Chapter VII

Summary, general discussion and future research

Although the overall caries prevalence and incidence have declined, pits and fissures, approximal surfaces, and surfaces next to deficient or overhanging restorations remain prone to caries. Limited access of saliva and inadequate oral hygiene procedures allow undisturbed and prolonged contact between plaque and tooth surface, which predisposes the respective site to being caries susceptible.

Our knowledge about plaque ecology within stagnation sites and plaque interactions with substratum (tooth surface) is still limited. In recent years, our group developed a simple model to simulate and to study these sites. The model involves narrow grooves, cut in bovine dentin discs that can be studied in either *in vitro* (Lagerweij *et al.*, 1996a) or *in situ* experiments (Lagerweij *et al.*, 1996b, 1997). This way it was shown that fluoride dentifrices have a maximum anti-caries effect at the entrance of the groove, while with decreasing groove width the caries-inhibiting effects of fluoride decreased.

Would higher fluoride concentrations prevent demineralization at the bottom of narrow dentin grooves? Do antimicrobials have an additional caries-preventive potential to fluoride? Does chlorhexidine affect plaque biofilms, young or established, within stagnation site? Is plaque metabolically active throughout the groove? How is this related to demineralization of the groove? This thesis describes experiments with the groove model in attempts to increase our knowledge about plaque stagnation sites.

In **Chapter II** the effects of fluoride released from freshly prepared resin-modified glass ionomer cement (RMGIC) on demineralization in narrow dentinal grooves was assessed *in vitro*. Control specimens and specimens partially covered with RMGIC were subjected to demineralization for 1 to 2 wks. Changes in mineral content throughout the groove were assessed by transversal microradiography (TMR).

The results showed that integrated mineral loss (IML) of subsurface lesions was reduced up to 60% in the RMGIC groups, while lesion depth (LD) was not significantly affected. However, IML in RMGIC groups did not decrease towards the base of the groove to the same degree as in the control groups.

We hypothesized that fluoride induced inhibition of demineralization led to less neutralization of acids at the entrance of the groove. Consequently acids may diffuse towards the bottom of the groove, which could result in the observed

changes in the demineralization pattern. This hypothesis was tested *in situ* in Chapter V.

In **Chapter III** the effects of groove pretreatment with fluoride- and chlorhexidine-containing varnishes on plaque and demineralization were assessed. To this end the proportions of mutans streptococci and lactobacilli in 3-wk old plaque in the grooves, and the degree of demineralization of dentin were determined. Two commercially available varnishes – Fluor Protector (0.1% fluoride varnish) and Cervitec (1% CHX and 1% thymol varnish), as well as the 1:1 mixture of both were compared to placebo. In each disc, the varnish was applied in and next to one groove, while two grooves were left untreated. Healthy volunteers (N = 23) each wore one disc at a time, fixed to their removable dentures, for four consecutive *in situ* periods.

Proportions of mutans streptococci in the treated grooves ranged from 0.005 to 41%, and the proportions of lactobacilli varied from below the detection level to 33% of the total colony forming units (cfu). None of the varnishes exhibited an inhibitory effect on the establishment or growth of mutans streptococci and lactobacilli at the end of 3-wk *in situ* period.

All varnished grooves, including the placebo, showed inhibitory effects on demineralization towards the bottom of the groove, where the varnish was likely to have been retained longer. Compared to placebo, the average IML reduction was the largest in the grooves treated with mixed varnish (74%), followed by Fluor Protector (63%) and Cervitec (30%). Subjects with the highest mineral loss during the placebo varnish period showed the largest inhibitory effects of the active varnish treatment. Interesting was the additional finding that in these subjects Fluor Protector inhibited mineral loss only in the varnished groove, while CHX varnish treatment (Cervitec and the mixture of Cervitec and Fluor Protector) affected also the two non-treated grooves.

Since the fluoride varnish had the most pronounced inhibiting effect on demineralization and CHX-containing varnishes showed a peripheral effect we concluded that a combination method could be the preferred treatment for caries-prone individuals.

The inhibitory effect of an antimicrobial varnish on dentin demineralization (Chapter III) was small compared to fluoride-containing varnishes, and we were

not able to show that dentin pretreatment with chlorhexidine prevents groove colonization by caries pathogenic species such as mutans streptococci. In the natural dentition, the conditions for successful antimicrobial treatments would be even less favorable, since it is not possible to obtain sterile 'baseline' surfaces as in our pretreatment study, and fast microbial re-growth would be determined by the residual microflora. Second, if dental plaque follows the properties of biofilms (Chapter I, Table 2), then, once established, plaque will be less susceptible to any treatment. In **Chapter IV** we aimed to assess directly the immediate bactericidal effect of CHX on young and established dental plaque.

Volunteers (N = 6) wore intra-oral appliances with dentin groove specimens for 6, 24 and 48 hrs to accumulate plaque *in situ*. Following this, one half of each specimen was treated with 0.2% CHX for 1 minute, while the other half served as untreated control. Samples were stained for vitality measurements and visualized by confocal laser scanning microscopy (CLSM). Plaque vitality, defined as the proportion of vital cells, was quantified by image analysis.

It was observed that the structure of 24- and 48-hr plaque exhibited the spatially complex channel system found in other biofilms (Costerton *et al.*, 1995). Both, the complexity of the structure, and the vitality increased with increasing plaque age and thickness. CHX significantly reduced the vitality in 6-hr samples and only in the outer layer of older plaque. With this study we showed that direct quantitative visualization of the bactericidal effects of antimicrobial treatment is possible. Furthermore the recalcitrant nature of established dental biofilms to a single CHX treatment was demonstrated.

Visualization of plaque deeper than 20-50 μm in the groove is not possible with conventional confocal laser scanning microscopy due to optical limitations. Additionally, vitality staining does not permit the assessment of metabolic activity of cells. In order to study plaque activity throughout the whole groove depth (at least 800 μm), a more robust approach was needed. Microsensor methods are successfully used to study biofilms (Chapter I), but have not been applied to dental plaque. In **Chapter V** we used H^+-selective microsensors to assess plaque pH in grooves made in dentin, enamel and polyacrylate – substrata of high, moderate and no solubility. The aim was, among others, to test the hypothesis that was formulated after the *in vitro* experiment described in Chapter II: mineral dissolution at the entrance of the groove neutralizes plaque pH.

Grooves in bovine dentin, bovine enamel and polyacrylate accumulated plaque *in situ* for 1 wk in healthy individuals (N = 5). Plaque pH profiles were registered *ex-vivo* before and after exposure to glucose both, spatially (at 10-μm steps throughout the depth of the groove) and temporally (up to 60 minutes).

The results showed that the resting pH was similar in all grooves irrespective of substratum. Glucose exposure resulted in the lowest and the most-prolonged pH drop at the bottom of the grooves made in an inert material – polyacrylate. In dentin, and to a lesser degree in enamel, a drop in plaque pH was observed mainly at the entrance of the groove. This gave evidence that, unless neutralized, hydrogen ions could reach the bottom of 0.8-mm deep groove filled with 7-day old plaque, thus supporting our hypothesis proposed in Chapter II and explaining the demineralization pattern in the grooves next to the RMGIC material.

Microradiographic assessment of dentin specimens showed demineralization along the walls of the groove with maximum mineral loss near the entrance of the groove. By subject, the mineral loss in dentin correlated negatively with the minimum pH at the bottom of polyacrylate grooves – the lower the pH dropped in acrylate, the more mineral was lost in dentin.

Since polyacrylate does not buffer plaque acids, it may be argued that a different, more aciduric and acidogenic flora might have been established in the inert grooves compared to the grooves in dentin and enamel. This might have contributed to the observed pH differences among substrata. However, the observation that resting pH was similar irrespective of groove substratum indicates that plaque in polyacrylate grooves was not exposed to continuous low pH periods. Nevertheless, the possibility for selective growth remains to be addressed in the future.

To avoid any effects of the three substrata on establishment of plaque, a similar experiment, though under standardized, *in vitro* conditions was performed (Deng *et al.*, 2001). Briefly, *S. mutans* cells were mixed with glucose and immobilized with agarose. This artificial plaque was exposed to dentin, enamel and polyacrylate, and pH profiles were recorded. The results were in agreement with our observations that plaque pH is neutralized by mineral dissolution.

What relevance may these findings have for the clinical situation? First, it prompts a closer look at the 'Stephan' curve. Since the 1940-ies (Stephan, 1944) the plaque pH-change in response to fermentable substrates is used to assess the

caries activity of an individual. However, when measuring pH with an electrode *in vivo*, one measures the resultant of two opposing processes: (1) acid production, and (2) acid neutralization by dissolving mineral. Consequently, any pH change measured on, in principle, dissolving substratum, is a conservative estimate of the amount of acid formed.

Second, our finding may explain the phenomenon of the "hidden caries" (Ricketts *et al.*, 1997). As mentioned in Chapter I, fissure demineralization is initially observed at the entrance of the fissures. As shown in Chapter VI, this is where carbohydrates are actively metabolized by the plaque microflora, and the organic acids formed will locally induce demineralization. However, if the solubility of enamel is reduced by fluoride, a low pH near the entrance of the plaque-filled fissure will not be neutralized. Hydrogen ions may then diffuse through plaque and expose deeper parts of the fissure to a low pH. Presuming that these parts of the fissures were less exposed to topical fluorides and thus were less acid-resistant, this might lead to lesion formation in the deeper parts of the fissure.

The three pilot experiments, described in **Chapter VI**, were performed to further assess the metabolic activity of plaque at a retention site. In experiment 1, glucose-exposed samples were challenged with urea, and plaque pH alkalization was monitored. The results showed that *in situ*-grown plaque has a large capacity to metabolize urea, which raised the pH throughout the 0.8-mm deep groove. However, while pH remained alkaline deep in the groove, plaque pH near the entrance of the groove decreased below pH 7 within 30 minutes after 2-minute exposure to urea.

In biofilms, gradients in nutrients and in metabolites result in physiological activity gradients within complex microbial communities (Chapter I). From experiment 1 (Chapter VI) and experiment described in Chapter V we could not discriminate whether the pH-changes observed were due to a gradient in substrate concentration or to differences in plaque metabolic activity at various depths in the groove. To study this, a new type of groove specimen was designed which allowed direct exposure of the bottom of the groove to external applications (Chapter VI, experiment 2). We showed that plaque at the bottom of the retention site only marginally metabolized carbohydrate: it did not respond to glucose application with a fast and significant pH drop, as did plaque at the entrance of

the groove. In contrast, the observed rise in pH after application of urea indicated that urease activity might be expressed in the cells at the bottom of the groove.

In the third experiment, described in Chapter VI, the effects of various dietary regimens on the plaque pH-response to glucose and on the demineralization of dentin specimens were assessed in one individual with high previous caries experience. It was shown that although frequent exposure to sugar solutions resulted in actively metabolizing plaque, a more retentive form of nutrients, such as cookies, was necessary to induce dentin demineralization within one week. This observation could have significant implications for the study of cariogenicity of foods and their acceptance as 'safe for teeth'.

As mentioned above, Chapter VI describes pilot experiments and no generalized conclusions from the individual findings should be drawn. However, with this work we illustrated the importance to study dental plaque as a biofilm and indicated the potential for full-scale future studies.

In summary, various approaches – from transversal microradiography of dentin to confocal laser scanning microscopy and micro-profiling of *in situ*-grown dental biofilm – were used in the research described in this thesis. The aim was to explore caries related parameters in dental plaque at stagnation sites as simulated by the groove model. We conclude that:

1) High amount of fluoride – released from fresh glass ionomer cement during acid exposure – inhibited dentin demineralization and changed the pattern of demineralization: the lesions formed were more homogeneous throughout the depth of the groove as opposed to non-fluoride control where demineralization was primarily observed at the entrance of the groove (Chapter II).

2) Dentin pre-treatment with the fluoride-containing varnishes had a greater inhibitory effect on *in situ* demineralization compared to chlorhexidine varnish (Chapter III). None of the varnishes tested had an effect on the proportions of mutans streptococci or lactobacilli in 3-wk old plaque.

3) With increasing plaque age, the plaque vitality, complexity of its structure and recalcitrance to chlorhexidine treatment increased (Chapter IV).

Judging from this, dental plaque can be regarded and should be studied as a biofilm.

4) Plaque pH-decrease after sugar challenge was partly neutralized by dissolution of the underlying dental hard tissue (Chapter V). The extent of demineralization of dentinal grooves correlated with the plaque pH-drop in grooves made in an inert material.

5) At the bottom of the grooves plaque did not metabolize glucose (Chapter VI). Urea was metabolized throughout the depth of the groove, and this resulted in alkalization of glucose-exposed plaque. Frequent exposure to sugar during plaque growth increased plaque acidogenicity, but food retentiveness rather than sugar content alone was the determining factor for the formation of caries-like lesions.

This thesis demonstrates that multiple experimental approaches, and the use of knowledge from other disciplines is essential for unraveling processes occurring at caries-prone plaque retention sites.

Selecting individuals based on the extent of demineralization during intra-oral experiments and analyzing their plaque properties, such as composition, structure, metabolic activity, diffusivity, resistance to treatments and buffering capacity would provide information on key-differences between highly caries-active and caries-resistant subjects. In the confocal microscopy study (Chapter IV) only one of our panelists (subject D) showed significant bactericidal effects of CHX throughout the depth of the older biofilm. The same individual, participating in the study described in Chapter V (subject D), differed again from the rest of the group with the lowest resting plaque pH and the largest mineral loss in dentin grooves. As opposed to the above mentioned case, one of our panelists (Chapter V, subject C) differed from the rest with high resting plaque pH and no or minor mineral loss in dentin grooves, while he belonged to the heavy plaque-formers in the confocal microscopy study (Chapter IV, subject C) not responding to CHX treatment. It would be revealing to use plaque of known 'intra-oral behavior' to produce *in vitro*-biofilms under controlled experimental conditions in order to study the mechanisms behind the observed differences. With respect to our setup for microsensor-measurements, more 'natural'

conditions for simulated salivary composition and flow should be introduced, since saliva covers most oral surfaces as a thin, slowly moving film (Collins and Dawes, 1987) rather than bathing the teeth in large volumes.

Dental plaque stagnation sites are complex, and far from fully understood habitats. The evolution of the oral flora as a biofilm over millions of years has resulted in complex interactions that are interrupted *in vitro* when dispersed samples are analyzed. About 50% of oral micro-organisms are not culturable on growth media, and even culturable organisms may enter a temporarily non-culturable or dormant state (Wade, 1999; Barer and Harwood, 1999). Future research should involve culturing-independent characterization of the stagnation site microflora. The interactions between dental plaque and tooth surface should be assessed with approaches that would respect the nature of the plaque biofilm.

Accepting dental plaque as a complex multicellular system makes one reconsider findings reported before the plaque 'biofilm' era. Can we rely on results from simplified *in vitro* models? An example of a topic urgently needed to be reevaluated: Are diffusion and reaction processes in undisturbed plaque biofilm comparable to findings obtained from dispersed or centrifuged plaque samples?

With the current advances in molecular technology, microscopy and analytical techniques, and by integrating various disciplines future investigations should be able to elucidate the questions raised.