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Application of stereological principles for quantification of bacteria in intact dental biofilms

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Introduction: Quantative confocal laser scanning microscopy (CLSM) in combination with fluorescent *in situ* hybridization (FISH) may help to increase our knowledge about biofilm formation. The purpose of this study was to develop and evaluate a stereological method for quantification of bacteria in intact biofilm. The method was applied in a quantitative study of the proportion of streptococci relative to other bacteria in initial *in-situ*-grown dental biofilms as a function of time.
Methods: Biofilms were collected on standardized glass slabs mounted in intra-oral appliances and worn by 10 individuals for 6, 12, 24, and 48 h. Biofilms were analysed using CLSM. Quantification of bacteria labelled with 16S ribosomal RNA oligonucleotide probes was performed with stereological tools: the unbiased counting frame and the two-dimensional fractionator.
Results: Results showed a notable increase in the total number of bacteria and streptococci over time, with a considerable inter-individual variation at each time-point. After 48 h there was a 12.5-fold difference between individuals in the total number of bacteria and a 12.6-fold difference in the number of streptococci. The number of streptococci exceeded that of other bacteria and over the examination period there was a relatively constant relationship between the number of streptococci and other bacteria (streptococci vs. non-streptococci: median 15.2; minimum 1.0; maximum 89.3).
Conclusion: The study demonstrates that the combined use of FISH and stereology is a relevant and reliable tool for obtaining unbiased information about the numerical contributions of specific bacterial populations during early biofilm formation.

Key words: biofilm; dental plaque; fluorescence *in situ* hybridization; quantification; stereology

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Recent development of molecular probes that enable the detection of specific bacteria by fluorescent *in situ* hybridization (FISH) and the introduction of confocal laser scanning microscopy (CLSM) have stimulated renewed interest in studies of intact natural biofilms. When combined, these methods make it possible to study spatial relationships and changes of specific members of complex microbial populations over time without disturbing the structure of the biofilm (1, 2). This development represents a major advantage over classical microbiological culture methods, in which the biofilm is removed from its original substrate. Furthermore, because of nutritional insufficiencies of growth media, microbiological methods are bound to underestimate several groups of bacteria (3, 4). For example, labelling with the DNA stain SYTOX® of 7-day-old dental biofilms was reported to give microbial counts 2.3 times higher than the number of colony-forming units obtained using culture methods (5).

In dental sciences, biofilms are a key research focus because of their etiological role in oral diseases. There is a specific interest in exploring the early stages of biofilm formation because understanding of the processes involved may open new avenues for interfering with the pathogenic properties of biofilms. Although members of the genus *Streptococcus* seem to predominate during the initial colonization of tooth surfaces (6, 7), results from cultivation studies are potentially biased (3, 4). So far, the new molecular methodologies have been infrequently applied to describe qualitative aspects of streptococcal colonization in natural biofilm development (8–11). After the advent of a variety of

specific 16S ribosomal RNA (rRNA) oligonucleotide probes there has been an increased focus on quantitative parameters (8, 10, 11). However, these studies have applied different methods of quantification, which makes comparisons between studies difficult. In two of the studies (8, 11) manual counting was performed, but it is not clear how the areas of counting were randomized. The study of Al-Ahmad et al. (10) quantifies the biomass of different targets relative to the total bacterial biomass, as detected by fluorescent staining of confocal micrographs. Other researchers use biofilm thickness as a quantitative measure of biomass (12–14). However, preparatory procedures, including dehydration and embedding, have been shown to reduce the biofilm thickness by about half (15).

The problem of estimating parameters such as number of bacteria in three-dimensional multilayered biofilms from two-dimensional section planes is an obvious obstacle in biofilm research. Automated digital image analysis can answer some quantitative questions but to our knowledge none of the currently available image analysis programs are suitable for counting clustered bacteria in multilayered biofilms (16). As an alternative to absolute counts, a microbial population can be quantified by determining the fraction of its biovolume relative to the volume of the total biomass. However, some research questions require absolute counts of bacteria, including those present in aggregates. These challenges may be addressed by the use of stereological methods, which are design-based sampling methods based on well-founded mathematical and statistical principles. Hence, stereology may be regarded as a sampling theory for populations with a geometric structure (17). The aim of this study was two-fold: first, to describe a stereological method for quantification of bacteria in intact oral biofilms *in situ*; second, to evaluate its usefulness in a quantitative study of the proportion of streptococci relative to other bacteria in initial *in-situ*-grown biofilms as a function of time.

Materials and methods

Experimental conditions

Oral biofilms were collected on custom-made glass slabs (Menzel, Braunschweig, Germany). The glass slabs were industrially manufactured (4 × 4 × 1 mm) with a surface roughness of 1200 grit. Six glass slabs were mounted slightly recessed in the buccal flanges of individually designed intra-oral appliances worn by 10 healthy volunteers (five female and five male, 23–36 years of age (median age 25 years) for 6, 12, 24, and 48 h. The 6-h specimens were obtained from 08.00 to 14.00 whereas the 12-h specimens were obtained from 20.00 to 08.00. The 24-h and 48-h cycles started at 08.00. The subjects retained the appliance intra-orally throughout the experimental period, except during tooth-brushing and intake of food or liquids other than water. The Ethics Committee of Aarhus County approved the protocol, and informed consent was obtained from all participants after they had received oral and written instructions about the study. A detailed description of the experimental model and the experimental conditions has been previously published (9).

Specimen preparation

FISH

Following *in situ* biofilm growth, FISH was performed as described by Dige et al. (9), using specific 16S rRNA probes against streptococci, *Actinomyces* spp., and all bacteria. Immediately after removal from the oral cavity, the glass slabs with the biofilms were fixed in 4% paraformaldehyde (3 volumes) in phosphate-buffered saline (PBS; 1 volume) (18) for 3 h at 4°C. The specimens were subsequently washed with sterile PBS and stored in a mixture of 100% ethanol and PBS (1 : 1) at −20°C. For permeabilization of the biofilm bacteria, the glass slabs were mounted on glass slides (Menzel, Braunschweig, Germany) with paraffin wax (GC Corporation, Tokyo, Japan) and treated with 25 μl lysozyme (Sigma, St Louis, MO) 70 U/μl [in 100 mM Tris–HCl, pH 7.5 (Sigma), 5 mM ethylenediamintetraacetic acid (EDTA; Merck, Darmstadt, Germany)] for 9 min at 37°C in a humid chamber. After that, the slides were rinsed with ultrafiltrated water and dehydrated in a series of ethanol washes (50, 80 and 100%; 3 min each wash) and dried for 10 min in a vertical position. The glass slabs were then exposed to 10 μl of hybridization buffer [0.9 M NaCl, 20 mM Tris–HCl, pH 7.5, 0.01% sodium dodecyl sulfate, 30% formamide (which according to pilot studies was found to be the optimal concentration for the probe–label combination used)] containing 100 ng of the designated oligonucleotide probe and incubated at 46°C for 2 h in a humid atmosphere in the dark. After hybridization, the slides were washed first in buffer (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.01% sodium dodecyl sulfate, and 112 mM NaCl) for 15 min in a water bath at 48°C, and then rinsed in ice-cold ultrafiltrated water. The oligonucleotide probe STR405 (5′-TAG CCG TCC CTT TCT GGT-3′) (MWG Biotech, Ebersberg, Germany) labelled with Alexa 488 was used to identify all *Streptococcus* spp. (19) and the oligonucleotide probe ACT476 (5′-ATC CAG CTA CCG TCA ACC-3′) (IBA, Göttingen, Germany) labelled with Atto 550 was used to identify *Actinomyces* spp. (20). The specificity of the probes used in the study was tested on smears of saline suspensions of the following strains: *Streptococcus mutans* NCTC 10449^{T,} *Streptococcus sanguinis* ATCC 10556^{T}, *Streptococcus gordonii* ATCC10558^{T}, *Streptococcus oralis* NCTC7864^{T}, *Streptococcus mitis* NCTC12261^{T}, *Streptococcus infantis* GTC849^{T}, *Streptococcus anginosus* NCTC10713^{T}, *Streptococcus constellatus* ATCC27823^{T}, *Streptococcus intermedius* ATCC27335^{T}, *Streptococcus salivarius* NCTC8618^{T}, *Streptococcus parasanguinis* CCUG27742, *Streptococcus cristatus* SK231, *Streptococcus pseudopneumoniae* SK674, *Streptococcus pneumoniae* TIGR4, *Streptococcus sinensis* CCUG48488^{T}, *Streptococcus pyogenes* clinical isolate, *Abiotrophia defectiva* SK892, *Globicatella adiacens* SK932, *Enterococcus faecalis* clinical isolate, *Lactobacillus acidophilus* ATCC4504, *Gemella haemolysans* CCUG37985^{T}, *Actinomyces naeslundii* ATCC12104^{T}, *Actinomyces naeslundii* genospecies 2 WVU627/75, *Actinomyces israelii* NCTC6826, *Actinomyces odontolyticus* NCTC9935, *Propionibacterium acnes* ATCC737, *Rothia dentocariosa* ATCC14189, *Bifidobacterium bifidum* ATCC15696, *Staphylococcus aureus* clinical isolate, *Staphylococcus epidermidis* clinical isolate, and *Veillonella parvula* clinical isolate. The probe STR405 gave a strong fluorescent reaction with all bacteria on the slides prepared with all *Streptococcus* species. Apart from a very weak staining of *A. defective*, easily distinguishable from the positive reactions seen with *Streptococcus* species, no other strains gave a positive reaction. The probe ACT476 gave a strong reaction from all bacteria in smears of *A. naeslundii* and *A. naeslundii* genospecies 2 but no reaction with other *Actinomyces* species or with other bacteria in the test panel. The DAPI reagent stained all bacteria in the panel. The oligonucleotide probe EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) (IBA) labelled with Atto633 was used as a positive control to identify all bacteria

(21). A search performed in the Ribosomal Database Project II at http://rdp.cme.msu.edu/index.jsp indicated that the probe EUB338 recognizes 241,803 out of 335,830 bacterial sequences in the database and did not miss any taxa of bacteria hitherto detected in the oral cavity.

CLSM

The glass slabs with the biofilm were examined on an inverted Zeiss LSM 510 META (Carl Zeiss, Jena, Germany) confocal microscope using 488-, 543-, and 633-nm laser lines for excitation of Alexa488, Atto550, and Atto633, respectively. Emission band-pass filters were set to 500–530 nm for detection of Alexa488, 565–615 nm for detection of Atto550, and 651–704 nm for detection of Atto633, respectively. Images were acquired using a ×63 (numerical aperture 1.2) water-immersible objective (C-Apochomat) and collected at electronic zoom ×0.7 (low magnification), ×2 and ×4, of which ×4 was used for quantification. Before microscopy a drop of Citifluor AF3 anti-bleaching reagent (Citifluor, Leicester, UK) was applied between the biofilm and the chambered cover-glass. Each biofilm was scanned using a systematic uniformly random sampling method (see description of the stereological analysis) and *z*-series of optical sections were generated by vertical sectioning at 0.5-μm distances through the biofilm. This interval was chosen to include the major bacterial groups.

Image analysis

Image analysis was performed using ImageJ 1.34 s (22, 23). ImageJ was used to adjust output levels within the individual channel of the 24-bit RGB merged images. Before merging of the images, biofilms were assembled into image stacks. In the merged images, streptococci, *Actinomyces* spp., and remaining bacteria were represented by yellow-green, purple-blue, and red colours, respectively. No other manipulation of the images was performed.

Stereological analysis

Systematic uniformly random sampling

Stereological analysis was performed as a systematic uniformly random sampling of fields of view (24). First, the area of interest for estimating the number of bacteria, was identified as a 2000 × 2000 μm quadrant in the centre of each glass slab to capture typical smooth surface biofilm (Fig. 1). Subsequently, based on the density and heterogeneity of bacteria recorded in pilot studies, four or eight systematic uniformly random sampling (SURS) fields were chosen. The first field of view was sampled using a random number table. From this random starting point within the area of interest, the remaining three or seven fields of view were sampled by moving the microscope stage with a fixed *x* and *y* distance from the previous field (in this case 1000 μm in the *x*-axis and 500 or 1000 μm in the *y*-axis depending on whether four or eight SURS fields were chosen). Bacteria in the individual SURS fields were not counted when the upper right corner of the superimposed counting frame (see later) was outside the area of interest (Fig. 1).

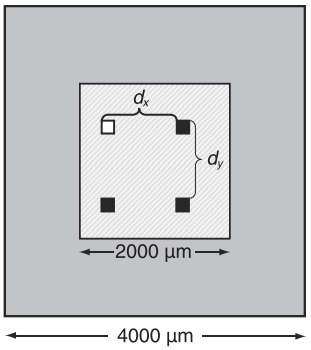

Fig. 1. Example of systematic uniformly random sampling of fields of view. The figure shows a square glass slab (grey), 4000 × 4000 μm. In the centre of the quadrant, the area of interest (hatching) is indicated by a 2000 × 2000 μm quadrant. Using a random number table the first field of view is sampled (white quadrant). The remaining sampling fields are found by moving the microscope stage in fixed steps (in this case 1000 μm) along the *x*-axis (d_x) and/or in the *y*-axis (d_y) (black quadrants). Counting of bacteria was only performed when the upper right corner of the counting frame was within the area of interest. If the upper right corner of the counting frame was outside the area of interest, bacteria were not counted.

Quantification of bacteria

The number of bacteria $\mathbf{Q}^-$ was counted using the unbiased counting frame originally described by Gundersen (25) adopting the following rule: only bacteria within the counting frame and bacteria intersecting the inclusion lines were counted (Fig. 2). Bacteria intersecting the exclusion lines were not counted (Fig. 2). The unbiased counting frame was superimposed on the images and fixed in the same position throughout subsequent focal planes. Bacteria were only counted the first time they came into focus in a section. Bacteria were counted manually and to remember which bacteria had already been counted, the point picker in the Particle Analysis plugin in the ImageJ software was used. The software also maintained a record of the number of cell markers placed by the operator.

The size and number of the unbiased counting frame varied according to cell density. For example, in specimens with low cell density (6 and 12 h), two counting frames covering the whole image except the outermost borders were used. On the other hand, when the cell density was high (multilayered biofilms) four counting frames of smaller areas were superimposed on the images. Therefore, the number of counting frames to be counted per specimen varied from eight (four SURS fields with two counting frames on each) to 32 (eight SURS fields with four counting frames on each). The size and number of counting frames also differed between counting of streptococci, *Actinomyces* spp., and remaining bacteria depending on the density and distribution of these bacterial groups. To ensure reliability of the quantitative data, a total of approximately 100–200 bacteria were counted per specimen (26, 27). The dimension of the counting frame(s) re-

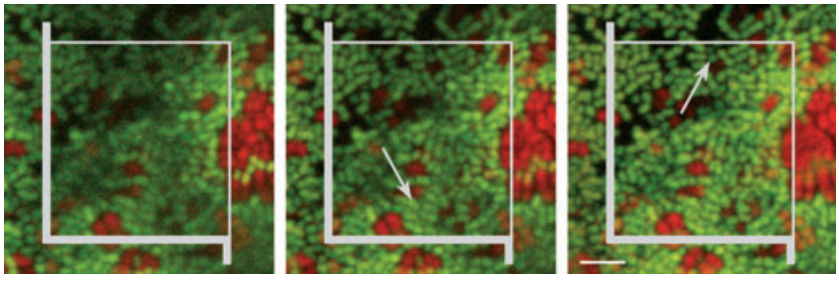

Fig. 2. Illustration of the unbiased counting frame and the principle of counting. The figure shows three consecutive horizontal sections of a 24-h biofilm, demonstrating streptococci (yellow-green) and other bacteria (red). Superimposed is a counting frame, which is placed at random. All bacteria within the frame were counted except for those touching the exclusion (bold) line. Bacteria were only counted the first time they came into focus in a section, as indicated by arrows in three consecutive sections. Scale bar, 5 μm.

mained the same for all images in one specimen.

The raw counts of streptococci, *Actinomyces* spp., and remaining bacteria were used to estimate the total bacterial number within the area of interest (2000 × 2000 μm). Subsequently, the total number of bacteria for each glass slab, *N*, was estimated by the two-dimensional fractionator, using the formula:

$$N = \frac{d_x \cdot d_y}{a(\text{frame})} \cdot \sum Q^-$$

where ΣQ^- is the total sum of counted bacteria, d_x and d_y are the sampling steps in the *x*- or *y*-direction (Fig. 1), respectively, and '*a*(frame)' is the area of the counting frame(s) corrected for magnification (μm^2) (28). For each volunteer, two glass slabs were analysed at each time-point and the mean value of the estimates was calculated.

Statistics

The coefficient of error (CE) was estimated as the counting noise (27), which means that the error variance due to systematic sampling of sections and fields of view was disregarded. An *F*-test was used to evaluate differences in variances between individuals using a level of significance of 0.01. Regression analysis was used to determine the relationship between the number of bacteria and time. Temporal changes in numbers of streptococci and total bacteria were analysed by Wilcoxon signed ranks test.

The error variance of the counting method was assessed by the same examiner counting three 24-h and three 48-h specimens twice according to Therkelsen (29).

Results

Tables 1 and 2 show the mean value of the estimates of the total number of streptococci and total number of bacteria at 6, 12, 24, and 48 h from the 10 individuals. As indicated by the minimum and maximum values, the speed of bacterial coverage of glass surfaces exposed in the oral cavity of human volunteers varied considerably between individuals at all time intervals. At 48 h there was a 12.5-fold difference between individuals in the total number of bacteria colonizing the surfaces and a 12.6-fold-difference in the number of streptococci. However, there was no statistically significant difference in the total variance CV(tot) between the two groups of bacteria (Tables 1 and 2). The biological variation, CV(bio), was determined from the CV(tot) and the CE(met), using the algorithm: $CV(bio) = \sqrt{CV^2(tot) - CE^2(met)}$. Tables 1 and 2 show the total variation [CV(tot)] in the number of bacteria, both as regards streptococci and total bacteria. Despite the fact that the CE(met) contains only part (counting noise) of the error variance, the ratio of CE^2/CV^2 was very small and, therefore, we conclude that the CV(tot) was mainly the result of biological variation [CV(bio)]. Moreover, the size of the CE(met) suggests diminishing returns for additional counting (27).

The error variance (CE) of the repeated counting was 0.05 and we consider this acceptable, also in view of the high biological variance.

Between 6 h and 48 h a notable increase was observed in the number of total bacteria and the number of streptococci (Fig. 3A,B). The increase could be described equally well by an exponential function or a power function. The number of all bacteria and the number of streptococci were lower at 12 h than at 6 h in eight individuals (cf. Fig. 3A,B); however, this temporal change in the numbers of streptococci and total bacteria was not significant. At 6 and 12 h the number of streptococci was higher than that of other bacteria in six individuals, whereas at 24 and 48 h the number of streptococci by far exceeded that of other bacteria in eight and seven persons, respectively.

When the estimated number of non-streptococci was plotted against the esti-

Table 1. Estimates of the number of streptococci at 6, 12, 24, and 48 h from 10 individuals

	Mean	[Min; max][1]	CV(tot)[2]	CE(met)[3]	CV(bio)[4]	$\frac{CE^2(met)}{CV^2(tot)}$
6 h	27.8×10^4	$[0.16 \times 10^4; 58.8 \times 10^4]$	0.70	0.13	0.69	0.03
12 h	17.3×10^4	$[0.60 \times 10^4; 107 \times 10^4]$	0.29	0.09	0.28	0.09
24 h	825×10^4	$[119 \times 10^4; 2200 \times 10^4]$	0.76	0.04	0.76	<0.01
48 h	1700×10^4	$[354 \times 10^4; 4470 \times 10^4]$	0.89	0.04	0.89	<0.01

[1]The lowest and highest estimates among the individuals [min; max]. The estimated numbers refer to a well-defined reference space that corresponds to an area of 4 mm^2.
[2]CV(tot), the observed total variation [standard deviation (SD) divided by the mean].
[3]CE(met), the coefficient of error.
[4]CV(bio), the biological variation.

Table 2. Estimates of the number of total bacteria at 6, 12, 24, and 48 h from 10 individuals

	Mean	[Min; max][1]	CV(tot)[2]	CE(met)[3]	CV(bio)[4]	$\frac{CE^2(met)}{CV^2(tot)}$
6 h	50.2×10^4	$[0.45 \times 10^4; 109 \times 10^4]$	0.57	0.08	0.56	0.02
12 h	28.4×10^4	$[2.58 \times 10^4; 155 \times 10^4]$	0.37	0.05	0.37	0.02
24 h	981×10^4	$[122 \times 10^4; 2290 \times 10^4]$	0.65	0.03	0.65	<0.01
48 h	2280×10^4	$[374 \times 10^4; 4670 \times 10^4]$	0.77	0.03	0.77	<0.01

[1]The lowest and highest estimates among the individuals [min; max]. The estimated numbers refer to a well-defined reference space that corresponds to an area of 4 mm^2.
[2]CV(tot), the observed total variation [standard deviation (SD) divided by the mean].
[3]CE(met), the coefficient of error.
[4]CV(bio), the biological variation.

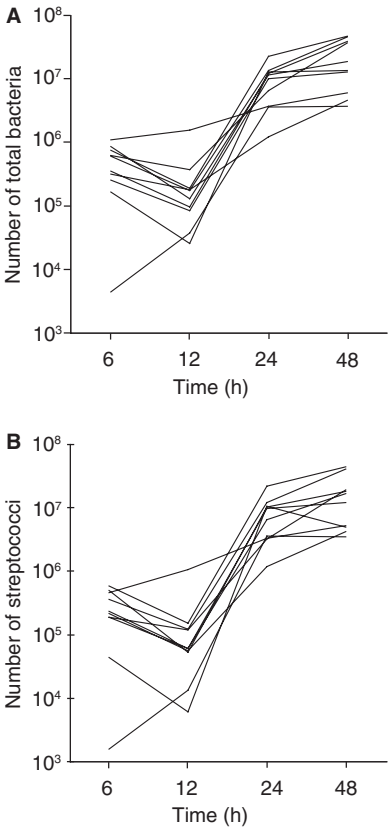

Fig. 3. The number of total bacteria (A) and the number of streptococci (B) recorded in dental biofilms developed within 6, 12, 24, and 48 h in 10 individuals. Note the logarithmic scale on the *y*-axis. The estimated numbers refer to a well-defined reference space that corresponds to an area of 4 mm^2.

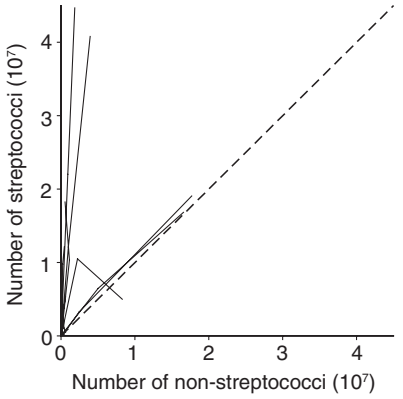

Fig. 4. Number of streptococci vs. number of non-streptococci at 6, 12, 24, and 48 h in 10 individuals. The dotted line indicates an equal amount of streptococci and other bacteria. The position of the thin lines shows that in most persons the number of streptococci exceeded that of other bacteria. Two individuals showed almost equal numbers of streptococci and non-streptococci and one individual showed an aberrant pattern. Over the examination period there was a relatively constant relationship between the number of streptococci and number of other bacteria for most individuals.

mated number of streptococci for each of the 10 individuals a relatively constant relationship over the entire examination period was observed – (streptococci vs. non-streptococci: median 15.2; minimum 1.0; maximum 89.2). For most individuals the number of streptococci exceeded that of other bacteria. Two individuals showed almost equal numbers of streptococci and non-streptococci and one individual showed an aberrant pattern (Fig. 4).

Discussion

To our knowledge this is the first time a mathematical design-based stereological approach has been used to quantify bacteria in *in situ* grown dental biofilms. We recorded changes in numbers and proportions of specific bacterial populations during initial biofilm development. Our method has some advantages compared with traditional methods for quantifying bacteria. It allows direct comparison between results obtained in different laboratories. Previously, quantification of bacteria in young developing oral biofilms has been performed by microbiological methods (colony-forming unit counts) (6, 30–32), by planimetric and/or gravimetric methods (33–36), or by the use of image analysis of sections following fluorescent *in situ* hybridization or immunofluorescence (8, 11, 37, 38). However, results from studies obtained by these methods are not directly comparable. Moreover, results acquired by the recently developed semi-automated (digital) image analysis tool for quantification of fluorescent signals should be interpreted with caution. To generate quantitative data from images based on fluorescent signals is a complex process. Some researchers perform quantitative analysis based on ratios of the area occupied by microorganisms to the whole area (8, 39, 40); others quantify the biomass of different targets (probes) in the biofilm by setting the EUB338-corresponding fluorescent volume at 100% and then calculate other targets as a percentage of the EUB338-fluorescing biomass (10). Irrespective of the strategy of quantification, fading of the fluorophore following optical sectioning, differences in fluorescence intensities depending on the dyes used for probe labelling, cell-specific uptakes of fluorescent stains, and varying thresholding are some of the problems of concern. Therefore, it has been recommended by Sekar et al. (41), that CLSM of biofilm area/volume should include size standards such as microbeads during image acquisition to minimize the error inherent in quantitative measurements.

In our study, bacterial populations were counted on the basis of whether individual bacteria were observed or not. Fluorescence intensities were not compared. This is in accordance with Peterson (42) who concluded that quantitative data are most informative when the targets represent absolute values of a parameter rather than the relative value. Absolute data allow comparison between experiments and between different investigators, provided that the overall methodology is the same. Nonetheless, every method has advantages and disadvantages and the choice of method must be adjusted for the research question addressed. The relative proportion of species/genera obtained by using quantitative semi-automated image analysis (8, 10, 39, 40, 43) may give different results from those obtained by our stereological method, which uses estimates of absolute numbers ignoring the size and morphology of the bacteria. This can be exemplified by experiments in individuals harbouring many of the previously shown large coccoid non-streptococci appearing in pairs and tetrads (9). Because of the size of these non-streptococci the proportion of these bacteria relative to streptococci may be larger using semi-automated analysis, whereas their absolute numbers may actually be smaller or the same as the number of streptococci when our stereological method is used. Therefore, when absolute numbers are the focus, for example in multiplication studies, the stereological method described in this study is a relevant alternative to semi-automated methods of quantifying fluorescence signals from histological samples.

Quantitative FISH has been reported to be tedious and time-consuming and to suffer from limited accuracy when applied to samples containing dense aggregates (44). Because of optical sectioning, confocal laser scanning microscopy may, to a certain degree, circumvent the problem of differentiating bacteria in aggregates by reducing interference by objects outside the focal plane. However, manual counting requires high-magnification images to allow differentiation between single cells. Furthermore, to obtain representative estimates, a large number of consecutive images need to be recorded and counted, which is time-consuming. Although the stereological approach described may not be as easy and time-saving as digital image analysis procedures, stereological methods enjoy the advantage of minimizing the workload to a manageable amount by systematic uniform sampling (in our case four or eight fields were analysed in each specimen depending on bacterial density) while still providing reliable quantitative information about the whole structure of interest (27). Moreover, the size and number of the unbiased counting frames varied according to bacterial density so that only a few hundred bacteria had to be counted, which according to the very low CE(met) is sufficient to get reliable results.

Manual counting of bacteria may sometimes be difficult. Even though different morphotypes of cocci and rods/filamentous bacteria could be clearly distinguished, and streptococci and non-streptococci could be easily differentiated in merged images (9), a good knowledge of bacterial cell morphology was required to be able to differentiate closely located bacteria and bacteria in division. Also, the fact that rod-shaped and filamentous bacteria are frequently oriented with their long axis perpendicular to the surface (45, 46) makes it more difficult to distinguish between coccoid and pleomorphic species in the *xy*-plane (9). This implies the risk of counting the same bacteria twice, thereby overestimating non-streptococci. However, we argue that this risk is minimal by the current method because bacteria were counted only the first time they appeared in a section. Counting of cells in aggregates is also complicated because densely packed cells cannot easily be separated during image segmentation (11, 43, 47, 48). We believe that automated

and semi-automated digital analysis tools are incapable of efficiently counting bacteria in dense clusters in biofilms because single-cell detection within these structures, including pleomorphic bacteria, cannot be automated. Therefore, we chose manual counting by an experienced investigator.

Another methodological aspect to be considered in fluorescence studies of intact biofilms is the problem of proper penetration of the fluorescent probes. We believe that probe penetration was not a problem in this study of young biofilms of limited thicknesses (up to 20–30 μm), but it may potentially be a problem if older biofilms were to be analysed. This view was recently supported by Paramonova et al. (49) who showed that for biofilms thicker than 120 μm, CLSM underestimates biofilm thickness noticeably because of poor penetration of the fluorescent dyes. An additional problem with thick biofilms is the risk of underestimating bacterial numbers because of considerable fading of the fluorophore when multiple optical sections are performed (9, 49). Degradation of the rRNA in dead bacteria or bacteria with a low metabolic activity may be another explanation for insufficient fluorescence of some bacteria (2, 11, 50, 51). The problems of poor probe penetration, fading, and insufficient fluorescence because of low cellular rRNA apply to automated digital image analysis as well.

The results of our stereological analysis corroborate previous quantitative evaluations of biofilm formation, showing a considerable inter-individual variation at each time-point (6, 11, 31, 35, 36). An exponential increase in the total number of bacteria and streptococci over time during the first 48 h of biofilm formation has been suggested (6, 30, 35). However, in the present study the bacterial growth was assessed at four time-points only, which is insufficient to determine whether the growth rate followed an exponential function or a power function. Furthermore, the fact that the number of streptococci far exceeded that of other bacteria at 24 and 48 h has been reported previously (6, 10, 31, 52). Of particular interest was the observation of a relatively constant relationship between streptococci and non-streptococci over the examination period for nine of the 10 individuals. The number of streptococci was about 15 times higher than the number of non-streptococci. Streptococci may therefore play a significant role for the total mass increase of developing young biofilms.

We found a lower number of streptococci and number of total bacteria at 12 h compared with 6 h. Although this observation was not significant, this temporal change was found in eight of the 10 individuals. The reason for this observation can only be speculated. One explanation could be that in this study the 6-h specimens were collected during the day whereas the 12-h specimens were obtained overnight. Such differences in the experimental conditions may possibly result from lower salivary secretion at night (53) and associated decreases in nutrient supplies (54) consistent with the observation by Quirynen and van Steenberghe (35) that the plaque growth rate, as determined by planimetric plaque growth, may be up to 50% lower at night. Combined with sustained shear forces from movements of the cheeks these explanations would, theoretically, result in reduced absolute numbers of bacteria as observed in the present study. Further studies are necessary to fully elucidate this interesting biological and potentially important phenomenon.

In conclusion, this study demonstrated that the combined use of CLSM/FISH and stereological methods is an attractive tool for quantification of bacterial populations in young intact biofilms. Although stereological methods are relatively time-consuming compared to automated and semi-automated digital image analysis and require prior knowledge of bacterial morphology, this principle allows unbiased and reliable determination of the numerical contribution of specific species in mixed bacterial communities. Importantly, application of stereological methods evades the inherent problems associated with varying intensity of the fluorophore, which is a possible bias in automated image analysis.

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