Automated biofilm morphology quantification from confocal laser scanning microscopy imaging

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Abstract In spite of the immediate visual appeal of confocal laser scanning microscopy images, the extraction of accurate reconstitutions of biofilm morphology requires a lengthy and computational intensive succession of processing steps. However, once performed, it provides ample reward by enabling the quantitative study of biofilm structure. A software suite of image processing tools for full automation of biofilm morphology quantification was developed by integrating preprocessing, segmentation and morphology quantification operations. This software toolbox was implemented in a web server and a user friendly interface was developed to facilitate image submission, storage and sharing, its access being unrestricted for scientific applications. The image bioinformatics tool which results from the integration of the processing operations can be accessed at *http://www.itqb.unl.pt:1111/clsmip/*. Its use is described in this paper and is illustrated with an example of processing of experimental data describing the growth of a mixed species denitrifying biofilm.

Keywords Biofilm; confocal laser scanning microscopy; imaging software

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Notation

V	Biovolume or biofilm volume [μm^3]
A	Biofilm to liquid medium interface area [μm^2]
Z	Vertical distance to solid substratum [μm]
Δz	Distance between adjacent CLSM optical cross-sections, or z-step [μm]
$C(z)$	Area of microbial colonization fraction profile
m_e	Number of horizontal cross-sections composing CLSM stack e
c_f	Biofilm colonization fraction at the solid substratum
h	Average height of biofilm micro-colonies [μm]

Introduction

From an engineering point of view, the effect of biofilm structure on process performance due to internal mass-transfer limitations has been a major concern since the early studies leading to the definition of the Thiele modulus. Biofilm bioreactors, in particular those using membrane supported growth, have been observed to lead to higher volumetric productivities than alternative designs. However, the biological constraints defining the boundaries for volumetric interface area, porosity and turtuosity are still poorly understood.

Confocal laser scanning microscopy (CLSM) is the method of choice for the monitoring of structure formation of live biofilms in laboratory flow-cell reactors. As a result of its non-invasiveness and non-destructive character, CLSM enables the *in vivo* reconstitution of the 3D structure of microbial biofilms in their naturally hydrated form (Lawrence *et al.*, 1991). Moreover, biofilm formation can be observed in detail by scanning the same site

repeatedly. However, the information captured in CLSM, in spite of its wide use, is only very partially quantified, and even then it consists mostly of direct measures on the unprocessed images. However, the potential of image analysis for the measurement of relevant morphology parameters that describe biofilm structure and architecture and relate to the underlying physiological processes has been demonstrated (Yang *et al*., 1999).

In this report, a set of computational tools for extraction of biofilm morphology from the three-dimensional structural data obtained from CLSM imaging, and the accompanying web-based interface, are described. The pre-processing tools requires normalization of contrast, alignment and tilting of stacks of CLSM optical cross-section, complemented by the recently developed objective threshold selection (OTS) method (Xavier *et al*., 2001). The latter is of critical importance for automated segmentation which is a necessary step for geometry processing operations, for quantification where integrated in a software suite for full automation of data processing for biofilm structure characterization. In this integration between image pre-processing and advanced quantitative structure characterization, the OTS procedure represents the, until recently, missing link for the full automation of biofilm image analysis. The quantification of morphology described here includes the measurement of: (i) area of microbial colonization profiles, (ii) biovolume, (iii) colonization fraction at the substratum, (iv) average height of micro-colonies, and (v) interfacial area.

The full integration of these operations allows the implementation of an automated image processing procedure for morphological quantification, which can be used for the processing of massive amounts of CLSM biofilm imaging data in a systematic way. The web-based application was also developed to provide access to the range of automated image processing algorithms while, at the same time, hosting a CLSM biofilm image database where images may be shared among researchers. This solution aims at offering a source of resources for the generalization of concepts and tools leading to unifying models of biofilm development, particularly suitable for continued monitorization of biofilm development in flow cell reactors.

Materials and methods

Biofilm growth in flow-cell bioreactors

CLSM biofilm images can be originated from several different experimental setups using laboratory flow-cell reactors (Palmer, 1999). For the example shown here, a biofilm from mixed species consortium was grown in denitrifying conditions in a flow-cell setup with growth medium continuously fed using a peristaltic pump (Watson-Marlow) at a flow rate of 1 $ml.min^{-1}$, corresponding to an approximate linear velocity of 18 $cm.min^{-1}$ (laminar flow) in the flow-cell channel where the images were acquired. Liquid culture medium composition, described previously (Xavier *et al*., 2001), provided suitable conditions for denitrification.

Image acquisition

The morphology quantification process is applicable to diverse formats data from CLSM imaging. Namely, variables such as optical cross-section resolution, area covered by image and vertical step between adjacent cross-sections (z-step) constitute part of the input of the quantitative analysis process. The example images were acquired for the denitrifying biofilm covering an area of $250 \times 250\ \mu m^2$ with a resolution of 512×512 pixels and a color depth of 8 bit (256 gray values). This corresponds to a pixel size of 0.488 μm (information provided by manufacturer). Each image acquisition event consisted of up to 31 optical cross-sections, evenly spaced by an exact vertical step of approximately 2 μm. The collective stack of horizontal cross-sections describes a volumetric loci within the biofilm at a

particular time. In the illustrative example presented below, the 3-dimensional monitoring of the same loci was repeated at regular time intervals. Consequently, a 4-dimensional matrix was acquired that captured the process of biofilm development. The biofilm was observed using a Leica TCS-NT laser confocal microscope operated as follows: dual excitation (488 and 568 nm), dual emission (530/30 BP into channel 1 to record Syto 9 fluorescence), 40 × 1.0 NA oil immersion lens at an Airy disc setting of 0.9. The flow-cell, placed under the CLSM, was not moved during the operation in order to always capture the same area. Image acquisition events took place at 16, 21, 24, 28, 32, 36 and 40 h after inoculation. The images were stored using the "tiff export" option on the Leica software, which saves each individual cross-section as a standard tiff-format digital image. Staining of the biofilm was preformed using 0.1 ml of a 6 µl.ml-1 aqueous solution of Syto 9 (Molecular Probes, Eugene, OR, USA), injected in the inflow stream 15 min prior to each image acquisition. Medium flow was halted for the 15 min following insertion of the stain to allow the dye to penetrate the biofilm. Syto 9 is a non-specific nucleic acid stain, with excitation/emission maxima of 480–500 nm. The use of Syto 9 for CLSM biofilm imaging has been reported previously (Xavier *et al*., 2000; Xavier *et al*., 2001). The non-destructive character of the use of Syto 9 together with CLSM in the conditions used here has also been confirmed in a study where biofilm growth in the presence of regular monitoring is compared with biofilm growth in control experiments, where the biofilm was grown in the absence of the referred stain (Xavier *et al*., submitted).

Image processing

The software suite of Image processing operations was implemented under Matlab programming environment (Matlab™ vers. 6.1, The Mathworks, Inc). Matlab was chosen due to convenience offered for matricial calculus. Since image processing algorithms are parallel processing operations, Matlab capability to emulate parallel processing operations greatly reduces algorithm implementation time. The resulting image bioinformatics tool can be accessed at *http://www.itqb.unl.pt:1111/clsmip/*.

Preprocessing operations

These include operations for three-dimensional tilting correction and stack leveling and contrast calibration. Correction of titling of the flow-cell during image acquisition was implemented using tri-linear interpolation for a 3D rotation transformation of the stacks of images, resulting in horizontal sections that are parallel to the plane defined by the solid substratum. Stack leveling, according to the height of the solid substratum, was implemented by determining the average gray value for each horizontal cross-section and detecting the substratum surface, identified by the cross-section with the maximum average gray value.

Image segmentation

Segmentation is the process of assigning pixels to distinct structural elements in images, e.g. biofilm, liquid media. Each stack of optical sections representing a 3D region of the space is processed to generate a volume filled with segmented voxels (3D pixels). The automated segmentation procedure was implemented following a two-step procedure: (i) direct thresholding using the OTS algorithm (Xavier *et al*., 2001) followed by, (ii) connect volume filtration (Heydorn *et al*., 2000). The use of the objective threshold selection (OTS) allowed the full automation of the segmentation, as opposed to visual threshold selections, which are common practice but lack reproducibility. Furthermore, the OTS method uses a statistical distribution of threshold values, which is used to determine confidence levels for the measurements that are computed downstream.

Quantification of morphology

Following preprocessing and segmentation, biofilm monitoring results from quantification of the following volumetric parameters.

1. Profiles of the area of microbial colonization: are defined as the profiles of the fraction occupied by biofilm at the longitudinal plane, e.g. along the direction perpendicular to the solid substratum surface (Kuehn *et al.*, 1998). For a given segmented CLSM stack, the area of microbial colonization at height z, $C(z)$, is derived from the fraction of pixels that have value = 1 in the optical cross-section taken at distance z to the solid substratum. This parameter, which has also been referred previously as "solids hold-up" (Kreft *et al.*, 2001), is related to the biofilm porosity profile, $P(z)$, by

$$C(z) = (1 - P(z)) \times 100\% \tag{1}$$

   The use of area of microbial colonization as a parameter for quantification of biofilm heterogeneity is, therefore, analogous to using biofilm porosity, the usefulness of which has been widely discussed (Lewandowski *et al.*, 1999; Lewandowski, 2000).
2. Biovolume, V: is measured from numeric integration of the area of microbial colonization profiles, following a method previously described (Kuehn *et al.*, 1998). This procedure accommodates the discrete nature of CLSM 3D rendering. Numerical integration uses the trapezoid sum described by Eq. (2), where m_e is the number of horizontal cross-sections in the stack and Δz is the z-step, the distance between adjacent cross-sections.

$$V = \int C(z)\, dz \approx \left[\frac{1}{2} \times C(z_1) + \sum_{m=2}^{m_e} C(z_m) + \frac{1}{2} \times C(z_{m_e}) \right] \times \Delta z \tag{2}$$

3. Colonization fraction at the substratum, c_f: is the fraction of the substratum surface colonized by the biofilm.
4. Average height of micro-colonies, h: measures the average height at which biofilm clusters rise from the solid-substratum. This value is computed as the ratio between biovolume and the colonized substratum area (Eq. (3)).

$$h = \frac{c_f}{V} \tag{3}$$

5. Interfacial area, A: is measured as the area of the interface between voxels representing biofilm and those of the culture medium. The interfacial area is the area summation of all biomass voxel surfaces exposed to the background (Heydorn *et al.*, 2000).

Web application interface

Integration of the operations described above results in full automation of image processing. The implementation of a web interface allows the use of the morphology quantification tools in an unsupervised manner, which is essential for data processing in projects that generate massive amounts data. The software suite constituted by the integrated image processing tools was implemented in a dual processor web server running GNU-Linux operating system with the web interface being built on PHP hypertext preprocessing language. An image database was set up in PostgreSQL to provide a repository of CLSM biofilm imaging. The experimental data for the illustrative mixed species denitrifying consortium grown in laboratory flow-cells is available in this repository. Therefore, through this application it is possible to repeat the morphology quantification operations described below.

Results

The formation of a biofilm from a mixed species consortium in denitrifying conditions in laboratory flow-cells was observed by analysis of serial image acquisition events. The automated web-based analysis of 3D stacks such as the one described in Figure 1B was used to follow biovolume distribution determined using the area of microbial colonization profiles (Figure 1C), microbial colonization of the solid substratum (Figure 1D), average height of micro-colonies (Figure 1E) and biofilm to liquid medium interfacial area (Figure 1F). Furthermore, using the OTS as the segmentation procedure automatically includes the determination of the sensitivity of measured volumetric parameters to the threshold value (Xavier *et al*., 2001).

The calculation of a particular morphological characteristic will automatically activate any preceding processing step. For example, the snapshot of the web interface in Figure 2A was generated in response to the single request of determining biovolume for the stack obtained at 13 hours of operation. As the results are computed and become available, the results for those preceding operations are also made available. For example, after a few

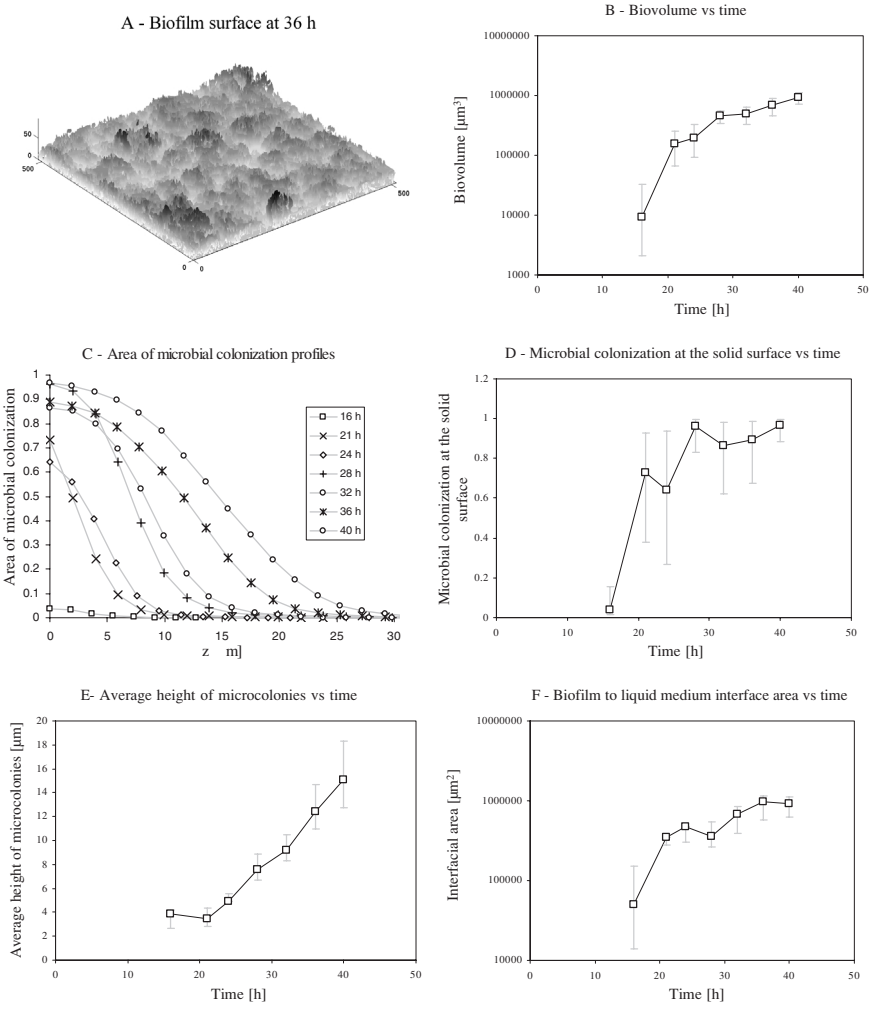

Figure 1 A – 3D rendering of the biofilm surface 36 h after flow cell inoculation; B – biovolume, V, time course; C – area of microbial colonization profiles; D – colonization fraction of the solid substratum, c_f, time course; E – average height of micro-colonies, h, time course; F – biofilm to liquid medium interface area, A, time course

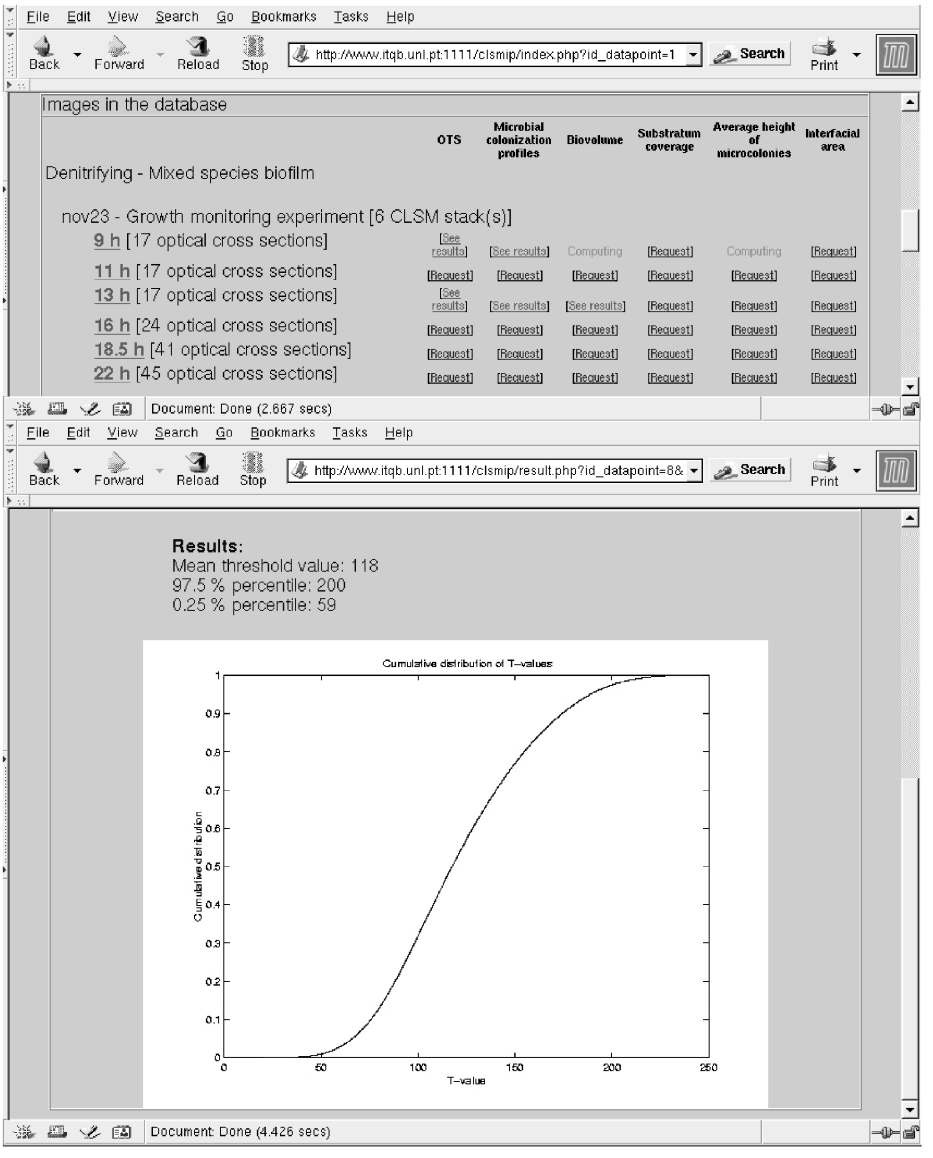

Figure 2 Snapshots of the web-based interface after biovolume calculation was requested for the 3D-stack obtained after 13 hours of flow-cell operation (above). After a few minutes the results become available and, for example, the preliminary results corresponding to the sensitivity of segmentation threshold to voxel value (left) can be inspected

minutes, the automated segmentation procedure is completed and the 3D volume (Figure 1A), the threshold sensitivity (Figure 2) and even the individual cross-sections are made available for inspection.

Discussion and conclusions

Multi-species microbial consortia, morphologically organized as biofilms, are the structural and functional unit of the microbial activity in natural environments. The quasi-organism nature of biofilms implies that its formation should be approached from a developmental biology perspective as has been stated before (Xavier *et al*., 2000, 2001). For example, the influence of biofilm structure in the mass transfer limitations properties will determine the

volumetric productivity of biofilm bioreactors. Accordingly, the study of biofilm formation requires the identification and quantification of the relevant morphological features in the 3D reconstitution of biofilm structure. Monitoring of morphology parameters of biofilm development in laboratory flow-cells through the method described here is made readily available in a user-friendly way through the use of a web based interface, and also provides means to systematically analyze massive amounts of data generated by CLSM. Quantification of biofilm morphology in a context of a large project will constitute an important source of knowledge for the generalization of principles geared towards the development of a unified biofilm model.

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