Chapter 1

General Introduction
In this chapter, an overview of the literature related to the topic of this thesis will be given. Emphasis will be put on the description of biofilms in general, in dentistry and on a summary of biofilm models which have been applied in dental research. The use of biofilm models in testing chlorhexidine will also be discussed. At the end, an outline of this thesis will be provided.

**Biofilm, Lifestyle of the Bacteria**

Bacterial biofilms, by definition, are matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces [Costerton et al., 1995]. Biofilms exist in nearly every aspect of our life. They are found on the surfaces of the teeth and implants, in food processing equipment, in water pipes, etc. Some biofilms are harmful to human beings; others, however, might be beneficial. Understanding the properties of biofilms can help us to control or to make use of biofilms more effectively. Traditionally, bacteria were studied in suspensions. In 1978, Costerton and colleagues speculated that the properties of bacterial cells change when the bacteria are co-existing in biofilms [Costerton et al., 1978]. Since then, more and more studies have shown that due to phenotypic changes of bacterial cells, diffusion barriers created by extracellular polysaccharides, or cell-to-cell communication, the properties of biofilms are significantly different from those of the traditionally studied planktonic cultures [Costerton et al., 1995; Marsh, 1995]. Now the importance of studying bacteria in their biofilm habitat is accepted worldwide.

**Dental Biofilm and Dental Plaque**

The term ‘biofilm’ was introduced to dentistry about ten years ago. From then on the research on dental biofilms has increased substantially, especially in the last few years. Nevertheless, it should be stressed that dental biofilms are not a completely new phenomenon in dentistry. Some researchers resembled ‘dental biofilm’ with ‘King’s new clothes’. Dental biofilm by definition is the same as dental plaque. In a symposium held in 1969, it was generally accepted that dental plaque consists predominantly of micro-organisms plus extracellular polysaccharides and usually an underlying pellicle of salivary origin [McHugh, 1999]. Dental plaque is recognized as the causal agent of caries and periodontal diseases. In the past, various aspects of dental plaque have been studied in depth. For example, the role of the extracellular
polysaccharides in caries formation [Gibbons, 1968] and in increasing the resistance of dental plaque to antimicrobials was already shown before 1990 [Wolinsky and Hume, 1985]. It might thus seem that the term ‘dental biofilm’ brought nothing new to dentistry.

However, recent biofilm studies indicate that the term ‘biofilm’ is not just a fashion. The introduction of dental biofilms broadened the concept of dental plaque. Viewing dental biofilms as one type of biofilm also facilitates us to apply new concepts and techniques from other biofilm areas to the research of dental biofilms. In fact, research on dental biofilms increased our understanding of the role of bacteria in oral diseases. It was confirmed that as for other biofilms, dental biofilms are more resistant to antimicrobial agents than planktonic cells. Moreover, it was revealed that the physiology of the cells changed when they were in a biofilm state. For instance, the cells in biofilms were more repressed in their respiratory activities [Nguyen et al., 2002] and possessed a better tolerance in an acidic environment [McNeill and Hamilton, 2003].

Dental Biofilm Models in Cariology

To study biofilms, the traditional bacterial batch culture systems are no longer suitable. Various biofilm models have therefore been developed. The type of model used in a biofilm study generally depends on the purpose of the study and the growth conditions required for the specific biofilm. In dentistry, equipment for biofilm growth can be as simple as a glass slide or a microtiter plate, or be rather complicated, such as a Constant Depth Film Fermentor (CDFF) or an in situ model. A brief review of in situ and in vitro biofilm models which are used in Cariology is given below.

In situ Model

In situ models involve the use of appliances or other devices which create defined conditions in the human mouth that simulate the process of dental caries [Zero, 1995]. Enamel/dentin slices, with or without grooves are used as substrata most frequently. Plaque is formed when carbohydrates are consumed, either experimentally controlled or provided by the subject’s normal diet. With this model, comprehensive studies have been carried out to explore the effects of various products, such as fluoride, food or milk products on the demineralization and remineralization processes [Zero, 1995].
Recently, *in situ* models have also been applied to explore the properties of *in situ* biofilms. The heterogeneous structure of dental plaque was discovered [Wood *et al.*, 2000] and the shifts in dental plaque ecology by antimicrobials were demonstrated [Gierts *et al.*, 2000]. The advantage of an *in situ* model is that it mimics the conditions in the mouth. But the disadvantage is that completing an experiment in the mouth increases the biological variation. As Featherstone [1992] pointed out, *in situ* models should not be used as an isolated test for studying caries or evaluating anticaries mechanisms but used as an intermediate stage between *in vitro* or animal laboratory studies and clinical trials.

The most popular *in vitro* biofilm models in caries studies are the microtiter biofilm model, the flowcell biofilm model and the Constant Depth Film Fermentor.

**Microtiter Biofilm Model**

Microtiter biofilm models make use of 24- or 96- wells polystyrene microtiter plates. Depending on the purpose of the study, biofilms may be grown on the surface of the polystyrene plates [Loo *et al.*, 2000], in hydroxyapatite (HAP) coated plates [Hazlett *et al.*, 1999], or on hydroxyapatite discs which are placed in the wells [Guggenheiem *et al.*, 2001]. The biofilm formation is evaluated by Gram-staining, fluorescent staining or plating. The microtiter biofilm model is a simple model which can be used as a rapid screening method for biofilm formation. In this model, cross contamination can be avoided since the biofilms grow independently in each well. So far, studies with microtiter models have indicated that the genes involved in cell-to-cell signaling [Li *et al.*, 2002], adherence [Froeliger and Fives-Taylor, 2001], and the regulation of fructose transport [Loo *et al.*, 2003] play a role in biofilm formation. Growth conditions, such as the nutrient supplied, pH, oxygen stress can affect the biofilm formation. It was also shown that caries preventive agents can be tested in this model [Shapi *et al.*, 2002]. However, one limitation of the model is that it is not practical to grow a biofilm for a long term because the medium has to be refreshed at least once daily manually. Also it is difficult to keep the conditions for biofilm growth constant [Li *et al.*, 2001].
Flowcell Biofilm Model

In this model, substratum is placed in a flow reactor and the growth medium is stirred or pumped to create a flow and thereby shear forces over the biofilm surface. The biofilms could grow on bovine enamel discs [Hodgson et al., 2001], microscopy glass slides or glass rods [Bleher et al., 2003]. The flowcell biofilm model is more complicated in design than the microtiter model. Unlike the microtiter model, the growth conditions can be controlled automatically and the biofilms could therefore grow for longer periods. In this model, the genes related to cell-to-cell communication [Bleher et al., 2003] and bacterial adhesion [Rogers et al., 2001] were again found to be involved in the early biofilm formation. Enhanced acid adaptation in biofilms, when compared to cells in suspension, was observed [Li et al., 2001]. Co-habitation among species in biofilm was also found, when compared to the results from traditional culture methods [Palmer et al., 2001]. More important for the study of dental caries, the effects of sugar on the structure of biofilm and enamel lesion formation underneath the biofilms could be shown in the flowcell model [Hodgson et al., 2001]. However, the influence of the biofilms on lesion formation could not be studied independently, since the enamel discs were not only covered by biofilms but they were also immersed in a big volume of growth medium that underwent pH changes.

Constant Depth Film Fermentor (CDFF)

Among in vitro biofilm models, the CDFF biofilm model is probably the most complicated one. A picture of the CDFF is shown in figure 1. The features of this model and the studies using this model are given in detail in the introduction of chapter 2. In summary, the advantage of the CDFF is that it contains a large number of identically grown biofilms and that it simulates the mouth condition. In particular, it is suitable for long term biofilm studies and for examining biofilms which induce de- and remineralization processes in dental
hard tissue, since only thin layer of medium covers the biofilm and the substrata during the growth of the biofilms.

**Biofilm De- and Remineralization Model**

Caries is the result of the dissolution of dental hard tissue covered by a biofilm. Acid production in the dental biofilms shifts the balance between demineralization and remineralization of dental hard tissue towards demineralization. Under oral conditions, caries formation is a slow process.

Fluoride has been proven to be a powerful caries preventive agent. A remarkable decline in caries prevalence has been seen after fluoride was introduced into dentistry [Fejerskov and Baelum, 1998]. The strategy of prevention was therefore changed. Currently more attention is being paid to high caries risk groups [ten Cate, 2001]. For those people, it seemed that fluoride alone, even when given at higher concentrations, can not prevent the occurrence of caries [Featherstone, 2000]. Antimicrobial agents might be formulated as an alternative or additional product for caries prevention. To test the efficacy of antimicrobials on caries prevention, a biofilm de- and remineralization model is needed before potential products could be tested in clinical studies.

Various biofilm models proved to be useful to study caries preventive agents on biofilm formation, but the concomitant effects on lesion formation have never been tested [Pratten et al., 1998b; Shapiro et al., 2002]. Furthermore, in some biofilm models it was possible to create lesion in enamel or dentin by growing biofilms, but the effects of caries preventive agents were not studied [Noorda et al., 1986; Hodgson et al., 2001]. It is therefore necessary to develop a model which includes the elements of the caries process, the presence of a biofilm and demineralization and remineralization, which processes can be studied simultaneously and in their mutual interactions.

**Chlorhexidine in Biofilm Studies**

**Mechanism of Chlorhexidine**

Chlorhexidine is 1,6-di(4-chlorophenyl-diguanido) hexane, a cationic bisbiguanide. It is incompatible (such as precipitation) with inorganic anions except in very diluted solutions and is also incompatible with organic anions, such as sodium lauryl sulphate,
sodium carboxymethyl cellulose, alginates and many pharmaceutical dyes. In certain combinations, there are no obvious signs of incompatibility, but the antimicrobial activity may be significantly reduced because the chlorhexidine is incorporated into micelles. At low concentrations, the action of chlorhexidine is bacteriostatic, and at higher concentrations, it is bactericidal, with the actual levels varying somewhat from species to species. The sequence of the lethal process is thought to be as follows: 1. rapid attraction toward the bacterial cell; 2. specific and strong adsorption to certain phosphate-containing compounds on the bacterial surface; 3. overcoming the bacterial cell wall exclusion mechanisms; 4. attraction toward the cytoplasmic membrane; 5. leakage of low-molecular weight cytoplasmic components, such as potassium ions, and inhibition of certain membrane-bound enzymes, such as adenosyl triphosphatase; 6. precipitation of the cytoplasm by the formation of complexes with phosphated entities, such as adenosine triphosphate and nucleic acids. Until step 5, this process is reversible. The cells could recover viability if the excess of chlorhexidine is removed by a neutralizing agent [Denton, 2000].

**Chlorhexidine Studies in Dentistry**

Chlorhexidine has been widely used in dentistry since it was introduced in the 1970s [Emilson, 1994]. Its popularity in dentistry was not only due to its broad antimicrobial spectrum, which includes Gram-positive and Gram-negative bacteria, but also due to its retention in the mouth, which prolongs its antimicrobial effect [Baca et al., 2003]. So far, it is considered the “golden standard” for antimicrobial efficacy among various antimicrobial agents. Clinical studies gave strong evidences that chlorhexidine could significantly reduce the number of salivary and plaque mutans streptococci, but the reduction of caries increment by chlorhexidine is controversial [Luoma, 1991; Gisselsson et al., 1994; van Strijp et al., 1997; Araujo et al., 2002; Dasanayake et al., 2002; de Soet et al., 2002]. Theoretically, the inhibition of caries by an antimicrobial agent could be achieved by inhibition of acid production or by inhibition of the formation of dental plaque. The clinical results do not seem to support either hypothesis. To further explore the relation of bacterial number, acid production and lesion formation, a biofilm model, which is easier to control and has less variation than clinical trials, might be a good alternative to a clinical study.
Chlorhexidine Studies in Biofilm Models

Chlorhexidine has been studied in various biofilm models. It was shown that the minimum concentration of chlorhexidine required to kill cells in a biofilm was considerably higher (10–100 times) than to kill cells in a suspension, regardless of the bacterial strain tested [Pratten et al., 1998b; Shapiro et al., 2002] or whether the cells were present as single species or multiple species [Kinniment et al., 1996b; Pratten et al., 1998a]. Furthermore, suprastructure of the biofilm on its resistance to chlorhexidine was demonstrated by comparing cells in undisturbed biofilms with cells dispersed from the same biofilms [Millward and Wilson, 1989]. It was also found that additional factors might control the efficacy of chlorhexidine on biofilms. The nature of the substratum might affect the susceptibility of the biofilms [Pratten et al., 1998b]; increasing biofilm age might increase the chlorhexidine resistance [Millward and Wilson, 1989]; growth condition of a biofilm, such as with or without sucrose, might influence the efficacy of chlorhexidine [Wilson et al., 1998]. Unfortunately, all the abovementioned studies used only bacterial viability as output parameter. No biofilm model so far has been used to test the effects of agents on the acidogenicity of the biofilm and lesion formation induced by the biofilm.

Chlorhexidine and Fluoride

The combination of chlorhexidine and fluoride was suggested in the 1970s [Luoma, 1972]. There might be two advantages of applying a combination of chlorhexidine and fluoride. First, it was thought that the antimicrobial effect could be enhanced when chlorhexidine was applied together with fluoride. A few in vitro studies showed that under certain concentrations, synergistic inhibitions of acid production and bacterial growth were observed [McDermid et al., 1985]. Second, the protection of fluoride on the teeth might be enhanced by the antimicrobial effect of chlorhexidine. However, in vitro and clinical studies did not convincingly show that the combination of these two agents reduced caries more effectively than when fluoride was given alone [van Loveren et al., 1996; Øgaard et al., 2001].

Objective of the Thesis

The purpose of this thesis was to develop the Constant Depth Film Fermentor as a demineralization and remineralization biofilm model and to use this model in
examining the effects of chlorhexidine and fluoride on both the properties of biofilms and lesion formation. In addition, some factors which might influence the efficacy of chlorhexidine were tested.

**Outline of the Thesis**

In *chapter 2* the potential of the CDFF as a biofilm demineralization model was tested. *Streptococcus mutans* biofilms were grown on dentin in the CDFF for 3 weeks and the effects of sucrose pulsing frequency in time on the demineralization process were studied.

In *chapter 3* the remineralization process in the CDFF was studied. Fluoride and a combination of fluoride and chlorhexidine treatments were examined for their capability to shift the de- and remineralization balance in the CDFF *S. mutans* biofilm model.

In *chapter 4* the CDFF biofilm model, as a caries model, was further studied for its application in testing caries preventive agents. Viability, acidogenicity of biofilms and lesion formation in dentin was examined in the CDFF *S. mutans* biofilm model when chlorhexidine with or without fluoride treatments were given. The effects of cessation of these treatments on the recovery of the biofilms and lesion formation were also tested.

In *chapter 5* the influence of the nature of the substratum on the properties of biofilms was studied. *S. mutans* biofilms were grown in either dentin or polyacrylate in the CDFF. pH profiles of the biofilms after sugar challenge and biofilm susceptibility to chlorhexidine were measured.

In *chapter 6* bacterial cells dispersed from biofilms were compared with those in undisturbed biofilms for their susceptibility to chlorhexidine. The viability and acid production after sugar challenge were measured. *S. mutans* biofilms were grown either on dentin surfaces or in grooves in dentin specimens to study the role of different types of surfaces that are present in the oral cavity.
In chapter 7 a summary of the studies presented in this thesis is given. The advantages and limitations of the CDFF biofilm model are discussed. Future studies with the CDFF are suggested.