation and high risk of selection bias as the authors had pointed out. The authors managed to correspond with Maslak (2004) to clarify some information regarding blinding and randomisation. The results concluded that the participants were

randomised on an individual level, and although the parents of participants were not blinded to the type of milk given, the outcome assessors and statistician involved were otherwise blinded. This included study is still considered to carry a high risk of bias, and its internal validity needs to be considered with caution. Three years after consumption of fluoridated milk, the mean difference in the reduction of DMFT was 0.13 (95% CI, 0.24-0.02) and the disease level was considered very low in the study leading to a small absolute effect size. The reduction in dmft showed a mean difference of 1.14 (95% CI, 1.86-0.42) which equates to a prevented fraction of 31%. The authors of the study also mentioned that no adverse effects of fluoridated milk consumption were reported. In general, the available evidence is considered of low quality, and recommendations include the need for more high-quality RCT's to draw definite conclusions about the effectiveness of milk fluoridation.

1.3 Bovine milk and dental health

There seems to be plenty of available research uniformly agreeing that milk is not considered to be a cause of dental caries. In the late 1980's, the Department of Health published a document stating that "Although lactose alone is moderately cariogenic, milk also contains factors which protect against dental caries, so that milk without added sugars may be considered to be virtually non-cariogenic" (DOH 1989). Milk's other components including minerals, lipids, other proteins i.e. casein play an important caries preventative role. Adding to that, milk also contains high concentrations of calcium and phosphorus which prevent the dissolution of enamel. The available literature assessing the caries preventative effects of milk consumption are mainly epidemiological studies and

despite the absence of controlled clinical trials, the evidence still support the consumption of milk which was associated with lower levels of caries experience (Rugg-Gunn and Woodward, 2011). Some epidemiological studies reported inconsistent results with regards to the association between intake of dairy products and dental caries among children (Petti et al., 1997; Petti et al., 2000; Kolker et al., 2007; Levine et al., 2007; Llana et al., 2008). The daily consumption of milk was reportedly related to lower caries prevalence among school children in Italy who were not using fluoride and had poor oral hygiene (Petti et al., 1997). Another cross-sectional study on Italian children between the ages of 3 – 5 mentioned that the consumption of yogurt and milk resulted in protective effects against dental caries (Petti et al., 2000). Milk consumption by low-income African American children had also been reported to reduce primary caries prevalence (Kolker et al., 2007).

1.3.1 Bioavailability of fluoride in milk

Similar to the effect of fluoridated water, fluoridated milk ingestion results in less demineralisation and more remineralisation of enamel and that is believed to be due to the increase in fluoride levels in enamel both pre- and post-eruptively (Banoczy et al., 2009). Banoczy et al. (2013) published an article investigating the history of fluoridated milk and its status on caries prevention. The authors reported that there are more than a 100 peer-reviewed papers on milk fluoridation, discussing the fact that most of the fluoride added to milk produces a soluble complex where the fluoride becomes bioavailable in ionic form. The calcium content in the diet affects the amount of fluoride consumed, as would simultaneous consumption with food.

Banoczy et al. (2013) also reported that the bioavailability of fluoride added to different milk preparations (whole milk, low-fat milk, fresh, sterilized, liquid or dried) were all investigated on the day of processing milk and after days of being stored, showing satisfactory results for all formulations. It is also important to mention that adding fluoride to milk does not alter its taste and would, therefore, be accepted by children. It would also seem suitable that since milk is an important part of a growing child's diet, adding an appropriate amount of fluoride exhibits additional caries preventative benefits.

1.3.2 Caries-preventive effect of milk fluoridation

The addition of fluoride to milk may cause it to partially interact with intrinsic calcium or else become associated with milk proteins. Some authors have felt that milk was a poor carrier of fluoride (Duff, 1981). However, others have disagreed with that. Rat studies carried out by Poulsen et al., 1976a, Banoczy et al., 1990 and Stosser et al., 1995 suggested that the caries-preventive effect of fluoride in milk had reached 40-50%.

Fluoridated milk delivers fluoride both topically and systemically. Milk and its derivatives have had a great impact on children's health status, including their dentition in the early 1930's (Roberts, 1931; Sprawson, 1932b). Therefore, milk is considered to be a feasible option that aids in providing fluoride to children. A number of studies were conducted that proved the caries preventive efficacy of fluoride in milk (Stephen et al., 1984; Banoczy et al., 1990; Banoczy et al., 2009). These studies followed the recommendations of the Swiss paediatrician Ziegler (1953) who proposed that milk could be used as an alternative vehicle for delivering fluoride. Furthermore, Ziegler (1956) described the technique used for

fluoridating milk, which was carried out by adding 1.0 ml of 0.22% NaF (1000 ppm F) solution to 1 litre of fresh milk. One of the initial clinical studies in USA where 6-9-year-old school children were provided with half a pint of fluoridated milk consisting of 1.0 mg F (in 285 ml =3.5 ppm F). The test group parents were also supplied with fluoridated milk to be taken home during vacation periods. The caries reduction after 3.5 years for the test group was 35% in permanent teeth where the most improvement was noted at 70% for children who were aged 6 years at the start of the study (Russof et al., 1962).

Subsequently, the Borrow Foundation was founded in 1971 by Dr. Edgar Borrow which was mainly established to ensure improvement of oral health in children by introducing milk as a vehicle for the delivery of fluoride and providing it in schools. The foundation was also concerned about supporting research, equipment, lectures and scientific knowledge for the sake of disseminating knowledge about this feasible preventative method. The Borrow Foundation went on to collaborate with the World Health Organization (WHO) to publish a book in 1996 that summarised the important research involved in milk fluoridation. This book was later revised and re-published in 2009 (Stephen et al., 1992; Banoczy et al., 2009).

Most of the human studies carried out on children using fluoridated milk are considered community based-programmes. Stephen et al. (1984) carried out a 5 year double blind trial in Scotland where fluoridated milk (1.5 mg/F in 200ml plastic pack) was distributed amongst 4-5-year-old school children to be consumed during school days only. After 5 years, the permanent teeth showed statistically significant reduction in DMFT (32.1%) in favour of the test participants (Stephen et al., 1984). Another community-based milk fluoridation

study in Bulgaria was designed as a field demonstration where non-randomised 1st grade and kindergarten school children were given 1 mg of fluoridated milk (200 ml) on weekdays only (180-200 days/year). Their results demonstrated the effect of fluoridated milk after three years of consumption on both primary and permanent dentitions with caries reduction of about 40% and 89% respectively (Pakhomov et al., 1995). The Chinese fluoridated programme providing 5-year-old school children with fluoridated milk over 5 years was carried out in 2 phases. The first phase was from 1994-1997 was not successful due to the increased amount of sugar added to the milk (7-10%) resulting in unfavourable outcomes. In their 2nd phase, this issue was rectified where little or no sugar was added to the milk containing fluoride at a concentration of 0.5 mg F (200 ml). The test children were given fluoridated milk to be taken on school days and also during weekends. This was also considered as a demonstration study as no randomisation was performed. Their results had improved after the 2nd phase, with an overall net caries increment of 0.4 dmft for the test children and 1.3 dmft for control children over the 21-month study period. This amounts to a statistically significant ($p < 0.001$) 69% reduction (Bian et al., 2003). Chile's community milk fluoridation varies considerably compared to other programmes. In addition to adding monfluorophosphate instead of sodium fluoride, they also provide their milk in powdered form and not liquid form. Marino et al. (2001) reported their results after 4 years of carrying out their community trial on 3-6-year-old children. The fluoride added to milk also varied depending on the children's age group and was provided over 365 days. The authors revealed that the proportion of caries-free children was statistically significantly higher ranging from 22-48% between 1994 and 1999. Three years following cessation of the

programme, there was an increase in caries levels in all children including the study group (Marino et al., 2001). The UK milk fluoridation programmes were initiated in 1993 in St Helens, Merseyside, involving 1600 children attending 40 local primary schools. There were 2 published evaluations including a longitudinal study in Knowsley (Ketley et al., 2003) and a cross-sectional study from the Wirral (Riley et al., 2005). The Knowsley study involved 3-5-year-old children who consumed milk containing 0.5 mg/F (189 ml) during school days only. After 4 years, the authors reported no reduction in caries increment following consumption of fluoridated milk, the justification was that there was a lack of statistical power (Yueng et al., 2005) or that the fluoride concentration might have been too low, the number of days where milk was consumed was only 180 days per year and that the caries experience was already too low in the primary dentition to notice any difference. Interestingly, the Wirral study had also reported that children consumed the same volume and concentration of fluoridated milk on school days only. However, their results showed that children who consumed fluoridated milk had 31% less DMFT compared to children who had plain milk. In January 2016, a proposal to introduce fluoridated milk to Blackpool children was accepted by the council as a method to deal with the elevated number of children presenting with very poor oral health. The feasibility of implementing this scheme would be through the free breakfast programme that will make it easier to reach all primary school children provided their parents were happy for their children to be involved in this programme (BDJ, 2016).

Table 1-2 from Banoczy et al. (2013) gives an idea about the international milk fluoridation schemes available and the number of children benefiting from them.

Table 1-1 International milk fluoridation schemes (Banoczy et al., 2013)

Country	Year the programme began	Number of children	Age range of children (y)	Amount of milk (ml)	Amount of fluoride (mg)
Bulgaria	1988	31000	3-7	100	0.5
				150	0.75
				200	0.5
UK	1993	32000	3-11	189	0.5
Russia	1994	39000	3-11	200	0.5
Chile	2000	220000	6-14	200	0.85
Thailand	2000	982188	3-12	200	0.5

It is clear from the literature that there are a number of variations between milk fluoridation schemes including the age groups of children involved in these programmes, the concentration of fluoride added to milk, the volume of milk, and the number of days per year where the milk is being provided to children.

A review of the literature on milk fluoridation schemes published by Banoczy et al. (2013) highlighted that the amount of fluoride added to milk commonly ranged between 0.5 – 1.0 mg per day. It was also reported that milk fluoridation should be considered when the fluoride concentration in drinking water was low, where a regular school milk programme existed, where there was significant caries experience in children, and when children were able to drink the fluoridated milk for at least 200 days in a year.

In vitro, in situ, and in vivo studies were also carried out in an attempt to study these variations. Malinowski et al. (2012a) published an in situ study assessing the efficacy of varying the concentrations of fluoridated milk on human dental enamel and concluded that both fluoride concentrations of 2.5 ppm and 5.0 ppm F exhibited the most protective effect on demineralised enamel. The same authors also demonstrated the benefits of adding 1.0 ppm F in milk using a pH cycling in vitro experiment where there was an evident increase in the advantages of fluoridated milk in concentrations of up to 5.0 ppm F but not beyond. It was also suggested that even concentrations as low as 2.5 ppm F in milk could show a beneficial effect in preventing against demineralisation of dental enamel (Malinowski et al., 2012b). As with the clinical studies, in vitro experiments also carried other multiple additional variations ranging from the pH cycling protocols to their assessment methods, making comparisons between studies difficult. The in vitro study by Ongtenco et al. (2014) was in agreement with other studies demonstrating the benefits of fluoridated milk on artificially created lesions compared to plain milk or deionised water. The authors also reported that 2.5 ppm F used twice daily exhibited the best remineralising potential compared to 5.0 ppm and 10.0 ppm F milk. This result was somewhat similar to what Itthagarun et al. (2011) concluded from their in vitro model study that 2.5 ppm F in milk seemed to show comparable remineralisation potential as higher fluoride concentrations and that increasing the volume of milk had little effect on remineralisation. It became clear that a dose-response effect can be identified when using fluoridated milk to prevent against carious lesions in situ and in vitro. Having said that, the number of studies that investigated the preventive effects of fluoridated milk under cariogenic conditions in situ and in

vitro are scarce and there is a need to carry out robust clinical studies especially those which use a randomised controlled design in order to obtain high-quality evidence similar to that provided for water fluoridation (Banoczy et al., 2013; Skold-Larsson et al., 2013; Cassiano et al., 2017).

1.4 The In vitro demineralisation/remineralisation model

Tooth decay is a consequence of continued mineral loss from the dental tissues. The in vitro demineralisation\remineralisation caries models were generally developed to help people understand complex procedures and factors that affect them. They aid in predicting a clinical outcome, under controlled and simplified conditions, which therefore assists in providing preventive measures against diseases. It is therefore important to first consider the research question in hand before choosing the appropriate model type that best interprets the results correctly. Different in vitro models have been developed and are continuously being enhanced to provide us with ways of carrying out reproducible tests under controlled conditions (Salli and Ouwehand, 2015).

The in vitro demineralisation models investigate the significant processes that are related to mineral loss from teeth (White, 1995). In vitro models that were used to determine the effects of remineralisation and demineralisation of enamel form an important part in the assessment of the cariogenic potential of a product. Advantages of using in vitro models are that they are easily standardised and therefore can be used to assess the effects of components added individually. Such experiments allow specific studies to be implemented, which were otherwise difficult or not possible to perform in vivo (Carey et al.,

1991). A useful in vitro model should also involve: The ability to easily sterilise different materials used, manipulation of the model components under sterile situations, accessing experiment specimens easily, reproducibility, simulation of the oral environment, possible low cost and ability to control contamination in the laboratory environment (Steiner-Oliveira *et al.*, 2007).

However, In vitro models can be limited in their capability to simulate biological conditions (White, 1992). They also lack the microorganisms naturally present in the oral environment which limits their ability to accurately reproduce the demineralisation process. Knowing that dental caries is a bacterial disease in which the causative bacteria are members of the dental biofilm that is absent from such experimental models. This would therefore eliminate the intra-oral bacterial interactions that characterise the caries process in vivo.

In order for in vitro models to simulate the pH changes that occur in the oral cavity, In vitro models consisting of a pH-cycling regime were used. The use of pH-cycling is considered to be the closest dynamic process for creation of a carious lesion (alternating periods of demineralisation and remineralisation) and first described by Featherstone *et al.* in 1986 (Fumes *et al.*, 2015). This process is achieved by subjecting the enamel to an array of demineralisation and remineralisation challenges to evaluate the reaction of products such as fluoride on the progression or regression of a lesion (ten Cate and Duisters, 1982; White, 1987). These pH-cycling experimental models where enamel is immersed in an acid medium are greatly used to replicate the cariogenic challenges that occur intra-orally (Steiner-Oliveira *et al.*, 2007). Such models have been broadly used with in vitro studies to evaluate remineralisation and demineralisation. The role of such pH cycling models is to aid in the production of adequate quantitative

data to supply investigators with enough confidence to design and carry out clinical trials appropriately. This is probably one of the main reasons why pH-cycling regimes are considered great tools aiding researchers in the assessment of the caries preventive effects of fluoridated milk using different fluoride concentrations (White, 1995).

Kahama et al., 1998 conducted an in vitro study demonstrating that 0.3 µg/ml fluoride in cow's milk provided protection of bovine enamel from demineralisation when comparing it to various fluoride concentrations in water. These types of milk studies have shown some limitations, one of which was described by McDougall, 1977 that the incubation of enamel with milk should not be for more than an hour due to bacterial fermentation of lactose that results in a decline in pH level. Studies that use the in vitro demineralisation models to assess the reaction of human dental enamel to fluoridated milk are scarce. However, Toth et al. (1997) demonstrated that 10 milligrams per litre fluoride in milk showed a profound decrease in acid solubility.

1.5 Types of enamel used in caries models

Ideally, human teeth are preferred for in vitro and in situ studies because they permit testing of the experiment hypothesis in a more clinically relevant substrate. Nonetheless, some limitations do exist when using human teeth which include their inadequate availability, insufficient quantities and appropriate quality, knowing that these teeth are mainly extracted due to extensive caries or other defects. It can also be difficult to control the source and age of the extracted human teeth which may impact on the outcome measures of the study. Added to that, the small and curved surface areas of human teeth may be

considered a limitation in some studies that require flat surfaces for experimentation. Ethical constraints and infection hazard awareness can also influence the choice of alternative substrates to be used in dental research (Yassen et al., 2011, Costa et al., 2015)). Therefore, obtaining and preparing human enamel can be a challenge because of its surface anatomical structure and broad variation in individual response to test conditions mainly if acquired from subjects of different ages (Featherstone and Zero, 1992a).

Bovine teeth have increasingly been used as an alternative to human teeth in dental studies. They can be more easily obtained in larger quantities, overall good quality and more unvarying composition than human teeth (Yassen et al., 2011). Bovine teeth present with an advantage in that they have flatter contours and are less variable in composition.

Feagin et al. reported that the calcium/phosphate ratio of the minerals removed from the enamel surface during demineralisation were similar in both human and bovine enamel, In addition to also displaying similar characteristics during remineralisation (Feagin et al., 1969). When comparing between the physical properties of human and bovine enamel, Spitzer and Bosch concluded that the refractive indices were not significantly different and that there were no substantial differences between the luminescence either (Spitzer and ten Bosch, 1975; Spitzer and ten Bosch, 1976). On the other hand, Featherstone and Mellberg compared the rates of artificial caries lesion production in permanent human and bovine enamel using different systems and they concluded that carious lesions seemed to progress twice as fast in bovine enamel compared to human enamel (Featherstone and Mellberg, 1981), this observation might be due to the fact that bovine enamel revealed higher porosity with bigger crystals

than human enamel (Attin *et al.*, 2007). Other studies have also showed that enamel of human primary teeth responded more rapidly to a cariogenic challenge than permanent teeth, but still responded in a similar manner to enamel of bovine teeth (Featherstone and Zero, 1992a). In terms of the mineral distribution characteristics of artificially created lesions on bovine versus human enamel, studies have reported that both were indistinguishable (Lippert and Lynch, 2014).

The type of hard tissue needed depends on the study that is to be carried out which varies between natural surfaces and those that need to be modified with the possibility of also creating a carious lesion. When lesions are created under laboratory conditions, the mineral loss can be controlled and measured. In order for the measurements to be reproducible, many assessment techniques require a flat surface (Koulourides *et al.*, 1974). It is therefore imperative that the morphological, chemical composition and physical property differences between both human and bovine enamel are considered during interpretation of results in experiments using bovine teeth.

1.6 Methods of evaluating demineralisation and remineralisation of dental tissues

Since the introduction of fluoridated products, there has been a remarkable increase in the time required for dental caries to advance from early demineralisation areas to obvious cavitations (Baelum *et al.*, 2006). This explains why it is important to develop detection methods that are able to identify the early stages of a carious process allowing a more conservative treatment approach to reverse or control the carious lesions. The methods mentioned in

this section work (TMR, PLM, QLF, SMH and CSMH) well as aids to clinical examinations as well as to improve how clinicians communicate with their patients to enhance oral hygiene and prevention (Gomez, 2015; van der Veen, 2015).

1.6.1 Transverse Microradiography (TMR) & Polarised Light Microscopy (PLM)

TMR and PLM have been used widely in tooth tissue demineralisation and remineralisation studies. They both involve cutting enamel slabs to sections that measure 100 µm in thickness which can be challenging and time-consuming. Another shortcoming would be that these methods both produce two-dimensional images of three-dimensional objects (Lo et al., 2010). These methods are able to detect lesion zones with different mineral content (Huysmans and Longbottom, 2004).

TMR is considered to be the gold standard method for the quantification of artificially created white spot lesions' mineral loss or gain and the preferred method for single in vitro measurement (ten Bosch and Angmar-Mansson, 1991). Since the development of a computer-aided video-image analysis feature, it has been utilised in the detection of small changes in mineral density over time (Damen et al., 1997). It involves the preparation of 80 and 150 µm plano-parallel sections of enamel and dentine specimens prior to obtaining radiographic images of the samples. The x-rays absorbed from the samples are compared to a simultaneously exposed standard (aluminum stepwedge). The lesion depth and mineral distribution profile are calculated to produce a parameter known as ΔZ which is the lesion mineral content (ten Bosch and

Angmar-Mansson, 1991). TMR is generally a very technique sensitive, laborious, and expensive method.

PLM requires more sample manipulation and is more difficult than TMR (Lo et al., 2010) but has been described to detect much smaller lesions than TMR (Huysmans and Longbottom, 2004). It involves preparing thin enamel sections prior to imbibing the sections in different mediums and examining them under a polarised microscope integrated with a high-resolution digital camera. The limited data provided on the tissues's mineral content which does not provide additional information to the detection of lesion depth (Wefel et al., 1985). PLM is usually used to assist TMR in the assessment of outer surfaces of lesions but it could also be used on its own to provide precise measurements if lesion depth is the main outcome measure as it could be difficult to outline a lesion's outer surface with TMR (ten Bosch and Angmar-Mansson, 1991).

1.6.2 Quantitative light-induced fluorescence (QLF)

With greater understanding of the dental caries disease process, fortunately, there have been great advancements in the comprehension of the methods that can be carried out to detect and measure the early stages of this disease (Pretty and Ellwood, 2013).

Quantitative light-induced fluorescence is a visible light system that allows the detection of early dental caries and then longitudinally monitors their progression or regression. Fluorescence is a phenomenon in which an object is excited by a specific light wavelength, and the fluorescent (i.e. reflected) light is of a larger wavelength. The fluorescence will be of a different colour when the

excitation light is in the visible spectrum which is detected using a high bandpass filter set at >540nm (Pretty, 2006).

Benedict first described enamel auto-fluorescence in 1928 and proposed its use in the detection of dental caries (Benedict, 1929). There is a reduction in enamel auto-fluorescence when enamel is demineralised. It was mentioned by de Josselin de Jong et al. in 1990 that the observed differences between sound and carious enamel fluorescence can be clarified by the different amounts of scattered and absorbed light. As dental enamel demineralises, it becomes porous, and the saliva then fills the pores resulting in a reduced light path in enamel (Tranæus et al., 2001).

When an area of demineralised enamel is present, fluorescence is reduced in two ways. Firstly, the demineralised area blocks the excitation light; secondly, the fluorescence from the dentine is back scattered as it tries to pass through the lesion. This loss in fluorescence can be quantified using proprietary software and is proven to relate well with actual mineral loss (Pretty and Ellwood, 2013).

QLF is a novel device in the analysis of early-demineralised enamel lesions. Kühnisch and Heinrich-Weltzien (2004) mentioned that QLF is a valuable device. They reported that it was non-destructive and useful for the early detection, quantification and longitudinal monitoring of early, non-cavitated carious lesions (Yin et al., 2007).

The QLF machine involves a light source with a band pass filter that produces a blue light at a wavelength of 370 nm attached to a camera containing another pass filter to decline the blue light and allow the passage of both green and red lights. The images obtained by the camera are displayed on the computer containing the software. Once the images of the teeth have been

captured, analysis of the lesion is then carried out to produce a quantitative evaluation of the demineralisation degree of the tooth. The analysis is done using the proprietary software where an area of sound enamel is defined around the lesion of interest, the software then utilises the pixel values of the sound enamel to recreate the surface of the tooth and then subtracts those pixels that are deemed to be the lesion. The software also allows for images taken on subsequent visits to be superimposed as precisely as possible in order to monitor progression or regression of lesions. This process is controlled by a limit of fluorescence loss which is set at 5%. Meaning that if pixels had a fluorescence loss of more than 5% of the average sound value, they are regarded as part of the lesion. After that, the software calculates the average fluorescence loss of the lesion (ΔF) in %, and the overall Area of the lesion in mm^2 . A third parameter is then calculated from ΔF and Area which represents the volume (size) of the lesion and is represented as ΔQ (Pretty and Ellwood, 2013).

The QLF method is a sensitive clinical procedure, able to detect occlusal caries, smooth surface caries and demineralisation adjacent to orthodontic brackets. It also detects more demineralised lesions than what is possible to detect by visual examination (Stookey, 2005). The sensitivity has been reported at 0.68 with a specificity of 0.70 for occlusal lesions, which means that there is a 68% chance that QLF will identify occlusal lesions as positive and a 70% chance that it will also identify caries-free occlusal surface as negative. Regarding correlations of the lesion depth and QLF metrics, it was reported to be up to 0.82 with substantial reliability for both the image capturing and analysis stages of the QLF process (Pretty and Ellwood, 2013). An earlier study by Ferreira Zandona et al. comparing different incipient caries detection methods,

reported that QLF is able to detect lesions with 5% mineral loss. The sensitivity and specificity levels were at 95.8% and 11% respectively, showing that QLF cannot distinguish between demineralised areas or developmental defects as they are both deficient in minerals resulting in high false-positive readings. However, the authors advised that combining QLF with a clinical examination method would help confirm the type of lesion, leading to increased specificity to 90.9% and decreased sensitivity to 49.9% (Ferreira Zandona et al., 2004; Stookey, 2005). A systematic review by Gomez et al. (2013) noted that a higher sensitivity value leading to higher true positive results in detection which is important when the intervention is mainly preventative.

This system has been validated when compared with transverse microradiography (TMR), showing acceptable correlation between QLF and TMR (Al-Khateeb et al., 1997) and thus indicating the validity of the QLF as a measure of mineral content (Higham et al., 2005). It has been shown to be reliable, accurate and reproducible in caries detection and monitoring. Repeatability and reproducibility have been tested clinically by Tranæus et al. where three examiners took 15 images of buccal white spot lesions on mandibular first permanent molars in 15 teenage patients and another single examiner analysed the images. They concluded that there were no significant differences between the examiners during the image capturing stage, with an inter-examiner reliability of 0.95 to 0.98 and intra-examiner reliability of 0.93 to 0.99 highlighting the excellent repeatability and reproducibility of the QLF method (Tranæus et al., 2002).

Studies have explained that QLF is able to detect demineralisation that resulted from an eight-hour exposure to a decalcification solution, and also

longitudinally follow the progression of the lesion in the same tooth (Ando et al., 1997).

The presence of confounding factors can affect the reliability of measurements by compromising the specificity (Gomez, 2015), care should be taken to work under standardised conditions to ensure valid results. In the case of in vitro studies, enamel and dentine thickness are the only confounding factors and it is of importance to ensure standardisation of thickness of enamel within the same group when carrying out in vitro fluorescence studies examining enamel lesion severity (Ando et al., 2003). Studies have suggested that by decreasing the thickness of enamel, more intense fluorescence can be noticed due to the reduced scattering of light (Pretty and Ellwood, 2013).

It is generally thought that the source of fluorescence is the dentine as well as the dentine-enamel junction. Literature has shown that if the underlying dentine is removed from the enamel, fluorescence is no longer present. However, it had been noted that only a small part of dentine is required for the fluorescence to be present (Pretty and Ellwood, 2013) as the dentine layer affects light scattering and the tooth's absorption properties (Gomez et al., 2014). Ambient light can also impact the QLF results. It was reported that a light level of 88 lux could be used without considerably affecting recorded values (Pretty et al., 2006).

1.6.3 Microhardness test

This method involves measuring the resistance of the enamel surface to indenter penetration, providing information on the amount of subsurface mineral

loss or gain by means of the porosity degree of the superficial enamel layer (Koulourides, 1971).

It initially requires the preparation of tooth tissue samples with flat polished surfaces. The samples are then subjected to a Knoop or Vickers diamond with a specific load for a certain duration to produce indentations on the flat surfaces. The length of these indentations is measured in μm representing the microhardness (Angmar-Mansson and ten Bosch, 1991).

Microhardness is sensitive to mineral density changes (Featherstone and Zero, 1992a) but the results that are obtained from the indentations represent only one surface of the sample and do not essentially provide information regarding the microhardness of all the other sites of that sample as microhardness value differs from one site to another (Caldwell et al., 1957).

The microhardness testing method can be divided into two types:

- 1) Surface microhardness (SMH): when used in caries related studies, it can only provide qualitative information on tooth tissue mineral changes while ensuring that the prepared enamel samples comprise flat surfaces to maintain measurement accuracy (Arends and ten Bosch, 1992). Despite that, it is still considered a sensitive assessment method for shallow lesions (depth $<50\ \mu\text{m}$), easy, consistent, and an economically acceptable testing method (Zero et al., 1990; Featherstone, 1992).
- 2) Cross-sectional microhardness (CSMH): Although this type is more technically complex, it has an advantage over SMH where it is able to assess the mineral changes quantitatively across the depth of enamel slab sub-surface lesion (Arends and ten Bosch, 1992).

Based on the what was previously mentioned, there is still a need for more fluoridated milk studies with in vitro demineralisation/ remineralisation models and pH-cycling designs to be carried out in order to investigate the best fluoride concentration in milk that would result in the highest reduction in early enamel carious lesions while minimising any possible adverse effects. There is also a lack of evidence identifying what the appropriate duration of administration of fluoridated milk would be in order to acquire the most benefits. School milk fluoridation schemes running in the UK are provided by 10 authorities including Leeds. There are around 1320 children in Leeds attending both primary schools and nurseries where 0.8 mg NaF is being added to bovine milk (189 ml) and consumed by children once a day during school days only (Leeds City Council, 2013). Both reviews provided by Banoczy et al. and the World Health Organization described that most milk fluoridation schemes generally offered the milk around 180-200 days/ year, assuming that children had a 100% school attendance (Banoczy et al., 2009; Banoczy et al., 2013; Yueng et al., 2015). Other international programmes, In Chile, distribute the fluoridated milk throughout the year to include all holidays and weekends (Marino et al., 2001). There is an obvious inconsistency in the number of days milk is being consumed by children and it would be interesting to attempt to investigate this point further in this in vitro designed study.

We have therefore proposed this 60 day in vitro randomised single blinded study design to investigate these concerns further in view of providing adequate quantitative data to supply future studies with enough confidence to design and carry out clinical trials appropriately.

2.0 AIM, OBJECTIVE & NULL HYPOTHESIS

2.1 Aim

To investigate the remineralisation and/or demineralisation of artificially created white spot lesions (WSL) on bovine enamel following a daily or intermittent exposure to 0.5 mg F or 1.0 mg F added to bovine milk using a pH-cycling in vitro model and whether the higher dose would result in a better preventive effect.

2.2 Objective

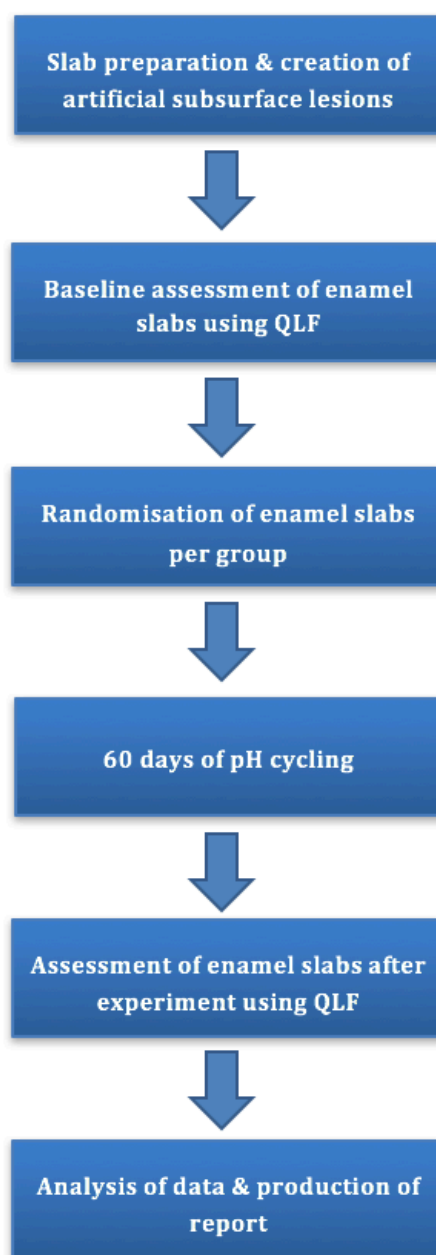
To investigate the dose response of two concentrations of fluoride added to milk (0.5 mg and 1.0 mg) on bovine enamel remineralisation and demineralisation under pH cycling conditions undergoing a daily or intermittent frequency of exposure by using quantitative light induced fluorescence (QLF) as the method of analysis.

2.3 Null hypothesis

There is no difference in the caries preventive effect on bovine enamel of increasing the fluoride dose from 0.5 mg F to 1.0 mg F in milk for both the daily or intermittent frequencies of exposure.

3.0 MATERIALS AND METHODS

This section will describe the methodology implemented in this in vitro study design including preparation of enamel samples, pH cycling regime, materials and equipment utilised to investigate the remineralising effect of fluoridated milk on bovine enamel subsurface caries-like lesions. This process is summarised in the following flow chart:



3.1 Sample size calculation

Statistical support was sought for the determination of the sample size required for this study. The sample size was based on data from a previous PhD thesis by Malinowski in 2010 entitled 'In situ studies with fluoridated milk for the remineralisation of dental enamel' with a similar pH cycling protocol and fluoride concentrations added to milk. The sample size was calculated by applying the data in an online sample size calculator website (Appendix 1), considering a significant level of 0.05 and 80% of power. The results suggested that at least 24 slabs were needed per group in order to find a significant difference between the control and treatment groups, yielding a minimum total of 144 enamel slabs.

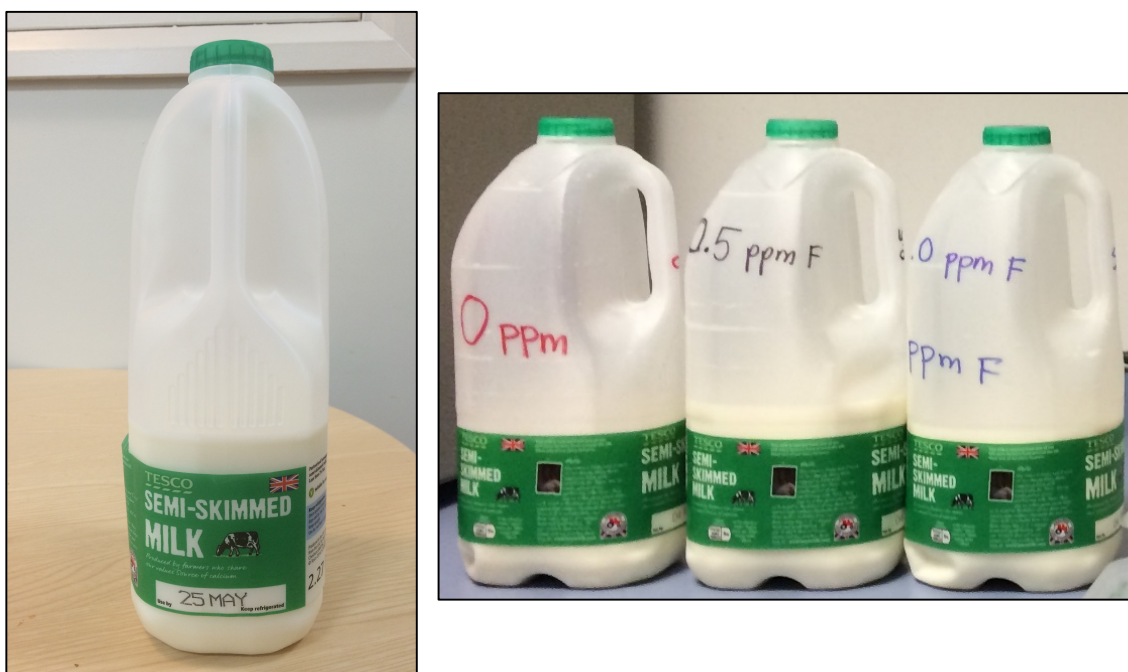
3.2 Randomisation and blindness

Random allocation of the slabs to the 6 experimental groups was performed using an online random number generator by creating a table of random numbers (Appendix 2). The study was designed as single blinded where the examiner was unaware of the group to which each enamel slab was assigned to during the QLF analysis stage.

3.3 Experimental materials (test and control products): (Figure 3-1)

- Milk (Tesco; cow's fresh semi-skimmed pasteurised) as the control
- Milk (Tesco; cow's fresh semi-skimmed pasteurised) + 0.5 ppm NaF (0.5 mgF/L)
- Milk (Tesco; cow's fresh semi-skimmed pasteurised) + 1.0 ppm NaF (1.0 mgF/L)

Figure 3-1: Test and control milks used



3.4 The study groups (Explained in detail in section 6.0)

Interrupted frequency:

- **Group 1:** 0.0 mg F was used to dip the enamel slabs on specific days throughout the 60-day period
- **Group 2:** 0.5 mg F was used to dip the enamel slabs on specific days throughout the 60-day period
- **Group 3:** 1.0 mg F was used to dip the enamel slabs on specific days throughout the 60-day period

Daily frequency

- **Group 4:** 0.0 mg F was used to dip the enamel every day throughout the 60-day period

- **Group 5:** 0.5 mg F was used to dip the enamel slabs every day throughout the 60-day period
- **Group 6:** 1.0 mg F was used to dip the enamel slabs every day throughout the 60-day period

3.5 Teeth selection and cleaning

Bovine dental enamel was obtained from a local abattoir in Bradford. Bovine teeth were extracted, collected and stored in distilled water and 0.1% thymol (Sigma Aldrich). This storage solution was based on the local protocol at the Leeds Dental Institute, and it had also been reported by Moura et al. that type of storage medium can have an effect on the way enamel reacts to a cariogenic challenge where a formaldehyde storage solution may reduce the enamel demineralisation caused by a pH cycling process compared to Thymol (Moura et al., 2004). The teeth were cleaned using a spoon excavator and a toothbrush with pumice powder and stone to remove any residual soft tissue. Prior to sectioning, the teeth were inspected using transillumination and transmitted light by low-power microscopy (Leitz, Wetzlar®, Germany) and teeth exhibiting caries, malformations or cracks were eliminated.

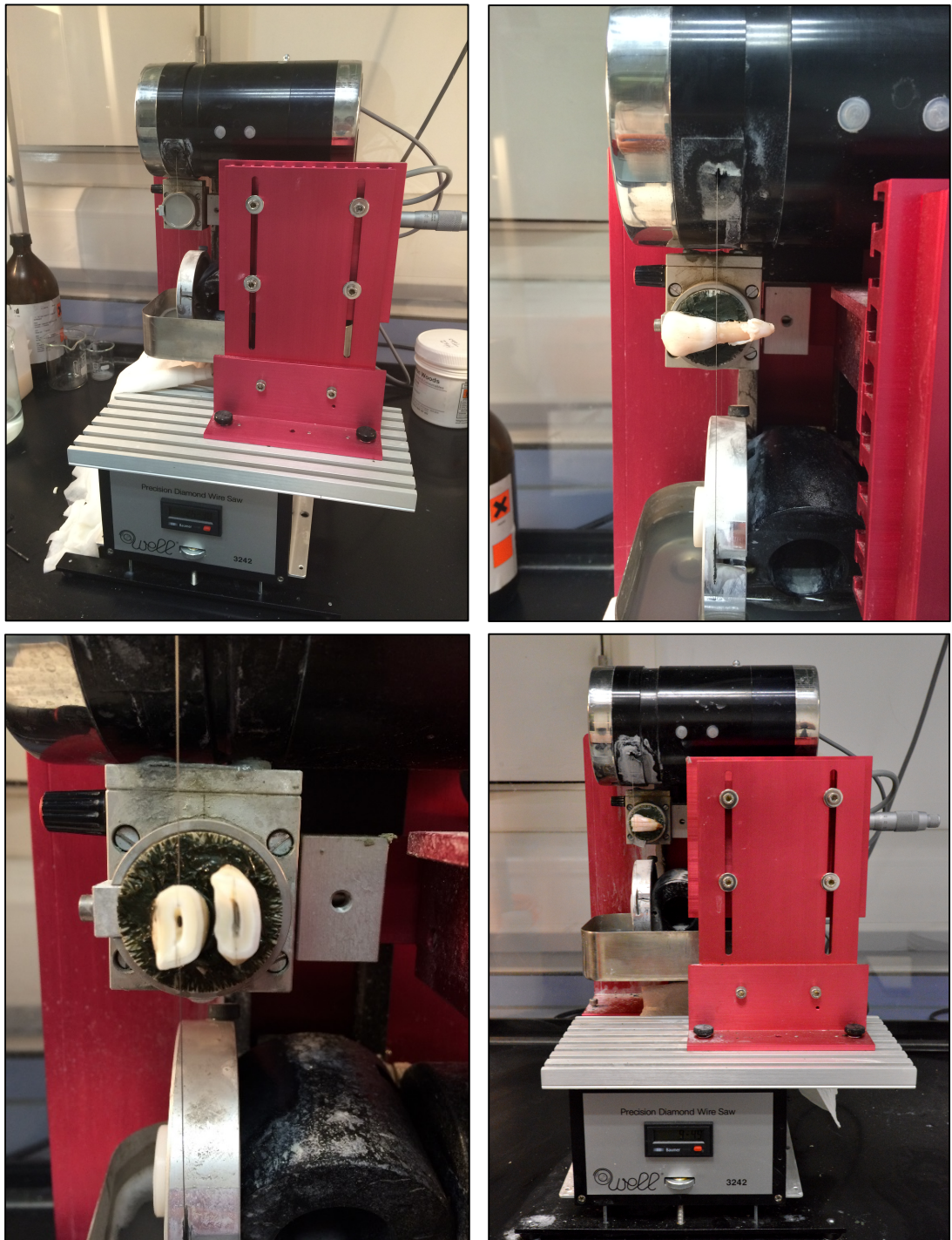
3.6 Enamel slab preparation for testing with QLF & creation of white spot lesions

Fine abrasive paper (P 1000 Wet and Dry paper, 3M) was used to lightly abrade the selected teeth to help remove the outermost enamel layer (100-200 μ m), attain a degree of flatness and achieve a level of standardisation since

the teeth might have accumulated some fluoride on the outer enamel layer from the buccal surface as the previous fluoride history of these teeth cannot be known.

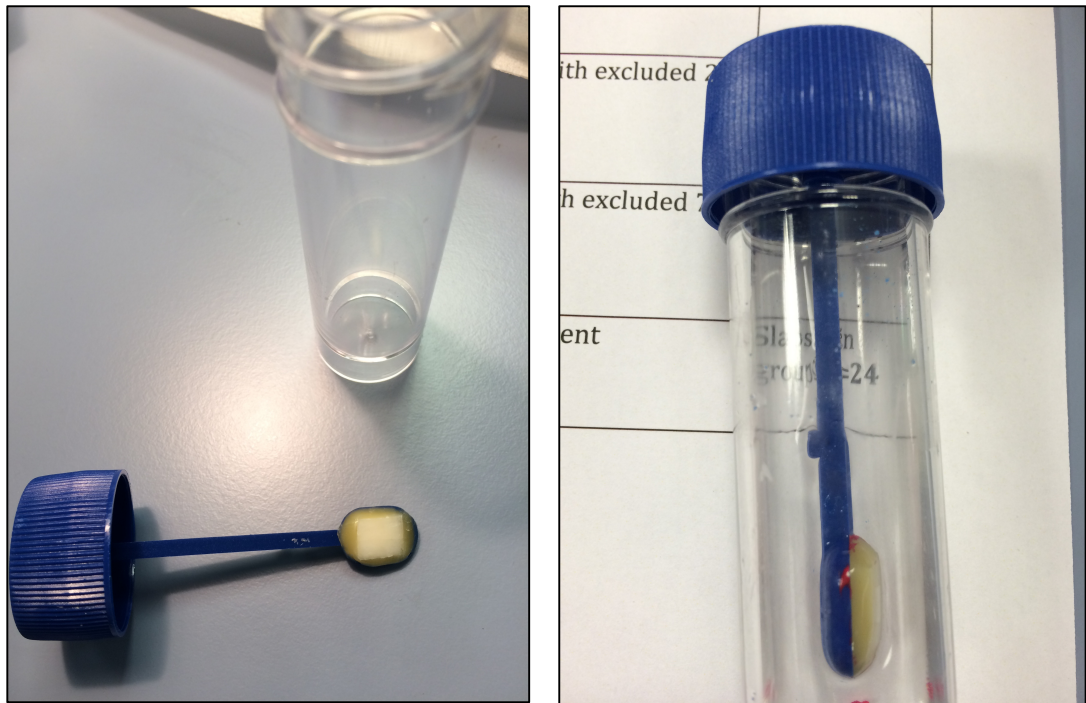
In order to obtain the enamel slab sections, each tooth was mounted to the cutting machine ceramic plate using green wax impression compound stick (Kerr, UK). Sectioning was performed using a water-cooled, Well Diamond Wire Saw cutting machine (Well® Walter EBNER, CH-2400 Le Loche) where only the buccal surface was utilised to create slabs measuring approximately 6x 5x 3 mms (Figure 3-2).

Figure 3-2: Well Diamond Wire Saw Cutting machine (Well® Walter EBNER, CH-2400 Le Loche)



In order to create artificial white spot lesions, each slab was then secured on a plastic rod attached to the lid of a universal type tube (Sterilin®, UK) using yellow dental “sticky wax” in order to hold the slabs while being immersed in the demineralising gel. This tube helped ensure that the slabs were suspended in the centre with free surrounding space (Figure 3-3).

Figure 3-3: Enamel slabs attached to universal (Sterilin®, UK) type tube using yellow dental wax “sticky wax”



The enamel slabs were then painted with two coats of an acid resistant nail varnish (Max Factor “Infinity”), except for a window of exposed enamel measuring approximately 4mm x 3mm in the centre of the buccal surface of each slab for the artificial carious lesion. The initial nail varnish coating was left to dry completely for at least 24 hours prior to applying the second coat (Figure 3-4).

On completion, the slabs were left in the plastic containers filled with distilled water in order to avoid dehydration and this process was also repeated at the end of the experiment.

Figure 3-4: Application of nail varnish coating with exposed enamel window



The demineralising gel mixture was then prepared by mixing 0.1 M lactic acid (Sigma Aldrich D/L GPR 87% Lactic acid) with 0.1 M sodium hydroxide (BDH Analar Grade) in proportion to give a pH value of 4.5. After that, 6% w/v hydroxyethyl cellulose (Sigma Aldrich) was added to the mixture whilst stirring, until a consistency which resembles “wallpaper paste” was achieved (Figure 3-5). The mixture was left to settle for 24 hours until it was ready to be used. The gel was then poured into the Universal “Sterilin” tubes into which the enamel slabs were submerged. The slabs were kept in the demineralising gel for 10

days at room temperature to create a clearly visible artificial white spot lesion (Figure 3-6). On the 11th day, the slabs were removed from the gel and rinsed with distilled water. Since the nail varnish was still moist, it was easily taken off using a Hollenbeck wax carver without scratching the created lesions or surrounding sound enamel. The other option for removing the nail varnish would have been to use methanol to rub it off, however, there was a risk of smearing the methanol over the newly created lesions. The slabs were then ready for the baseline QLF measurements.

Figure 3-5: Preparation of the demineralising gel mixture

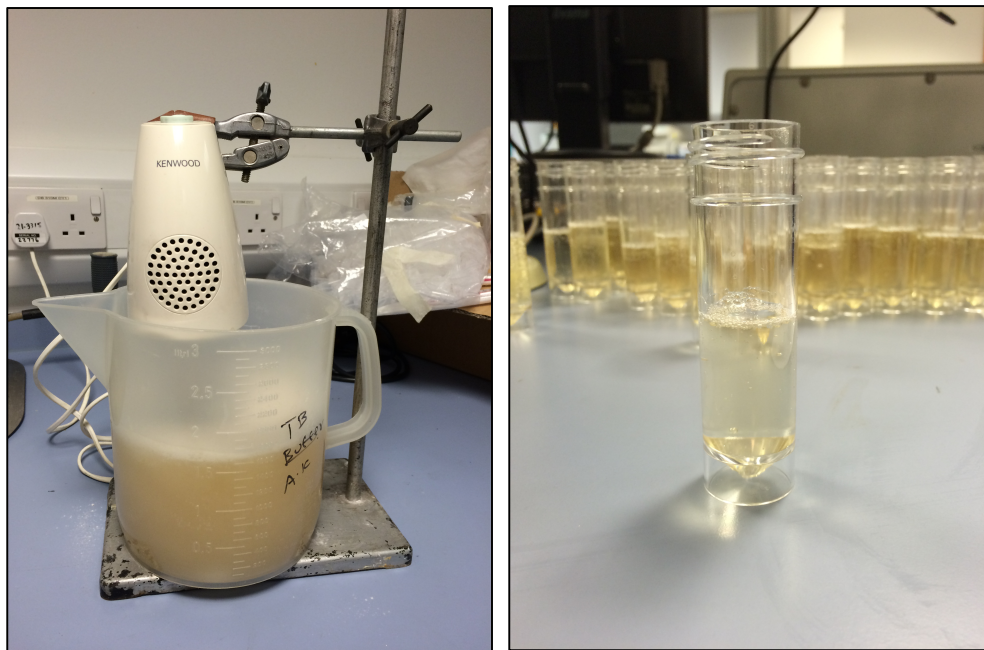
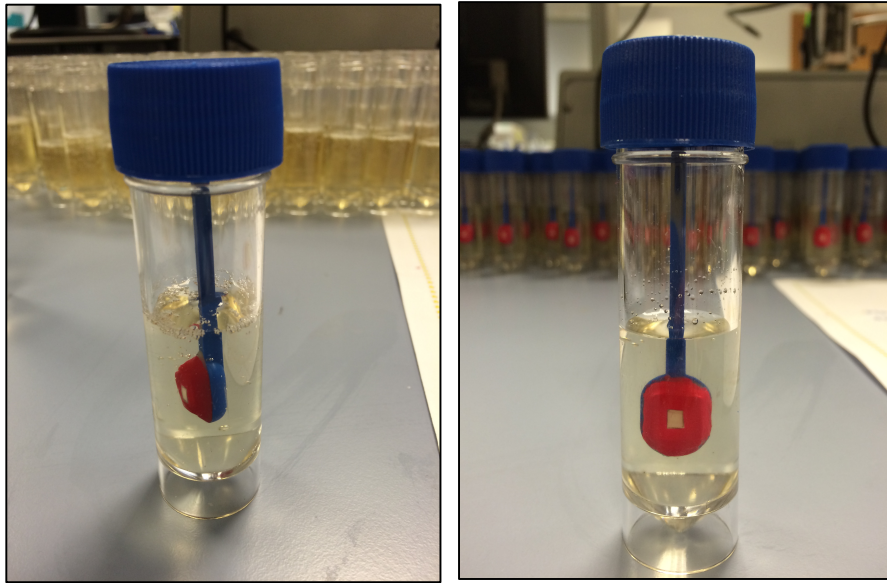


Figure 3-6: Enamel slabs submerged in the demineralising gel



3.7 Quantitative light-induced fluorescence (QLF) analysis

Baseline measurements were obtained using the QLF machine (QLF-D Biluminator™ 2 - Inspektor Research Systems B.V., Amsterdam, The Netherlands) after the creation of enamel subsurface lesions and each slab was also re-analysed at the end of the 60-day period of the experiment under standard conditions (Figure 3-7). Prior to obtaining the images and as the slabs were stored in moist conditions, they were initially dried with compressed air for 15 seconds. The process of photo capturing and QLF analysis were carried out in a darkened room (Figure 3-8).

Figure 3-7: QLF machine. SLR camera mounted on the stand with a fixed distance from the enamel slab

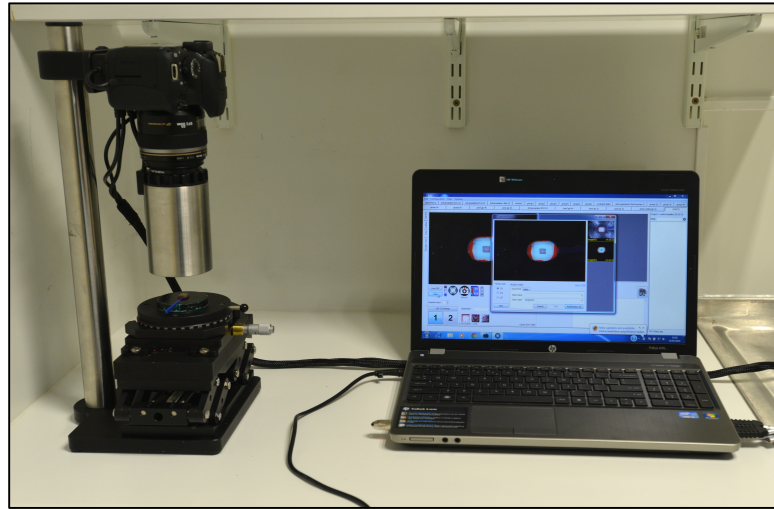
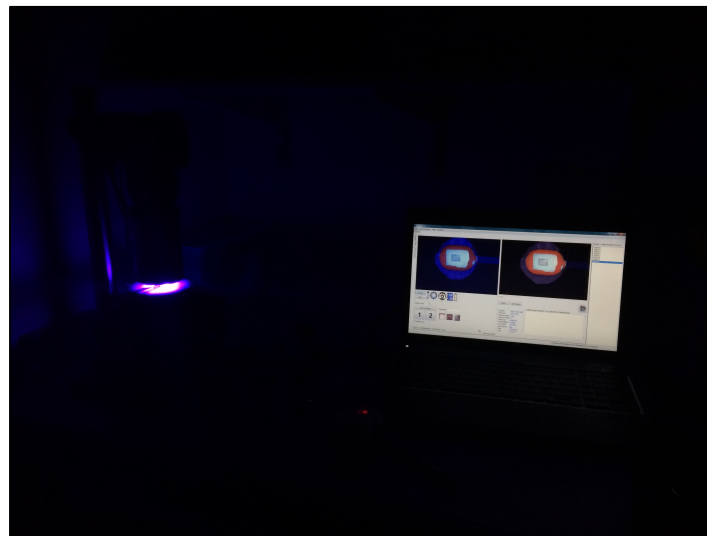


Figure 3-8: Darkened room setting



The QLF system comprised of a BiluminatorTM apparatus attached to a Single Lens Reflex (SLR) camera equipped with a 60mm macro lens. The light sources and filters producing white-light, blue-light and QLFTM images are produced by the BiluminatorTM. The camera used to capture all the fluorescence images of the enamel slabs was a digital full-sensor SLR Canon (Figure 3-9)

camera (model 550D, Canon, Tokyo, Japan) while enabling the “Live- view” feature.

Figure 3-9: Canon camera used with attached lens and Biluminator™ apparatus

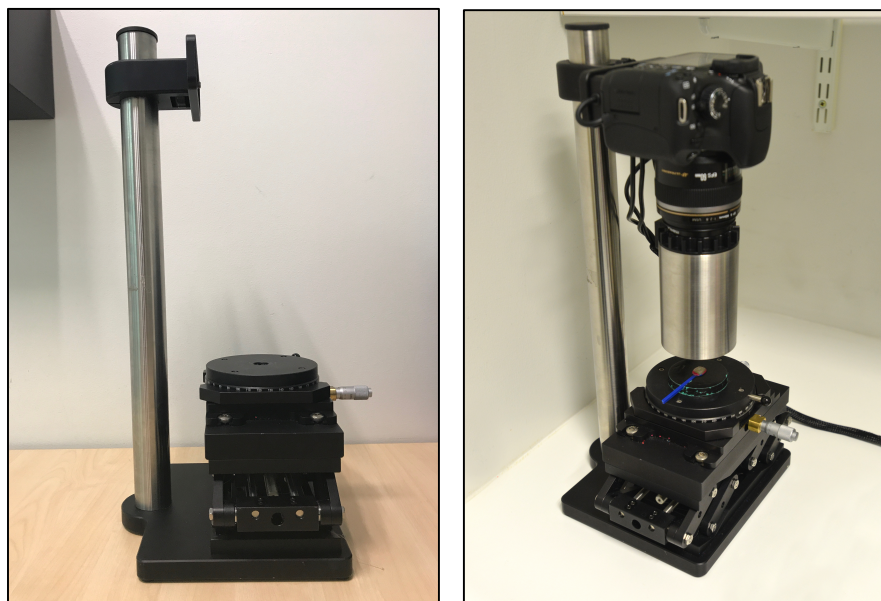


The images were taken while the camera system was set at a shutter speed of 1/30, aperture value of white-light at 18, aperture value of blue-light at 6.7, and an ISO speed of 1600. As the images were captured, they were automatically saved on a laptop computer (HP ProBook 4530s) installed with an image-capturing software (C3 version 1.24.0.0; ©2011-2013 Inspektor Research System). The laptop also included the analysis software (QA2 version 1.24.0.0; Inspektor Research Systems B.V., Amsterdam, The Netherlands) where the single trained examiner studied and analysed the captured fluorescence images.

The distance between the enamel slabs and the camera was fixed throughout the analysis to ensure the ability to repeat the measurements consistently from the same angle and position. The camera was attached to a stand in a position that ensured optimum illumination of the enamel slab surface. The camera stand

also consisted of a jack with a flat platform which was maintained at the same level throughout the experiment (Figure 3-10) therefore controlling the stability of the enamel slabs, image magnification, and light intensity.

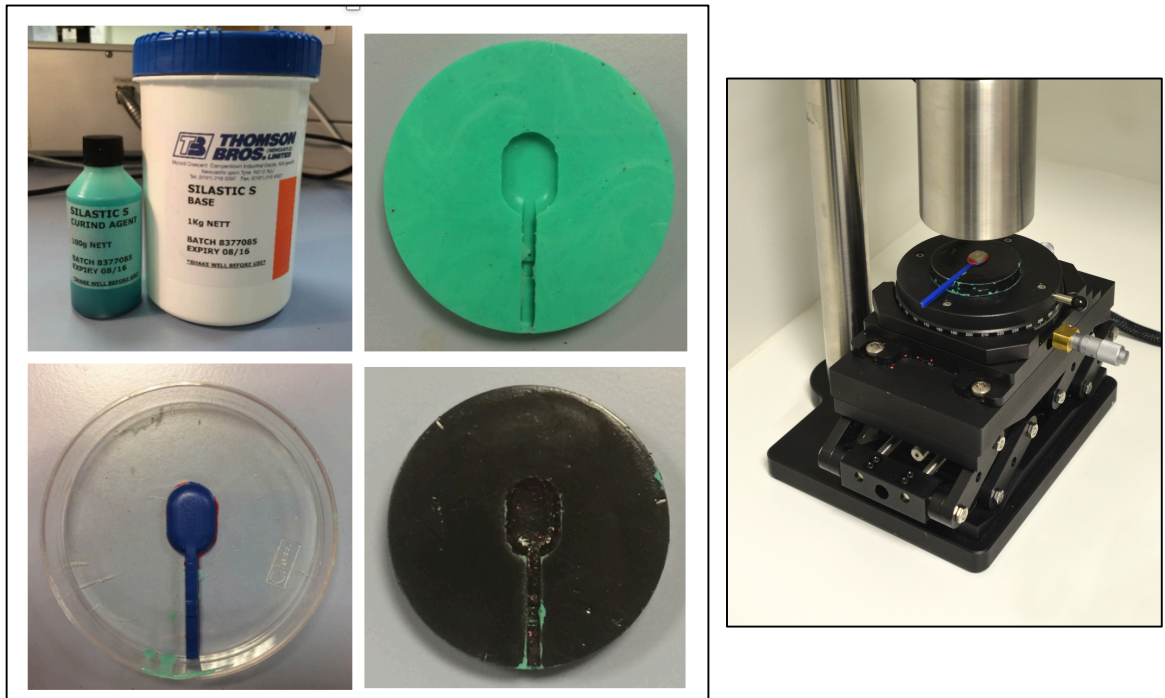
Figure 3-10: Camera stand and jack set at a standardised position



As an additional measure, a silicone base was created to guarantee that the slabs on the camera jack's platform were secured in the same exact place under the camera lens throughout the analysis. This silicone mould was made by mixing the silicone rubber base with a green curing catalyst agent (Dow Corning Silastic® S RTV; Thompson Bros. Ltd; Newcastle-upon-Tyne, UK.) in a 9:1 ratio, pouring it into a petri dish with a plastic spoon handle stabilised to ensure creation of space for later placement of the enamel slabs with their holders during the image capturing process (Figure 3-11). Once the silicone mould had set, it was then painted black to avoid any effect the fluorescent green

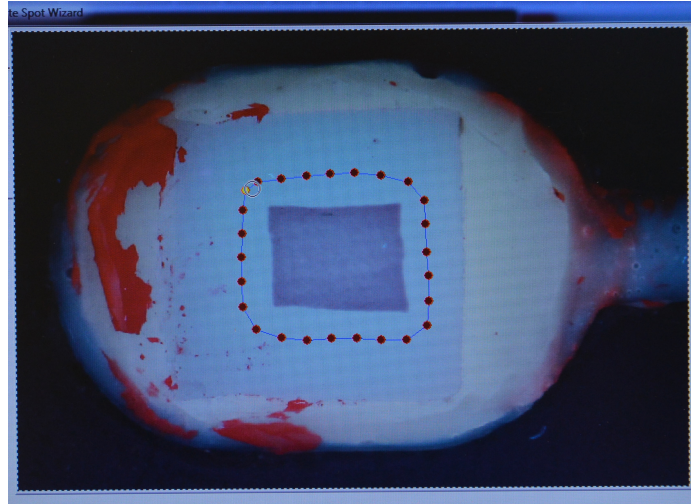
colour might have on image capturing (Wilko, black spray paint with matte finish).

Figure 3-11: Creation of silicone mould



The QLF analysis process was carried out by the examiner, where a patch was drawn to encircle the white spot lesion while ensuring that the borders remained on sound enamel (Figure 3-12). The system used the fluorescence radiance of the surrounding sound enamel to reconstruct the fluorescence level of the white spot lesion to calculate the percentage difference. The QLF system enables the replication of images on subsequent visits by its video re-positioning system, this helped ensure that the same area of interest was used for both baseline and final analysis.

Figure 3-12: QLF image (with blue-light) during analysis, shows the darkened demineralised lesion surrounded by sound enamel. The image also shows the patch encircling the lesion



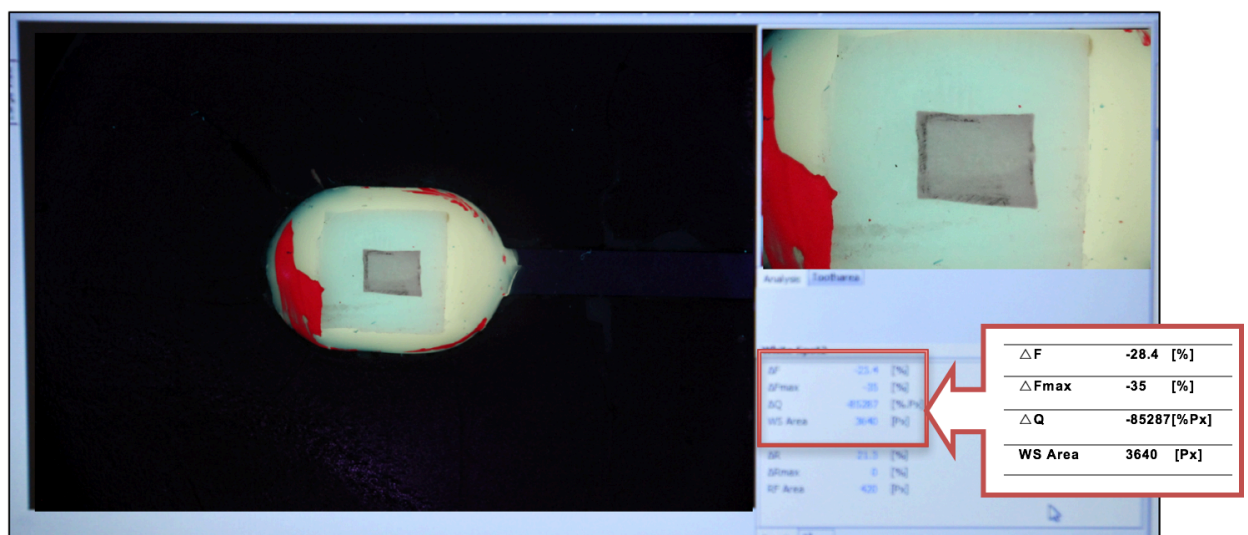
3.8 Data handling

As previously explained, since the demineralised lesion appears darker than sound enamel, its fluorescence appeared lower than that of sound enamel when analysed by the QLF system. To calculate the loss of fluorescence, the fluorescent radiance in the lesion site was reconstructed by interposition from the radiance of sound enamel surrounding the lesion. When the lesions fluorescence radiance levels are less than 95% of the reconstructed sound fluorescence radiance, it is then considered to be an artificial early carious lesion displayed in shades of grey where the darker shades correspond to higher levels of fluorescence loss. Therefore, the fluorescence loss in the lesion is the result of difference between the measured and reconstructed values.

The QLF parameters obtained during the analysis are mainly the following three metrics (Figure 3-13):

- 1) ΔF is the percentage loss of fluorescence pertaining to the fluorescence of sound tooth tissue and is related to lesion depth [%]
- 2) ΔQ is the percentage fluorescence loss pertaining to the fluorescence of sound tissue times the Area ($\Delta F \times \text{Area}$) and is related to the lesion volume [%px²]
- 3) Lesion Area is the lesion's surface area conveyed in pixels [px²]

Figure 3-13: An example of the analysis software displaying QLF parameter values (Image captured with blue-light feature)



3.9 Included artificial lesions depending on the ΔF ranges

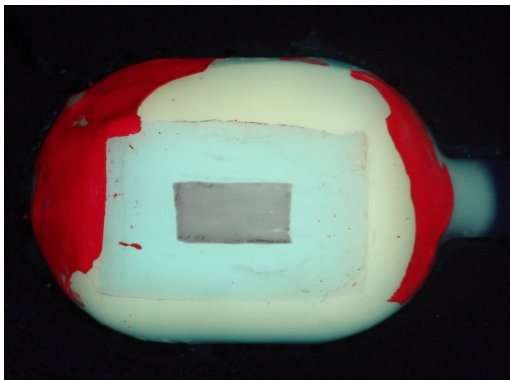
On completion of all baseline QLF analyses, the artificial white spot lesions created included an average ΔF of approximately 30.4 +/-5 (range 25-35). This was done by calculating the average number for all the ΔF values measured (30.4) and the standard deviation (+/-5) on Microsoft® Excel version 15.33. However, that range would only involve a total of 127 slabs which is below the

required sample size for our study. It was therefore decided to expand the average ΔF range to 22-37, which included a total of 151 slabs.

The following images are examples of enamel slabs from the daily frequency groups analysed under the QLF blue-light showing demineralised enamel lesions at baseline and after completion of the 60-day pH cycling regime for each group (Figures 3-14, 3-15, 3-16). The interrupted frequency group slabs also display a similar appearance.

Figure 3-14: Showing an enamel slab from group 4 (0.0 ppm F; daily frequency)

Lesion before pH cycling



Lesion after pH cycling

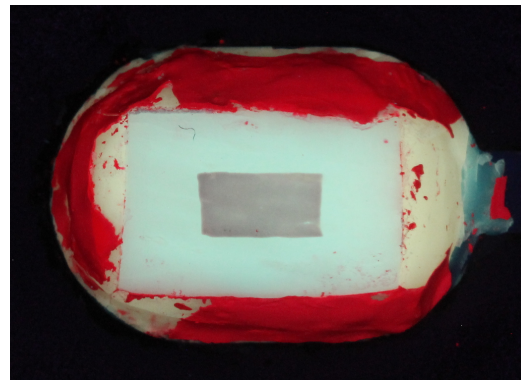
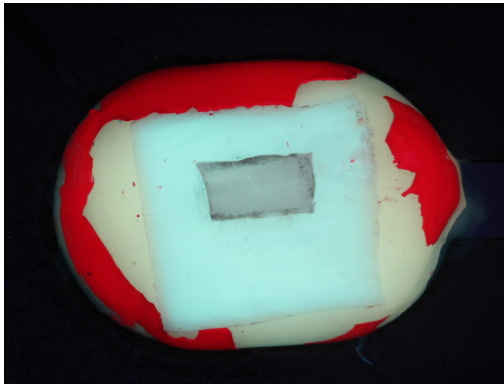


Figure 3-15: Showing an enamel from group 5 (0.5 ppm F; daily frequency)

Lesion before pH cycling



Lesion after pH cycling

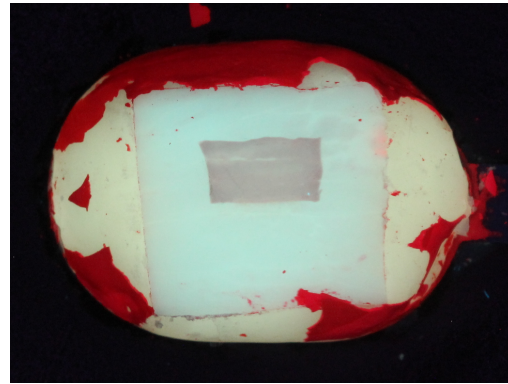
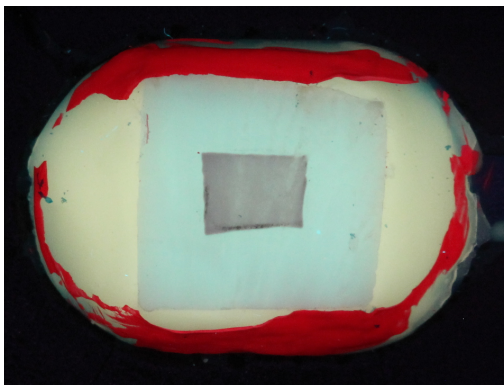
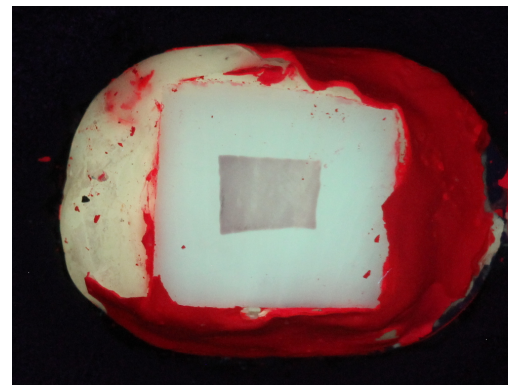


Figure 3-16: Showing an enamel slab from group 6 (1.0 ppm F; daily frequency)

Lesion before pH cycling



Lesion after pH cycling



3.10 Training and calibration

Training on using QLF machine was provided to the investigator prior to initiating the study. The supplier in Amsterdam was contacted for advice on training and in cases where difficulties were encountered, an explanation was provided. The training included the process of image capturing as well as

analysing the images. The assessment of sound and demineralised enamel as well the lesion border was calibrated by the investigator during image analysis. The QLF softwares for both image capturing and analysis include PDF instruction files with an easily followed step by step explanation on setting up the system as well as how the process works.

3.11 Intra-examiner reproducibility

This was evaluated using the intra-class correlation coefficient (ICC) and the Bland-Altman plot where 15 % of the enamel slabs were randomly selected by the investigator and retested with the QLF system at the beginning of the experiment.

3.12 Study groups

Two concentrations of fluoride were used in the study groups: 0.5 mg F and 1.0 mg F. While non-fluoridated milk was used for the control groups. Those concentrations were divided into the 6 groups according to each of the interrupted and non- interrupted frequencies.

Group 1: The enamel slabs were dipped into 0.0 mg F (0.0 ppm F) milk, for 60 days in an interrupted frequency manner.

Group 2: The enamel slabs were dipped into 0.5 mg F (0.5 ppm F) milk, for 60 days in an interrupted frequency manner.

Group 3: The enamel slabs were dipped into 1.0 mg F (1.0 ppm F) milk, for 60 days in an interrupted frequency manner.

Group 4: The enamel slabs were dipped into 0.0 mg F (0.0 ppm F) milk, for 60 days in a daily frequency manner.

Group 5: The enamel slabs were dipped into 0.5 mg F (0.5 ppm F) milk, for 60 days in a daily frequency manner.

Group 6: The enamel slabs were dipped into 1.0 mg F (1.0 ppm F) milk, for 60 days in a daily frequency manner.

Dipping the enamel slabs in fluoridated milk with different concentrations was meant to resemble the consumption of milk (200ml) by children at school under cariogenic conditions either with an interrupted or daily frequency of consumption. The 60-day period of this experiment was chosen in an attempt to represent one whole year (i.e. 365 days). This seemed as an appropriate duration to investigate the amount of remineralisation and/or demineralisation of bovine enamel and pick up any differences using the QLF system in this in vitro study. It would have been interesting to carry out such a study for a whole year, however that is not possible due to the clinical obligations of the investigator. So, a duration of 60 days was agreed on which is still longer than what has been carried out in previous in-vitro studies as explained later on in the discussion.

3.13 Frequencies used (Daily and Intermittent)

3.13.1 Daily frequency of consumption during 60 days.

Three groups of enamel slabs were dipped in 0.0 mg F, 0.5 mg F or 1.0 mg F milk once per day every day, at the same time of day for the duration of 60 days.

60-day period:

MTWTFSS MTWTFSS MTWTFSS MTWTFSS

MTWTFSS MTWTFSS MTWTFSS MTWTFSS

MTWT

60-day period represented one year i.e. 365 days.

MTWTFSS - represented each day of the week where the enamel slabs were dipped in fluoridated milk.

3.12.2 Interrupted frequency of consumption during 60 days.

The other remaining 3 groups of enamel slabs were dipped in 0.0 mg F, 0.5 mg F or 1.0 mg F milk once per day, at the same time of day on marked days during the 60-day period. The continuous application of milk took place for 60 days. However, for the interrupted frequency, the following were days on which there was no milk exposure (Weekends (17 days), Half term (3 days), Christmas (2 days), Easter (2 days) and Summer holiday (5 days)).

The designated days were marked on a calendar according to the following calculations made, Figure 3-17 illustrates an example.

60-days period:

M T W **T** F **S** **S** M T W T **F** **S** **S** M T **W** T **F** **S** **S** M T W **T** F **S** **S**
M T W T F **S** **S** M T **W** T **F** **S** **S** M T W **T** F **S** **S** M T **W** T **F** **S** **S**
M **T** **W** T

60 days period represents one school year i.e. 365 days

M T W T F S S – Letters that are not highlighted in colour represented each day of the week where the enamel slabs were dipped in fluoridated milk.

S – represented the total number of weekends in one year, which is 104 days during 365 days. The total number of weekends in this study per 60 days was 17 days.

Calculation:

$$365 - 104$$

$$60 - x$$

$$X = 60 \times 104 / 365 = 17 \text{ days}$$

T – represented the total number of half-term days in one year, which is 15 days (3 weeks, without weekends) during 365 days. The total number of half-term days in our study per 60 days is 3 days.

Calculation:

$$365 - 15$$

$$60 - x$$

$$X = 15 \cdot 60 / 365 = 3 \text{ days}$$

W T – represented the total number of days at Christmas holiday in one year, which is 10 days (2 weeks, without weekends) during 365 days. The total number of days at Christmas holiday in our study per 60 days is 2 days.

Calculation:

$$365 - 10$$

$$60 - x$$

$$X = 10 \cdot 60 / 365 = 2 \text{ days.}$$

W T – represented the total number of days at Easter holiday in one year, which is 10 days (2 weeks, without weekends) during 365 days. The total number of days at Easter holiday in our study per 60 days is 2 days.

Calculation:

$$365 - 10$$

$$60 - x$$

$$X = 10 \cdot 60 / 365 = 2 \text{ days.}$$

F S S M T – represented the total number of days on Summer holiday in one year, which is 30 days (6 weeks, without weekends) during 365 days. The total number of days on summer holiday in our study per 60 days is 5 days.

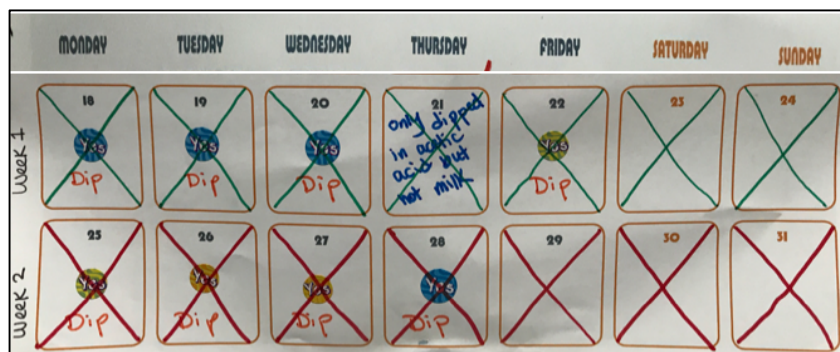
Calculation:

$$365 - 30$$

$$60 - x$$

$$X = 30 \cdot 60 / 365 = 5 \text{ days.}$$

Figure 3-17: An example of marking the calendar with labels to highlight which days the interrupted frequency groups should be dipped in milk



3.14 The pH cycling regime in the study

All enamel slabs within both the interrupted and daily frequency patterns had undergone the pH cycling regime (As per the Leeds Dental Institute Protocol which was based on the work of ten Cate and Duijsters 1982 and Malinowski et al., 2012b).

The preparation had involved labelling 6 plastic containers for each group, where the enamel slabs were dipped during each step throughout the pH cycling scheme in order to avoid contamination between dippings (Figure 3-18). The artificial saliva solutions (day time and night time), acetic acid solution, fluoridated milk and milk/saliva slurry had all been prepared before initiating the experiment and were changed on a daily basis each morning before the start of each cycle (the process of preparation of the solutions used will be explained in detail in the following section). The acetic acid and artificial day time saliva were also changed twice during the cycling process as a precaution.

Figure 3-18: Each group had separate labelled containers. Each step of the cycle required the use of a designated container to prevent contamination



The cycle started each day by dipping the enamel slabs with the attached plastic holders (rods), depending on their group and to which frequency they were allocated, in fluoridated milk (0.0 ppm F as the control and 0.5 ppm F and 1.0 ppm F as the test groups) first for 5 minutes followed by a 10-minute dipping in a bovine milk/ artificial day time saliva slurry (Figure 3-19). Although the groups that were assigned to the interrupted frequency pattern of dipping were not exposed to fluoridated milk or the milk/artificial day time saliva slurry on some days, they had still undergone the other steps of the cycle on those days as normal.

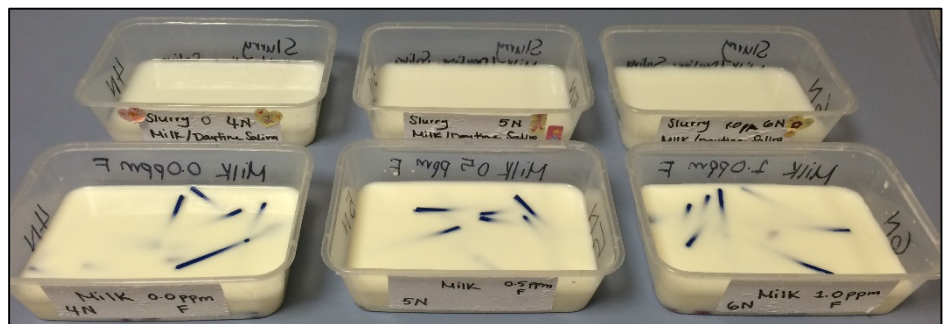
Figure 3-19: Dipping the slabs in fluoridated milk followed by the milk/saliva slurry

Interrupted and Daily frequency days



Timers

Daily frequency days only



After that, the enamel slabs were rinsed with distilled water for 1 minute and placed in the plastic containers filled with artificial day time saliva and left in an incubator for at least 60 minutes (Figure 3-20). The incubator was set at 37°C to resemble the normal human body temperature.

Figure 3-20: Day time saliva containers and incubator



Enamel slabs were then rinsed with distilled water for 1 minute before they were exposed to their first demineralisation challenge dipping of acetic acid solution (pH 4.8) which lasted for 2 minutes. Following the acetic acid exposure, the slabs were rinsed with distilled water for 1 minute, placed in artificial day time saliva, and kept in the incubator for at least 60 minutes. The slabs had undergone a total of 5 times of the same repeated process of acetic acid challenge exposure cycle (Figure 3-21). This process was carried out based on local Leeds Dental Institute protocol for pH-cycling which was an attempt to mimic what happens in the oral cavity when consuming meals with snacks during the day (demineralisation) with two episodes of fluoride exposure similar to two episodes of tooth brushing during the day. The locally agreed protocol was developed based on the research work done by Duggal et al., 2001 and Issa et al., 2003.

Figure 3-21: All groups undergoing the acetic acid challenge



Finally, on completion of the demineralising challenge, the slabs were placed in fluoridated milk again for 5 minutes followed by a 10-minute exposure to milk/artificial day time saliva slurry before rinsing them with distilled water and placing them in artificial night time saliva until the next day cycle (Figure 3-22). A checklist was designed and followed each day to avoid overlooking any of the steps (Appendix 3). Figure 3-23 illustrates the steps of the experiment.

Figure 3-22 Artificial night time saliva containers

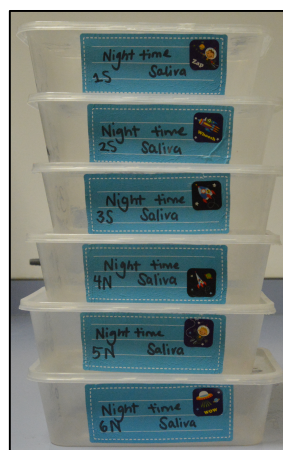
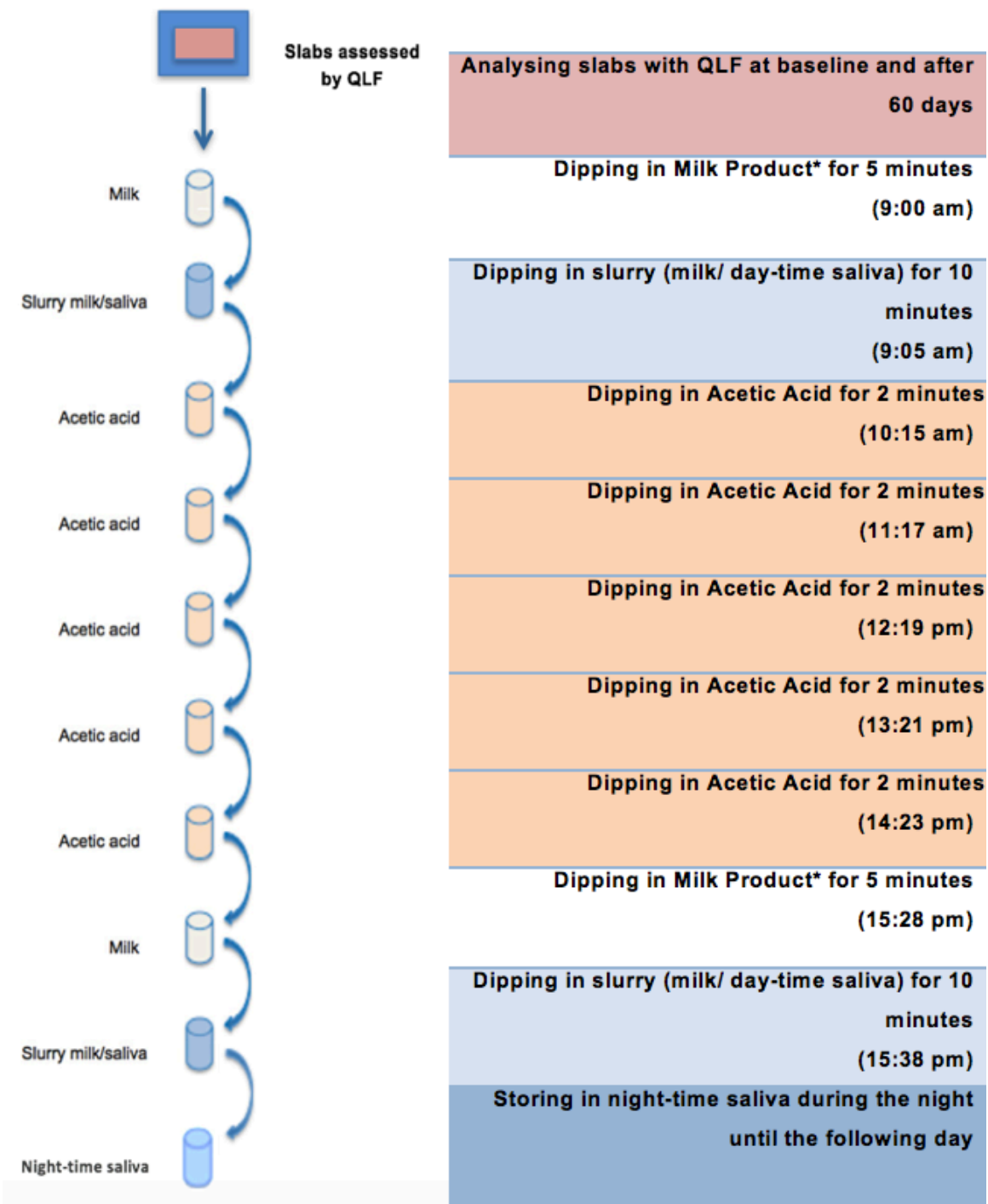


Figure 3-23: Daily Regime followed for 60 days:



3.15 Preparation of solutions used for pH cycling

3.15.1 Preparation of fluoridated milk with different concentrations

An aqueous solution of sodium fluoride was added to the pasteurised milk in a fixed ratio in order to produce the required concentration of fluoridated milk. A 100 ppm aqueous stock solution of sodium fluoride was prepared by dissolving 1.105 grams of sodium fluoride (extra pure, BP grade) per litre in distilled water. Therefore, 1 ml of aqueous solution of sodium fluoride added to 1 litre of milk would give a final concentration of 1 ppm F. The pH of prepared fluoridated milk was 6.7-6.8.

The milk cartons used contained 2.272 L of milk. In order to produce the required concentrations of 0.5 ppm F and 1.0 ppm F used in this study, the following calculations were made by adding Na F from the 100 ppm solution stock using a pipette set at 3.79 ml:

0.5 ppm F = 3.79 ml x 3 (Na F from 100 ppm aqueous stock solution was added 3 times using the pipette set at 3.79 ml) = 11.36 ml in 2.272 L of milk.

1.0 ppm F = 3.79 ml x 6 = 22.71 ml in 2.272 L of milk.

3.15.2 Preparation of the milk/ saliva slurry

This was simply done by mixing 1 part of the milk (+/-fluoride) to 3 parts of artificial day-time saliva.

3.15.3 Artificial saliva

Two types of artificial saliva solutions were prepared for this study, one was used in between dippings during the day and the other solution was used

overnight as a storage solution for the slabs. The day-time saliva was a supersaturated solution that allowed remineralisation of enamel slabs. While the night saliva was a saturated solution that maintained the enamel condition without providing any mineral exchange. The composition of the artificial saliva solution was based on the electrolyte composition of natural saliva. The preparation was based on the same composition used by Malinowski et al. (2012b) as provided by Dr. RP. Shellis (Department of Oral and Dental Science, University of Bristol, Bristol, UK).

3.15.3.1 Artificial day-time saliva solution

The composition was as shown in Table 3-1 (Figure 3-24):

Table 3-1: Composition of synthetic day-time saliva solution

Contents	Concentration g/L	g/L X5
Calcium carbonate	0.07	0.35
Magnesium carbonate (hydrated basic)	0.019	0.095
Potassium di-hydrogen phosphate	0.554	2.77
HEPES buffer (acid form)	4.77	23.85
Potassium chloride	2.24	11.2

In 900 ml of distilled water, 1.8 ml 1 mol/L HCL (Hydrochloric acid) was added. The above components were then added in and stirred with a magnetic stirrer until they were completely dissolved. The pH was then adjusted to 6.8 by adding KOH (Potassium hydroxide) to the solution which was made up to 1 L

with de-ionised water. A stock solution of 5 L was made as the solution was changed twice during each cycle, this was done by multiplying all the contents' concentrations by 5.

Figure 3-24: Preparation of artificial saliva



3.15.3.2. Artificial night-time saliva solution

The composition was as shown in Table 3-2:

Table 3-2: Composition of synthetic night-time saliva solution

Contents	Concentration g/L	g/L X 5
Calcium carbonate	0.05	0.25
Magnesium carbonate (hydrated basic)	0.019	0.095
Potassium di-hydrogen phosphate	0.068	0.34
HEPES buffer (acid form)	4.77	23.85
Potassium chloride	2.24	11.2

In 900 ml of distilled water, 1.8 ml 1 mol/L HCL was added. The above components were then added in and stirred with a magnetic stirrer until they were completely dissolved. The pH was then adjusted to 6.8 by adding KOH to the solution which was made up to 1 L with de-ionised water. A stock solution of 5 L was made as the solution was changed twice during each cycle, this was done by multiplying all the contents' concentrations by 5.

3.15.4 Preparation of acetic acid solution

The following components were used (Table 3-3) based on the same preparation done in a number of studies by ten Cate and more recently in a thesis carried out by Bataineh in 2014 at the Leeds Dental Institute.

Table 3-3: Acetic acid solution components

Contents	Concentration g/L	g/L X 5
Calcium chloride	1.66	8.3
Potassium di-hydrogen phosphate	1.22	6.1
Acetic acid	30.02	150.1

These contents were added to 1 L of distilled water and stirred with a magnetic stirrer until fully dissolved. The pH was adjusted to 4.8 with a pH meter (Orion-model 920A- Figure 3-25) by adding NaOH (Sodium hydroxide) to the solution.

Figure 3-25: Orion pH meter



3.16 Statistical analysis

Statistical analysis of the collected data from the baseline and final QLF analyses was carried out using the SPSS statistical software package for windows (SPSS Inc. version 20.0) and included all three parameters of QLF.

Descriptive statistics included the mean, median, range, and standard deviation. Shapiro-Wilk test was used to test the normality distribution of the data.

The paired sampled t-test was carried out to compare between the baseline and final changes in remineralisation and also when comparing the fluoride concentrations between the interrupted and daily frequencies.

Analysis of variance (ANOVA) was used to compare the 3 different fluoride concentrations within each frequency separately if the data were normally distributed, if not then Kruskal-Wallis test was used instead. In order to assess the significant difference between the fluoride concentrations, the Bonferroni (used when carrying out multiple comparisons test on normally distributed data where the p value is adjusted to a very small value (0.001) to reduce the overall chance of coming up with the wrong conclusion) or Mann-Whitney U test was carried out. The significance level was set at $p < 0.05$ with a 95% confidence interval.

4.0 RESULTS

4.1 Quantitative Light induced fluorescence results (QLF)

Statistical analysis was carried out for the three main parameters:

ΔF : Defined as the fluorescence loss percentage with respect to the fluorescence of sound tooth tissue. In relation to lesion depth (%).

Area: Defined as the lesions' surface area expressed in pixels² (px²).

ΔQ : Defined as ΔF times the Area. Which is the fluorescence loss percentage with respect to the fluorescence of sound tooth tissue times the Area in relation to the lesion volume (%px²).

4.2 Descriptive statistics and normality tests

The difference (change) was calculated for each QLF parameter (ΔF , ΔQ and Area) on excel (version 15.33) using the formula:

Difference (change) = Final reading - Baseline reading

e.g $\Delta F \text{ diff} = \Delta F \text{ Final reading} - \Delta F \text{ Baseline reading}$

4.2.1 Interrupted frequency

4.2.1.1 Normality tests for Interrupted Frequency:

The normality test (Shapiro-Wilk test) was carried out for the variables (ΔF , ΔQ and Area) and for all the three fluoride concentration levels (0.0 ppm F as the control, 0.5 ppm F and 1.0 ppm F) (Table 4-1). The data were normally distributed if the p-values were greater than 0.05 (p-value>0.05). The

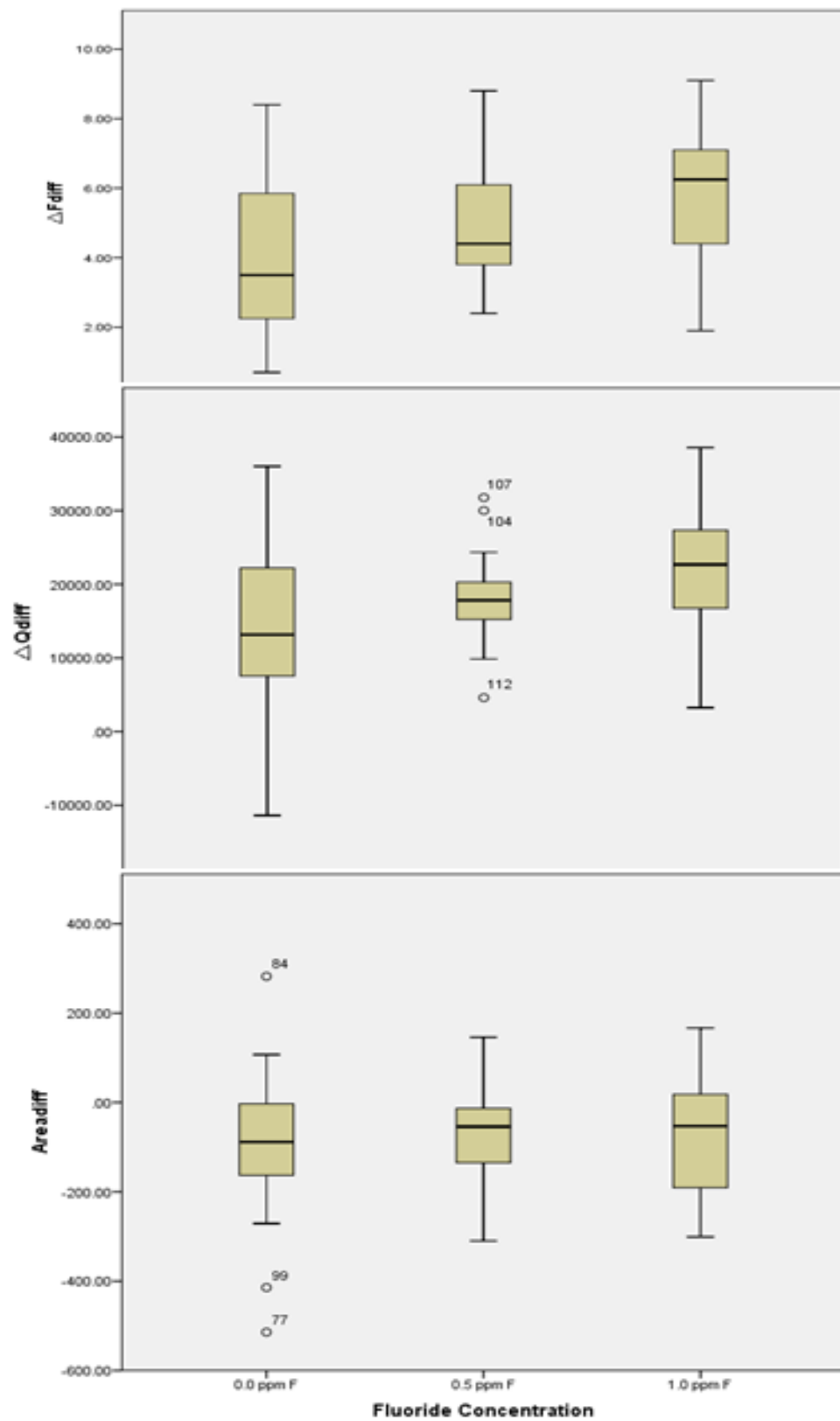
significance values for the three QLF parameters in the interrupted frequency were greater than 0.05 which meant that the data were considered to be normally distributed.

Table 4-1: Normality test (Shapiro-Wilk) p-values for the Interrupted frequency

Fluoride concentration	Variable		
	ΔF diff	ΔQ diff	Area diff
0.0 ppm F	0.417	0.607	0.393
0.5 ppm F	0.200	0.495	0.189
1.0 ppm F	0.445	0.751	0.622

The box-plots (Figure 4-1) show the distribution of data across the three levels of fluoride concentrations. When looking at ΔF boxplots for the interrupted frequency, 0.0 ppm F concentration seems to display a wider range of distribution of the samples compared to the other two concentrations. The median on the other hand, seems to be at a higher level for 1.0 ppm F concentration level. The ΔQ boxplots show the smallest range of data distribution at the 0.5 ppm F concentration level, compared to the other fluoride concentration levels, with three extreme values evident. Again, 0.0 ppm F seems to show a wider range similar to ΔF in addition to the median being higher at concentration level 1.0 ppm F. However, the boxplots for Area show some extreme values at the 0.0 ppm F concentration level and a slight difference in the range of data distribution between the three levels of fluoride concentrations. Also, the median appears to be almost at the same level for all fluoride concentration levels.

Figure 4-1: Box-plots for ΔF diff, ΔQ diff, and Area diff (Interrupted Frequency)



4.2.1.2 Descriptive statistics and mean comparisons

Table 4-2 shows the descriptive statistics for all QLF parameters ΔF , ΔQ and Area. The means for both ΔF & ΔQ increased as the fluoride concentration level became higher. On reviewing the Area means, there seems to be no clear pattern.

Table 4-2 Descriptive statistics for ΔF diff, ΔQ diff and Area diff for the interrupted frequency

Variable	Fluoride conc.	N	Mean	Std. Deviation	Minimum	Maximum
ΔF diff	0.0 ppm	24	3.846	2.214	0.700	8.400
	0.5 ppm	25	4.832	1.553	2.400	8.800
	1.0 ppm	26	5.889	1.987	1.900	9.100
ΔQ diff	0.0 ppm	24	14208.904	9948.610	-11386.700	36027.300
	0.5 ppm	25	18020.856	5819.320	4609.700	31765.000
	1.0 ppm	26	21665.212	7761.864	3243.000	38550.000
Area diff	0.0 ppm	24	-98.417	164.826	-514.300	282.300
	0.5 ppm	25	-79.680	114.911	-310.000	146.000
	1.0 ppm	26	-70.819	120.610	-300.700	166.600

4.2.2 Daily frequency

4.2.2.1 Normality test for the Daily frequency:

The normality test (Shapiro-Wilk) was carried out for all variables (ΔF , ΔQ , Area) for all fluoride concentration levels (0.0 ppm F as the control, 0.5 ppm F and 1.0ppm F). Both tests showed that ΔF was the only variable that satisfied normality throughout all the concentration levels (Table 4-3). However, the variables Area (all fluoride concentration levels) and ΔQ at concentrations 0.0

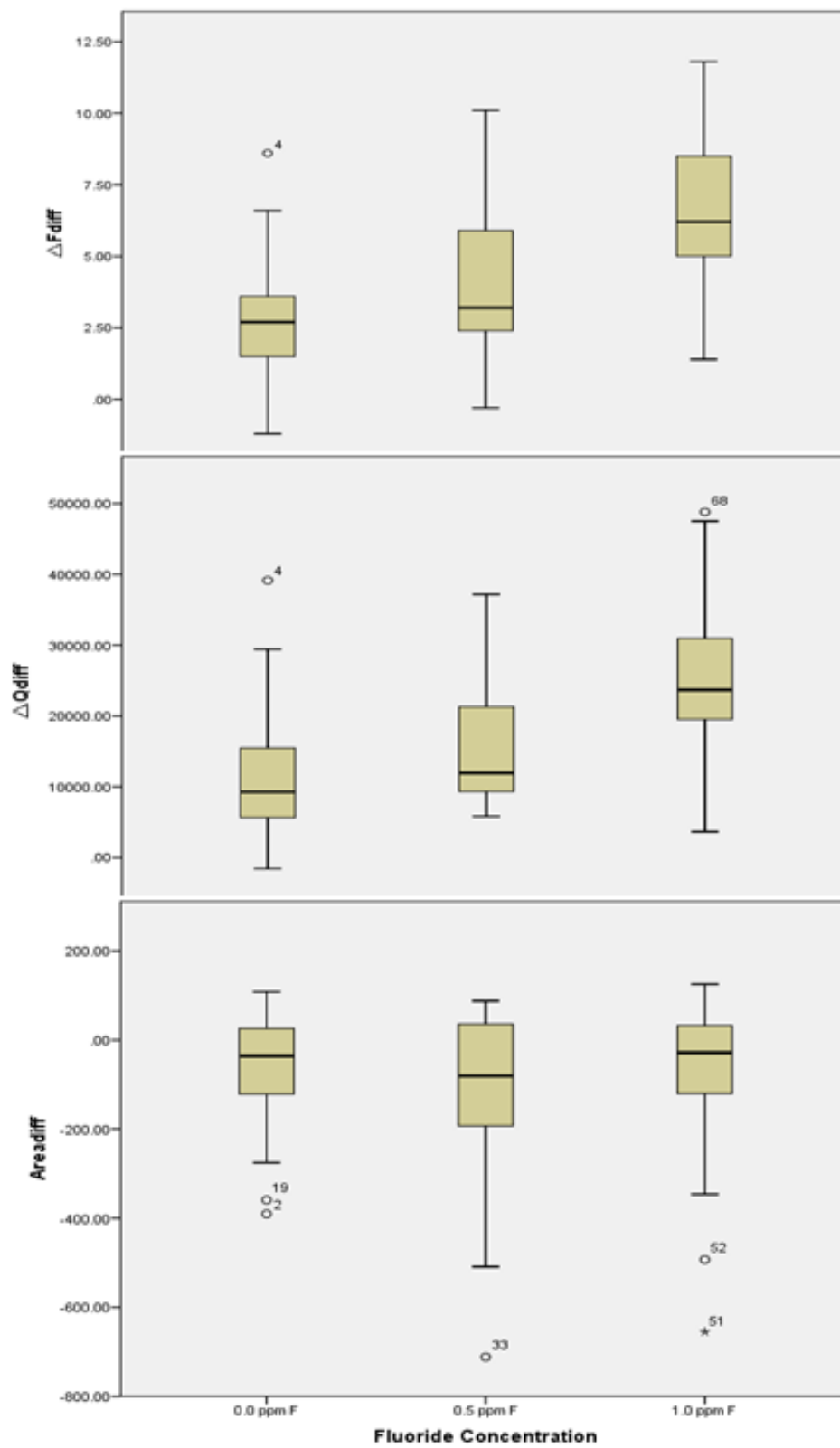
ppm F and 0.5 ppm F were not normally distributed. Thus, the non-parametric tests for ΔQ and Area were used.

Table 4-3: Normality test (Shapiro-Wilk) p-values for the Daily Frequency

Fluoride conc.	Variable		
	ΔF diff	ΔQ diff	Area diff
0.0 ppm	0.581	0.004	0.035
0.5 ppm	0.151	0.002	0.003
1.0 ppm	0.779	0.227	0.001

The boxplots in (Figure 4-2) illustrate the distribution of data across the three levels of fluoride concentrations. For variable ΔF , the data at 0.0 ppm F seems to have the narrowest range with one extreme value evident, while data at 0.5 ppm F and 1.0 ppm F display almost equal range of data distribution with a skew to the right. The median is higher at the 1.0 ppm F concentration level. Data distribution for ΔQ showed the widest range at fluoride concentration level 1.0 ppm F where there was one extreme value present at that level while a second extreme value was at the level of 0.0 ppm F. As for Area, the widest range of data distribution appears to be at 0.5 ppm F concentration level where one extreme value was apparent. However, there are two extreme values present at fluoride concentration levels 1.0 ppm and 0.0 ppm F.

Figure 4-2: Box-plots for ΔF diff, ΔQ diff, and Area diff (Daily Frequency)



4.2.2.2 Descriptive statistics and means comparisons

Table 4-4 shows the descriptive statistics for all QLF parameters ΔF , ΔQ and Area and it is evident that the mean for all concentration levels seemed to increase as the fluoride concentration level in milk increased for both parameters ΔF & ΔQ . However, when considering the Area of the lesion, the means showed no obvious pattern which could be due to the presence of extreme values or other factors.

Table 4-4: Descriptive statistics for ΔF diff, ΔQ diff and Area diff for the daily frequency

Variable	Fluoride conc.	N	Mean	Std. Deviation	Minimum	Maximum
ΔF diff	0.0 ppm	25	2.820	2.349	-1.200	8.600
	0.5 ppm	25	3.804	2.465	-0.300	10.100
	1.0 ppm	26	6.565	2.410	1.400	11.800
ΔQ diff	0.0 ppm	25	11020.036	8862.540	-1582.000	39157.400
	0.5 ppm	25	15177.372	9227.148	5821.000	37193.400
	1.0 ppm	26	25720.835	11688.749	3640.000	48829.700
Area diff	0.0 ppm	25	-70.052	136.042	-390.300	108.600
	0.5 ppm	25	-120.900	196.257	-711.600	87.700
	1.0 ppm	26	-92.673	178.753	-654.700	125.300

4.2.3 Difference between baseline values

The one way ANOVA test was performed to evaluate if there was any statistically significant difference in all the QLF parameter values at baseline between the three fluoride concentration levels to be tested. Appendix 5 outlines

the results, considering the interrupted and daily frequencies, where no statistically significant difference was found.

4.3 Testing the differences within each group (Baseline & After treatment)

This was carried out to in order to establish whether there was a statistically significant difference between the means, when comparing the baseline and final readings for all parameters (ΔF , ΔQ and Area), while considering all concentration levels of fluoride in milk (0.0 ppm F as the control, 0.5 ppm F and 1.0 ppm F).

4.3.1 Difference in ΔF within each fluoride concentration (Interrupted & Daily Frequency)

Table 4-5 shows the baseline and final readings for both the interrupted and daily frequencies at all fluoride concentration levels. There is evidence of improvement in ΔF values for both the interrupted and daily frequencies at all fluoride concentration levels.

Table 4-5: The mean values of ΔF at baseline and after treatment for all ΔF concentration levels

Fluoride conc.	Frequency	ΔF Baseline		ΔF Final	
		Mean	Std. Deviation	Mean	Std. Deviation
0.0 ppm	Daily	-27.532	3.229	-24.712	2.639
	Interrupted	-27.913	3.233	-24.067	3.92
0.5 ppm	Daily	-26.796	4.769	-22.992	3.691
	Interrupted	-26.800	3.624	-21.968	3.743
1.0 ppm	Daily	-26.762	3.683	-20.196	3.514
	Interrupted	-26.389	4.064	-20.502	3.939

The normality assumption was satisfied for all ΔF values for both the interrupted and daily frequencies (Tables 4-1, 4-3). Based on the results of the paired sampled T-Test (Table 4-6) it can be concluded that ΔF final readings show a statistically significant improvement compared to ΔF baseline readings for both the interrupted and daily frequencies at all concentration levels of fluoride in milk.

Table 4-6: Paired Sampled T-Test results for ΔF values at baseline and after treatment

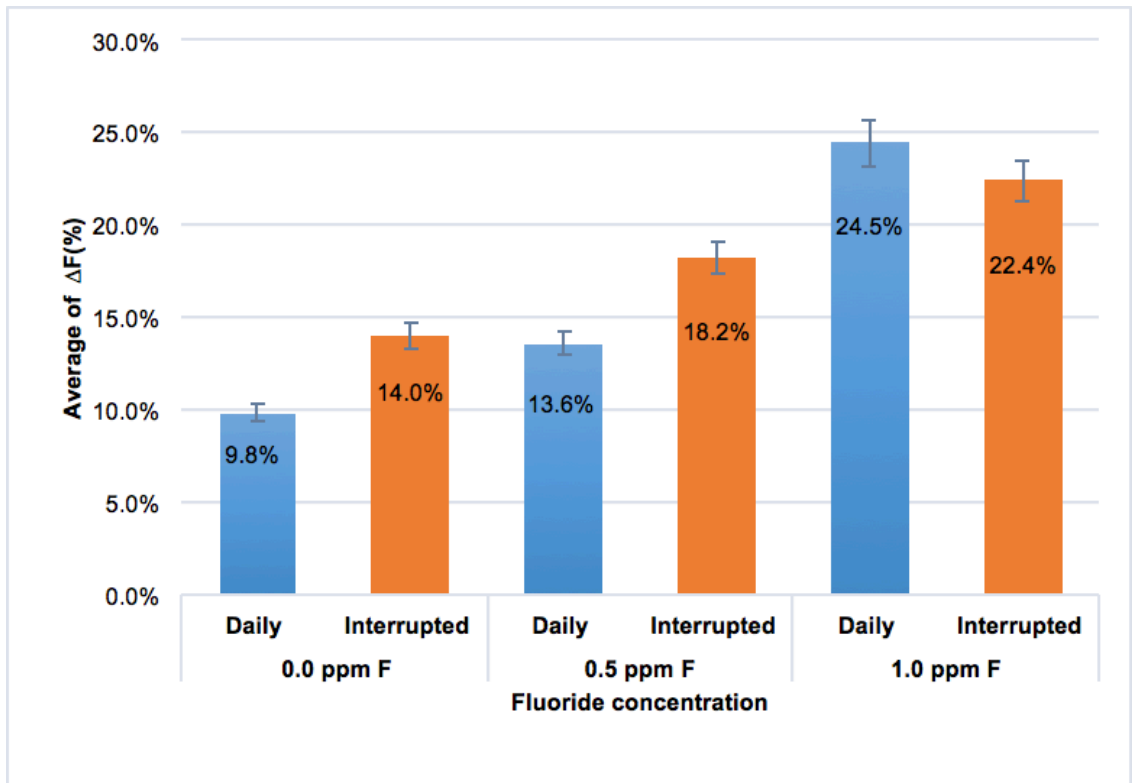
Fluoride conc.	Frequency	Mean	95% Confidence Interval of the Difference		p-value
			Lower	Upper	
0.0 ppm	Daily	2.820	1.851	3.789	<0.001
	Interrupted	3.846	2.911	4.781	<0.001
0.5 ppm	Daily	3.804	2.787	4.821	<0.001
	Interrupted	4.832	4.191	5.473	<0.001
1.0 ppm	Daily	6.565	5.592	7.539	<0.001
	Interrupted	5.888	5.086	6.691	<0.001

The percentage change in ΔF at baseline and after treatment (%F) was assessed by using the following formula:

$$\text{Difference in } \Delta F \text{ at baseline and after treatment} / \Delta F \text{ at baseline) } \times 100$$

Graph 4-1 shows the %F by the level of fluoride concentration in milk which seems to demonstrate a consistent pattern of improvement as the level of fluoride in milk increased for both the interrupted and daily frequencies. It is also clear that the interrupted frequency groups for both 0.0 and 0.5 ppm F concentration levels showed more improvement than their counterparts in the daily frequency. However, the daily frequency exhibited more improvement at the 1.0 ppm F level as opposed to the interrupted frequency.

Graph 4-1: The mean percentage change in ΔF



4.3.2 Difference in ΔQ within each fluoride concentration (Interrupted and Daily frequency)

Table 4-7 shows the baseline and final readings for both the interrupted and Daily frequencies at all fluoride concentration levels. It is clear that ΔQ values indicate improvement for both the interrupted and Daily frequencies at all fluoride concentration levels.

Table 4-7: The mean values of ΔQ at baseline and after treatment for all F concentration levels

Fluoride conc.	Frequency	ΔQ Baseline		ΔQ Final	
		Mean	Std. Deviation	Mean	Std. Deviation
0.0 ppm	Daily	-88086.840	20854.547	-77066.804	16200.562
	Interrupted	-92133.933	19738.765	-77925.029	16378.035
0.5 ppm	Daily	-87748.040	20010.038	-72570.668	16078.879
	Interrupted	-90760.232	17499.674	-72739.376	16207.852
1.0 ppm	Daily	-93949.073	20530.467	-68228.238	13771.507
	Interrupted	-90644.592	18055.049	-68979.380	16283.215

Based on the results of the paired sampled T-Test (Table 4-8) it can be concluded that ΔQ final readings show statistically significant improvement compared to ΔQ baseline readings for both the interrupted and daily frequencies at all concentration levels of fluoride in milk. However, since the normality assumption for fluoride concentration levels 0.0 ppm F and 0.5 ppm F for the daily frequency were not satisfied (Table 4-3), the Wilcoxon Signed-Rank Test (Table 4-9) was carried out. This test confirmed that there was statistically significant improvement for ΔQ final readings compared to baseline for both 0.0 ppm F and 0.5 ppm F concentration levels within the daily pattern of frequency. This reaffirms the same conclusion shown by the paired sampled T-Test.

Table 4-8: Paired Sampled T-Test results for ΔQ values at baseline and after treatment

Fluoride conc.	Frequency	Mean	Std. Deviation	95% Confidence Interval of the Difference		p-value
				Lower	Upper	
0.0 ppm	Interrupted	14208.904	9948.610	10007.975	18409.834	<0.001
0.5 ppm	Interrupted	18020.856	5819.320	15618.759	20422.953	<0.001
1.0 ppm	Daily	25720.835	11688.750	20999.647	30442.024	<0.001
	Interrupted	21665.212	7761.864	18530.127	24800.296	<0.001

Table 4-9: Wilcoxon Signed-Rank Test for ΔQ values at baseline and after treatment

Fluoride conc.	Frequency	Z	Asymp. P-value
0.0 ppm	Daily	-4.319 ^b	<0.001
0.5 ppm	Daily	-4.372 ^b	<0.001
b based on negative ranks			

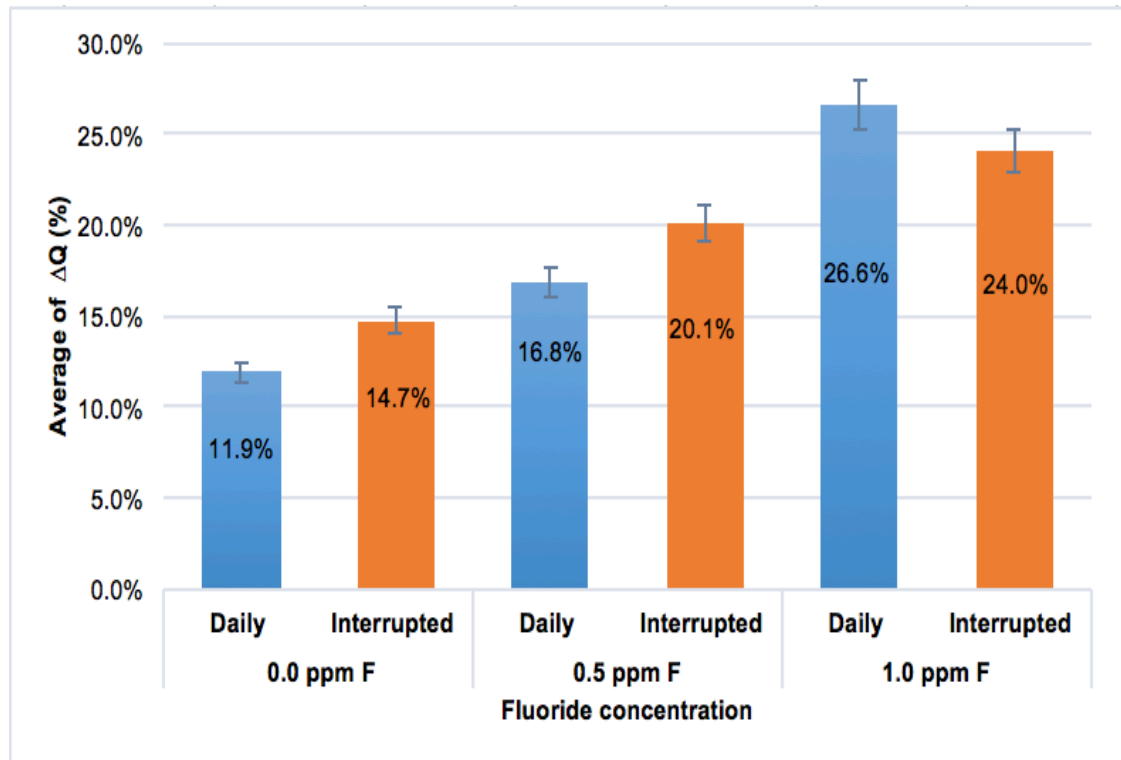
The percentage change in ΔQ at baseline and after treatment (%Q) was assessed by using the following formula:

$$\text{Difference in } \Delta Q \text{ at baseline and after treatment} / \Delta Q \text{ at baseline) } \times 100$$

Graph 4-2 shows the %Q by the level of fluoride concentration in milk which seems to demonstrate a consistent pattern of improvement as the level of fluoride in milk increased for both the interrupted and daily frequencies. It is also clear that the interrupted frequency groups for both the 0.0 and 0.5 ppm F concentration levels showed more improvement than their counterparts in the

daily frequency. However, the daily frequency exhibited more improvement at the 1.0 ppm F level as opposed to the interrupted frequency.

Graph 4-2: The mean percentage change in ΔQ



4.3.3 Difference in Area within each fluoride concentration (interrupted and daily frequency)

Table 4-10 shows the baseline and final readings for both the interrupted and daily frequencies at all fluoride concentration levels. It is clear that the values for the Area of the lesion indicate improvement for both the interrupted and daily frequencies at all fluoride concentration levels.

Table 4-10 The mean values of Area at baseline and after treatment for all F concentration levels

Fluoride conc.	Frequency	Area Baseline		Area Final	
		Mean	Std. Deviation	Mean	Std. Deviation
0.0 ppm	Daily	3193.400	586.725	3123.348	578.361
	interrupted	3373.721	613.602	3275.304	611.689
0.5 ppm	Daily	3279.376	540.983	3158.476	565.058
	interrupted	3383.164	390.335	3303.484	386.655
1.0 ppm	Daily	3524.904	691.832	3432.231	737.813
	interrupted	3452.981	611.592	3382.161	614.937

Considering the normality assumption was satisfied for all of the fluoride concentration levels in the interrupted frequency (Table 4-1), the paired sampled T-Test was used (Table 4-11). However, since the normality was not satisfied for any of the fluoride concentration levels in the daily frequency (Table 4-3), the Wilcoxon Signed-Ranks Test (Table 4-12) was carried out instead. It can be concluded from the results of both tests that there was a reduction in the lesion Area between baseline and final readings which was statistically significant for both the interrupted and daily frequencies and for all the fluoride concentration levels.

Table 4-11 Paired Sampled T-Test results for Area values at baseline and after treatment

Fluoride conc.	Frequency	Mean	Std. Dev.	95% Confidence Interval of the Difference		p-value
				Lower	Upper	
0.0 ppm	Interrupted	-98.416	164.825	-168.016	-28.819	0.008
0.5 ppm	Interrupted	-79.680	114.910	-127.112	-32.247	0.002
1.0 ppm	Interrupted	-70.819	120.609	-119.534	-22.103	0.006

Table 4-12 Wilcoxon Signed-Rank Test for Area values at baseline and after treatment

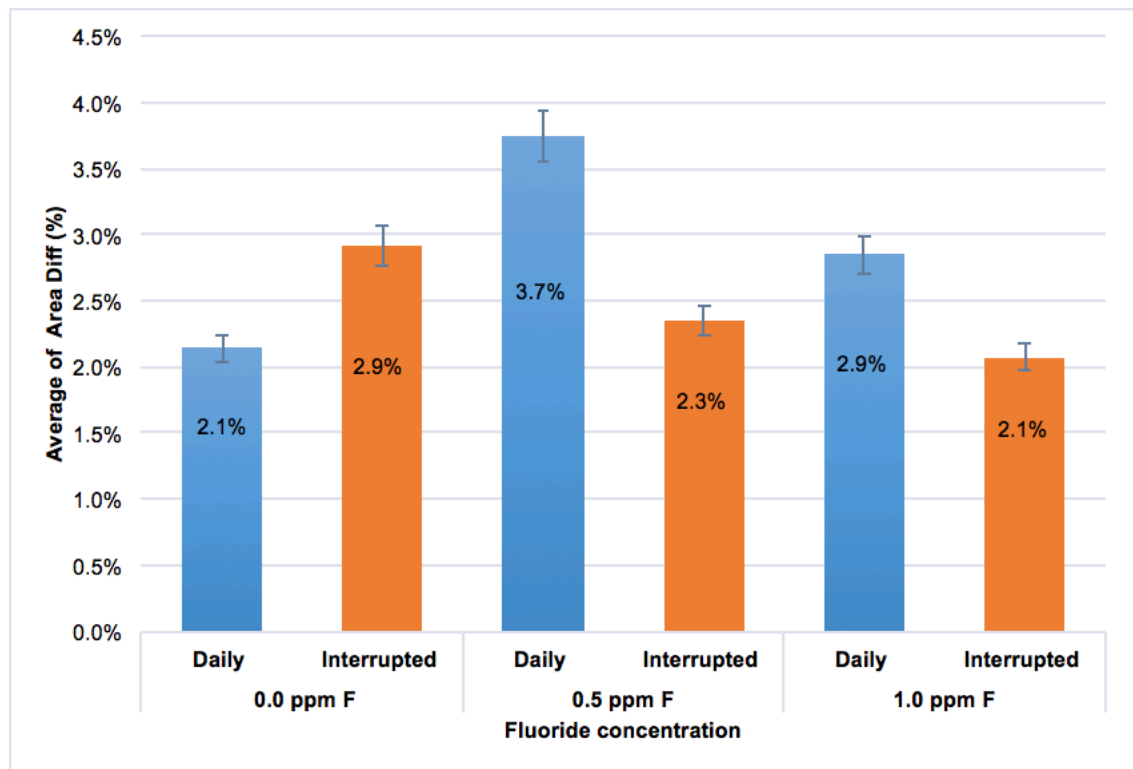
Fluoride conc.	Frequency	Z	Asymp. p-value
0.0 ppm	Daily	-2.058 ^c	0.040
0.5 ppm	Daily	-2.489 ^c	0.013
1.0 ppm	Daily	-2.273 ^c	0.023
c based on positive ranks			

The percentage change in Area at baseline and after treatment (%Area) was assessed by using the following formula:

$$\text{Difference in Area at baseline and after treatment/Area at baseline} \times 100$$

Graph 4-3 shows that the %Area decreased as the fluoride concentration level in milk increased for the interrupted frequency which displayed an inconsistent pattern of change. However, there seemed to be no clear pattern when looking at the daily frequency.

Graph 4-3: the mean percentage change in Area



4.4 Comparing the fluoride concentration levels in milk for each frequency (Interrupted & Daily)

Each frequency was considered separately for the QLF parameters (ΔF , ΔQ , Area). As previously mentioned in section 4.2, the difference (change) for each parameter was initially calculated for baseline and final readings which was then used in order to compare between the fluoride concentration levels for each of the frequencies (Interrupted & Daily) separately.

4.4.1 The Interrupted frequency:

The values for ΔF , ΔQ and Area were analysed to determine if there was a statistically significant difference between the different fluoride concentration

levels (0.0ppm as the control, 0.5 ppm and 1.0 ppm) when taking into account the interrupted frequency pattern separately.

To assess if there was a statistically significant difference between the fluoride concentration levels, the one-way ANOVA test (Table 4-13) was performed to compare the different concentrations in the interrupted frequency using QLF variable means (ΔF , ΔQ , Area).

The results demonstrated a statistically significant difference for ΔF & ΔQ only, which meant that at least one of the means of the fluoride concentration levels were significantly different. However, there was no statistical difference between the means for all fluoride concentration levels when considering the lesion Area.

Table 4-13: One-Way ANOVA results between fluoride concentration levels for the difference in all QLF parameters at baseline and after treatment

Variable		Sum of Squares	df	Mean Square	F	p-value
ΔF diff	Between Groups	52.167	2	26.083	6.972	0.002
	Within Groups	269.361	72	3.741		
	Total	321.527	74			
ΔQ diff	Between Groups	693915694.017	2	346957847.009	5.436	0.006
	Within Groups	4595332056.078	72	63824056.334		
	Total	5289247750.095	74			
Area diff	Between Groups	9825.615	2	4912.808	0.271	0.763
	Within Groups	1305424.514	72	18130.896		
	Total	1315250.129	74			

To identify which of the fluoride concentration levels were statistically significantly different within parameters ΔF & ΔQ , the pairwise comparisons test was carried out using the Bonferroni adjustment (Bonferroni, 1936; Strassburger and Bretz, 2008). Table 4-14 demonstrates the results, confirming that there was a statistically significant difference between fluoride concentration levels 0.0 ppm and 1.0 ppm only for both ΔF & ΔQ where the higher concentration exhibited better results.

Table 4-14: Multiple comparisons of the difference in ΔF and ΔQ at baseline and after treatment between all levels of fluoride concentrations

Variable	Fluoride conc.		Mean Difference (I-J)	Std. Error	95% Confidence Interval		p-value
	(I)	(J)			Lower Bound	Upper Bound	
ΔF diff	0.0 ppm	0.5 ppm	-0.986	0.552	-2.341	0.368	0.236
		1.0 ppm	-2.043*	0.547	-3.384	-0.700	0.001
	0.5 ppm	0.0 ppm	0.986	0.552	-0.368	2.341	0.236
		1.0 ppm	-1.056	0.542	-2.384	0.271	0.165
	1.0 ppm	0.0 ppm	-2.043*	0.547	0.700	3.384	0.001
		0.5 ppm	1.0564	0.542	-0.271	2.384	0.165
ΔQ diff	0.0 ppm	0.5 ppm	-3811.952	2283.045	-9408.151	1784.247	0.298
		1.0 ppm	-7456.307*	2261.439	-12999.544	-1913.070	0.005
	0.5 ppm	0.0 ppm	3811.952	2283.045	-1784.247	9408.151	0.298
		1.0 ppm	-3644.356	2237.796	-9129.640	1840.929	0.323
	1.0 ppm	0.0 ppm	-7456.307*	2261.439	1913.070	12999.544	0.005
		0.5 ppm	3644.356	2237.796	-1840.929	9129.640	0.323
* The mean difference is significant at the 0.05 level.							

4.4.2 For the Daily frequency

ΔF , ΔQ and Area were analysed to determine if there was a statistically significant difference between the different fluoride concentration levels (0.0 ppm as the control, 0.5 ppm and 1.0 ppm) when taking into account the daily frequency pattern. Similar to the interrupted frequency, the difference was calculated for each QLF variable for the baseline and final readings.

The one- way ANOVA test (Table 4-15) was performed to compare the fluoride concentration levels in the daily frequency for ΔF means to confirm if there was a statistically significant difference between the fluoride concentration levels. It was clear from the results that there was a statistically significant difference between the means, therefore indicating that at least one of the fluoride concentration levels was different. The Bonferroni test results for ΔF (Table 4-16) confirmed that there was a statistically significant difference between fluoride concentration levels 0.5 ppm and 1.0 ppm as well as between 0.0 ppm and 1.0 ppm which indicated that the latter was more effective in lesion remineralisation. On the other hand, there was no statistically significant difference between concentration levels 0.0 ppm and 0.5 ppm F which may indicate that they were both equally effective in lesion remineralisation.

Table 4-15: One-way ANOVA results between fluoride concentration levels for the difference in ΔF at baseline and after treatment

	Sum of Squares	df	Mean Square	F	p-value
Between Groups	193.154	2	96.577	16.652	<0.001
Within Groups	423.368	73	5.800		
Total	616.522	75			

Table 4-16: Multiple comparisons of the difference in ΔF at baseline and after treatment between all levels of fluoride concentrations

Fluoride conc.		Mean Difference (I-J)	Std. Error	95% Confidence Interval		p-value
Mean	Mean			Lower Bound	Upper Bound	
0.0 ppm	0.5 ppm	-.984	.681	-2.653	.685	0.459
	1.0 ppm	-3.745*	.674	-5.398	-2.092	<0.001
0.5 ppm	0.0 ppm	.984	.681	-.685	2.653	0.459
	1.0 ppm	-2.761*	.674	-4.414	-1.108	<0.001
1.0 ppm	0.0 ppm	3.745*	.674	2.092	5.398	<0.001
	0.5 ppm	2.761*	.674	1.108	4.414	<0.001
* The mean difference is significant at the 0.05 level.						

Due to the fact that at least one concentration level did not satisfy the normality assumption within both ΔQ and Area variables, a non-parametric test (Kruskal-Wallis test) was used to test the difference between the three concentration levels for ΔQ and Area. The results (Table 4-17) show that there was a statistically significant difference between the fluoride concentration levels

for the ΔQ variable, indicating that at least one of the means were different. However, there seems to be no statistically significant difference between the fluoride concentration levels for the Area of the lesion.

Table 4-17: Kruskal-Wallis test results between fluoride concentration levels for the difference in ΔQ and Area at baseline and after treatment

Variable	Asymp. P-value
ΔQ diff	<0.001
Area diff	0.816

In order to identify which of the fluoride concentration levels showed a statistically significant difference for ΔQ , the Man-Whitney U test carried out. The results (Table 4-18) would suggest that the mean at fluoride concentration level 1.0 ppm was significantly greater compared to the means for both concentration 0.5 ppm and 0.0 ppm. However, there was no statistically significant difference between 0.0 ppm and 0.5 ppm F.

Table 4-18: Pairwise comparison test of the difference in ΔQ at baseline and after treatment between all levels of fluoride concentrations

Fluoride conc.	Asymp. P-value (2-tailed)
0.0 ppm vs 0.5 ppm	0.059
0.5 ppm vs 1.0 ppm	0.001
0.0 ppm vs 1.0 ppm	<0.001

4.5 Comparing the fluoride concentration levels in milk between both frequencies (Interrupted vs. Daily)

When considering the null hypothesis: there is no significant difference in the clinical effectiveness on enamel remineralisation when comparing between the interrupted and daily frequencies at fluoride concentration levels 0.0 ppm F (as the control), 0.5 ppm F and 1.0 ppm F.

The difference was calculated for each parameter, as mentioned in the previous section, and was used in the analysis.

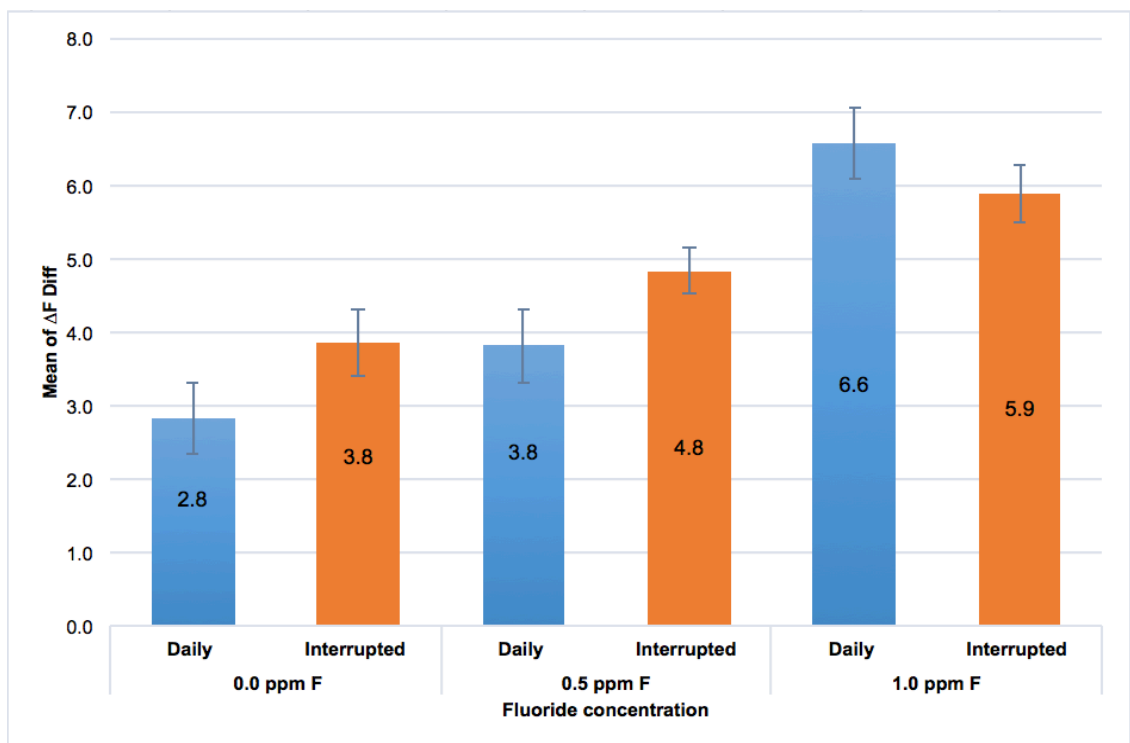
4.5.1 Difference in ΔF (ΔF diff) values at baseline and after treatment between the interrupted and daily frequencies

Table 4-19 shows the means and standard deviations for ΔF values by frequency (interrupted, daily) and fluoride concentration levels (0.0 ppm, 0.5 ppm and 1.0 ppm). Graph 4-4 illustrates the means for ΔF by frequency and fluoride concentration levels, which seems to show an increase (i.e. improvement in remineralisation) as the fluoride concentration level increases for both frequencies. The figure also demonstrates that the mean values for the interrupted frequency were higher than those from the daily frequency pattern for both concentration levels 0.0 and 0.5 ppm F, while the mean was higher for the daily frequency when the concentration level was at 1.0 ppm F.

Table 4-19: Descriptive statistics for the difference in ΔF (ΔF diff) at baseline and after treatment for all fluoride concentration levels

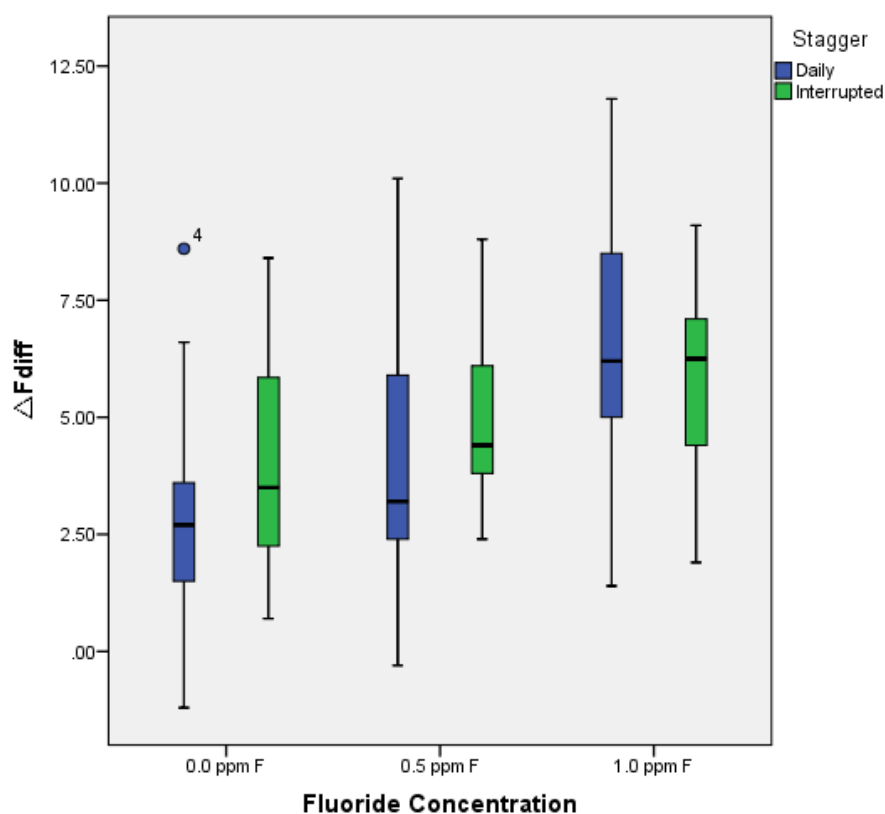
Fluoride conc.	Frequency	N	Mean	Std. Deviation
0.0 ppm	Daily	25	2.820	2.349
	Interrupted	24	3.846	2.214
0.5 ppm	Daily	25	3.804	2.465
	Interrupted	25	4.832	1.553
1.0 ppm	Daily	26	6.565	2.410
	Interrupted	26	5.889	1.987

Graph 4-4: Mean of the difference in ΔF (ΔF diff) by frequency and level of concentration



The box-plots (Figure 4-3) show that the range of data distribution is wider for the daily frequency at all fluoride concentration levels compared to the interrupted frequency. It also demonstrates that the median for the interrupted frequency is higher than the daily frequency for both concentration levels 0.0 and 0.5 ppm F, while it was almost at the same level for both frequencies when the concentration level was at 1.0 ppm F

Figure 4-3: Boxplots for mean of ΔF diff by frequency and level of fluoride concentration



Since the normality assumption was satisfied for both the interrupted and daily frequencies for all fluoride concentration levels in milk (Table 4-1, Table 4-3), the independent sample T-Test (Table 4-20) was used to test the mean differences between both the interrupted and daily frequencies for each fluoride

concentration level. It was evident that there was no statistically significant difference between the fluoride concentration levels when comparing between the interrupted and non- interrupted frequencies of dipping the slabs into fluoridated milk regardless of the concentration level. Meaning that 1.0 ppm fluoride in milk which underwent the interrupted frequency had the same effect on enamel lesion remineralisation as the concentration of 1.0 ppm F in milk when enamel slabs were dipped in a daily frequency manner.

Table 4-20: Independent two-sample T-Test results for the difference in ΔF (ΔF diff)

Fluoride conc.	Mean Difference D-I	Std. Error Difference	95% Confidence Interval of the Difference		p-value
			Lower	Upper	
0.0 ppm	-1.026	0.653	-2.339	0.287	0.123
0.5 ppm	-1.028	0.583	-2.199	0.144	0.084
1.0 ppm	0.677	0.613	-0.553	1.907	0.274

4.5.2 Difference in ΔQ (ΔQ diff) values at baseline and after treatment between the interrupted and daily frequencies

Table 4-21 shows the means and standard deviations for ΔQ values by frequency (interrupted, daily) and fluoride concentration levels (0.0 ppm, 0.5 ppm and 1.0 ppm).

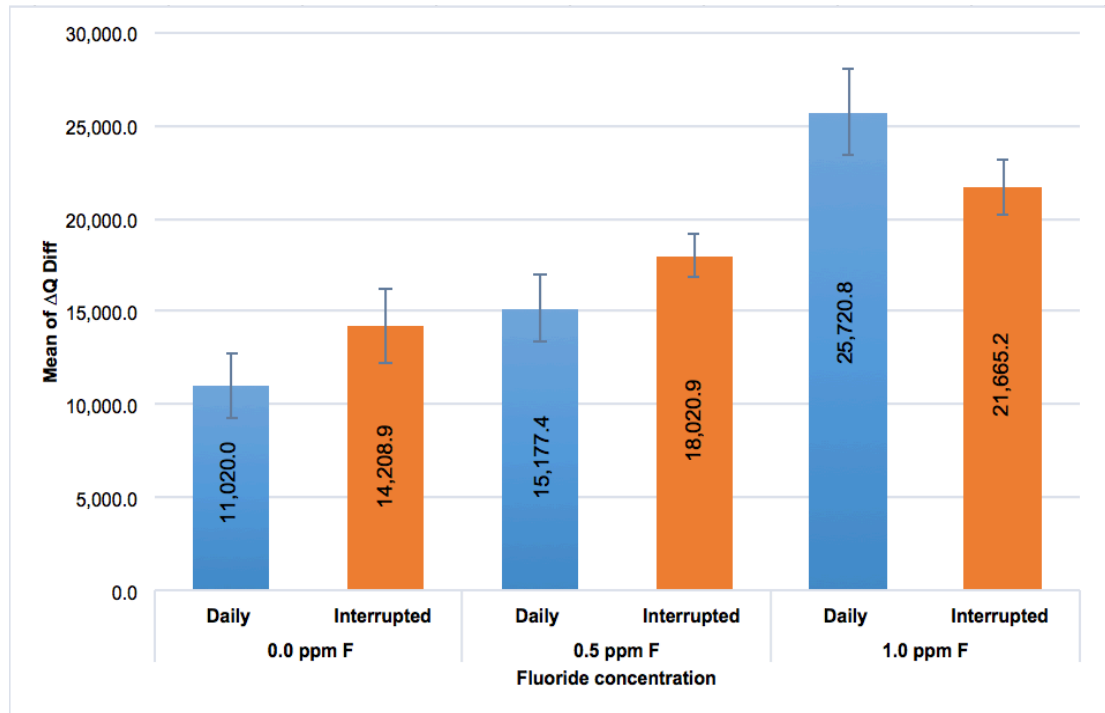
The means for the parameter ΔQ seemed to increase as the fluoride level in milk increased for both the interrupted and daily frequencies (Graph 4-5). It can also be concluded from the graph that the mean values for the interrupted

frequency are higher than those of the daily frequency at both fluoride concentration levels 0.0 ppm and 1.0 ppm. However, the mean was higher for the daily frequency when the level of fluoride in milk increased to 1.0 ppm.

Table 4-21: Descriptive statistics for the difference in ΔQ (ΔQ diff) at baseline and after treatment for all fluoride concentration levels

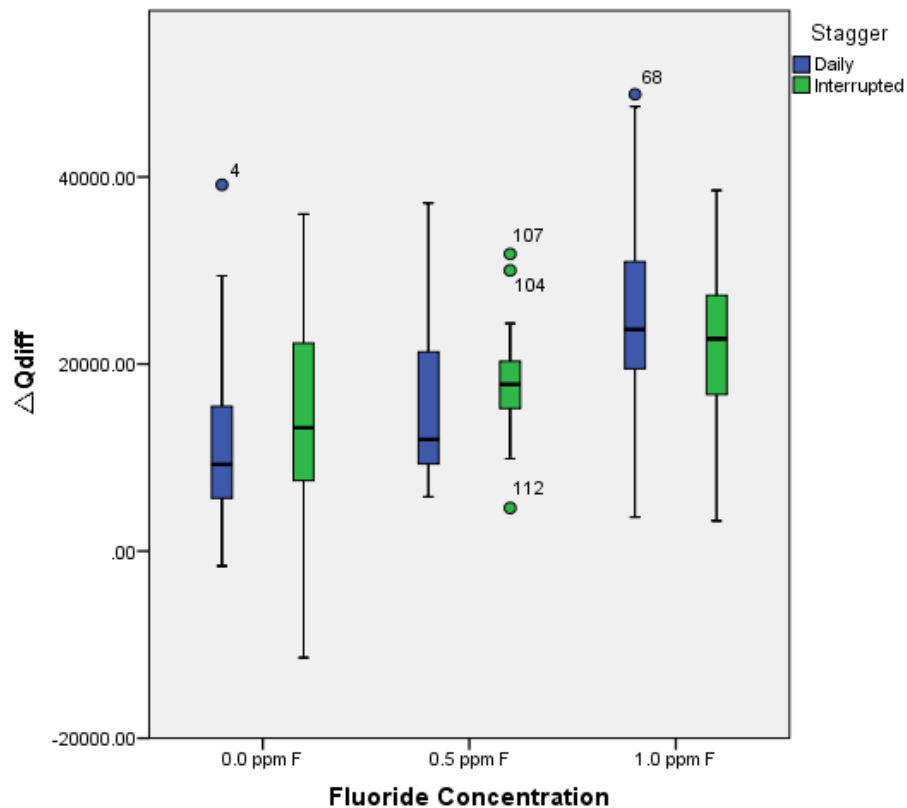
Fluoride conc.	Frequency	N	Mean	Std. Deviation
0.0 ppm	Daily	25	11020.036	8862.540
	Interrupted	24	14208.904	9948.609
0.5 ppm	Daily	25	15177.372	9227.148
	Interrupted	25	18020.856	5819.319
1.0 ppm	Daily	26	25720.834	11688.749
	Interrupted	26	21665.211	7761.864

Graph 4-5: Mean of the difference in ΔQ (ΔQ diff) by frequency and level of fluoride concentration



The box-plot (Figure 4-4) shows that the range of data distribution is the widest at fluoride concentration level 1.0 ppm for the daily frequency, while the smallest spread of data for the interrupted frequency is apparent at concentration level 0.5 ppm F with three outliers present. The median for the interrupted frequency, on the other hand, was at a higher level for both concentration levels 0.0 ppm and 0.5 ppm F but was slightly higher for the daily frequency when the fluoride concentration level was at 1.0 ppm.

Figure 4-4: Boxplots for mean of ΔQ diff by frequency and level of fluoride concentration



Since the normality assumption was satisfied for both the interrupted and daily frequencies when the concentration level of fluoride in milk was at 1.0 ppm F (Tables 4-1, 4-3), the independent sampled T-Test was used to compare the mean differences between both the interrupted and daily frequencies (Table 4-22). The non-parametric test (Mann-Whitney U test) was used for both concentration levels 0.0 ppm and 0.5 ppm F as the normality assumption was not satisfied.

Based on the independent samples t-test results (Table 4-22) it can be concluded that there is no statistically significant difference between the interrupted and daily frequency means at the 1.0 ppm F level. The non-parametric test (Table 4-23) showed evidence of a statistically significant

difference in favour of the interrupted frequency when fluoride concentration level in milk was at 0.5 ppm F ($p < 0.05$) but not at 0.0 ppm F.

Table 4-22: Independent two-sample T-Test results for the difference in ΔQ (ΔQ diff) for fluoride concentration level 1.0 ppm

Fluoride conc.	Mean Difference D-I	Std. Error Difference	95% Confidence Interval of the Difference		p-value
			Lower	Upper	
1.0 ppm	4055.623	2751.736	-1471.402	9582.648	0.147

Table 4-23: Mann-Whitney U test for the difference in ΔQ (ΔQ diff) for fluoride concentration levels 0.0 ppm and 0.5 ppm

Fluoride conc.	Asymp. p-value
0.0 ppm	0.101
0.5 ppm	0.043

4.5.3 Difference in lesion Area (Area diff) values at baseline and after treatment between the interrupted and daily frequencies

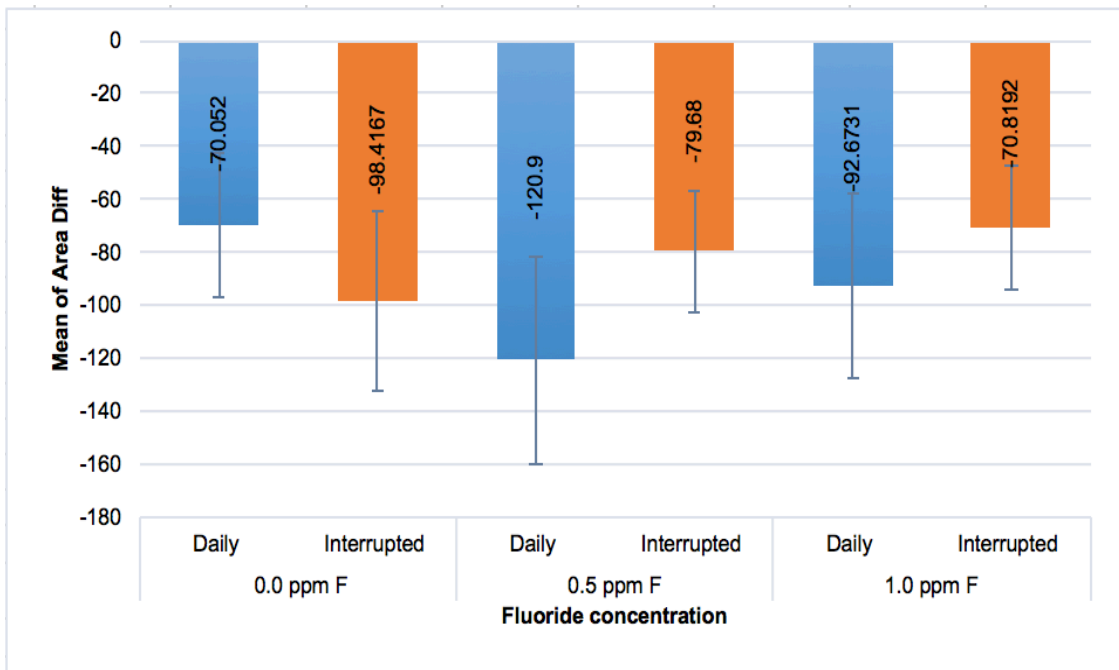
Table 4-24 shows the means and standard deviations of the lesion Area values by frequency (interrupted, daily) and fluoride concentration levels (0.0 ppm, 0.5 ppm and 1.0 ppm). There seems to be no specific pattern of change within the mean values of the lesion Area which could be attributed to a number of factors including the presence of outlier values (Graph 4-6).

The most improvement in lesion Area with the highest mean value appears to be at concentration level 0.5 ppm F within the daily frequency pattern (Graph 4-6).

Table 4-24: Descriptive statistics for the difference in lesion Area (Area diff) at baseline and after treatment for all fluoride concentration levels

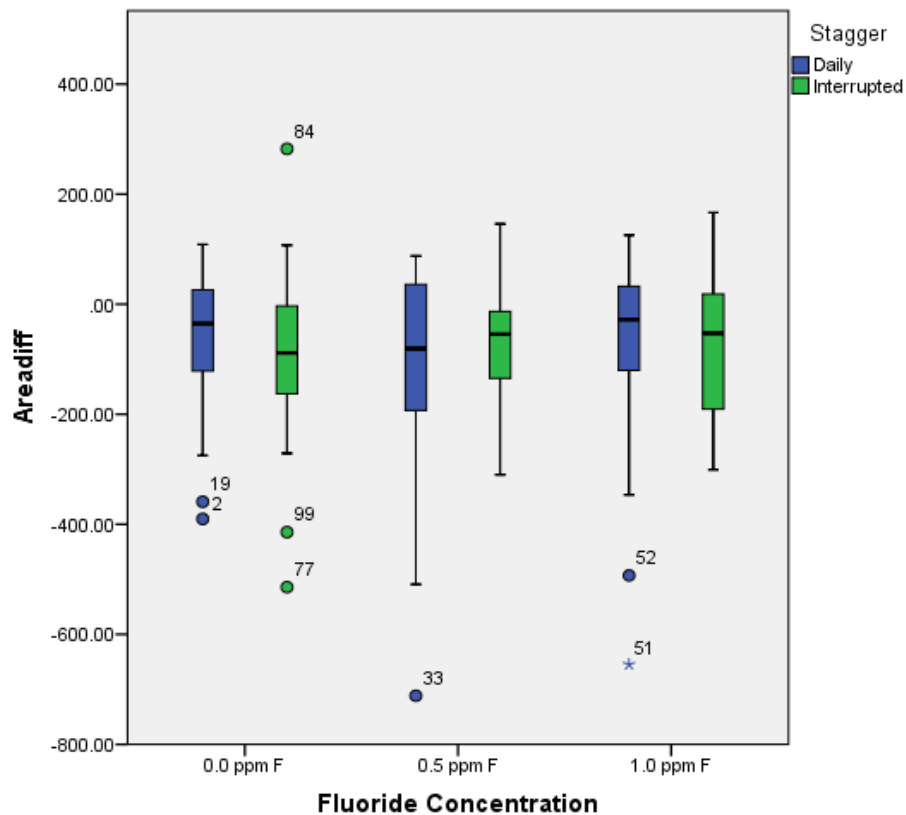
Fluoride conc.	Frequency	N	Mean	Std. Deviation
0.0 ppm	Daily	25	-70.052	136.042
	Interrupted	24	-98.417	164.826
0.5 ppm	Daily	25	-120.900	196.257
	Interrupted	25	-79.680	114.911
1.0 ppm	Daily	26	-92.673	178.753
	Interrupted	26	-70.819	120.610

Graph 4-6 Mean of the difference in lesion Area (Area diff) by frequency and level of fluoride concentration



The boxplot in Figure 4-5 shows the distribution of extreme values for both frequencies. It also shows the range of data distribution over the three fluoride concentration levels, where the broadest variation is at the 0.5 ppm F level for the daily frequency. The other data distribution ranges seem to be comparable to each other.

Figure 4-5: Boxplots for mean of lesion Area diff by frequency and level of fluoride concentration



Since the normality assumption was not satisfied for any of the fluoride concentration levels within the interrupted frequency pattern (Tables 4-1, 4-3), the non-parametric test (Mann-Whitney U test) was carried out to assess the mean difference between both frequency patterns for all levels of fluoride concentrations (Table 4-25). Based on these results it can be concluded that there is no statistically significant difference (no significant effect on lesion remineralisation) when comparing between the means of both the interrupted and daily frequencies at any of the fluoride concentration levels in milk).

Table 4-25: Mann-Whitney U test for the difference in lesion Area (Area diff)

Fluoride Conc.	Asymp. p-value
0.0 ppm	0.327
0.5 ppm	0.900
1.0 ppm	0.869

As previously suggested that one of the causes for the inconsistent pattern of improvement shown in Graph 4-5 might have been due to the presence of extreme values. Therefore, the analysis was repeated after removing all the extreme values and the results confirmed the same conclusion as mentioned in Table 4-25 (Table 4-26) suggesting that the extreme values had no significant impact on the outcome.

Table 4-26: Mann-Whitney U test for the difference in lesion Area (Area diff) (without the extreme values)

Fluoride Conc.	Asymp. p-value
0.0 ppm	0.177
0.5 ppm	0.904
1.0 ppm	0.497

4.6 Intra-examiner reproducibility

4.6.1 Intra-examiner reproducibility for ΔF

Twenty-three enamel slabs (15%) were randomly selected and reanalysed at the beginning of the experiment. The bias or the mean of the difference was 7% which is close to 0 (Table 4-27), meaning that there was a good level of agreement.

The 95% levels of agreement were -0.33 and 0.19. This variation was not considered of clinical or statistical importance therefore showing good agreement between readings.

The Intra-class Correlation Coefficient (Table 4-28) was 0.99 which shows excellent reproducibility. The Bland-Altman plot (Figure 4-6) also illustrates there was good agreement when the sample was re-tested.

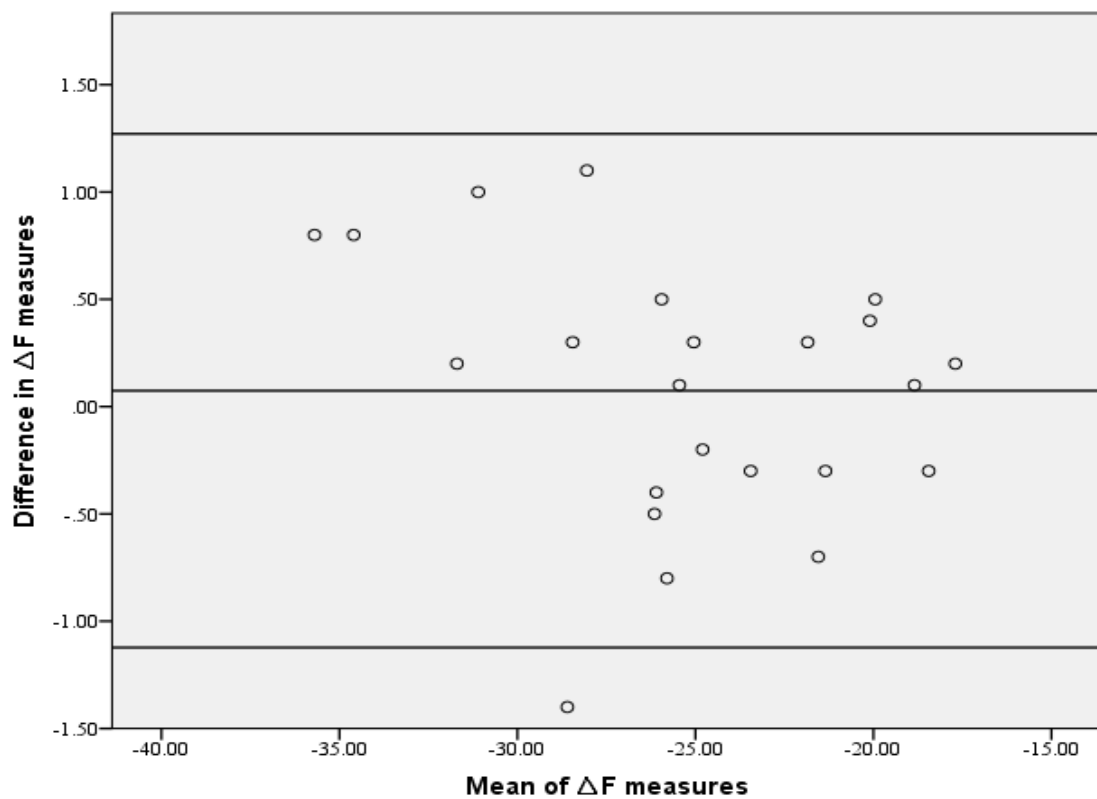
Table 4-27: Paired sampled Test for ΔF measures

	Mean	Std. Deviation	95% Confidence Interval of the Difference		p-value
			Lower	Upper	
ΔF 2 nd reading - ΔF 1 st reading	0.0739	0.610	-0.190	0.337	0.568

Table 4-28: Intra-Class Correlation Coefficient for ΔF measures

	Intra-Class Correlation	95% Confidence Interval		F Test with True Value 0
		Lower Bound	Upper Bound	p-value
Single Measures	.993	.983	.997	<0.001
Average Measures	.996	.992	.998	<0.001

Figure 4-6: Bland-Altman Plot for reproducibility of ΔF measures at the beginning of the experiment



4.6.2 Intra-Examiner Reproducibility for ΔQ

The same 15% randomly selected slabs as in ΔF were also reanalysed at the beginning of the experiment for ΔQ . The bias or the mean of the difference was -449.8 \%px^2 (Table 4-29). The 95% levels of agreement were -1814.87 and 915.25 . This variation was not considered to be of clinical or statistical importance therefore showing good agreement between the readings.

The intra-class correlation coefficient (Table 4-30) was 0.99 which shows excellent reproducibility. The Bland-Altman plot (Figure 4-7) again showed good agreement when re-tested.

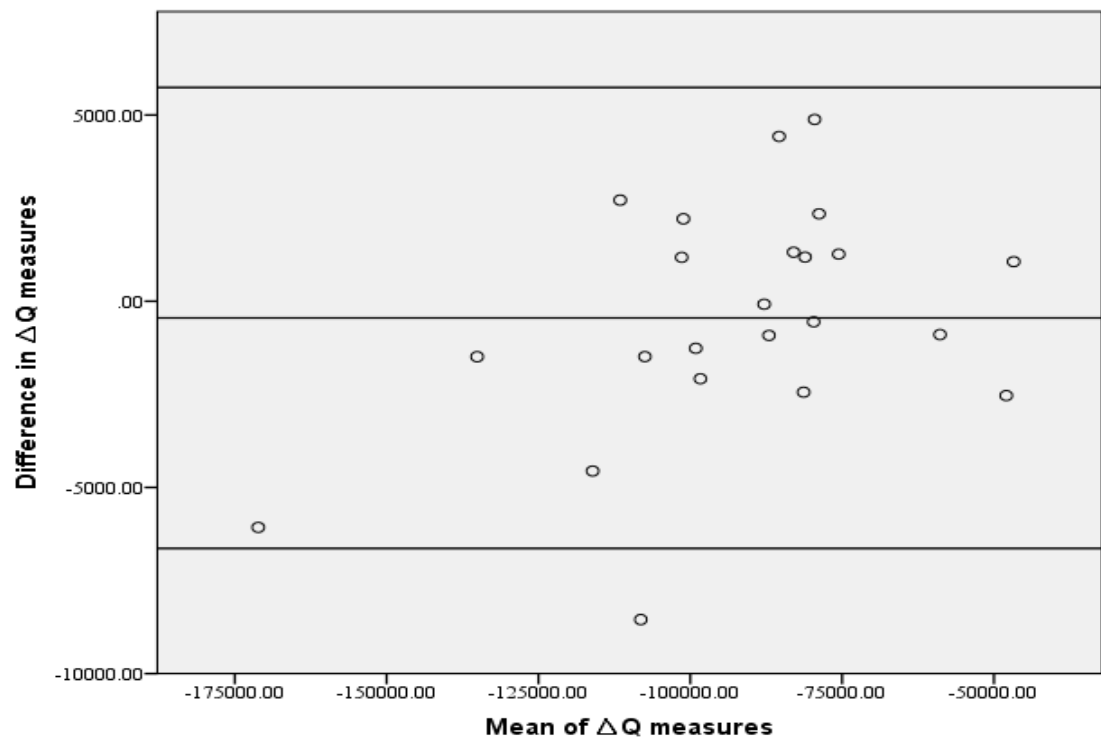
Table 4-29: Paired Samples Test for ΔQ measures

	Mean	Std. Deviation	95% Confidence Interval of the Difference		p-value
			Lower	Upper	
ΔQ 2nd reading - ΔQ 1st reading	-449.809	3156.701	-1814.869	915.259	0.502

Table 4-30: Intra-Class Correlation Coefficient for ΔQ measures

	Intra-Class Correlation	95% Confidence Interval		F Test with True Value 0
		Lower Bound	Upper Bound	p-value
Single Measures	0.993	0.985	0.997	<0.001
Average Measures	0.997	0.992	0.999	<0.001

Figure 4-7 Bland-Altman Plot for reproducibility of ΔQ measures at the beginning of the experiment



4.6.3 Intra- Examiner Reproducibility for Area

The same 15% randomly selected slabs as in ΔQ were also reanalysed at the beginning of the experiment for the lesion Area. The bias or the mean of the difference was 16.29 px² (Table 4-31). The 95% limits of agreement were -16.69 and 49.26. This variation was not considered to be of clinical or statistical importance therefore showing good agreement between the readings.

The intra-class correlation coefficient (Table 4-32) was 0.99 which shows excellent reproducibility. The Bland- Altman plot (Figure 4-8) also showed good agreement as the two previous QLF parameters.

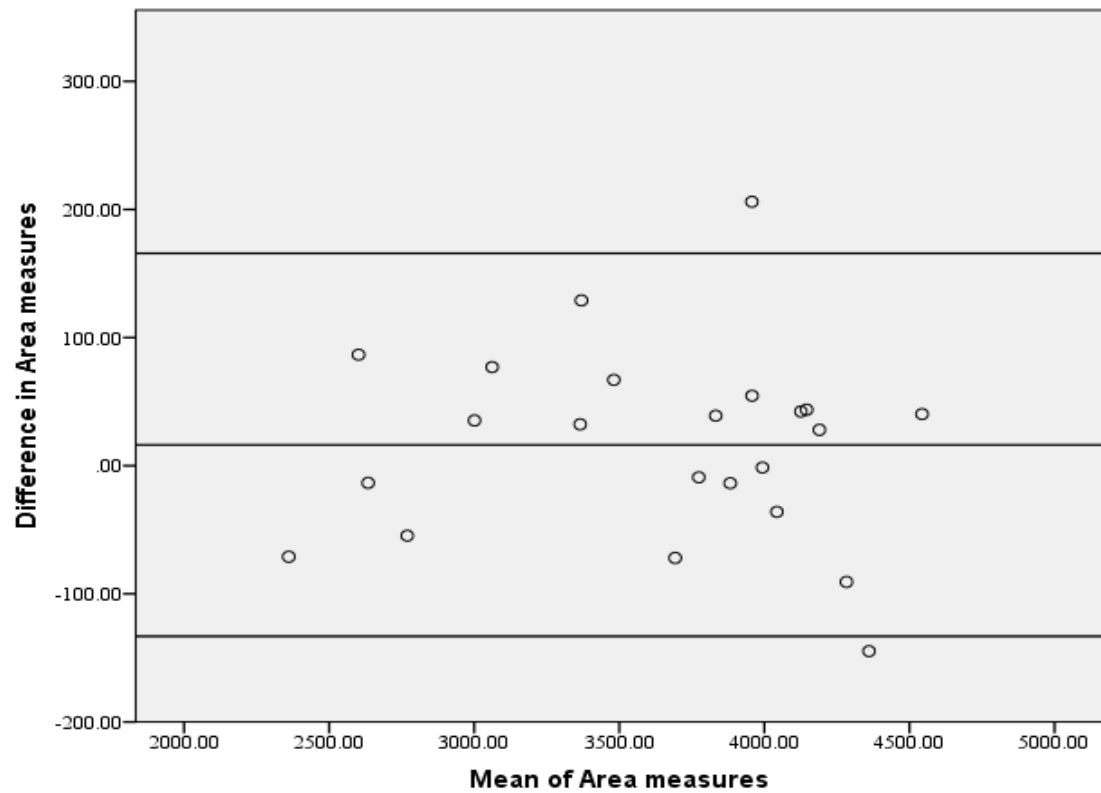
Table 4-31: Paired Samples Test for Area measures

	Mean	Std. Deviation	95% Confidence Interval of the Difference		p-value
			Lower	Upper	
Area 2 nd reading - Area 1 st reading	16.287	76.250	-16.686	49.260	0.317

Table 4-32: Intra-Class Correlation Coefficient for Area measures

	Intra-Class Correlation	95% Confidence Interval		F Test with True Value 0
		Lower Bound	Upper Bound	p-value
Single Measures	0.993	0.983	0.997	<0.001
Average Measures	0.996	0.991	0.998	<0.001

Figure 4-8 Bland-Altman Plot for reproducibility of Area measures at the beginning of the experiment



4.7 Summary of the results

ΔF (Fluorescence loss in %):

1. With regards to comparing between the different fluoride concentration levels in milk within each frequency separately:
 - a) For the Interrupted frequency, it was evident that there was a statistically significant difference in the ΔF values between concentrations 0.0 ppm F and 1.0 ppm F in milk. The greatest improvement was noted at the 1.0 ppm F concentration level followed by the 0.5 ppm F concentration. However, there was no statistically significant difference when comparing 0.5 ppm F to the other concentrations (0.0 ppm F & 1.0 ppm F).
 - b) As for the Daily frequency, the improvement seemed to increase as the level of fluoride was higher and it was clear that there was a statistically significant difference when comparing between the concentrations 1.0 ppm and 0.0 ppm F as well as between 1.0 ppm F and 0.5 ppm F. However, there was no statistically significant difference between 0.0 ppm F with 0.5 ppm F.
2. In relation comparing between the frequencies, there seemed to be no statistically significant difference when comparing between the frequencies at the three levels of fluoride concentrations. This would suggest that although both frequencies showed improvement

(remineralisation of the lesion) as the level of fluoride concentration was higher, the ΔF results were insignificant as to which frequency pattern resulted in the most improvement.

3. When comparing between baseline and final readings for the ΔF values, there was an overall statistically significant improvement between all the baseline and final readings for all the concentration levels within both the interrupted and daily frequencies. In general, there was more improvement as the fluoride concentration level increased. The percentage improvement was higher for the interrupted frequency than the daily frequency at levels 0.0 ppm F and 0.5 ppm F, while it was higher for the daily frequency at 1.0 ppm F.

ΔQ ($\Delta F \times \text{Lesion Area}$):

1. With regards to comparing between the different fluoride concentration levels in milk within each frequency separately:
 - a) For the Interrupted frequency, it was clear that there was a statistically significant difference between concentration levels 1.0 ppm F and 0.0 ppm F where the higher fluoride concentration level was better.
 - b) As for the Daily frequency, there was a statistically significant difference between fluoride concentration levels 0.5 ppm and 1.0 ppm as well as between 0.0 ppm and 1.0 ppm. However, there

was no statistically significant difference between 0.0 ppm and 0.5 ppm. This is similar to ΔF in conclusion where the highest fluoride concentration level in milk shows the most improvement in lesion remineralisation.

2. Considering the difference between the two frequencies, there was a general improvement in ΔQ values with evidence of lesion remineralisation progress as the level of fluoride concentration increased but there was no statistically significant difference between the frequencies at concentration levels 0.0 ppm F or 1.0 ppm F. The results did show evidence of a statistically significant difference between the interrupted and daily frequencies when fluoride concentration level in milk was at 0.5 ppm F ($p < 0.05$). This was in favour of the interrupted frequency and could be attributed to the apparently low standard deviation compared to the other concentration levels.
3. When comparing between baseline and final readings for ΔQ values, it was clear that there was a statistically significant improvement between all the baseline and final readings at all the fluoride concentration levels for both the interrupted and daily frequency patterns. In general, there was more improvement as fluoride concentration level increases. The percentage improvement was higher for the interrupted frequency than the daily frequency at levels 0.0 ppm F and 0.5 ppm F, while it was higher for the daily frequency at 1.0 ppm F.

Lesion Area:

1. With regards to comparing between the different fluoride concentration levels in milk within each frequency separately:
 - a) Within the Interrupted frequency, the lesion area values showed no statistically significant difference between the means for any of the fluoride concentration levels. There was no consistent pattern of change in the lesion area between the concentration levels.
 - b) For the Daily frequency, there was no significant difference between the concentration levels and like the interrupted frequency, there seemed to be no clear pattern of improvement for the lesion area. This might be due a number of confounding factors other than the presence of extreme values.
2. For the difference between the two frequencies, the inconsistent pattern of the lesion area readings is also evident when comparing the fluoride concentration levels between the frequencies. There was no statistically significant difference found between any of the fluoride concentration levels when compared with their counterparts in the opposing frequency pattern.
3. When comparing the baseline and final readings, there was a statistically significant difference between baseline and final readings for lesion Area showing general improvement in remineralisation.

The percentage improvement was the highest for the daily frequency at concentration levels 0.5 ppm F.

5.0 DISCUSSION

Following the development of community-based schemes in the late 1980's, it was evident from the available literature that milk can provide an alternative cost-effective vehicle for the delivery of fluoride owing to the cariostatic properties of the mineral, protein and fat contents of the milk. However, it is of importance to also consider the applicability of such programmes where neither water nor salt fluoridation is possible (Banoczy et al., 2009; Banoczy et al., 2013). By increasing the concentration of fluoride ion at the enamel/plaque junction, this results in a decrease in enamel demineralisation rate, increase in its remineralisation rate as well as a decrease in the rate of plaque acid production leading to caries prevention (Yeung et al., 2015).

When searching through the literature, it was clear that the majority of studies that investigated the effects of providing fluoridated milk to school children varied from randomised controlled trials to community based caries preventive programmes reporting statistically significant reductions in caries experience compared to the control groups. (Banoczy et al., 2009). The amount of fluoride added to milk was generally in the range of 0.5 to 1.0 mg per day (Banoczy et al., 2013). It was also evident from those studies that the duration of fluoridated milk consumption by children was between 180-200 days per year (i.e. school days only), although there were a few studies that had supplied the children in their test groups with fluoridated milk for up to 300 days, these studies had shown significant clinical effectiveness in caries reduction but they were not considered to be of high-quality (Banoczy et al., 2009).

The available studies suggested obvious clinical effectiveness on caries prevention of fluoridated milk in both the primary and permanent dentitions in school children and is most beneficial when started at an early age (Banoczy et al., 2009). However, there still seems to be a shortage of good quality evidence with definite conclusions on the risks and benefits of milk fluoridation and what the optimal duration of fluoridated milk consumption should be.

This current in vitro study, therefore, aimed to investigate the clinical effectiveness on caries prevention from increasing the amount of fluoride in milk from 0.5 mg F to 1.0 mg F per school day as well as to evaluate the effects of varying the frequency of drinking fluoridated milk (i.e. interrupted and daily frequency).

5.1 The in vitro model

The present study had used an in vitro pH-cycling model to assess the anti-cariogenic effect of different concentrations of fluoridated milk on enamel remineralisation and demineralisation where minerals that are lost or gained are proof of the efficacy of such experiments.

In-vitro models are well established in the literature; they allow conducting experiments under well-controlled conditions which can be easily modified (White, 1995). In-vitro models were used to facilitate the understanding of the caries process by explaining how fluoride applies its anti-caries properties. These models then aided in the creation of adequate quantitative data to properly design clinical trials in the future. Another advantage of in-vitro models is that they involve a smaller sample size (Buzalaf et al., 2010).

On the other hand, in vitro studies carry potential limitations which include

the inability to mimic the complicated intra-oral conditions that lead to development of carious lesions bearing in mind that the oral environment changes over time and differs from one person to another (Steiner-Oliveira et al., 2007). The in vitro models cannot simulate solid surface area to solution ratios or saliva to plaque fluid configurations found in vivo, and they are also unable to properly imitate topical use and clearance from the oral cavity (Buzalaf et al., 2010). Another limitation is the absence of intra-oral microorganisms which results in focusing on the physical-chemical aspects of dissolution (Oliveira et al., 2007).

5.2 Design of the study

This study comprised of six groups in total, where three groups were allocated to either the Interrupted or Daily frequency. The groups were divided up as follows:

Group 1 (control, 0.0 ppm F in milk), Group 2 (0.5 ppm F in milk), and Group 3 (1.0 ppm F in milk) included the enamel slabs that were dipped into milk on allocated days (Interrupted frequency manner), while the remaining three groups: Group 4 (control, 0.0 ppm F in milk), Group 5 (0.5 ppm F in milk) and Group 6 (1.0 ppm F in milk) included the enamel slabs that were dipped into milk every day (Daily frequency manner) over the 60-day period of this experiment.

This study was designed to be randomised and single blinded where the enamel slabs were randomly allocated into one of the six groups using an online random number generator, and the blindness was implemented to try and prevent the creation of bias.

5.2.1 The bovine teeth caries model

Various studies have used bovine enamel as a substitute for human dental enamel in experiments investigating demineralisation and remineralisation protocols (Attin et al., 2007). It would have been both ideal and preferable to use human teeth for this current study. However, due to the limitations in obtaining them in large numbers for in vitro models along with significant challenges with regards to supplying human teeth and the related costs that accompanied such a process, it was decided and agreed upon, after reviewing the available literature, that the second-best alternative, in this case, would be the enamel of bovine origin. Bovine teeth have the advantage of being readily available from local abattoirs as well as their larger sizes which make them easier to handle and process in the laboratory in addition to maximum use of their large flat surface area (Laurance-Young et al., 2011).

It has been proven that human and bovine enamel behaved in a similar manner to acidic challenges and remineralisation conditions. Mellberg had also deemed bovine teeth to be a suitable replacement in studies evaluating the demineralisation of enamel (Mellberg, 1992; Attin et al., 2007).

Milk fluoridation programmes mainly involve young school children and research in this field aims to prove the benefits of using milk as a vehicle for providing fluoride aiming to reduce demineralisation and enhancing remineralisation of enamel. Although bovine teeth were used in this study, it has been mentioned in the literature that these teeth seem to resemble primary human enamel with regards to faster caries progression as opposed to adult human enamel (Featherstone and Mellberg, 1981; Attin, 2007). Apart from the obvious macroscopic dimensional dissimilarities between bovine and human

teeth, their morphological microstructures closely resemble one another including the number and density of dentinal tubules and collagen matrices in both primary and permanent dentitions (Laurance-Young et al., 2011).

Although differences exist between human and bovine enamel, the available evidence suggests that these dissimilarities are not so significant so as to exclude the use of bovine teeth in research considering that both substrates react in the same way to acidic challenges and remineralisation conditions (Mellberg, 1992; Laurance-Young et al., 2011).

5.2.2 Preparation and storage of enamel slabs

The type of storage media chosen in this study for the extracted teeth was distilled water and 0.1% thymol (Sigma Aldrich). A variety of methods have been used to preserve the structural integrity of extracted teeth, in order to maintain their baseline conditions, these include formalin, sodium hypochlorite, alcohol, glutaraldehyde, autoclaving and thymol (Dominici et al., 2001; Kumar et al. 2005; Shellis et al., 2011). Thymol has largely been used in in vitro and in situ studies due to its bacteriostatic properties and ability to inhibit growth of fungi. Humel et al. also reported that thymol showed no effect on dentine permeability, microleakage or bond strength (Humel et al., 2007).

The enamel slabs in this study were prepared from bovine incisor teeth where the buccal surfaces were only used as they offer larger flat surfaces to be utilised for the experiment in addition to providing a more uniform thickness of enamel and dentine compared to the palatal surfaces. It has already been mentioned that bovine enamel is a suitable substitute to human enamel in laboratory based studies as both possess potentially the same physiochemical

characteristics and chemical constituents. No significant differences were found in terms of the content of carbonate, polishing ability, indices for refraction and luminescence, therefore, proving comparable demineralisation and remineralisation features (Feagin et al., 1969; Putt et al., 1980).

5.2.3 Creation of artificial carious lesions

Reports by a number of studies mentioned that bovine teeth had been used for assessing and monitoring the prevention or reversal of the carious process in vitro (Mellberg and Loertscher, 1979; Mellberg, 1980).

Artificially created carious lesions were produced under various settings to evaluate the types of lesions and their progression (Featherstone and Mellberg, 1981). These methods include dipping the enamel slabs in either an acidified demineralisation gel (hydroxyethyl, methylcellulose), solution (Carbopol) or buffer gel (ten Cate et al., 1996; Toth et al., 1997; Amaechi et al., 1998; Lippert et al., 2011; Malinowski et al., 2012; Lippert and Juthani., 2015). It was evident that the creation of artificial caries-like lesions using buffers was faster, 1.5 times deeper with more loss of minerals (Amaechi et al., 1998) as opposed to the shallower lesions produced by the acidified demineralisation gels which required a longer duration to develop at similar pH and concentration levels (Featherstone and Rodgers, 1981). These studies also reported that the surrounding temperature plays a role in the degree of demineralisation with 37°C having a greater impact than 20°C room temperature. However, the susceptibility of the tooth surface to caries among different tooth types is also related to the salivary composition, flow rate and the morphology of teeth in addition to the intrinsic susceptibility differences (Shellis, 1984).

There seemed to be notable variation in the duration used for creation of artificial carious lesions varying between 7 -14 days in some studies (Toth, 1997; Lippert et al., 2012; Malinowski et al. 2012b; Gomez et al., 2014; Lippert and Juthani, 2015) while others used up to 4 days only (Itthagaran et al., 2011; Ongtenco et al. 2014).

The demineralisation gel used to create artificial white spot lesions in this study was the hydroxyethyl cellulose acidified gel which produces artificial lesions that can be compared to natural lesions histologically (Wefel et al., 1995). The slabs were immersed in universal tubes containing the hydroxyethyl gel and were left at room temperature for up to 10 days until the lesions were visible. This method was used by Malinowski et al. in their in vitro study, however, they kept the slabs in the demineralising gel for a shorter (7 day) duration (Malinowski et al., 2012b). There is no consensus as to what the optimal duration is for the creation of artificial lesions, and the local protocol currently being applied by the Leeds Dental Institute was followed which is a 7-10 days period until a white spot lesion can be visually identified (ten Cate and Duijsters, 1982).

The artificial white spot lesion created included an average ΔF of approximately 30.4 \pm 5 (range 25-35). However, that range would only involve a total of 127 slabs which is below the required sample size for this current study and that would result in a smaller sample size leading to an increase in the chances of a Type II error skewing the results, which could possibly decrease the power of the study. It was therefore decided to alter the average ΔF range to 22-37. The average range in this study was higher than that reported by Bataineh, 2014 (20.7) but less than what was described by Lippert, 2011 (55.6). Although these

studies used the same QLF system, their aims, objectives and remineralisation methods were different.

5.2.4 The pH cycling experimental model

The different pH cycling protocols available have played a role in providing much-needed understanding of the caries process and ways to prevent it. These protocols essentially consist of exposing enamel and or dentine to a series of remineralisation and demineralisation attacks that simulate the dynamic intra-oral processes (White, 1995). Although they are widely used, these models still carry numerous limitations, one of which is that they cannot replicate the microbiological environment of the oral cavity (Steiner-Oliveira et al., 2007).

The pH cycling model used in this study was based on the work of ten Cate and Duijsters, (1982), which is now considered the method of choice in the analysis of caries pathogenicity, to investigate different methods for caries prevention and also for the development of new products (ten Cate, 2015). This method involves cyclic interruptions of the pH neutral condition with acid/ demineralisation challenges, to resemble the oral environment during sugar intake (ten Cate and Duijsters, 1982; White, 1995; ten Cate, 2015). The pH cycling protocol carried out for this study was developed at the University of Leeds and previously used at the Leeds Dental Institute, Paediatric Dentistry Department (ten Cate and Duijsters, 1982; White, 1995).

The duration of this study's pH cycling model was carried out over a 60-day duration period which is longer than that mentioned by previous studies. Other studies have carried out their pH cycling regimes mostly over 14 days (ten Cate and Duijsters, 1982; Malinowski et al. 2012; Ongtenco et al., 2014). While

others had chosen 10, 15, 20, 21, 30 and up to 99 days to assess the remineralisation effects of different concentrations of fluoridated milk, fluoridated tooth paste or CPP-ACP products (Arnold et al., 2003; Itthagarun et al., 2011; Lippert et al., 2012; Bataineh, 2014; Oliveira et al., 2014).

It was decided that a 60-day period would provide sufficient time to demonstrate changes on the prepared, pre-demineralised enamel slabs. At the beginning of each day, depending on which frequency (interrupted, daily) and fluoride concentration the slabs were randomly allocated to, the slabs were dipped in fluoridated milk (0.0 ppm, 0.5 ppm or 1.0 ppm) for 5 minutes followed by 10 minutes of a milk/saliva slurry (1:3). The enamel slabs were exposed to the test or control milk and milk/saliva slurry twice a day, before and after the acid challenges, to replicate how the natural saliva dilutes the milk intra-orally.

After that, the slabs were subjected to 5 subsequent challenges of acetic acid solution (pH 4.8), each lasting for 2 minutes to simulate the cariogenic attack (Duggal et al., 2001; Issa et al., 2003). The slabs were placed in supersaturated artificial saliva between the cariogenic challenges during the day for not less than 60 minutes each time while kept in a saturated solution of artificial saliva overnight. The difference in both formulations is that the morning saliva is supersaturated with calcium and phosphate to permit the remineralisation of the slabs, while the night time saliva was mainly to keep the slabs in a neutral state without allowing any mineral exchange.

5.2.5 Study materials used

The milk used for this experiment included fresh, semi-skimmed pasteurised cow's milk (Tesco British Semi Skimmed Milk). The non-fluoridated milk groups (0.0 ppm F) were used as the negative controls to evaluate the remineralisation effects from the artificial saliva solutions. The other fluoride concentrations added to milk included 0.5 ppm F and 1.0 ppm F. The solution was made up so that 1 ml of aqueous sodium fluoride (NaF) solution added to 1 Litre of milk would result in a final concentration of 1 ppm F. Banoczy et al. reported that the bioavailability of added fluoride to milk is shown to be satisfactory in all types of milk consumed (whole, low-fat, fresh, pasteurised, sterilised, liquid or powder) (Banoczy et al., 2013).

Community-based milk fluoridation programmes offer school children 189-200ml of milk with added fluoride to be ingested during school days (approximately 180-200 days/year). The fluoride concentrations added usually vary between 2.5 and 5 ppm F depending on the programme and location/country. These fluoride concentrations entail that the total amount of fluoride ion ingested by children equals 0.5 and 1.0 mg F/day respectively. Which is equivalent to the amount of fluoride used for dipping the pre-demineralised enamel slabs during the pH cycling experiment. As 1mg F/ L equals 1ppm F, then 1mg F/0.2 L (200ml) results in 5 ppm F and 0.5mg F in 200ml would equate to 2.5 ppm F. Alternatively, If the fluoride concentration levels in this study were to have been increased to 2.5 and 5 ppm F instead of 0.5 and 1.0 ppm F then that would imply that the children would end up ingesting 2-5 times more fluoride than what is proposed by other studies.

In some districts in the United Kingdom where dental caries is reported to be high, and water fluoridation schemes are not possible, the implementation of community milk fluoridation programmes were initiated as a method for improving dental health. Although the evidence of caries reduction effectiveness from the UK programmes are equivocal, it was suggested that the possible reasons these programmes might be having issues, include: some faults within the delivery systems and the number of days the children are absent from school (Ketley et al., 2003; Foster and Tickle, 2013).

Despite that, there are now over a million children receiving fluoridated milk as a form of prevention against dental caries based on evidence from clinical studies worldwide as fluoridated milk provides both topical and systemic effects resembling the effects of fluoridated water (Banoczy et al., 2013).

5.3 Method of analysis: Quantitative light Induced Fluorescence (QLF)

With the development of new sensitive caries detection methods, researchers have been able to study small, early mineral changes using less invasive methods (Nyvad, 2004). QLF has been reportedly used in pH cycling studies (Al-Khateeb et al., 1997) as well as in vivo studies (Tranaeus et al., 2001; Karlsson, 2010) to monitor the white spot lesions by quantifying the changes in the mineral content of the lesions during demineralisation and/or remineralisation for both primary and permanent teeth (Ando et al., 2001). Other benefits of this method, in addition to facilitating longitudinal monitoring of an early lesion's demineralisation and/or remineralisation, is that it does not involve destruction of the enamel slabs as well as considerably reducing the time

needed to analyse a large number of samples as opposed to TMR method (Gmür et al., 2006).

QLF analysis of the slabs was performed at the beginning of the experiment, and on completion of the 60- day pH cycling regime (final measures were analysed on the 61st day). The images of the lesions were randomised for the analysis to ensure that the examiner was blinded to the groups by recoding the groups on the QLF machine as well as the slab numbers in each group.

The distance between the enamel slabs and the camera was fixed throughout the analysis to ensure the ability to repeat the measurements. A silicone base mould was developed in our study to guarantee that the placement of the slabs on the camera jack's platform were secured in the same position throughout the analysis. This ensured that each one of the pictures were taken in the exact same way. The analysis was done using the proprietary software where a patch was drawn around the white spot lesion, making sure the patch was on sound enamel but as close to the lesion as possible, which then allowed the software to reconstruct the enamel surface using the pixel values from the sound enamel and subtracting them from the pixels of the lesion of interest.

This QLF method has been validated against TMR which is considered to be the gold standard in the analysis of mineralisation, where QLF has proven to show high levels of sensitivity. A good correlation to TMR was reported for the measurement of early artificial carious lesions for human teeth ($r=0.84$) and bovine teeth ($r=0.74$ and 0.83) (Al-Khateeb et al., 1997; Hall et al., 1997).

To say that QLF is reliable, it needs to be able to reproduce the same results when the same system is being used in numerous other instances at different points in time. This is usually reported as inter or intra-examiner

reproducibility, shown statistically as intra-class correlation coefficient (ICC) or Kappa score. ICC is the preferred statistical method for reporting as the QLF data are continuous data rather than categorical. Pretty et al. reported that according to previous data, QLF is considered to be overall objective apart from when the images are being captured and during successive analysis. Nonetheless, these instances have not had a negative impact on the results (Pretty et al., 2003). Inter- and intra-examiner reproducibility/repeatability were assessed both in vitro and in vivo demonstrating excellent results. Tranaeus et al. reported the intra-class coefficient calculation (ICC) for both image capturing and analysis stages in vivo. The inter-examiner reproducibility was between 0.95-0.98 for the image capturing stage. As for the analysis stage, the ICC for the inter-examiner repeatability was close, being between 0.95 and 0.99 and the intra-examiner reproducibility for this stage was also between 0.93 and 0.99 (Tranaeus et al., 2002; Pretty et al., 2003). Pretty et al. described the repeatability of QLF in vitro for the analysis stage only, where the ICC was 0.93 and 0.92 for the intra-examiner and inter-examiner reliability tests respectively (Pretty et al., 2002a; Pretty et al., 2003). The intra-examiner repeatability for our current study was limited to the analysis stage of the QLF examination, showing an ICC value of 0.99 for intra-examiner reproducibility which is very good.

In an attempt to eliminate potential operator bias in the present study, the investigator received the appropriate training of the system and software prior to initiation of the experiment. However, there are a number of confounding factors that have been described in previous studies to affect the QLF readings. The angulation of the samples during analysis (Ando et al., 2004), the morphology of the slabs since they are curved rather than flat surfaces (Tranaeus et al.,

2002), delineation of the lesion area patch during analysis i.e. a small patch produces a large lesion area while a large patch results in a small one (Tranaeus et al., 2002), dehydration of the lesion (Pretty et al., 2004), underlying thickness of enamel and dentine (Rousseau et al., 2002; Ando et al., 2003), and the effect of ambient light during QLF analysis (Pretty et al., 2002; Higham et al., 2005).

The QLF images and analyses can be influenced by the amount of dehydration of the lesion being tested causing increase in the fluorescence loss in vitro occurring within a few minutes in atmospheric air (Angmar-Månsson et al., 2001). The light scattering increases due to the greater index of refraction of dry enamel crystals as opposed to wet crystals, it is therefore, important to maintain consistency with the drying time in vitro (Wu et al., 2010). This highlights the importance of having a skillful operator with prior experience in the system as a novice would probably require a longer time to analyse the slabs risking more dehydration of the slabs (Tranaeus et al., 2002). The slabs in our study were dried using compressed air for 15 seconds prior to image capturing as recommended by Pretty et al. (2004).

As for the effect of ambient light, Pretty et al. confirmed that the use of QLF in a room where the ambient light is 88 lux is appropriate, which means that the lighting is sufficient enough to write notes (Pretty et al., 2002). The QLF system in this study was placed in a completely darkened room during image capturing in an attempt to control the effect of lighting.

Some studies have speculated that bovine enamel might be covered with a thin layer of cementum (Ainamo, 1970). However, the smoothening of the enamel surface, in this study, using grit abrasive paper following tooth sectioning would have eliminated that cementum layer if it were present. Rousseau et al.

did state that although the underlying dentine might play a role in the fluorescence radiance, this did not impact the QLF analysis greatly as all of the fluorescence radiance identified was produced from the enamel structure. This would imply that the dentine and/or DEJ do not significantly alter the mineral loss during the QLF analysis (Rousseau et al., 2002). This factor was not completely standardised in our study as the bovine teeth were sectioned where only the buccal surfaces were utilised and no alterations were carried out on the dentine thickness.

Ando et al. advised to place the slabs perpendicular (90°) to the camera when the white spot lesion images were being captured on the smooth enamel surfaces, they also mentioned that it is important to try and control these conditions as angulations more than 20° had an effect on the QLF parameter analysis (Ando et al., 2004). In order to standardise the conditions in our study, the slabs were stabilised on plastic rods using “sticky wax” while trying to ensure that each slab was flat and perpendicular to the working bench as much as practicable. The silicone base mould created in this study also helped maintain the slabs perpendicular to the camera lens.

Some authors have reported that the presence of surface defects or stains around the artificial carious lesion may also alter the analysis with QLF (Meharry et al., 2012). It is, therefore, important to ensure that the enamel slabs of the selected teeth to be used comprise smooth, intact surfaces. For this reason, all the extracted teeth were screened using trans-illumination and transmitted light with a low power microscope, and the ones that presented with cracks, caries, defects or surface loss were therefore excluded from our study.

5.3.1 QLF results

As previously mentioned in the results part (section 4.0), QLF gives information on the loss of fluorescence (ΔF), lesion Area in mm² and the lesion volume (ΔQ) which is an integration of these two parameters. Hence, ΔQ indicates the amount of mineral loss relative to the lesion's size and severity. While the results for all the parameters were statistically analysed, it was evident that both ΔF and lesion Area results showed different outcomes when taken into account separately and could not be considered good indicators of lesion progression or regression (Ando et al., 2004). For this reason, it was decided that the lesion volume (ΔQ) was chosen to be the main guide of mineral loss and demineralisation or remineralisation of the lesions in our study.

5.4 The effect of fluoridated milk on enamel subsurface lesion remineralisation

It was clear from the results of this current study that fluoridated milk (among all concentrations) exhibited a clear dose-response effect as even the control groups (0.0 ppm F) showed evidence of remineralisation of the subsurface lesion, although not as substantial as the higher concentrations. This phenomenon could be attributed to the supersaturated artificial saliva remineralising solution effect against the demineralisation caused by acetic acid challenges in addition to the calcium and phosphate present in the 0.0 ppm F which might have also contributed to remineralisation. Moreover, the fluoridated milk's influence on tipping the demineralisation/ remineralisation gradient towards mineral gain was clear as the total time the slabs were in contact with fluoridated milk per day was equivalent to 30 minutes (15 minutes/ twice daily)

compared to the total of 10 minutes they were in contact with the acetic acid solution.

The literature has previously reported that the use of natural saliva causes a more rapid effect on remineralisation of artificial lesions than artificial saliva. The artificial saliva did, however, show a remineralising effect beyond the superficial lesion layers (Silverstone, 1972).

One of the limitations of in vitro models is the absence of plaque or the overlying pellicle, acting as a reservoir for fluoride and mineral deposits which are later released when the plaque pH level is lowered during an acidic attack. The addition of plaque was not possible in the present study due to its design, and therefore acetic acid was used instead of the carbohydrate challenge normally considered in in situ models. Two experimental designs have highlighted the impact of saliva and plaque when fluoridated milk was being investigated. Petersson et al. (2002) reported a significant increase in fluoride level within saliva ($p < 0.05$) after consuming fluoridated milk and water. The salivary fluoride concentrations had returned to baseline values after 2 hours compared to the fluoride storing potential of plaque which showed a two-fold statistical increase evident after 2 hours of fluoridated milk and water consumption. In addition, the study by Whitford et al. also indicated that although the mode of drinking fluoridated milk did not affect the concentration of fluoride in children's plaque or saliva, these fluoride levels in plaque or saliva were altered according to the fluoride concentration in milk (Whitford et al., 2016).

The available evidence suggests that the most common fluoride concentrations used in milk related in vitro/situ/vivo studies or school schemes were 2.5 ppm and 5.0 ppm F within a 200ml milk carton which account for 0.5

mg F and 1.0 mg F/Day of the total amount of ingested fluoride ion respectively. This amount is equivalent to the concentrations chosen in this study as it was taken into account that if higher concentrations were to be used in vitro then comparison to what is currently being provided to school children would be difficult and it would also exceed the total amount of fluoride ion ingested as the milk needs to be swallowed by children. Care needs to be taken, and a limit of up to 1 mg F in 200 ml milk for children should be employed depending on the child's age and body weight which can be assessed using questionnaires and monitoring the urinary fluoride excretion. It is also important to highlight that a small proportion of fluoride interacts with the intrinsic ions in milk (i.e. calcium) or combines with the available milk proteins. Even though the calcium content in milk is 1200 mg/l, only a small proportion is available in the form of free calcium ions (80 mg/l) as most of the calcium is already combined with milk constituents such as citrate and casein. This amount will not result in calcium fluoride precipitation at fluoride concentration levels of 2.0 – 5.0 ppm F (Holt et al., 1981). Nevertheless, it was also evident that there is still no verification as to what the effective fluoride concentration level in milk school programmes should be in order to provide a positive preventive effect on the dentition while avoiding any harmful adverse effects.

Regardless of which frequency pattern, the groups were assigned to during the pH cycling. It was concluded that although all fluoride concentrations provided remineralisation, including the negative control groups (0.0 ppm F), the most significant improvement was clear with milk containing the highest fluoride concentration level (1.0 ppm F) for both ΔF and ΔQ parameters but not for the lesion Area. There was a significant difference between the negative control (0.0

ppm) and 1.0 ppm F only for both ΔF and ΔQ that had undergone the interrupted frequency pattern, compared to the significant difference found between 0.5 ppm and 1.0 ppm F as well as between 0.0 ppm and 1.0 ppm F for the daily frequency pattern.

Malinowski et al. also carried out an in vitro, single-blinded study following a similar pH cycling protocol for 14 days, where they investigated the dose-response effect of fluoridated milk containing 0.0, 0.25, 0.5, 1.0, 5.0 and 10.0 ppm F using TMR as their method of analysis. The results had shown that there was a trend of increased remineralisation as the fluoride in milk increased except for the negative control (0.0 ppm F) which resulted in demineralisation. Although TMR is more complex, it provides a more robust measurement of the lesion's progression or regression. There was a significant difference between the groups with the highest fluoride concentration levels (5.0 ppm and 10.0 ppm F) in this study but not less, with a clear benefit on enamel lesion remineralisation from 1.0-5.0 ppm F but not more. It was also reported that there was no significant difference when 0.5 ppm was compared to 1.0 ppm F (Malinowski et al., 2012b).

Finding comparable in vitro studies that had employed the same pH cycling methods, with similar methods of analysis has proven to be challenging. It is therefore very important to be cautious while extrapolating the results due to these differences in addition to other in vitro limitations mentioned earlier. Hence, validating those studies by using in situ models prior to making any recommendations is imperative.

Other in vitro models have exhibited the effective protective properties of fluoridated milk against enamel demineralisation using a 99-day pH cycling

model. The authors measured the effects by a computerised reconstruction of the artificial lesions after sectioning the samples. The conclusion was that milk containing fluoride (2.2 mg NaF/l =1ppm) was protective against acidic challenges. A loosely bound fluoride reservoir is formed by calcium and fluoride binding combined with the protective effects of calcium and phosphate in milk, and the formation of a hard surface layer by intrinsic milk proteins contributed to the fluoridated milk mechanism of action as the authors concluded in the study (Arnold et al., 2003).

The study by Ongtenco et al. had used higher levels of fluoride concentrations in milk than what had been applied in our present study (2.5 ppm F, 5 ppm F and 10.0 ppm F). Their pH cycling method and analysis techniques were also different, the polarized light microscope yields a qualitative evaluation while microradiography quantitatively assesses the changes in lesion depth and mineral content. Interestingly, they had found that there was no statistically significant difference when slabs were dipped once a day versus dipping on alternate days. The most significant reduction in lesion depth was shown with the lowest fluoride concentration level of 2.5 ppm used twice a day ($p<0.05$) rather than once a day or on alternate days over the 20-day study period. The higher concentrations of 5 ppm and 10 ppm did not show any significant difference or any increased improvement in lesion remineralisation. However, when compared to their negative controls, all fluoride concentration levels showed significant improvement in lesion depth (Ongtenco et al., 2014). Similarly, Itthagarun et al. also carried out an in vitro study investigating the effects of fluoridated milk on enamel's remineralisation efficacy. They also reported a significant decrease in lesion depth for all the study groups following

the 20-day experimental period. Although not significant, they had also noticed that the lesion depth showed slightly more decrease when the volume of fluoridated milk was doubled (from 25 ml to 50 ml). This was speculated as being due to the added availability of calcium, phosphate, milk proteins and fluoride to react with enamel which showed an 8% decrease in lesion progression. The other important aspect of their experiment was that the lowest fluoride concentration of 2.5 ppm in milk provided similar remineralisation potential as the other higher concentrations (Ittharagun et al.,2011). These studies did not assess concentration levels lower than 2.5 ppm F, and their pH-cycling protocol involved prolonged periods of demineralisation, milk treatment followed by remineralisation. Their protocol was completely different to our study's regime of alternating episodes of demineralisation and remineralisation which is considered a true pH cycling model.

Effects of concentrations and temperatures of fluoridated milk on enamel caries lesion re-hardening were also investigated. Lippert et al. prepared their fluoridated milk from powdered milk mixed with de-ionised water instead of the fresh pasteurised milk used in our study. The reason why tap water was not used in their study was because it contained up to 0.7 ppm F. Their pH cycling comprised of exposing the pre-demineralised enamel slabs to four hours of an acid attack followed by four separate episodes of fluoridated milk exposure (2.5, 5.0, 10.0 and 20.0 ppm F) each lasting up to 10 minutes, slabs were kept in a remineralising solution of a natural and artificial saliva mixture in between. Following their 15-day experiment, the authors have concluded that microhardness testing provided a more sensitive technique to detect the ability of the different treatment groups to re-harden incipient caries lesions. In addition,

higher fluoride concentrations and temperatures of milk resulted in more fluoride uptake and re-hardening of enamel. Increasing the temperature of fluoridated milk improved its positive effect which the authors explained could be due to the enhanced mobility of the fluoride ion as the temperature became higher, providing that the results from aqueous solutions can be generalised to milk (Lippert et al., 2012). Chilean milk fluoridation programmes currently add fluoride to powdered milk as part of the 'natural nutrition complementing programme' (PNAC) where every Chilean child from age 0-6 years is provided with a monthly supply of cow's milk powder; this scheme currently covers up to 90% of children living in rural communities in Chile. The trial ran for 4 years, and the authors have emphasised the important health and economic advantages of providing fluoridated milk products to children living in rural communities of Chile (Marino et al., 2001).

Another in vitro experiment by Toth et al. in 1997 confirmed the topical effect of fluoridated milk on enamel lesions. They incorporated 1 mg F and 10 mg F into their 14-day experiment, where the lesser concentration did not show any effect probably due to the short duration of the study. The most significant increase in enamel's fluoride content was exhibited by the group exposed to 10 mg F for 14 days. Interestingly, the fluoride ion value was mentioned in this study compared to others, and it seems that there is a general inconsistency in the reporting of fluoride doses in vitro studies which results in the confusion of the reader. It would be of benefit if future studies could standardise the reporting of fluoride doses to eliminate any misunderstandings. Fluoride doses up to 8-10 mg/l can be provided to children over eight years old according to Lippert et al. (2012) where the dose depends on the child's body weight. This is based on the

American Academy of Pediatrics (1986) report that the accepted daily fluoride dose intake for children should be 0.05-0.07 mg F/kg body weight per day.

There have also been reports from in situ studies on the preventative effect of fluoridated milk on artificial enamel carious lesions. It can be challenging to replicate all aspects of any milk fluoridation programme into an in situ experiment without any compromises being made such as the duration and timing of milk consumption. In situ models are generally cost effective and provide a better understanding of the important aspects of the caries process including the impact from intra-oral bacteria, diet, saliva and salivary flow. In order to create conditions that resemble the caries process, the subjects are required to use intraoral appliances with embedded enamel slabs. It was apparent that in situ models assessing the effects of fluoridated milk involve older subjects rather than children which are the ideal target population as they are the main subjects being provided milk during school programmes. If children were to be recruited to an in situ study, alterations to the appliances' design will have to be made, considering that children are not denture wearers. Poor compliance would be a major issue bearing in mind the difficulties in speech, discomfort and the inconsistent use of the appliances during the day and at night.

There were two in situ studies that had incorporated the same fluoride concentrations used in the current research study, 0.5 mg F and 1.0 mg F, except their subjects were provided with a volume of 200 ml of milk each day. Malinowski et al. (2012a) evaluated the effects of 2.5 ppm and 5.0 ppm F concentrations on preventing against enamel demineralisation with a cariogenic challenge when compared to 0.0 ppm F. All their treatment groups showed significant change in hardness from baseline and the percentage hardness for

the 5.0 ppm F group was higher than other groups although not significantly. A significant difference was found when comparing both 2.5 and 5.0 ppm F groups to 0.0 ppm F confirming that even low concentrations of fluoride produce an enamel surface that is more resistant to a strong cariogenic challenge. More recently, Cassiano et al. carried out a double blind randomised cross over in situ experiment, similar to the work done by Malinowski et. However, in addition to examining the amount of fluoride in milk, the authors have also assessed the impact of alternating the frequency of intake of fluoridated milk on the remineralisation of carious lesions of enamel and dentine. The percentage surface hardness recovery of enamel showed significant increase in the groups treated with fluoridated milk regardless of the concentration used with no significant difference reported between these groups. The highest remineralisation was obvious when 0.5 mg F and 1.0 mg F were added to 200 ml milk and consumed daily (Cassiano et al., 2017). Their results were in agreement with other in vitro studies as milk containing 2.5 ppm F improved enamel remineralisation significantly and higher concentrations did not result in any additional effects (Ittharagun et al., 2011, Malinowski et al., 2012b). This was also in agreement with our findings, as ΔQ showed a significantly higher improvement in favour of the interrupted frequency for 0.5 mg F. However, when each frequency was considered separately, 1.0 mg F in milk provided more significant remineralisation.

The other in situ study investigating the remineralising effects of fluoridated milk with different concentration was performed by Lippert et al. (2014), they had added 1.5 mg F and 3.0 mg F into their milk which was higher than what is normally incorporated into milk fluoridation programmes. Their overall point was

that the benefit/risk ratio can be enhanced by increasing the dose of fluoride in a smaller volume of milk. A comparison to their results could not be made due to their high fluoride doses.

An attempt to compare in vitro to in vivo studies might not be valid. Nevertheless, a randomised controlled trial with two parallel groups carried out in Denmark investigated the remineralising effect of daily intake 1.0 mg of fluoridated milk on enamel adjacent to orthodontic appliances. Their study was over two and half months and QLF was used to assess mineral loss or gain at baseline and after treatment. Final ΔF readings displayed significant reduction in demineralisation within the test group compared to the significant increase in the control group ($p < 0.05$). The authors concluded that fluoridated milk consumed on a daily basis may assist the remineralisation of white spot lesions adjacent to orthodontic appliances (Sköld-Larsson et al., 2013).

A number of systematic reviews have been published discussing the effectiveness of milk fluoridation on the reduction of dental caries in children receiving fluoridated milk (Cagetti et al., 2013, Yeung et al., 2015).

In the updated Cochrane review, only one unpublished randomised controlled trial was included. Although the study (Maslak et al., 2004) carried a high risk of bias, it showed considerable reduction in caries level within the primary dentition (dmft) of the test groups with a preventive fraction of 31% in 3 year old children following three years of consumption of fluoridated milk (0.5 mg F) compared to the control children who were provide with non-fluoridated milk. The reduction in caries was also evident in the permanent dentition but not as substantial as the primary dentition due to the very low level of disease in the study. Despite its methodological limitations and lack of information on potential

risks, the provision of fluoridated milk for school children did succeed in reducing the level of caries within the primary dentition (Yeung et al., 2015).

A broader review of the applicability of milk fluoridation programmes was reported by Banoczy et al. The clinical studies included 18 studies carried out in 12 countries demonstrating the effectiveness of fluoridated milk on both primary and permanent teeth of school children (Banoczy et al., 2013). More recently, a 5-year cohort study by Peterson et al. evaluated the clinical effectiveness of Bulgarian community milk fluoridation school programmes over seven cities where children in the intervention sites were provided with 0.5 mg in 100 or 200 ml milk or yoghurt every school day. There was a 46% caries reduction (dmft) in the fluoridated group than the non-fluoridated group (30%) in the primary dentition compared to the 61% and 53% caries reduction in the permanent dentitions (DMFT) of fluoridated vs. non-fluoridated groups respectively (WHO, 1997). Over those five years, there has been a drop in the level of dmfs and DMFS by 43% and 68% respectively. Fluoridated milk was not provided to children to take home, regardless, this scheme proved to be an effective public method in caries prevention using milk fluoridation as a valuable vehicle (Petersen et al., 2015). Recommendations from these clinical studies is to ideally provide children with fluoridated milk before the age of 4 and to continue until the eruption of first permanent molars for the ideal protection of primary and permanent dentitions while insuring a risk benefit balance (O'Mullane et al., 2016). Fluoride concentrations in teeth reflect the overall availability of fluoride during tooth formation and varies depending on the time of exposure to and intake of fluoride (Pessan and Buzalaf, 2011). The accumulation of fluoride continues throughout life with bone and dentine but not enamel as once the

human enamel is fully formed its fluoride concentration can only be permanently altered at subsurface levels (O'Mullane et al., 2015).

Overall, although more high-quality evidence is required on the clinical effectiveness of milk fluoridation in caries prevention. A need to highlight the ideal concentration of fluoride to be added to milk while minimizing any possible risks is still necessary.

5.4.1 Interrupted vs. Daily frequency patterns

In an attempt to mimic the frequency of providing fluoridated milk in school programmes (every day or on school days only), slabs undergoing the pH cycling model in this study were divided into either interrupted or daily frequency as previously explained. Different school fluoridation schemes offer fluoridated milk to children over variable frequencies. Currently, the UK programmes deliver 0.5 mg fluoride every school day to children (200 days/year) whereas children in Chile receive their fluoridated milk throughout the year (365 days) with concentrations ranging between 0.25-0.75 mg/day depending on the age of the child (Banoczy et al., 2013). This is in line with our understanding of the need for a constant low-level of fluoride intra-orally as part of an effective mechanism of action against carious attacks (Buzalaf et al., 2010).

Ongtenco et al.'s in vitro study had investigated the effect of altering the frequency of fluoridated milk exposure on the improvement of enamel remineralisation. The enamel slabs were placed in milk either once a day, twice a day or on alternate days during their 20-day pH cycling regime. The authors stated that increasing the frequency of exposure to fluoridated milk twice a day resulted in higher remineralisation compared to once a day or on alternating

days (Ongtenco et al., 2014). This was consistent with our results as the slabs were exposed to fluoridated milk and a milk-saliva slurry twice daily over the 60-days. Although on comparison, the interrupted (on specific days) frequency in our study showed more superiority over the daily (everyday) frequency. It is better to confirm those findings with an in situ follow up study prior to extrapolating these results or making any recommendations. On the other hand, our outcomes were not in agreement with the results from an in situ study by Cassiano et al. since they reported that the use of fluoridated milk every day provided better enamel remineralisation effect than its use every other day. They had suggested that providing school children with fluoridated milk throughout the year rather than just during weekdays is more beneficial. However, more studies need to be carried out in order to confirm those findings prior to making any changes to current milk fluoridation schemes (Cassiano et al., 2017). Our current study is considered the first in vitro study to carry out a pH-cycling regime over 60 days, it was therefore difficult to compare to other studies with regards to the frequency of exposure of enamel slabs to different fluoride concentrations of milk.

More research is required to assess this novel approach of daily and interrupted frequency patterns of exposure of enamel slabs to fluoridated milk. It could have been of benefit if the analysis was carried out after 30 days as an interim point and then at 60 days to assess if there was a difference and whether a longer duration of pH-cycling showed a more pronounced effect for both the treatment and control groups. The 60 day period was locally agreed on in proportion to simulate 365 days due to the clinical obligations of the examiner in this study. The reason why 365 days was considered is because milk fluoridation

programmes provide the milk to be consumed during school days only (200 days a year) which does not account for the days where children are absent so the question is: would the remaining 165 days/year have an effect on enamel remineralisation if children were to consume fluoridated milk on those missed days? and that was calculated in our current in vitro to try and accommodate a school year into 60 days. This novel approach has not been done before, nor has it been carried out for this length of time. We do not know what the best duration is for pH-cycling to show an effect on enamel (whether demineralisation or remineralisation) but the hope was 60 days would be enough to notice any changes occurring on enamel.

6.0 CONCLUSION

The present study's design can further add to the understanding of the caries preventive benefits of providing school children with milk containing 0.5 mg F/day or 1 mg F/day. It also aids in providing qualitative data for future in situ studies in order to formulate an informative decision as to the ideal number of days the fluoridated milk should be provided in school milk fluoridation programmes. This current study presented that both fluoridated and non-fluoridated systems in this pH cycling experiment exhibited a trend towards a concentration gradient, as fluoride added to milk showed a significant effect on the model. A dose response effect was evident with increased remineralisation as the fluoride dose in milk became higher. This system also concluded that the interrupted frequency pattern was at least as good as the daily frequency when the frequencies were compared ($p < 0.043$), suggesting that the provision of fluoridated milk to school children during school days only would therefore be sufficient. However, due to the methodological limitations of in vitro studies and the small sample size in this current study, it would be difficult to explain why these findings were observed and what the rationale is as ΔQ at 0.5 mg F had only just reached significance (0.043). We can only speculate that the reason might be related to the interrupted frequency being exposed to more demineralisation episodes (Acid attacks) than remineralisation (fluoridated milk) and that might have resulted in the enamel being more porous which could have enhanced the uptake of fluoride compared to the frequent exposure of the daily groups fluoridated milk. Having said that, it is important to extrapolate these findings with caution.

Consequently, the first part of the hypothesis can be rejected as there was a significant benefit of increasing the fluoride dose in the daily (every day) frequency from 0.5 mg/l to 1.0 mg/l. When comparing the frequencies it could also be possible to reject the second part of the hypothesis as ΔQ was significant in favour of the interrupted frequency.

7.0 RECOMMENDATIONS AND FUTURE RESEARCH

- Although QLF is well established in the literature, it helps in providing additional information when used as an adjunct to conventional clinical methods and as this method of analysis was unable to confirm whether the remineralisation of the lesion was occurring in the body of the lesion or the surface layer, it might be of benefit to combine QLF with another clinical method of analysis for example, ICDAS. As QLF is highly sensitive and ICDAS is highly specific. Another suggestion would be to include microhardness as an outcome measure.
- A larger sample size might have contributed to the detection of more difference between samples. This could be incorporated in future larger scale randomised controlled in situ studies.
- With the aim of improving the delivery of fluoridated milk to school children, there is room for future intra-oral in situ and in vitro studies to confirm the results obtained from our current data regarding the frequency of administration.
- Due to the limitations in our current model, further Intra-oral clinical studies would also be beneficial to confirm the effectiveness of increasing the fluoride dose up to 1.0 mg F. Considering that the significant improvement between 0.5 and 1.0 was clear when milk was used every day in this study. It would therefore be interesting to compare the ingested dose of fluoride in fluoridated milk (0.5 and 1.0 mg F/L) with the

concentration of fluoride in 200 ml milk given to school children (2.5 and 5.0 ppm F/L) in future in vitro and in situ studies.

- The 60-day period of this current study might have not been a sufficient duration to detect changes caused by demineralisation and/or remineralisation and it might be of interest to extend this period in future research to more than 60 days, for example 6 months, and compare the outcomes with our results.

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9.0 APPENDICES

Appendix 1: Online sample size calculator

- ☒ Calculate Sample Size (for specified Power)
- ☐ Calculate Power (for specified Sample Size)

Enter a value for μ_1 :

Enter a value for μ_2 :

Enter a value for σ :

- ☒ 1 Sided Test
- ☐ 2 Sided Test

Enter a value for α (default is .05):

Enter a value for desired power (default is .80):

The sample size (for each sample separately) is:

Reference: The calculations are the customary ones based on normal distributions. See for example *Hypothesis Testing: Two-Sample Inference - Estimation of Sample Size and Power for Comparing Two Means* in Bernard Rosner's **Fundamentals of Biostatistics**.

(<https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html>)

Appendix 2: Random number generator table

(<http://stattrek.com/statistics/random-number-generator.aspx>)

Random Number Generator

Use the Random Number Generator to create a list of random numbers, based on your specifications. The numbers you generate appear in the [Random Number Table](#).

For help in using the Random Number Generator, read the [Frequently-Asked Questions](#) or review the [Sample Problems](#).

- Enter a value in each of the first three text boxes.
- Indicate whether duplicate entries are allowed in the table.
- Click the **Calculate** button to create a table of random numbers.

Note: The seed value is optional. Leave it blank to generate a new set of numbers. Use it to repeat a previously-generated set of numbers.

How many random numbers?

Minimum value

Maximum value

Allow duplicate entries

Seed (optional)

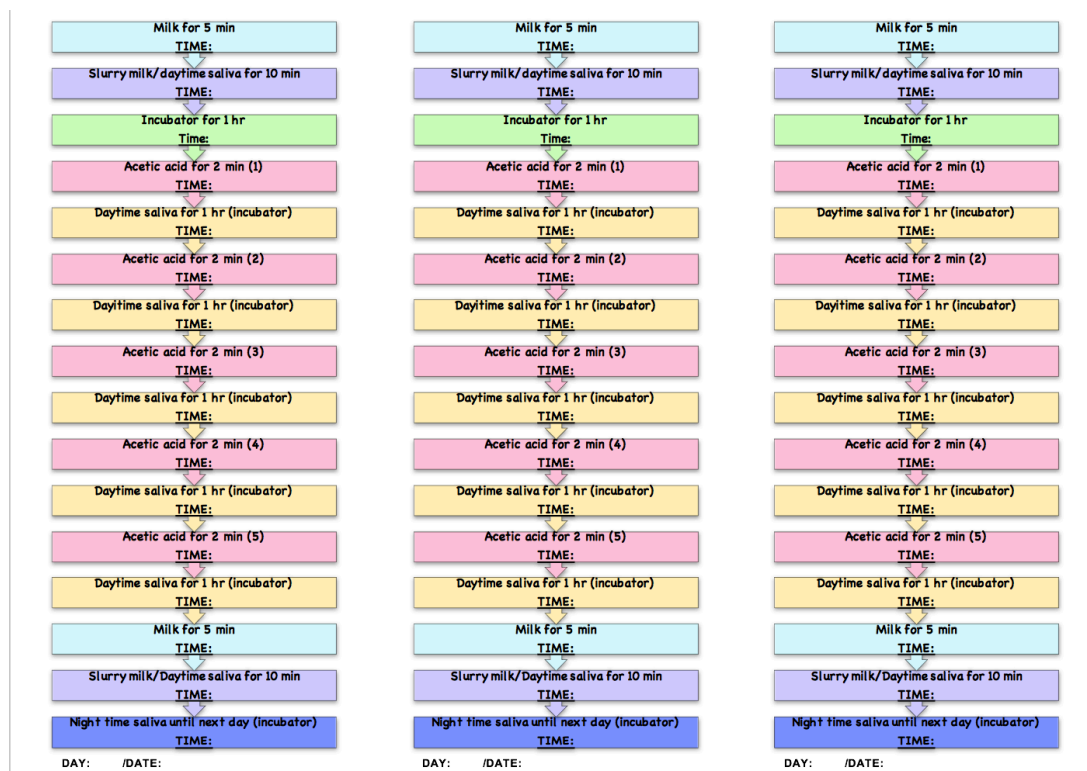
Random Number Table

[Random Number Generator](#) | [Frequently-Asked Questions](#) | [Sample Problems](#)

151 Random Numbers

```
090 076 078 073 087 044 023 132 059 017 064 074 082
058 131 002 107 095 061 121 093 066 137 086 049 009
088 038 080 026 077 036 048 123 081 063 116 108 007
024 012 046 056 099 010 014 042 070 055 141 035 148
053 006 098 124 125 135 092 071 029 067 065 111 122
130 106 028 072 050 004 102 109 018 101 114 003 033
094 133 096 057 136 128 083 020 129 013 134 054 031
103 146 022 118 037 043 045 060 084 145 021 032 119
140 115 112 008 019 027 075 120 052 150 149 011 051
041 142 030 104 091 143 144 025 062 079 068 151 097
015 085 034 040 147 016 100 047 089 039 001 105 069
139 126 110 127 113 117 138 005
```

Appendix 3: Daily flow chart used during pH-cycling



Appendix 4: One way ANOVA between fluoride concentrations for QLF parameter values at baseline (considering interrupted and daily frequencies)

Stagger			Sum of squares	df	Mean Square	F	Sig.
Non-staggered	ΔF baseline	Between Groups	9.542	2	4.771	.307	.737
		Within Groups	1135.246	73	15.551		
		Total	1144.787	75			
	ΔQ baseline	Between Groups	623734513.356	2	311867256.678	.744	.479
		Within Groups	30585032366.531	73	418973046.117		
		Total	31208766879.887	75			
	Area baseline	Between Groups	1516264.348	2	758132.174	2.031	.139
		Within Groups	27251623.015	73	373309.904		
		Total	28767887.364	75			
Staggered	ΔF baseline	Between Groups	30.694	2	15.347	1.141	.325
		Within Groups	968.533	72	13.452		
		Total	999.227	74			
	ΔQ baseline	Between Groups	33667235.425	2	16833617.713	.050	.952
		Within Groups	24460580840.086	72	339730289.446		
		Total	24494248075.511	74			
	Area baseline	Between Groups	95225.408	2	47612.704	.158	.854
		Within Groups	21667444.698	72	300936.732		
		Total	21762670.106	74			

Appendix 5: Oral presentation abstract for research day at the Leeds Dental Institute presented on Research Day 11/7/2016

Abstract: The Effect of Varying the Amount of Fluoride in Milk and The Frequency of Its Consumption on Demineralisation/Remineralisation of Dental Enamel

R AlSourani, M Malinowski and KJ Toumba
Leeds Dental Institute, Clarendon Way, Leeds

Introduction:

The clinical effectiveness of varying the frequency of consumption of fluoridated milk has not been clearly established. There is still a need to determine what the minimum threshold of fluoridated milk consumption might be and could programmes which offer milk on less than 180-200 days per year provide a suitable vehicle for the delivery of fluoride.

Aims:

- 1) Clinical effectiveness in caries prevention of increasing the amount of fluoride in milk from 0.5 mg F per school day to 1.0 mg F.
- 2) Clinical effectiveness in caries prevention of varying the frequency (staggered and non-staggered) of drinking fluoridated milk.

Materials and methods:

Suitable bovine teeth were prepared and sectioned so that a margin of sound enamel surrounded the white spot lesion that was created.

Two concentrations of fluoride were used in the study groups: 0.5 mg F and 1.0 mg F. For control groups, non-fluoridated milk was used. Those concentrations were divided into 6 groups according to staggered or non- staggered frequency. The slabs were randomly assigned.

Dipping the enamel slabs in the fluoridated milk with different concentrations was carried out over a 60 days time period which represented one school year (i.e. 365 days). Quantitative Light-induced Fluorescence was carried out at baseline and at the end of the study.

Statistical Analysis:

Statistical analysis on the collected data will be carried out using the SPSS statistical package (SPSS Inc. ver.17). Shapiro-Wilk test will first be used to test the normality of the data. Analysis of variance (ANOVA) will be used to compare the different groups with a Bonferroni correction.