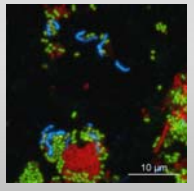
Multi-species dental biofilms



Irene Dige

School of Dentistry, Aarhus University, Denmark

Overview

- Introduction to oral and dental biofilms
- Previous studies of dental biofilms
- Recent studies of multispecies dental biofilms
- Methodological considerations

Biofilm

- The oral cavity is home to ca. 700 bacterial species
- Dental plaque is an archetypical example of a biofilm

(Nyvad *1983)*

Dental biofilm bacteria

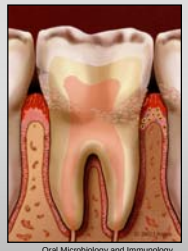
Table 1 Bacterial genera found in dental plaque.

GRAM-POSITIVE	GRAM-NEGATIVE
COCCI	
Abiotrophia	*Neisseria*
Peptostreptococcus	*Veillonella*
Streptococcus	
Staphylococcus	
RODS	
Actinomyces	*Actinobacillus*
Bifidobacterium	*(Bacteroides)**
Corynebacterium	*Campylobacter*
Eubacterium	*Cantonella*
Lactobacillus	*Capnocytophaga*
Propionibacterium	*Centipeda*
Pseudoramibacter	*Desulfovibrio*
Rothia	*Desulfobacter Eikenella*
	Fusobacterium
	Haemophilus
	Johnsonii
	Leptotrichia
	Porphyromonas
	Prevotella
	Selenomonas
	Treponema
	Wolinella

(Marsh & Bradshaw *1999)*

Supra- and subgingival biofilms

Caries and gingivitis



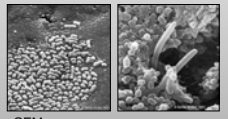
Oral Microbiology and Immunology

Periodontitis

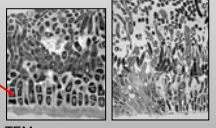
Oral Microbiology and Immunology

Classical methods

- Ultrastructural studies (TEM/SEM)
  (Listgarden *et al.* 1975, Theilade & Theilade 1970, Nyvad & Fejerskov 1987)
  - provide high resolution structural imaging
  - offer detailed information about bacterial structure and extra-cellular material is easily identified
  - described the basic patterns of microbial colonization on tooth surfaces



SEM (Nyvad & Fejerskov *1987)*



TEM (Nyvad & Fejerskov *1989)*

Classical methods

- Microbiological culture-methods
  (Ritz 1967, Socransky *et al.* 1977, Syed & Loesche 1978, Theilade *et al.* 1982, Nyvad & Kilian 1987, 1990)
  - documented which bacteria were primarily found in initial dental biofilm
  - the bacteria removed from their original substratum
  - do not include yet-uncultured organisms
  - studies showed dominance of *Streptococcus* and *Actinomyces* spp.

Additional methods

- Confocal Laser Scanning Microscopy (**CLSM**)
  - allows for analyses of intact biofilms in 3D
- Fluorescence *in situ* Hybridization (**FISH**)
  - specific identification of bacteria in microbial communities (Diaz *et al.* 2006, Al-Ahmad *et al.* 2007, Hannig *et al.* 2007)
- Combined use of CLSM and FISH
  - allows analysis of spatial and temporal dynamics of individual microbial populations in intact biofilms
- Methods of quantification
  - automated digital image analysis (Daims *et al.* 2006)
  - classical stereological methods (Gundersen *et al.* 1988)

New perspectives

- Methods such as CLSM and FISH
  - Make it possible to further investigate how the bacteria colonize and interact
- Need for increased knowledge about streptococci and *Actinomyces*
  - the pattern of colonization
  - spatial relationsships
  - numerical changes

- **Dige I**, Nilsson H, Kilian M, Nyvad B. In situ identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence *in situ* hybridization. *Eur J Oral Sci* 2007; 115: 459–467.
- **Dige I**, Nyengaard JR, Nyvad B. Application of stereological principles for quantification of bacteria in intact dental biofilms. *Oral Microbiol Immunol* 2009: 24: 69-75.
- **Dige I**. Initial dental biofilm formation studied by confocal laser scanning microscopy and fluorescence *in situ* hybridization. Århus Universitet 2008. Ph.d.-afhandling.
- **Dige I**, Nyengaard JR, Raarup M, Kilian M, Nyvad B. *Actinomyces naeslundii* in initial dental biofilm formation. *Microbiology* 2009; 155: 2116-2126.

In situ biofilm growth

- Ten healthy volunteers.
- Intra-oral appliance worn for 6, 12, 24, and 48 hours.

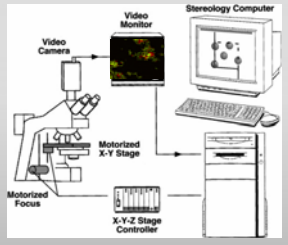
Fluorescence *in situ* hybridization

- After intra-oral exposure the biofilms were labelled with 16S rRNA-targeted oligonucleotide probes

Probe	Target	Sequence of probe (5' → 3')	Reference	Fluorescent dyes
EUB338	All biofilm bacteria	GCT GCC TCC CGT AGG AGT	Amann et al. 1990	Alexa 546 / Atto633
STR405	All streptococci	TAG CCG TCC CTT TCT GGT	Paster et al. 1998	Alexa 488
ACT476	*Actinomyces naeslundii*	ATC CAG CTA CCG TCA ACC	Gmür & Lüthi-Schaller 2007	Atto 550

CLSM

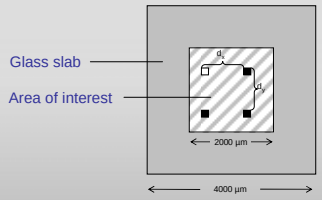
- Specimens were analysed by Confocal Laser Scanning Microscopy (CLSM).



Petersen, DA., *1999*

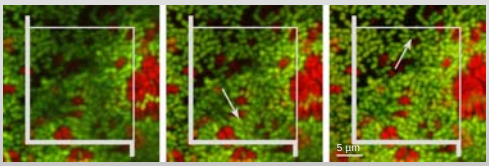
Stereological methods

- Systematic uniformly random sampling of fields of view



Stereological methods

- Quantification of bacteria was performed by the stereological tools:
  - unbiased counting frame



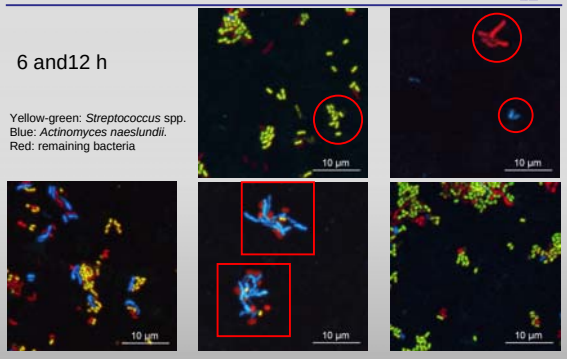
  - 2D fractionator

$$N = \frac{dx \cdot dy}{a(frame)} \cdot \sum Q^{-}$$

Qualitative results

6 and12 h

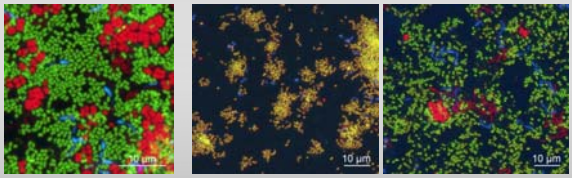
Yellow-green: *Streptococcus* spp.
Blue: *Actinomyces naeslundii.*
Red: remaining bacteria



Qualitative results

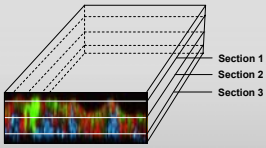
12 hours

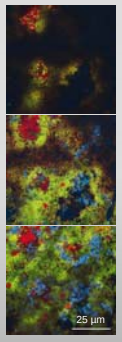
24 hours



Yellow-green: *Streptococcus* spp.
Blue: *Actinomyces naeslundii*.
Red: remaining bacteria

Qualitative results





Section 1

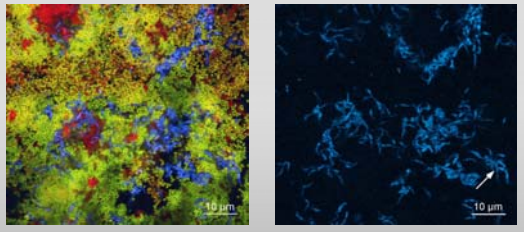
Section 2

Section 3

Yellow-green: *Streptococcus* spp.
Blue: *Actinomyces naeslundii*.
Red: remaining bacteria

Qualitative results

xy-section through a 24-h biofilm

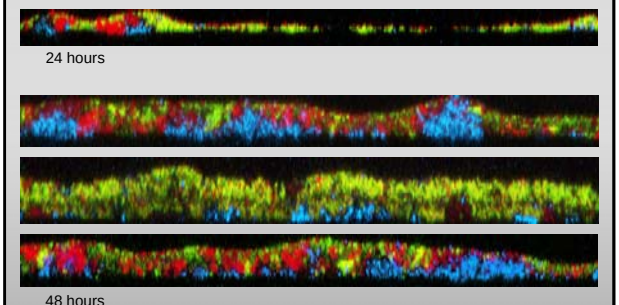


All colour channels

Blue colour channel

Qualitative results

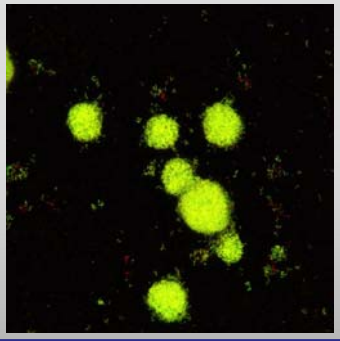
Sagittal sections



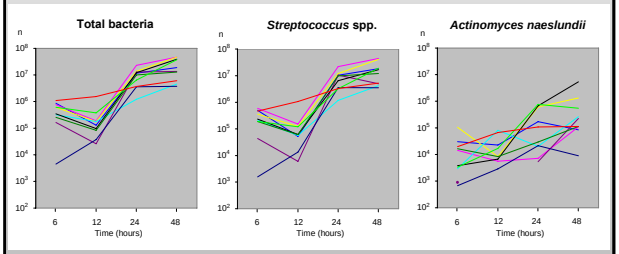
24 hours

48 hours

3D imaging



Quantitative results



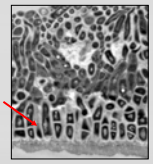
Time-dependant growth patterns

Conclusions

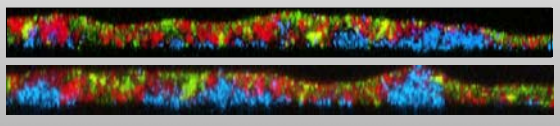
- The first time applied a systematic approach for examination of oral biofilm formation within the initial 48h by using a combination of CLSM and FISH.
- Differentiation of streptococci from *Actinomyces naeslundii* and other bacteria and description of their spatio-temporal organization.
- *Actinomyces naeslundii* predominantly occupy the inner part of multilayered biofilms.
- Observation of columnar patterns in developing biofilms and led to new information about the multi-species architecture.

Actinomyces colonization patterns

- *Actinomyces naeslundii* predominantly occupying the inner part of multilayered dental biofilms



Nyvad & Fejerskov *1989*



Actinomyces

- Diverse physiological characteristics
- Ability of *Actinomyces* to utilize different energy sources
  - Carbohydrates and lactate

    → make *Actinomyces* spp. particularly well fitted to live and survive in substrate-limited environments deep in the biofilm.
- *Actinomyces* have been documented to have pH-modulating activities
  - Produce ammonia via ureolysis
  - Can convert lactate to weaker acids

    → may therefore have a controlling effect on the dental caries processes by reducing the acidogenic potential of the biofilm. (Takahashi & Yamada 1996)

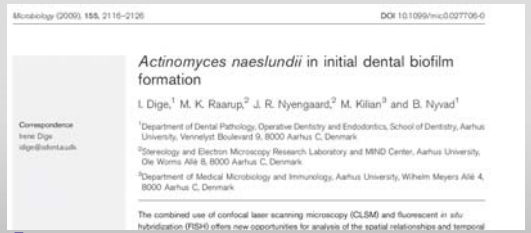
Methodological considerations

- FISH
  - Fading of the fluorophores
  - Penetration of the probes
  - Insufficient fluorescence due to low cellular rRNA
  - Difference in intensity of fluorescent signal
- CLSM
- Stereology vs. semi-automated digital image analysis

Acknowledgements

- **Supervisors and co-authors**
  - Bente Nyvad, dr. odont
  - Mogens Kilian, dr. odont
  - Holger Nilsson, dr. med
  - Jens Nyengaard, dr. med
  - Merete Raarup, PhD
- **Statistical advice**
  - Vibeke Bælum, dr. odont
  - Michael Væth, PhD
- **Technical assistance**
  - Lene Grønkjær
  - Anette Larsen
- **Financial support**
  - Aarhus University Research Foundation
  - The Swedish Patent Revenue Fund for Research in Preventive Odontology,
  - The Danish Dental Association

Thank you for your attention!



Actinomyces naeslundii in initial dental biofilm formation

I. Dige,[1] M. K. Raarup,[2] J. R. Nyengaard,[2] M. Kilian[3] and B. Nyvad[1]

[1]Department of Dental Pathology, Operative Dentistry and Endodontics, School of Dentistry, Aarhus University, Vennelyst Boulevard 9, 8000 Aarhus C, Denmark

[2]Stereology and Electron Microscopy Research Laboratory and MIND Center, Aarhus University, Ole Worms Allé 8, 8000 Aarhus C, Denmark

[3]Department of Medical Microbiology and Immunology, Aarhus University, Wilhelm Meyers Allé 4, 8000 Aarhus C, Denmark

Irene Dige

School of Dentistry, Aarhus University, Denmark