THE EFFECT OF AMINE FLUORIDE PREPARATIONS ON THE FORMATION AND VIABILITY OF ORAL BACTERIAL BIOFILMS

Thesis submitted by
Jason Embleton BSc (Hons)
for the degree of
DOCTOR OF PHILOSOPHY
in the
Faculty of Medicine
University of London

Department of Microbiology
Eastman Dental Institute
for Oral Health Care Sciences
256 Gray's Inn Road
London, WC1X 8LD

-1999-
Abstract

In this study, the anti-plaque activities of amine and tin fluorides were investigated in laboratory models that were designed to simulate specific components of microbial ecology in the oral cavity. The efficacy of the agents applied topically in the form of a solution was determined. The influence of growth mode and sucrose on the bactericidal activity towards *Streptococcus sanguis* was determined using a constant depth film fermenter (CDFF). *S. sanguis* cultures growing as biofilms were less susceptible to the amine fluorides than planktonic cultures. When sucrose was added to the growth medium, the susceptibility of both planktonic and biofilm cultures were reduced. The smallest kills, which were measured in sucrose-grown biofilms, were not significantly different from control groups. Inorganic fluorides had no bactericidal effect on *S. sanguis* biofilms regardless of the presence of sucrose in the growth medium. The CDFF was also used to investigate the effect of olaflur/tin fluoride on the formation of microcosm biofilms over a 12 hour period. In the absence of sucrose, the structures of control and test group biofilms were observably different.

A parallel flow cell system was developed to investigate the anti-adhesion properties of the amine and tin fluorides. The effect on the net adhesion of *S. sanguis* to mucin-conditioned surfaces over a one hour period was determined. Only one of the amine fluorides tested was able to reduce the net adhesion of *S. sanguis* over a one hour period. Tin (II) and tin (IV) fluorides were found to reduce the net adhesion after one hour of culture flow.

The flow cell system was also used to investigate the effects of olaflur/tin fluoride on bacterial desorption and co-adhesion. The amine/tin combination
had no effect on either the level of *Streptococcus gordonii* desorption, or the co-adhesion of the streptococci with *Actinomyces naeslundii*.

In summary, the study has shown that the CDFF model was useful in determining the effect of anti-plaque agents on the susceptibility of intact biofilms of oral bacteria. The parallel flow cell system was a good model for investigating processes involved in adhesion of oral bacteria to conditioned surfaces and enabled the determination of the effects of the anti-plaque agents on bacterial adhesion, desorption and co-adhesion. Additionally, the system allowed the comparison of the substantivity of the different anti-plaque agents.
Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated.
Acknowledgements

I would like to sincerely thank my supervisors Prof. Mike Wilson and Prof. Hubert Newman, and my industrial sponsors Dr. V. Bieri for their support and guidance throughout this project.

I would also like to thank all of my colleagues at the Eastman Dental Institute for all their help and encouragement throughout this thesis. I would particularly like to thank Mr Iain Allan, Dr. Jon Pratten, Dr. Dave Spratt, Mrs Nikki Morgan, Dr Jonathon Knowles, Dr Sean Nair and Miss Chanothai Hengtrakool.

I am very thankful to my Mum and Dad for their continued support over my student years.
## Index of contents

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Declaration</td>
<td>4</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>5</td>
</tr>
<tr>
<td>Index of contents</td>
<td>6</td>
</tr>
<tr>
<td>List of figures</td>
<td>14</td>
</tr>
<tr>
<td>List of tables</td>
<td>18</td>
</tr>
<tr>
<td>List of equations</td>
<td>19</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>20</td>
</tr>
<tr>
<td>Publications as a result of this thesis</td>
<td>21</td>
</tr>
</tbody>
</table>

## Chapter One

### Introduction

1.1. Epidemiology and economics of caries 23
1.2. Biofilms
   1.2.1. The substratum and conditioning film 24
   1.2.2. Microbial adhesion 25
      1.2.2.1. Non-specific interactions 25
      1.2.2.2. Specific interactions 29
   1.2.3. Co-adhesion and co-aggregation 30
1.2.4. Biofilm structure 33
1.2.5. Environmental influence on biofilm structure 38
1.2.6. Biofilm nutrition 39
1.2.7. Biofilms and cell-to-cell signalling 41
1.2.8. The bacterial composition of the oral biofilm 42
1.3. Dental caries.
   1.3.1. The pathogenesis of dental caries 45
   1.3.2. Caries and oral biofilms 47
1.3.3. Bacterial metabolism in pathogenesis 49
1.3.4. Host control of pathogenesis 50
1.3.5. External influences on pathogenesis 52

1.4. Caries prevention 52
1.4.1. Fluoridation 52
1.4.2. Anti-plaque chemicals 53
   1.4.2.1. Bactericidal agents and biofilm resistance 54
   1.4.2.2. Anti-adhesion activities 58

1.5. Amine fluorides and stannous fluorides 58
   1.5.1. Overview of amine and stannous fluoride use 58
   1.5.2. The fluoride ion 59
   1.5.3. Amine fluoride 61
   1.5.4. Stannous fluoride 62
   1.5.5. Amine and stannous fluoride combinations 63

1.6. Models for testing of anti-plaque agents 63
   1.6.1. Overview 63
   1.6.2. Biofilm-based models 64
      1.6.2.1. The Modified Robbins Device 64
      1.6.2.2. Perfused biofilm fermenter 65
      1.6.2.3. The Constant Depth Film Fermenter 66
      1.6.2.4. Artificial mouths 67
      1.6.2.5. Analytical procedures for biofilm models 68
   1.6.3. Models for the study of adhesion processes 69
      1.6.3.1. Parallel flow cells 70
      1.6.3.2. Analytical procedures for adhesion studies 71

1.7. Aims of the study 73

Chapter Two
Materials and Methods 75

2.1. Organisms 76
   2.1.1. Single species experiments 76
2.1.2. Microcosm experiments
2.1.3. Co-adhesion experiments
2.2. Growth media
2.3. The Constant Depth Film Fermenter
  2.3.1. Fermenter design
  2.1.1. Sampling of the fermenter
2.4. Substrata for CDFF investigations
  2.4.1. Substratum for single species experiments
  2.4.2. Substratum for microcosm CDFF experiments
2.5. Continuous culture
2.6. Glass packed tube culturing device
2.7. Analytical procedures
  2.7.1. Viable counts
  2.7.2. Fluorescence microscopy
  2.7.3. Photomicrography
  2.7.4. Digital capture of microscopical images
  2.7.5. Measurement of pH of biofilm and planktonic cultures
  2.7.2. Measurement of total protein in biofilms
2.8. Parallel plate flow cell
  2.8.1. Key concepts in flow cell design
  2.8.2. The flow cell
  2.8.3. Running temperature
  2.8.4. Viscosity of mucin-containing medium (MCM)
  2.8.5. Measurement of MCM density
  2.8.6. Shear rate and shear stress
  2.8.7. Calculation of Reynolds number
  2.8.8. Flow cell length requirements
2.9. Culture methods for flow cell investigations
2.10. Peripherals for flow cell experiments
2.11. Analysis of adhesion
Chapter Three

Influence of sucrose on the susceptibility of planktonic Streptococcus sanguis to amine fluorides and amine-inorganic fluoride combinations.

3.1. Aims
3.2. Materials and Methods
3.2.1. Growth of planktonic culture
3.2.2 Microscopy of planktonic cultures
3.2.3. Agents and controls used in the study
3.2.4. Effect of agents on planktonic cultures
3.3. Results
3.4. Discussion

Chapter Four

The susceptibility of Streptococcus sanguis biofilms to amine and inorganic fluorides

4.1. Aims
4.2. Materials and Methods
4.2.1. Bacterial strain and growth medium
4.2.2. CDFF set-up and operation
4.2.3. Analytical procedures for determination of biofilm structure
4.2.4. Agents used in the study
4.2.5. Susceptibility testing of biofilms
4.2.6. Statistical analysis of results
4.3. Results
4.3.1. Biofilm structure
4.3.2. Susceptibility of S. sanguis biofilms to test solutions
4.4. Discussion
Chapter Five

Factors affecting the susceptibility of *S. sanguis* to amine fluorides.

5.1. Aims

5.2. Materials and Methods

5.2.1. Influence of specific growth rate and biofilm source on *S. sanguis* susceptibility

5.2.2. Cell free exopolymer (EPS) preparations and reaction with amine fluoride

5.2.3. Interactions of amine/tin fluoride with MCM and sterile saliva

5.3. Results

5.4. Discussion

Chapter Six

Effect of surface treatment with amine and inorganic fluorides on *S. sanguis* biofilm formation using the CDFF.

6.1. Aims

6.2. Materials and Methods

6.2.1. Set-up and inoculation of CDFF

6.2.2. Agents used in the study

6.2.3. Analytical procedures

6.3. Results

6.4. Discussion

Chapter Seven
The effect of surface treatment with amine and inorganic fluorides on adhesion of *S. sanguis* to parallel flow cells.

7.1. Aims

7.2. Materials and Methods

7.2.1. General equipment used in the study

7.2.2. Antimicrobial agents used in the study

7.2.3. Treatment of pellicle-coated glass (MCM-glass) with test solutions

7.2.4. Treatment of glass with the test solutions

7.2.5. Adhesion to surface with pellicle formed from MCM mixed with test solutions

7.2.6. Adhesion of material to MCM-glass from culture mixed with test solutions

7.2.7. Adhesion of salivary bacteria, and planktonic *S. sanguis*, to flow cells

7.2.8. Statistical analysis of results

7.3. Results

7.3.1. GPT culture analysis

7.3.2. Adhesion of *S. sanguis* to MCM-glass treated with the test solutions

7.3.3. Adhesion of *S. sanguis* to glass treated with the test solutions prior to culture flow

7.3.4. Adhesion of *S. sanguis* to flow cells with a pellicle formed from MCM mixed with test solutions

7.3.5. Adhesion of material to MCM-glass from flow of culture mixed with test solutions

7.3.6. Adhesion of salivary bacteria, and planktonic *S. sanguis* cells, to flow cell surfaces

7.4. Discussion
Chapter Eight

The effect of amine/tin fluoride on microcosm biofilm formation.

8.1. Aims
8.2. Materials and Methods
  8.2.1. Inoculum and growth medium
  8.2.2. CDFF set-up and operation
  8.2.3. Viable counts of biofilms
  8.2.4. Fluorescence microscopy of intact biofilms
  8.2.5. Identification of bacterial genera in biofilms
8.3. Results
8.4. Discussion

Chapter Nine

Amine/tin fluorides and adhesion processes involving Streptococcus gordonii and Actinomyces naeslundii.

9.1. Aims
9.2. Materials and Methods
  9.2.1. General equipment
  9.2.2. Antimicrobial agents used in the study
  9.2.3. Experimental protocol
  9.2.4. Statistical analysis of results
9.3. Results
9.2. Discussion

Chapter Ten

Final Discussion

10.1 Aims of study.
10.2. Bactericidal activity of AmFs 277
10.3. Anti-adhesion properties of amine and tin fluorides 286
  10.3.1. The effect of AmFs on adhesion 286
  10.3.2. The effect of AmF/tin fluoride on adhesion 288
10.4. Amine/tin fluoride combination and bacterial desorption and 289
  co-adhesion
10.5. The combined effects of the anti-plaque activities of the 291
  agents
10.6. Final summary 292

References 294
List of figures

1.2.2.1. Interactions involved in adhesion in DLVO theory 28
1.2.3. Diagrammatic representation of proposed bacterial accretion in dental plaque by Kolenbrander & London, 1993 33
1.2.4.1. Generalised biofilm model 34
1.2.4.2. Heterogeneous mosaic biofilm model 35
1.2.4.3 Consensus model according to Costerton et al (1994). 36
1.2.6. Schematic representation of possible fates of a nutrient entering a biofilm 41
1.3.1. Tooth structure and sites of carious lesions 46
1.3.3. The Stephan Curve response of plaque pH to bacterial metabolism of dietary sugars. 50
1.6.2.1. Modified Robbins Device 65
1.6.2.3. The Constant Depth Film Fermenter 67
1.6.3.1. Schematic diagram of a flow cell as described by Bos et al, 1999. 71
2.3.1.1. The Constant Depth Film Fermenter. 79
2.3.1.2. Schematic horizontal section through the fermenter 81
2.3.1.3. Schematic vertical section through the fermenter 82
2.5. Chemostat apparatus 86
2.6. Glass-packed tube (GPT) device and peripherals 87
2.8.2. Internal measurements of Camlab microslide flow cell. 92
2.8.4. The capillary U-tube viscometer 93
2.11. Scheme of image analysis system. 100
3.3.1. Gram stain of Streptococcus sanguis chemostat culture grown in MCM. 109
3.3.2. Bacterial and exopolymer aggregate from chemostat culture of S. sanguis, grown in MCM plus sucrose and stained with Baclight live/dead fluorescent stain. 110
3.3.3. The susceptibility of MCM-grown planktonic S. sanguis to 111
amine fluorides and amine fluoride / inorganic fluoride combinations.

3.3.4. The susceptibility to amine fluorides and amine fluoride / inorganic fluoride combinations of planktonic S. sanguis grown in MCM plus sucrose.

4.2.2. Apparatus set-up

4.2.3. Focus wheels on microscope

4.3.1.1. Surface view of 7 day-old S. sanguis biofilm grown in MCM.

4.3.1.2. Surface view of 7 day-old biofilm grown in MCM plus sucrose

4.3.1.3. Side-view of a 7 day-old S. sanguis biofilm grown in MCM plus sucrose

4.3.1.4. Representative growth curves for biofilms grown in the presence and absence of sucrose.

4.3.2.1. Susceptibility to test solutions of MCM-grown S. sanguis biofilms

4.3.2.2. Susceptibility to test solutions of MCM plus sucrose-grown S. sanguis biofilms

5.3.1. Viable counts of GPT-grown S. sanguis cultures after incubation with GABA agents.

5.3.2. Absorbance of amine fluoride after additions of EPS

5.3.3. Images of MCM solution mixed with olaflur/tin (IV) fluoride

5.3.4. Images of saliva mixed with olaflur/tin (IV) fluoride

6.2.1. Apparatus set-up for pre-treatment experiment using the CDFF

6.3.1. Viable counts of 4 hour old S. sanguis biofilms on MCM-HA substratum treated with test solutions prior to culture flow.

6.3.2. Viable counts of 4 and 8 hour old S. sanguis biofilms on MCM-HA substratum treated with test solutions prior to culture flow

6.3.3. Viable counts of 4 and 8 hour S. sanguis biofilms on MCM-
HA substratum treated with steraflur, or, deionised water prior to culture flow.

6.3.4. Gram stain preparations of (A) *S. sanguis* chemostat culture, and, (2) human saliva

7.2.3. Apparatus set-up for flow cell experiments adding agents to pellicle.

7.2.5. Schematic diagram of apparatus set-up for experiments mixing MCM with agents prior to pellicle formation.

7.2.6. Schematic diagram of apparatus set-up for experiments mixing culture with agents prior to running through flow cell.

7.3.2.1. Images of adherent *S. sanguis* following treatment of MCM-glass with deionised water, Olaflur and Olaflur/tin (IV) fluoride.

7.3.2.2. The percentage surface coverage of MCM-glass by *S. sanguis* following pre-treatment with GABA agents and controls.

7.3.3.1. Images of *S. sanguis* adhered to glass treated with deionised water and olaflur/tin (IV) fluoride

7.3.3.2. The percentage surface coverage of glass and MCM-glass treated with deionised water and olaflur/tin (IV) fluoride prior to *S. sanguis* culture flow.

7.3.4.1. *S. sanguis* adhered to flow cells with a pellicle formed from MCM mixed with deionised water, olaflur and olaflur/tin (IV) fluoride.

7.3.4.2. *S. sanguis* coverage of flow cells with a pellicle formed from MCM mixed with deionised water, olaflur and olaflur/tin (IV) fluoride combination.

7.3.5.1. Material adhered to MCM-glass after flow of *S. sanguis* GPT culture mixed with either deionised water, olaflur or olaflur/tin (IV) fluoride combination.

7.3.5.2. Percentage of surface area of MCM-glass covered by material following one hour flow of culture mixed with either deionised water, olaflur or olaflur/tin (IV) fluoride combination.

7.3.6. Adhesion to glass flow cells of (A) *S. sanguis* grown by
batch, and, (B) salivary bacteria

8.2.2. Apparatus set-up

8.3.1. MCM-grown microcosm biofilms growing on surfaces pretreated with deionised water (control)

8.3.2. MCM-grown microcosm biofilms growing on surfaces pretreated with olaflur/tin (IV) fluoride

8.3.3. S-MCM-grown microcosm biofilms growing on surfaces pretreated with deionised water (control)

8.3.4. S-MCM-grown microcosm biofilms growing on surfaces pretreated with olaflur/tin (IV) fluoride

8.3.5. Filamentous structure observed in an S-MCM-grown biofilm

9.2.3. Apparatus design for co-adhesion experiments.

9.3.1. Adhesion and co-adhesion of S. gordonii and A. naeslundii.

9.3.2. PSC by A. naeslundii in relation to PSC by S. gordonii.

9.3.3. Measurement of different processes for A. naeslundii adhering to surfaces with adherent S. gordonii.

9.3.4. The relationship between S. gordonii PSC and the percentage of total A. naeslundii co-adhering

9.3.5. Measurements of adhesion and co-adhesion processes involving S. gordonii and A. naeslundii.

10.2.1 Clinical and laboratory testing of the bactericidal activity of amine fluorides towards oral bacteria prior to this study.

10.2.2. Clinical and laboratory testing of the bactericidal activity of amine fluorides towards oral bacteria following this study
List of tables

1.2.8. Bacteria isolated from the oral cavity 44
1.4.2. Oral antimicrobial agents and there assigned grouping 54
2.2. Composition of mucin-containing medium 77
2.4.1. Formula of mineralising solution. 84
3.2.3. Amine fluoride and inorganic fluoride concentrations in solutions used for antimicrobial activity testing. 105
4.2.4. Amine fluoride and inorganic fluoride concentrations in solutions used for antimicrobial activity testing. 125
4.3.1.1. Measurements of pH and mean viable count of S. sanguis biofilms. 132
5.2.1. Amine fluoride and inorganic fluoride concentrations in solutions used in the study. 133
6.2.2. Amine fluoride and inorganic fluoride concentrations in solutions used for treating surfaces. 170
6.3.1. Mean viable counts of 4 hour old S. sanguis biofilms grown on MCM-HA treated with test solutions prior flow. 173
7.2.2. Amine fluoride and inorganic fluoride concentrations in solutions used in the study. 190
7.3.1.1. GPT concentrations and pH pre- and post-flow cell experiments. 199
8.3.1. Identification of the predominant microcosm biofilm bacterial strains. 240
8.3.2. Mean viable counts of bacteria recovered from microcosm biofilms grown in the CDFF 243
8.3.3. Mean viable counts of organisms recovered from pooled saliva samples. 244
9.3.1. Mean concentration of A. naeslundii and S. gordonii GPT cultures 260
9.3.2. Correlation coefficients for adhered S. gordonii and percentage of total A. naeslundii co-adhering. 265
List of equations

2.8.2. Width to height ratio of flow cell 92
2.8.4. Calculation of viscosity of MCM at 25°C 94
2.8.5. Calculation of density of MCM at 25°C 94
2.8.6.1. Calculation of shear rate 95
2.8.6.2. Calculation of shear stress 96
2.8.7. Calculation of Reynolds number 96
2.8.8. Calculation of flow cell length requirement 97
List of Abbreviations

°C    degrees centigrade
µl    Microlitre
µm    Micrometre
AmF   amine fluoride
CDFF  constant depth film fermenter
CFU   colony forming unit
CHX   chlorhexidine digluconate
Cp    Centipoise
GPT   glass packed tube
H     Hour
L     Litre
MBC   minimum bactericidal concentration
MCM   mucin-containing medium
MIC   minimum inhibitory concentration
PSC   percentage surface coverage
PTFE  Polytetrafluoroethylene
S     Second
Chapter 1 - Introduction
1.1. Epidemiology and economics of caries

Dental caries is a progressive destructive disease of teeth associated with bacterial plaque. Early reports associated the disease with children (Burt 1981), however, more recent studies in the US have observed attack rates in adults similar to those of children aged 7-12 years (Glass 1987). Social, cultural, economic and behavioural factors affect caries incidence although their impact is unclear. From the time of commencement of dental records, to 1988, 268 million filled teeth and 32 million decayed teeth had been recorded in England and Wales alone (Downer 1993). In developed countries the disease is on the decline (Marthaler et al 1990) but it is still a substantial burden to a nation’s health and economy. Estimations of European dental expense as a percentage of gross national product in 1982 ranged from 0.26% in the UK to 0.87% in Sweden (Von der Fehr & Schwarz 1994). This relates to $22.4 and $101.7 dental expenditure per capita per annum respectively. Obviously there is much interest in preventative measures against the disease. Oral hygiene procedures have long been a component of caries prevention programmes. Increasing disease awareness and commercial advertising has generated a large market sector specialising in oral hygiene products. In 1997 the UK market for oral hygiene products was worth £512 million (Mintel Marketing Intelligence 1998). Toothpastes and mouthwashes represented 67% of total sales. A growth area of this market is the incorporation of anti-plaque agents into oral hygiene products. It is hoped that such a strategy will improve the effectiveness of caries prevention programmes.
1.2. Biofilms

Dental caries is preceded by the formation of dental plaque, i.e., microorganisms growing in a matrix consisting of host, exogenous and microbiologically-derived molecules. This growth strategy, known as a biofilm, is observed in many different habitats (Costerton et al 1995, Marsh & Bradshaw 1995, Peyton & Characklis 1995). In the development of effective caries prevention programmes an understanding of oral biofilms and their association with the disease is fundamental. The following sections discuss the processes involved in biofilm formation and homeostasis with the emphasis on the oral environment.

1.2.1. The substratum and conditioning film

In the human oral cavity hard crystalline (enamel) surfaces and epithelial linings exist and both may be colonised by micro-organisms. Desquamation of epithelial cells prevents adhered bacteria forming biofilms, whereas bacteria adhering to enamel are able to progress to form mature biofilms. Micro-organisms do not generally adhere directly to the enamel surface but to a film of host-derived organic molecules (the pellicle). The molecules adsorb to the hydroxyapatite (the crystalline portion) in the enamel surface (Jensen et al 1992). These proteins adsorb from saliva flowing over the teeth. The saliva flow has a depth of around 0.01 - 0.02 mm for incisors (Disabato-Mordarski and Kleinberg 1996). Acidic proline-rich proteins, statherins, histatins, cystatins and α-amylase are all known to readily adsorb to hydroxyapatite (HA). On clean hydroxyapatite the initial rate of adsorption is rapid (seconds) followed by a slower change in adsorption over the next two hours (Lamkin et
al 1996). The two different adsorption rates may be due to direct adsorption to HA and interactions with pre-adsorbed proteins respectively. The rates of adsorption of the different proteins differ as do the concentrations of each protein in the pellicle.

1.2.2. Microbial adhesion


1.2.2.1. Non-specific interactions

Non-specific interactions can take place over greater distances than specific interactions and are presumably the first form of interaction between micro-organism and a surface. Non-specific interactions have been difficult to evaluate experimentally thus theoretical approaches based on colloid and surface chemistries have been applied in an attempt to explain experimental observations.

The classical DLVO theory (Derjaguin & Landau 1941, Van Loosdrecht 1989), named after the creators Derjaguin, Landau, Verwey, and Overbeek,
describes microbial interactions with surfaces in terms of lifshitz-van der waals forces and electrostatic interactions. Lifshitz-van der waals forces are weak attractive forces between bacteria and surfaces which operate over distances of 50 nm and greater (Busscher et al 1990). Electrostatic interactions, which operate over shorter distances may be repulsive or attractive and are affected by the ionic strength of the medium. As the ionic strength of the medium increases the distance and magnitude of electrostatic interactions decrease becoming negligible at high ionic concentrations (Bos et al 1999). At low ionic strengths, two zones (known as the primary and secondary minima) can exist where the net balance between lifshitz-van der waals forces and electrostatic forces is attractive (Mills and Powelson 1996). The secondary minimum occurs at the greater distance and forces are relatively weak forming a reversible attraction. Between the primary and secondary minimum zones, net forces are repulsive due to the net negative surface charge on both the bacterium and the substratum. At the primary minimum net forces between the bacterium and substratum are attractive and stronger than at the secondary minimum. The Classical DLVO theory does not take into account other forces that may be involved in bacterium/substratum interactions. The theory has been developed by Van Oss et al, 1986, to include Lewis acid-base interactions and Brownian motion forces (figure 1.2.2.1.). The forces of Lewis acid-base interactions are huge in comparison to the forces of lifshitz-van der waals forces and electrostatic interactions. They may include hyrophobic/hydrophilic interactions and displacement of surface bound water molecules (Bos et al 1999). The distance at which Lewis acid-base interactions are operative are less than 5
nm, making it unlikely that they are involved in attractions at the secondary minimum. However, it is likely that Lewis acid-base interactions will be involved in attractions at the primary minimum. Brownian motion forces were not considered in the classical DLVO theory. Such forces may allow bacteria to overcome repulsive forces and reach distances where interactions are favourable for adhesion. Once a bacterium is held in an attached state, other interactions can occur leading to a strengthening of adhesion. These include hydrogen bonding, calcium bridging and adhesin-receptor interactions (Busscher et al 1990). The strength of attachment can be increased by the production of exopolymers (Costerton et al 1987) which may effectively cement the bacteria to the surface.
1.2.2.1 Interactions involved in adhesion in DLVO theory

- Lifshitz-van der Waals forces operate over long distances (>50 nm)
- As bacterium approaches surface repulsive/attractive electrostatic forces influence adhesion at low ionic strengths
- At distances less than 5 nm strong Lewis acid-base interactions influence attachment. Interfacial water may be removed by hydrophobic groups on bacterium
- At distances less than 1 nm hydrogen bonding, cation bridging and adhesin-receptor interaction occur

Not all experimental observations are explainable by the DLVO theory. An alternative model to explain bacterial adhesion is the thermodynamic theory (Fletcher and Loeb 1979). This approach views attachment as a spontaneous event accompanied by a reduction in the free energy of the system. The bacterium and surface are assumed to physically contact each other under conditions of thermodynamic equilibrium i.e. reversible adhesion (Bos et al 1999). Calculations are based on surface free energy measurements made by contact angle measurements. As with the DVLO model, thermodynamic predictions do not explain experimental observations (Bellon-Fontaine et al 1990). Production of exopolymers, pellicle maturation, bacterial grazing of
pellicle and bacterial cell surface modification will all affect the surface free energy of either the substratum or the bacterium and therefore affect calculations based on a thermodynamic approach.

1.2.2.2. Specific interactions

The characterisation of specific interactions of bacteria with substrata has been the subject of much investigation and many have been described in functional terms. Streptococci, especially members of the sanguis group, are the predominant colonisers of the salivary pellicle (Jenkinson & Lamont 1997), and not surprisingly, have received the most attention in the investigation of surface adhesins. Various distinct adhesins have been characterised, many of which may be expressed on the same cell (Jenkinson & Lamont 1997). At least 10 different distinct types/families of streptococcal adhesins which mediate attachment to salivary pellicles have been studied. The two largest families of streptococcal adhesins are the Antigen I/II family (Jenkinson and Demuth 1997) and the Lral family (Jenkinson 1994). Adhesins belonging to the Antigen I/II family have been reported in the mutans group (Lee et al 1989) and in the mitis group (Jenkinson et al 1993) of streptococci. The Antigen I/II family adhesins have a molecular mass of around 160-175 kDa and are anchored to the cell wall at the C-terminal end. The salivary pellicle receptor for the adhesins is salivary agglutinin glycoprotein (SAG) (Demuth et al 1990). Different regions on the adhesins mediate adhesion to immobilised and fluid phase SAG (Jenkinson & Demuth 1997). The adhesins also mediate adhesion to other oral bacteria. The Antigen I/II adhesin on S.
*gordonii*, SspA, mediates adhesion to receptors on type II fimbriae of *Actinomyces naeslundii* (Jenkinson et al 1993).

The Lrl family of adhesins are associated with the sanguis group of oral streptococci. The adhesins mediated binding to the salivary pellicle (Jenkinson & Lamont 1997). However, the exact receptors within the pellicle have yet to be determined.

Other adhesins of oral streptococci include amylase binding proteins (Douglas 1990) and surface lectins that bind salivary glycoproteins (Murray et al 1986). *S. mutans* Ingbritt has been reported to possess an adhesin that binds to dextran and 1,6-α-linked glucans (Banas et al 1990). Hence streptococcal extracellular polysaccharides adhered to the enamel may act as additional receptors for bacterial adhesion. The glucosyl transferases that catalyse the production of extracellular polysaccharides may themselves mediate adhesion to the substratum (Fukishima et al 1992).

**Actinomyces** are known to possess adhesins that mediate adhesion to the salivary pellicle (Clark et al 1981). The adhesin is situated on type 1 fimbriae (Clark et al 1984). The salivary pellicle receptors for the adhesin have been shown to be proline-rich proteins (Clark et al 1989, Leung et al 1990).

Evidence for specific adhesins for salivary pellicle components on other oral bacteria has been published. This includes promoted adhesion of *F. nucleatum* to hydroxyapatite following conditioning with proline-rich glycoproteins (Gillece-Castro et al 1991) and fimbriae-mediated attachment of *P. gingivalis* to saliva-coated hydroxyapatite (Lee et al 1992).
The specific interactions discussed so far have been between pellicle components and bacteria. The bacteria that adhere to the pellicle can grow and divide to produce micro-colonies. Typical doubling times for oral streptococci and actinomyces during the first four hours of biofilm formation are less than one hour (Weiger et al 1995). Besides growth, biomass is increased by the adhesion of planktonic phase organisms to adhered bacteria. This phenomenon is termed co-adhesion (Busscher and van der Mei 1997). This feature of biofilm formation is important in the species diversification and succession within oral biofilms. Co-aggregation, adhesion of micro-organisms to each other in the planktonic phase, increases the species diversity of bacterial aggregates involved in co-adherence. Co-adhesion and co-aggregation are widely studied phenomena and many specific interactions have been identified. The primary co-adhesion interactions occur mainly between the cleaned tooth colonisers and other bacteria. However, it should be noted that even on cleaned tooth surfaces residual bacteria may remain. Oral streptococci adhere intragenerically and intergenerically (Whittaker et al 1996). Figure 1.2.3. shows a diagrammatic representation of proposed bacterial accretion in dental plaque by Kolenbrander & London, 1993. Oral streptococci have been reported to adhere to Actinomyces spp. (Kolenbrander and Anderson 1990, Mc Nab et al 1994, Jenkinson and Easingwood 1990), Fusobacterium nucleatum (Kaufman and DiRienzo 1989), Haemophilus parainfluenzae (Lai et al 1990), Porphyromonas gingivalis (Lamont et al 1994), Prevotella loescheii (Weiss et al 1988), Veillonella atypica (Hughes et al 1992) and Candida albicans
(Jenkinson et al 1990). Primary colonisers tend to co-aggregate with early and late colonisers whereas late colonisers do not tend to co-aggregate with other late colonisers (Whittaker et al 1996). Fusobacteria as a group co-aggregate with almost all oral bacteria thereby mediating the incorporation of fastidious secondary colonisers into biofilms. Co-adhesion and co-aggregation allow the incorporation of species within the biofilm that are unable to grow in the early biofilm environment. As the biofilm develops and micro-habitats diversify, these bacteria can take advantage of suitable environments, for example, anaerobic conditions. Co-aggregation can occur between partners that share a commensal relationship. *Veillonella atypica* and *Veillonella dispar* are able to utilise lactate produced by several species of oral streptococci that are co-aggregation partners (London and Kolenbrander 1996). Frequently observed structures in biofilms are often the result of co-aggregation or co-adhesion, for example, “corn cob” structures in oral biofilms (Mouton et al 1980, Vrahopoulos et al 1992).
1.2.4. Biofilm structure

Attachment of bacteria to the pellicle, cell growth, replication, co-aggregation and co-adhesion lead to the formation of diverse biofilms that are heterogenous in nature. Figure 1.2.4.1. shows a generalised model of biofilm structure at a solid/liquid interface. The biofilm extends from the conditioning film out to the surrounding bulk fluid. The structure of a biofilm will be
influenced by nutrient availability, the micro-organisms present and the physico-chemical properties of the environment.

**Figure 1.2.4.1. Generalised biofilm model**

Techniques such as confocal scanning laser microscopy-CSLM (Lawrence et al 1991), nuclear magnetic resonance (NMR) visualisation (Costerton et al 1994) and electron microscopy (Barber 1994) have aided more detailed investigations of biofilms. Using these techniques the structure of biofilms from a wide range of habitats has been explored. Using differential interference contrast microscopy, Walker et al, 1995, studied the structure of biofilms in water distribution systems. The biofilm model proposed by their investigations is shown in figure 1.2.4.2.. The biofilm consists of stacks of micro-colonies extending from a base film with an approximate thickness of 5
\( \mu \)m. The stacks are sufficiently spaced to allow free movement of liquid and micro-organisms in between.

**Figure 1.2.4.2.** Heterogeneous mosaic biofilm model

The diagram shows microcolonies of bacteria forming towers that rise from a thin basal layer of bacteria. The basal layer is adhered to the substratum.

Using CSLM, Costerton et al 1994, have studied many pure culture and natural microbiological communities. From their studies they developed a consensus model for biofilm structure (figure 1.2.4.3.) The model proposes microcolonies of organisms rising from a base film held together by an extracellular matrix. The micro-colonies can form stacks which may fuse with one another. Running between these microcolonies are water channels in which the movement of liquid follows convective flow patterns (Costerton et al 1994). The surface, the hydrodynamics of the environment, nutrients, antagonistic chemicals and the microbial consortia involved will affect the structural properties of the model (Stoodley et al 1989).
The model shows microcolonies of micro-organisms that extend from the substratum into the liquid phase. These microcolonies sometimes take on mushroom shapes and the top of these structures may fuse with other colonies. Some of the microcolonies may be sloughed from the surface and flow away in the liquid flow.

All the models previously discussed are for biofilms formed at solid/liquid interfaces. Supra-gingival plaque forms at the solid/liquid interface of enamel and saliva. In the oral environment, the mechanical action of the tongue influences biofilm structure. At inaccessible sites, the biofilm may become densely packed due to a squashing action by the tongue. In contrast, thin films containing few bacteria may develop on accessible sites due to the constant removal of the biofilm by mechanical shearing, i.e. chewing and tongue movement. The structure of dental plaque has been studied using electron microscopy. The use of electron microscopy for determining biofilm
structure is limited due to the level of sample manipulation that the procedure requires. However, electron microscopy does provide valuable information on the specific arrangement of micro-organisms within biofilms, and the cell density within different regions of the biofilm. Figure 1.2.4.4. shows a transmission electron micrograph of *in vivo* dental plaque (Vrahapoulos 1989). The base film consists of dense regions of bacteria. As the distance of the biofilm from the substratum increases, the density of micro-organisms in the biofilm decreases. Micro-organisms in small stacks at the surface may extend into the bulk fluid.

**Figure 1.2.4.4.** Transmission electron micrograph of *in vivo* dental plaque. From: Vrahopoulos (1989). Bar represents 2 \( \mu \text{m} \). The base is the substratum.
Reportedly over 500 bacterial taxa have been retrieved from the human oral environment (Moore et al. 1988). Over 200 bacterial species can be found inhabiting an individual oral cavity. Specific structural features are often encountered within oral biofilms (Listergarten, 1976). These structures include microcolonies, parallel alignment of bacteria, and specific interactions, e.g. corn-cobs.

1.2.5. Environmental influence on biofilm structure

The specificity of surface attachment may influence the microbial consortia especially in young biofilms and may be responsible for species stratification in the biofilm near the surface. Surface roughness influences the process of microbial adhesion. Microbial adhesion to enamel in vivo initiates at sites of surface irregularities (Nyvad & Fejerskov 1987). This was demonstrated by electron microscopy of teeth pieces attached to intra-oral acrylic appliances for 4 to 12 hours. At such sites the micro-organisms are protected, to an extent, from shear forces allowing a higher frequency of transition from reversible to irreversible adhesion (Quirynen & Bollen, 1995).

Exopolymers are produced by micro-organisms that may increase the cohesive strength between cells and the substratum (Zobell 1943, Boyd & Chakrabarty 1995). The cohesive strength between the biofilm and the surface is partly responsible for the level of sloughing, that is, the detachment of large masses from the biofilm, which in turn affects the structure. The hydrodynamics of the environment will modify the profile of the biofilm by the processes of erosion and sloughing thereby altering the mass transfer rates. Biofilm erosion is a product of the shear stresses exerted on the biofilm. This is due to dissipating kinetic energy from the liquid phase breaking bonds within the biofilm (Characklis et al. 1990). Sloughing has been observed at low
shear stresses and during substrate loading (Jansen & Kristerson 1980). Erosion, which increases with shear rate, tends to produce smooth biofilms whereas sloughing increases the roughness of the biofilm. Mass transfer rates will be a function of slow molecular diffusion in smooth biofilms but a function of slow molecular and fast turbulent diffusion in rough biofilms (Characklis et al 1990).

Erosion and sloughing rates will continually alter. This is due to the modulating biofilm profile altering the hydrodynamics of the immediate environment. Biofilms growing in the oral cavity are also influenced by forces unique to the environment. The action of the tongue and other muscular movements will increase the removal of the biofilm (Wilson 1996) and may also increase the density of the biofilm through a squashing action.

The mass transfer properties of the biofilm are affected by erosion and sloughing processes as previously mentioned. Mass transfer is also controlled by the concentration of nutrients in the liquid phase and within the biofilm.

1.2.6. Biofilm nutrition

The fate of a nutrient entering a biofilm is diverse and inevitably affects the structure and organisation of a biofilm (Bowden and Li 1997). Figure 1.2.6. is a schematic representation showing the possible fates of a nutrient entering a biofilm. The products of a metabolised nutrient may serve as substrates for further microbial metabolism, may act as an antimicrobial, may contribute to the matrix (Costerton et al 1981), may alter the pH, or simply diffuse out of the biofilm. Utilisation of gases during metabolism may also alter the environment. The products of metabolism and their influence on the
environment are a controlling factor in the relationships between members of the microflora, that is, competition, mutualism, synergism, commensalism and amensalism. In thick, mature biofilms the utilisation of nutrients leads to the establishment of gradients of nutrients, light, gases and products of metabolism (Gilbert et al, 1993). This not only affects mass transfer rates but encourages succession by providing habitats suitable for microflora with distinct requirements thus increasing the heterogeneity of the biofilm. In mature biofilms, motility can strengthen the opportunities to find a desirable habitat. Predation is also a feature of mature biofilms. All the factors mentioned will influence the composition, organisation and structure of biofilms. Under normal circumstances the endogenous biofilms protect the surfaces from colonisation by exogenous bacteria.
1.2.7. Biofilms and cell-to-cell signalling

Cell signalling may affect the formation and structural organisation of microbial biofilms. The role of cell-to-cell signalling in the development of *Pseudomonas aeruginosa* biofilms has been investigated by Davies et al, 1998. They investigated biofilm formation by wild type *P. aeruginosa* and mutant strains with a deficient *lasI* gene. The *lasI* gene directs the synthesis of N-(3-oxododecanoyl) homoserine lactone (PAI-2) (Pearson et al 1997). Wild type biofilms consisted of distinct microcolonies composed of clusters of cells. These micro-colonies were interspersed by water channels. In contrast, *lasI* gene mutants formed biofilms consisting of thin continuous sheets of cells only 20% of the thickness of wild type biofilms. Furthermore, addition of 0.2% sodium dodecyl sulphate for five minutes removed virtually all *lasI* mutant
biofilm from surfaces but had no detectable effect on wild type biofilms. Such dramatic changes to the biofilm structure will alter the effects of erosion, sloughing, mass transfer, biofilm susceptibility, etc.

The majority of bacterial signalling mechanisms have been reported from Gram-negative organisms. Many involve homoserine lactones as in the previous example. Gram-positive bacteria have been shown to use peptides as signalling molecules. These may stimulate responses in other genera. For example, *Staphylococcus aureus* produces a peptide that induces the expression of plasmid-borne *Enterococcus faecalis* adhesin resulting in the clumping of *E. faecalis* cells (Muscholl-Silberhorn et al 1997). Signalling molecules that alter the phenotype of other genera may influence the inclusion/exclusion of species within biofilms. This will affect the chemical composition of the biofilm matrix through utilisation of nutrients and metabolite production. Hence the properties of the biofilm as a whole may be altered.

1.2.8. The bacterial composition of the oral biofilm

It is estimated that only 1% of the planet's bacterial species can be cultivated by traditional techniques (Pace 1996). In the oral cavity it is estimated that 40% of the flora remain to be cultivated (Tanner et al 1994). Recently the characterisation of uncultivable micro-organisms from the oral cavity has been assisted by the development of molecular techniques, in particular, 16S ribosomal RNA probes (Tanner et al 1994). The use of 16S and 23S probes has also proven useful as a tool in species level identification of cultivable oral bacteria (Whiley et al 1995). Classification of micro-organisms using 16S
rRNA probes is based on phylogeny rather than a phenotypic approach and helps to clarify previous taxonomic problems.

The bacterial microflora of the human oral cavity incorporates species from four of the 10 bacterial phyla, namely Proteobacteria, the Gram positives, the Spirochaetes and the Flavobacter-Bacteroides group. Most of these species may constitute part of the microflora of either sub- or supra-gingival plaque. One area may act as a reservoir for colonisation of the other. Table 1.2.8. lists the genera found in sub and supragingival plaque.

The main primary colonisers in the formation of supragingival plaque are members of the mitis group of oral streptococci, *Actinomyces* spp., *Neisseria* spp, and *Haemophilus* spp. In mature supragingival plaque, *Corynebacterium* spp, *Veillonella* spp, *Fusobacterium* sp, *Lactobacillus* spp, *Peptostreptococcus* spp, *Prevotella* spp, and *Propionibacterium* spp are commonly isolated. Oral streptococci comprise the largest proportion of the supragingival plaque microflora constituting around 28% of cultivable microorganisms (Marsh & Martin 1992). The major proportion of streptococci on sound enamel is comprised of members of the mitis group, although members of the milleri (Whiley et al 1987) and mutans group are frequently isolated. The main eukaryotic organisms to be recovered from supragingival plaque are yeasts of the *Candida* genus. The site of supragingival plaque will influence the composition of its microflora. Smooth areas, where mechanical shear is the greatest, will possess thin sporadic plaque that will comprise mainly primary colonisers, for example, *S. sanguis*. Fissure and interproximal sites that are subject to less mechanical shear than smooth sites will possess thicker plaque. Thicker plaques favour a more diverse microflora due to the
gradients of nutrients, waste products and gases, supplying a wider range of micro-environments.

Table 1.2.8. Bacteria isolated from the oral cavity

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>lactate production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eikenella</td>
<td><em>E. corrodens</em></td>
<td>+</td>
</tr>
<tr>
<td>Kingella</td>
<td><em>K. orale, K. denitrificans</em></td>
<td></td>
</tr>
<tr>
<td>Neisseria</td>
<td>several including <em>N. gonorrhoeae</em></td>
<td></td>
</tr>
<tr>
<td>Actinobacillus</td>
<td><em>A. actinomycetemcomitans</em></td>
<td></td>
</tr>
<tr>
<td>Haemophilus.</td>
<td>Several species</td>
<td></td>
</tr>
<tr>
<td>Campylobacter</td>
<td>several species</td>
<td></td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>several including <em>F. nucleatum</em></td>
<td>+</td>
</tr>
<tr>
<td>Selenomonas</td>
<td>several</td>
<td>+</td>
</tr>
<tr>
<td>Centipeda</td>
<td><em>C. peridontii</em></td>
<td></td>
</tr>
<tr>
<td>Mitsuokella</td>
<td><em>M. dentalis, M. multiacidus</em></td>
<td>+</td>
</tr>
<tr>
<td>Veillonella</td>
<td><em>V. parvula, V. atypica, V. dispar</em></td>
<td></td>
</tr>
<tr>
<td>Eubacterium</td>
<td>several</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>several</td>
<td>+</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>several</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>mainly members of mitis and mutans group</td>
<td>+</td>
</tr>
<tr>
<td>Rothia</td>
<td><em>R. dentocariosa</em></td>
<td>+</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td><em>B. dentium</em></td>
<td>+</td>
</tr>
<tr>
<td>Propionibacterium</td>
<td>several including <em>A. naeslundii</em></td>
<td>+</td>
</tr>
<tr>
<td>Actinomyces</td>
<td><em>C. matruchotii</em></td>
<td></td>
</tr>
<tr>
<td>Treponema</td>
<td>several including <em>T. denticola</em></td>
<td></td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>several including <em>P. gingivalis</em></td>
<td>-</td>
</tr>
<tr>
<td>Prevotella</td>
<td>several</td>
<td>+</td>
</tr>
<tr>
<td>Capnocytophaga</td>
<td>several including <em>C. ochracea</em></td>
<td>-</td>
</tr>
<tr>
<td>Bacteroides</td>
<td><em>B. heparinolyticus, B. zoogloeiformans</em></td>
<td>+</td>
</tr>
<tr>
<td>Peptostreptoccus</td>
<td>several including <em>P. micros</em></td>
<td>-</td>
</tr>
</tbody>
</table>

Individuals with no obvious signs of gingivitis may harbour sub-gingival plaque. In such individuals, the sub-gingival plaques comprise of mostly (approximately 95%) non-motile rods and cocci (Listgarten & Hellden, 1978). At sites of inflammation, i.e. sites of gingivitis, the numbers of motile rods and spirochaetes increase and the proportion of streptococci decreases (Lindhe et al 1980). Potentially pathogenic bacteria are frequently isolated from sub-
gingival plaque, for example, *Eikenella corrodens*, *A. actinomycetemcomitans* and *Treponema denticola*, *Porphyromonas gingivalis*, *F. nucleatum*, *Prevotella intermedia*. The protozoan, *Entamoeba gingivalis* has been found in the oral cavities of subjects with gingivitis and periodontal disease (Linke et al 1989, el Hayawan & Bayoumy 1992).

**1.3. Dental caries**

1.3.1. *The pathogenesis of dental caries*

Caries is the slowly progressing decomposition of teeth through acid-induced demineralisation. The disease occurs at various sites on the tooth (figure 1.3.1.) and common forms are smooth surface (especially in nursing infants), approximal, occlusal and root surface caries. The latter group differs slightly as the surface at the solid/liquid interface is cementum rather than enamel.
The enamel at carious sites is usually covered by an aciduric biofilm containing high proportions of *S. mutans* and *S. sobrinus* (Lang et al 1987). The enamel consists of tightly packed hydroxyapatite crystals in a uniform rod packing structure with inter-crystalline spaces (micropores) containing a solution of organic molecules. If the plaque is left undisturbed there is acidic demineralisation of the crystals along the rod increasing the porosity of the enamel. The sub-surface tissue is demineralised at a faster rate than the surface enamel. The protection of the surface from demineralisation is not fully understood. However, proline-rich proteins and statherin present in saliva have been shown to inhibit demineralisation (Hay 1984, Featherstone et al 1993). These large molecular weight proteins are unable to diffuse through the relatively small micropores possibly explaining the difference in demineralisation at and below the surface. The structural properties of the
surface could also be responsible for the reduced demineralisation (Weatherall et al 1984). At this stage, arrest of the enamel lesion can be caused by frequent plaque removal from the lesion site. On arrest, the surface of the lesion is eroded by mechanical processes to produce a shiny hard finish to the enamel. Precipitation of crystals may occur within the enamel due to the supersaturated state of the water in the micropores. Without plaque removal the lesion will progress reaching the dentin where two distinct areas of bacterial flora develop. The necrotic area contains a mainly gram positive aciduric flora frequently containing S. mutans. The flora at the advancing end of the lesion contains a less diverse flora dominated by gram positive rods such as Lactobacillus spp., Propionibacterium spp. and Eubacterium spp. (Edwardsson 1974). The metabolism of lactic acid by eubacteria and propionibacteria indicates some bacterial succession. Dissolution of the mineral content of dentin allows access to the collagen portion that can be degraded by microbial proteases and used for metabolism. When demineralisation approaches within 0.5 - 1.0 mm of the pulp cavity, inflammatory responses are triggered by microbial products (Shovelton 1972).

1.3.2. Caries and oral biofilms

The formation of biofilms on the tooth surface is a naturally-occurring process. The pathogenicity of oral biofilms is controlled by external, host and microbiological interactions. Generally the balance results in avirulent biofilms that are advantageous to the host by preventing colonisation by exogenous pathogenic micro-organisms. This balance may be affected by an increase or
decrease in one of the controlling factors (refer to sections 1.3.3., 1.3.4. and 1.3.4.) leading to the formation of a pathogenic microflora (Hardie 1992). The most prevalent of oral diseases associated with micro-organisms is caries. Several hypotheses have been proposed to describe the microbial aetiology of caries. The “specific plaque hypothesis” (reviewed by van Houte, 1980) proposes specific members of the biofilm community to be the causative agents. The association of high proportions of *S. mutans* and *S. sobrinus* with carious lesions in many clinical trials (van Houte 1980, Loesche 1986) suggests the mutans streptococci may be the cariogenic members of the community. An alternative hypothesis, the “non-specific plaque hypothesis” (reviewed by Loesche, 1976), describes the aetiology of caries as a result of the biofilm/host interactions in their entirety. Neither hypothesis is able to fully explain the aetiology of caries (Marsh 1993). The association between caries and mutans streptococci suggests a level of specificity in the disease, however, caries can develop in the absence of these bacteria. The “ecological plaque hypothesis” of Marsh, 1991, is perhaps better able to explain the aetiology of caries. It is proposed that "a factor (or factors) triggers a shift in the proportions of the resident microflora and so predisposes a site to disease." The pathogenic process is therefore a result of a shift in the flora favouring species that are potentially cariogenic. The factors that may induce a shift in the microflora are many and are often interconnected. The following section describes some of the main factors involved in the control of the plaque microflora.

1.3.3. *Bacterial metabolism in pathogenesis*
Metabolism of host and external nutrients affects the balance of the plaque flora. In resting plaque, acetic, butyric and propionic acids are the predominant acids produced from the metabolism of salivary mucins and amino acids (Geddes 1975). These acids do not significantly lower plaque pH which has a range 5.6-7.0 in resting plaque. The metabolism of low molecular weight carbohydrates from the external environment leads to the formation of high concentrations of lactate and a corresponding drop in plaque pH. The curve associated with this pH drop and its gradual restoration to that of resting plaque is termed the Stephan curve (Stephan 1940) (figure 1.3.3.). The return of the pH to that of resting plaque which takes around one hour is predominantly due to the action of salivary buffers and diffusion of lactate out of the plaque. Further lactate may be removed by its metabolism to propionate, acetate, hydrogen and carbon dioxide by oral bacteria including *Veillonella* spp., *Neisseria* spp., *Eubacterium* spp. and *Propionibacterium* spp.. Base production through metabolism of proteins (Reynolds and Riley 1989) and by bacterial urease activity may also help to elevate the pH level. The production of intracellular polysaccharides in times of nutrient excess can lengthen periods of lactate production thereby decreasing the rate of return to resting plaque pH (Spataforra et al 1995). Glucan, fructan and heteropolysaccharide exopolymers (EPS) produced by oral bacteria are major components of the plaque matrix. The EPS strengthens adhesion of plaque to the pellicle coated-enamel (London & Kolenbrander 1996) and lessens physical disruption thereby favouring thicker biofilms. Increased lactate production is observed in biofilms containing high amounts of exopolymer (Van Houte et al 1989) due to increased diffusion rates of substrate. EPS is a
possible nutrient source for oral bacteria (Burne 1991) and may also promote adhesion of oral streptococci (Hiroi et al 1992).

Figure 1.3.3. The Stephan Curve response of plaque pH to bacterial metabolism of dietary sugars.

1.3.4. Host control of pathogenesis

The principal host factors controlling the plaque flora are saliva and the tongue. Movement of the tongue produces shear on oral biofilms resulting in their mechanical removal. Saliva exercises control over the microflora by a variety of different mechanisms, the most important being its buffering action. The salivary buffers, the carbonic acid-bicarbonate and phosphate systems buffer at around pH 7.0 favouring the non-acidogenic members of the biofilm community. As plaque thickness increases, the influence of salivary buffers is compromised in the lower strata of the plaque and therefore its capacity to control the microflora. The organic constituents of saliva can be metabolised by some members of the microbial community (De Jong and Van der Hoeven
Acetic, butyric and propionic acids are the predominant acids produced from the metabolism of salivary mucins and amino acids (Geddes 1975). These low pKa acids have little impact on plaque pH. Saliva also contains specific and non-specific antibacterial systems. The specific factor is the immunoglobulin, IgA, which inhibits adhesion of mutans streptococci (Tyler & Cole 1998). The non-specific antimicrobial factors are the lactoperoxidase system, lysozyme and histidine-rich peptides. The importance of these factors in regulating plaque bacteria is unclear (Kirstila et al 1996), however, lactoferrin and lysozyme have been shown to possess antimicrobial activity against *Candida* species (Samaranayake et al 1997). Finally, salivary flow removes exogenous food sources from the oral cavity and shear of the flow can reduce the biological load of plaques. Gingival crevicular fluid (GCF) is a serum-like fluid secreted in the gingival crevice (Marsh & Martin 1992). The protein concentration of GCF is over ten-fold higher than that of saliva. The defence molecules in GCF include the immunoglobulins, IgG, IgM and IgA. GCF also contains leucocytes of which approximately 95% are neutrophils. The crevicular fluid can mix to some extent with saliva and act as a food source and provide immunoglobulins (Brandtzaeg 1989) and host defence cells that may affect the balance of the flora.

1.3.5. *External influences on pathogenesis*

The principal external factors affecting plaque flora are diet and mechanical cleansing. Of extreme importance in the shift to potentially cariogenic populations is the fermentation of low molecular weight sugars producing
lactic acid. Lactic acid lowers the pH of the plaque allowing the acidogenic members of the flora to thrive. The retention time of fermentable sugars in the oral cavity, and therefore the duration of lactic acid production, influences the balance of the flora. The retention time of nutrients depends to a large extent on the rheological properties of the nutrients. Liquids pass through the oral cavity rapidly whereas baked carbohydrate snacks, for example, crisps, may adhere to the teeth.

The implementation of oral hygiene procedures, for example, toothbrushing, results in disruption of the microbial community. This generally results in thin plaques in which the environment can be efficiently controlled by salivary buffers. Mechanical removal increases initial biofilm forming processes, that is, adhesion and colonisation, and favours primary colonisers.

1.4 Caries prevention

1.4.1. Fluoridation

The use of fluoride in the prevention of caries gained scientific support in the 1930's when it was identified as the causative agent in drinking waters responsible for mottled enamel and reduced caries incidence. The methods of administration of fluoride in caries prevention programmes include water fluoridation, fluoride tablets, salt fluoridation, milk fluoridation and topically-applied fluoride and slow release devices.

Topically-applied fluoride includes professionally-applied gels, solutions and varnishes and self-applied gels, toothpaste and mouthwashes. Self-applied sodium fluoride mouthwash (0.2%) reduced caries incidence in studies in

1.4.2. Anti-plaque chemicals.
The use of fluoride in caries prevention programmes has been a successful cost-effective method of reducing the incidence of the disease. However, the disease is still prevalent and alternative prevention and treatment programmes are sought. A growth area in caries prevention is the use of alternative antimicrobials incorporated into toothpastes and mouthwashes for daily use. Any such agent that is used daily should possess few or no side effects and bacteria should not develop resistance to the drug. For the latter reason, antibiotics are not used for prevention programmes but restricted to use in disease treatment. Many different agents have been suggested or developed for the prevention of caries. These agents have been categorised on the basis of their retention in the oral cavity (i.e. their substantivity) and their mode of action (Brecx 1997). Table 1.4.2. lists the chemicals used as oral antimicrobial agents and their assigned groups.
First generation antimicrobials are agents that possess activity but have little or no substantivity. These agents tend to act by a bactericidal or bacteriostatic mechanism. Second generation antimicrobials are mainly bacteriostatic or bactericidal agents that interact with available surfaces to increase retention time. Third generation agents are those that possess some degree of bactericidal or bacteriostatic activity but also possess anti-plaque activity. This may take the form of adhesion prevention, disruption of adhesive forces within biofilms and properties that block specific inter-generic and intra-generic adherence between bacteria. Except for chlorhexidine, the grouping of the agents is based on results from clinical trials and on very little evidence from mechanistic studies in vitro.

1.4.2.1. Bactericidal agents and biofilm resistance

The most widely used property of chemicals used to control plaque and thus prevent dental caries is bactericidal or bacteriostatic activity which is usually referred to as antimicrobial activity. A distinction between the two is difficult as some agents, for example, chlorhexidine, can act as either depending on concentration and the identity of the target bacteria (Jones 1997).
Second generation antimicrobials that are bactericidal or microbicidal agents with a level of substantivity (Brecx 1997) are currently the favoured antimicrobials used in mouthwashes and toothpastes. Bactericidal agents work by various mechanisms and the target organism(s) can be the community as a whole or a specific member. The killing of biofilm bacteria attempts to (1) prevent an increase in the biofilm mass by preventing growth, and, (2) prevent further acid production.

Unfortunately there is a major problem associated with the agents developed and used in current mouthwashes and toothpastes. It is well documented that biofilm bacteria are invariably less susceptible to bactericidal agents than their planktonic counterparts and the subject has received many reviews (Costerton et al 1987, Brown & Gilbert 1993, Bradshaw 1995). However, the bactericidal agents currently in use in oral hygiene products have been developed and tested predominantly on planktonic bacteria. The result is that the activity of these agents towards oral biofilms in vivo is often poor when compared to that observed during laboratory development. Various suggestions have been proposed to explain the differences in susceptibility of biofilm and planktonic cultures. The importance of diffusion limitation has been investigated as a cause of reduced efficacy of antimicrobial agents. Diffusion limitation has been calculated to occur in biofilms (Nichols 1993), however, viability has been observed in biofilms after hours or days of antibiotic delivery (Nickel et al 1985). Besides diffusion limitation, it has been suggested that the glycocalyx may hinder the penetration of antimicrobials by ionic and chemical interactions. The polyanionic nature of polysaccharide matrices (Costerton et al 1987, Hoyle et al 1990) may influence the access of...
cationic antimicrobials. Chemical bonding between the glycocalyx and antimicrobials could occur (Nichols 1991) which may neutralise agents and protect underlying cells (Brown & Gilbert 1993). Alternatively, the glycocalyx may repel agents and thus prevent access to inner parts of the biofilm (Costerton et al 1987). The biofilm matrix may alter the micro-environment of associated cells through nutrient limitation or trapping, causing increased localised concentrations of metabolites and exclusion of gases (Gilbert et al 1993). Changes in the micro-environment may cause a phenotypic change in biofilm cells conferring increased resistance. Nutrient availability may influence metabolic pathways, cell surface composition and growth rate (Brown & Gilbert 1993) which in turn may alter susceptibility to antimicrobial agents. The effect of growth rate on the sensitivity of Bacillus megaterium to chlorhexidine and 2-phenoxyethanol has been studied (Gilbert & Brown 1980). Under conditions of carbon, magnesium or phosphate limitation the sensitivity of chemostat cultures of B. megaterium to chlorhexidine increased with an increase in growth rate. The sensitivity to 2-phenoxyethanol was only increased when the cultures were limited by carbon and magnesium. The susceptibility of Escherichia coli to quaternary ammonium compounds (Wright & Gilbert 1987) has been investigated in relation to specific growth rate. The susceptibility of the organism to an alkyltrimethylammonium bromide with a carbon chain length of 16 increased with an increase in the specific growth rate. This was observed under magnesium, carbon, phosphate and nitrogen limitation.

A change in the susceptibility of micro-organisms to antimicrobial agents may occur immediately after adhesion to a substratum. Fujiwara et al, 1998,
determined the minimum bactericidal concentrations (MBC’s) of various antibiotics toward *Pseudomonas aeruginosa*, *Serratia marcescens* and *Proteus mirabilis* planktonic and adhered cells. The MBC’s of 6 different antibiotics were increased for all three species after adhesion of cells to tissue culture plates. In most cases the MBC was increased more than 128 times after the cells had adhered to the substratum. In a similar model, the attachment of *Staphylococcus epidermidis* and *Escherichia coli* to polystyrene was shown to affect the level of susceptibility to a variety of chemical agents (Das et al 1998). The cause of reduced susceptibility of biofilms to bactericidal agents is likely to be a function of many of the factors discussed above. Clearly, major problems exist in the development of agents, and ecological factors should be taken into account when developing new compounds.

The effectiveness of agents that work by bactericidal or bacteriostatic mechanisms is compromised for reasons other than the reduced susceptibility of biofilms. Acid can remain in killed biofilms and continue to demineralise enamel. Cell death and lysis may provide further nutrients for bacteria that survive treatment. If the members of the microflora are not equally susceptible to the agent, a shift in the proportions of species could occur which could either be beneficial or undesirable. Despite the problems associated with the use of bactericidal agents they have proved effective in disease prevention in clinical trials and remain an economically viable method for plaque control and caries prevention.
1.4.2.2. Anti-adhesion activities

The use of chemicals with activities capable of preventing bacterial adhesion may be of potential use in caries prevention. Agents may prevent adhesion to the surface, or inhibit specific interactions between bacteria. Agents that disrupt mature biofilms may also be beneficial in reducing caries incidence. The effectiveness in preventing microbial adhesion is dependent on the substantivity of the agent. The duration of activity is likely to be better than observed with bactericidal agents, as anti-adhesion agents do not generally react with the microbial cell. Thus the agent is not depleted as a result of its activity. A downside to the use of anti-adhesion agents is that they provide no protection against plaque re-growth from areas where mechanical removal of the biofilm was unsuccessful. The use of anti-adhesion agents in conjunction with bactericidal chemicals is desirable due to possible additive effects.

1.5. Amine fluorides and stannous fluorides

1.5.1. Overview of amine and stannous fluoride use

Amine fluorides, usually in combination with stannous fluoride, are used in mouthwash and toothpaste formulations on the European continent. Meridol (Wybert GmbH, Freiberg, Germany) is an example of a commercially available mouthrinse containing both amine and stannous fluorides. There have been over 60 clinical trials investigating the effects of amine and stannous fluorides in the human oral cavity. Around forty of these trials investigated the effect of the agents on supra-gingival plaque. In contrast, there are only around 25 in vitro investigations into the effects of the agents on oral bacteria. The majority of these have determined the in vitro
susceptibility of bacteria grown in a planktonic state. The many clinical trials
have found beneficial anti-caries activity when using the agents alone (Mercer

1.5.2. The fluoride ion

The effects of fluoride on the structural properties of enamel and the
metabolism of oral bacteria have been investigated in an attempt to resolve
the mechanisms involved in caries prevention by fluoridation. However the
general observations are applicable to the action of topically applied fluoride-
containing compounds. Fluoride incorporated into apatite lattices alters the
structural conformation of the crystals increasing the stability and decreasing
acid solubility (Brown et al 1977). Fluoride in solution can be taken up by the
outermost layers of enamel or adsorb to the surface and decrease
demineralisation (ten Cate & Duijsters 1983).

Fluoride not only alters the structural properties of enamel but affects oral
bacterial metabolism. Central to fluoride activity is the inhibition of enolase,
the enzyme catalysing the conversion of 2-P-glycerate to P-enolpyruvate
(PEP) in the glycolytic pathway. Inhibition has been observed in vivo
(Hamilton 1990) for oral streptococci which increase in sensitivity to fluoride
as the pH falls (Hamilton & Ellwood 1978). PEP synthesis is central to oral
streptococcal biosynthesis and its inhibition will affect other processes. Sugar
transport into the cells of acidogenic bacteria is largely by the P-enolpyruvate
phosphotransferase system. This pathway will be indirectly inhibited due to
the unavailability of PEP, the phosphoryl donor for the reaction.
Maintenance of cellular pH at a level higher than the surrounding medium is essential for enzymatic function in acidogenic bacteria. Lactic acid efflux and ATPase proton pumping (Hamilton & Buckley 1991) remove protons from the cell creating an electrical difference between the internal and external environment. Inhibition of PEP production lowers the cellular ATP and lactic acid concentrations and thus compromises the ability to maintain the proton gradient and cellular pH. Fluoride can also directly affect the ATPase of oral bacteria (Sutton et al 1986).

The potential energy in the proton gradient (proton motive force-PMF) is used by cells for biological work, for example, flagellum motion, solute transport, and removal of the pH gradient. Thus disruption of the proton gradient will affect any mechanism relying on PMF. The effect on pH homeostasis by impairment of the proton extruding processes is alleviated to some extent by physiological adaptation to the acidic environment (Hamilton & Buckley 1991). Extracellular polysaccharides (EPS) produced mainly through sucrose metabolism are important in the protection of streptococci in the oral cavity. It appears that the enzymes involved in EPS production are fluoride-insensitive. The importance of fluoride in the inhibition of bacterial metabolism with regard to caries inhibition is a matter of debate. The fluoride concentration in plaque has been estimated to be lower than the concentrations that were required to inhibit metabolism in vitro (Tatevossian1990). However the response of metabolic processes to fluoride in vivo has yet to be determined and thus cannot be discounted.
1.5.3. Amine fluoride

It is well documented that amine fluorides possess bactericidal and bacteriostatic activity towards oral bacteria (Kay & Wilson 1988, Bansal et al 1990, Shapira et al 1991). The mechanism of bactericidal activity is unclear, but it is thought to involve the surfactant properties of the chemicals (Shern et al 1970). Amine fluorides are also known to inhibit bacterial acid production (Capozzi et al 1967) and block enzymes involved in bacterial metabolism (Bramstedt & Bandilla. 1966)

The adsorption of amines to enamel and pellicle-coated enamel has been reported to reduce enamel dissolution rates in vitro (Rosemann et al 1969). It is thought that the amine layer reduces ion diffusion rates from the surface and it has been shown that the diffusion rate decreases with increasing chain length of amine (Borggreven & Driessens 1986). The presence of a protein pellicle is reported not to affect adsorption of amine (Sefton et al 1996). The surface free energy of enamel is lowered by amine adsorption (Busscher et al 1988), a feature that has been shown to reduce adhesion of oral streptococci (Busscher 1986) in vitro. Anti-adhesion activity has been reported in models using topically-applied amine fluoride (Meurman 1987) and with the agent incorporated into a resin (Bapna et al 1988). Both investigations were operated in batch systems.

Fluoride uptake by enamel is increased by treatment with amine fluorides compared to sodium fluoride (Mok et al 1990) in vitro. Uptake has also been shown to increase in areas of demineralised enamel in artificial lesions (Chan et al 1991).
1.5.4. Stannous fluoride

Stannous fluoride was first used in oral hygiene products as far back as the 1950's (Gaffar et al 1997). The agent has been demonstrated to prevent acid formation by oral bacteria (Lilienthal 1956). Inhibition is thought to arise from the affinity of the cation for thiol groups (Oppermann 1981). An additional reduction in acid production may be a result of fluoride interfering with the glycolytic pathways of oral bacteria. The inhibition of acid production possibly explains the pronounced reductions in S. mutans concentrations (as a proportion of the total cultivable flora) in clinical trials (Schaeken et al 1986, Svanberg & Rolla 1982). It has been suggested that plaque inhibition in clinical trials may be due in part to an anti-adsorption activity (Skorland et al 1978). Presumably this is due to an increase in the hydrophobicity of enamel when treated with stannous fluoride (Rolla & Ellingsen 1994). In contrast, Olsson and Odham 1978 did not detect any anti-adhesion activity of stannous fluoride using whale dentin surfaces attached to human teeth but did show a bactericidal activity at concentrations as low as 0.8%. Anti-adhesion activity has been reported in vitro (Gross & Tinanoff 1977, Ota et al 1989). However closed systems were used in the models which do not take into account the substantivity of the agent. Stannous fluoride has been reported to mildly inhibit cell-free S. mutans glucan production (Scheie & Kjeilen 1987). In contrast stannous fluoride has been reported to enhance glucan production from whole cells of S. mutans and other oral streptococci (Zameck & Tinanoff 1987).
1.5.5. Amine and stannous fluoride combinations

There have also been studies that have investigated the properties of amine and stannous fluoride in combination. Weiger, (1998), reported bactericidal activity but no anti-adhesion activity when using the combination in clinical trials. In contrast, Soderling, et al (1991), did report \textit{in vivo} anti-adhesion activity of the combination. However Soderling’s study group consisted of just three females and is unlikely to be statistically reliable. Inflammatory response may be affected by amine and stannous fluorides. Both agents have been reported to increase \textit{in vitro} superoxide release from human neutrophils stimulated with fMLP, and combining the agents enhances the effect (Shapira et al 1997).

1.6. Models for testing of anti-plaque agents

1.6.1. Overview

The previous section highlights the lack of laboratory-based investigations into the modes of action of amine and stannous fluorides. The recent progress in the understanding of microbial ecology, for example, the decreased susceptibility of biofilms compared to planktonic bacteria, has led to the development of models and microcosm investigations that better simulate the habitat under investigation. It is probable that the decline in the use of animal testing has also benefited the development of improved \textit{in vitro} models. Models have been developed to study the antimicrobial and anti-plaque properties and the following sections will discuss different innovations in more detail.
1.6.2. Biofilm-based models

With the inherent problems associated with testing antimicrobial agents it is perhaps not surprising that there are many different laboratory models in use for the study of microbial biofilms. The different models have been reviewed by Gilbert & Allison 1994, Wilson, 1996, and Wimpenny, 1997. Closed systems such as agar plates and submerged substrata in test tubes are useful in preliminary investigations on novel antibacterial strategies and in developing protocols for more complex devices. However, as dental plaque forms in an environment characterised by a continuous flow, this section will concentrate on devices designed to incorporate this property.

1.6.2.1. The Modified Robbins Device

The modified Robbins device (Nickel et al 1985) (figure 1.6.2.1.) has been an invaluable tool for determining the antimicrobial susceptibility of biofilm bacteria. The equipment is essentially a long tube enclosed in an acrylic block with twenty-five sampling plugs along its length. Substrata can be fitted to the plugs so that they lie flush with the lumen. Temperature control is simple, by water bath or incubator. The device can either be inoculated and then run on sterile media, run on the effluent of a chemostat or on a batch loop system. Favorable elements of the device are the multiple samples per operation, its robustness and the flexibility in substratum choice. The device has proven useful in the study of lumen biofilms but has not gained favour in oral biofilm studies.
Diagram A shows a cross-section showing the lumen within the perspex block, the sample ports and a stud and B represents the view from above. C is a cross-section of a stud and D, the view of the 1 cm rim of the stud within which the discs are inserted.

1.6.2.2. Perfused biofilm fermenter

Biofilm devices have been developed specifically to investigate the effects of growth rate on bacterial susceptibility to antimicrobial agents. Early devices (perfused biofilm fermentor - Gilbert et al 1989) consisted of bacteria attached to the underside of filters impermeable to the bacteria that were then perfused from above with medium. At steady state, growth rate is regulated by controlling the concentration and flow rate of medium to the device. Investigations using the device with a selection of bacteria and antimicrobial agents have demonstrated the importance of growth rate when considering the differences in susceptibility of planktonic and biofilm bacteria (Brown & Gilbert 1993). A modification of the system using Sorbarod filters has been
described by Hodgson et al, 1995. Using this model Gilbert et al, 1997a, demonstrated a novel mode of inclusion of ‘foreign’ planktonic Enterococcus faecalis cells into pre-formed biofilms. The device, or similar models, have yet to be used to determine the effect of growth rate on the susceptibility of oral bacteria to amine fluorides.

1.6.2.3. The Constant Depth Film Fermenter

The constant depth film fermenter-CDFF (Peters & Wimpenny 1988) (figure 1.6.2.3.) is an autoclavable laboratory model with the distinction of producing biofilms of known thickness. Specially-designed polytetrafluoroethylene (PTFE) pans fit in 15 holes bored in a steel turntable on which media drips. Up to six PTFE plugs can be fitted to each pan. Biofilms can grow on the substrata held over the plugs (or on the plugs themselves) recessed to a depth of choice. Biofilm growth above the level of the turntable, and excess media, is removed by two PTFE spring loaded scraper blades. The CDFF has been used to grow single species (Pratten et al 1998a), mixed species (Kinniment et al 1996) and microcosm oral biofilms (Pratten et al 1998b). The number of replicate biofilms (up to 90) and the control over biofilm thickness makes the CDFF ideal for susceptibility testing. The thin film of media trickling over the turntable and the mechanical action of the scraper blades affords the device physical characteristics similar to those found in the human oral cavity.
1.6.2.4 Artificial mouths

Devices termed ‘artificial mouths’ have been designed and developed specifically for studying oral biofilms (Dibdin et al 1976, Sissons & Cutress 1987) with the intention of simulating conditions in vivo as closely as possible.
Artificial mouths are designed for microcosm studies although single species studies are possible. The devices have been used to investigate metabolism and pH of artificial plaques and their involvement in mineralisation (Sissons et al 1991). Due to the complexity of artificial mouth systems the number of replicate biofilms is small. Screening of chemicals for bactericidal activity requires a large number of replicate biofilms. Hence artificial mouths are probably not the most suitable models for screening of chemical agents for antimicrobial activity.

1.6.2.5. Analytical procedures for biofilm models

The different devices discussed in this section have all proved useful in studying bacterial biofilms. The biofilms can be analysed using a variety of techniques. These include classical analytical methods, for example, viable counts, direct enumeration, electron microscopy and radioactive labelling. More recent technological advances in microbial analysis include scanning confocal microscopy (Costerton et al 1995) and specialised fluorescent probes and reporter genes to mark particular species within biofilms (Skillman et al 1998).

Devices that produce reproducible biofilms are desirable for testing of antimicrobial activity. The use of these systems in the study of anti-plaque agents and adhesion events is compromised by the lack of sensitivity over short time periods. This is in part due to the difficulty of using real time analysis with the devices. However, once the effects of an agent on adhesion processes have been characterised, biofilm models are suitable to study the long term effect of the agent.
1.6.3. Models for the study of adhesion processes

To investigate the anti-plaque properties of chemical agents, models are required that are able to detect extremely low numbers of micro-organisms. Simple models for studying adhesion processes include substrata covered with saliva (Shahal et al 1988), test materials submerged in broth cultures (Eifuku-Koreeda et al 1991, Neeser et al 1994) and adhesion to the bottom of micro-well plates (McNab & Jenkinson 1998). These systems are useful in the investigation of the effects of surface properties on adhesion and in the characterisation of adhesion and co-adhesion mechanisms. Unfortunately, there are limitations to the value of such models in the study of anti-plaque agents. The closed batch design of the systems does not allow for the substantivity of the agents to be assessed nor do they allow for simple shear force control. With many batch models it is difficult to connect analytical devices for continuous monitoring.
1.6.3.1. Parallel flow cells

Parallel flow cells are well-defined models that have been developed for the study of microbial adhesion (Berg & Block 1984, Christersson 1991, Herles et al 1994, Busscher & van der Mei 1995). Unlike the simple models mentioned above, parallel flow cells offer a continuous system that can measure the substantivity of anti-plaque agents under controlled conditions. Flow cell models vary slightly from laboratory to laboratory but all contain certain essential components. Figure 1.6.3.1. shows a schematic representation of a flow cell as described by Bos et al, 1999. The flow cell in all cases has parallel sides and is long enough to establish laminar flow. Flow cells may differ in cross-sectional area, however, the view path is usually a small distance for the benefit of detection procedures. The cell may be reusable or disposable. Reusable cells often possess recessed areas where test substrata can be fitted. Temperature may be controlled by incubators, fitted thermostats or water baths. The flow cell has an inlet for conditioning fluids, test agents and microbial culture and an effluent outlet. The flow rate of culture through the cell is pre-determined and controlled by means of a pump.
1.6.3.1. Schematic diagram of a flow cell as described by Bos et al, 1999.

1.6.3.2. Analytical procedures for adhesion studies

A variety of analytical techniques have been used in combination with flow cells for continuous monitoring of adhesion events. The use of confocal laser microscopy-CLM in microbial ecology was developed using biofilms grown in flow cells (Caldwell et al 1992). More recently, computer software has been designed to allow semi-automated image processing using scanning CLM (Kuehn et al 1998). Light microscopy has been used in conjunction with flow cells by numerous workers (Lawrence et al 1992, Barton et al 1996, Habash et al 1997). The development of digital imaging technology and image analysis software benefits light microscopy techniques (Nivens et al 1995) as has improvement in the resolution of long working distance optical lenses. Fluorescence microscopy has been used in conjunction with flow cells to investigate the ability of preparations of mutanase and dextranase to remove
oral bacterial biofilms (Johansen et al 1997). Other continuous non-disruptive monitoring systems include bioluminescence (McElroy & DeLuca 1983), spectrometry and electrochemical measurement (Nivens et al 1995). Their use in the study of adhesion processes may be limited due to the relatively low number of cells involved in such events.

Flow cell systems have been used successfully to study the processes of microbial adhesion (Christersson et al 1989, Yu et al 1996), co-adhesion (Sjollema et al 1990, Bos et al 1996) and desorption (Christersson et al 1988). However there are few publications describing the use of flow cell systems to elucidate the action of anti-plaque agents on these processes. Rundegren et al, 1992, investigated the effects of delmopinol pulsing on the formation of S. mutans biofilms grown in parallel flow cells. They found that the agent not only reduced the biofilm biomass at 29 hours but also increased biofilm disruption by ultra-sonication. Flow cell studies have been used in the development of triclosan as an anti-plaque agent (Gaffar et al 1994). They were able to show that copolymer addition to triclosan formulations increased the agent’s ability to reduce plaque formation in vitro.

The reported use of flow cells to determine the effectiveness and mode of action of anti-plaque agents is very limited. However the results obtained thus far suggest the system could be of great use for in vitro studies.
1.7. Aims of study

The previous sections have discussed the microbial ecology of human dental caries and the methods exercised in prevention programmes. One method of prevention is the use of topically-applied chemical agents to control dental plaque. The mechanism of control may be antimicrobial, anti-adhesion, anti-coadhesion or biofilm-disrupting.

Antimicrobial activity has been characterised for many chemical agents. However the activity observed in vivo is often greatly reduced compared to that seen in laboratory trials. This is mainly because most in vitro testing has been performed on planktonic bacteria that are inherently more susceptible to antimicrobial agents than their counterparts growing as biofilms. This is true for the long chain aliphatic antimicrobial compounds, the amine fluorides. Extensive clinical trials have shown that the agents possess plaque-reducing properties although less so than chlorhexidine. In contrast, the in vitro results published on planktonic bacteria observed greater kills of oral bacteria with amine fluorides than chlorhexidine digluconate.

The object of the first part of the study was to determine the influence of various factors on the susceptibility of oral bacteria to a range of amine fluorides. Testing of the agents grown in identical media but using different growth modes combined with testing of organisms grown in the presence or absence of sucrose allows a stepwise progression of increasing complexity of models. This should prove useful in determining the relative importance of each factor in microbial susceptibility to the agents.

There are few investigations of the anti-plaque properties of amine and stannous fluorides. The publications that do exist are somewhat varied in
observed activities due to differences in the design of the models used to investigate the properties.

The second part of the study attempted to determine what anti-plaque properties the agents possess using continuous systems that investigate the different possible modes of action and the relative substantivity of the agent. The agents were studied in combination to determine any net benefits to their anti-plaque properties.
Chapter 2 - Materials and Methods
2.1 Organisms

2.1.1. Single species experiments

The organism used in all single species experiments was *Streptococcus sanguis* NCTC 10904. The reference culture was rehydrated and streaked in quadruplicate on tryptone soya agar (Oxoid, Basingstoke, UK). The plates were incubated aerobically at 37°C for 24 hours. Four to five discrete colonies were aseptically transferred from an agar plate and transferred to 10 ml of nutrient broth (Oxoid). After 24 hours aerobic incubation at 37°C the 10 ml culture was aseptically transferred to 100 ml of sterile nutrient broth. The broth was incubated for 24 hours at 37°C aerobically. The culture was then Gram stained and, if no sign of contamination, was aseptically split into 1.0 ml aliquots in sterile Nunc cryovials (Fisher Scientific, Loughborough, UK). The cryovials were stored at -70°C.

2.1.2. Microcosm experiments

The microcosm plaque communities were grown from pooled human saliva. The saliva was pooled from three to four individuals. This was split into 10 ml aliquots and frozen at -70°C.

2.1.3. Co-adhesion experiments

The organisms used in co-adhesion experiments were provided by Dr Rod McNab, Department of Microbiology, Eastman Dental Institute, London. The organisms were *Streptococcus gordonii* Challis (Pakula & Walczak 1963) and *Actinomyces naeslundii* ATCC 12104. The *S.gordonii* strain was originally
obtained from Dr DJ Leblanc (University of Texas Health Science Centre at San Antonio, San Antonio, Texas, US) and was designated DL1 Challis.

### 2.2 Growth medium

The growth medium in all experiments was a mucin-based formulation. The components are listed in table 2.2. The composition was based on the work of Russell and Coulter (1975), Shellis (1978) and Pratten et al (1997).

**Table 2.2. Composition of mucin-containing medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Lab-lemco’ powder</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Proteose Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Mucin Type III: Partially Purified from Porcine Stomach</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.35 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

After autoclaving 12.5ml of 40 % filter sterilised urea was added. All chemicals were obtained from Sigma, Poole, UK.

For rinse and washing procedures, medium containing only the salts of MCM was used. This was termed MCM-salts and contained per litre: 0.83 g Nacl, 0.2 g CaCl₂, 0.2 g KCl.
2.3. The Constant Depth Film Fermentor

2.3.1 Fermenter design

The introduction discussed the relative merits of various models to study the efficacy of antimicrobial compounds in killing biofilm bacteria. The model chosen for such testing was the constant depth film fermentor (CDFF). This was because of the relatively large number of biofilms grown per fermentor run and the ability to control the thickness of the biofilm. Devices using a scraper bar to control biofilm thickness were first described by Atkinson and Daoud (1970). The idea was developed by Wimpenny (1981) to investigate oral biofilms, and subsequently improved by Peters and Wimpenny (1988). A modified version of this model was used in the present study (figure 2.3.1.1). The equipment was manufactured by John Parry-Jones Engineering, Cardiff.
The stainless steel turn-table (figure 2.3.1.2) holds fifteen polytetrafluoroethylene (PTFE) pans. Each plan is held flush with the top of the turntable by two silicone rubber 'O' rings. A pan contains 5 cylindrical holes, 5 mm in diameter, which accommodate PTFE plugs. The pan also has a central threaded hole into which a special tool is inserted for removal of pans from the fermentor. Discs of various materials can be placed on, and are supported by, the PTFE plugs. These discs act as the substratum for biofilms (refer to section 2.4 for details of disc materials). The discs sit on the PTFE
plugs and can be accurately recessed using pre-moulded tools. For the purpose of these studies, depths of 100 μm and 300 μm were used.

The steel turn-table is set on a central shaft that passes through the base plate (figure 2.3.1.3). The turn-table is powered by a motor fitted to the bottom of the shaft. The motor is driven by a 15 V power supply (BBH Power Products). Flush with the top of the turn-table are two spring-loaded PTFE scraper blades extending from the top plate. The top plate is fitted with three medium inlet ports, a sample port and air inlet port. Two Whatman Hepa-vent 0.2 μm pore filters (Fisher Scientific) are attached in series to the air inlet port. Between the top and bottom plates are a QVF borosilicate glass housing and two PTFE seals. The bottom plate contains the turntable shaft housing and an effluent outlet port.
Figure 2.3.1.2. Schematic horizontal section through the fermenter

- PTFE angled scraper blade
- Position of main medium inlet
- PTFE pan
- Position of silicone rubber ring
- PTFE pan plug
- QVF glass cylinder
- Direction of disc revolution
- Stainless steel disc
The peripherals in the operation of the CDFF include a nutrient supply, an inoculum and effluent collection. The flow lines between peripherals and the CDFF were composed of silicone rubber tubing (Fisher). Flow lines were attached to one another by quick disconnect fittings (Sigma). Unless otherwise stated liquids were pumped by a Watson Marlow 101U/R peristaltic pump (Watson Marlow Limited, Cornwall, UK) using Watson Marlow 0.8 mm bore pump tubing. The peripherals varied from experiment to experiment.
hence a detailed description will be given in the materials and methods section of subsequent chapters.

2.3.2. Sampling of the fermentor

As previously mentioned, a special sampling tool was used to remove pans during operation. The first step in sampling was to stop the turntable whilst a pan was positioned underneath the sampling port. The port was flamed and unscrewed. A sterilised sampling tool was lowered through the sampling port and screwed in to the threaded hole in the centre of the pan. The pan was carefully pulled out of the turntable and lifted from the fermenter. The sampling port was screwed shut.
2.4. Substrata for CDFF Investigations

2.4.1. Substratum for single species CDFF experiments

In all single species experiments 5mm hydroxyapatite (HA) discs were used. These were obtained from US Bio-interfaces inc. (San Diego, US). The discs were re-used three times before discarding. After each use, the discs were autoclaved, suspended in methanol (>5 minutes) and wiped free of adherent material with isopropyl alcohol BP wipes. (Vernon Carus, Preston, UK). The discs were then suspended in a solution designed to promoted mineralisation (Causton & Johnson, 1982). The formula of the mineralising solution is listed in table 2.4.1.

Table 2.4.1 Formula of mineralising solution.

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>amount (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.16</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>0.02</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate 1-hydrate</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
</tr>
</tbody>
</table>

All chemicals were obtained from Sigma, Poole, UK.
2.4.2. Substratum for microcosm CDFF experiments

Pyrex glass disc were used in microcosm CDFF experiments. The 5 mm diameter discs were supplied by Hampshire glassware (Southampton, UK) and were discarded after one use.

2.5. Continuous culture

Planktonic cultures for antimicrobial testing were grown by continuous culture. The apparatus set-up is shown in figure 2.5.. The volume of the chemostat culture was 500 ml. The flow rate of medium to the chemostat was 0.03 L h\(^{-1}\). The dilution rate for the chemostat was 0.06 h\(^{-1}\). The chemostat was inoculated with an overnight culture of \(S.\ sanguis\) grown in tryptone soya broth (Oxoid), aerobically at 37°C. The achievement of steady state was determined by viable counts (section 2.7.1.). Liquids were pumped by a Watson Marlow 101U/R peristaltic pump (Watson Marlow Limited, Cornwall, UK) using Watson Marlow 0.8 mm bore pump tubing.
The chemostat vessel was incubated at 37°C.

2.6. Glass packed tube culturing device

Biofilm-derived suspended cells were used for antimicrobial susceptibility testing and for bacterial adhesion experiments. The physiological state of such cells would be more like that of bacteria in saliva, which are mostly derived from biofilms on oral surfaces, than that of cells grown by traditional planktonic culture methods. The biofilm-derived suspended cells were obtained from the effluent of a glass-packed tube (GPT) device. The device consisted of a 280-cm length of silicone rubber tubing (Fisher Scientific) filled with 710- to 1,180-μm-diameter glass beads (Sigma) inoculated with an overnight nutrient broth (Oxoid) culture of *S. sanguis* (tryptone soya broth [Oxoid] was used as the inoculum broth for GPT's used in adhesion studies). The apparatus set-up is shown in figure 2.6. Following a 4-h static attachment
phase, MCM (section 2.2.) was pumped through the device at a flow rate of 0.03 L h\(^{-1}\) (dilution rate = 2.31 h\(^{-1}\)). The achievement of a steady CFU concentration in the MCM exiting the device was determined by viable count (section 2.7.1.). Liquids were pumped by a Watson Marlow 101U/R peristaltic pump (Watson Marlow Limited, Cornwall, UK) using Watson Marlow 0.8 mm bore pump tubing.

**Figure 2.6.** Glass-packed tube (GPT) device and peripherals

The GPT device was incubated at 37°C.
2.7. Analytical procedures

2.7.1. Viable counts

Viable count procedure were used for enumeration of colony forming units (CFU) in biofilms and planktonic cultures. Prior to viable count procedures, biofilms were dislodged from the substratum and suspended in sterile medium. Refer to the respective sections for details of suspension methodology. Biofilm suspensions and planktonic cultures were serially diluted (100μl:900μl) in phosphate buffered saline (dulbecco “A”) (PBS) (Oxoid). Twenty μl aliquots of each dilution and the neat suspension/culture were spread in quadruplet on quarters of agar plates. Unless otherwise stated, tryptone soya agar (Oxoid) was used for plating procedures. Inoculated plates were incubated aerobically at 37°C.

2.7.2. Fluorescence microscopy

The fluorescent stain used was the BacLight live/dead viability kit (Molecular Probes Inc. Eugene, US). The kit contains two nucleic acid stains, SYTO 9 and propidium iodide. SYTO 9 stains live bacteria, with intact membranes, green. Propidium iodide stains dead bacteria, with compromised membranes, red. The two stains supplied in two dye solutions. The first contained, 1.67 mM SYTO 9 and 18.3 mM propidium iodide, in DMSO. The second contained, 1.67 mM SYTO 9 and 1.67 mM propidium iodide, in DMSO. The solutions were used in equal volumes for staining procedures.

Refer to respective chapters for details of staining protocols. The stained specimens were observed by epi-fluorescence microscopy using a Zeiss microscope fitted with a mercury-arc lamp (serial number-46 72 59-9901).
objective lens were a Zeiss plan 10/0.22 (x10) (Germany), a Leitz Wetzlar II 25/0.35 (x25) (Germany), an Olympus ulwd CDPlan 40 (x40) (Japan), and a Zeiss Plan 100/1.25 Oil (x100) (Germany). The first three lenses were selected for ultra-long working distance. The Zeiss Plan 100 lens was used in conjunction with oil immersion.

2.7.3. Photomicrography

Photographs of microscopical specimens were taken using a winder m 35 camera and a Zeiss MC63 exposure box. The film used for photomicrography was Fujichrome Sensia 100 135.36 (Fuji, Japan).

2.7.4. Digital capture of microscopical images

A JVC TK-C1381 colour video camera (BRSL, Newbury, UK) was attached to the microscope. The camera output was sent to a Dell 466 DL personal computer fitted with a Mutech Image/VGA-composite board (Optimum Vision LTD, Hampshire, UK). The board allows individual digital frames to be captured from the video camera and to be saved onto disc.

2.7.5. Measurement of pH of biofilm and planktonic cultures

pH was determined by using Whatman type CS pH strips (pH 3.8 to 5.5 and 5.2 to 6.8) and a Shindengen (Camlab) pH boy-p2 pH meter for the chemostat and GPT cultures. Only the pH meter was used for determining biofilm pH. All pH measurements were made at room temperature.

2.7.6. Measurement of total protein in biofilms
The protein content of *S. sanguis* biofilms was measured using a Micro BCA Protein Assay Reagent Kit (Pierce, Illinois, USA). The kit uses a two step colourmetric assay for protein. The first step is the biuret reaction, the reaction of protein with Cu$^{2+}$ in an alkaline environment to produce Cu$^{1+}$. The Cu$^{1+}$ ions are then reacted with bicinchoninic acid to form a water soluble reaction product that exhibits strong absorbance at 562 nm. Sample preparation adhered to the protocol supplied with the kit. Absorbance was measured at 570 nm.

### 2.8. Parallel Plate Flow Cell

#### 2.8.1. Key concepts in flow cell design

Laminar flow provides constant experimental flow conditions aiding the study of processes including cell adhesion, adsorption and de-adsorption. For laminar flow conditions to develop several criteria need to be met (Busscher et al 1995):

1. The width to height ratio of the chamber should be larger than 5 to exclude side wall effects.
2. The Reynolds number must be below 2000.
3. The length of the chamber must be long enough for effects on flow from the inlet to have been lost.

The primary concern in flow cell design was that these criteria were met and laminar flow was established. The secondary concern when developing the system was to establish a hydrodynamic shear rate comparable to the shear rate of saliva *in vivo*, and the shear rate used in other published research. From the research published by Vissinck et al 1984, and Dawes et al 1989, Bos et al, 1996 estimated the shear rate of saliva flowing over teeth to be approximately 10 s$^{-1}$ under resting conditions. In published papers using parallel flow cells to study oral bacterial adhesion, the hydrodynamic properties in terms of shear rate range from 0.9 s$^{-1}$ (Herles et al 1994) to 10 s$^{-1}$.
In this investigation we aimed to achieve a shear rate within this range. The properties of the suspending medium and the flow conditions are important in calculating the hydrodynamic forces exerted on adhering cells. The following section describes the measurements and subsequent calculations that were required to establish the correct hydrodynamic forces within the flow cell. It should be noted that pulsing from the peristaltic pump, to some extent, affected the dynamics of the flow. The pumps used in the study were Watson Marlow 101U/R pumps (Watson Marlow, Cornwall, UK). This model was designed to minimise pulsing effects. For the sake of the following calculations, pulsing effects were considered to have negligible effect on flow conditions.

2.8.2 The flow cell
Disposable, rectangular glass capillary tubes (microslides) manufactured by Camlab, U.K. were chosen for the study. The factors contributing to this choice were:

(1) dependability of supply—as a large number of flow cell runs will be necessary. These tubes come in packs of 40 and supply is essentially limitless.

(2) The inexpensive nature of the product allows a new cell to be used for each run. This should aid the reproducibility of experiments.

(3) Glass is an accepted material for oral adhesion studies.
The dimensions of the cell fit the first criterion for laminar flow:

**Equation 2.8.2** Width to height ratio of flow cell

\[
\frac{w}{h} \geq 5
\]

where:

- \( h \) = chamber height
- \( w \) = chamber width

The width to height ratio for our flow cells was 10. The second and third criteria depend not only on cell dimensions but fluid flow rate and physical properties.

### 2.8.3 Running temperature

The flow cell was operated at room temperature. The literature values of water at 25°C (Weast 1975) were used for calculations.
2.8.4. Viscosity of mucin-containing medium (MCM)

Viscosity measurements were made, with the help of Peter Winlove, at the Physiological Flow Studies Group, Centre for Biological and Medical Systems, Imperial College of Science Technology and Medicine, London. SW7 2BY.

The instrument s used for viscosity measurement were:
1. A Brookfield Model DV-111 Programmable Rheometer.

The viscosity of the MCM was close to water and the accuracy of the Brookfield Rheometer is poor at such viscosities. The capillary tube method was therefore used to make accurate viscosity measurements. The Brookfield rheometer was still useful as a validation of the capillary tube method and to determine whether viscosity was constant over a wide torque range.

**Figure 2.8.4. The capillary U-tube viscometer**

1. upper reservoir
2. upper mark
3. lower mark
4. lower reservoir
5. 37°C waterbath

Water or MCM was added to the tube so that both reservoirs were half full. The temperature of the fluid was allowed to equilibrate with the waterbath temperature. The fluid was then allowed to run from the top reservoir to the bottom reservoir. \( T_0 \) was recorded as the meniscus reached the top mark.
$T_1$ was recorded as the point when the meniscus passed the lower mark. The time taken for the meniscus to travel from the top mark to the lower mark was recorded in seconds and this information, along with the standard viscosity of water at the desired temperature, was used to calculate the viscosity (centipoise) of the MCM. The mean time for deionised water was 270 ± 1.0 s. The mean time for mucin-containing medium was 313 ± 0.2 s.

**Equation 2.8.4 Calculation of viscosity of MCM at 25°C**

\[
\frac{\text{time (s)}_{MCM}}{\text{time (s)}_{dH_2O}} = \frac{\text{viscosity (cp)}_{MCM}}{\text{viscosity (cp)}_{dH_2O}}
\]

** viscosity of water (cp ) at 25°C (Weast 1975)

Using equation 2.8.4 the viscosity of MCM at 25°C was calculated to be 1.03 centipoise

2.8.5. Measurement of MCM density

The density of MCM was measured against the density of deionised water. The mass of equal volumes of each was measured at the same temperature and from this information, and standard values for water (Weast 1975), the density of MCM can be calculated. The mean mass of deionised water was 0.984 ± 0.0007 g. The mean mass of MCM was 0.975 ± 0.0025 g.

**Equation 2.8.5. Calculation of density of MCM at 25°C**

\[
\frac{\text{mass (g)}_{MCM}}{\text{mass (g)}_{dH_2O}} = \frac{\text{density (gml}^{-1})_{MCM}}{\text{density (gml}^{-1})_{dH_2O}}.
\]

* Density of water (g ml$^{-1}$) at 25°C (Weast 1975)
Using equation 2.8.5, the density of MCM at 25°C was calculated to be 0.989 g ml\(^{-1}\).

2.8.6. Shear rate and shear stress
The next stage in system design was to decide on a suitable shear rate. This allowed the calculation of the flow rate. This in turn allowed confirmation of whether the second and third criteria for laminar flow were achieved. The shear rate we decided upon was 7.5 s\(^{-1}\) or as near as flow rate calibration would allow. Equation 2.8.6.1. can be used to calculate the flow rate.

Equation 2.8.6.1. Calculation of shear rate

\[
\sigma = \frac{3}{2} \frac{Q}{b^2 w}
\]

where:
- \(Q\) = volumetric flow rate ml second\(^{-1}\).
- \(b\) = half depth of cell
- \(w\) = width of cell
- \(\sigma\) = shear rate (s\(^{-1}\))

For a shear rate of 7.5 s\(^{-1}\) the volumetric flow rate was calculated to be 0.0008 ml second\(^{-1}\) (0.048 ml minute\(^{-1}\)). This figure was approximated to 0.05 ml minute\(^{-1}\) for ease of pump calibration. The shear rate derived using this figure is 7.78 s\(^{-1}\).

The shear stress, a measurement of the force acting on adhering particles (Busscher et al 1995), was calculated using equation 2.8.6.2.
Equation 2.8.6.2. Calculation of shear stress

\[ \tau_x = \sigma(\mu) \]

where:
\( \sigma \) = wall shear rate \( s^{-1} \)
\( \mu \) = absolute viscosity of MCM (poise at 25°C)

The shear stress for a shear rate of 7.78 \( s^{-1} \) was calculated to be 0.08 dynes \( cm^{-2} \).

2.8.7. Calculation of Reynolds number

The second criterion for laminar flow to be achieved was that the Reynolds number for the flow must be below 2000. This can be determined using equation 2.8.7.

Equation 2.8.7. Calculation of Reynolds number

\[ Re = \rho \frac{Q}{(w + 2b)\mu} \]

where:
\( \rho \) = fluid density (g ml\(^{-1}\))
\( Q \) = volumetric flow rate (ml second\(^{-1}\))
\( w \) = width of chamber (cm)
\( b \) = half depth of chamber (cm)
\( \mu \) = absolute viscosity g cm\(^{-1}\) second\(^{-1}\)
The Reynolds number was calculated to be 2.09. This is well within limits required for laminar flow.

### 2.8.8. Flow cell length requirements

The final criterion for laminar flow to be achieved is that the length of the cell must be sufficient to overcome inlet effects. The length of cell required to overcome inlet effects can be calculated from equation 2.8.8.

**Equation 2.8.8. Calculation of flow cell length requirement**

\[ Le = (0.044)2b(Re) \]

Where:
- \( Le \) = required length
- \( b \) = half gap distance (cm)
- \( Re \) = Reynolds number
- 0.044 = Proportionality constant (Busscher et al 1995)

The length required to overcome inlet effects was calculated to be 0.0037 cm. The length of the microslide tubes is 10 cm thus the third criterion for laminar flow is achieved.
2.9. Culture Methods for flow cell investigations
Cultures for flow cells experiments were grown in the GPT device. Refer to section 2.6. for details.

2.10. Peripherals for flow cell experiments
The set-up of peripherals in the flow cell model was slightly different for each series of experiments. Refer to the relevant sections of chapters 7 and 9 for details of apparatus set-up for each series of experiments.

2.11. Analysis of adhesion
Adherence was measured using computer-aided image analysis techniques. The scheme of the analysis system is shown in figure 2.11. The flow cell was mounted in a specially made holder attached to the microscope platform. The inner bottom surface of the flow cell was viewed using an Olympus ulwd CDPlan 40 objective lens. Images were stored on a PC by the procedure described in section 2.7.4.. Images were stored in grayscale. The image analysis software used to measure adhesion was Scion image (Scion Corporation, US). This software was obtained from the internet. A stored image was opened in the programme. The colour of each pixel in the image was assigned a value of 0-256. A zero value indicated a white pixel and a value of 256 indicated a black pixel. Values in between indicated the grey intensity of the pixel. The image was converted to binary. The threshold between white and black could be set at any of the 256 greyscale values. A value was selected that showed the bacterial cells as white and the background as black. Any artefacts within the image were converted to black.
The threshold was recorded and the pixel values were saved as numerical data. The data were opened in a simple spread sheet written in Microsoft XL. The spread sheet calculated the number of pixels below and above the image threshold level. From this information the spread sheet calculated the percentage of the surface that was white, hence the percentage of the surface covered with bacterial cells. The images recorded were taken along the central axis of the lower surface.
Figure 2.11. Scheme of image analysis system.
Chapter 3 -
Influence of sucrose on the susceptibility of planktonic *Streptococcus sanguis* to amine fluorides and amine fluoride-inorganic fluoride combinations.
3.1. Aims

The purpose of this study was to investigate the effects of sucrose on the susceptibility of *Streptococcus sanguis* to amine fluorides and amine fluoride-inorganic fluoride combinations. In the oral cavity sucrose is frequently available for bacterial metabolism. The metabolism of sucrose may affect the physiology of streptococcal cells conferring decreased susceptibility to antimicrobial agents. This study attempts to determine the effect of sucrose metabolism on susceptibility and to identify changes in culture physiology concurrent with altered susceptibility.
3.2. Materials and Methods

3.2.1. Growth of planktonic culture

*S. sanguis* was grown by continuous culture using a simple chemostat apparatus (section 2.5). Refer to section 2.1.1. for strain and storage details. The chemostat was operated aerobically at 37°C. Bacteria were grown in MCM (section 2.2), or MCM supplemented with 36 g sucrose litre⁻¹, at a flow rate of 0.03 L h⁻¹ (dilution rate = 0.06 h⁻¹). The achievement of the steady state was determined by viable count (section 2.7.1.). After reaching a steady state, the pH of the culture was determined using a pH boy-P2 meter (Camlab) and Whatman type CS pH strips (Fisher) (section 2.7.5)

3.2.2. Microscopy of planktonic cultures

The cultures were Gram stained (Difco) and observed at 1000x magnification under oil immersion. Cultures were also observed by epi-fluorescence microscopy following staining with the Baclight live/dead viability kit (section 2.7.2). To 1 ml of suspension 1.5 μl of each reagent was added. The solution was vortexed and incubated in the dark for 15 minutes. Five μl of culture was transferred to a Thoma bacterial counting chamber (Weber, England) and a coverslip was placed over the top. Images of Gram-stained and fluorescent preparations were recorded using a standard exposure camera (section 2.7.3).
3.2.3 Agents and controls used in the study.

The antimicrobial agents used in the study were obtained from GABA International, Therwil, Switzerland. The agents are listed in table 3.2.2. Other than the active agents, preparations contained (per litre) 2 g of polyethylene glycol-hydrogenated castor oil (GABA International), 50 g of ethanol (BDH), 0.25 g potassium acesulfame (Hoechst, Frankfurt, Germany), and 25 g of xylitol (Sigma). This solution, and deionised water, were used as negative controls. Chlorhexidine digluconate (0.2%) (CHX) (Sigma) was used as a positive control.
Table 3.2.3 Amine fluoride and inorganic fluoride concentrations in solutions used for antimicrobial activity testing.

<table>
<thead>
<tr>
<th>Active ingredients of solutions*</th>
<th>mg L⁻¹ amine concentration</th>
<th>Molar of amine</th>
<th>mg L⁻¹ inorganic concentration of cation</th>
<th>Molar inorganic cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>olaflur</td>
<td>3250</td>
<td>0.0071</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*(C₂₇H₆₀F₂N₂O₃)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oleaflur</td>
<td>4671</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*(C₂₂H₄₅FNO₂)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steraflur</td>
<td>4697</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*(C₂₂H₄₇FNO₂)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olaflur / tin (II) fluoride</td>
<td>1625</td>
<td>0.0035</td>
<td>390</td>
<td>0.0033</td>
</tr>
<tr>
<td>olaflur / zinc fluoride</td>
<td>1625</td>
<td>0.0035</td>
<td>215</td>
<td>0.0033</td>
</tr>
<tr>
<td>zinc fluoride</td>
<td>-</td>
<td>-</td>
<td>430</td>
<td>0.0066</td>
</tr>
</tbody>
</table>

All solutions had a fluoride concentration of 250 mg L⁻¹ F⁻.
3.2.4. Effect of agents on planktonic cultures.

For both MCM and MCM plus sucrose cultures, 3.0 ml of agent was incubated with 2.4 x 10^7 CFU for 1 minute at room temperature. This corresponds to a mean fluoride/CFU ratio of 1.6 x 10^{-5} mol of fluoride per 1 x 10^7 CFU of S. sanguis. The agents and cells were vortexed to mix in the last second of the incubation, and then 100 µl was immediately transferred to 900 µl of neutralising solution (Difco). The suspension was serially diluted in phosphate buffered saline (PBS) (Oxoid) and viable counts of the resulting suspensions were performed (section 2.7.1).
3.3. Results

The CFU formed per CFU per hour for chemostat cultures grown in MCM was 0.06 h⁻¹ (n = 4). Chemostat cultures grown in MCM (pH 6.0 ± 0.2) contained long chains of bacteria that frequently extended to over 70 cocci in length (figure 3.3.1). In contrast, cultures grown in MCM plus sucrose (pH 5.4 ± 0.2) contained aggregates of cells and exo-polymeric material (EPS) (figure 3.3.2.).

No viable bacteria were retrieved from MCM-grown cultures incubated with amine fluoride containing solutions corresponding to at least a 3.5 log₁₀ reduction (figure 3.3.3.). Viable bacteria were retrieved from cultures incubated with deionised water (4.8 ± 0.1 x 10⁶ CFU), zinc fluoride (4.6 ± 1.3 x 10⁶ CFU), the basal medium of amine fluoride preparations (5.0 ± 0.3 x 10⁶ CFU) and CHX (7.8 ± 1.9 x 10⁴ CFU). The variance (log₁₀) between the test groups containing viable cells was compared using the f-test. As the variance was not statistically different (p≤0.01) the viability of the cultures were compared using the parametric single factor analysis of variance. There was no significant difference (p≤0.05) in the number of CFU recovered from cultures incubated with the deionised water, the basal medium or zinc fluoride solution. The viability of cultures treated with CHX was significantly different (p≤0.05) from the zinc fluoride and negative control groups.

Viable bacteria were recovered from all the MCM plus sucrose-grown cultures following incubation with the agents (figure 3.3.4.). There was no significant difference (single factor anovar p≤0.05) between the viable counts of the MCM plus sucrose grown cultures treated with deionised water (5.4 ±
1.5 \times 10^6 \text{ CFU}), the basal medium (4.0 \pm 1.5 \times 10^6 \text{ CFU}), zinc fluoride solution (4.4 \pm 1.6 \times 10^6 \text{ CFU}) and 0.2\% \text{ CHX} (4.7 \pm 2.6 \times 10^6 \text{ CFU}). Significant reductions (p\leq0.05) in CFU concentration were achieved by incubating cultures with all amine-fluoride containing solutions. The number of CFU recovered from cultures treated with Olaflur (4.1 \pm 8.1 \times 10^5 \text{ CFU}), Oleaflur (6.0 \pm 8.4 \times 10^3 \text{ CFU}) and Steraflur (5.3 \pm 6.5 \times 10^4 \text{ CFU}) were not statistically different from each other. The kills were achieved by incubating cultures with Olaflur/zinc fluoride (7.4 \pm 5.8 \times 10^4 \text{ CFU}) and Olaflur/tin (II) (7.8 \pm 2.3 \times 10^5 \text{ CFU}) fluoride combinations were significantly different from the kills achieved using the pure amine fluoride solutions.
Figure 3.3.1. Gram stain of *Streptococcus sanguis* chemostat culture grown in MCM.

Bar = 20μm
Figure 3.3.2. Bacterial and exopolymer aggregate from chemostat culture of *S. sanguis*, grown in MCM plus sucrose and stained with Baclight live/dead fluorescent stain.

Bar = 5μm
Figure 3.3.3. The susceptibility of MCM-grown planktonic *S. sanguis* to amine fluorides and amine fluoride / inorganic fluoride combinations.

Fluoride-containing solutions were adjusted to 250 mg L\(^{-1}\) F\(^-\). Error bars are standard deviations. pH 6.0 ± 0.2.
Fig 3.3.4. The susceptibility to amine fluorides and amine fluoride / inorganic fluoride combinations of planktonic *S. sanguis* grown in MCM plus sucrose.

Fluoride containing solutions were adjusted to 250 mg L$^{-1}$ F$^-$. Error bars are standard deviations. pH 5.4 ± 0.2.
3.4. Discussion

The aim of this investigation was to determine the importance of sucrose metabolism on the susceptibility of *S. sanguis* to amine and inorganic fluorides. Planktonic cultures grown in the absence of sucrose were highly susceptible to the amine fluorides and amine fluoride/inorganic fluoride combinations. This observation is consistent with the strong *in vitro* activity previously reported for oral bacteria grown in batch-culture (Kay & Wilson 1988, Bansal et al 1990). The presence of sucrose in the growth medium significantly reduced the susceptibility of *S. sanguis* to the amine fluorides. Zinc fluoride solution did not exhibit microbicidal activity regardless of the presence or absence of sucrose in the growth medium. Combining the inorganic fluorides with Olaflur did not appear to improve the activity of the amine fluoride. However comparison with the pure amine solution is difficult as the combination contains half the concentration of amine cation. The lack of activity of zinc fluoride solution and the reduced activity of the combinations implies that the active agent is the amine cation and not the fluoride ion.

The reduced susceptibility of *S. sanguis* to the agents when grown in sucrose-containing medium suggests an alteration in cell/culture physiology. The morphology of the planktonic culture grown without sucrose was distinct to that of cells grown in media containing sucrose. Sucrose-grown cells existed singularly and in small units often surrounded by extensive extra-cellular polymer (EPS). In contrast, cells grown in MCM without sucrose existed in long chains and EPS was not observable by light microscope techniques. The EPS produced by cultures grown in sucrose-containing medium may possibly protect cells from the chemical agent. This could be due to ionic binding of
cationic antimicrobial agents to polyanionic exopolymers (Costerton et al 1987, Hoyle et al 1990) or chemical bonding between agent and exopolymer (Nichols 1991). The standard deviations for the number of CFU recovered from cultures treated with amine fluorides were large. This may have been due to a large range in size of cell/EPS flocs that are broken up on spreading. This observation would suggest the EPS is involved in the reduced susceptibility of *S. sanguis* cultures grown in sucrose.

Another possible explanation for the reduced susceptibility of sucrose-grown cultures is that sucrose metabolism leads to a change in the surface associated components of the cells. Alteration of cell surface components during sugar metabolism explained the reduced susceptibility of an oral *Streptococcus* sp. to an antimicrobial agent (Knox et al 1979). Increasing the growth medium glucose concentration from 0.2 to 0.5% decreased the lysis of *S. mutans* by *Streptomyces globisporus* N-acetyl-muramidase. The decrease in susceptibility was independent of pH, cell wall composition (as opposed to cell surface) and specific growth rate. The influence of sucrose metabolism on surface associated components was not studied in this investigation.

Another factor to consider is pH. Cationic antimicrobials are thought to exhibit greater activity at alkaline pH (Baker et al 1941). This agrees with the observation that cultures grown in MCM were more susceptible than cultures grown in MCM plus sucrose. However if pH did affect the activity of the agents, it is unclear whether this effect was due to reduced activity of the agents at the lower pH, or, due to a physiological change at the lower pH conferring decreased susceptibility.
The viable counts of S. sanguis cultures treated with amine fluorides were significantly lower than the viable counts of cultures treated with chlorhexidine digluconate (0.2%). The testing of in vitro planktonic cultures is a very simple model for antimicrobial testing. Such experiments simplify the microbiology of the in vivo habitat in terms of growth mode, growth rate and interactions. In addition, the impact of host factors is, to a large extent, eliminated. Under these conditions the amine fluorides were observed to be far more active than chlorhexidine. However in clinical trials, chlorhexidine is equal to (Oosterwaal et al 1991, Netuschil et al 1995) or outperforms amine fluorides (Hefti & Huber 1987, Etemadzadeh et al 1989) in terms of antimicrobial efficacy. In future chapters investigations will include experiments aimed at identifying factors compromising the efficacy of amine fluorides. The effects of increasing the microbial complexity of the system and interaction with host derived molecules will be investigated.

The results of this investigation stress the influence of growth medium on the susceptibility of microbes to antimicrobial agents. This is particularly relevant when assessing agents intended for use in the oral cavity. The nutrients available for microbial metabolism in the mouth are diverse and are frequently available at concentrations in excess of growth requirements. Under these conditions the microflora may utilise nutrients for specialised survival strategies. This can affect the results of antimicrobial testing and as such lead to erroneous conclusions as to the potential effectiveness of an agent.

Summary:

- Growth in sucrose-containing medium alters the S. sanguis culture morphology.
• Growth in sucrose-containing medium significantly reduces the susceptibility of *S. sanguis* to amine fluorides.

• The activity of amine fluorides toward planktonic *S. sanguis* cultures was significantly greater than that of chlorhexidine digluconate (0.2%).
Chapter 4 - The susceptibility of *S. sanguis* biofilms to amine and inorganic fluorides
4.1. Aims

Further to the investigations in Chapter 3, the aim of this part of the study was to determine the susceptibility to amine and inorganic fluorides of *Streptococcus sanguis*, grown as biofilms. The effect of the presence of sucrose during growth was investigated. The biofilm structure in the presence and absence of sucrose was characterised.
4.2. Materials and Methods

4.2.1. Bacterial strain and growth medium

*Streptococcus sanguis* was used in all experiments. Refer to section 2.1.1 for strain and storage details. The growth medium used in all experiments was mucin-containing medium (MCM). Refer to section 2.2. for details of formulation and preparation. Sucrose was prepared as a 10% solution in deionised water.

4.2.2. CDFF set-up and operation

Figure 4.2.2. shows the apparatus set-up used in the investigation. Refer to section 2.3.1. for general details of fermenter design. The set-up for experiments using sucrose is shown. Hydroxyapatite discs, (refer to section 2.4.1. for details) recessed to a depth of 300 μm, were used as the substratum for biofilm growth. The CDFF was inoculated with a 10 ml overnight culture of *S. sanguis* in nutrient broth (Oxoid) via the sampling point. Two litres of fresh MCM was then re-circulated through the CDFF at a flow rate of 0.03 L h⁻¹. After 24 hours, the re-circulating flask was replaced with a fresh supply of MCM for continuous culture of the biofilms. The CDFF was operated aerobically at 37°C, the turntable speed was 3 rpm, and MCM was delivered to the CDFF at a flow rate of 0.03 L h⁻¹. For biofilms grown in MCM plus sucrose, a 10% solution of sucrose was pulsed into the CDFF three times a day for 33 minute periods at a flow rate of 0.6 L h⁻¹. A steady level CFU per biofilm was determined by viable count (refer to section 2.7.1.).
The set-up shown is the apparatus used for growing MCM plus sucrose-grown biofilms. The CDFF and the recirculating flask were incubated aerobically at 37°C. The recirculating flask was removed 24 hours after inoculation.
4.2.3. Analytical procedures for determination of biofilm structure.

Biofilms were suspended in 1 ml of PBS for enumeration procedures. Biofilms were removed from the substratum by vortexing for one minute. Bacteria were enumerated by viable count. Refer to section 2.7.1. for methodology.

The BacLight live/dead viability kit (Molecular probes) was used for structural staining of intact biofilms. The biofilms were stained according to the procedure in section 2.7.2. Images of the stained biofilms were recorded using the photomicrography techniques detailed in section 2.7.3.

The pH of the biofilms was measured using a Camlab pH boy pH electrode (refer to section 2.7.5. for methodology).

The height of biofilm structures from the surface was measured using a Zeiss microscope. The focus wheels on the microscope possess graduations for measurement of movement in focal plane. Figure 4.2.3. shows the marking system on the two focal wheels. The fine focus wheel and rough focus wheel revolve at different rates for the same movement in focal plane. The marker on the rough focus wheel will move past (x) graduations on the fine focus wheel for (y) movement in focal plane. The distance moved can be calibrated for known distances of focal plane movement. A pack of one hundred cover slips (Chance Proper LTD, UK.) were used for the calibration procedure. The width of the total number of slides in one pack was measured and divided by the total number of slides in a box to find the width of one slide. A coverslip was placed on top of a microscope slide marked with black ink. The top of the coverslip was also marked with black ink. The microscope was focused on the ink on the coverslip using 1000x magnification under oil immersion. The value on the scale of the fine focus wheel aligned with the mark on the rough focus
was recorded. The focal plane was moved until the ink mark on top of the coverslip was in focus. The new value on the fine focus wheel that was aligned with the rough focus wheel mark was recorded. This step was repeated five times. The whole process was then repeated with two, three and four coverslips. The information from the experiment was used to construct a calibration curve of the distance moved in focal plane (micrometres) and the distance moved on the fine focus wheel scale. The highest and lowest measurements for each set of measurements were discounted and the remaining three values averaged. The calibration curve was used to calculate the height of biofilm structures. Measurements were made on the Baclight stained biofilms that had been used for photomicrography. The distance from the substratum to the top of the highest points of biofilms was measured.
The rough and fine focus wheels move at different rates. As the focal plane is moved, the mark on the rough focus wheel will move around the scale on the fine focus wheel.
The total protein in biofilms grown in the presence and absence of sucrose was determined. The Micro BCA Protein Assay reagent Kit (Pierce) was used for this purpose. A description of the kit and the protocol for use is given in section 2.7.6. Five biofilms were pooled for determination by suspending in 1 ml of PBS. Samples were stored before use at -70°C. Prior to use, the samples were washed four times. For wash steps, cells were pelleted by centrifugation for 30 minutes (Stratagene ProFuge 10K micro-centrifuge, US) and resuspended in deionised water. The absorbance of prepared samples was measured at 570 nm. The experiment was repeated three times for both MCM and MCM plus sucrose-grown biofilms.

4.2.4. Agents used in the study

The agents used in the study are listed in table 4.2.4. Other than the active agents, preparations contained (per litre) 2 g of polyethylene glycol-hydrogenated castor oil (GABA International), 50 g of ethanol (BDH), 0.25 g potassium acesulfame (Hoechst, Frankfurt, Germany), and 25 g of xylitol (Sigma). All the agents in the table were tested with MCM-grown biofilms. The background solution and deionised water were used as negative controls. Chlorhexidine digluconate (0.2%) was used as a positive control. For MCM plus sucrose-grown biofilms, olaflur, steraflur and oleaflur were tested. Deionised water was used as a negative control.
Table 4.2.4. Amine fluoride and inorganic fluoride concentrations in solutions used for antimicrobial activity testing.

<table>
<thead>
<tr>
<th>Active ingredients of solutions*</th>
<th>mg L(^{-1}) amine concentration of amine</th>
<th>Molar</th>
<th>mg L(^{-1}) inorganic cation</th>
<th>Molar concentration of inorganic cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>olaflur (C(<em>{27})H(</em>{60})F(_2)N(_2)O(_3))</td>
<td>3250</td>
<td>0.0071</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>oleafur (C(<em>{22})H(</em>{45})FNO(_2))</td>
<td>4671</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>steraflur (C(<em>{22})H(</em>{47})FNO(_2))</td>
<td>4697</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>olaflur / (C(<em>{27})H(</em>{60})F(_2)N(_2)O(_3))</td>
<td>1625</td>
<td>0.0035</td>
<td>390</td>
<td>0.0033</td>
</tr>
<tr>
<td>tin (II) fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olaflur / (C(<em>{27})H(</em>{60})F(_2)N(_2)O(_3))</td>
<td>1625</td>
<td>0.0035</td>
<td>195</td>
<td>0.0017</td>
</tr>
<tr>
<td>tin (IV) fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olaflur / (C(<em>{27})H(</em>{60})F(_2)N(_2)O(_3))</td>
<td>1625</td>
<td>0.0035</td>
<td>215</td>
<td>0.0033</td>
</tr>
<tr>
<td>zinc fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zinc fluoride</td>
<td></td>
<td></td>
<td>430</td>
<td>0.0066</td>
</tr>
</tbody>
</table>

All solutions had a fluoride concentration of 250 mg L\(^{-1}\) F\(^-\).
4.2.5. Susceptibility testing of biofilms

A pan containing five biofilms grown in MCM was aseptically removed from the CDFF and placed in a sterile tube. Agent (3.0 ml) was then carefully added to the tube. After 1 minute at room temperature, the pan was removed from the agent and three separate biofilms were suspended in 1.0 ml of sterile PBS and vortexed for 1 minute. Viable counts were performed according to the procedure in section 2.7.1. For biofilms grown in MCM plus sucrose, a pan containing five biofilms was aseptically removed from the CDFF and placed in a sterile tube. Test solution (5.7 ml) was carefully added to the tube (to achieve the same CFU-to-fluoride ratio used with MCM-grown biofilms). After 1 minute at room temperature, the pan was removed from the agent and three separate biofilms were suspended in 1.0 ml PBS with 0.1 g of 710-to 1,180-μm-diameter glass beads (Sigma) added. Tubes were vortexed for five minutes to remove and break up the biofilm. Viable counts were performed on the suspensions according to the procedure in section 2.7.1. For both MCM and MCM plus sucrose-grown biofilm experiments, 100 μl aliquots of the agents that the biofilms were incubated with were transferred to 900 μl of neutralising solution. Viable counts were performed on the samples as before.

4.2.6. Statistical analysis of results

The protein determinations, viable counts of seven day-old biofilms and viable counts for biofilms treated with the antimicrobial agents were tested for significant differences in measurements. F-test analysis revealed no significant difference in the variance of the grouped measurements. Thus a
parametric test was used to compare the results. The test used was the Single factor analysis of variance. The tests were performed on Microsoft Excel on the Windows 95 platform.
4.3. Results

4.3.1. Biofilm structure

Figure 4.3.1.1. shows a representative surface view of a MCM-grown *S. sanguis* biofilm. The 7 day-old biofilm was stained with the Baclight stain. The biofilm consists of distinct microcolonies of various sizes. Figures 4.3.1.2. and 4.3.1.3. show the surface and side view of a MCM plus sucrose-grown biofilm respectively. The biofilm covered a far greater area of the substratum than the MCM-grown biofilm, and consisted of stacks of microcolonies surrounded by extensive extra-cellular material.
The figure shows a representative image of a *S. sanguis* biofilm grown in MCM on hydroxyapatite (HA). The biofilm was stained using the Baclight stain. The bar represents 50 μm.
Figure 4.3.1.2. Surface view of 7 day-old biofilm grown in MCM plus sucrose

The figure shows a representative image of a *S. sanguis* biofilm grown in MCM on hydroxyapatite (HA). The biofilm was stained using the Baclight stain. The bar represents 50 μm.
Figure 4.3.1.3. Side-view of a 7 day-old *S. sanguis* biofilm grown in MCM plus sucrose

The figure shows a representative image of a *S. sanguis* biofilm grown in MCM on hydroxyapatite (HA). The biofilm was stained using the Baclight stain. The bar represents 50 μm.
Table 4.3.1.1. lists the pH and mean viable count of *S. sanguis* biofilms grown in MCM with and without sucrose. The viable counts are the means from replicate fermenter runs. The pH of MCM plus sucrose grown biofilms was 5.6 ± 0.2. The MCM-grown biofilms were too thin to obtain accurate readings using the pH meter. The mean viable count for seven day old biofilms grown in MCM was 6.0 ± 1.9 x 10^6 CFU/biofilm. The mean viable count for biofilms grown in MCM plus sucrose was 9.2 ± 5.4 x 10^6 CFU/biofilm. The viable counts for MCM and MCM-plus sucrose grown biofilms were not significantly different (p>0.01).

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>Mean viable count (CFU/biofilm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM</td>
<td>-</td>
<td>6.0 ± 1.9 x 10^6 (n=5)</td>
</tr>
<tr>
<td>MCM plus sucrose</td>
<td>5.6 ± 0.2</td>
<td>9.2 ± 5.4 x 10^6 (n=3)</td>
</tr>
</tbody>
</table>

For viable counts, three biofilms were used from each run. The figures in brackets are the number of replicate fermenter runs used to perform counts.

All measurements were made on seven day-old biofilms.

Table 4.3.1.2. shows the protein determinations for MCM and MCM plus sucrose-grown biofilms and the respective mean viable counts. The protein measurements for biofilms were made from one run for each group. The mean total protein for a seven day-old MCM-grown biofilm was 3.0 ± 1.0 x 10^-9.
mg protein. The mean viable count in the same fermenter run on the same day was \(4.23 \pm 2.4 \times 10^6\) CFU/biofilm. For MCM plus sucrose-grown biofilms the mean total protein was \(1.5 \pm 0.2 \times 10^{-2}\) mg protein. The mean viable count was \(1.5 \pm 0.8 \times 10^7\) CFU/biofilm. After correction for the difference in the mean viable counts there was no significant difference (\(>0.01\)) in the total protein in MCM and MCM plus sucrose grown biofilms.

Table 4.3.1.2 Protein determinations of MCM and MCM plus sucrose-grown \textit{S. sanguis} biofilms

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Mean viable count (CFU/biofilm)</th>
<th>Mean protein (mg/biofilm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM</td>
<td>(4.23 \pm 2.4 \times 10^6)</td>
<td>(3.0 \pm 1.0 \times 10^{-3})</td>
</tr>
<tr>
<td>MCM plus sucrose</td>
<td>(1.5 \pm 0.8 \times 10^7)</td>
<td>(1.5 \pm 0.2 \times 10^{-2})</td>
</tr>
</tbody>
</table>

For each group, the measurements were made on triplicate samples from one run. All measurements were made on seven day old biofilms.

Figure 4.3.1.4. shows biofilm growth curves for MCM and MCM plus sucrose-grown \textit{S. sanguis} biofilms. The values are mean viable counts from one run for each group. The same response is seen in both curves. On day one there is a rapid increase in bacterial numbers, presumably due to colonisation of the surface. The number of bacteria then remains approximately constant over days two to four. This is followed by an increase in cell numbers over days 5 and 6. The increase declines by day seven. Figure 4.3.1.5. illustrates the heights of MCM plus sucrose-grown biofilms measured using the graduated scale on the microscope focal wheels. The shape of the curve is
similar to the growth curve for MCM plus sucrose-grown *S. sanguis* biofilms. The mean height of the eight tallest stacks on day 6 was $73.8 \pm 14.8 \, \mu m$. The MCM-grown *S. sanguis* biofilms did not exhibit stack formation making the height of the large microcolonies difficult to measure. The highest point measured on the MCM-grow biofilms was $32.3 \, \mu m$. This measurement was made on a 5 day-old biofilm.
Figure 4.3.1.4. Representative growth curves for biofilms grown in the presence and absence of sucrose.

The values are the means of counts from three biofilms. The curves correspond to values obtained from a single fermenter operation for each group.
Figure 4.3.1.5. Measurement of biofilm stack heights for MCM plus sucrose-grown biofilms

The measurements were made on biofilms stained with the Baclight fluorescent stain kit. The highest points of the stacks in the biofilms were measured. Each value is the mean of >5 measurements.
4.3.2. Susceptibility of S. sanguis biofilms to test solutions

Figure 4.3.2.1. illustrates the viability of MCM-grown S. sanguis biofilms after one minute exposure to the test solutions. There was no significant difference in the viable counts of the negative controls, deionised water (7.0 ± 4.2 x 10^6 CFU/biofilm) and the basal solution (2.0 ± 1.0 x 10^6 CFU/biofilm). The viable count for the positive control, 0.2% chlorhexidine digluconate (9.2 ± 3.7 x 10^5 CFU/biofilm), was not significantly different to the viable counts of biofilms treated with the negative controls. Zinc fluoride was used alone and in combination with olaflur to treat MCM-grown biofilms. The viable counts of biofilms treated with zinc fluoride (5.8 ± 1.7 x 10^6 CFU/biofilm) and olaflur/zinc fluoride (3.8 ± 2.2 x 10^5 CFU/biofilm) were not significantly different to the viable counts of the negative controls. The viable counts of olaflur in combination with tin (IV) fluoride (5.3 ± 3.7 x 10^5 CFU/biofilm) and tin (II) fluoride (5.3 ± 2.6 x 10^6 CFU/biofilm) were not significantly different to the viable counts of the negative control groups. The viable counts of biofilms following treatment with the pure amine fluoride solutions were significantly lower than the counts in the control biofilms. The order of activity of the amine fluorides against MCM-grown biofilms was olaflur>oleaflur>steraflur. The mean count of viable bacteria recovered from biofilms treated with olaflur was (1.1 ± 0.6 x 10^3 CFU/biofilm). The mean count of viable bacteria recovered from biofilms treated with oleaflur was 4.5 ± 0.5 x 10^3 CFU/biofilm. The mean viable count of biofilms following treatment with steraflur was 1.7 ± 1.4 x 10^5
CFU/biofilm. Viable bacteria were recovered from the deionised water, basal solution and zinc fluoride solution used to incubate biofilms. No viable bacteria were recovered from the amine fluoride containing solutions used to treat biofilms.
Figure 4.3.2.1. Susceptibility to test solutions of MCM-grown *S. sanguis* biofilms

Biofilms were incubated with the agents at room temperature for one minute. The mean colony forming units per biofilm are illustrated. Counts are the means of values from three separate fermenter runs. Fluoride-containing solutions were adjusted to 250 mg litre$^{-1}$ fluoride. Chlorhexidine digluconate (CHX) was adjusted to 0.2%.
Figure 4.3.2.2. illustrates the viability of MCM plus sucrose-grown *S. sanguis* biofilms after one minute exposure to the test solutions. The mean viable count for biofilms treated with deionised water was $1.1 \pm 0.8 \times 10^7$ CFU/biofilm. The mean viable counts of biofilms treated with amine fluorides were all lower than the mean viable count in the deionised water control group. However the decrease in the viable counts of the biofilms after treatment with olaflur ($5.6 \pm 0.7 \times 10^6$ CFU/biofilm), oleaflur ($2.8 \pm 1.8 \times 10^6$ CFU/biofilm) and steraflur ($7.9 \pm 6.3 \times 10^3$ CFU/biofilm) were not statistically significant ($p>0.01$). Viable bacteria were recovered from the deionised water used to treat the biofilms in the control group. No viable bacteria were recovered from the amine fluoride solutions used to treat the biofilms.
Figure 4.3.2.2. Susceptibility to test solutions of MCM plus sucrose-grown *S. sanguis* biofilms

Biofilms were incubated with the agents at room temperature for one minute. The mean total colony forming units per biofilm are illustrated. Counts are the means of values from three sets of samples taken from two separate fermenter runs. Fluoride-containing solutions were adjusted to 250 mg litre\(^{-1}\) fluoride.
4.4. Discussion

The aim of this part of the investigation was to determine the importance of both the biofilm growth mode and sucrose on the susceptibility of *S. sanguis* to amine fluoride and amine fluoride/inorganic fluoride combinations. The biofilm growth mode reduced the susceptibility of MCM-grown *S. sanguis* compared to planktonic cultures tested in chapter 3. The addition of sucrose to the growth medium further reduced the susceptibility of *S. sanguis* biofilms. Zinc fluoride alone, and in combination with olaflur, did not possess significant bactericidal activity. Neither did the tin (II) and (IV) fluorides combined with olaflur. These observations suggest that the bactericidal activity is derived primarily from the amine cations. The double-charged amine cation from the olaflur preparation was more effective than the singly-charged cations, as has been previously demonstrated with other cationic species (Baker et al 1941). However, the activity of the cations studied was also shown to be affected by the hydrophilic-hydrophobic balance, chain length, and specific chemical structure. Thus the double charge on the olaflur cation cannot be regarded as the definitive reason for the greater activity of the agent compared to oleaflur and steraflur.

Several explanations have been proposed to explain the reduced susceptibility of biofilms to antimicrobial agents when compared to planktonic cultures. The biofilm matrix may alter the micro-environment of associated cells through nutrient limitation or trapping, causing increased localised concentrations of metabolites and exclusion of gases (Gilbert et al 1993). Such changes to the micro-environment are likely to affect the growth rate of
bacteria. Growth rate is known to affect the physiology of bacterial cells (Williams, 1988) and such changes in the physiology of cells might confer decreased susceptibility to antimicrobial agents. The relationship between specific growth rate and antimicrobial susceptibility has been examined with different bacterial species and antimicrobial agents. The effect of growth rate on the sensitivity of *Bacillus megaterium* to chlorhexidine and 2-phenoxyethanol has been studied (Gilbert & Brown 1980). Under conditions of carbon, magnesium or phosphate limitation the sensitivity of chemostat cultures of *B. megaterium* to chlorhexidine increased with an increase in growth rate. The sensitivity to 2-phenoxyethanol was only increased when the cultures were limited by carbon and magnesium. The susceptibility of *Escherichia coli* to quaternary ammonium compounds (Wright & Gilbert 1987) was investigated in relation to specific growth rate. The susceptibility of the organism to an alkyltrimethylammonium bromide with a carbon chain length of 16 increased with an increase in the specific growth rate. This was observed under magnesium, carbon, phosphate and nitrogen limitation. We were unable to determine the specific growth rate of biofilms grown in the CDFF. Hence it is not known whether the differences in susceptibility observed for the planktonic cultures tested in chapter 3 and the biofilm tested in this chapter were affected by the specific growth rate of the cells.

Viable cells were not recoverable from the amine-containing solutions in which biofilms were incubated. It could be construed that this is evidence that the cells are not inherently less susceptible to agents than planktonic cells and that it is the biofilm structure that protects the cells. However the growth mode of the cells lost to the solution is not known. It is possible that the
majority may have been planktonic cells living in the volume of liquid above the biofilm. We were unable to determine how these cells were grown in the set of CDFF experiments.

Another factor to consider is pH. Cationic antimicrobial agents are thought to exhibit greater activity at alkaline pH (Baker et al 1941). We were unable to measure the pH of the MCM-grown biofilms accurately due to their thin nature. Hence we do not know if there was a difference in the pH of the biofilms when grown with and without sucrose. The pH of planktonic cultures grown with sucrose (chapter 3) and the sucrose-grown biofilms were measured. The pH of the planktonic culture was lower than that of the biofilms. The biofilms were less susceptible to amine fluorides than the sucrose-grown planktonic culture. Thus it is not possible to explain the reduced susceptibility of biofilms to amine fluorides by pH alone.

Growth in sucrose-containing medium decreased the susceptibility of *S. sanguis* biofilms to amine fluorides. The sucrose-grown biofilms contained high concentrations of exopolymer compared to the biofilms grown without sucrose. Chemical bonding, i.e. reaction, between agent and exopolymer may protect cells within biofilms from antimicrobial agents (Nichols 1991). This could explain the decreased susceptibility of sucrose-grown biofilms. The large volumes of exopolymer surrounding the cells may neutralise the agents. Thus the majority of cells will be unaffected by the amine fluorides. This chapter did not investigate whether the amine fluorides react with the *S. sanguis* exopolymers produced by sucrose metabolism. Interactions are investigated in the next chapter.
The observed difference in the susceptibility of MCM and MCM plus sucrose-grown biofilms may be due to diffusion limitation. The structures of the two types of biofilm may profoundly affect the time-related ability of the agents to kill cells. The MCM-grown biofilms contained dense populations of *S. sanguis* cells in relatively wide convex masses. Conversely, sucrose-grown biofilms contained less densely packed cells surrounded by large quantities of exopolymeric material. The cells were arranged in units that form tall thin stacks. In sucrose-grown biofilms agents must diffuse through the polymers of the matrix to reach the cells. In contrast, there is no barrier to reach the outer cells of MCM-grown biofilms. The diffusion of small cationic species such as the amine cations should not be greatly impeded by the polymers due to the highly hydrated nature of the polymers. However when using very short incubation periods (one minute in our experiment) the diffusion barrier provided by the exopolymer may be sufficient to produce a significant change in the kill achieved. It is possible that cationic agents such as the amines ionically interact with polyanionic exopolymers (Costerton et al 1987). Such ionic interactions would increase diffusion limitation and enhance differences in kill. If biofilms were treated with agents for long time periods the efficacy of killing in the two types of biofilm could be reversed. In the tall thin stack structure of MCM plus sucrose-grown biofilms the majority of cells would presumably be equally accessible to antimicrobial agents. Thus the time required for all cells to be reached by the agents will be approximately equal. In contrast, the time taken to kill cells in the densely packed cell masses of MCM-grown biofilms will be controlled by their position within the colony. Cells in the outer areas will be killed very quickly. However cells in the centre of the
colony will be protected from agents by the outer cells. The rate of diffusion through densely packed cell masses would presumably be slower than through the hydrated polymers. Additionally, the amines reacted with the outer cells may help to exclude fresh agent from the biofilm.

This chapter has discussed possible causes for the decreased susceptibility of (1) biofilms compared to planktonic cultures, and, (2) sucrose-grown biofilms compared to biofilms grown without sucrose. There are several explanations for the reduced susceptibility. One or all may be responsible for the observations in this investigation. It would be necessary to investigate each factor independently to ascertain the role in the decreased susceptibility of biofilms, and, sucrose-grown cells.

**Summary:**

- *S. sanguis* biofilms were less susceptible to amine fluorides than planktonic cultures
- The incorporation of sucrose in the growth medium decreased the susceptibility of biofilms to amine fluorides
- Inorganic fluoride compounds did not significantly affect the viability of *S. sanguis* biofilms.
Chapter 5 - Factors affecting the susceptibility of *S. sanguis* to amine fluorides.
5.1. Aims

The previous chapter investigated the effect of biofilm growth mode on the susceptibility of *S. sanguis* to amine fluorides. The effect of sucrose on biofilm susceptibility was also determined. The biofilms of *S. sanguis* were less susceptible to the agents than planktonic cultures. In the previous chapter possible factors that may affect the level of susceptibility of the biofilms were discussed. In this investigation the aim was to determine the significance of some of the factors with regard to the susceptibility of *S. sanguis* biofilms. The effect of specific growth rate, and a biofilm source for suspended cells, were investigated as reasons for the decreased susceptibility of biofilms.

The sucrose grown biofilms were less susceptible to amine fluorides than biofilms grown without sucrose. Sucrose-grown biofilms contained relatively large amounts of exopolymeric material. In this investigation we attempted to produce cell free preparations of the polymer(s). We aimed to use these to investigate whether the polymers chemically react with the amine fluoride-olaflur.

This study also investigated interactions between olaflur/tin fluoride combination and the mucin-containing medium, MCM. This was compared to the interaction of the amine with fresh sterile saliva.
5.2. Materials and Methods

5.2.1. Influence of specific growth rate and biofilm source on S. sanguis susceptibility

Biofilm derived suspended cells were obtained from the exit flow of a glass packed tube (GPT) device. Refer to 2.6. for a description of the device. The specific growth rate of the culture was determined by viable count (refer to section 2.7.1. for methodology). The device was operated at 37°C, aerobically, using a flow rate of 0.03 L h^-1. The susceptibility of cells in the exit flow of the GPT to amine and inorganic fluorides was tested. Three ml of agent was incubated with 2.4 x 10^7 CFU for 1 minute at room temperature. This corresponds to a mean fluoride/CFU ratio of 1.6 x 10^{-5} moles of fluoride per 1 x 10^7 CFU of S. sanguis. The agents and cells were vortexed to mix in the last second of the incubation, and then 100 μl was immediately transferred to 900 μl of neutralising solution (Difco). Viable count procedures were performed on the suspension (refer to section 2.7.1. for methodology). The agents used in the study are listed in table 5.2.1. Other than the active agents, preparations contained (per litre) 2 g of polyethylene glycol-hydrogenated castor oil (GABA International), 50 g of ethanol (BDH), 0.25 g potassium acesulfame (Hoechst, Frankfurt, Germany), and 25 g of xylitol (Sigma). This basal solution and deionised water were used as negative controls.
Table 5.2.1. Amine fluoride and inorganic fluoride concentrations in solutions used in the study.

<table>
<thead>
<tr>
<th>Active ingredients of solutions*</th>
<th>mg L$^{-1}$ amine concentration of amine</th>
<th>Molar</th>
<th>mg L$^{-1}$ inorganic cation</th>
<th>Molar</th>
<th>mg L$^{-1}$ inorganic cation</th>
<th>Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olaflur</td>
<td>3250</td>
<td>0.0071</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C$<em>{27}$H$</em>{60}$F$_2$N$_2$O$_3$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleaflur</td>
<td>4671</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C$<em>{22}$H$</em>{45}$FNO$_2$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steraflur</td>
<td>4697</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C$<em>{22}$H$</em>{47}$FNO$_2$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olaflur / tin (II) fluoride</td>
<td>1625</td>
<td>0.0035</td>
<td>390</td>
<td>0.0033</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olaflur / zinc fluoride</td>
<td>1625</td>
<td>0.0035</td>
<td>215</td>
<td>0.0033</td>
<td></td>
<td></td>
</tr>
<tr>
<td>zinc fluoride</td>
<td>-</td>
<td>-</td>
<td>430</td>
<td>0.0066</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All solutions had a fluoride concentration of 250 mg L$^{-1}$ F$^-$. 

150
5.2.2. Cell free exopolymer (EPS) preparations and reaction with amine fluoride

Cell-free glucosyl transferase preparations were used to produce the EPS. A stationary phase S. sanguis culture (5ml) in MCM was filter-sterilised into 15 ml of 10% sucrose in MCM salts (refer to 2.2. for details of MCM and MCM-salts). This was incubated at 37°C for five days. The resulting suspension was centrifuged at 3,500 rpm (15 minutes in a Centaur 2 centrifuge [Fisons]), and the pellet was re-suspended in 10 ml of MCM salts. Olaflur (1 ml) (250 mg L⁻¹ fluoride) or the basal solution (refer to section 5.2.1.) was added either to 1 ml of MCM salts or EPS (up to 7 mg dry weight) made up to 1-ml volume with MCM salts. After vortexing, the suspensions were filter-sterilised to remove the EPS. The absorbance was measured at 270 nm (the amine absorbance peak, determined by scanning spectrophotometry) by using the negative control plus MCM salts (50:50) to zero the spectrophotometer.

5.2.3. Interactions of amine/tin fluoride with MCM and sterile saliva.

MCM and MCM salts medium were prepared and sterilised according to the protocol in section 2.2.. Saliva was collected from one healthy individual. The sample was filter-sterilised through a 0.8 µm pore syringe filter (Nalgene), and then a 0.2 µm pore filter (Nalgene). One ml of each medium was transferred to a compartment of a sterile polystyrene well plate (Bibby Sterilin). An image of each liquid was recorded using the digital capture system described in section 2.7.4.. One ml of olaflur/tin (IV) fluoride combination (250 mg L⁻¹ fluoride) was added to each of the liquids in the wells. After 1 and 10 minutes, images were retaken of the solutions. After 10 minutes, the wells were emptied and rinsed twice with 1 ml of MCM-salts. One ml of MCM-salts was then added to each of
the wells and the images were taken of the base surface of the well. In addition to the above experiments, the production of precipitates was monitored in the following mixtures. Firstly, olaflur without tin (IV) fluoride was added to MCM in a sterile tube (100µL:1000µL). Secondly, the amine/tin (IV) fluoride combination was added (100µL:1000µL) to MCM medium with the proteose peptone, lab lemco and yeast extract absent.
5.3. Results

Bacteria in the exit flow of the GPT device existed as dense clumps, short chains and single cells, rather than the long chains associated with planktonic cultures of *S. sanguis*. Figure 5.3.1. illustrates the effect of amine and inorganic fluorides on the susceptibility of *S. sanguis* grown in the GPT device. The mean specific growth rate of the cells was 0.012 h\(^{-1}\) (n=4). The cells were incubated with the agents for one minute. The mean viable count of cultures following treatment with deionised water was 1.4 ± 0.1 x 10\(^7\) total CFU. The mean viable count for cultures treated with the basal solution was 7.3 ± 1.5 x 10\(^6\) total CFU. The difference in counts between the deionised water and basal solution negative controls was not significant (p>0.01). The mean viable count of cultures treated with zinc fluoride was 4.6 ± 1.3 x 10\(^6\) total CFU and was not significantly different to the counts of the negative controls. It was not possible to recover any viable organisms from cultures treated with any of the amine fluoride-containing solutions.
Figure 5.3.1 Viable counts of GPT-grown *S. sanguis* cultures after incubation with GABA agents.

All amine fluoride containing solutions were adjusted to 250 mg L\(^{-1}\) fluoride. Error bars are the standard deviations of mean counts from three repetitions of the experiment.
Figure 5.3.2. illustrates the absorbance of olaflur after treatment with different amounts of EPS. There was no reduction in the concentration of amine left in solution following the addition of up to 7 mg (dry weight) of exopolymer to the solution of olaflur (3.25 mg of amine). The absorbance of the amine fluoride solution actually increased slightly after the addition of the EPS.
The amount of amine cation used in each test was 3.25 mg. The EPS was removed by filter sterilisation prior to absorbance measurements. Error bars are standard deviations of replicate samples.
Figure 5.3.3. shows the effect of adding the olaflur/tin (IV) fluoride combination to MCM medium. Image (A) shows the MCM medium prior to adding the agent. Images (B) and (C) show the MCM medium 1 and 10 minutes after addition of the amine/tin combination. The solution contained large amounts of precipitated material. Image (D) shows the surface of the well plate 10 minutes after addition of amine/tin fluoride. The plate had been rinsed twice with MCM-salts. Some of the precipitated material is clearly shown adhered to the surface.

Figure 5.3.4. shows the effect of the olaflur/tin fluoride combination on filter-sterilised saliva. As with the MCM medium, material precipitated immediately after addition of amine/tin fluoride. The precipitate remained adhered to the surface of the well plate after rinses with MCM-salts. In the control group, the olaflur/tin fluoride combination was added to MCM-salts. No precipitates were observed. In the test tube experiments, olaflur precipitated components of MCM and saliva in the absence of tin (IV) fluoride. Precipitates were also observed in MCM medium with the proteose peptone, lab lemco powder and yeast extract absent.
Figure 5.3.3. Images of MCM solution mixed with olafluor/tin (IV) fluoride

One ml of olafluor/tin (IV) fluoride combination was added to one ml of MCM. The solutions were incubated together at room temperature. (A): MCM prior to addition of amine/tin fluoride. (B): MCM one minute after the addition of amine/tin fluoride. (C): MCM ten minutes after the addition of amine/tin fluoride. (D): Precipitate adhered to the bottom surface of the container.
Figure 5.3.4. Images of saliva mixed with olaflur/tin (IV) fluoride

One ml of olaflur/tin (IV) fluoride combination was added to one ml of filter-sterilised saliva. The solutions were incubated together at room temperature. (A): Saliva prior to addition of amine/tin fluoride. (B): Saliva one minute after the addition of amine/tin fluoride. (C): Saliva ten minutes after the addition of amine/tin fluoride. (D) Precipitate adhered to the bottom surface of the container.
5.4. Discussion

In chapter four, possible explanations for the decreased susceptibility of *S. sanguis* biofilms to amine fluorides were discussed. In this investigation we attempted to determine the importance of some of these factors. We also investigated interactions between the amine fluoride and the growth medium.

The importance of specific growth rate on phenotype and susceptibility of bacteria to antimicrobial agents was discussed in chapter 4. The phenotype of cells may be affected by other factors associated with biofilm growth. These include gradients of pH, waste products, nutrients and gases (Gilbert et al 1993). There may also be a change in phenotype in response to adhesion to a surface. Fujiwara et al, 1998, determined the minimum bactericidal concentrations (MBC's) of various antibiotics toward *Pseudomonas aeruginosa*, *Serratia marcescens* and *Proteus mirabilis* planktonic and adhered cells. The MBC’s of 6 different antibiotics were increased for all three species after adhesion of cells to tissue culture plates. In most cases the MBC was increased more than 128 times after the cells had adhered to the substratum. In a similar model, the attachment of *Staphylococcus epidermidis* and *Escherichia coli* to polystyrene was shown to affect the level of susceptibility to a variety of chemical agents (Das et al 1998).

In this chapter we investigated the susceptibility of biofilm-derived *S. sanguis* grown using the GPT device. The cells in the fluid phase existed as dense clumps, short chains and single cells rather than the long chains associated with planktonic cultures. The specific growth rate was five times lower than that of the planktonic cultures used in chapter three. The cultures were highly
susceptible to the amine fluorides at the concentrations used in the study. No cells were recovered from any of the amine-fluoride containing solutions. This observation was consistent with that for planktonic cultures grown in the same medium (refer to chapter 3). In our investigation we used amine fluoride concentrations similar to those used in commercially-available amine fluoride preparations. Thus a change in cell phenotype was not a dominant factor in the reduced susceptibility of *S. sanguis* biofilms to amine fluorides at concentrations used *in vivo*.

The presence of sucrose in the growth medium decreased the susceptibility of planktonic cultures (chapter 3) and biofilms (chapter 4) of *S. sanguis*. The decrease in susceptibility was accompanied by the production of large volumes of extracellular polymer (EPS). In this chapter we investigated whether the EPS reacted with amine fluorides. In the experiments we added EPS to olaflur, and after removal of the EPS, measured the absorbance of the solution. We were unable to show a reduction in the absorbance at the wavelength of the amine peak. Hence the decreased susceptibility of *S. sanguis* grown in the presence of sucrose is unlikely to be due to neutralisation of amine cations by the EPS surrounding the cells.

In this investigation we showed that the amine/tin fluoride combination precipitated salivary components. We also showed that the amine fluoride precipitated the glycoprotein component of the medium used in our growth experiments. The finding of this experiment is similar to work of Voegel & Belcourt, 1980, which investigated the interactions between saliva and inorganic cations. They added inorganic cations to saliva to reach a final concentration of 0.1M. Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$ and Fe$^{2+}$ and Al$^{3+}$ precipitated mostly
glycoproteins. Cu$^{2+}$ and Zn$^{2+}$ precipitated the majority of salivary proteins. The concentration of tin in our preparation was 0.003M. This was over 50-fold lower than the 0.1M concentration of inorganic cations used in their study. It is not known if inorganic cations at this concentration would precipitate salivary components. However we do know that the amine cation used in our preparation did precipitate salivary components.

The precipitation of salivary molecules is important in relation to the activity of the amine fluorides. Such interactions could possibly inactivate the agents. There is no available data on the activity of amine fluorides following interactions with salivary proteins in the bulk fluid phase. However Sefton et al, 1996, did investigate the activity of amine fluorides after adsorption to a salivary pellicle formed on hydroxyapatite (HA) substratum. They found that adsorption to the pellicle did not inactivate the activity of the amine ion. It is not known whether the immobilisation of the saliva molecules had modified the interactions with amine fluorides. However the observations do suggest that precipitation may be due to repulsive interactions between the amine and saliva leading to micelle formation. If this is the case, the potential activity of the amine would depend on whether the amine is trapped inside the micelles or remains in the bulk fluid. Further investigations into the interactions between saliva and amine fluorides are warranted to determine whether the activity of the agent is compromised.

**Summary:**

- Biofilm-derived *S. sanguis* cells with a growth rate five-fold lower than chemostat cultures were similarly susceptible to the amine fluoride, olaflur.
• The EPS produced when cells were grown in sucrose did not react with olaflur
• Components of saliva precipitate immediately after the addition of olaflur/tin fluoride.
Chapter 6 - Effect of surface treatment with amine and inorganic fluorides on *S. sanguis* biofilm formation using the CDFF
6.1. Aims

The bactericidal activity of amine fluorides and amine/inorganic fluoride combinations towards formed *S. sanguis* biofilms was investigated in chapter 4. This study attempted to investigate the adsorption of agents to the surface and their effect on biofilm formation. The study aimed to investigate bactericidal activity towards adherent bacteria and anti-adhesion activity.
6.2. Materials and Methods

6.2.1. Set-up and inoculation of CDFF

Refer to section 2.3.1 for general details of fermenter design. The *S. sanguis* culture used in the experiment was grown by continuous culture (section 2.5.) using MCM medium (section 2.2.). The chemostat culture was allowed to reach steady state before use in experiments. The achievement of steady state was determined by viable plate count and pH (section 2.7.5.). The substratum used for the experiment was hydroxyapatite (US Bio-interfaces) (section 2.4.1.). The substratum discs were recessed to a depth of 100 μm. The CDFF was sterilised by autoclaving at 121°C for fifteen minutes. Afterwards the pans were aseptically removed from the CDFF and transferred to a sterile container. Sterile MCM was added to the container until it covered the substratum in the PTFE pans. After two minutes the MCM was drained off. A pan was then aseptically transferred to 10 ml of filter sterilised agent or control. The pan was incubated with the agent at room temperature for one minute (refer to following section for agents used in the study). The agent was then drained off and the pan aseptically transferred to the CDFF. The pan was levelled flush with the top of the turntable. The CDFF was aseptically attached to the peripherals as shown in figure 6.2. The chemostat culture was pumped into the CDFF at a flow rate of 0.03 L h⁻¹. The CDFF turntable speed during the experiment was 3 rpm. The fermenter was incubated at 37°C under aerobic conditions during the experiment. Two different experimental strategies were tried with the system. Firstly, one pan was treated with each solution and the CDFF was operated for four hours. Samples were removed at the end of this period biofilm analysis (refer to section 6.2.3. for analysis...
procedures). In the second experiment two pans were treated with each test solution and the CDFF was operated for 8 hours. Pans were removed for biofilm sampling after 4 hours of culture flow and at the end of the 8 hour period. Refer to section 6.2.2. for details of which agents were used in each experiment.
Figure 6.2.1. Apparatus set-up for pre-treatment experiment using the CDFF.

The outflow of the chemostat could be switched to an effluent bottle or the CDFF by the use of clamps. The 500 ml chemostat vessel and CDFF were housed in an incubator set at 37°C.
6.2.2. Agents used in the study

The agents used in the study are listed in table 6.2.2. Other than the active agents, preparations contained (per litre) 2 g of polyethylene glycol-hydrogenated castor oil (GABA International), 50 g of ethanol (BDH), 0.25 g potassium acesulfame (Hoechst, Frankfurt, Germany), and 25 g of xylitol (Sigma).

In the four hour experiment, all the agents listed in table 6.2.2. apart from steraflur, were tested. Deionised water and the basal solution for GABA preparations were used as negative controls. Chlorhexidine digluconate (0.2%) (CHX) (Sigma) was used as a positive control. Two different sets of agents were used in experiments investigating biofilm formation after 4 and 8 hours. In the first series of experiments olaflur, zinc fluoride and steraflur solutions were tested. Deionised water was used as the negative control. In the second series of experiments only steraflur and deionised water were used.
Table 6.2.2. Amine fluoride and inorganic fluoride concentrations in solutions used for treating surfaces.

<table>
<thead>
<tr>
<th>Active ingredients of solutions*</th>
<th>mg L$^{-1}$</th>
<th>Molar concentration of amine</th>
<th>mg L$^{-1}$</th>
<th>Molar concentration of inorganic cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>olaflur</td>
<td>3250</td>
<td>0.0071</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C$<em>{27}$H$</em>{60}$F$_2$N$_2$O$_3$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steraflur</td>
<td>4697</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C$<em>{22}$H$</em>{47}$FNO$_2$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tin (IV) fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olaflur / tin (IV) fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1625</td>
<td>0.0035</td>
<td>195</td>
<td>0.0017</td>
<td></td>
</tr>
<tr>
<td>tin (II) fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olaflur / tin (II) fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1625</td>
<td>0.0035</td>
<td>390</td>
<td>0.0033</td>
<td></td>
</tr>
<tr>
<td>zinc fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olaflur / zinc fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1625</td>
<td>0.0035</td>
<td>215</td>
<td>0.0033</td>
<td></td>
</tr>
<tr>
<td>zinc fluoride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All solutions had a fluoride concentration of 250 mg L$^{-1}$ F$^-$. 


6.2.3 Analytical procedures

Chemostat samples were collected before and after each experiment and the *S. sanguis* concentration was determined by viable count (section 2.7.1). A Gram stain preparation of the chemostat culture was prepared. This was recorded using the photomicrography system discussed in section 2.7.3.. A Gram stain preparation of bacteria in saliva was also prepared for comparison. This was recorded using the digital procedure described in section 2.7.4.. Viable counts were performed on biofilms removed from the CDFF (Refer to section 2.7.1. for methodology). Viable counts were performed on three separate discs per run.
6.3. Results

Table 6.3.1. lists the mean viable counts of CDFF biofilms on surfaces treated with test solutions. The surface was hydroxyapatite with a pellicle formed from mucin-containing medium (MCM-HA). Counts were performed after 4 hours of culture flow. The experiment was repeated 6 times. There was no statistically significant difference (p>0.01) in viable count between any of the test groups regardless of which solution was used to treat MCM-HA prior to culture flow. The mean viable counts are represented graphically in figure 6.3.2.. The mean viable count of the chemostat culture prior to the experiment was $1.07 \pm 1.0 \times 10^9$ CFU/biofilm (n=6). The mean viable count of the chemostat culture following the experiment was $2.78 \pm 3.1 \times 10^9$ CFU/biofilm (n=4).
Table 6.3.1. Mean viable counts of 4 hour old *S. sanguis* biofilms grown on MCM-HA treated with test solutions prior flow.

<table>
<thead>
<tr>
<th>Test agent</th>
<th>mean viable count (CFU/biofilm)</th>
<th>No. of runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olaflur</td>
<td>$2.54 \pm 1.1 \times 10^4$</td>
<td>6</td>
</tr>
<tr>
<td>Olaflur / tin (IV) fluoride</td>
<td>$3.68 \pm 1.9 \times 10^4$</td>
<td>6</td>
</tr>
<tr>
<td>Olaflur / tin (II) fluoride</td>
<td>$4.12 \pm 1.9 \times 10^4$</td>
<td>5</td>
</tr>
<tr>
<td>Olaflur / zinc fluoride</td>
<td>$3.54 \pm 1.0 \times 10^4$</td>
<td>6</td>
</tr>
<tr>
<td>zinc fluoride</td>
<td>$4.53 \pm 3.2 \times 10^4$</td>
<td>6</td>
</tr>
<tr>
<td>deionised water</td>
<td>$4.03 \pm 1.5 \times 10^4$</td>
<td>6</td>
</tr>
<tr>
<td>chlorhexidine (0.2%)</td>
<td>$3.02 \pm 1.3 \times 10^4$</td>
<td>6</td>
</tr>
<tr>
<td>basal solution</td>
<td>$4.36 \pm 1.5 \times 10^4$</td>
<td>6</td>
</tr>
</tbody>
</table>

All fluoride containing solutions were adjusted to 250 mg L$^{-1}$ fluoride. Three biofilms were sampled for each run.
Figure 6.3.1. Viable counts of 4 hour old *S. sanguis* biofilms on MCM-HA substratum treated with test solutions prior to culture flow.

Fluoride-containing solutions were adjusted to 250 mg litre\(^{-1}\) fluoride. Chlorhexidine digluconate (CHX) was prepared in basal solution to a concentration of 0.2%. Error bars are standard deviations of means of triplicate discs from >4 replicate runs.
Figure 6.3.2. illustrates the mean viable counts of biofilms on MCM-HA treated with test solutions. Three different test solutions and one control were used in each CDFF run. Viable counts were performed after 4 and 8 hours of culture flow. After 4 hours there was no significant difference between the viable counts of biofilms growing on MCM-HA treated with deionised water (4.99 ± 1.3 x 10^4 CFU/biofilm), zinc fluoride (3.5 ± 1.8 x 10^4 CFU/biofilm), olaflur (1.29 ± 0.4 x 10^4 CFU/biofilm) and steraflur (6.08 ± 5.2 x 10^4 CFU/biofilm). Similarly, after 8 hours of culture flow there was no significant difference between the viable counts of biofilms growing on MCM-HA treated with deionised water (1.04 ± 0.1 x 10^5 CFU/biofilm), zinc fluoride (7.65 ± 3.7 x 10^4 CFU/biofilm), olaflur (4.69 ± 4.4 x 10^4 CFU/biofilm) and steraflur (7.35 ± 7.6 x 10^4 CFU/biofilm). The mean viable count of the chemostat culture prior to the experiment was 1.28 ± 1.5 x 10^9 CFU/biofilm (n=2). The mean viable count of the chemostat culture following the experiment was 1.27 ± 0.2 x 10^9 CFU/biofilm (n=2).
Figure 6.3.2. Viable counts of 4 and 8 hour old *S. sanguis* biofilms on MCM-HA substratum treated with test solutions prior to culture flow.

Fluoride-containing solutions were adjusted to 250 mg litre$^{-1}$ fluoride. Error bars are standard deviations of means from two runs.
Figure 6.3.3. illustrates the mean viable counts of biofilms on MCM-HA treated with deionised water or steraflur. Only one GABA agent was used in the experiment. Viable counts were performed after 4 and 8 hours of culture flow. After 4 hours there was no significant difference in the viable counts of biofilms growing on MCM-HA treated with deionised water ($3.37 \pm 2.8 \times 10^4$ CFU/biofilm) and steraflur ($1.91 \pm 0.6 \times 10^4$ CFU/biofilm). Similarly, after 8 hours of culture flow there was no significant difference in the viable counts of biofilms growing on MCM-HA treated with deionised water ($5.19 \pm 3.8 \times 10^5$ CFU/biofilm) and steraflur ($1.76 \pm 0.1 \times 10^4$ CFU/biofilm). The mean viable count of the chemostat culture prior to the experiment was $1.6 \pm 0.5 \times 10^8$ CFU/biofilm ($n=2$). The mean viable count of the chemostat culture following the experiment was $6.10 \pm 5.5 \times 10^8$ CFU/biofilm ($n=2$).
Figure 6.3.3. Viable counts of 4 and 8 hour *S. sanguis* biofilms on MCM-HA substratum treated with steraflur, or, deionised water prior to culture flow.

Fluoride-containing solutions were adjusted to 250 mg litre$^{-1}$ fluoride. Error bars are standard deviations of means from two runs.
Figure 6.3.4. shows representative Gram stained preparations of bacteria. Picture A shows the chemostat culture of *S. sanguis* used in our study. Picture B shows a Gram stain of freshly collected saliva from one healthy individual. The chemostat culture contained long chains of streptococci that sometimes folded to form tangled masses. In contrast the Gram stain of saliva contained cells singularly, in pairs and in dense aggregates. Long chains and large masses were absent.
Figure 6.3.4. Gram stain preparations of (A) *S. sanguis* chemostat culture, and, (2) human saliva
6.4. Discussion

This study attempted to investigate the effect of surface treatment with amine and inorganic fluorides on biofilm formation. The surface was hydroxyapatite with a pellicle formed from mucin-containing medium (MCM-HA). Biofilms were analysed after 4 and 8 hours of culture flow. The study was unable to show any significant effect on biofilm formation. This result was consistent for all fluoride compounds (250 mg litre$^{-1}$) and chlorhexidine digluconate (0.2%) (CHX). Both amine fluorides (AmF’s) and CHX have been shown to reduce biofilm formation \textit{in vivo} (Netuschil et al 1995) when used as mouthwashes. The time periods used by Netuschil et al were comparable to those used in our study. The lack of agent activity in our investigation may be the result of differences between the experimental model and the \textit{in vivo} situation. In our model we used hydroxyapatite as the substratum. This constitutes only part of the enamel in the oral cavity. We operated a single species system in our model. This is an over simplified model of the microflora in the oral cavity. It does not take into account interactions between members of the community which may be involved in biofilm formation, e.g., co-adhesion. Hence potential anti-plaque activities of the agents tested may be missed. The medium used in our experiment was a mucin-containing medium. This medium was formulated in an attempt to simulate the nutrients available in saliva. It does not take into account other components of saliva, e.g., IgA, lysozyme, salivary buffers. It is not known if components of saliva absent in our model would influence the activity of the agents.
The previous paragraph discussed the major differences between our model and the conditions *in vivo*. In addition to these differences, there may have been deficiencies in our model attributable to the design of components in the system. The first area of consideration was that too many agents were being used in each fermenter run and that mixing of agents, or their effects, occurred. The scraper bars in the CDFF may have caused the flow of free agent from one pan to another within the device. Equally, unattached bacteria flowing over the surfaces may mix with a particular agent in one pan and then adhere in another of the pans. Thus the bactericidal activity of an agent may not be confined to the particular discs that were treated. In the series of experiments we reduced the number of agents tested in each run until just one agent, steraflur, and the deionised water control were used. The difference in mean viable counts between biofilms on MCM-HA treated with deionised water and steraflur did increase as the number of agents per run decreased. However the difference was never statistically different. If the agents had needed to be tested separately the workload would have greatly increased. Assuming 15 solutions (including controls) are to be tested 15 operations of the CDFF are required rather than 1 run if agents can be tested together. This results in not only a fifteen-fold increase in CDFF operation time but a fifteen-fold increase in down-time between fermenter runs. One of the favourable design aspects of the CDFF is the large number of biofilms that can be produced per operation (Wilson 1996). Obviously, this feature is compromised if solutions need to be tested separately. The method of bacterial measurement may not have been suitable for the experiment. Viable count procedures possess a relatively large inherent error.
The procedure may not have been sensitive enough to measure differences. Additionally, viable counts only detect viable bacteria and do not measure the concentration of dead bacteria. Hence viable counts do not differentiate between anti-adhesion and bactericidal/bacteriostatic activities. Measurement of the two activities together may increase the experimental error. An analytical procedure that measures just one, or differentiates between anti-plaque effects, would improve the sensitivity of the experiment. In this study the anti-adhesion activity is the preferred area of interest. Microscopic techniques offer a direct method for measurement of adhesion. With the advancement in computer technology, the reliability of direct microscopic procedures is greatly improved. This is due to the ability to store and analyse large amounts of data compared to traditional photomicrography techniques (Nivens et al 1995). Procedures involve little or no manipulation of the specimen thus experimental error is minimised. Direct microscopic techniques are able to detect and measure low cell concentrations which is extremely advantageous when studying bacterial adhesion to surfaces.

The culturing procedures used to provide the bacterial suspension used in the experiment may have compromised the evaluation of the efficacy of anti-plaque properties of the agents. The morphology of the S. sanguis chemostat culture differed from the morphology of bacteria in saliva. The chemostat culture contained long chains and large aggregates (which appear to be tangled chains) of streptococcal cells. In contrast bacteria in saliva existed singly, in pairs and in small dense clusters. The long chains and large aggregates in the chemostat cultures may affect the anti-adhesion properties of agents. In the recessed areas above the substratum in the CDFF, the flow
and turbulence are likely to be low. Brownian motion and gravity may influence the adhesion of bacteria at turbulence and flow speeds. The movement of small particles will be affected by both Brownian motion and gravitational pull. As particle size increases the effects of Brownian motion will decrease. Gravity will become the dominant force acting on the particles. Brownian motion moves particles in all directions whereas gravity only moves particles downwards. The action of Brownian motion on long chains and large aggregates of cells may be negligible compared to single cells. Thus the influence of gravity will be greater. This will increase the rate of sedimentation of particles. Increased sedimentation will increase the frequency of contact between bacteria and the treated surface. This may compromise both the anti-adhesion and the bactericidal activities of agents. Sedimented cells do not have to adhere to the surface. The majority will just lie on top of it. This will mask the activity of anti-adhesion agents incorporated in the pellicle. The bactericidal activity of the agents will be affected by the increased frequency of contact between cells and the surface. The rate of neutralisation of reactive species, e.g. amine cations, will be increased reducing the substantivity of the active agent.

There is much evidence that biofilm cells differ from planktonic cells in their physiology (refer to reviews by Bradshaw 1995, and, Gilbert et al 1997b). Changes to the surface physiology of cells may profoundly affect the efficacy of anti-adhesion agents. Bacteria in saliva are predominantly derived from biofilms. The bacteria we used in this study were not. Cultures derived from biofilm-growing cells would therefore provide a more suitable alternative.
When pans are removed from the fermenter, a small volume of liquid remains on the disc. If the liquid volume is large enough the count may be greater than the true value for adherent bacteria. The planktonic cells could be removed in the CDFF or following removal of pans. Immersion of removed pans in fresh medium would remove the majority of planktonic cells. However this manual procedure is subject to inherent variation in shear force. This will increase the experimental error of viable counts. Alternatively, fresh medium could be pumped into the CDFF at a flow rate high enough to wash away unattached cells. This would be a more consistent method of removing planktonic cells. Unfortunately the flow rates that would remove unattached bacteria but not shear off adherent bacteria are unknown. To measure such flow rates using the CDFF would be an extensive investigation in itself.

The shear rates in the CDFF acting on adhering bacteria are unknown. It is possible to estimate the shear forces exerted on the surface of the biofilm flush with the surface. It is not as straightforward to measure the shear forces in the space between the substratum and the top of the turntable. A system incorporating shear rate control would be advantageous for studying adhesion processes. This would aid comparison with other investigations and adhesion in vivo. It would also facilitate the study of flow forces on substantivity of anti-plaque agents.

The use of a shorter time period may improve the validity of adhesion experiments. Bloomquist et al, 1996, studied bacterial adhesion in vivo and showed that most bacterial adhesion to enamel pieces attached to teeth occurred in the first 60 minutes after pellicle formation. Following this period, other biofilm-forming processes, e.g. co-adhesion and growth, will
predominate. A shorter time period for experiments should reduce error in adhesion measurements derived from other processes.

In conclusion, an alternative system to study anti-adhesion activity is required. Such a system would not be modelled on the oral environment in the same way as the CDFF model. Instead it would be designed specifically for the control of shear forces and direct observation of adhesion events. The information gathered from the operation of such a system can then be used to design experiments for the more complex CDFF model with a better understanding of what operating conditions are appropriate.

**Summary:**

- An alternative model to the CDFF is sought for studying the effect of surface-treatment with anti-plaque agents
- Analysis should distinguish between bactericidal/bacteriostatic activity and anti-adhesion activity.
- The system should allow control of hydrodynamic shear.
- The culturing strategy for bacteria used in adhesion studies needs to be improved so that the cells resemble salivary bacteria more closely.
Chapter 7 - The effect of surface treatment with amine and inorganic fluorides on adhesion of *S. sanguis* to parallel flow cells.
7.1. Aims

The purpose of the investigation was to develop a method to study anti-adhesion properties of amine and inorganic fluorides. The system needed to incorporate direct real time analysis, ease of replication and a sensitive means of determining adhesion. A flow cell system was developed to meet these requirements. Once the system had been developed the aim was to determine if amine and inorganic fluorides possess anti-adhesion properties towards *S. sanguis*. The property was to be assessed in a continuous system for a period long enough in duration to assess whether the agents possess substantivity. The effect of treating the surface with the agents in the presence and absence of a pellicle was compared. The effect of mixing the agent with the solution used to produce the pellicle was also investigated. Finally, the effect on adhesion of mixing the agents with the bacterial culture was investigated.
7.2. Materials and Methods

7.2.1. General equipment used in the study

The equipment used in the flow cell system is discussed in section 2.8.. The GPT device was used to culture *S. sanguis* for use in flow cell experiments (section 2.6.). The pH and *S. sanguis* concentration in the GPT culture were determined before the initial, and after the final, use for flow cell work for each GPT (sections 2.7.1. and 2.7.5.). The equipment set-up for each set of experiments is described in the respective sections. MCM was used for pellicle formation and growing of GPT culture. MCM-salts was used for all rinse procedures (refer to section 2.2. for media details).

7.2.2 Antimicrobial agents used in the study

The antimicrobial agents used in the study were obtained from GABA International, Therwil, Switzerland. The agents are listed in table 7.2.2.. Other than the active agents, preparations contained (per litre) 2 g of polyethylene glycol-hydrogenated castor oil (GABA International), 50 g of ethanol (BDH), 0.25 g potassium acesulfame (Hoechst, Frankfurt, Germany), and 25 g of xylitol (Sigma). Deionised water was used as the negative control. Chlorhexidine digluconate (0.2%) (CHX) (Sigma) was also tested for anti-adhesion activity. Not all agents were use for each experiment. Refer to experiment section for details of which agents were tested.
Table 7.2.2. Amine fluoride and inorganic fluoride concentrations in solutions used in the study.

<table>
<thead>
<tr>
<th>Active ingredients of solutions*</th>
<th>mg L(^{-1})</th>
<th>Molar concentration of amine</th>
<th>mg L(^{-1})</th>
<th>Molar concentration of inorganic cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>olaflur</td>
<td>3250</td>
<td>0.0071</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C(<em>{27})H(</em>{60})F(<em>{2})N(</em>{2})O(_{3}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oleaflur</td>
<td>4671</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C(<em>{22})H(</em>{45})FNO(_{2}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steraflur</td>
<td>4697</td>
<td>0.013</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(C(<em>{22})H(</em>{47})FNO(_{2}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olaflur / tin (IV) fluoride</td>
<td>1625</td>
<td>0.0035</td>
<td>195</td>
<td>0.0017</td>
</tr>
<tr>
<td>olaflur / tin (II) fluoride</td>
<td>1625</td>
<td>0.0035</td>
<td>390</td>
<td>0.0033</td>
</tr>
<tr>
<td>zinc fluoride</td>
<td>-</td>
<td>-</td>
<td>430</td>
<td>0.0066</td>
</tr>
</tbody>
</table>

All solutions had a fluoride concentration of 250 mg L\(^{-1}\) F\(^-\).
7.2.3. Treatment of pellicle-coated glass (MCM-glass) with test solutions.

Figure 7.2.3. shows the equipment set-up when the agents were added to the pellicle or directly to the glass. Prior to use, the supply lines to the flow cell were clamped off. One of the flow cells was also clamped off. The following protocol was used in all determinations:

1. MCM salts solution was pumped through the system at maximum pump flow.
2. Three ml of MCM from the tube prior to the flow cell was pumped through the cell at a flow rate of 0.91 ml minute\(^{-1}\) (shear rate =142 s\(^{-1}\))
3. MCM salts was pumped through the system at maximum flow rate for five minutes to remove unattached MCM.
4. Five ml of agent was pumped through the flow cell from the agent reservoir at a flow rate of 0.91 ml minute\(^{-1}\) (shear rate = 142 s\(^{-1}\)).
5. MCM salts was pumped through the flow cell at maximum pump flow rate for 5 minutes.
6. The GPT culture was pumped through the flow cell at maximum flow rate until it passed through the flow cell
7. The culture was pumped through the flow cell at a flow rate of 0.05 ml minute\(^{-1}\) (shear rate = 7.8 s\(^{-1}\)).
8. After one hour of culture flow, the pump was switched to the MCM-salts supply which was passed through the flow cell at a flow rate of 0.46 ml minute\(^{-1}\) (shear rate = 72 s\(^{-1}\)) for ten minutes.
9. The flow rate of MCM-salts was increased to 0.91 ml minute\(^{-1}\) (shear rate = 142 s\(^{-1}\)).
10. After twenty minutes, images of the bottom flow cell surface were taken following the procedure in section 2.7.4. and analysed for surface coverage.
The room temperature at the beginning and end of each run was recorded. All the agents listed in table 7.2.3. were tested in the experiment. The experiment was repeated three times for each agent.

Figure 7.2.3. Apparatus set-up for flow cell experiments adding agents to pellicle.

The GPT device was incubated at 37°C.
7.2.4. Treatment of glass with the test solutions.

The procedure followed the protocol in section 7.2.3. but the 3 ml MCM medium supply for pellicle formation was replaced with 3 ml of MCM salts. The agent used in the experiment was Olaflur/tin(IV) fluoride at the concentration in table 7.2.2.. Deionised water was used as the negative control. The experiment was repeated three times for deionised water and twice for olaflur/tin (IV) fluoride solution.

7.2.5. Adhesion to surface with pellicle formed from MCM mixed with test solutions.

The apparatus set-up for the experiment is shown in figure 7.2.5.. The mixing reservoir was specially made by Hampshire glassware, UK. The volume of the reservoir was 25 ml. The following protocol was used in all determinations:

1. MCM salts solution was pumped through the system at maximum pump flow rate.
2. Three ml of MCM from the tube prior to the flow cell was pumped into the mixing reservoir at a flow rate of 0.91 ml minute\(^{-1}\) (shear rate = 142 s\(^{-1}\)).
3. Five ml of agent was pumped into the mixing reservoir at a flow rate of 0.91 ml minute\(^{-1}\) (shear rate = 142 s\(^{-1}\)).
4. The agent/MCM mix was pumped through the flow cell at a flow rate of 0.91 ml minute\(^{-1}\) (shear rate = 142 s\(^{-1}\)).
5. MCM salts was then pumped through the flow cell at maximum pump flow rate for 5 minutes.
6. The GPT culture was pumped through the flow cell at maximum flow rate until it passed through the flow cell.

7. The culture was pumped through the flow cell at a flow rate of 0.05 ml minute\(^{-1}\) (shear rate = 7.8 s\(^{-1}\)).

8. After one hour of culture flow, the pump was switched to the MCM-salts supply which was passed through the flow cell at a flow rate of 0.46 ml minute\(^{-1}\) (shear rate 72 s\(^{-1}\)) for ten minutes.

9. The flow rate of MCM-salts was increased to 0.91 ml minute\(^{-1}\) (shear rate 142 s\(^{-1}\)).

10. After twenty minutes, images of the bottom flow cell surface were taken following the procedure in section 2.7.4. and analysed for surface coverage.

The room temperature at the beginning and end of each run was recorded. Olaflur and olaflur/tin (IV) fluoride combination were used in the experiment. Deionised water was used as the negative control. The experiment was repeated three times for each solution.
Figure 7.2.5. Schematic diagram of apparatus set-up for experiments mixing MCM with agents prior to pellicle formation.

The GPT device was incubated at 37°C.
7.2.6. Adhesion of material to MCM-glass from culture mixed with test solutions.

Figure 7.2.6. show the apparatus set-up for this set of experiments. The following protocol was used in all determinations:

1. MCM salts solution was pumped through the system at maximum pump flow rate.

2. 5 ml of culture was pumped from the GPT device to the reservoir at the maximum pump flow rate (NB. 5 ml was sufficient culture to run the flow cell for one hour).

3. Three ml of MCM from the tube prior to the flow cell was pumped through the flow cell at a flow rate of 0.91 ml minute$^{-1}$ (shear rate = 142 s$^{-1}$)

4. MCM salts solution was pumped through the flow cell at maximum pump flow rate for five minutes.

5. One ml of agent was pumped into the mixing reservoir at a maximum pump flow rate.

6. The agent/culture mix was pumped from the mixing reservoir at a flow rate of 0.91 ml minute$^{-1}$ (shear rate = 142 s$^{-1}$) until it reached the end of the flow cell.

7. The culture was then pumped through the flow cell at a flow rate of 0.05 ml minute$^{-1}$ (shear rate = 7.8 S$^{-1}$).

8. After one hour of culture flow, the pump was switched to the MCM-salts supply which was passed through the flow cell at a flow rate of 0.46 ml minute$^{-1}$ (shear rate 72 s$^{-1}$) for ten minutes.

9. The flow rate of MCM-salts was increased to 0.91 ml minute$^{-1}$ (shear rate = 142 s$^{-1}$).
10. After twenty minutes, images of the bottom flow cell surface were taken following the procedure in section 2.7.4. and analysed for surface coverage.

The room temperature at the beginning and end of each run was recorded. Olaflur and Olaflur/tin(IV)flouride combination were used in the experiment. Deionised water was used as the negative control. The experiment was repeated three times for each solution.

Figure 7.2.6. Schematic diagram of apparatus set-up for experiments mixing culture with agents prior to running through flow cell.

The GPT device was incubated at 37°C.
7.2.7. Adhesion of salivary bacteria, and planktonic S. sanguis, to flow cells

The adhesion of S. sanguis grown in the GPT was compared to the adhesion from a sample of saliva and a S. sanguis batch culture. The sample of saliva was collected immediately before use. Saliva was obtained from one healthy individual. The batch culture was grown for 24 hours at 37°C in aerobic conditions. MCM was used as the growth medium. Both cultures were pumped through clean flow cells at a rate of $3 \times 10^{-4} \text{ L h}^{-1}$ (shear rate=7.8 s$^{-1}$) for one hour. The flow rate was increased 0.05 L h$^{-1}$ (shear rate=142 s$^{-1}$). Images were then collected using the video capture system described in section 2.7.4.

7.2.8. Statistical analysis of results

The percentage surface coverage values for controls groups were normally distributed. In test groups where adhesion was reduced it was common for bacteria to be absent from captured frames. Due to a high frequency of recorded zero values the distribution of values was not normally distributed. Thus a non-parametric test assuming unequal variance was used to measure the significance of experimental values. The test used for comparison of data was the the Kruskal Wallis test. This was performed using SPSS (standard version) on the Windows 95 platform.
7.3. Results

7.3.1. GPT culture analysis

Table 7.3.1.1 Lists the mean concentration and pH of GPT cultures pre and post use for flow cell experiments. The mean CFU count for GPT culture prior to use (1.18 ± 0.7 x 10^7 CFU ml^{-1}) was not significantly different (t-test p>0.01) from the mean CFU concentration after use for flow cell experiments (1.71 ± 1.1 x 10^7 CFU ml^{-1}). The mean pH of GPT culture prior to use for experiments (5.87 ± 0.1) was significantly different (t-test p>0.01) from the mean pH of culture following flow cell experiments (5.74 ± 0.1).

<table>
<thead>
<tr>
<th></th>
<th>Pre-flow cell experiments</th>
<th>Post-flow cell experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean count</td>
<td>number of measurements</td>
</tr>
<tr>
<td></td>
<td>1.18 ± 0.7 x 10^7</td>
<td>11</td>
</tr>
<tr>
<td>CFU ml^{-1}</td>
<td></td>
<td>1.71 ± 1.1 x 10^7</td>
</tr>
<tr>
<td>PH</td>
<td>5.87 ± 0.1</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.74 ± 0.1</td>
</tr>
</tbody>
</table>

7.3.2. Adhesion of S. sanguis to MCM-glass treated with the test solutions.

Figure 7.3.2.1 shows S. sanguis adhered to MCM-glass treated with deionised water, olaflur and olaflur/tin(IV). The frames with the median value of surface coverage for each test group are shown. The cells adhered to the surface exist singularly, in pairs, in short chains and small clumps. Figure 7.3.2.2. illustrates
the percentage surface coverage (PSC) of MCM-glass. The measurement is the net PSC after one hour of culture flow following MCM-glass treatment with agents. There was no significant difference in the net PSC of MCM-glass when treated with either deionised water (0.63 ± 0.2%), zinc fluoride (0.45 ± 0.1%), olaflur/zinc fluoride combination (0.7 ± 0.2%), olaflur (0.41 ± 0.2%) or oleaflur (0.53 ± 0.3%) prior to culture flow. The PSC of MCM-glass was significantly different from the previous groups following treatment prior to culture flow with 0.2% chlorhexidine digluconate (CHX) (0.86 ± 0.1%), olaflur/tin (II) fluoride (0.11 ± 0.05%), steraflur (0.05 ± 0.03%) and olaflur/tin (IV) fluoride (0.05 ± 0.01%). With respect to the control, the PSC increased following treatment with CHX and decreased for the other groups. The rate of adhesion of S. sanguis to surfaces treated with olaflur and oleaflur appeared to be reduced in comparison to controls. The apparent decrease in adhesion rate was short lived (minutes) and was not measured however, the net PSC after one hour was not statistically different from controls. The mean room temperature prior to the start of the experiments was 23.2 ± 1.3°C (n = 31). The mean room temperature at the end of the experiments was 23.5 ± 1.1°C (n = 28).
Figure 7.3.2.1. Images of adherent *S. sanguis* following treatment of MCM-glass with deionised water, Olaflur and Olaflur/tin (IV) fluoride.

The frames with the median value of percentage surface coverage (PSC) for each test group are shown. The measurements are the net PSC after one hour of culture flow following MCM-glass treatment with agents.
Figure 7.3.2.2. The percentage surface coverage of MCM-glass by *S. sanguis* following pre-treatment with GABA agents and controls.

![Graph showing percentage surface coverage](image)

The percentage surface coverage is the net coverage after one hour of culture flow. All fluoride-containing solutions were adjusted to 250 mg litre\(^{-1}\) fluoride. Chlorhexidine digluconate (CHX) was adjusted to 0.2%. Error bars are standard deviations of the values for frames analysed from three separate runs.
7.3.3. Adhesion of S. sanguis to glass treated with test solutions prior to culture flow.

Figure 7.3.3.1 shows the bacteria adhered to glass treated with deionised water and olaflur/tin(IV) fluoride combination prior to culture flow. The frames with the median value of surface coverage for each test group are shown. The cells adhered to the surface exist singularly, in pairs, in short chains and small clumps. Figure 7.3.3.2 illustrates the percentage surface coverage of glass and MCM-glass by S. sanguis following surface treatment with deionised water and olaflur/tin (IV) fluoride. The measurements are the net PSC after one hour of culture flow. There was a significant difference in the PSC of glass pre-treated with deionised water (0.55 ± 0.2%) compared to when the glass was pre-treated with olaflur/tin (IV) fluoride (0.22 ± 0.2%). There was no significant difference in the PSC of glass treated with deionised water or MCM-glass treated with deionised water. The PSC of glass treated with olaflur/tin (IV) fluoride (0.22 ± 0.2%) was significantly different to the PSC of MCM-glass treated with olaflur/tin (IV) fluoride (0.05 ± 0.01%). The mean room temperature prior to the start of the experiments was 23.9 ± 0.5°C (n = 4). The mean room temperature at the end of the experiments was 24.3 ± 0.3°C (n = 3).
Figure 7.3.3.1. Images of *S. sanguis* adhered to glass treated with deionised water and olaflur/tin (IV) fluoride

A glass pre-treated with dH$_2$O.
B glass pre-treated with olaflur/tin (IV) fluoride combination
Bar = 10 microns

The frames with the median value of percentage surface coverage (PSC) for each test group are shown. The measurements are the net PSC after one hour of culture flow following glass treatment with agents.
Figure 7.3.3.2. The percentage surface coverage of glass and MCM-glass treated with deionised water and olaflur/tin (IV) fluoride prior to S. sanguis culture flow.

Fluoride-containing solutions were adjusted to 250 mg litre$^{-1}$ fluoride. Error bars are standard deviations of values for frames analysed from three separate runs.
7.3.4. Adhesion of S. sanguis to flow cells with a pellicle formed from MCM mixed with test solutions.

Figure 7.3.4.1. shows the S. sanguis to adhered to glass with a pellicle formed from deionised water/MCM, olaflur/MCM and olaflur/tin(IV) fluoride/MCM. The frames with the median value of surface coverage for each test group are shown. The cell units adhering to the interface appear similar to those adhering to MCM-glass (figure 7.3.2.1.). Figure 7.3.4.1. illustrates the PSC of flow cells with a pellicle formed from GABA agents or deionised water mixed with MCM. The measurement is the net PSC after one hour of culture flow. The PSC of flow cells with a deionised water/MCM pellicle (0.7 ± 0.2%) and an olaflur/MCM pellicle (0.66% ± 0.2) was not significantly different (p>0.01). The PSC in flow cells with a pellicle formed from olaflur/tin(IV) fluoride/MCM (0.02 ± 0.01%) was significantly lower than for the other two groups. The PSC of MCM-glass treated with deionised water was not significantly different (p>0.01) to the PSC on flow cells with a pellicle formed from MCM mixed with deionised water. The PSC of MCM-glass treated with olaflur/tin (IV) fluoride was statistically different to the PSC of flow cells with a pellicle formed from MCM mixed with olaflur/tin (IV) fluoride. The mean room temperature prior to the start of the experiments was 23.7 ± 0.6°C (n = 7). The mean room temperature at the end of the experiments was 24.1 ± 0.6°C (n = 8).
Figure 7.3.4.1. *S. sanguis* adhered to flow cells with a pellicle formed from MCM mixed with deionised water, olaflur and olaflur/tin (IV) fluoride.

The frames with the median value of percentage surface coverage (PSC) for each test group are shown. The net PSC after one hour's culture flow was measured.
Figure 7.3.4.2. *S. sanguis* coverage of flow cells with a pellicle formed from MCM mixed with deionised water, olaflur and olaflur/tin (IV) fluoride combination.

Fluoride-containing solutions were adjusted to 250 mg litre$^{-1}$ fluoride. Error bars are standard deviations of values for frames analysed from three separate runs.
7.3.5. Adhesion of material to MCM-glass from flow of culture mixed with test solutions.

Figure 7.3.5.1 shows material adhering to MCM-glass flow cells from *S. sanguis* GPT culture mixed with either deionised water, olaflur or olaflur/tin(IV) fluoride. The frames with the median value of surface coverage for each test group are shown. The cell units adhering to the interface when the culture is mixed with deionised water appear similar to those adhering to flow cells when the pellicle is treated with the agents (figure 7.3.2.1). In contrast, material adhering to MCM-glass when the culture was mixed with olaflur included aggregations of material that did not resemble streptococcal cells. Small units were also adhered to the surface that could possibly be bacterial cells. When tin (IV) fluoride was used in combination with olaflur the majority of the aggregated material was prevented from adhering to the surface. Figure 7.3.5.2. illustrates the PSC of MCM-glass from GPT culture mixed with either deionised water, Olaflur or Olaflur/tin(IV) fluoride. The measurement is the net PSC after one hour of culture/agent flow. The PSC by material on MCM-glass following culture/deionised water flow (0.89 ± 0.1%) was significantly different to the PSC on MCM-glass following culture/Olaflur (2.8 ± 0.8%) and culture/Olaflur/tin(IV) fluoride (0.1 ± 0.03%) flow. The PSC of MCM-glass after culture/deionised water flow was not statistically different to the PSC of MCM-glass treated with deionised water prior to the culture flow. Likewise there was no significant difference between the PSC of MCM-glass following olaflur/tin (IV) fluoride/culture flow and the PSC of MCM-glass treated with olaflur/tin (IV) fluoride prior to the culture flow. The PSC of MCM-glass treated with olaflur prior to the culture flow was significantly
different to the PSC of MCM-glass when the culture was mixed with olaflur. The mean room temperature prior to the start of the experiments was 23.9 ± 0.3°C (n = 9). The mean room temperature at the end of the experiments was 24.6 ± 0.3°C (n = 9).
Figure 7.3.5.1. Material adhered to MCM-glass after flow of *S. sanguis* GPT culture mixed with either deionised water, olaflur or olaflur/tin (IV) fluoride combination.

A culture mixed with dH$_2$O
B culture mixed with olaflur
C culture mixed with olaflur/tin (IV) fluoride combination
Bar = 10 microns

The frames with the median value of percentage surface coverage (PSC) for each test group are shown. The net PSC after one hours culture flow was measured.
Figure 7.3.5.2. Percentage of surface area of MCM-glass covered by material following one hour flow of culture mixed with either deionised water, olaflur or olaflur/tin (IV) fluoride combination.

Fluoride-containing solutions were adjusted to 250 mg litre$^{-1}$ fluoride. Error bars are standard deviations of the values for frames analysed from three separate runs.
7.3.6. Adhesion of salivary bacteria, and planktonic S. sanguis cells, to flow cell surfaces.

Figure 7.3.6 shows bacteria adhering to flow cells from saliva, and S. sanguis batch culture. Organisms were allowed to adhere at a shear rate of 0.78 s\(^{-1}\). The shear rate was turned up to 142 s\(^{-1}\) prior to video capture of images. This removed large amounts of bacteria from the surface in the S. sanguis batch culture experiment. In contrast, few of the organisms at the surface in the saliva experiment were removed by increasing the shear rate. The pattern of adhesion for salivary bacteria was similar to the adhesion of biofilm-derived S. sanguis grown in the GPT device. In contrast, chains of cells from the S. sanguis batch culture adhered and sedimented in large quantities. Chains of cells in the flow frequently became entangled with chains at the surface producing large clusters of bacteria.
Figure 7.3.6 Adhesion to glass flow cells of (A) *S. sanguis* grown by batch, and (B) salivary bacteria

(A) Adherent *S. sanguis* grown by batch culture in MCM. (B) Adherent bacteria from a fresh sample of human saliva. Bacteria were allowed to adhere at a shear rate of 7.8 s\(^{-1}\) for one hour. The shear rate was increased to 142 s\(^{-1}\) prior to collection of images. Bar = 10 \(\mu\)m.
7.4. Discussion

The reason for the development of the GPT culturing device was to produce a method of culturing *S. sanguis* in a state similar to that found in human saliva. The adhesion of *S. sanguis* cells grown using the GPT culture was similar to that of bacteria from saliva. In contrast, *S. sanguis* cells grown by batch culture sedimented and adhered in large quantities. The chains of cells frequently became entangled further increasing the amount of immobilised cells. In chapter 6, it was discussed how the morphology of cultures grown by planktonic methods may affect the level of efficacy of anti-plaque agents. The results of this investigation show that there is a distinct difference in the deposition pattern of planktonic and biofilm derived *S. sanguis* cells. Such differences could profoundly affect the efficacy of anti-plaque agents. The use of fresh saliva to grow organisms for wide scale testing of anti-adhesion agents is inappropriate as it is far to troublesome to collect enough saliva. Fresh saliva may also vary in composition and concentration of solutes. The use of GPT cultures for testing of anti-adhesion agents is a convenient alternative. The cultures are cheap, the medium is reproducible and the cells adhere in a similar manner to cells from saliva.

The GPT culture never actually reached a steady state. The viable count of GPT cultures was not significantly different before and after their use for flow cell work. The pH of GPT cultures was significantly different before and after their use for a set of experiments. This change was over a period of about 10 days. The change in the culture was small and the effect on adhesion was
insufficient to cause a statistically significant difference in the counts from the three separate control runs for each experiment.

The shear rate for adhesion in the experiments was 7.8 s$^{-1}$. Rinse steps with MCM salts increased shear up to 72 s$^{-1}$ and 142 s$^{-1}$. At these shear rates cells did not adhere to the surface. Busscher et al, 1992a, hypothesised that the shear forces fluctuate in the oral cavity and that bacteria adhere to surfaces during periods when the shear is low. The in vivo work of Quirynen et al, 1993, supports this theory. They found increased complexity of supra-gingival plaque on roughened abutements and suggested this was due to the initial reversible binding of cells occurring in the surface irregularities where shear forces are decreased. The in vitro work in our study is consistent with the hypothesis. The retention of adhered bacteria as shear force was increased suggests reinforcement of initial adhesion. This is supported by the observation that bacteria adhering in the last few seconds of flow at a shear rate of 7.8 s$^{-1}$ were frequently removed when the shear rate was increased to 72 s$^{-1}$.

The amine fluoride, steraflur, and combinations of tin (II) fluoride and tin (IV) fluoride with olaflur were the only agents able to reduce the net adhesion of S. sanguis to MCM-glass after one hour of culture flow. Solutions containing olaflur, olaflur and zinc fluoride alone did not prevent adhesion of S. sanguis to MCM-glass. This indicates that the ions responsible for anti-adhesion activity were the tin ions and the steraflur amine ion. The background solution, fluoride ions and the other amine ions were not responsible for the inhibition of adhesion over a one hour period.
Pre-treatment of MCM-glass with tin (II) and tin (IV) fluoride decreased the net adhesion of *S. sanguis* to the surface after one hour of culture flow. The experiment showed that besides tin fluoride preventing adhesion the component responsible also exhibited a level of substantivity. This agrees with *in vivo* work by Elworthy et al 1996. They assessed substantivity indirectly by measuring the suppression of salivary bacterial concentrations. Tin fluoride was shown to be substantive within the oral cavity for 430 minutes compared to water. A similar effect was recorded by Jenkins et al 1990. Attramadal & Svatun 1984 demonstrated raised tin levels in saliva 4 hours after rinsing or toothbrushing. The tin (IV) ions possessed a greater anti-adhesion activity towards *S. sanguis* than tin (II) ions. The mechanism by which tin fluorides prevent bacterial adhesion is not known. Tin ions may interfere with hydrophobic interactions. The hydrophobicity of *S. sanguis* cells has been studied in relation to bacterial adhesion. Nesbitt et al, 1982, used an aqueous/hydrocarbon mixture to measure the hydrophobicity of *S. sanguis* cells. They found that the bacteria preferably bound to the hydrocarbon component (whether toluene or hexadecane) suggesting the cell surface was hydrophobic. They also showed that chemicals known to interfere with hydrophobic interactions were able to prevent binding between *S. sanguis* and hydroxyapatite. One of the chemicals used was the lithium cation. It is not known if tin ions exhibit hydrophobic bond disrupting properties. If they do, it may explain the inhibition of adhesion observed in our experiments. Alternatively, the tin ions may interfere with specific adhesin-receptor interactions. Oral streptococci are known to possess multiple adhesins for a variety of components of salivary pellicles (Rudney et al 1999). These include
acidic proline-rich proteins, statherin, alpha-amylase, lactoferrin and lysozyme and mucin glycoproteins. In our experiment we used MCM medium to condition surfaces. This medium does not contain significant concentrations of the majority of salivary components. However the medium does contain a high concentration of glycoprotein in the form of hog gastric mucin. Murray et al, 1992, investigated interactions of *S. sanguis* strains with glycoproteins isolated from saliva by SDS-PAGE electrophoresis. They found that all three strains of *S. sanguis* tested, bound to low molecular weight salivary mucins. One strain, *S. sanguis* ATCC 10556, also bound to proline-rich glycoproteins and amylase. If the tin ions interfered with adhesin-receptor interactions in our experiments, it is possible that the hog gastric mucin in our medium was the receptor for *S. sanguis* adhesin(s).

Laying a pellicle from a mixture of MCM and tin (IV) fluoride was more effective at preventing adhesion than adding tin (IV) fluoride to a pre-formed MCM pellicle. This was presumably due to increased opportunity for the tin (IV) fluoride and MCM components to interact. This observation indicates a possible improvement that could be made to agent delivery systems. A two solution mouthwash could be used to accompany standard dental hygiene procedures. One solution could contain the anti-plaque agent whereas the other could contain a protein/glycoprotein solution. The two solutions could be mixed prior to use and then used in a similar fashion to traditional mouthwashes. The aim of the treatment would be to produce an artificial pellicle on the teeth that is enriched in tin ions. The mouthwash would have to be used immediately after tooth brushing as the salivary pellicle forms in seconds on cleansed enamel (Lamkin et al 1996).
Treatment of glass with olaflur/tin (IV) fluoride prior to pellicle formation significantly reduced net *S. sanguis* adhesion over one hour compared to treatment of the glass with deionised water. However the reduction in adhesion was significantly less when the glass was treated with the combination than when MCM-glass was treated with olaflur/tin (IV) fluoride. The results indicate that the tin ions are able to adhere to glass and the MCM pellicle. The enhanced reduction in *S. sanguis* adhesion when adding the agents to the pellicle suggests that (1) tin ions adhere in greater concentrations to MCM-glass than glass, and/or, (2) the substantivity is greater when tin is bound to the pellicle rather than glass.

Treatment of MCM-glass with either olaflur or oleaflur prior to culture flow did not reduce the net adhesion of *S. sanguis* to the surface over a one hour period. Previous studies have reported an *in vitro* anti-adhesion activity of amine fluoride towards *S. mutans* and *S. sobrinus* (Altenhofen et al 1989), and, *S. mutans* and *S. sanguis* (Meurman 1987). However the systems used in their studies were closed and did not take into account substantivity. We did observe a reduction in the initial rate of adhesion of *S. sanguis* to olaflur-and oleaflur- treated MCM-glass compared to MCM-glass treated with deionised water, however, the net adhesion after one hour was no different suggesting poor substantivity of the agents. The work of Altenhofen et al, 1989, and Meurman, 1987 suggest that olaflur and oleaflur have the ability to prevent adhesion if they are in some way immobilised at the surface. Bapna et al 1988 have shown that incorporation of the amine, dodecylamine, into dental adhesive resins prevented adhesion of *S. mutans in vitro.*
Unlike the olaflur and oleaflur preparations, steraflur did inhibit *S. sanguis* net adhesion over a one hour period when used to treat MCM-glass prior to culture flow. The reason for the increased activity of the agent was not investigated in this study and remains unclear. It is possible that the reduced reactivity of steraflur may be the cause of its increased anti-adhesion properties. Steraflur has been shown to possess a lower bactericidal activity towards *S. sanguis* biofilms than olaflur and oleaflur (Embleton et al 1998). If the agent is reduced in its rate of reaction with *S. sanguis* cells and medium components then it will be removed from the pellicle at a slower rate and hence possess increased substantivity. Alternatively the structure of steraflur may increase the stability of its binding to pellicle components.

Mixing the amine fluoride with the *S. sanguis* culture caused material to precipitate and adhere to MCM-glass. The percentage of the surface covered by the material was significantly greater than the percentage covered by *S. sanguis* cells when MCM-glass was treated with olaflur prior to culture flow. The nature of the material adhering to MCM-glass was not determined in the experiment. In chapter 5 it was shown that olaflur precipitated components of both MCM and filter-sterilised saliva. However, mixing of olaflur with MCM did not cause large amounts of precipitated material to adhere to the surface. Thus it is probable that the material is a mixture of precipitated MCM components and *S. sanguis* cellular material. The adherence of precipitated material is a concern with regard to oral use. Adherence of the material will increase the area of the surface. Additionally, bacterial adherence may increase disproportionately to the increase in surface area due to the nature of the material. The material is thought to be a mixture of precipitated medium.
components and *S. sanguis* cells. Such material may offer a large increase in available receptors for oral bacterial adhesins. The adhered material may also impart an undesirable mouthfeel for users. This is obviously of concern when trying to market a product for oral use.

When tin (IV) fluoride was used in combination with olaflur most of the material was prevented from adhering to the surface. This observation provides new reasons to support the use of tin fluoride as an adjuvant to amine fluorides.

There was no significant difference in the surface coverage of glass and MCM-glass by *S. sanguis* when the surfaces where treated with deionised water prior to culture flow. There have been a number of studies on the adhesion of oral streptococci to surfaces with and without a pellicle. The adhesion of *S. anginosus*, *S. oralis* and *S. salivarius* to titanium beads (Edgerton et al 1996), the adhesion of mutans streptococci to glass (Busscher et al 1992b) and the adhesion of *S. sanguis* and *S. mutans* to enamel (Pratt-Terpstra et al 1989) were all reduced if a salivary pellicle was allowed to form prior to adhesion. In our study we used MCM to form the pellicle. This is the same medium which was used to grow GPT cultures used for adhesion studies. Pellicles can form in seconds (Lamkin et al 1996) and it is likely that a pellicle could have formed from the MCM in the culture medium faster than cells could adhere. Thus the adhesion would be expected to be the same in both experiments.

Pre-treatment of MCM-glass with 0.2% CHX increased the net adhesion of *S. sanguis* to the surface after one hour of culture flow. CHX has been shown to possess a strong affinity for salivary glycoproteins *in vitro* (Rolla et al 1970).
Hence it would be sensible to assume that CHX would also bind to the hog gastric mucins present in MCM which is used to produce the pellicle in this experiment. CHX binds to the phosphate groups of components in the negatively-charged bacterial cell wall (Hugo & Longworth 1966). If CHX incorporated into our pellicle is still able to bind bacterial cells, the number of available attachment sites for \textit{S. sanguis} may be increased. Alternatively the positive charge of CHX may increase the overall positive charge of the pellicle and increase the attractive force for negatively-charged bacterial cells. Although the number of adhering bacteria was increased by treatment of the pellicle with CHX the experiment did not investigate the viability of the adhered cells. CHX is known to be bactericidal at high concentrations and bacteriostatic at lower concentrations (Hennessey 1973). Thus if CHX is incorporated into the pellicle then it would probably be able to prevent bacterial growth and replication, one of the processes leading to biofilm formation. It is widely accepted that CHX decreases plaque formation \textit{in vivo}. The results of this study show that CHX does not inhibit bacterial adhesion but is incorporated into the pellicle where it may be exhibit bactericidal or bacteriostatic activity. Thus in early biofilm formation bacterial replication may be the rate determining factor of biofilm growth rather than co-adhesion.

\textbf{Summary:}

- The GPT device and flow cell system provided an effective reproducible system for studying anti-adhesion properties of anti-plaque agents
- Tin fluorides and steraflur reduced adhesion of \textit{S. sanguis} to flow cells.
• Incorporation of tin (IV) fluoride into the pellicle was more effective at reducing adhesion than treating glass with the agent.

• When olaflur was mixed with the bacteria culture large aggregates of precipitated material from the growth medium and bacterial cells adhered to the surface. Addition of tin (IV) fluoride prevented the adhesion of the majority of the material.
Chapter 8 - The effect of amine/tin fluoride on microcosm biofilm formation
8.1. Aims

In previous chapters, the bactericidal and anti-adhesion properties of an amine/tin (IV) fluoride combination were investigated. It was shown that the combination of agents can kill *S. sanguis* in biofilms grown using the constant depth film fermenter (CDFF). It was also shown that the combination prevented adhesion of *S. sanguis* to mucin-coated glass surfaces in a parallel flow model. In this chapter, the effect of amine/tin fluoride on biofilm formation by bacteria present in pooled human saliva was examined.

In chapter 6, the effect of surface treatment with GABA agents on *S. sanguis* biofilm formation was investigated. In that study, several problems with the experiment methodology were discussed. The system in this investigation was designed to take into account the problems previously encountered.
8.2. Materials and Methods

8.2.1. Inoculum and growth medium

Pooled human saliva was used as the inoculum in all experiments. The saliva was pooled from healthy individuals working at the Eastman Dental Institute. The saliva from four individuals was used as the inoculum to grow biofilms in mucin-containing medium (MCM). For biofilm formation in the presence of sucrose, a fresh supply of saliva was collected from three of the four individuals previously used. Pooled saliva was stored prior to use at -70°C. The growth medium used in all experiments was mucin-containing medium (MCM). MCM-salts was used in rinse steps. Refer to section 2.2. for details of media formulation and preparation. In experiments investigating biofilm formation in the presence of sucrose, the sugar was added to MCM to give a concentration of 36 g L\(^{-1}\). This was designated S-MCM.

8.2.2. CDFF set-up and operation

Two CDFF’s were operated simultaneously in the series of experiments. Figure 8.2.2. illustrates the apparatus set-up. The saliva inoculum (10 ml) was diluted ten-fold with MCM salts prior to use. This was stirred continually using an IKA color squid magnetic stirrer (Fisher). The GABA agent used in the experiments was olaflur/tin (IV) fluoride combination (250 mg litre\(^{-1}\) fluoride). This and the negative control (deionised water) were filter sterilised into their respective supply tubes. Pyrex glass was used as the substratum (refer to section 2.4.2. for details). The glass discs were recessed to a depth of 100 μm.
The MCM/S-MCM and saliva supplies were pumped toward the CDFF’s at maximum flow rate. The pumps were switched off just before the supplies entered the fermenters. The CDFF turntables were operated at 3 RPM. MCM/S-MCM was run into both of the fermenters at maximum pump flow rate (approximately 0.3 L h\(^{-1}\)) for five minutes. The flow rate was then reduced to 0.03 L h\(^{-1}\). Fifty ml of agent was supplied to one of the CDFF’s at a flow rate of 3.0 L h\(^{-1}\). Deionised water was supplied to the other CDFF in the same manner. The saliva supply was then pumped into each of the CDFF’s at a flow rate of 0.003 L h\(^{-1}\). The fermenters were enclosed in an incubator at 37°C in an aerobic environment. PTFE pans were removed from the CDFF’s for biofilm analysis after 6 and 12 hours. Prior to removal, the MCM/S-MCM and saliva supplies were turned off. MCM-salts medium was pumped into the CDFF’s at a flow rate of 6.0 litres h\(^{-1}\) for 3 minutes. This was to remove unattached and loosely-attached cells from the CDFF. After pan removal the MCM/S-MCM and saliva supplies were turned back on.
The CDFF’s were incubated aerobically at 37°C. The saliva sample was stirred continuously throughout the experiments.

8.2.3. Viable counts of biofilms

Aerobic counts were performed on blood agar (blood agar base [Oxoid], 7.5% defibrinated horse blood [TCS Microbiology]) and tryptone soya agar (Oxoid). Duplicate blood agar plates were incubated in a carbon dioxide enriched atmosphere. Anaerobic counts were performed on fastidious anaerobe agar (FAA) (Lab M) supplemented with 7.5% defibrinated horse blood. Counts of gram negative anaerobes were performed on FAA supplemented with 2.5 mg L$^{-1}$ vancomycin hydrochloride (Sigma) and 10 mg L$^{-1}$ nalidixic acid (Sigma) prepared in 5 ml of 70% ethanol (Sigma). Tryptone soya agar supplemented with 20% sucrose (Sigma) and 0.1 unit per ml bacitracin (Sigma) was used to
selectively grow mutans streptococci. Viable counts were performed on three separate biofilms per sample (refer to section 2.7.1 for methodology). Viable counts were also performed on the saliva inoculum at the beginning and end of each experiment.

8.2.4. Fluorescence microscopy of intact biofilms

Two biofilms per pan were used for fluorescence microscopy. The biofilms were stained using the Baclight live/dead viability kit (Molecular Probes) (refer to section 2.7.2.). Images of the stained biofilms were recorded using the system described in section 2.7.4..

8.2.5. Identification of bacterial genera in biofilms

The bacteria on the mixed species agar plates were isolated by streaking colonies on fresh blood agar plates. These were incubated aerobically at 37°C for 24 hours. The plates were then stored at 4°C. Colonies from the streak plates were aseptically transferred to 10 ml tryptone soya broths (Oxoid) which were then incubated aerobically at 37°C overnight. The broth cultures were then Gram stained. Catalase and oxidase tests were performed on colonies from the streak plates of the isolated bacteria. The bacteria strains were sub-cultured onto 9 blood agar plates. The plates were split into three sets. One set was incubated aerobically, one set was incubated in a carbon dioxide enriched atmosphere, and the final set was incubated anaerobically. The strains were also sub-cultured on to mitis salivarius (MS) agar plates (Difco, Detroit, US) which were incubated aerobically at 37°C.
8.3. Results

Figure 8.3.1. shows representative images of MCM-grown microcosm biofilms. The substratum was conditioned with MCM prior to culture flow. The images were taken 12 hours after the start of biofilm formation. The biofilms consisted of micro-colonies of varying size. The largest micro-colony shown (picture A) had a diameter of approximately 446 \( \mu \text{m} \) (horizontal measurement). Some of the micro-colonies appeared to fuse (picture C). Void areas were visible within the large micro-colonies.
Biofilms were stained with the Baclight live/dead viability kit. The biofilms were observed by epi-fluorescence microscopy. The substratum used was pyrex glass. This was conditioned with MCM medium and then treated with deionised water prior to culture flow. The bar represents 50 μm. The images are random fields of view from replicate biofilms.
Figure 8.3.2. shows representative images of MCM-grown microcosm biofilms formed on MCM-glass treated with olaflur/tin (IV) fluoride. The images were taken 12 hours after the start of biofilm formation. The biofilms consisted of distinct micro-colonies of varying size. On average the micro-colonies were smaller than in the MCM-grown biofilms in the control group (deionised water pre-treatment). The micro-colony in picture (C) was the largest on that particular disc. The diameter was approximately 163 µm (horizontal measurement).
Biofilms were stained with the BacLight live/dead viability kit. The biofilms were observed by epi-fluorescence microscopy. The substratum used was pyrex glass. This was conditioned with MCM medium and then treated with olaflur/tin (IV) fluoride (250 mg L\(^{-1}\) fluoride) prior to culture flow. The bar represents 50 µm. The images are random fields of view from replicate biofilms.
Figure 8.3.3. illustrates S-MCM-grown microcosm biofilms formed on MCM-glass pre-treated with deionised water. The images were taken 12 hours after the start of biofilm formation. The biofilms consisted of distinct micro-colonies of varying size. Compared to the MCM-grown biofilms, there was a greater proportion of the surface where micro-colonies were absent. The largest micro-colonies were slightly smaller in diameter than those in MCM-grown biofilms. However the micro-colonies were greater in height than MCM-grown biofilms. Hence small micro-colonies on the substratum were out of focus and only just visible when the focal plane was set on the top of a large micro-colony (figure 8.3.3. pictures A and B). This was not the case with MCM-grown biofilms (figure 8.3.1. pictures A and B). The micro-colonies did not appear to contain the void spaces that were observed in the micro-colonies of MCM-grown biofilms.
Figure 8.3.3. S-MCM-grown microcosm biofilms growing on surfaces pre-treated with deionised water (control)

Biofilms were stained with the Baclight live/dead viability kit. The biofilms were observed by epi-fluorescence microscopy. The substratum used was pyrex glass. This was conditioned with S-MCM medium and then treated with deionised water prior to culture flow. The bar represents 50 μm. The images are random fields of view from replicate biofilms.
Figure 8.3.4. illustrates S-MCM-grown microcosm biofilms formed on MCM-glass pre-treated with olaflur/tin (IV) fluoride combination. The images were taken 12 hours after the start of biofilm formation. The biofilms consisted of distinct micro-colonies of varying size. The size and frequency of the micro-colonies did not appear different to those in the control group biofilms.
Figure 8.3.4. S-MCM-grown microcosm biofilms growing on surfaces pretreated with olaflur/tin (IV) fluoride

Biofilms were stained with the Baclight live/dead viability kit. The biofilms were observed by epi-fluorescence microscopy. The substratum used was pyrex glass. This was conditioned with S-MCM medium and then treated with olaflur/tin (IV) fluoride (250 mg L\(^{-1}\) fluoride) prior to culture flow. The bar represents 50 µm. The images are random fields of view from replicate biofilms.
Figure 8.3.5. shows part of a biofilm grown in S-MCM. The pictures show a strand type structure which contained filamentous and spherical cell morphologies. This type of structure was not seen on any of the other biofilms used for microscopy.

Figure 8.3.5. Filamentous structure observed in an S-MCM-grown biofilm

Biofilms were stained with the Baclight live/dead viability kit. The biofilms were observed by epi-fluorescence microscopy. The substratum used was pyrex glass. This was conditioned with S-MCM medium and then treated with deionised water prior to culture flow. The bar represents 50 μm.
Table 8.3.1. lists the tests used to identify the genera of the predominant microcosm biofilm strains. There were five species/strains of bacteria that comprised the majority of the biofilm flora. One of these was identified as a *Staphylococcus* sp.. The other four species were found to be *Streptococcus* spp.
Table 8.3.1. Identification of the predominant microcosm biofilm bacterial strains.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gram stain</th>
<th>Catalase test</th>
<th>Oxidase test</th>
<th>Aerobic/Anaerobic growth</th>
<th>Growth on MS plates</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>colony type 1</td>
<td>+ve, grape-like clusters</td>
<td>+ve</td>
<td>-ve</td>
<td>both</td>
<td>-ve</td>
<td>Staphylococcus</td>
</tr>
<tr>
<td>colony type 2</td>
<td>+ve, chains and single cocci</td>
<td>-ve</td>
<td></td>
<td>both</td>
<td>+ve</td>
<td>Streptococcus</td>
</tr>
<tr>
<td>colony type 3</td>
<td>+ve, chains and single cocci</td>
<td>-ve</td>
<td></td>
<td>both</td>
<td>+ve</td>
<td>Streptococcus</td>
</tr>
<tr>
<td>colony type 4</td>
<td>+ve, chains and single cocci</td>
<td>-ve</td>
<td></td>
<td>both</td>
<td>+ve</td>
<td>Streptococcus</td>
</tr>
<tr>
<td>colony type 5</td>
<td>+ve, chains and single cocci</td>
<td>-ve</td>
<td></td>
<td>both</td>
<td>+ve</td>
<td>Streptococcus</td>
</tr>
</tbody>
</table>
Table 8.3.2. lists the mean concentrations of bacteria recovered from microcosm biofilms. The bacteria comprised almost entirely of streptococci and staphylococci. These bacteria grew on the aerobically incubated plates, the blood agar plates growing in the CO₂ enriched environment, and the fastidious anaerobe agar plates incubated anaerobically. The values in table 8.3.2. for total streptococci and staphylococci are the counts on the TSA plates. Other genera represented in the biofilm comprised less than 1% of the cultivable microflora. It was impossible to count these bacteria on plates with aliquots of lower dilution due to confluent growth of the staphylococci and streptococci. There was no growth on any of the Gram negative anaerobic agar plates. The most abundant genus in the biofilms was the *Streptococcus*. In 6 hour-old control MCM-grown biofilms 97% of the recoverable bacteria were streptococci of which 3% belonged to the mutans group. Staphylococci comprised 4% of the total flora recovered. In 6 hour-old MCM-grown biofilms formed on olaflur/tin fluoride-treated surfaces, 87% of the recoverable bacteria were streptococci of which 14% belonged to the mutans group. Staphylococci comprised 13% of the total flora recovered. In 12 hour-old control MCM-grown biofilms 86% of the recoverable bacteria were streptococci of which 8% belonged to the mutans group. Staphylococci comprised 7.8% of the total flora recovered. In 6 hour-old MCM-grown biofilms formed on olaflur/tin fluoride-treated surfaces, 50% of the recoverable bacteria were streptococci of which 40% belonged to the mutans group. Staphylococci comprised 50% of the total flora recovered. In the biofilms grown in MCM plus sucrose, The proportion of the recovered bacteria that were streptococci was always greater than 99%. The proportion
of the streptococci which belonged to the mutans group was always less than 1%. Staphylococci represented less than 1% of the flora in the biofilms.

Table 8.3.3. lists the viable counts of bacteria in the inocula used in the investigation. In the inoculum for the MCM-growth experiments, prior to biofilm formation, streptococci represented 53% of the flora. Twelve percent of the streptococci belonged to the mutans streptococci group. Staphylococci comprised 47% of the total flora.

In the inoculum for the MCM plus sucrose-growth experiments, prior to biofilm formation, streptococci represented 93% of the flora. Twelve percent of the streptococci belonged to the mutans streptococci group. Staphylococci comprised 7% of the total flora.
Table 8.3.2. Mean viable counts of bacteria recovered from microcosm biofilms grown in the CDFF

<table>
<thead>
<tr>
<th>test group</th>
<th>time</th>
<th>presence/absence of sucrose</th>
<th>staphylococci (CFU/biofilm)</th>
<th>streptococci (CFU/biofilm)</th>
<th>mutans streptococci (CFU/biofilm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6 hours</td>
<td>absent</td>
<td>$2.9 \pm 1.7 \times 10^3$</td>
<td>$6.3 \pm 8.1 \times 10^4$</td>
<td>$2.0 \pm 1.7 \times 10^3$</td>
</tr>
<tr>
<td>olaflur/tin (IV) fluoride</td>
<td>6 hours</td>
<td>absent</td>
<td>$5.3 \pm 0.9 \times 10^3$</td>
<td>$3.4 \pm 4.4 \times 10^4$</td>
<td>$5.5 \pm 4.5 \times 10^3$</td>
</tr>
<tr>
<td>Control</td>
<td>6 hours</td>
<td>present</td>
<td>$4.2 \pm 3.1 \times 10^1$</td>
<td>$1.1 \pm 0.8 \times 10^5$</td>
<td>$6.7 \pm 5.3 \times 10^2$</td>
</tr>
<tr>
<td>olaflur/tin (IV) fluoride</td>
<td>6 hours</td>
<td>present</td>
<td>$1.2 \pm 1.4 \times 10^2$</td>
<td>$1.8 \pm 1.7 \times 10^4$</td>
<td>$1.4 \pm 0.3 \times 10^2$</td>
</tr>
<tr>
<td>Control</td>
<td>12 hours</td>
<td>absent</td>
<td>$4.2 \pm 2.8 \times 10^5$</td>
<td>$2.6 \pm 3.3 \times 10^6$</td>
<td>$2.2 \pm 1.2 \times 10^5$</td>
</tr>
<tr>
<td>olaflur/tin (IV) fluoride</td>
<td>12 hours</td>
<td>absent</td>
<td>$1.5 \pm 0.9 \times 10^6$</td>
<td>$1.5 \pm 1.6 \times 10^6$</td>
<td>$9.8 \pm 9.4 \times 10^5$</td>
</tr>
<tr>
<td>Control</td>
<td>12 hours</td>
<td>present</td>
<td>$1.2 \pm 0.6 \times 10^2$</td>
<td>$3.6 \pm 1.2 \times 10^6$</td>
<td>$7.1 \pm 0.7 \times 10^2$</td>
</tr>
<tr>
<td>olaflur/tin (IV) fluoride</td>
<td>12 hours</td>
<td>present</td>
<td>$3.6 \pm 3.4 \times 10^2$</td>
<td>$2.4 \pm 1.0 \times 10^6$</td>
<td>$9.0 \pm 11.3 \times 10^3$</td>
</tr>
</tbody>
</table>

Olaflur/tin (IV) fluoride was adjusted to 250 mg L$^{-1}$ fluoride. The values for MCM-grown biofilms are the means of triplicate counts from three separate runs. The values for S-MCM-grown biofilms are the means of triplicate counts from two separate runs. All counts are CFU biofilm$^{-1}$. Standard deviations of the counts are shown.
Table 8.3.3. Mean viable counts of organisms recovered from pooled saliva samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>staphylococci (CFU/ml)</th>
<th>total streptococci (CFU/ml)</th>
<th>mutans streptococci (CFU/ml)</th>
<th>Gram negative anaerobes (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-experiment (MCM)</td>
<td>4.6 ± 5.5 x 10^7</td>
<td>5.1 ± 2.1 x 10^7</td>
<td>6.3 ± 1.3 x 10^6</td>
<td>1.1 ± 0.5 x 10^6</td>
</tr>
<tr>
<td>Post experiment (MCM)</td>
<td>3.4 ± 1.7 x 10^8</td>
<td>3.2 ± 2.5 x 10^8</td>
<td>3.0 ± 2.0 x 10^8</td>
<td>4.2 ± 4.2 x 10^5</td>
</tr>
<tr>
<td>Pre-experiment (S-MCM)</td>
<td>5.8 ± 5.3 x 10^5</td>
<td>7.9 ± 2.9 x 10^6</td>
<td>9.7 ± 0.9 x 10^5</td>
<td>1.7 ± 1.8 x 10^5</td>
</tr>
<tr>
<td>Post experiment (S-MCM)</td>
<td>9.8 ± 14.0 x 10^9</td>
<td>1.8 ± 2.5 x 10^9</td>
<td>3.2 ± 4.3 x 10^8</td>
<td>1.3 ± 1.5 x 10^6</td>
</tr>
</tbody>
</table>

The standard deviations of means from replicate runs are shown. Three separate counts were performed on the inoculum used to grow MCM-biofilms. Two counts were performed on the inoculum used to grow S-MCM-biofilms. All counts are the mean CFU ml⁻¹.
8.4. Discussion

This study attempted to investigate the effect of surface treatment with olaflur/tin (IV) fluoride on the formation of microcosm biofilms. Biofilm formation after 6 and 12 hours in MCM and S-MCM growth medium was investigated. The biofilms were analysed by viable count and micrography of stained specimens.

The dominant genus isolated in all our biofilms was Streptococcus. The majority of the streptococci were non-mutans streptococci. The genus that comprised the second highest proportion of bacteria isolated from the biofilms and inocula was Staphylococcus. Staphylococci are known to inhabit saliva and plaque in the human oral cavity. In published work, values for the proportions of staphylococci in the recoverable salivary flora are 0.7% (>3 x 10^4 CFU ml⁻¹) (Hase et al 1998), and 0.08% (>1000 CFU ml⁻¹). The numbers and proportions of staphylococci in the saliva samples used in this study were far higher. In the first pooled saliva sample, staphylococci represented 47% (4.6 ± 5.5 x 10⁷ CFU ml) of the recoverable microflora. In the second sample of pooled saliva sample, staphylococci comprised 6.84% (5.8 x ± 5.3 10⁶ CFU ml) of the recoverable microflora. The cause of the elevated staphylococcal concentrations was not determined. It may have been due to infection of the nasal-pharyngeal tract in one of the subjects from which the saliva was collected. In a clinical study by Emilson & Fornell, 1976, the staphylococcal concentration in saliva collected from 6 individuals was determined. One individual had complained of a sore throat at the time of sample collection. Although the actual figures were not quoted the workers noted that the salivary staphylococcal concentration of the individual was elevated
compared to the other subjects. A lower concentration of staphylococci in the second collection of saliva may have been due to infection during the first sample collection. The two samples of saliva were collected more than two weeks apart.

In the clinical trial of Marsh et al 1992, the number of viable staphylococci recovered from the plaque was <0.2% of total cultivable bacteria. In this study the mean proportion of staphylococci was less than 0.2% in 3 out of the 8 total sets of biofilms. The proportion of cultivable flora comprising staphylococci was below 15% in 7 of the 8 sets of biofilms. In the set of MCM-grown biofilms analysed after 12 hours formation on olaflur/tin fluoride-treated surfaces, the proportion of staphylococci reached 50% of the recoverable flora. The high number of staphylococci in some of the CDFF-biofilms may have been due to the absence of factors that control staphylococci in the oral environment. Oral streptococci have been shown to produce bacteriocin-like substances that are able to inhibit growth of staphylococci (Tzannetis et al 1991). Staphylococci from oral samples were inhibited less than isolates from nasal samples. In addition one of the oral strains was not inhibited by the bacteriocin-like substances. The relatively high staphylococcal concentrations in our biofilms may have been due to our sample containing a strain that was inhibited poorly or not at all by such substances. Other staphylococci-controlling factors may have been absent in our model and allowed for establishment of large numbers in some of the biofilms.

In the study we were unable to show any significant difference in the viable counts of control and test group biofilms grown in the same medium. We were also unable to show significant differences in the viable counts of
bacteria in control and test group biofilms after 6 and 12 hours. This was
despite the fact that there was a unilateral increase in the mean count from 6
to 12 hours irrespective of experimental group. This pattern of increase would
suggest that the number of bacteria in the biofilms was increasing but the
viable count procedure was not sensitive enough to measure it. The reason
for the poor sensitivity of measurements was the high variability in the viable
counts of the biofilms. The standard deviations for the counts were frequently
equal to, or greater than, the mean CFU value.

The reason for the high variability in the counts, and hence poor sensitivity of
the procedure, is likely to be a feature of the nature of bacteria in saliva. In
the saliva sample most of the bacteria are derived from biofilms. The mode by
which bacteria may end up in saliva varies. Daughter cells may be shed by
the biofilm as a method of colonising new areas. Alternatively, parts of the
biofilm may end up in the saliva due to erosion and sloughing of biofilms.

Bacteria may be present in saliva attached to shed human epithelial cells
(Frandsen et al 1991). The size and composition of bacterial units in saliva
will be a function of the different mechanisms by which they came to inhabit
the suspended phase. Co-aggregation in saliva may serve to increase the
diversity of bacterial units further. Saliva can thus be thought of as a
heterogenous suspension of bacteria in varying sized units with a variety of
different flora compositions. This is highlighted by the variability of viable
counts performed on the saliva inocula used in our experiments. In 6 out of
20 groups of replicate counts the standard deviations were larger than the
mean viable counts.
If the saliva contains a range of bacterial aggregates, it is likely that the biofilms grown from such a mixture will reflect its heterogeneity. The bacteria adhering to each substratum piece may be unique in their variety, growth rate and proportions of different species. This will obviously affect the biofilm forming processes of adhesion, co-adhesion and growth. During the early stages of biofilm formation the differences may be pronounced and cause the variation observed in the viable counts. An excellent example of the variation which may be found in the biofilms is shown in figure 8.3.5. This biofilm contained a large filamentous structure. The structure was unique to that particular biofilm. The sensitivity of the viable count procedure could probably have been improved by homogenising the saliva inoculum. This would provide a more homogenous inoculum in terms of unit size, spread of bacterial species and growth rate. However this is less like the situation in vivo and is perhaps not of any real utility.

This study investigated the structure of intact biofilms by fluorescent microscopy. Biofilms were stained with a BacLight live/dead viability kit. The biofilms were observed at low power (x10 objective) and representative images of biofilms stored digitally. Marked differences in the MCM-grown control and agent group biofilm structures were observed. The biofilms of both groups consisted of distinct micro-colonies with channels within the units. However micro-colonies on surfaces treated with olaflur/tin (IV) fluoride prior to biofilm formation did not reach the sizes of the largest micro-colonies in control biofilms. In the control biofilms a far greater proportion of the substratum was covered with micro-colonies than in the test group biofilms. It is not clear which property(ies) of the agent combination were responsible for
this change. It is probable that the different properties of the agents acted
synergistically. The anti-adhesion properties of the tin (IV) ion may have
prevented or decreased the adhesion of bacteria. Alternatively, the amine
ions in the agent combination may have killed primary colonisers of the
surface. Both properties, if occurring, would be lost over a period of time.
Thus the biofilms will eventually form but only after a lag period. Hence the
level to which the biofilm had formed after 12 hours was different on the
control and agent treated surfaces.
The reduction in biomass and micro-colony size is important in relation to
dental caries. Smaller units of biofilm are controlled more efficiently by
salivary buffers than thicker biofilms. Additionally, treatment of smaller units
with chemical agents will be more efficient as factors such as diffusion
limitation and sequestration by the outer biofilm will be less pronounced.
Biofilms grown in S-MCM consisted of large distinct micro-colonies which
extended further away from the surface than those in MCM-grown biofilms.
The micro-colonies appeared smooth and without water channels. This was
presumably due to the production of exopolymers. In the S-MCM-grown
biofilms, there was no structural differences between control and test groups.
It is not clear how the addition of sucrose to the growth medium decreased
the activity of the agent. The exopolymers formed by sucrose metabolism
may have quenched or masked the amine and tin ions. Alternatively, the
specific growth rate and/or co-adhesion frequency may have been elevated in
the presence of sucrose. This may have reduced the differences in biofilm
structure if a stationary phase of biofilm development is approached around
the 12 hour period.
In clinical trials, viable count and plaque index scoring procedures are frequently used to determine the effect of antimicrobial agents on supragingival plaque formation. Viable count procedures may be employed which all measure the level of colony-forming units either in the plaque itself or in saliva. In addition to CFU measurements, plaque is often assessed in terms of accumulation. One such method of measuring plaque build-up is known as the plaque index. There are many variations on this scoring system although they are all similar to the procedure of Silness & Loe, 1964. Essentially, the system involves scoring the level of plaque accumulation on tooth surfaces. The scale includes zero values where plaque is absent, low values where plaque is only visible by staining, and high values where the plaque is clearly visible and can be removed in thick pieces. The use of plaque index scoring may be thought of as being analogous to the fluorescent microscopy used in our investigation. In both procedures the amount of biofilm is simply compared in control and test groups by visualisation. The only difference is that the observations in this study were made at a magnification 100 fold greater than that used in plaque index scores.

In this study, different conclusions could be drawn on the anti-plaque activity of amine/tin (IV) fluorides depending on whether viable counts or fluorescence microscopy were used to analyse biofilms. As the microscopy technique is similar to plaque index scoring it is interesting to compare the measured anti-plaque activity in clinical trials when viable counts and plaque indices were used. Netuschil et al, 1995, used both plaque indices and a viable count procedure to investigate the effect of amine/tin fluoride mouth-rinsing on dental plaque over a three day period. The viable counts were
performed on samples of the plaque. They found a significant difference in viable counts of viable bacteria per mm$^2$ of enamel between subjects using control mouthwashes and amine/tin fluoride mouthwash. In their study they also found large differences in the plaque index scores between the test and control groups. In the clinical trial of Hefti & Huber, 1987, equivocal but different results were obtained. They investigated the effect of amine/tin fluoride in a mouthwash over a seven day period. They measured the viable counts of total salivary bacteria and *S. mutans*. The concentration of bacteria in the saliva would obviously be a function of the plaque accumulation in the oral cavity. They also evaluated plaque build up by analysing images of stained teeth. Although they referred to this procedure as planimetric analysis, the procedure was essentially the same as that of plaque index scoring. In their investigation they were unable to show a statistically significant difference in viable counts between control and test groups. However they were able to show a statistical difference by planimetric analysis. The results of these clinical trials suggest that viable count procedures can be a useful method of measuring the effect of amine/tin (IV) fluoride on dental plaque formation. However visual assessment of plaque formation is probably a more sensitive method for detecting anti-plaque activity of the amine/tin fluoride combination. In this study, the viable count procedures were not sensitive enough to demonstrate activity of the agents. However, differences in biofilm structure were observed by a direct, visual technique which was analogous to plaque index measurements.

The use of fluorescence microscopy could have been extended by collecting a greater quantity of images and measuring biofilm features, e.g., microcolony
size, percentage of surface covered by bacteria. However it is notable that these measurements would only be two dimensional. Biofilm microcolony size could be measured using the microscope focal scale by the procedures described in chapter 4. Dry weight measurements could be used to validate these measurements.

Summary:

- Viable count procedures were subject to large variation when used for microcosm biofilms
- Differences in the structure of biofilms forming on surfaces treated with olaflur/tin (IV) fluoride and deionised water were observed when biofilms were grown in MCM medium
- No differences in the structure of biofilms forming on surfaces treated with olaflur/tin (IV) fluoride and deionised water were observed when biofilms were grown in S-MCM medium
Chapter 9 - Amine/tin fluorides and adhesion processes involving

*Streptococcus gordonii* and *Actinomyces naeslundii.*
9.1. Aims

The study investigated the effect of an amine/tin fluoride combination on biofilm-forming processes using the flow cell model. The aim was to establish the effect of the agents on biofilm-forming processes in a dual species system. The species used for the study were *Streptococcus gordonii* and *Actinomyces naeslundii*. The effect of the agents on the adhesion, desorption and co-adhesion of these species was investigated.
9.2. Materials and Methods

9.2.1. General equipment

The flow cell system used to study the effect of amine/tin fluoride on adhesion processes is described in section 2.8.. The strains of *S. gordonii* and *A. naeslundii* used in the study are described in section 2.1.3.. Cultures were grown in GPT devices (refer to section 2.6.). Separate GPT devices were used to culture each species. The pH and bacterial concentration of GPT cultures were determined before the initial, and after the final, use for flow cell work (refer to sections 2.7.1. and 2.7.5.). MCM medium was used for pellicle formation and growing of GPT cultures. MCM salts was used for all rinse procedures (refer to section 2.2. for details of media).

9.2.2 Antimicrobial agents used in the study

The anti-plaque agent used in the study was olaflur/tin (IV) fluoride combination. This was adjusted to 250 mg litre\(^{-1}\) fluoride. Other than the active agents, preparations contained (per litre) 2 g of polyethylene glycol-hydrogenated castor oil (GABA International), 50 g of ethanol (BDH), 0.25 g potassium acesulfame (Hoechst, Frankfurt, Germany), and 25 g of xylitol (Sigma). Deionised water was used as the negative control.
9.2.3. Experimental protocol

The equipment set-up for the investigation is shown in figure 9.2.3.

The following protocol was used in all determinations:

1. MCM salts solution was pumped through the system at maximum pump flow rate.
2. Three ml of MCM from the tube prior to the flow cell was pumped through the cell at a flow rate of 0.91 ml minute\(^{-1}\) (shear rate=142 s\(^{-1}\)).
3. MCM salts was pumped through the system at maximum flow rate for five minutes to remove unattached MCM.
4. *S. gordonii* GPT culture was pumped through the system at maximum flow rate until it exited the flow cell.
5. The flow rate was then adjusted to 0.05 ml minute\(^{-1}\) (shear rate=7.8 s\(^{-1}\)).
6. The pump was switched to the MCM-salts supply so that MCM-salts followed the culture through the system.
7. After one hour of culture flow, the flow was increased to 0.46 ml minute\(^{-1}\) (shear rate=72 s\(^{-1}\)) for ten minutes.
8. The flow rate of MCM salts was increased to 0.91 ml minute\(^{-1}\) (shear rate=142 s\(^{-1}\)) for a further fifty minutes.
9. During the period of MCM salts flow, the syringe line was filled with olafur/tin (IV) fluoride (or deionised water for control runs).
10. The flow was switched from the MCM salts supply to the syringe line and the agent was pumped at maximum flow rate towards the flow cell.
11. Just before the agent started to enter the flow cell, the pump was switched back to the MCM salts supply.
12. The agent, followed by MCM salts, was pumped through the flow cell at a flow rate of 0.91 ml minute\(^{-1}\) (shear rate=142 s\(^{-1}\)) for thirty minutes.

13. During the period of MCM salts flow, images of the bottom surface of the flow cell were taken (refer to section 2.7.4. for method). The positions of fields of view were recorded.

14. During MCM salts flow, the syringe line was filled with A. naeslundii culture freshly-collected from the exit flow of the GPT device.

15. The pump was switched from the MCM salts supply to the syringe line and the culture was pumped towards the flow cell at maximum flow rate.

16. When the culture reached the flow cell the flow rate was turned down to 0.05 ml minute\(^{-1}\).

17. The pump was switched from the syringe line to the MCM salts supply so that MCM salts would pass through the flow cell after the A. naeslundii culture.

18. The A. naeslundii culture was allowed to flow for one hour at a rate of 0.05 ml minute\(^{-1}\) (shear rate=7.8 s\(^{-1}\)).

19. The flow rate was increased to 0.46 ml minute\(^{-1}\) (shear rate=72 s\(^{-1}\)) for ten minutes.

20. After the ten minute period the flow rate was increased to 0.91 ml minute\(^{-1}\) (shear rate=142 s\(^{-1}\)) for a further twenty minutes.

21. Images of the bottom surface of the flow cell were taken as before. The positions of images collected were the same as for the images taken after the period of S. gordonii culture flow.
The GPT device shown in the diagram was for culturing *S. gordonii*. The GPT device used for culturing *A. naeslundii* was not attached to the flow cell system and is omitted for clarity. The GPT devices were incubated at 37°C aerobically. The rest of the system was operated at room temperature. Only one flow cell was used per run. The other cell was clamped off.
9.2.4. Statistical analysis of results

The Kruskal-Wallis test was used to measure the significance of differences in the adhesion of *S. gordonii* between the control and test groups. Provided the groups were not significantly different, the same test was used to determine the significance of differences in values for adhesion processes involving *A. naeslundii*. These processes were the direct adhesion of *A. naeslundii* to the surface and co-adhesion with *S. gordonii*. 
9.3. Results

Table 9.3.1. lists the mean concentrations of the GPT bacterial cultures used in the study. The mean concentration of the *S. gordonii* culture was $1.4 \pm 0.5 \times 10^7$ CFU ml$^{-1}$. The mean concentration of *A. naeslundii* culture was $1.5 \pm 0.8 \times 10^9$ CFU ml$^{-1}$.

Table 9.3.1. Mean concentration of *A. naeslundii* and *S. gordonii* GPT cultures

<table>
<thead>
<tr>
<th>bacterial species</th>
<th>mean count</th>
<th>number of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. gordonii</em></td>
<td>$1.4 \pm 0.5 \times 10^7$</td>
<td>2</td>
</tr>
<tr>
<td><em>A. naeslundii</em></td>
<td>$1.5 \pm 0.8 \times 10^9$</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 9.3.1. shows *S. gordonii* cells adhered to the surface after treatment with deionised water (1A) and olaflur/tin (IV) fluoride (2A). The images shown for each group are those with the median value for percentage surface coverage (PSC). Images (1B) and (2B) show the same areas after one hour of *A. naeslundii* flow. The actinomyces adhered directly to the surface and co-adhered with the *S. gordonii* cells.
Figure 9.3.1. Adhesion and co-adhesion of *S. gordonii* and *A. naeslundii*.

(1A) Adhesion of *S. gordonii* after one hour of culture flow, and, treatment with deionised water. (1B) Adhesion and co-adhesion of *A. naeslundii* to the same surface after one hour of *A. naeslundii* flow. (2A) Adhesion of *S. gordonii* after one hour of culture flow and treatment with olaflur/tin (IV) fluoride. (2B) Adhesion and co-adhesion of *A. naeslundii* to the same surface after one hour of *A. naeslundii* flow. Figure (1A) and (2A) are the images from the control and test group series with the median values for PSC by *S. gordonii*. The olaflur/tin (IV) fluoride combination was adjusted to 250 mg litre$^{-1}$ fluoride. Bar = 10 μm.
Figure 9.3.2. illustrates the relationship between the PSC by *S. gordonii* and the PSC by *A. naeslundii*. The PSC by *A. naeslundii* refers to co-adhering and independently adhering actinomyces. As surface coverage by *S. gordonii* increased the PSC by *A. naeslundii* decreased. The proportions of independently adhered and co-adhered *A. naeslundii* are shown in figure 9.3.3.

In both the control and olaflur/tin (IV) fluoride groups the total independent adhesion was greater than the total co-adhesion. In the control group, 75% of the actinomyces adhered independently of *S. gordonii*. In the olaflur/tin (IV) fluoride group 67% of the actinomyces adhered independently of *S. gordonii*. 


Figure 9.3.2. PSC by *A. naeslundii* in relation to PSC by *S. gordonii*.

XY scatter of PSC by *A. naeslundii* adhered to the surface against PSC by *S. gordonii*. Each point represents the PSC in one image. The olaflur/tin (IV) fluoride combination was adjusted to 250 mg litre\(^{-1}\) fluoride. The experiment was performed three times for the control group and twice for the olaflur/tin (IV) fluoride test group. PSC by *A. naeslundii* is the sum of independent adhesion and co-adhesion with *S. gordonii*. Exponential trendlines for the relationships are shown.
Figure 9.3.3. Measurement of different processes for A. naeslundii adhering to surfaces with adherent S. gordonii.

The pie charts show the proportions of the total actinomyces adhering independently and co-adhering with S. gordonii. The values are the means for percentage surface coverage for each group. The experiment was performed three times for the control series and twice for the olaflur/tin fluoride combination. The olaflur/tin (IV) fluoride combination was adjusted to 250 mg litre\(^{-1}\) fluoride.
The balance between independent adhesion and co-adhesion was related to the PSC with *S. gordonii*. The greater the proportion of surface occupied by *S. gordonii*, the greater the co-adhered percentage of total *A. naeslundii* present (figure 9.3.4.). The correlation coefficients for this relationship are presented in table 9.3.2.. There was a positive correlation coefficient for the control group (deionised water treatment) of 0.82. The correlation coefficient for the test group in which *S. gordonii* cells were treated with olaflur/tin (IV) fluoride was 0.78.

**Table 9.3.2.** Correlation coefficients for adhered *S. gordonii* and percentage of total *A. naeslundii* co-adhering.

<table>
<thead>
<tr>
<th>test group</th>
<th>correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.82</td>
</tr>
<tr>
<td>olaflur/tin(IV) fluoride</td>
<td>0.78</td>
</tr>
</tbody>
</table>
Figure 9.3.4. The relationship between *S. gordonii* PSC and the percentage of total *A. naeslundii* co-adhering

XY scatter illustrating the relationship between *S. gordonii* PSC and the percentage of actinomyces co-adhering. The olaflur/tin (IV) fluoride combination was adjusted to 250 mg litre$^{-1}$. 
Figure 9.3.5. illustrates the adhesion and co-adhesion measurements for *S. gordonii* and *A. naeslundii*. The mean PSC by *S. gordonii* after the cells were treated with the control solution (deionised water) was 1.5 ± 1.1% n=13. The mean PSC by *S. gordonii* after adhered cells were treated with olaflur/tin(IV) fluoride was 1.0 ± 0.7% n=10. The difference in *S. gordonii* adherence after treatment with deionised water and tin (IV) fluoride was not statistically significant (p>0.01). The mean PSC by the total *A. naeslundii* (adhered plus co-adhered) was 1.11 ± 0.70% (n=13) for the control group (deionised water). The mean PSC by the total *A. naeslundii* on olaflur/tin(IV) fluoride treated surfaces was 0.61 ± 0.36% (n=10). The PSC by *A. naeslundii* at the interface was not significantly different (p>0.01) between the control and test group.

The mean PSC by independently adhering (PSC-I) *A. naeslundii* was 0.83 ± 0.78% (n=13) on surfaces treated with the control (deionised water). The mean PSC-I by *A. naeslundii* on olaflur/tin(IV) fluoride treated surfaces was 0.41 ± 0.22% (n=10). The independent adhesion (PSC-I) of *A. naeslundii* to the surface was not significantly different (p>0.01) between the control and test group.

The mean PSC by co-adhered (PSC-C) *A. naeslundii* was 0.28 ± 0.19% (n=13) on surfaces treated with deionised water. The mean PSC-C by *A. naeslundii* on olaflur/tin(IV) fluoride treated surfaces was 0.21 ± 0.15% (n=10). There was no significant difference (p>0.01) in the level of *A. naeslundii*/*S. gordonii* co-adhesion between the control and test group.
Figure 9.3.5. Measurements of adhesion and co-adhesion processes involving *S. gordonii* and *A. naeslundii*.

*S. gordonii* was allowed to adhere for one hour. The surface and adhered cells were treated with either deionised water (control) or olaflur/tin (IV) fluoride combination. Measurement of PSC by the bacteria was then made. *A. naeslundii* was then allowed to adhere/co-adhere for a period of one hour. Measurements were remade of the same areas as before to determine the level of *A. naeslundii* adhesion/co-adhesion. The olaflur/tin (IV) fluoride combination was adjusted to 250 mg litre\(^{-1}\) fluoride. The experiment was performed three times for the deionised water control group and twice for the olaflur/tin (IV) fluoride agent group.
9.4. Discussion

Co-aggregation/co-adhesion between bacterial species has been suggested to be an important factor in the formation of microbial biofilms (Kinder & Holt 1994). The use of the terms co-aggregation and co-adhesion varies between research groups and the date of publication of the work. For the purpose of this discussion, the terms will be used according to the definition of Busscher & Van der Mei 1997. Principally, co-aggregation is the interaction between two different bacterial species/strains when suspended in the liquid phase. Co-adhesion is the interaction between a micro-organism adhered to the surface and a different species/strain suspended in the liquid phase.

Streptococci and actinomyces are among the predominant primary colonisers of the human oral cavity. Nyvad & Kilian, 1987, examined the microflora of plaque, forming on enamel test pieces inserted in the mouths of four subjects. After four hours of plaque formation, a mean of 77% of the recoverable microflora comprised of streptococci. The mean proportion of recoverable actinomyces in four hour-old plaque was 5%. There has been extensive research on the co-aggregation of the two genera. S. gordonii has been shown to posses multiple adhesins that are able to mediate adhesion to A. naeslundii (Whittaker et al 1996, Jenkinson & Lamont 1997). SspA is a 210 kDa polypeptide expressed on the surface of S. gordonii that belongs to the Antigen I/II family of streptococcal surface polypeptides (Jenkinson et al 1993). With the aid specially-constructed mutants Jenkinson et al, 1993, showed that the polypeptide mediates adhesion to A. naeslundii. CshA is
another surface polypeptide that binds actinomycoses (McNab et al 1999). The 259 kDa polypeptide is present on the cell surface in fibril structures. Another surface component of *S. gordonii* cells, a 34.7 kDa surface lipoprotein termed ScaA, has also been shown to mediate binding to *A. naeslundii* (Whittaker et al 1996). Research on the receptors of *A. naeslundii* that bind the streptococcal adhesins has received less attention. However, *A. naeslundii* is known to possess two distinct types of fimbriae (type I and type II) on the cell surface. Both types of fimbriae consist of subunits approximately 60 kDa in size. Type II fimbriae are thought to be responsible for lactose inhibitable binding to oral streptococci (Whittaker et al 1996).

In our study we showed that *S. gordonii* adhered to pellicle-coated glass was able to co-adhere with *A. naeslundii*. The co-adhesion of the two species occurred in a continuous flow at a shear rate of 7.8 s⁻¹. *A. naeslundii* was also able to adhere to the surface independently of *S. gordonii* cells.

The total number of adhering actinomycoses was related to the PSC by *S. gordonii*. The PSC by *A. naeslundii* decreased as PSC by streptococci increased. Surfaces on which the PSC by *S. gordonii* was below 1% generally had the highest levels of PSC by *A. naeslundii*. The balance between independent adhesion and co-adhesion of *A. naeslundii* was also dependent on the concentration of *S. gordonii* adhered to the test surfaces. As the percentage surface coverage by *S. gordonii* cells increased so did the percentage of the total *A. naeslundii* that co-adhered. In this study, co-adhesion became dominant over independent adhesion when the percentage of surface area covered by *S. gordonii* cells was greater than 2 percent. Thus in the formation of the dual species biofilms in the flow cell, co-adhesion was
not the dominant force in early biofilm formation. This result is consistent with measurements made in vivo (Skopek et al 1993). Skopek et al studied the co-adhesion of oral bacteria to streptococci adhered to enamel chips. After four hours, the concentration of *A. naeslundii* on surfaces covered with a non co-aggregating *S. gordonii* strain was not significantly different from the concentration on surfaces covered with a co-aggregating strain of *S. oralis*. The decrease in the total number of actinomyces adhering to the surface as the percentage surface coverage by *S. gordonii* increased was primarily due to a decrease in the independent adhesion. At the levels of coverage by *S. gordonii* where this trend was observed, the majority of the surface was still available for adhesion by the actinomyces. Thus the adhered *S. gordonii* cells must be influencing adhesion of *A. naeslundii* directly to the surface. There are physical and microbiological factors that may explain the decline in *A. naeslundii* adhesion when surface is still available. From a physical standpoint, the action of the adhered streptococcal cells on the hydrodynamics of the flow local to the surface may be important. As the flow reaches the posterior (relative to direction of flow) area of adhered bacteria eddies in the flow may occur. Such turbulence may reduce the time dependent adhesion of actinomyces. From a microbiological standpoint, the reduction in *A. naeslundii* adhesion to the surface as *S. gordonii* surface coverage increased may have been enhanced by competition for a receptor in the pellicle. Both *S. gordonii* (Gibbons et al 1991) and *A. naeslundii* (Stromberg et al 1992) have been shown to possess adhesins for proline-rich proteins (PRP’s) in pellicles on hydroxyapatite. However, the mucin-containing medium used for preparation of the pellicle in this study contained
only a low concentration of PRP’s and so this factor is unlikely to be important. Nevertheless, it does show that competition for receptors may be a contributing factor. Oral bacteria are known to possess multiple adhesins and it is quite possible that the adhesins involved in competition may not yet have been identified. The MCM medium used contained a high concentration of high molecular weight glycoprotein (hog-gastric mucin-HGM). *S. gordonii* has been shown to bind salivary agglutinin, a high molecular weight glycoprotein (Duan et al 1994) not dissimilar to HGM. It is not known whether the strain of *A. naeslundii* used is able to bind high molecular weight glycoproteins. If it is, the mucin in the pellicle would be an obvious choice as a receptor for competitive adhesion.

The reduction in actinomyces adhesion when the concentration of adhered *S. gordonii* increased could possible be due to cell-cell signalling. Gram positive bacteria have been shown to produce pheromones that are able to stimulate responses in other species of bacteria. For example, *S. aureus* produces a peptide that induces the expression of plasmid borne *Enterococcus faecalis* adhesin (Muscholl-Silberhorn et al 1997). This results in the clumping of *E. faecalis* cells. Although speculative, it is possible that *S. gordonii* cells may produce a pheremone that is able to control the adhesion of *A. naeslundii*.

A possible mode of action for anti-plaque agents is the ability of the agent to cause desorption of adhered cells/biofilms. One of the few studies to investigate such properties used a flow cell model similar to the one used in the present study. Rundegren et al, 1992, observed a decrease in the cohesion of glucan-containing plaque when pulsed with delmopinol. In this study flow cells were used to investigate whether an olaflur/tin (IV) fluoride
combination possessed an activity able to cause desorption of *S. gordonii* cells. The agents did not cause a significant removal of cells when compared to treatment with deionised water. However, they may have affected the viability of adherent cells although this was not determined.

The study also investigated the ability of the olaflur/tin (IV) fluoride combination to prevent the attachment of *A. naeslundii* to the surface. Two different attachment processes were measured. Firstly, the independent adhesion of *A. naeslundii* following treatment of the surface with olaflur/tin (IV) fluoride was investigated. There was a reduction in the mean adhesion of *A. naeslundii* to surfaces treated with the test agent, although this was not statistically significant. The second attachment strategy investigated was co-adhesion of *A. naeslundii* to *S. gordonii* adhered to the surface. The effect on co-adhesion of treating the adhered *S. gordonii* cells with olaflur/tin (IV) fluoride was measured but it was found that the agent had no significant effect on the level of co-adhesion between the two species.

The relationships between *S. gordonii* surface coverage and the adhesion of *A. naeslundii* (independent adhesion and co-adhesion with *S. gordonii*) indicate that a reproducible experimental system has been developed. In vitro models systems have previously been used for studying the effects of anti-plaque agents on oral bacterial co-aggregation (Smith et al 1991, Fine et al 1996). Both models involved mixing planktonic cultures of different bacterial genera in the presence and absence of anti-plaque agents. Co-aggregation was measured by scoring the change in turbidity of the mixtures. Smith et al also measured the level of co-aggregation by a radioactive assay.
The model developed in this study was designed to measure co-adhesion rather than co-aggregation. Co-adhesion was investigated under controlled conditions of hydrodynamic shear. The use of video capturing techniques allowed data to be stored rapidly for subsequent analysis.

**Summary:**

- Total adhesion of *A. naeslundii* at the interface decreased as the coverage of the surface by *S. gordonii* increased
- In total, independent adhesion of *A. naeslundii* was dominant over co-adhesion with *S. gordonii*
- Olaflur/tin (IV) fluoride did not cause desorption of *S. gordonii* cells
- Olaflur/tin (IV) fluoride did not reduce net *A. naeslundii* adhesion directly to the surface after one hour of culture flow
- Olaflur/tin (IV) fluoride did not reduce co-adhesion of *S. gordonii* and *A. naeslundii*
Chapter 10 - Final Discussion
10.1 Aims of study

The main purpose of this study was to investigate the antibacterial and anti-plaque properties of amine and tin fluorides. AmFs have been reported to exhibit bactericidal activity towards oral bacteria \textit{in vitro} (Kay & Wilson 1988) and prevent adhesion of bacteria in closed systems \textit{in vitro} (Bapna et al 1988). Tin fluorides have been reported to possess bacteriostatic activity towards oral bacteria \textit{in vitro} (Zameck & Tinanoff 1987), and, anti-adhesion activity in closed systems (Gross et al 1977, Ota et al 1989). In comparison to the strong activities of the agents reported \textit{in vitro}, the activities of the agents in clinical trials have been disappointing. Bactericidal activity has been reported \textit{in vivo} (Weiger et al 1998), however the level of activity was greatly reduced compared to that exhibited \textit{in vitro}. There are no reports of the agents exhibiting a beneficial anti-adhesion activity \textit{in vivo}.

The laboratory models used in published work to test the antimicrobial properties of amine and tin fluorides have relied on traditional techniques that do not take in to account environmental influence on the activity of the chemicals. In this study, the main aims were to investigate antimicrobial properties using systems that were designed to model specific aspects of bacterial ecology in the oral cavity. For bactericidal studies, the influence of sucrose and growth mode were of primary concern. For anti-adhesion studies, the effect of conditioning films and a continuous flow were investigated.
Besides killing of bacteria, and the prevention of bacterial adhesion, the agents may possess other anti-plaque activities such as the ability to inhibit co-adhesion and to effect desorption of adherent bacteria. In the final part of this study the agents were tested to determine if they possessed such properties.

10.2. Bactericidal activity of AmFs

The antagonistic action of AmFs towards oral bacteria was recorded as early as 1958 (Hermann & Muhlemann 1958). In more recent publications, a strong \textit{in vitro} activity has been reported (Kay & Wilson 1988, Oosterwaal et al 1989). In the study of Kay & Wilson, 1988, the minimum bactericidal concentration (MBC) of three different AmFs was determined for 40 strains of oral bacteria including \textit{Streptococcus} spp., \textit{Actinomyces} spp., and \textit{Bacteroides} spp.. The concentration of AmF required to kill bacteria depended on the bacterial strain and which amine was used. Concentrations of 55 mg L$^{-1}$ and upwards were reported to be bactericidal. Approximately 85% of tested bacteria were killed by AmF concentrations of 250 mg L$^{-1}$. All tested bacteria were killed by an AmF concentration of 1440 mg L$^{-1}$. The MBC of AmF towards oral bacteria in the study of Oosterwaal et al, 1989, was within the range reported by Kay & Wilson, 1988. The values quoted for MBCs in the two studies were for 100% kills.

Clinical trials have been used to investigate the bactericidal activity of AmF solutions towards bacteria in supra-gingival plaque. The clinical trials frequently involve subjects rinsing twice daily with mouthwashes for a pre-determined period of time. Netuschil et al, 1995, monitored the bactericidal
activity of an AmF toward supra-gingival plaque bacteria over a period of 3 days. Subjects rinsed twice daily, for one minute, with a commercial AmF mouthrinse, Meridol. The proportion of micro-organisms in supra-gingival plaque that were viable was determined by vital fluorescence microscopy. The percentage vitality of plaque bacteria was 48.2% after three days of AmF rinsing, compared to 91.8% in the control group. In a similar trial using the same agent formulation and delivery regime, the vitality of plaque bacteria was measured over a period of twenty one days (Brecx et al 1990). The percentage vitality of plaque bacteria was between 67% and 70% for both control and agent groups prior to the trial. In the control group, the percentage vitality did not change over the period of the trial. In the AmF test group, the percentage vitality was reduced to 54% on day one of the trial. The percentage vitality then increased over the period of the trial and was 64% on day 21. In a clinical trial by Riep et al, 1999, the effect of the Meridol AmF preparation on supra-gingival plaque was assessed by determining plaque indices (refer to chapter 8 for explanation of procedure). Over a 4-day period, subjects rinsed twice daily for one minute with Meridol. Although the score for the test group was slightly lower than the control group, the mean plaque index for control and agent groups were not statistically different. Plaque index scoring is not solely a measurement of bactericidal activity but the combined effects of all anti-plaque activities. Even so, the small reduction in plaque index does imply that the bactericidal efficacy was poor.

Comparison of the bactericidal activity of AmFs in clinical trials, and the bactericidal activity of the agents in vitro, is difficult. Subject compliance, variations in the AmF concentration, duration of treatment times, and time
course of trials, may all affect the level of bactericidal activity measured. In addition to these problems, clinical trials frequently use a commercial AmF containing preparation, Meridol, that also contains stannous fluoride. Nevertheless, the reduced activity of AmFs in clinical trials when compared to the *in vitro* activity may be influenced by factors other than the experimental differences. In the development of chemical agents for use *in vivo*, ten Cate & Marsh, 1994, proposed that a hierarchy of tests should be established to determine, stepwise, environmental effects on agent activity. The knowledge of bacterial susceptibility to AmFs prior to this study is summarised in figure 10.2.1. The strong *in vitro* activity was shown using systems that were designed for widescale screening of potential agents, and as a benchmark for comparison of agents tested by different groups of workers. Although useful as a preliminary screening measure, the design of these procedures does not take account of the microbial ecology of the oral cavity. Hence, when agents are used in clinical trials, the bactericidal activity is often poor in comparison to what is implied by results of antimicrobial testing *in vitro*. Figure 10.2.1. shows the stepwise progressions that can be made to increase the complexity of the system, from simple MBC measurements to clinical trials.
Prior to this study, it was not known what factors contributed to the reduction in the bactericidal activity of AmFs in the clinical environment. This study investigated the effect of growth mode and sucrose on the susceptibility of *S. sanguis* to amine and inorganic fluorides.

Three separate AmFs were tested for their bactericidal activity towards *S. sanguis*. These were olaflur (MW 498), oleaflur (MW 374) and steraflur (MW 376). Olaflur contains two fluoride molecules per molecule of amine cation. All amine fluoride solutions were adjusted to 250 mg L\(^{-1}\) prior to use. Olaflur was also tested in combination with zinc and tin fluorides. The fluoride in the preparations was derived 50:50 from the amine and inorganic compound.
Hence the solution contained half the concentration of the amine ion that was used alone.

Planktonic cultures of *S. sanguis*, grown in mucin-containing medium (MCM), were treated with the AmF solutions. No viable bacteria were recovered from cultures after one minute treatment with any of the three AmFs. The olaflur/inorganic fluoride combinations also killed all bacteria. Zinc fluoride solution had no effect on the viability of planktonic *S. sanguis* cells. Hence the bactericidal activity was not due to the fluoride ions. Biofilms grown in MCM using the constant depth film fermenter (CDFF) were also treated for one minute with the AmF’s. Bacterial kills were achieved with all the AmF-containing solutions, however a proportion of the biofilm bacteria always remained viable. The pure AmF solutions were more effective than the olaflur/inorganic fluoride combinations which effectively contained half the amine ion concentration. The kills achieved with the amine/tin and amine/zinc combinations were similar. These observations, together with the fact that pure zinc fluoride solution had no effect on viability, imply that the amine ions were responsible for the bactericidal activity. The three different amine ions produced different levels of bacterial kills in the biofilms. The order of bactericidal activity of the amines toward *S. sanguis* biofilms was olaflur>oleaflur>steraflur.

The effect of incorporating sucrose into the growth medium on the susceptibility of *S. sanguis* to these agents was investigated. The kills of both planktonic and biofilm cultures grown in the presence of sucrose were reduced when compared to the kills in MCM-grown cultures. Viable bacteria were recoverable from MCM plus sucrose-grown planktonic cultures.
irrespective of test solution, however, highly significant kills were still achieved. The kills of bacteria in MCM plus sucrose-grown biofilms were much reduced compared to the kills in MCM-grown biofilms. The order of greatest reduction to smallest was the same as that for the kills achieved for biofilms grown in MCM. However, the reductions in the mean viable counts of MCM plus sucrose-grown biofilms were not statistically significant to the control group.

Following the observations that the presence of sucrose and the biofilm growth mode reduced the susceptibility of *S. sanguis* to AmFs, the study investigated some possible factors that may have been responsible. Factors that may influence the susceptibility were discussed in chapter 3, 4 and 5. One factor investigated was the influence of phenotype change due to biofilm-growth mode and specific growth rate (Gilbert et al 1993). Biofilm-derived *S. sanguis* cultures with a specific growth rate 5 times lower than the planktonic cultures were tested with the AmFs and were found to be highly susceptible. As with the planktonic cultures grown in MCM, no viable bacteria were recoverable after one minute incubation with AmFs.

Large amounts of insoluble exopolymer(s) were observed in biofilms and planktonic cultures grown in the presence of sucrose. Using cell-free preparations, it was investigated whether these exopolysaccharides reacted with AmFs hence protecting the bacterial cell. A reaction between the AmFs and the insoluble exopolysaccharide could not be shown.

At the concentration of AmF used in this study, we were unable to show an influence on susceptibility through a change in bacterial physiology, or by
exopolymer neutralisation of the AmFs. Thus, the factor(s) responsible for the reduced susceptibility of S. sanguis biofilms has yet to be determined.

One of the factors (discussed in chapters 4) which may account for the reduced susceptibility of the biofilms to short (1 minute) exposure to AmFs was diffusion limitation. Unfortunately, it was not possible to investigate the effect of diffusion limitation on susceptibility. Such investigations would have required the sensitive measurement of small differences in amine ion concentration over relatively short distances (<50 μm). The duration and funding of this study did not permit the investigation of diffusion limitation.

The experiments investigating the susceptibility of planktonic S. sanguis to AmFs showed that the amine ions are reactive species under favourable conditions. If the access of amine ions into the biofilm is compromised, e.g., through diffusion limitation and/or repulsion, the agents may be neutralised before they are able to enter the biofilm. In chapter 5 we showed that olaflur precipitated salivary components immediately on mixing. Although it is not known whether the precipitation of the salivary components neutralised the amine ions it does show that the amines can interact with different components in the oral cavity. Thus prolonged exposure of the AmFs to the environment external to the biofilms may neutralise the agents before they are able to perform their desired function.

In sucrose-grown cultures of S. sanguis, additional factors may affect the killing of bacteria by AmFs. Apart from the insoluble exopolymer products of streptococci previously discussed, streptococci also produce soluble exopolymers during periods of sucrose metabolism (Montville et al 1977, Kuramitsu & Ingersoll 1977). It is unknown whether these soluble polymers
interact with AmFs. If they do, they may help to neutralise the agents prior to reaction with plaque bacteria.

The bactericidal investigations in this study have shown the effect of nutrients and growth mode on the susceptibility of *S. sanguis* to AmFs. Figure 10.2.2. illustrates the information gathered in this study, and previous studies, on the susceptibility of oral bacteria to AmFs. Prior to this study, the susceptibility of planktonic bacteria in the absence of sucrose and the susceptibility of bacteria in clinical trials were known. This study has examined steps of increasing complexity between these two extremes as proposed by ten Cate & Marsh, 1995.

**Figure 10.2.2.** Clinical and laboratory testing of the bactericidal activity of amine fluorides towards oral bacteria following this study

- Clinical trials
  - Microcosm studies
    - ± sucrose
  - Mixed species biofilm
    - ± sucrose
  - Single species biofilm
    - + sucrose
    - − sucrose
  - Planktonic studies
    - + sucrose
    - − sucrose

Some trials reported no significant bactericidal activity whilst other trials did report bactericidal activity

Unknown effects

Unknown effects

Kills in biofilms, however, total counts were not significantly different to those of controls

Strong activity reported in this study

Strong activity reported in this study

Strong activity consistently reported
The investigation has shown that the susceptibility of a single species, *S. sanguis*, to AmFs was affected by sucrose metabolism and biofilm growth. When the factors were investigated separately, the *S. sanguis* cells were still highly susceptible to the agents. However, when the effects of sucrose and biofilm growth were investigated together, the susceptibility of *S. sanguis* was greatly decreased. If the complexity of the oral environment (refer to figure 10.2.2.) is compared with the model used in this study it is apparent that the activity of the AmFs was significantly compromised in very simple systems. The use of stepwise modelling has allowed the determination of the ecological factors that account for the reduced susceptibility in clinical trials. If the activity of an agent is shown to be lost in simplified models of the oral cavity then programmes can be terminated before the step of expensive and time consuming clinical trials.

This section has discussed the bactericidal activity of AmF in relation to formed bacterial biofilms. This is only one potential anti-plaque activity of the agents. In addition to the killing of formed biofilms, it is desirable if agents are able to prevent biofilm formation.

In the hierarchical testing of agents proposed by ten Cate & Marsh, 1994, it was proposed that all anti-plaque properties of chemicals be investigated. In addition, the substantivity of the agent in the human cavity should be determined. In the following section, the investigation of activities of amine and tin fluorides that prevent bacterial adhesion are discussed. Observations of the *in vitro* substantivity of the chemical agents on conditioned surfaces are also discussed.
10.3. Anti-adhesion properties of amine and tin fluorides

Bacterial adhesion to conditioned surfaces is the first step in biofilm formation. A possible anti-plaque activity of chemical agents is the ability to interfere with the adhesion process. In this study, a parallel plate flow cell system was used to investigate adhesion processes. The study investigated the effects on adhesion of treating conditioned and unconditioned surfaces with amine fluorides and AmF/tin fluoride combinations. The effect on adhesion of mixing the agents with the conditioning medium prior to pellicle formation was also investigated. Finally, the effect on adhesion of mixing the agents with the bacterial culture was determined. Adhesion experiments were performed for one hour periods using S. sanguis. The medium used for conditioning surfaces was the mucin-containing medium, MCM.

10.3.1. The effect of AmFs on adhesion

The three AmFs used for bactericidal studies were also used in the adhesion studies. The only amine to significantly reduce net adhesion of S. sanguis over a one hour period was steraflur. It was observed that olaflur and oleaflur did reduce the initial adhesion, however, the net adhesion after one hour was no different to that in control groups. It has previously been shown that AmFs prevent adhesion in a closed system (Meurman 1987). The results of this study show that all three amines possessed activity capable of preventing adhesion, however, the only agent to show a measurable level of substantivity was steraflur. The reason for the increased substantivity of steraflur,
compared to olaflur and oleaflur, was not determined in the study. The reactivity, strength of adsorption and relative stability may all have affected the substantivity of the agents. It is notable that steraflur was shown to be less bactericidal than the other two amines in the first part of the study. Sefton et al, 1996, showed that two different AmFs were still bactericidal when bound to protein-coated hydroxyapatite. It would be reasonable to assume that the more reactive an agents is, the faster it will be neutralised, even if adsorbed to a conditioning film. The low reactivity of steraflur compared to the other amines may thus explain its substantivity. Alternatively, the three amines may be desorbed from the conditioning film at different rates. Sefton et al, 1996, measured desorption of two AmFs (molecular weights 297 and 335) from protein-coated hydroxyapatite during rinsing with water. They showed that the level of desorption was low, the concentrations released being below the minimum inhibitory concentrations of the amines. Unfortunately, the shear forces on the absorbed amines during the rinse procedures were not determined. Busscher & Van der Mei, 1995, reported that the forces involved in dipping and rinsing procedures are large enough to remove tightly-attached cells. The shear forces in this experiment were far lower, i.e. they allowed adhesion of bacteria rather than removal. If the AmFs used in this study exhibited similar desorption properties as the amines used by Sefton et al, then it would be unlikely that much desorption took place at the low shear forces used in our study. Thus the desorption rate may not have had much influence on the levels of substantivity in our study. The effect of pH on the adsorption of the AmFs used by Sefton et al, 1996, was shown to be different for the two amines. It was suggested that this was due to different levels of
hydrophobicity of the two amines. The hydrophobicity of the three amines used in this study are unknown. Hence it cannot be discounted that the pH of the culture medium affected the substantivity of the AmFs.

The effect of mixing olaflur with the MCM used for pellicle formation and with the *S. sanguis* culture were investigated. Mixing of olaflur with the MCM resulted in the formation of a precipitate. The adhesion of the precipitate to the surface in the flow cell was negligible. Mixing of the amine fluoride with *S. sanguis* culture resulted in the aggregation of material in the culture milieu, large quantities of which adhered to the substratum. The material deposited onto the substratum is likely to consist of precipitated medium components and bacterial cells/cell debris. The large amounts of precipitated material are of concern when considering the anti-plaque properties of AmFs. The material increases the surface area of the substratum for adhesion. This material may serve as a binding site for bacterial adhesion. It may also act as a food source for future adherent micro-organisms. The effect of tin ions on this deposition will be discussed in the following section.

10.3.2. The effect of AmF/tin fluoride on adhesion

As with the AmFs, tin fluoride has been shown to prevent bacterial adhesion in closed systems (Gross et al 1977, Ota et al 1989). In the previous section we discussed the effect of AmFs on bacterial adhesion. The amine fluoride, olaflur, was shown to have no effect on net adhesion after one hour. It precipitated material from the growth medium and aggregated bacterial cells. This amine was used in conjunction with tin (II) and tin (IV) fluorides.
Both the tin (II) and tin (IV) fluoride combination with olaflur significantly reduced the net adhesion of *S. sanguis* to MCM-conditioned glass after one hour. The tin (IV) fluoride solution was more effective than the tin (II) fluoride solution. Mixing of the tin (IV) fluoride/olaflur combination with the MCM prior to pellicle formation increased the activity of the agent. The olaflur/tin (IV) fluoride combination caused the formation of large aggregates when mixed with the *S. sanguis* culture. However, tin (IV) fluoride prevented the adhesion of this material to the surface.

The investigation has shown that tin fluorides, in combination AmF, reduced adhesion of *S. sanguis in vitro* over a one hour period. The next step in testing would be to determine the *in vivo* substantivity of the agents to see if similar levels occur *in vivo*.

### 10.4. Amine/tin fluoride combination and bacterial desorption and co-adhesion.

The final part of this study investigated whether the olaflur/tin (IV) fluoride combination was able to desorbe bacteria from surfaces and whether the agents interfered with inter-species co-adhesion. Using the flow cell system that had been developed for adhesion studies, the effect of the chemicals on interactions between *S. gordonii* and *A. naeslundii* were investigated. Prior to testing of the agents, the importance of adhesion and co-adhesion on biofilm formation were determined. The results of the investigation showed that on surfaces with adherent *S. gordonii, A. naeslundii* co-adhered with the *S. gordonii* cells and also adhered independently of the streptococci. The
Actinomyces were more likely to adhere to the surface independently of the streptococci rather than co-adhere. Treatment of adherent *S. gordonii* cells with olaflur/tin fluoride did not cause desorption of the streptococcal cells. Nor did it affect the level of co-adhesion of *S. gordonii* and *A. naeslundii*.

The observation that the agents did not interfere with co-adhesion and did not cause bacterial desorption reveals an area in which there is potential for improvement of agent combinations. The incorporation of agents that prevent co-adhesion, or cause bacterial desorption, may enhance the anti-plaque properties of amine/tin combinations. Agents that could be used in combination include traditional agents such as chlorhexidine digluconate or new agents. Chlorhexidine has been shown to inhibit co-aggregation of many oral bacterial pairs (Smith et al 1991). Adhesion between Gram negative and Gram positive bacteria was inhibited at chlorhexidine concentrations as low as 0.01%. The inhibition of co-aggregation of Gram positive pairs was less effective. The use of chlorhexidine in conjunction with amine and stannous fluorides would lend the added benefit of the bactericidal properties of chlorhexidine. Alternatively, a novel inhibitor of microbial co-adhesion could be incorporated into combinations. A high molecular weight constituent of cranberry juice has been shown to possess anti-co-aggregation activity (Weiss et al 1998). The cranberry juice constituent reversed 58% of 84 different co-aggregation interactions tested.
10.5. The combined effects of the anti-plaque activities of the agents

In this study, the bactericidal and anti-adhesion activities of amine and stannous fluorides have been investigated. The bactericidal activity of AmF varied from amine to amine. However biofilm growth mode and the presence of sucrose in the growth medium decreased the bacterial kills achieved with all of the AmFs. The least active AmF, in terms of bactericidal activity, was the amine that possessed the greatest substantivity and anti-adhesion effects. The tin fluorides did not exhibit bactericidal properties in the study but did show substantivity over one hour and anti-adhesion activity. When considering the formulation of amine/tin combinations, the properties of the AmF which are the most useful should be considered. A choice can be made about the activity supplied by the AmF. A stronger bactericidal activity will result in a loss of substantivity and anti-adhesion activity. However, due to the tin exhibiting the latter properties, the stronger bactericidal activity may be more advantageous. Alternatively a combination of different AmFs may offer the most effective anti-plaque properties.

Substantivity is a measure of the retention of the active agent in the oral cavity. Obviously the longer an agent is active the better. However the substantivity is not the only factor that may govern the duration of agent effects in the oral cavity. Sooner or later the anti-plaque activity of the chemical agents will be lost, either by neutralisation or clearance from the mouth. After the loss of activity, the plaque levels will be restored to that on untreated teeth. The rate at which the levels are restored is obviously of importance. The different anti-plaque activities may affect the plaque
restoration rate differently. Hence, when considering the best formulation of amine/tin fluoride combinations the effect of the different agents on plaque restoration levels should be investigated.

In this study we investigated the effects of tin (IV) fluoride combined with the most bactericidal of the amines fluorides, olaflur, on biofilm formation. Biofilms were analysed by fluorescence microscopy after 12 hours. It is doubtful if the agents would exhibit any activity after 12 hours but the prolonged effects could be examined. In the absence of sucrose, the amount of biofilm formation was reduced on surfaces treated with olaflur/tin fluoride. The micro-colonies were smaller on treated surfaces and less numerable. It is not known whether the effects were due to the bactericidal activity of the amine, anti-adhesion activity of the tin fluoride, or both. In biofilms forming in the presence of sucrose there was no apparent difference in biofilm formation on treated and control surfaces.

10.6. Final summary

This study has shown that the CDFF model was useful in determining the effect of anti-plaque agents on the susceptibility of intact biofilms of oral bacteria. The parallel flow cell system was a good model for investigating processes involved in adhesion of oral bacteria to conditioned surfaces and enabled the determination of the effects of the anti-plaque agents on bacterial adhesion, desorption and co-adhesion. Additionally, the system allowed the comparison of the substantivity of the different anti-plaque agents.

The results showed that all the amine fluorides exhibited bactericidal activity towards S. sanguis and that the activity was affected by biofilm growth and
sucrose. The agent with the greatest bactericidal activity was olaflur. Tin (II) fluoride, tin (IV) fluoride and steraflur were shown to reduce the adhesion of *S. sanguis* to conditioned surfaces. The agent which exhibited the greatest anti-adhesion was tin (IV) fluoride.


Berg HE & Block SM A miniature flow cell designed for rapid exchange of media under high-power microscope objectives. J Gen Microbiol 1984; 130: 2915-20.


Bos R, van der Mei HC, Busscher HJ. Influence of ionic strength and substratum hydrophobicity on the co-adhesion of oral microbial pairs. Microbiology 1996, 142 (Pt 9):2355-61


Jenkinson HF & Easingwood RA. Insertional inactivation of the gene encoding a 76-kilodalton cell surface polypeptide in *Streptococcus gordonii* Challis has


McBride BC, Gisslow MT. Role of sialic acid in saliva-induced aggregation of 

McElroy WD, DeLuca MA. Firefly and bacterial luminescence: basic science 

McNab R, Forbes H, Handley PS, Loach DM, Tannock GW, Jenkinson HF. 
Cell wall-anchored CshA polypeptide (259 kilodaltons) in Streptococcus 
gordonii forms surface fibrils that confer hydrophobic and adhesive properties. 

McNab R & Jenkinson HF. Altered adherence properties of a Streptococcus 
gordonii hppA (oligopeptide permease) mutant result from transcriptional 
effects on cshA adhesin gene expression. Microbiology 1998, 144 ( Pt 1):127-
36.

McNab R, Jenkinson HF, Loach DM, Tannock GW. Cell-surface-associated 
polypeptides CshA and CshB of high molecular mass are colonization 

Mercer VH & Muhler JC. Comparison of a single application of stannous 
fluoride with a single application of sodium fluoride or two applications of 


