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REVIEW ARTICLE

Advances in mathematical modeling of biofilm structure

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ABSTRACT

Mathematical modeling of spatial biofilm structure has been in development for the past 10 years, its main goal being to derive the dynamics of biofilm structure from first-principle descriptions of the various physical, chemical and biological processes involved in biofilm formation. Early efforts described development of unrestricted monospecies consortia, often considering diffusion and reaction of a single solute species. Multi-dimensional modeling of biofilms has presently reached a stage where multi-species systems with any number of bacterial and solute species, reactions and arbitrary detachment scenarios may be readily implemented using a general-purpose software framework introduced recently. The present work presents motivations for the mathematical modeling of biofilm structure and provides an overview on major contributions to this field from pioneering efforts using cellular automata (CA) to more recent methods using the preferred individual-based modeling (IbM). Recent examples illustrate how biofilm models can be used to study the microbial ecology in: (a) development of multi-species nitrifying biofilms with anammox bacteria, (b) interspecies hydrogen transfer in anaerobic digestion methanogenic consortia, (c) competition between flock-formers and filamentous bacteria influenced by environmental conditions and its effect on morphology of activated sludge flocs, and (d) a two-species biofilm system with structured biomass describing extracellular polymeric substances (EPS) and internal storage compounds. As recent efforts from direct comparison of structure predicted by three-dimensional modeling with that observed by confocal laser scanning microscopy imaging of biofilms grown in laboratory flow cells show a good agreement of predicted structures, multi-dimensional modeling approaches presently constitute a mature and established methodology to enhance our understanding of biofilm systems.

INTRODUCTION

During biofilm development, a large number of phenomena occur simultaneously and interact over a wide range of length and time scales. As a result of nutrient conversions, the biofilm expands on the basis of bacterial growth and production of extracellular polymeric substances (EPS). Chemical species need to be continuously transported to and from the biofilm system by physical processes such as molecular diffusion and convection. Fluid flow influences biofilm growth by determining the concentrations of available substrates and products. On the other hand, the flow also shears the biofilm surface, and determines biofilm detachment processes. In the case of multi-species systems, microorganisms of different species interact in complex relationships of competition or cooperation. All these linked phenomena create a dynamic picture of the biofilm three-dimensional (3D) structure. The large number of localized interactions poses an important challenge for experimentalists. Mathematical models can prove useful because they allow testing of hypotheses and, in addition, can direct experimental efforts to complex regions of operation that can easily confound the general intuition. Although the word "modeling" is used for different purposes, the final result is invariably the same: models are no more than a simplified representation of reality based on hypotheses and equations used to rationalize observations. By providing a rational environment, models can lead to deeper and more general understanding. Ultimately, understanding the underlying principles becomes refined to such a state that it is possible to make accurate predictions.

MICROBIAL GROWTH IN BIOFILMS

Activity of microbes in biofilms is often notably different from that observed when they are in the suspended planktonic phase. Microbial cells enclosed in a biofilm matrix show significant advantages in relation to their planktonic counterparts, namely in the resistance to aggressive agents, such as increased resistance to disinfectants and antibiotics (Ceri *et al.*, 2001) and to ultraviolet radiation (Elasri & Miller, 1999), drying (EPS are highly hydrated) and protection from grazing by predators such as protozoa. Conversely, there are also notable disadvantages for bacteria when growing in a biofilm, such as an increased competition for limiting resources and increased mass transfer resistance, interference competition by production of antibiotics, overgrowth, and increased pressure from parasites. Most of such observed characteristics of microbial growth in biofilms can be explained by invoking transport phenomena, i.e. the physical implications of growth in densely packed environments where fluid flow is reduced. In sufficiently thick biomass clusters, as are generally the case in biofilms, diffusional distances are long enough that solute transport to inner bacterial cells becomes slow in comparison with the bioconversion kinetics of the microorganisms. In such situations, solute gradients are formed throughout the biofilm and mass transport becomes the rate-limiting process of the various biotransformations occurring (Characklis *et al.*, 1990). In these environments, solute gradients provide favorable conditions for the creation of functional micro-niches. For example, the depletion of oxygen in proportion to depth observed in activated sludge flocs (Schramm *et al.*, 1999; Meyer *et al.*, 2003) and biofilms (de Beer *et al.*, 1993) can create microenvironments suitable for the proliferation of anaerobic organisms, despite the presence of dissolved oxygen in the surrounding liquid phase. Solute gradients in oral biofilms also account for the local acidity that causes caries. In dental plaque, acidogenic and aciduric (acid-tolerating) bacteria rapidly metabolize dietary sugars to acids, which gradually accumulate, creating acidic microenvironments that are at the same time responsible for enamel demineralization (tooth decay) and for inhibiting competition from species associated with enamel health (Marsh, 2003). Mass transport limitations that impede efficient antibiotic penetration in biofilm matrices are frequently appointed as possible mechanisms responsible for the mentioned resistance to antibiotics (for references, see Mah & O'Toole, 2001). In light of these facts, an interpretation of the biofilm behavior from the extrapolation of the planktonic cell is not possible without knowledge of the mass transfer processes, in this complex morphology, responsible for the creation of the microenvironments (de Beer & Schramm, 1999).

BIOFILM STRUCTURE

The term "biofilm structure" includes (a) the 3D shape of the biofilm matrix, together with (b) the spatial distribution of fixed substances, both biotic and abiotic, in the biofilm matrix. The shape of the biofilm matrix defines the interface of the biofilm with the surrounding liquid phase, through which all solute transport takes place, and also it defines the diffusion distances. The spatial distribution of fixed substances refers to the spatial distribution of EPS, bacterial cells of different species and eventually inorganic particles. Biofilm structure can be described by simple properties such as thickness or density, but in many cases more complex measures are needed (Table 1).

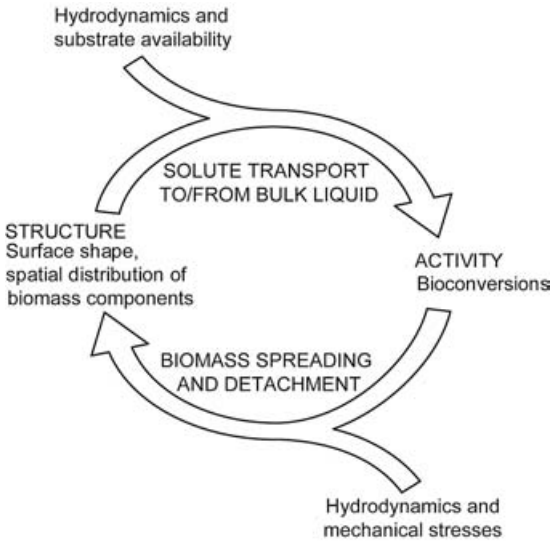

Fig. 1: Structure/activity relationships in biofilms.

The interest of scientists from several fields in studying biofilms has led to the investigation of numerous diverse biofilm systems, accordingly using a vast number of diverse techniques. This multi-disciplinary effort has led to the reporting of various types of biofilm structures and to the formulation of conceptual models for biofilm structure (for references, see Stoodley *et al.*, 1999). The observation that every microbial biofilm community is unique (Tolker-Nielsen & Molin, 2000) and that structure variability can occur even within the same system by changing operation conditions (Van Loosdrecht *et al.*, 1995) has led to a consensual view that structure in biofilms can show a great degree of spatial and temporal heterogeneity (Stoodley *et al.*, 1999).

The structure of biofilms plays a very important role in their activity. Together with the liquid flow, the shape of the biofilm surface influences the transport of all solutes from the bulk liquid to the microbial cells where the bioconversions occur. The structure, in turn, is itself defined by the biofilm activity. Bacterial cell growth and division, and EPS production and secretion, together with external factors such as shear forces defined by hydrodynamics and other mechanical forces (e.g. scrapping from cleaning or other procedures, grazing by protozoa, etc.) shape the biofilm structure (Fig. 1). Moreover, there are experimental indications of active,

Table 1: Parameters used for morphological quantification of biofilm structure

Parameter	Description	References
Biovolume	The volume occupied by the biofilm matrix, excluding voids	Kuehn *et al.*, 1998
Porosity	Ratio between biovolume and the total volume, including voids	Zhang & Bishop, 1994; Picioreanu *et al.*, 1998
Solids hold-up	1-porosity, also called filled space fractions (Heydorn *et al.*, 2000b) or area of microbial colonization (Kuehn *et al.*, 1998; Xavier *et al.*, 2003). Can be used as a profile along the biofilm depth	Picioreanu *et al.*, 1998
Thickness	Mean or maximum thickness of the biofilm	Heydorn *et al.*, 2000b
Volume of microcolonies	Mean volume of individual biofilm clusters	Heydorn *et al.*, 2000b
Surface to volume ratio	The ratio of biofilm interfacial area to biovolume	Heydorn *et al.*, 2000b
Surface enlargement	The ratio of biofilm interfacial area to solid surface area	Picioreanu *et al.*, 1998
Surface coverage	The fraction of solid surface area colonized by biofilm	Xavier *et al.*, 2003
Diffusional distance	The mean or the maximum distance from any point in the biofilm to the biofilm surface	Yang *et al.*, 1999
Textural entropy	A measure of the randomness of the spatial distribution of biomass density in the biofilm	Yang *et al.*, 1999
Heterogeneity	The standard deviation of substrate concentrations as experienced by the cells (determined by modeling)	Kreft *et al.*, 1998
Contrast	Similar to textural entropy	Kreft *et al.*, 2001
Fractal dimension	A measure of the irregularity of the biofilm–liquid interface	Hermanowicz *et al.*, 1995; Picioreanu *et al.*, 1998
Developmental axis	Complexity of the branching patterns in biofilm clusters	Xavier *et al.*, 2000
Roughness	Related to standard deviation of biofilm thickness, higher values indicate more variable (and therefore rougher) biofilm surface	Murga *et al.*, 1995; Picioreanu *et al.*, 1998

regulated detachment due to quorum sensing or starvation (Schooling *et al.*, 2004). Understanding this relationship between biofilm structure and activity and the factors that physically shape the biofilm is crucial in order to effectively utilize and control biofilms in industrial and medical settings (Stoodley *et al.*, 1999).

MODELING BIOFILM STRUCTURE

Modeling of biofilm structure has been driven by the aim of describing the structure/activity relation in a formal (i.e. mathematical) way. Mathematical description of the processes acting simultaneously in the course of biofilm development can help in understanding their interdependence. A model that predicts biofilm structure from environmental conditions and activity from the biofilm structure is a valuable tool for testing hypotheses. Such a model can be especially important in the understanding of biofilm systems where operation parameters have implications on each other and which pose significant difficulties in the design of experiments. For instance, studying the direct effect of imposed detachment forces on the bioconversions in a biofilm airlift suspension (BAS) reactor is a challenging task (Kwok *et al.*, 1998). Changing the concentration of basalt particles in the reactor, for example, to increase abrasion forces also increases the carrier area available for biofilm growth. In models, in turn, there is full control over the system parameters, which allows study of the direct effects of changing specific parameters (see Xavier *et al.*, 2004c).

The most widely used biofilm model at the moment is perhaps the stratified 1D dynamic multi-species model of Wanner and Gujer (Wanner & Gujer, 1986; Wanner & Reichert, 1996). This model is implemented in AQUASIM, a software package for the simulation of aquatic systems (Reichert, 1994). AQUASIM constitutes a valuable tool for describing macroscopic conversions in biofilm systems and, therefore, to the understanding of biofilm processes in a quantitative way. Modeling biofilm structure is even possible using this model, although limited to 1D profiles, i.e. distribution of species, biofilm density and porosity throughout the biofilm depth.

To further understand how other aspects of structure may develop from environmental conditions, time-dependent models in a two-dimensional (2D) or even 3D space are required (Van Loosdrecht *et al.*, 2002). Multi-dimensional (2D or 3D) models provide insight on the structure/activity relationship in conditions closer to reality. The 1D models assume a planar geometry, i.e. homogeneous structure in all directions parallel to the surface of attachment. The 2D or 3D models, in turn, account for the spatial heterogeneity of state variables in multiple directions (substrate concentrations, bacterial species composition, EPS, liquid flow velocities, etc.) and, from this, the biofilm structure is implicitly emerging.

Multi-dimensional models are computer simulations aimed at explaining biofilm development by describing all factors of relevance using first principles. Besides contributing to understanding the role of environmental conditions in structure formation, the effects of lateral gradients and structural elements, such as pores, in the

overall biofilm conversions may also be described. The 2D and 3D models follow a bottom-up approach where large-scale structure results from actions and interactions taking place at a smaller scale. The behavior of the parts at small scale should preferentially be defined without using assumptions of a completely hypothetical nature. These modeling approaches, however, typically require more sophisticated numerical methods and significant amounts of computational power for performing the simulations.

The 2D model of Wimpenny & Colasanti (1997) was the first to attempt a theoretical confirmation that different biofilm structures can be observed for the same microbial system by changing environmental conditions. In this study, a simple cellular automata (CA) model was used, having a fixed set of rules for biofilm growth, to demonstrate that changing the concentration of a rate-limiting substrate can cause morphology to be either penetrated by water channels (for low substrate concentrations) or dense and confluent (for high substrate concentrations). In spite of the ground-breaking merits of this pioneer model, it had serious shortcomings, namely by considering that growth occurs only in the outermost layer of the biofilm, neglecting any growth occurring inside the biomass matrix (Van Loosdrecht *et al.*, 1997). This unrealistic limitation was overcome in a hybrid discrete-differential biofilm model, also based on CA, that used the uncoupling of diffusion–reactions processes from the much slower process of biomass spreading (Picioreanu *et al.*, 1998, 1999). In the CA biofilm model by Picioreanu *et al.* (1998), the pressure exerted by biomass growing in the biofilm depth will generate displacement of cells towards the biofilm–liquid interface. Studies using this diffusion–reaction–growth model and another model including biofilm detachment (Picioreanu *et al.*, 2001) showed that the previously assumed influence of the concentration of a limiting substrate (Wimpenny & Colasanti, 1997) is in fact a particular case of a more general rule where the gradient of substrate concentration together with the biomass detachment forces determine the emergent structure (Van Loosdrecht *et al.*, 1997). Biofilm growth in diffusion-limited conditions originates a rough structure, whereas biofilm growth in growth-limited conditions produces smooth structures.

Multi-dimensional modeling approaches to biofilm structure have since been reported to describe processes such as convective flow (Picioreanu *et al.*, 2000), biomass detachment either due to shear forces (Picioreanu *et al.*, 2001) or chemically mediated (Hunt *et al.*, 2003), shear-induced deformation (Klapper *et al.*, 2002; Stoodley *et al.*, 2002), autoinhibitory behaviour (Chang *et al.*, 2003), EPS production (Kreft & Wimpenny, 2001), a nitrifying system (Kreft *et al.*, 2001; Picioreanu *et al.*, 2004) and an anaerobic biofilm comprising a sulfate reducer and a methanogen (Noguera *et al.*, 1999), and membrane-attached biofilm growth (Noguera *et al.*, 2000).

Some of these approaches to multi-dimensional modeling use techniques other than the previously mentioned "pure" cellular automata (Wimpenny & Colasanti, 1997), where diffusion and reaction of substrates is simulated by Brownian random walks and is coupled to biomass growth (Fig. 2a), and the hybrid discrete-differential cellular automata (Picioreanu *et al.*, 1998), where diffusion and reaction of solutes is represented by partial differential equations while biomass representation is still discrete in space (Fig. 2b). A viscoelastic fluid description of biofilms (Klapper *et al.*, 2002; Stoodley *et al.*, 2002) assumes biomass as a continuum (Fig. 2c) for which the properties of a viscoelastic fluid apply. Another model used a continuum approach based on spreading described by a non-linear density-dependent diffusion mechanism (Eberl *et al.*, 2001).

Individual-based modeling (IbM; Kreft *et al.*, 2001; Picioreanu *et al.*, 2004) describes biomass as comprising spherical particles with positions in space defined by continuous coordinates (Fig. 2d). Each of these biomass particles is an individual that, throughout the process of biofilm development, grows, moves and divides (generating new individuals), but maintains its original identity. IbM allows for individual variability, i.e. each "individual" has its own set of variable parameters. This modeling approach goes one step closer to the aim of modeling biofilm systems from first principles, when compared with cellular automata approaches in which biomass is represented using a discrete grid. The cost to be paid for this detailed level of description is in more computationally demanding simulations.

The fact that several approaches for multi-dimensional modeling of biofilms can presently be found in the literature is indicative of the booming interest in the field. This interest is, on one hand, inspired by new experimental findings and, on the other hand, stimulated by the results achieved by pioneer approaches and, in addition, facilitated by the increasing availability of affordable computational power and new, efficient, numerical methods. Simulations that previously required high-end mainframe computers (e.g. massively parallel machines) run nowadays on an ordinary desktop computer. However, the existing number of different modeling implementations is at the same time indicative of the immaturity of this relatively recent research field. A standardization of methods used for multi-dimensional modeling is highly desirable for the near future, possibly culminating in the adoption of a common modeling framework by many researchers working in this field. Efforts for such standardization are already underway, an example of which is a recently proposed framework that integrates the spherical-particle biomass spreading mechanism of IbM and uncoupled diffusion–reaction processes (Fig. 2e). The framework allows the definition of (2D and 3D) biofilm systems with an arbitrary number of microbial and solute species, providing a structure for multi-dimensional, multi-species dynamic modeling of biofilm systems (Xavier *et al.*, 2004b). The choice of the IbM method of biomass description to be adopted in this framework was based on the fact that this method is better suited to the description of multi-species systems, when compared with other modeling approaches (Kreft *et al.*,

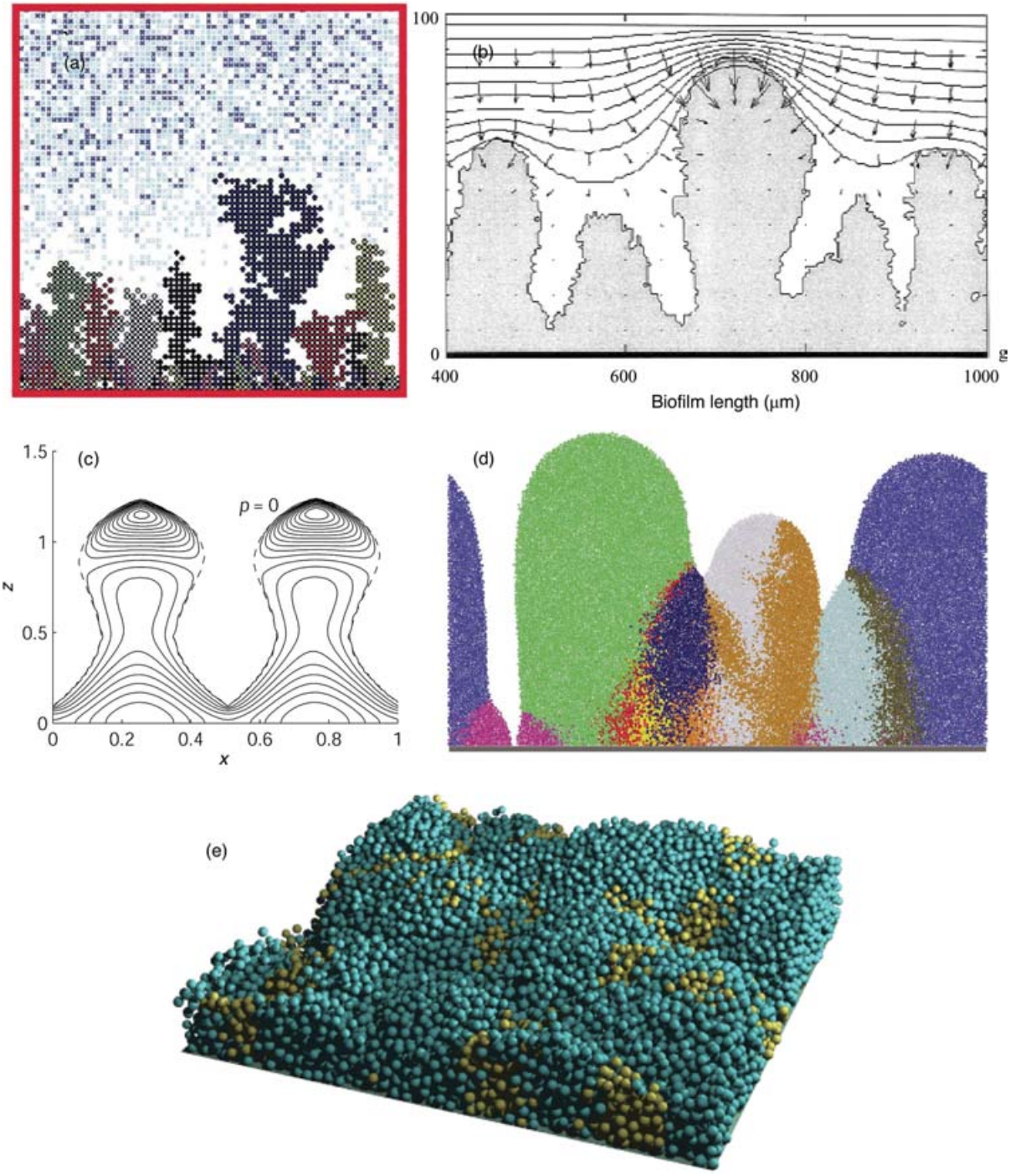

Fig. 2: Examples of multi-dimensional modeling of biofilm structure. (a) A 2D cellular automata model using reaction and Brownian motion for diffusion of solute particles, combined with biomass division at the biofilm surface only (from Wimpenny & Colasanti, 1997). (b) 2D concentration fields for a limiting substrate (lines) and biofilm shape (gray area) computed with a hybrid discrete (CA)-differential model (from Picioreanu *et al*., 1998). (c) Finger formation in 2D continuum model with contour lines showing the pressure (*p*) field (from Dockery & Klapper, 2001). (d) Individual-based model (IbM) simulation showing growth of 10 clones of species 1 (ammonia oxidizer) in differential colours and 10 clones of species 2 (nitrite oxidizer) all in magenta (from Kreft *et al*., 2001). (e) 3D structure obtained using a modeling framework introduced recently (Xavier *et al*., 2004b), which allows both 2D and 3D simulation of multi-species, multi-solute systems, here showing a system of two species (in blue and yellow) competing for the same substrate.

2001). Moreover, IbM allows the inclusion of easily structured biomass features such as the production and excretion of EPS (Kreft & Wimpenny, 2001). Ecological studies of competition and cooperation in mixed species biofilms are also presented in Kreft (2004a,b).

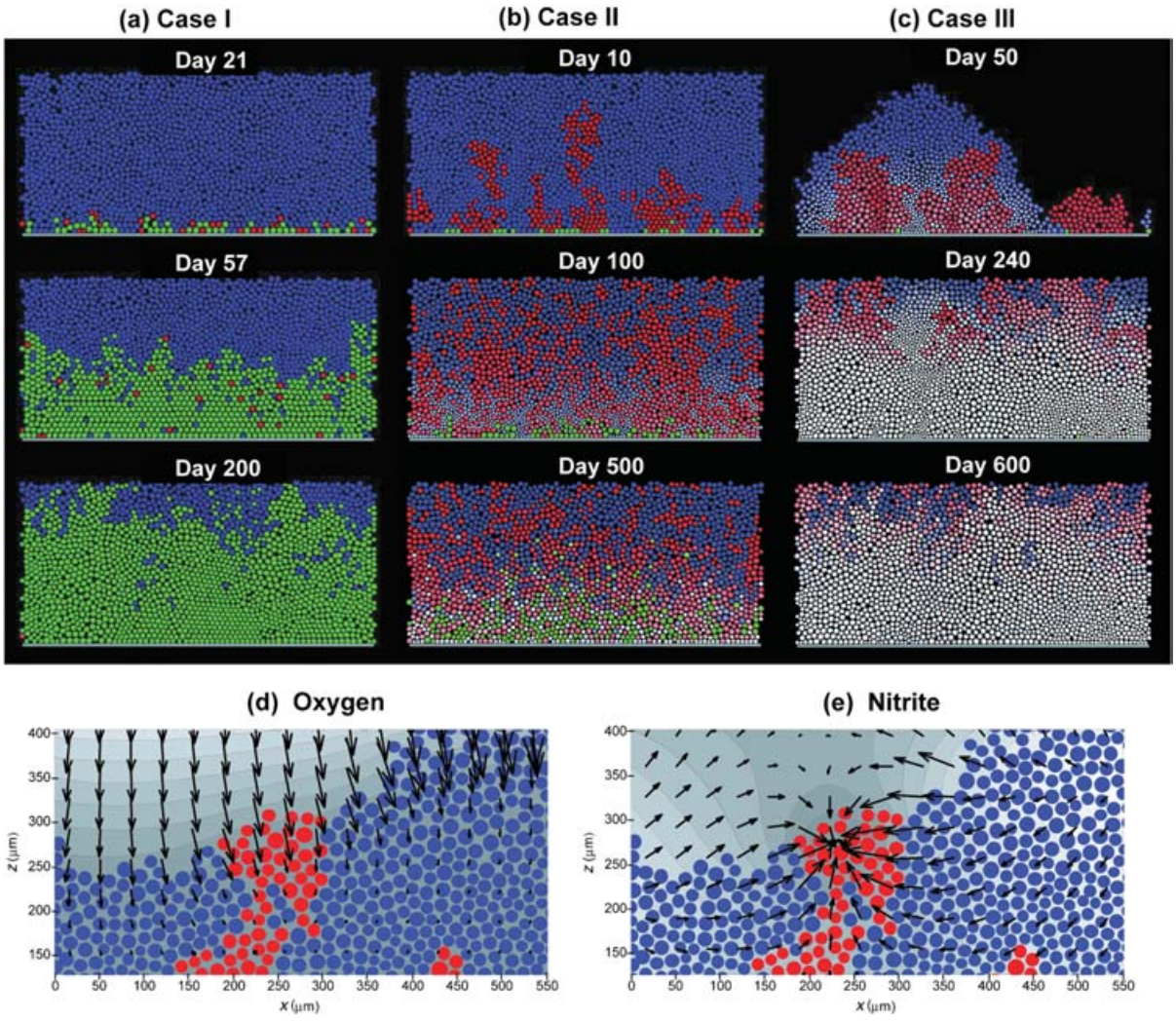

Fig. 3: Modeling a multi-species nitrifying biofilm with aerobic ammonium oxidizers (blue), aerobic nitrite oxidizers (red) and anaerobic ammonium oxidizers (green). The presence of inerts in biomass particles is represented by increasingly lighter colors. (a to c) Results from simulations carried out at different scenarios of nutrient availability. Concentrations of nutrients in the reactor influent are: Case I: oxygen limitation, $C^{(in)}_{S,O_2} = 2\,kg/m^3$, $C^{(in)}_{S,NH_4} = 30\,kgN/m^3$, $C^{(in)}_{S,NO_2} = C^{(in)}_{S,NO_3} = 0$. Case II: mostly aerobic biofilm, $C^{(in)}_{S,O_2} = 10\,kg/m^3$, $C^{(in)}_{S,NH_4} = 30\,kgN/m^3$, $C^{(in)}_{S,NO_2} = C^{(in)}_{S,NO_3} = 0$. Case III: nitrogen limitation, $C^{(in)}_{S,O_2} = 10\,kg/m^3$, $C^{(in)}_{S,NH_4} = 4\,kgN/m^3$, $C^{(in)}_{S,NO_2} = 3\,kgN/m^3$, $C^{(in)}_{S,NO_3} = 23\,kgN/m^3$. (d and e) Detail on oxygen (d) and nitrite (e) fluxes (indicated by the length and direction of arrows) and 2D concentration fields. These pictures illustrate that, although gradients are mainly vertical for solutes that are only consumed by the biofilm, such as oxygen, for solutes that are both produced and consumed, such as nitrite, significant multi-directional gradients may be present (reproduced from Picioreanu *et al.*, 2004).

The following sections provide examples of recent applications of individual-based multi-dimensional modeling in describing structure and microbial ecology in diverse biofilm systems.

MODELING A MULTI-SPECIES NITRIFYING BIOFILM

A nitrifying biofilm comprising three microbial species – aerobic ammonium oxidizers, aerobic nitrite oxidizers and anaerobic ammonium oxidizers – was implemented in a particle-based variant of the IbM approach (Picioreanu *et al.*, 2004). The system described also the decay of active biomass to inert materials and included five solute species – oxygen, ammonium, nitrite, nitrate and N_2. Simulations were performed in three different scenarios of availability of ammonium and oxygen, to evaluate the evolution of the three microbial species present. Stratification of the biofilm according to the presence of electron acceptor and the growth rate of microorganisms was observed, showing different distributions at steady state for the three differently analyzed scenarios (Fig. 3). Comparisons carried out in the same study between 1D (implemented in AQUASIM), 2D and 3D modeling alerted us to the presence of strong multi-directional gradients likely to be found for the concentration of intermediate solutes. This was the case for nitrite, which, being produced by ammonium oxidizers and consumed by nitrite oxidizers, showed significant gradients in the

directions parallel to the solid surface in 2D and 3D simulations, a characteristic obviously not observed in 1D modeling. This comparative study, nevertheless, showed good agreement between all modeling approaches in respect of the overall conversion rates. This indicates that 1D modeling approaches may be sufficient to accurately describe bioconversions occurring in a planar (flat surface) biofilm system. The 2D and 3D approaches, however, provide further insight with respect to local microbial interactions of competition and cooperation occurring in the system. Other recent modeling studies arrived at the same conclusion for monospecies biofilms (Morgenroth *et al.*, 2004) and for heterotrophic–autotrophic biofilm systems (Noguera & Picioreanu, 2004).

MODELING A MULTI-SPECIES METHANOGENIC BIOFILM

Another problem with great potential for spatial modeling is the ecology of methanogenic communities (Picioreanu *et al.*, 2005). Multi-dimensional models are exciting tools for evaluating existing structured models of anaerobic digestion (particularly kinetics), and interpreting results obtained from advanced microbial ecology analysis (using molecular methods). Formation of a layered structure of the granules or biofilms has been proposed, on the basis of the relative kinetic rates of the different steps in anaerobic digestion (AD). AD is a multi-step process, with (in order) extracellular hydrolysis, acidogenesis, acetogenesis, and hydrogenotrophic and aceticlastic methanogenesis. Detailed information on the energetics of methanogenic processes can be found in an excellent review by Schink (1997). A good base to think about these interactions is the anaerobic digestion model proposed by an International Water Association (IWA) task group (Batstone *et al.*, 2002). Acidogenic bacteria convert monosaccharides, amino acids and long-chain fatty acids into short-chain fatty acids such as valerianic, butyric or propionic. Then, in a second step, syntrophic organisms produce acetic acid and hydrogen, which are finally converted to methane. An example of such a simulation including seven bacterial groups and 12 chemical species is presented in Fig. 4. The growth of syntrophic acetogens (propionate, and also butyrate utilisers) is of particular interest. When growing near the biofilm surface (or the edge of the methanogenic granule), these microbes can live without the presence of hydrogenotrophic methanogens, as hydrogen can be wasted to the bulk. It should be noted that releasing hydrogen to the bulk helps only if hydrogen is continuously removed from the bulk, and this is the case assumed here. When growing in the biofilm depth, they survive only in the presence of hydrogen utilizers. This is explained by the fact that the syntrophic propionate consumers are inhibited by the hydrogen produced by themselves and by the sugar-converting acidogens. Therefore, they prefer to grow in the vicinity of the hydrogenotrophs, which in turn depend on the acidogens for hydrogen production. Multi-dimensional fluxes of hydrogen in such a methanogenic biofilm can be seen in the simulation presented in Fig. 4b.

MODELING THE COMPETITION BETWEEN INTERNAL STORAGE COMPOUND-PRODUCING AND EPS-PRODUCING BACTERIA IN BIOFILMS

The potential of the individual-based approach to model biofilm systems with structured biomass was further illustrated in a case study describing the competition between two autotrophic bacterial species differing in their ability to produce either polyhydroxybutyrate (PHB), an intracellular storage polymer that constitutes a reserve of carbon and energy, or EPS. A hypothetical system was proposed (Xavier *et al.*, 2004b), in which the two competing heterotrophs, PHB-producers and EPS-producers, possess the same kinetics for growth and production of a polymer, PHB or EPS, respectively (Fig. 5). For this two-species system, if growth were carried out in a chemostat, identical kinetics would provide microorganisms of the two species with equal competitiveness. In a biofilm, however, if growth were limited by diffusion of oxygen, EPS-producers would have a competitive advantage, as shown by simulations. By spreading more rapidly as a result of the production of a voluminous EPS matrix, EPS-producing organisms would take advantage of higher oxygen concentration at the top of the biofilm, eventually overcoming the PHB-producers. It was further shown by simulations that this competition could be inverted if growth was carried out in feast–famine regimens, where the carbon source was provided in pulses. In such cases, PHB-producers would become the most competitive species thanks to their ability to produce the internal storage compound during the feast phase and later use the stored PHB for growth during the famine phase, when an external carbon source was no longer available. EPS-producers, not possessing the capability to produce storage compounds, would grow only in the feast phase, being subject to starvation and detachment under non-growth conditions in the famine phase. Biomass detachment by continuous surface erosion is implemented using an approach explained by Xavier *et al.* (2004b,c). In a structured representation of biomass, this system considered (a) two solute species (oxygen and a soluble carbon source) and (b) five particulate species (active mass of PHB-producers, PHB, active mass of EPS producers, EPS and inert biomass).

MODELING OF ACTIVATED SLUDGE FLOCS

In spite of not being attached to a surface, microbial aggregates in the form of flocs and granules are often viewed as biofilms, owing to the similarities in terms of physical processes involved in their formation and

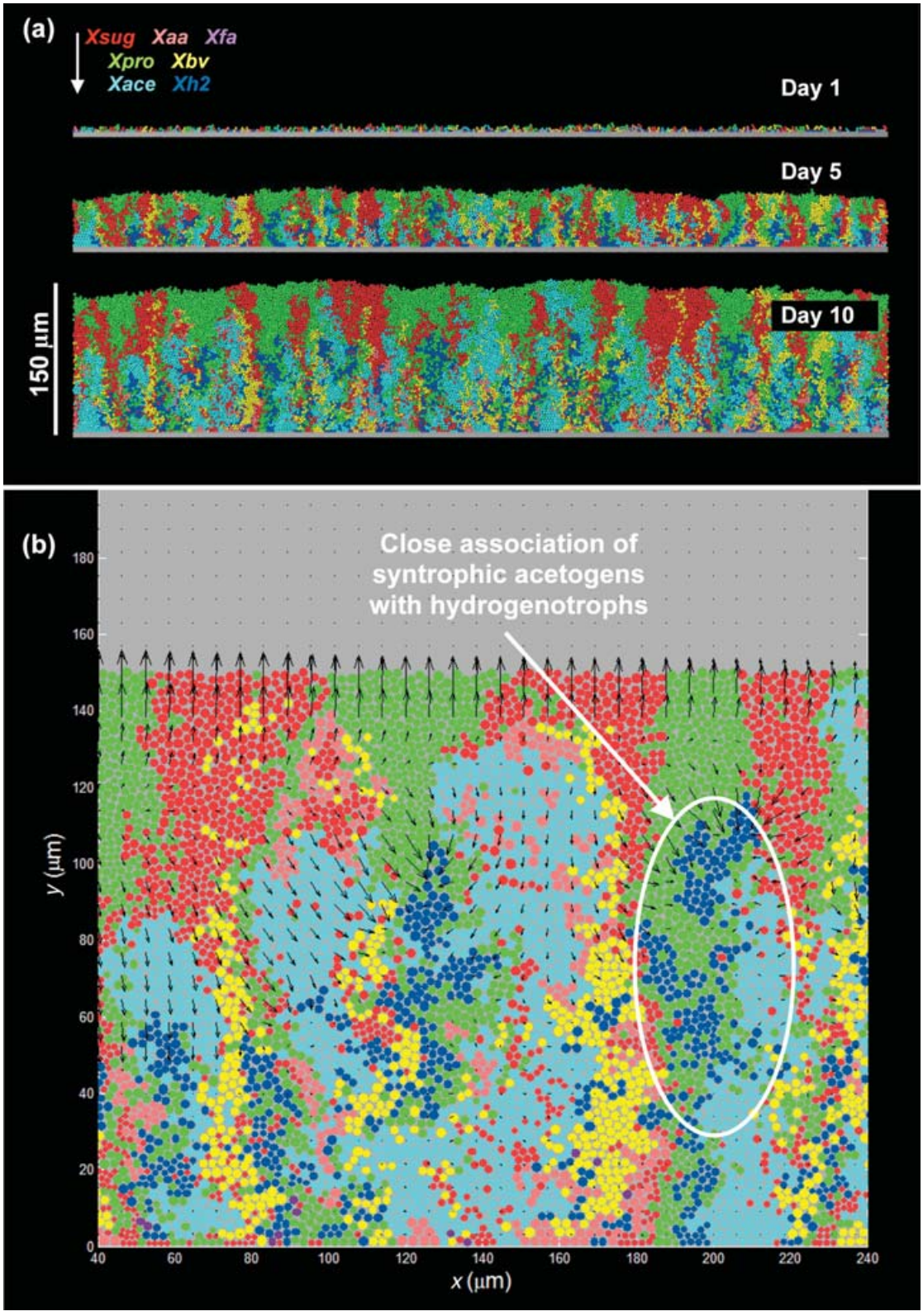

Fig. 4: (a) Simulated development of an anaerobic digestion (methanogenic) biofilm. The seven microbial groups are growing on: sugar (Xsug, red), amino acids (Xaa, pink), fatty acids (Xfa, purple), butyrate/valerate (Xbv, yellow), propionate (Xpro, green), acetate (Xace, cyan) and hydrogen (Xh2, blue). Formation of vertical bacterial clusters and horizontal stratification with fast growing microorganisms near the biofilm surface (sugar and propionate consumers) can be observed. (b) Close-up in a methanogenic biofilm shows intimate association of syntrophic acetogens, inhibited by H_2 (green and yellow), with hydrogenotrophic methanogens consuming H_2 (blue). Arrows indicate the direction and magnitude of hydrogen fluxes.

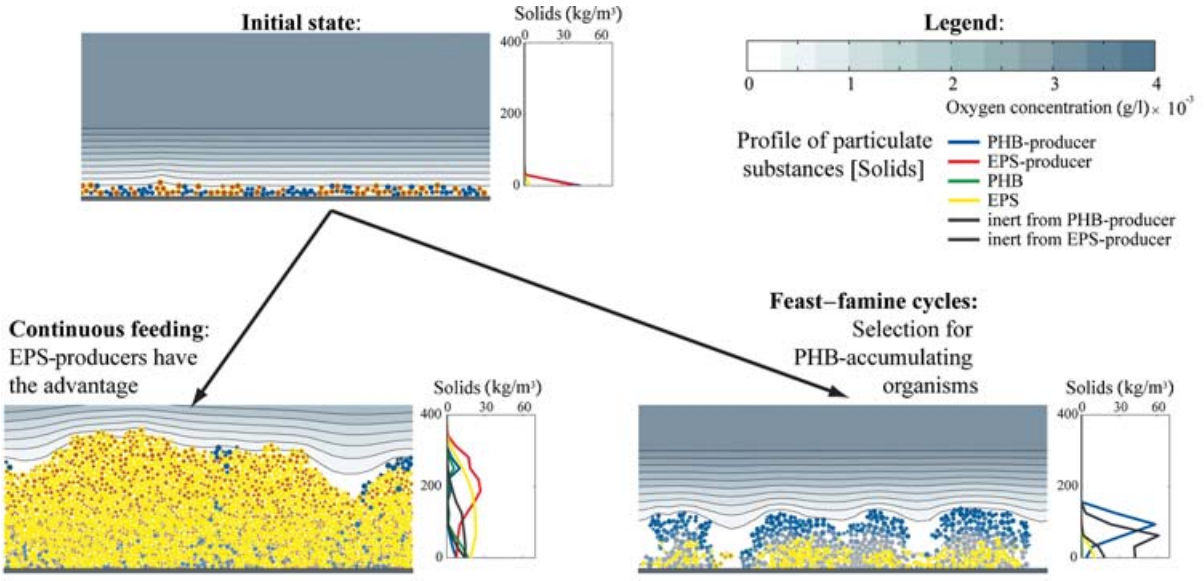

Fig. 5: Results from simulations carried out for a two-species biofilm where the competition between PHB-producing and EPS-producing heterotrophic microorganisms is observed under different substrate feeding regimens (Xavier *et al.*, 2004b). The color-coding is the same for the concentration profiles and for biomass particles in the 2D distribution. The biomass colors become increasingly lighter as the fraction of inert material increases. EPS-producers (in red) have a competitive advantage over PHB-producers (in blue) in the case where carbon source feeding is constant, in spite of having similar kinetics. This advantage is a consequence of the faster spreading EPS-producers producing an EPS matrix (in yellow). By spreading faster, EPS-producers occupy a superior position in the biofilm structure, taking advantage of the of the higher oxygen concentrations at the top. The competition is reversed by growing the community in a feast–famine regimen: PHB-producer organisms accumulate PHB as internal storage during the feast phase and use this for growth during the famine phase, when growth of EPS-producers is halted due to the absence of an external carbon source.

development. This is especially true in respect of the importance of microgradients of solute concentration occurring in these aggregates for the operation of activated sludge processes used in biological nutrient removal systems (Schramm *et al.*, 1999; Meyer *et al.*, 2003). The development of filamentous bacterial structures leads to biological aggregates with low density and poor settling properties, usually called "bulking sludge". Bulking sludge is highly undesirable in activated sludge systems and it is assumed to occur in nutrient-limiting conditions. The individual based model for biofilms was adapted (Martins *et al.*, 2004b) to simulate the formation of activated sludge flocs, including two different bacterial morphotypes: floc-forming bacteria and filamentous bacteria. The different morphotypes were implemented by defining different rules for cellular division. For floc-forming bacteria, directions for cellular division are equiprobable, i.e. there is an isotropic growth mechanism that leads to spherical colonies of floc-former bacteria when no growth limitations occur (e.g. owing to diffusion). For filamentous bacteria, division occurs following a preferential direction. This preferential direction is kept in the "memory" of the filamentous organisms and is invariable in time such that growth originates filament-like colonies. Attachment of individual cells to the aggregate is also considered by periodically letting a fraction of the detached cells of any of the two types stick randomly (after a Brownian motion through the liquid) to cells of the same or another morphotype. Simulations carried out at different regimens of nutrient availability and of attachment showed results in line with experimental observations on the importance of diffusion-limited substrate uptake for the development of filamentous structures responsible for the occurrence of bulking sludge (Martins *et al.*, 2003, 2004a), unwanted in the operation of activated sludge processes (Fig. 6).

MODEL COMPARISON WITH CONFOCAL MICROSCOPY IMAGING

In spite of described progress in the multi-dimensional modeling of biofilms, the necessity of comparing model predictions with experimental data has been raised (Kreft *et al.*, 2001). This was approached in a recent study (Xavier *et al.*, 2004a) where the performance of a 3D model was assessed by comparison with biofilm structure observed experimentally from confocal laser scanning microscopy (CLSM) monitoring of a biofilm grown in a laboratory flow cell.

The model implementation used in this study was the referred framework for multi-dimensional biofilm modeling using the spherical-particle biomass-spreading mechanism of IbM and mass balances for the limiting soluble substrate, including processes of diffusion and reaction. Although the processes involved in the diffusion–reaction processes, i.e. transport and transformation of solute species, are governed by sufficiently well–known physical laws, the biomass spreading, which results from

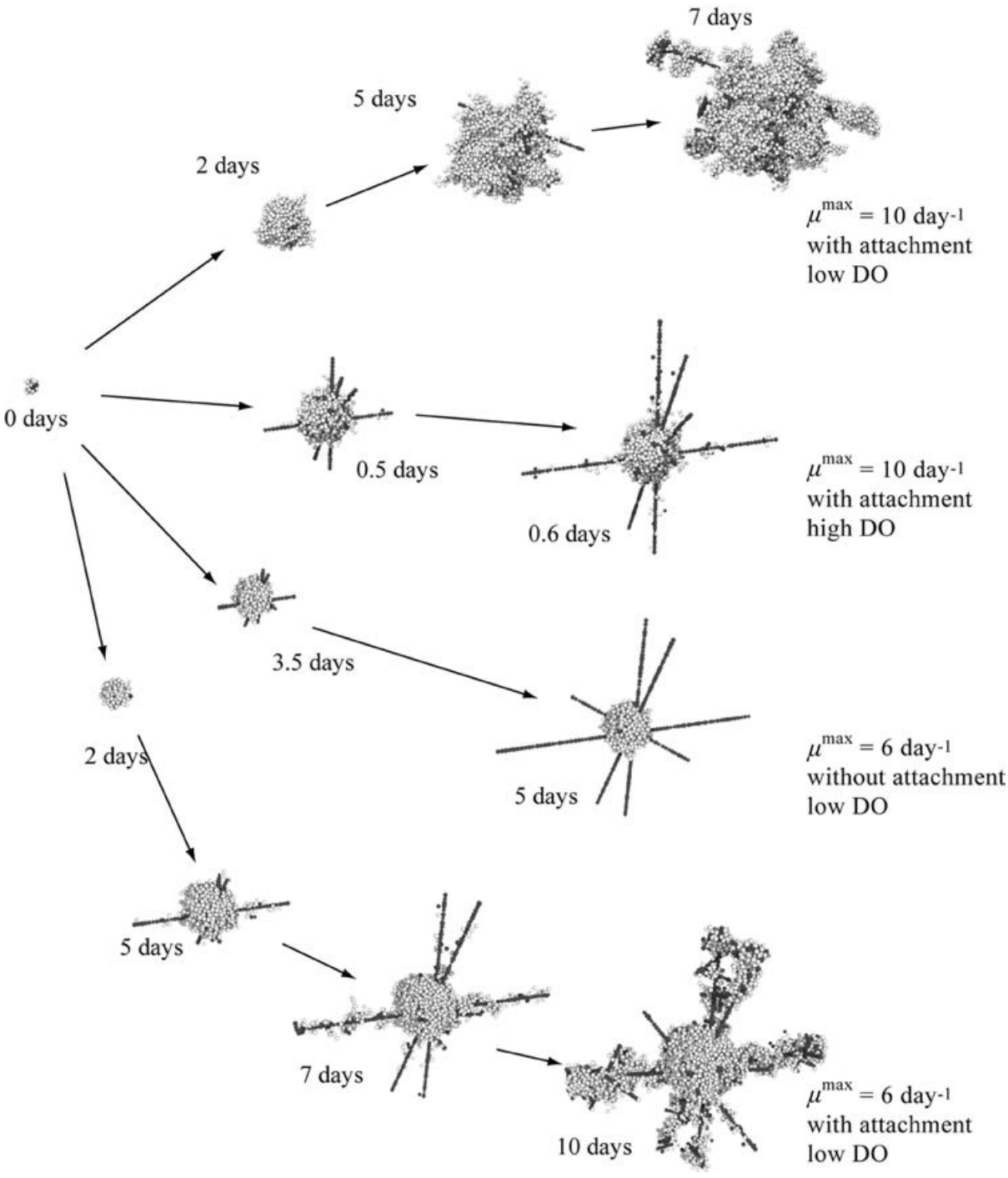

Fig. 6: Simulated activated-sludge floc with dual-morphotype bacteria for four scenarios of different diffusion limitation of solutes (by changing microorganisms maximum specific growth rate, μ^{max}, and concentration of dissolved oxygen, DO) and presence of biomass attachment. Floc-forming biomass is represented by white spheres and filamentous bacteria by dark spheres (adapted from Martins *et al.*, 2004b).

interactions at cellular or cell-cluster scale is much less well understood. However, the biomass-spreading mechanism is of chief importance in biofilm structure prediction. The comparison with experimental data by Xavier *et al.* (2004a) provided a first evaluation of the biomass-spreading mechanism.

CLSM imaging of biofilms is an ideal source of data for the evaluation of 3D biofilm structure model predictions, owing to its dynamic but non-destructive characteristics. However, because of the stochastic nature of biofilm development (Heydorn *et al.*, 2000a), evaluation cannot be carried out by direct comparison

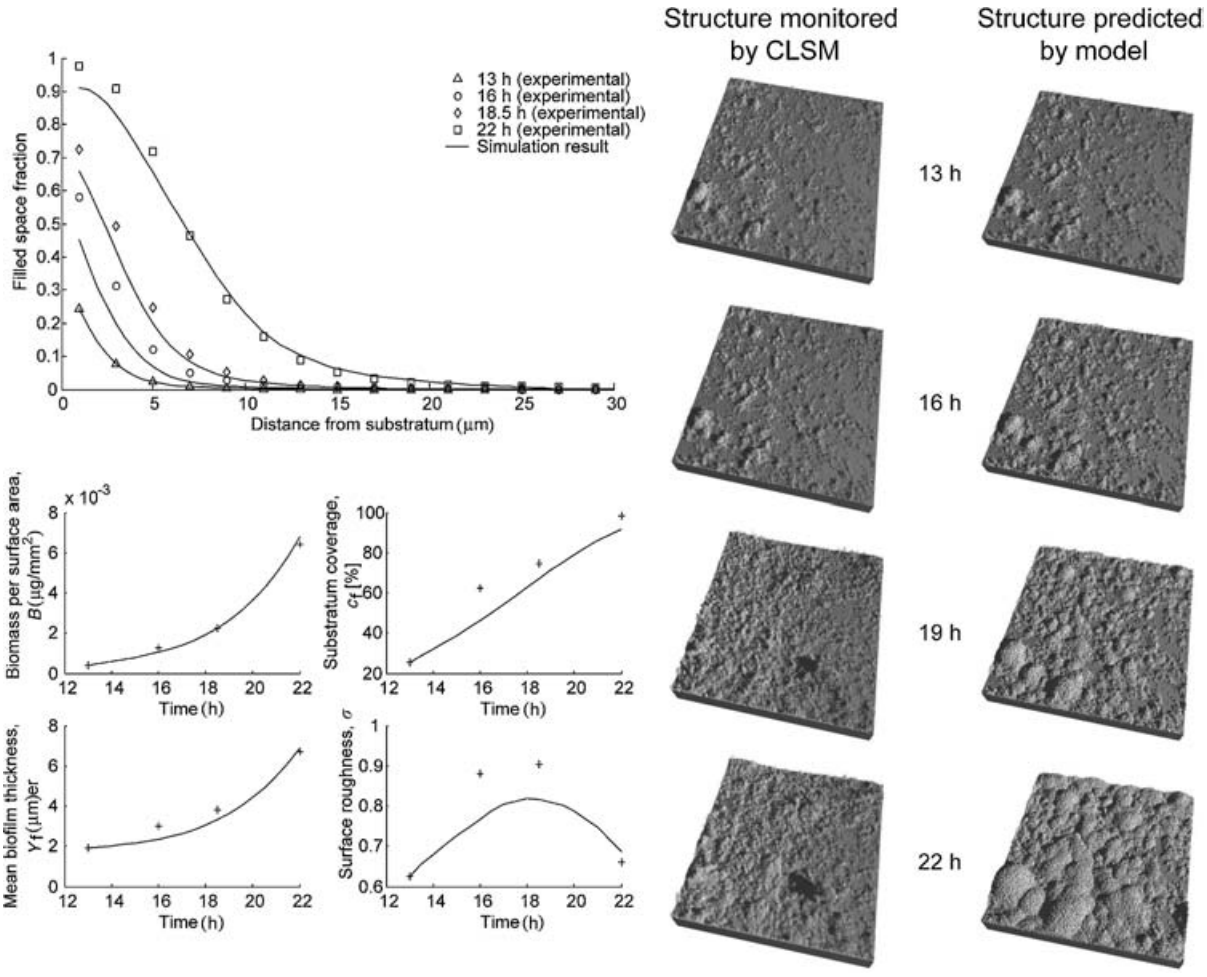

Fig. 7: Results from a study where structure predicted by 3D modeling is compared with data from biofilm monitoring using CLSM (Xavier *et al*., 2004a). The stochastic process of biofilm development, which is also implemented in the model, requires the use of morphology parameters to perform the comparison. The initial biomass distribution for the simulated biofilm is taken from the experimental biofilm.

of the 3D spatial structure. This stochastic nature of the morphogenetic process is also implemented in the biofilm model, namely by using random number generation in some operations involved in biomass spreading (see also Xavier *et al*., 2004c). A quantitative comparison, therefore, imposes the use of morphological parameters such as those described in the literature (Table 1), which, thanks to the similar nature of the 3D data obtained both from CLSM imaging and the model simulations, could be readily determined by procedures derived from automated image analysis (Xavier *et al*., 2003).

This study concluded that the model used is capable of accurate prediction of biofilm structure, as described by a set of morphological parameters such as filled space fraction, total biomass, substratum coverage, mean biofilm thickness and surface roughness (Fig. 7). The study reported by Xavier *et al*. (2004a) further supports the use of the spherical-particle biomass-spreading mechanism as a tool for biofilm modeling.

CONCLUSIONS

Owing to the involvement of biofilms in a large range of human activities, the aim of controlling biofilm formation and growth is common to several areas of research, including bioengineering, public health and microbiology. To effectively control biofilm activity, the processes involved must be understood. Understanding the structure/activity relation, and the numerous processes involved, is at the core of understanding biofilms. In this context, multi-dimensional modeling of biofilm structure constitutes a valuable tool for the investigation of this dynamic structure/activity relation. Since pioneer cellular automata models representing unrestricted growth of single-species biofilms were introduced about eight years ago, multi-dimensional models have progressed to include a large variety of physical processes such as biomass attachment, detachment and fluid flow. In parallel, biofilm models have evolved to increasingly complex representations of mixed-species consortia, including multiple solute species

and bioprocesses and are now suitable for microbial ecology studies. At present, several implementations of multi-dimensional biofilm models may be found in the literature. From these, the individual-based approach is in our view the closest to the goal of modeling biofilm dynamics from first principles. This is reflected in the convenience with which it may be used to implement diverse multi-species scenarios, including the examples mentioned here and the study reported by Xavier *et al.* (2004c).

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