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Biofilm Structure and Activity

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Editorial: advances and challenges in biofilm research

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Introduction *(Prepared by Zbigniew Lewandowski)*

This special edition of *Water Science and Technology* is dedicated to publishing selected papers presented at the IWA conference on Biofilm Structure and Activity, Las Vegas, Nevada, USA, held on 24-26 October, 2004. The title of the conference departed from the conventional phrase referring to biofilm structure and function and, instead, referred to biofilm structure and activity. That departure was intentional, and motivated by the fact that it is difficult to define biofilm function; it can be almost anything we want it to be, such as remediation of toxic substances, microbially influenced corrosion or tooth decay. Biofilm activity, on the other hand, can be defined as the rate of substrate consumption per unit area of the substratum, for example, and it can be quantified. Since parameters characterizing biofilm structure can be quantified as well, biofilm structure and activity can be correlated. Much work has been done recently to quantify the relations between structure and activity of biofilms, and some results of that work are presented here. Quantifying relations between biofilm structure and the rates of various biofilm processes, such as microbial growth and detachment, has developed to a major field of exploration in biofilm engineering, and it affects many other fields of biofilm research. The conference acknowledged the importance of such studies.

Conferences dedicated directly or indirectly to biofilm research are organized by several large national and international associations, such as ASM, NACE and IBBS. In addition, elements of biofilm research can be found at conferences organized by several other large organizations. Biofilm research has become very diverse and includes contributions dedicated to environmental, industrial, and biomedical research from individuals with diverse backgrounds. Because of that, it is difficult for individual researchers to see the entire field of biofilm research, and to identify the most urgent research needs and future studies. We should therefore use our biofilm conferences not only as an opportunity to present recent advances, but also as an opportunity to summarize our knowledge of biofilm processes, to define gaps in understanding, and to suggest directions of future explorations. Such summaries will help us to monitor progress, and will improve communication between our group and other biofilms groups. This paper presents such a summary.

The chairpersons of the various sessions of the conference agreed to prepare informal statements presenting their views on recent accomplishments and on remaining challenges in the areas of biofilm research defined by the titles of their sessions. The following text presents all these opinions assembled, as an overview of recent advances and future needs in biofilm research, or at least in the areas of biofilm research presented at our conference. Although this paper is not a systematic literature review, and the opinions expressed here are personal and informal, since these opinions have come from acknowledged experts in biofilm research they provide a valuable summary of our knowledge of biofilm processes at the time of the conference, and this entire collage of contributions may serve as a guide to the papers presented in the remaining parts of this volume, and as a tool to communicate our position to other groups active in biofilm research.

Biofilm modeling *(Prepared by Eberhard Morgenroth, Oskar Wanner, and Bruce Rittmann)*

Progress

As part of the biofilm modeling session, the IWA Task Group on biofilm modeling presented selected chapters of their report "Mathematical Modeling of Biofilms," which will soon be released as a *Scientific and Technical Report* from IWA Publishing. This report includes a quantitative comparison of existing biofilm models, supports model-based design of biofilm reactors, can be used as basis for teaching biofilm-system modeling, and provides the foundation for researchers seeking to use biofilm modeling or to develop new biofilm models. In the report, each model is described in a common format: (i) the essential features of the model; (ii) the applications for which the model can be used; (iii) the limitations of the model; and (iv) the mathematical treatment of the model.

The Task Group provided an overview on the large number of biofilm models that have been developed in the last two decades. These models range from simple one-substrate, one-species models to complex multi-substrate, multi-species models and from simple one-dimensional analytical expressions to sets of three-dimensional partial differential equations. The Task Group demonstrated applications of the models for the solution of typical problems and also presented the results of the performance of the models in a number of comparative studies (benchmark studies). The Task Group underscored that the question "which biofilm model is appropriate to be used to solve a given problem?" has no single and clear-cut answer. The best answer depends on the specific modeling objective and on the data and the computational resources available.

Challenges

Advanced mathematical models for biofilms help us understand the complex phenomena that occur in biofilms. In recent years, biofilm modeling has rapidly advanced, resulting in a diversity of modeling approaches and tools. Presentations in the modeling sessions of Biofilms 2004 demonstrated the achievements, but also the future challenges of biofilm modeling. Mathematical models are able to describe distribution and competition of different microbial species. Results from mathematical modeling need to be evaluated based on experimental observations, such as by using molecular microbial methods to quantify and localize the distribution of different microbial groups within the biofilm. Mathematical models are able to predict biofilm morphology and how this influences hydrodynamics and external mass transfer. These modeling results need to be evaluated based on experimental observations that evaluate fluid flow and mass transfer at the microscale. Mathematical models are able to provide other outputs: e.g., the development of mechanical strength within the biofilm matrix that could help predict biofilm detachment. Other models describe the porosity of the biofilm, which could result in advective mass transport within the biofilm matrix. Presentations of experimentalists and modelers approached the challenges related to bringing mathematical modeling and experimental observations closer together.

Advances in biofilm modeling have mostly focused on biofilm processes and phenomena at the micrometre or millimetre scale. What are needed now are models able to simulate and predict the behavior of larger biofilm systems and reactors. This could be achieved through the process of up-scaling. Up-scaling is the process whereby macro-scale conservation equations are developed based on averaging or homogenizing the micro-scale phenomena over a representative region of the system. Up-scaling inherently results in the loss of microscale information, but the important features of the micro-scale are kept through the identification of macroscopic parameters that come naturally from the up-scaling itself.

Biofilm reactors *(prepared by I. F. Melo, C. Picioreanu, B. E. Rittmann)*

Progress

The Biofilms 2004 conference in Las Vegas focused on the technological perspective of industrial biofilm systems (biofilm reactors, bioremediation), including the advances obtained in subjects such as modeling/simulation and biofilm structure-activity relationships. The fundamental biological aspects of biofilm formation and activity were also present, but were not the chief purpose of the meeting. The sessions dedicated to biofilm reactors lasted one whole day and included 15 presentations, although several presentations in other sessions also reported important research work on bioreactors.

During the conference, it became clear that modeling has taken a leadership role in biofilm research. Now, models are beginning to include EPS, hydrodynamics in and around the biofilm, shear stress effects, mechanical properties, multiple species performing different roles (e.g., nitrifiers and heterotrophs), and distinctions among microorganisms with supposedly similar metabolisms (e.g.,, antibiotic resistant versus non-resistant bacteria). These added mechanisms make it possible to predict complexity in terms of community structure, community function, and biofilm architecture. We already are gaining important insights into why biofilms are dense versus fluffy, mushroom-like versus block-like, single-species versus multi-species, and strong versus weak.

Experimental work is increasingly employing the rapidly evolving tools of molecular microbial ecology. While not yet routine, these powerful methods are becoming part of the toolkits of many biofilm researchers. While the advances reached with modeling and molecular methods are very encouraging, even more encouraging is a burgeoning trend to combine several types of tools. In particular, combining molecular microbial ecology with mathematical modeling, which is beginning to take hold, help us understand community structure and function together. The systematic and quantitative nature of modeling is the perfect complement to the information obtained from the molecular tools.

As regards Biofilm Reactors, a large diversity of applications is now available in terms of water and wastewater treatment: anaerobic processes, nitrification, denitrification, detoxification, etc. Apart from the traditional biofilm reactors, such as biofilters, fixed-bed and fluidized-bed systems, a significant weight was put this time on the research of membrane reactors as an efficient way to deliver gaseous substrates (H_2, O_2, volatile hydrocarbons). Other interesting engineering applications appeared, such as pressurized biofilters. In the N-removal field, two relatively new areas (reported also last year at the Cape Town Biofilm conference) were the CANON process involving anammox bacteria to convert ammonium to N_2 via nitrite only and the still intriguing nitrification at low pH. Although empiricism and trial-and-error approaches can be seen in many of the studies reported at this conference, the use of mathematical modeling is now visible both to explain the experimental results and to predict biofilm reactor behavior.

The performance of a biofilm reactor is dependent on the structure of the biofilm. Biofilm imaging techniques were further developed. A note can also be made on the establishment of magnetic resonance imaging as a very valuable tool in investigating the biofilm-flow interactions. Another note is on the increasing number of software packages developed for biofilm image analysis, which at this point obviously would require some standardization.

Challenges

The last decade was very rich in advancements on the fundamental knowledge about biofilm biology and biochemistry, served by impressive improvements on the laboratorial research techniques (microelectrodes, confocal laser microscopy, molecular biology methodologies, image processing, etc.) The physical and microbial structure of biofilms was revealed and, in many cases, quantified. Modeling tools enabled researchers to visualize and simulate the effects of external variables on biofilm structure and activity (1, 2 and 3-D modeling, multi-substrate and multi-species modeling, artificial neural networks and individual-based methodologies such as cellular automata, etc.). Developments in biochemical processes occurring in biofilms (e.g., advanced nutrient removal, xenobiotics degradation, interaction with biocides, detachment mechanisms, quorum sensing, role of particulates) and diverse reactor configurations (e.g., moving bed reactors, membrane attached bioreactors, sequencing biofilm batch reactors) continued.

Meanwhile, what has happened in the applied world of reactor design and operation? Did research accomplish anything in terms of fulfilling the needs of practicing engineers? One clear outcome is that many engineering firms are moving away from the paradigm of laminar flow systems (e.g., trickling filters and "fat" biofilms) to turbulent flow reactors (e.g., airlift circulating-bed reactors), which often are more compact and efficient. However, design and operational control of biofilm reactors are still largely based on accumulated empirical knowledge, often leading to over-sizing and instability problems (e.g., biomass excessive growth and sloughing off, gradual or sudden reduction of microbial activity over time).

We are now equipped with more or less sophisticated models that allow us to predict changes in biofilm behavior and in bioreactor performance. But, although predicting such trends is an important step forward, these computational tools do not give us yet absolute values needed to design and operate biofilm reactors in a rational way. In fact, a recurring theme among those who use models is that they need better values for key parameters. More to the point, they need means to gain good estimates of these parameters without having to measure them for every system.

For the purpose of designing and operating biofilm reactors, engineers still have limited knowledge on:

- how to predict the biofilm thickness and density
- how to estimate realistic values of both the mass transfer parameters and the reaction kinetic parameters in biofilms
- how to quantitatively predict the active biomass inside biofilms

Because these parameters change with the hydrodynamic, chemical, and microbial patterns in the reactor, their mathematical description and quantification is needed. Coordinated efforts on these issues should be made in order to accompany the advances that are being (and will go on being) obtained in the modeling, microbiological, biochemical and structural studies of biofilms. An IWA task group focused on such subject might help in organizing the research goals of the different laboratories involved and accelerate the production of reliable and useful data.

Another important issue is the combination of biofilm studies with modern materials, which has been emphasized by the recent developments in membrane-attached biofilm systems. Higher reactor efficiency levels and new biofilm applications will be certainly obtained by increased interfacing with the rapidly innovating field of smart materials (DNA transfer via these materials and rational use of biocides are two possible examples).

There is no doubt that innovation and scientific knowledge in biofilm reactors has been clearly expanding in the fields of water and effluent treatment, as shown in the Biofilms 2004 meeting. Although it does not fit within the scope of IWA, another challenge for the biofilm community is to transfer the advances achieved in the water field to biofilm reactors used for the production of biotechnology specialties. Because pure cultures are often used, prediction of biofilm properties and activity probably can be achieved with good accuracy using the tools already developed for the wastewater treatment reactors.

Bioremediation *(Prepared by Al Cunningham and Erik Arvin)*

Progress

In the 1970s and early 1980s, a large number of ground water sites were beginning to be identified which were contaminated with petroleum hydrocarbons, chlorinated organics, metals, and radionuclides. Remediation protocols for these contaminated ground water sites were gradually established driven by the Resource Conservation and Recovery Act. (RCRA) and the Superfund legislation administered by EPA. By the mid 1980s, leaking underground fuel tanks became one of the most ubiquitous of all subsurface contamination issues. In addition, chlorinated hydrocarbon sites were recognized as some of the most difficult to remediate due to the presence of newly discovered non-aqueous phase liquids (NAPLs).

Remediation protocols which emerged during this period tended to focus on invasive, engineered systems such as pump and treat, excavation, and land farming. But as these sites and others were being investigated and remediation systems were being designed and installed across the country, it became clear that many of these systems were not suitable to clean up aquifers to drinking water standards. By the early 1990s, EPA and the National Research Council found that the nation was wasting large sums of money on ineffective remediation systems.

During the 1990s attention has focused more and more on natural bioremediation as the preferred technology for treating contaminated ground water and soil. Bioremediation relies on subsurface microorganisms, either naturally present or injected, to biotransform organic contaminants into harmless by products. In the case of radionuclides and heavy metals, microbial activity can result in removal of the contaminants from solution by chemical precipitation and sorption to soil or microbial biomass (Lau *et al.*, presented at this conference). A proper application of natural bioremediation requires a thorough understanding of the physical, chemical, and biological mechanisms which affect rates of migration, degradation, and ultimate remediation. Many of these complex mechanisms are being evaluated at actual field sites or in supporting laboratory studies although many challenges remain.

Challenges

The IWA International Conference, Biofilms 2004, Biofilm Structure and Activity, addressed some of the important biofilm-related challenges in natural and engineered systems. In porous media, as in other aqueous environments, microbial consortia dynamics, mass transport and hydrodynamics are intrinsically interrelated. Microbial cells may exist in suspension or adsorb firmly to solid surfaces comprising the effective pore space. If favorable transport of growth nutrients persist cells growth will occur and biofilm accumulation will increase. If organic contaminants serve as growth nutrients then the rate of contaminant biotransformation (i.e. bioremediation) may increase. However the overall rate of biotransformation kinetics is affected by a variety of specific environmental conditions such a contaminant biodegradability (Arvin and Broholm, presented at this conference), microbial inhibition, (Choi *et al.*, presented at this conference), and interspecies competition (Jiang *et al.*, presented at this conference).

If biofilm accumulation causes a reduction in pore space, local changes in mass transport and pore velocity will result thereby further influencing consortial dynamics including cell detachment and transport. The complexity of these interactions is further amplified by the introduction of a variety of xenobiotic materials (contaminants) resulting from human activity. The response of microbial consortia to xenobiotic challenges, like many complex biological phenomena, involve interrelated flow, transport, and reaction processes which occur over several different length scales. Typically, the contaminants consist of a complex mixture of specific compounds (oil, creosote, etc.) which makes quantification of the removal processes very difficult. These and other related interactive processes must be more clearly understood to advance the science of bioremediation.

Scientists and engineers continue to conduct research which addresses the multiphase, and multi-component nature of bioremediation. In the subsurface contaminants can partition between the soil, vapor, aqueous and NAPL phases there by creating source zone areas with non-aqueous phase liquids (NAPLs), soil-sorbed organics, and vapors in the unsaturated zone. LNAPLs, which float on the water table, and DNAPLs, which sink to the bottom of an aquifer, can leach multiple contaminant species (i.e. multicomponents) into ground water aquifers for decades. The potential for bioremediation is profoundly affected by the nature of the multiphase partitioning process. Specialized remediation schemes, which might involve a variety of methods for a mixture of chemicals, must now be evaluated in complex ground water settings. New and emerging methods and models must be considered in order to address and possibly control complex NAPL source zones and the dissolved contaminant plumes which they generate.

Considering the complexity of bioremediation in the subsurface, as well as in engineered systems, it is not surprising that bioremediation often does not lead to compliance with drinking water applications. Therefore in practice, the goal may be maximum mass removal within the economic resources given. In order to apply the treated water for water supply purposes, it is important to develop cost effective methods - including biofilm processes - for in-situ and ex-situ remediation of contaminated groundwater. This also requires thorough understanding of the fundamental processes that control treatment efficiency.

Biofilm structure *(Prepared by Per Halkjær Nielsen and Haluk Beyenal)*

Progress

The term "biofilm structure" is usually applied to describe either the composition of the microbial population in the biofilm community or to describe the physical structure of the biofilm. Here we focus on recent developments and perspectives related to the measurements and quantification of the heterogeneous physical structure of biofilms. Over the years the physical shape of biofilms has been characterized as flat, dense, tulip or mushroom-like, filamentous with streamers, and mosaic. In order to investigate in detail factors of importance for the physical structure of biofilms and to compare results from different biofilm studies the need arose for methods to compare different types of biofilms in a reproducible manner.

The recent development in quantification of numerical structure parameters can now be used 1) to compare the structures of different biofilms; 2) to test the reproducibility of biofilm structure; 3) in monitoring temporal variations in biofilm structure; 4) in testing the effects of various substances, including antimicrobials, on biofilm structure; 5) to quantify the effects of environmental factors on biofilm structure; and 6) in computing parameters characterizing biofilm structure for biofilm modeling or using biofilm structure in biofilm modeling.

In particular the use of CSLM can provide data about the physical heterogeneity of biofilms. The data sets require digital image analysis for both visualization, quantification and to obtain structural parameters such as volumetric porosity, fractal dimension of the cell clusters, roughness of the biofilms, diffusion distance etc. However, the calculations resulting in these parameters are not trivial. Several software packages have been developed for biofilm structure quantification such as Image Structure Analyzer (ISA-2), COMSTAT, Confocal Analysis (ConAn), PHLIPS, Microstat, Quantimet and Image

Bioinformatics. The software calculates various parameters; biovolume, volume to surface area ratio, porosity, average and maximum diffusion distances, surface area between biomass and voids, fractal dimension, average run lengths (in X, Y and Z directions), aspect ratio, mean thickness, maximum thickness (averaged over a defined percentage of maximums), roughness coefficient, textural entropy, homogeneity and energy. It seems that the number of the calculated parameters is increasing as well as the number of software available on the market. Although there are many parameters calculated by the different software packages, we still do not know which parameters are most representative of biofilm structure, and likewise, their relevance to the underlying biofilm processes is not clear. In the future there will most likely be more parameters to be calculated and the correlation between calculated parameters and the overlying process needs to be investigated. However, interestingly, currently none of the biofilm models use the quantified structural parameters. It will be very useful if the current biofilm models accept the quantified structural parameters and also predict the activity of the biofilms. This approach may help us to better understand the relevance of these parameters to biofilm processes.

Challenges

A future challenge is to relate differences and changes in the physical structure of the biofilm to the actual microbial populations, as well as their heterogeneity and response to various environmental parameters. It is known from studies of pure cultures that different species (or different mutants of the same species) form different types of biofilms.Therefore, it is important to know whether changes in biofilm structure caused by for example variations in shear or loading are related primarily to a change of the microbial population or to changes of the physico-chemical properties of the same population in the biofilm.

In order to quantify the physical structure of the biofilm it is essential to apply reliable "probes" such as specific stains and oligonucleotides or other methods to detect the heterogeneity and, in some cases, also specific components in the biofilm. Although many probes are available, a great challenge is to develop new probes that can better quantify various components, such as specific components in the EPS matrix. Such probes can be used together with a quantitative description of the microbial population composition by FISH for a detailed study of the heterogeneity.

Besides CSLM other novel methods are very promising in obtaining data on biofilm structure. Recent developments in nuclear magnetic resonance (NMR) imaging are enabling the researchers to visualize and quantify liquid flow in biofilms at higher resolutions compared to previous studies. Detachment rates and local flow velocities can be measured. NMR can also be combined with CLSM to visualize spatial and temporal variations of metabolites within biofilms, but the relatively low sensitivity still makes it difficult to analyze concentrations typical of many biofilms.

Biofilm ecology and biofouling *(Prepared by Stefan Wuertz and Eugene Cloete)*

Progress

Microbial processes and interactions in biofilms are relevant to technical applications in terms of materials and energy flow leading to either the formation of biofilm or its sustained activity. They are important for alleviating conditions that may lead to unwanted biofilms and understand their relative contributions, for example, to deterioration of drinking water distribution systems or heating systems. For biofilms in water or wastewater treatment applications the ability to exploit microbial processes is crucial to pushing technical limits of existent facilities.

Recent advances in our understanding of fundamental processes like adhesion to interfaces and how microbial populations are organized in biofilms have come from a polyphasic approach that utilizes complementary experimental techniques. Polyphasic means that in addition to describing diversity and numerical abundance of microbial species some measure of function and eco-physiological interactions among community members is explored.

The combination of fluorescent in situ hybridization (FISH) for numerical and spatial characterization with ex situ population analysis tools like denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) for microbial diversity profiles has become more common in lab-scale or higher scale bioreactor studies. A recent example is the discovery of the abundance of *Crenarchaeota* in low-temperature anaerobic wastewater treatment biofilms (Collins *et al.*, presented at this conference). Knowledge concerning the presence or absence of microbial cells alone does not account for their functional role in a biofilm, even if the exact spatial location is known from images obtained by confocal laser scanning microscopy.

The increasing use of functional probes like microelectrodes to measure chemical gradients (e.g. Okabe *et al.*, presented at this conference) and micro-autoradiography (MAR) to demonstrate specific substrate uptake population (e.g. Nielsen and Nielsen, presented at this conference) in conjunction with FISH and other population analysis methods has opened the door to experimental verification of microbial processes that involve different nutritional groups and which are presently included in biofilm models. An example is the utilization of microbial soluble products released during decay of ammonium and nitrite oxidizing organisms by filamentous heterotrophic bacteria belonging to the *Chloroflexi* (green non-sulfur bacteria), which formed in close proximity to nitrifiers (Okabe *et al*, presented at this conference).

Challenges

Microbial ecologists studying biofilms seek to understand the structure and function of the complex biofilm community, the long-term population dynamics, and the activity of individual organisms within the community. Progress has been slowed by the lack of suitable techniques available to experimentalists.

New methods continue to arrive, and one that deserves attention is field emission scanning electron microscopy (FE-SEM). Two different types of appendages have been demonstrated in *Acinetobacter* sp. Tol 5 that can facilitate long-distance and shorter-distance interactions between cells and substratum (Ishii *et al*, presented at this conference). The action of the long, straight and unbranched anchor-like appendage appears to be independent of ionic strength in the surrounding medium and hence cannot be explained by the DLVO theory. Importantly, the appendages were produced only in the presence of a carrier material and adhesion was strongly dependent on the presence of toluene (Ishii *et al.*, presented at this conference). This recent discovery and the previous examples of polyphasic approaches exemplify the impact that advanced experimental methods can have on biofilm ecology research, either because they provide evidence for microbial interactions that could otherwise only be surmised or because they lead to truly new insights that spark further investigations.

In the future nuclear magnetic resonance microscopy (NMR) may provide a significant contribution, in combination with spectroscopy, toward the understanding of adherent cell metabolism. NMR has a lower spatial resolution than confocal laser scanning microscopy, but as noninvasive technique nevertheless offers a wealth of insights. For live cells, NMR can provide information about metabolite content useful for metabolic pathway and solute flux studies. It also measures diffusive and convective mass transport and water compartmentalization, without being subject to opacity losses and light scattering effects.

Ecology, when studied from this vantage point, can be simplified as an attempt by engineers to either deprive microbial communities of those constituents that lead to significant biofilm formation and diversification, or to optimize biofilm microbial structure and architecture so that pollutant removal processes can occur at higher rates. In this regard the chemical-physical conditions within which the biofilm is formed play an important role. These factors include hydrodynamics that influence, for example, shear stress, the chemical quality of the water, temperature, the presence or absence of oxygen, light conditions and the surface onto which the microorganisms will attach.

The challenges that lie ahead are best seen when turning to evolving biofilm models. Extracellular polymeric substances are beginning to be modeled and their visualization is changing our view of biofilm architectures. Some efforts are underway by experimentalists to account for these important structural components (e.g. Neu and Staudt, presented at this conference). Yet much needs to be done to bring experimental work closer in line with the impressive advances in biofilm modeling. For example, there is an opportunity for inter-laboratory comparisons to study the reproducibility of basic biofilm architectures. Even small flow cells that can be operated on a movable microscope stage may lead to diverging biofilm structures due to unpredictable sloughing events over time, or when different flow cells that have been operated as replicate experiments are viewed under high magnification (GrayMerod *et al.*, presented at this conference). The reasons for the variability of nascent biofilm architectures must be understood before the role of microbial community structure and microbial interactions at the microscopic level can be fully explored. Biofilm modeling involves a variety of spatial and temporal scales and the greatest challenge lies in accounting for microbial ecology in all those different situations where biofilms are important in technical systems, ranging from biofouling and biocorrosion to a full-scale wastewater treatment system.