

# QUANTIFICATION OF BIOFILMS IN MULTI-SPECTRAL DIGITAL<sup>1</sup> VOLUMES FROM CONFOCAL LASER-SCANNING MICROSCOPES

KARSTEN RODENACKER<sup>1</sup>, ANDREAS BRÜHL<sup>2</sup>, MARTINA HAUSNER<sup>3</sup>, MARTIN KÜHN<sup>3</sup>,  
VOLKMAR LIEBSCHER<sup>1</sup>, MICHAEL WAGNER<sup>2</sup>, GERHARD WINKLER<sup>1</sup> AND STEFAN WUERTZ<sup>3</sup>

<sup>1</sup>GSF-National Research Centre for Environment and Health, Institute of Biomathematics and Biometry, 85764 Neuherberg, Germany, <sup>2</sup>Department of Microbiology, Technische Universität München, Germany, <sup>3</sup>Institute for Water Quality Control and Waste Management  
(Accepted May 5, 2000)

## ABSTRACT

Populations of bacteria in sludge flocs and biofilm marked by fluorescence marked with fluorescent probes are digitised with a confocal laser scanning microscope. These data are used to analyse the microbial community structure, to obtain information on the localisation of specific bacterial groups and to examine gene expression. This information is urgently required for an in-depth understanding of the function and, more generally, the microbial ecology of biofilms. Methods derived from quantitative image analysis are applied to digitised data from confocal laser scanning microscopes to obtain quantitative descriptions of volumetric, topological (and topographical) properties of different compartments of the components under research. In addition to free-moving flocs, also biofilms attached to a substratum in an experimental environment are analysed. Growth form as well as interaction of components are quantitatively described. Classical measurements of volume and intensity (shape, distribution) and distance dependent interaction measurements using methods from mathematical morphology are performed. Mainly image (volume) processing methods are outlined. Segmented volumes are globally and individually (in terms of 3D-connected components) measured and used for distance mapping transform as well as for estimation of geodesic distances from the substrate. All transformations are applied on the 3D data set. Resulting distance distributions are quantified and related to information on the identity and activity of the probe-identified bacteria.

Keywords: biofilm, activated sludge floc, fluorescent *in situ* hybridisation, green fluorescent protein, confocal laser-scanning microscopy, mathematical morphology, 3D image analysis.

## INTRODUCTION

In most natural and engineered systems micro-organisms grow as spatially organised, matrix-enclosed multi-species communities in biofilms or aggregates (e.g. activated sludge flocs). The advent of the fluorescent *in situ* hybridisation (FISH) using rRNA-targeted gene probes in combination with confocal laser-scanning microscopy (CLSM) allows to directly analyse the microbial community structure of such systems and to obtain information on the localisation of specific bacterial groups. Similarly, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* provides a label for living bacteria and can be used as a biological probe and reporter molecule for the investigation of gene expression or protein localisation (Chalfie *et al.*, 1994). Here we report on the development of a tool to quantitatively address the spatial interaction of probe identified bacterial

populations with each other within their natural biofilm habitat (co-localisation). Such data are urgently required for an in-depth understanding of the function and, more generally, the microbial ecology of biofilms.

Methods derived from quantitative image analysis are applied to digitised data from confocal laser scanning microscopes (CLSM) to obtain quantitative descriptions of volumetric, topological (and topographical) properties of different compartments of the components under research. Free-moving flocs and biofilms attached to the substrate in an experimental environment are analysed. Growth form as well as interaction of components are quantitatively described. Classical measurements of volume and intensity (shape, distribution) and distance dependent interaction measurements by use of methods from mathematical morphology are performed.

<sup>1</sup> Supported in parts by DFG SFB 411

*Biofilms* - are structurally and functionally organised colonies, microorganisms embedded in a matrix of extracellular polymeric substances (EPS). These ubiquitous life-forms accumulate on interfaces under favourable physiological conditions. The establishment of novel genetic traits in diverse natural environments is to a large extent the result of horizontal gene transfer. Transfer of plasmids by conjugation, a gene transfer mechanism which requires cell to cell contact, is likely to be prevalent in biofilms due to the relative spatial stability of bacterial cells within the EPS matrix. Until now, gene transfer frequencies have mostly been evaluated by plating transconjugants (recipient cells which have received a plasmid from a donor cell) on selective agar plates. This method is thus dependent on the subsequent cell division and colony formation by transconjugants. A more elegant approach is the detection of gene transfer events on a single cell level with *in situ* methodology (Hausner and Wuertz, 1999). In this study, a plasmid encoding a gene for the expression of a GFP-mutant, the enhanced yellow fluorescent protein (EYFP), and CLSM were used to quantitatively evaluate gene transfer frequencies in model bacterial biofilms.

*Activated sludge flocs* - represent suspended bacterial aggregates which strongly differ in size (from a few to several hundreds  $\mu\text{m}$  in diameter) and architecture. The majority of the sewage treatment plants world-wide apply the activated sludge process in order to efficiently treat municipal and industrial sewage. Due to their outstanding biotechnological importance activated sludge flocs have been intensively studied using classical microbiological techniques. However, the recent application of the cultivation independent full-cycle rRNA approach has revealed that the majority of the bacteria present in the sludge flocs have yet not been cultivated. Fluorescent *in situ* hybridisation (FISH) with rRNA-targeted oligonucleotide probes and CLSM were used to quantitatively analyse the microbial community structure of activated sludge flocs and to study the spatial interaction of bacteria identified by different probes (Wagner *et al.*, 1998). Such data are used to identify mutualistic interactions of still uncultured bacterial species within their natural environment. Together with results from a recently developed technique for the analysis of the *in situ* physiology of probe-identified bacteria by combination of FISH and micro-autoradiography (Lee *et al.*, 1999), key questions of microbial ecology can now directly be addressed.

## MATERIAL AND METHODS

### BIOFILM

In order to quantify gene transfer frequencies *in situ* on a single cell level, model *Alcaligenes eutrophus* recipient biofilms were grown on cover slips (0.2 mm thick) supported on a stainless steel frame in continuous flow-through cells ("flow cells"; 40 mm long, 4 mm wide, 8 mm in height). During biofilm development, the biofilms were irrigated with two different types of medium: Luria-Bertani (LB) broth and 0.2% glucose in a mineral medium. Biofilm development took place at room temperature for 5 days with a continuous supply of the medium at a flow rate of 0.016 ml/min. After the initial 5-day period, an overnight culture of donor cells (an *Escherichia coli* S17-1 lambda pir strain donor carrying the tra<sup>-</sup>mob<sup>+</sup> pRK415 plasmid with an insertion of the gene for EYFP) was added to the recipient biofilm in the flow cell. After a contact time of 2 hrs between recipient and donor cells, during which plasmid transfer occurred, the biofilm was irrigated with a buffer solution for additional 12 hrs in order to allow for the expression of EYFP in transconjugant cells. Next, the biofilms were fixed with a 4% paraformaldehyde (PFA) solution and hybridised *in situ* with a Cy5-labelled rRNA-directed oligonucleotide probe (BET 42a) which targets the Beta-subgroup of *Proteobacteria*, to which the recipient *A. eutrophus* strain also belongs. The hybridised biofilm could then be observed with a Zeiss LSM410 CLSM. Upon excitation with the 633 nm laser line recipient cells emit fluorescence in the far-red spectral range ( $> 665$  nm) whereas the donor cells fluoresce greenish-yellow (525-540 nm) upon excitation with the 488 nm laser line. Transconjugants emit both in the greenish-yellow (EYFP expression) and red (BET 42a probe) spectral ranges and can be detected by co-localisation. In each case, 9 vertical stacks of images (xy-sections) were collected (x,y,z: 3x3x60 images for the LB biofilm and 3x3x30 images for the glucose biofilm).

### SLUDGE FLOCS

Activated sludge from an industrial treatment plant receiving sewage with high ammonium concentrations (up to 5000 mg l<sup>-1</sup>) were fixed with 4% PFA and stored in 50% EtOH/1xPBS at -20°C until use. Samples were hybridised with a set of rRNA-targeted probes previously developed based on a phylogenetic inventory of the microbial population occurring within

this plant. Probes were labelled with FLUOS, Cy3 and Cy5. Probe-labelled cells were detected using by recording vertical stacks of images with a Zeiss LSM510 CLSM using the 488 nm laser line (FLUOS), 543 nm laser line (Cy3), and the 633 nm laser line (Cy5), respectively (Fig. 1).

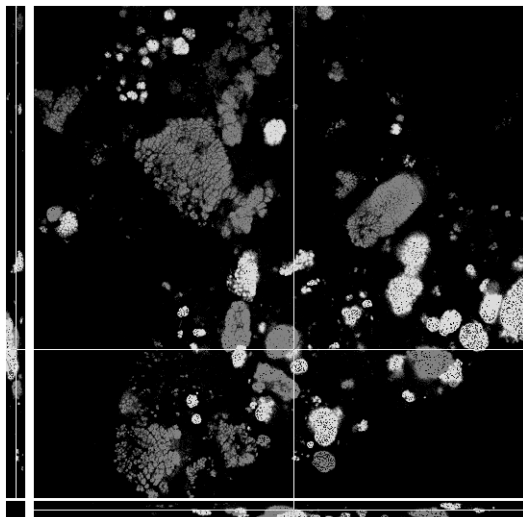


Fig. 1. *Sludge flocs: double marked from CLSM.*

## IMAGE PROCESSING METHODS

For a correct segmentation of the components a rough depth (Z-direction) intensity correction is developed. Segmentation is done by a threshold method. Cleaning of the binary volume data is performed by size and other features including user interaction.

Typically CLSM data are gathered with different resolution in X-Y and Z direction. To allow distance related transformations the data are re-binarised using linear interpolation. This increases the data size (and computing time) considerably, however the methods applied are much more simple, reliable and straight forward.

Data are available with voxel values for up to three different fluorescent spectral bands. Each voxel represents the distribution of the probe(s) reaction at this location. A threshold is a minimum necessary amount of probe reaction to be accepted as existent. For biochemical reasons some probe reactions must not be at the location of another; hence a pre-processing of segmented probe regions has to be performed. Vice versa different probe reactions at the same location can describe certain types or behaviours of bacteria (double hybridisation with rRNA-targeted probes, donors, transconjugants).

Segmented volumes are globally and individually (in terms of 3D-connected components) measured

and used for distance mapping transform as well as for estimation of geodesic distances from the substrate (growth range). All transformations are applied on the 3D data set. Resulting distance distributions are quantified and related to information on the identity and activity of the probe-identified bacteria.

All image analysis (IA) and featuring operations are performed using *idl* (Research System Inc., Boulder) on a HP Visualize workstation. All operations from mathematical morphology are based on works of Serra (1982), Sternberg (1986), Rodenacker *et al.* (1983), Meyer and Beucher (1990), Jeulin (1986), for the watershed algorithm on Vincent and Soille (1990) and Stoyan and Ohser (1982) for random structures.

## SHORT DESCRIPTION OF IA METHODS APPLIED

### *Depth intensity correction*

Several factors influence the intensity of the measured fluorescent signal. Beside the transparency of the medium there is also very important the bleaching behaviour of the fluorochromes used. Under the assumption of similar intensities of the marked organisms for each Z-co-ordinate a mean intensity is estimated. The resulting function is fitted and used as depth intensity correction function (Fig. 2).

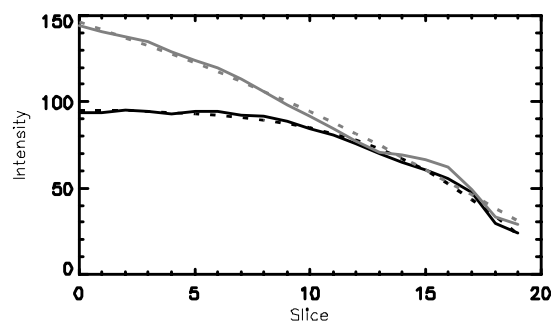


Fig. 2. *Sludge flocs: depth correction function.*

### *Thresholding*

The threshold for each spectral channel is automatically detected by a Gaussian estimate of the background voxel distribution.

### *Cleaning*

Derived from different bacteria populations the possibility to co-exist allows it to delete impossible combinations by application of logical AND and XOR on the binary volumes. Remaining small objects are deleted by an opening of given size of the structuring element.

### *Labelling*

Binary digital volumes are labelled by uniquely identifying each connected component (6- or 26-connected in 3D) by one number. This is performed by a 3-dim. watershed transformation, which is actually the quickest labelling method (Vincent and Soille, 1990).

### *Re-binarisation for isotropy*

The whole volume data set is re-binarised in Z-direction and linearly interpolated, so that Z-voxel spacing becomes equal to X- and Y-voxel spacing (Fig. 3).

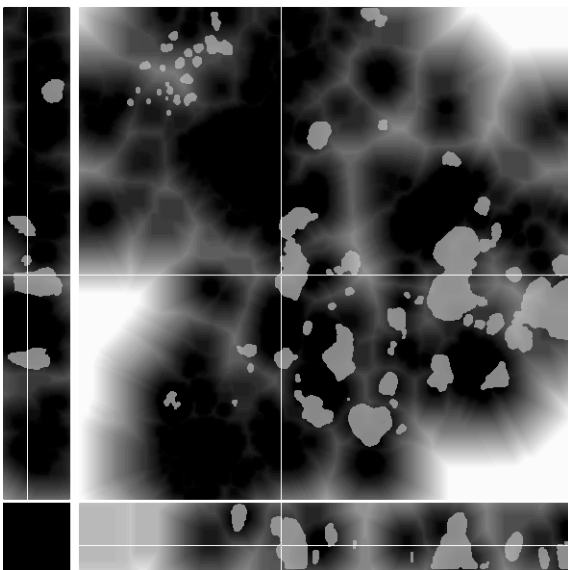


Fig. 3. *Sludge flocs: distance mapping (away from red phase) with green phase overlaid.*

### *Interaction and display*

The resulting data sets can be displayed by orthogonal sections. Connected components can either be deleted by clicking with the mouse at it or by cutting with vertical sections.

### *Clustering for individual analysis*

Regions of interest often consist of scattered signals building clusters of organisms. These clusters are defined by means of closings with a spherical structuring element of given radius. This radius denotes the maximum distance between scattered data inside of a cluster. Small clusters are deleted by application of openings with appropriate structuring element (Fig. 5).

### *Distance mapping for co-localisation measurement*

Given a binary 3-dim. data set with voxels set ("1") for the regions of interest (ROI) the distance transform is generated by successive 3-dimensional dilation with a small (radius 1) approximated digital sphere and subsequent addition. The procedure is terminated if the dilated binary volume has reached the border of the volume or if a terminating maximum distance is reached. The maximum value of the resulting data set minus the resulting data set itself is the distance transformed data set. Each voxel represents now the minimum distance to a voxel set. Voxels with distance 0 are inside the ROI(s) (Fig. 3).

### *Geodesic distances*

are only applied for biofilm. Starting from the substrate, typically at  $Z=0$  the segmented biofilm fibres are traced by successive dilation. After the transformation each voxel value represents the geodesic distance to the substrate.

### *Measuring methods*

For biofilm and sludge several different measurements are appropriate to describe quantitatively the amount, localisation and co-localisation of the participating micro-organisms. In the following the applied methods are shortly outlined.

### *Global and individual measurements*

Volumes are the number of voxels of the regions of interest (ROI), either globally or per unique label number. Beside the volumes mean intensities per ROI can be measured too (Fig. 6).

### *Co-localisation measurements*

This means the estimation of probabilities or likeliness of one probe reaction being near to another. Starting from the binary volume of one marked organism the shortest distance from any location to another volume set is called distance mapping. Considering a second binary volume the distribution of distances of all voxels set in the second volume describes the likeliness to be near to the first binary volume (Fig. 4).

### *Geodesic distance measurements*

For objects grown up from a substrate the length of the objects can be described by their distribution of geodesic distances.

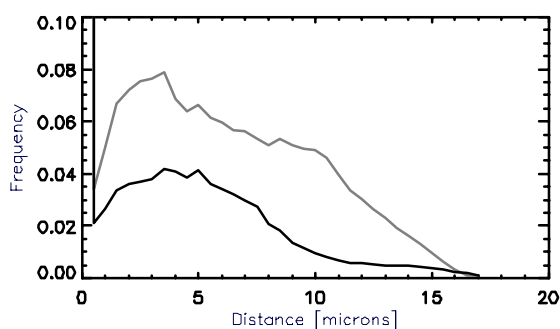


Fig. 4. Sludge flocs: distance distribution.

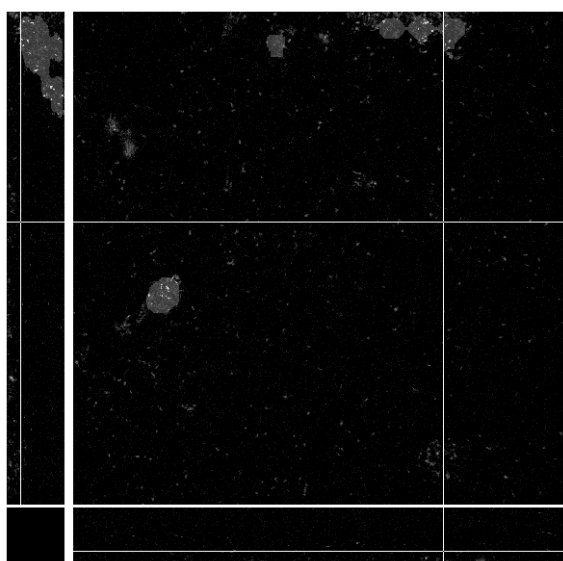


Fig. 5. Biofilm: with clusters marked in grey.

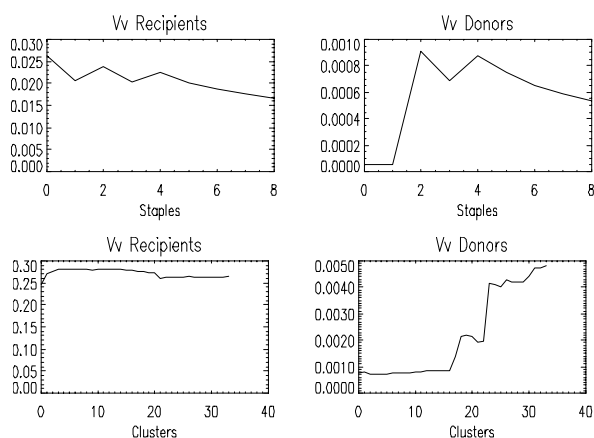


Fig. 6. Biofilm: volume density for increasing number of (top) volumes, (down) clusters of recipients and donors.

## RESULTS AND DISCUSSION

### BIOFILM

Previously, quantitative *in situ* determination of conjugative gene transfer in defined bacterial biofilms grown on microscopic slides revealed conjugation frequencies (transconjugant/ recipient-cell ratios) a thousand-fold higher than those determined by classical plating techniques. The experiments showed that conjugation was not affected by nutrient concentration alone but was influenced by time and biofilm structure (Hausner and Wuertz, 1999). However, methods for 3D image analysis are required in order to elucidate the relationships between conjugation events and biofilm development. In this study, image analysis of the digitised data disclosed a significantly higher volume of transconjugants in the biofilm supplied with LB-medium. Similarly, the conjugation frequency was also higher in this biofilm. Cluster and co-localisation analysis as described above showed that even though small clusters (less than 1% of the reference volume) dominated in both biofilm types, larger clusters (up to 10% of the reference volume) were formed only in the LB-biofilm. In this biofilm, transconjugants were clearly associated with large clusters. In contrast, no obvious trend was evident in the glucose-biofilm. In both biofilm types, however, the highest conjugation frequencies were detected in small clusters in the lower regions of both biofilms. The image analysis methods developed here will be used in subsequent investigations to provide information on the positioning of transconjugants with respect to recipient as well as to donor cells within defined clusters. The complete data set will facilitate insight into the complex interactions during exchange of genetic information in bacterial biofilms.

### SLUDGE FLOCS

Preliminary experiments were performed focusing on ammonia-oxidising and nitrite-oxidising bacteria which convert by mutualistic interaction ammonia via nitrite to nitrate. Interestingly, we could show in a recent study that *Nitrosococcus mobilis*, previously only reported for marine systems is the dominant ammonia-oxidiser within the analysed plant while hitherto uncultured members of the genus *Nitrospira* were implicated with nitrite-oxidation (Juretschko *et al.*, 1998). Both, the ammonia- and the putative nitrite-oxidisers did occur as densely packed clusters within the sludge flocs. The novel *Nitrospira*-

like bacteria were considered as nitrite-oxidisers since (i) they are most closely related to recognised nitrite-oxidisers and (ii) no other known nitrite-oxidisers (for example *Nitrobacter*) could be detected *in situ*. However, further data are required to understand the *in situ* physiology of these obviously important but previously overlooked bacteria and to provide hints for future successful enrichment and isolation. In order to understand their interactions with other bacterial groups several co-localisation analysis using the image analysis methods described above were performed. Initial results suggest that the *Nitrospira*-like bacteria are indeed spatially most closely arranged with ammonia-oxidisers confirming their assumed physiology (ammonia-oxidisers produce nitrite from ammonium which is subsequently used as electron donor by nitrite oxidisers). In future studies we will apply the developed methods for detailed studies of spatial interactions of uncultured probe-identified bacteria within sludge flocs and biofilms. In parallel the question of *in situ* physiology of such organisms will be tackled by performing FISH in combination with micro-autoradiography (Lee *et al.*, 1999). This battery of tools should allow a significantly more detailed understanding of microbial ecology and simultaneously provide valuable information for improving cultivation techniques in order to be able to harvest the natural bounty of the microbial world.

## SUMMARY

A short overview of image analysis methods are outlined for the quantitative description of 3D data sets from CLSM imaging from microbial origin. Especially the localisation, tracing and interaction of different (marked) populations of organisms is considered. The methods applied are only a starting point for the theoretical and practical penetration of the highly complicated complexes of marked micro-organisms mapped to digital data.

A preliminary report of some of the data was presented at the X<sup>th</sup> International Congress for Stereology, Melbourne, Australia, 1-4 November 1999.

## REFERENCES

- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994). Green fluorescent protein as a marker for gene expression. *Science* 263:802-4.
- Hausner M, Wuertz S (1999). High initial frequencies of conjugation in biofilms as determined by quantitative *in situ* analysis. *Appl Environ Microbiol* 65:3710-3.
- Jeulin D (1986). Study of spatial distributions in multicomponent structures by image analysis. *Acta stereol* 5:233-9.
- Juretschko S, Timmermann G, Schmid M, Schleifer K.-H, Pommerening-Röser A, Koops HP, Wagner M (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl Environ Microbiol* 64:3042-51.
- Lee N, Nielsen PH, Andreasen KH, Juretschko S, Nielsen JL, Schleifer K-H, Wagner M (1999). Combination of fluorescent *in situ* hybridization and microautoradiography - a new tool for structure-function analyses in microbial ecology. *Appl Environ Microbiol* 65:1289-97.
- Rodenacker K, Gais P, Jütting U, Burger G (1983). Mathematical morphology in grey-images. In: Schüssler H, ed. *Signal Processing II: Theories and Applications*. Amsterdam: Elsevier Science Publ, 131-4.
- Sternberg SR (1986). Grayscale Morphology, *Computer Vision. Graphics and Image Processing* 33:333-55.
- Stoyan D, Ohser J (1982). Correlation between planar random structures, with an ecological application. *Biom J* 24:631-47.
- Serra J (1982). *Image Analysis and Mathematical Morphology*. London: Academic Press.
- Meyer F, Beucher S (1990). Morphological segmentation. *J Visual Comm and Image Repres* 1:21-45.
- Vincent L, Soille P (1990). Watersheds in digital spaces: an efficient algorithm based on immersion simulations. *IEEE Trans Patt Anal Mach Intell* 13:583-98.
- Wagner M, Hutzler P, Amann R (1998). 3-D analysis of complex microbial communities by combining confocal laser scanning microscopy and fluorescence *in situ* hybridization (FISH). In: Wilkinson MHF, Schut F, eds. *Digital Image Analysis of Microbes*. Chichester: John Wiley & Sons, 467-86.