Biofilms (2005) **2**, 245–273 © 2006 Cambridge University Press
doi:10.1017/S1479050506001992 First published online 26 May 2006 Printed in the United Kingdom

Abstracts of an international conference "Biofilms II: Attachment and detachment in pure and mixed cultures", held at the Leipziger Kubus UFZ Centre for Environmental Research, Leipzig, Germany, from 23 March to 24 March 2006

The effect of growth and detachment on formation of large-scale biofilm structure

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The importance of biofilm structure on the performance of biofilms is well known. However, how structures develop during the maturation of biofilm is not clear. In this study, we evaluated the effect of biomass development on the evolution of biofilm structure at the square centimeter level. Specifically we focused on the development of large-scale structural features such as elongated colonies or streamers, ripple structures and areas with particularly little biomass (e.g. previously detached areas). The term "structure" is defined here as the three-dimensional morphology of a biofilm whereas "texture" reflects the structure of a biofilm in an image.

Biomass and textural information were obtained by digital image analysis from a time series of images grabbed with a desktop scanner. The scanning method allows the rapid acquisition of images covering areas at the square centimeter level and was verified elsewhere. It could be shown that the grey scale on images correlated well with average biomass and biofilm thickness. The challenge in the collection of thousands of images lies in the evaluation of the information they contain. Without automated and systematic quantification of features of interest their informational content may be reduced to anecdotal evidence based on the manual interpretation of a limited number of images. Using the image analysis software Visilog Xpert 6.2, procedures were developed to perform fast Fourier transforms (FFT) and construct and analyse spatial grey-level dependence matrices (SGLDM) on images. These procedures allowed automated texture analysis of the biofilm images.

As a result of the analysis of FFT spectra, the development of texture gradients could be detected and quantified on slides incubated in an annular reactor. Directionality of biofilm growth at an angle of $69.5^\circ \pm 0.5^\circ$ (mean ± standard error) became more pronounced over time, mirroring the direction of flow. The growth direction could be confirmed by textural analysis of biofilm images. Texture on biofilm images was evaluated using SGLDM constructed for various directions and feature lengths. The SGLDM approach used in this study significantly extends the reported procedures for textural analysis of biofilm images in the literature. We showed that textural properties such as energy, inertia and textural entropy calculated from the images are sensitive towards directions and length. Distinctive behaviour of these parameters in the 70° direction indicated elongated features with a length between 100 and 200 μm and corresponds well with FFT analysis. Values for directionality and length calculated from the SGLDM reflected features visible on the biofilm images. Thus the results encourage the use of textural descriptors for the automated evaluation of structure development. Currently the correlation

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

between the development of textural properties and biomass development is being evaluated.

Biofilm cohesive energy density determination using a novel atomic force microscopy methodology

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It is essential to understand biofilm stability both to encourage biofilm maintenance in some applications, such as waste treatment, and effectively to remove undesired biofilm in others, such as biofilms covering medical implants. Whereas factors responsible for biofilm growth are well studied, those controlling the detachment process are not clearly understood. Understanding the cohesive interactions in the biofilm matrix could lead to the design of new strategies for controlling biofilm development. In this study we developed a novel atomic force microscopy method and reproducibly measured, *in situ*, the cohesive energy density of moist biofilms via the frictional force by inducing abrasion with a raster-scanned tip under an elevated load. The volume of biofilm displaced and the corresponding frictional energy dissipated were determined at different biofilm depths and the cohesive energy density was then calculated. Our results showed two distinct values of cohesive energy density with biofilm depth. We found that the cohesive energy density was approximately 20 times higher at the cell surface as compared with the material, probably extracellular polymeric substances, surrounding the cells. Ongoing experiments in our laboratory are focussed on applying this method to verify this hypothesis and to study the cohesive energy density of biofilms grown under different conditions. This method could also be used to investigate biofilms subjected to treatment with different biocides in order to determine how best to remove them. Such research will improve our understanding of biofilm cohesion and help the design of new strategies for controlling biofilm development.

Fluorescence correlation spectroscopy under two-photon excitation for the study of diffusion and reactivity of bacteriophage inside bacterial biofilms

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Fluorescence correlation spectroscopy (FCS) has emerged as an ultrasensitive technique for monitoring molecular dynamics operating at the single-molecule level. In association with two-photon excitation (TPE), this fluorescence emission analysis has opened new opportunities for the study of biological systems. Indeed, owing to the very weak concentration of fluorophores used and the small volumes probed, FCS allows non-invasive *in vivo* studies. We have recently demonstrated that FCS under TPE can be applied successfully to characterize the penetration and diffusion capabilities of fluorescent nanoprobes inside microbial biofilms. The formation of biofilms is an

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

extremely common phenomenon associated with significant problems in medical, industrial and environmental areas. These problems include the possibility of biofilms trapping toxicants or hostile microorganisms such as bacteriophage or viruses, depending on the steric and physicochemical interactions with the adherent cells and the organic matrix.

Our method of FCS under TPE has been applied in this context to analyse the diffusion and reactivity of phage C2 (a prolate-type bacteriophage with a long and non-contractile tail) with *Lactococcus lactis* biofilms selected for their different sensitivity to the bio-contaminant. The study was extended to a *Stenotrophomonas maltophilia* biofilm in order to characterize the role of the exopolymer matrix in phage retention inside the microbial structure. Results of fluorescence intensity vectors and autocorrelation curves for phage C2 through the biofilms was compared with the curves obtained for the free molecule.

Biothermodynamic characterization and dynamic analysis of biofilms using calorimetry

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Microbial communities grow more stable when they are associated on surfaces or in aggregates. This advantage of biofilms may be exploited technically in the degradation of xenobiotics or biocatalysis, where fixed biomass has the added advantage of easier separation of excreted products. Biofilm on technical surfaces often incurs costs owing to, for example, microbially introduced corrosion, fouling, restricted flow or reduced heat conductance. In all these cases biofilm control requires cheap, reliable and non-invasive real-time monitoring methods.

One idea for a real-time detector is based on the fact that the maintenance of highly ordered biofilm structures gives rise to the permanent dissipation of the Gibbs energy of the assimilated substances, mostly as heat. The heat production thus reflects changes in the biofilm activity in real time. Furthermore, enthalpy balances have the potential to reveal unexpected material fluxes because they fulfil Hess's law, i.e. the amount of heat involved in the reaction is the same, whether the reaction takes place in one or several steps.

To test the applicability of calorimetric analysis, a pure culture biofilm of *Escherichia coli* DH5α was investigated in an established flow-through calorimeter. Biofilm poisoning and detachment events were indeed reflected by measurable changes of heat production within a few minutes.

Although these results are promising, established calorimeters are not flexible enough and are too large and too costly for routine biofilm control in technical installations. Miniaturized, silicon-chip-based calorimeters appear to be better suited because they combine small size with extremely short response times and potentially low costs, all of which facilitate their integration and use in technical systems [1]. Furthermore, their modular set-up allows the establishment of biofilms on surfaces exposed to environments of interest, followed by their integration and measurement inside the central calorimeter unit [2]. This facilitates the high throughput analysis of biofilm samples. Experiments demonstrating the great potential of chip-calorimetry were presented.

REFERENCES

1. Lerchner, J., Wolf, A., Wolf, G., Baier, V., Kessler, E., Nietzsch, M. & Krügel, M. (2006) A new micro-fluid chip calorimeter for biochemical applications. *Thermochimica Acta*, in press. (On-line 2005: doi 10.1016/j.tca.2005.07.011.)

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

2. Maskow, T., Lerchner, J., Peitzsch, M., Harms, H. & Wolf, G. (2006) Chip-calorimetry for the monitoring of whole cell biotransformation. *Journal of Biotechnology* **122**, 431–442.

Biomimetic antifouling coatings for sensor surfaces for water monitoring: performance control in defined biofilm cultures and under real environmental conditions

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The goal of this study was to create a thin but very stable antifouling barrier on different sensor surfaces. With this aim, we used biomimetic surface coatings on a base of archaebacterial tetraetherlipid, which is the material of choice because it has a highly effective specific anti-fouling action with the respective mediums. The new tetraetherlipid-coated sensor surfaces showed a successful reduction in biofilm formation in a mixed culture of water-dwelling bacteria, as well as under real environmental conditions [1].

The tetraetherlipid (caldarchaeol) was isolated directly from a lipid extract from the archaeon *Thermoplasma acidophilum* via column chromatography followed by pre-activation procedures that allowed chemical coupling of the lipid to glass surfaces. Subsequently, the outward-facing head groups of the lipid layer were further modified in order to obtain surfaces with different physical and chemical properties, for example by introducing a flexible, hydrophilic polyethylene glycol spacer or charged molecules such as cholamine or taurine [2].

A comprehensive material characterization using confocal scanning laser microscopy and surface energetic and electrokinetic analyses confirmed the surface modification. The evaluation of the established physicochemical surface qualities was performed by means of existing models that describe biofilm formation and growth. Performance control was based on the application of a water model for the simulation of a specific microbiological situation and the realization of defined *in vitro* cultures using laboratory bioreactors. A new laboratory test system for the assessment of antifouling coatings was based on different primary colonists of river water, drinking water and waste water. Dynamic bioadhesion tests on lipid-modified surfaces were realized for pure and mixed cultures. In addition, field tests under real environmental conditions of water obtained from the Neustadt/Thuringia dam were carried out to analyse long-term biofilm formation.

Biomimetic coatings consisting of monolayers of modified tetraetherlipid molecules (caldarchaeol) could be prepared on sensor surfaces. In the dynamic test procedure under defined laboratory conditions a reduced bacterial adhesion for the different modified surfaces could be shown. Results of biofilm tests under real environmental conditions confirmed the observed antifouling effect. The selected experimental approaches based on pure and mixed biofilm cultures could therefore be directly evaluated in comparison with the established performance control under real environmental conditions.

When we looked at the chemical flexibility and the mechanically stable covalent fixation on sensor surfaces, a significant improvement in sensor quality caused by the antifouling capacity of the new coatings was demonstrated. Furthermore, it was shown that the complexity of the established biofilm model is of great importance in obtaining reproducible results corresponding to the practice.

This work was supported by the BMBF (02WU0294–8).

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

REFERENCES

1. Frant, M., Stenstad, P., Johnsen, H., Dölling, K., Rothe, U., Schmid, R. & Liefeith, K. (2006) Anti-infective surfaces based on tetraether lipids for peritoneal dialysis catheter systems. *Materials Science and Engineering Technology*, in press
2. Bakowsky, U., Rothe, U., Antonopolous, E., Martini, T., Henkel, L. & Freisleben, H. (2000) Monomolecular organization of the main tetraetherlipid from *Thermoplasma acidophilum* at the water–air interface. *Chemistry and Physics of Lipids* **105**, 31–42.

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The contribution of *rpos* to formation of *Escherichia coli* biofilms

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The creation of starved, stationary-phase zones in biofilms seems to be a significant factor in biofilm formation. In this study, the role of the *rpoS* gene in *Escherichia coli* biofilms was investigated because it is known to be expressed during entry into stationary phase and stress conditions.

To assess the importance of the *rpoS* gene in biofilm formation, we used *E. coli* K-12 mutant strain MG1655 *rpoS* in our flow chamber experiment. We applied confocal laser scanning microscopy (CLSM) with LIVE/DEAD staining to show the role of the *rpoS* gene in *E. coli* biofilms. We found that the *rpoS* mutant can form only thin biofilms with large void spaces inside. We also found many actively moving and rotating cells on glass surfaces at the early stages of biofilm formation. To further assess the role of the *rpoS* gene in *E. coli*, we performed DNA microarray analysis, which revealed that the pattern of gene expression of the *rpoS* mutant was different from that of the wild-type strain. In the stationary phase, 193 genes were significantly down-regulated in the *rpoS* mutant, including genes induced in starvation conditions (*dps, psiF, phoH*), genes encoding heat shock proteins (*ibpA, hslJ, dnaK, clpB*), genes induced at high temperature (*rpoD, rpoH*), and osmotically inducible genes (*osmY, osmB, osmC*). On the other hand, 86 genes were significantly up-regulated in the *rpoS* mutant, and 19 of those were genes involved in energy metabolism.

These results suggest that the *rpoS* mutant is less capable of response and adaptation to stresses than is the wild-type strain in stationary phase, which might be the reason for the formation of only thin biofilms and the presence of void spaces inside the biofilms. In addition, they also suggest that the *rpoS* mutant shows too much motility even in the stationary phase. It could explain the presence of the actively moving and rotating cells in the early stages of biofilm formation, which may be the reason why the *E. coli rpoS* mutant is incapable of establishing mature biofilms.

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Synergistic effects in mixed *Escherichia coli* biofilms: conjugative plasmid transfer drives biofilm expansion

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Bacterial biofilms, often composed of multiple species and genetically distinct strains, develop under complex influences of cell–cell interactions. Although detailed knowledge about the mechanisms underlying the formation

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

of single-species laboratory biofilms has emerged, little is known about the pathways governing the development of more complex heterogeneous communities. In this study we established a laboratory model where biofilm-stimulating effects due to interactions between genetically diverse strains of *Escherichia coli* were monitored and characterized. Synergistic induction of biofilm formation resulting from the co-cultivation of 403 undomesticated *E. coli* strains with the characterized *E. coli* K-12 strain MG1655 in LB medium was detected at a significant frequency: 189 (47%) of the 403 *E. coli* strains generated a significantly stronger biofilm in the presence of MG1655 as compared to monoculture ($P < 0.05$). In 56 strains (14%), the accumulated biofilm in co-culture was significantly higher than the sum of the biofilms formed in the individual monocultures ($P < 0.05$). Interestingly, synergistic effects of co-cultivation of pairs of natural isolates were also observed, demonstrating that biofilm promotion in this system is not dependent on the laboratory strain.

To analyse the nature and variety of mechanisms underlying the induction phenomenon, we focussed on the subset of 56 test strains that displayed the strongest induction in the initial screening. We found that *E. coli* MG1655 is the dominant strain in the great majority of these induced biofilms (87%). Moreover, the capacity of an isolate to promote the biofilm through co-cultivation was acquired by the K-12 strain in most cases, since it was further transferable from that K-12 derivate to another strain upon co-cultivation (93%). The transmissibility of the capacity to induce biofilm formation in co-culture suggested a significant role for horizontal gene transfer in the phenomenon. Therefore, we performed a polymerase chain reaction survey for specific conjugative transfer genes for the most prevalent conjugative plasmids in *E. coli*, F-like and IncIα plasmids. We found that synergistic development of biofilm within the 56 *E. coli* isolates exhibiting the strongest effects was most often linked to conjugative transmission of natural plasmids carried by the *E. coli* isolates (70%). These results provide the first evidence for a role of conjugative plasmids in biofilm formation of natural *E. coli* isolates. Detection of enhanced biofilm formation with natural strains apparently requires the opportunity for plasmid transmission as co-culture-induced biofilm formation was inhibited through the presence of a related conjugative plasmid in the co-cultured K-12 strain – presumably due to surface exclusion functions. This finding argues against the notion that gene transfer in this system is an indirect consequence of favourable cell densities provided by a developing biofilm, and instead is involved in promoting its expansion. Additional mechanisms may contribute to the observed induction phenomenon.

While there are obvious limitations to a direct extrapolation of these *in vitro* observations to natural *E. coli* biofilms, the utility of this approach is to provide initial insights into synergistic effects, the relevance of which can then be evaluated in successively complex *in vitro* or *in vivo* model systems.

The universal stress protein PA3309 in *Pseudomonas aeruginosa* is induced in biofilms

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Pseudomonas aeruginosa is an important opportunistic pathogen that causes persistent lung infections in the cystic fibrosis lung. It forms microcolonies within mucus plaques and generates an anaerobic environment. Survival under these oxygen-free conditions requires arginine fermentation or denitrification by the use of nitrate or nitrite. Recently we showed that pyruvate fermentation sustains anaerobic survival but no anaerobic growth.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

Two-dimensional gel electrophoresis revealed strong up-regulation of two hypothetical proteins, PA3309 and PA4352. Analysis of both proteins with the Pfam (Protein Families) database showed the presence of one or two universal stress protein (Usp) domains. Usps belong to a superfamily of proteins present in bacteria, archaea, fungi, flies and plants and are grouped according to their Usp domain sequence similarity. *Escherichia coli* Usps are well characterized and seem to be involved in resistance to diverse stresses and survival in the stationary phase. A PA3309 knock-out mutant was constructed and characterized phenotypically. This mutant showed a 2-log ratio reduced survival rate during pyruvate fermentation as compared with the PAO1 wild type.

Besides pyruvate fermentation, we found the PA3309 protein to be present in different biofilm models. The induction of the PA3309 promoter was monitored with a transcriptional PA3309-*gfp* fusion. *Pseudomonas aeruginosa* harbouring the PA3309-*gfp* fusion was grown in a flow cell biofilm set-up. Promoter activity was detected only in the deeper layers of the biofilm. Two-dimensional gel analysis of different regulatory mutants in the biofilm showed induction of the PA3309 promoter to be independent of Anr, the oxygen-sensing global regulator in *P. aeruginosa*. However, further studies with a transcriptional PA3309-*lacZ* fusion suggested induction of PA3309 to be dependent on a currently unknown stationary-phase regulator. Currently, we are investigating the contribution of PA3309 protein to the survival of *P. aeruginosa* in the deeper layers of biofilm.

Extracellular polymeric substances from biofilms on membranes in waste-water treatment plants

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The formation of bacterial biofilms on membrane surfaces is dependent on several factors such as the ability of the attached microorganisms to build extracellular polymeric substances (EPS). Thus membranes used in waste-water treatment suffer from biofilm formation, subsequent biofouling and pore blockage caused by EPS.

We have investigated membranes from bioreactors and waste-water treatment plants in North-Rhine Westphalia, Germany, with a focus on EPS and their effect on membrane fouling. In addition, cleaning procedures for removing the EPS and thereby recovering the membrane performance have been studied. Our experimental approach includes quantification of EPS found in membrane surface layers and in membrane pores (proteins, carbohydrates, DNA, lipids), analysis of molecular weight fractions by size-exclusion chromatography (HP-SEC), scanning electron microscopy of membranes, determination of elemental distribution on membrane surfaces by energy dispersive X-ray spectroscopy and verification of EPS and microorganisms attached to the membrane by confocal laser scanning microscopy (CLSM).

In waste-water treatment plants, regular mixing in the aeration tank in combination with continuous back-washing cycles and cleaning of the hollow fibre membranes prevents the formation of thick biofilm layers. Nevertheless, a flux decline and pore blockage is observed. By HP-SEC we were able to show that hollow fibre membranes used in a real-scale plant effectively reject the high molecular weight fraction of EPS that is also present in the activated sludge polymers. Within this high molecular weight fraction we detected carbohydrates. However, the analysis of the cleaning solutions after usage of different chemicals showed that the high molecular weight fraction clearly is

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

not bound to the membrane pores. The pore blockage and subsequent flux decline seem to be caused by smaller molecules such as humic acids, proteins and lipids. We are currently investigating the more detailed composition of the particular SEC-fractions.

Another approach was tested by using fluorescence stains that bind to EPS that we assume is present on the fouled membrane as part of a biofilm. Biofilm investigations of pure bacterial cultures by CLSM has been described; however, the application to environmental samples with unknown composition and mixed populations is difficult. We were able to successfully detect polysaccharides by CLSM on a fouled hollow fibre membrane from a waste-water treatment plant and we showed that these EPS are non-homogeneously distributed but clearly closely associated with microorganisms attached to the surface.

Our on-going research and future work includes filtration of model water aimed at the determination of substances most likely to cause fouling phenomena in general.

Biofilm-to-planktonic cell yield: a strategy for proliferation

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The phenomenon that biofilm-associated cells grow significantly more slowly than planktonic cells has been discussed in numerous books, reviews and research articles, and is frequently mentioned as a physiological attribute of biofilm organisms that confer resistance to antimicrobial agents and survival during other adverse conditions.

We propose that, in addition to their role in survival, biofilms also play an important role in microbial proliferation. This hypothesis is based on the presence of cell numbers often exceeding 10^9 cells/ml in the effluent from continuous flow cells in which pure or mixed culture biofilms were cultivated under a range of nutrient conditions, and at dilution rates up to 50 times greater than the maximum specific growth rate measured in batch culture. This cell yield from biofilms to the planktonic phase greatly exceeded the yield predicted on the basis of the biofilm footprint area when batch maximum growth rates were used in the calculations. Subsequent direct and indirect photometrical measurements of biofilm growth indicated faster growth rates during early-stage biofilm formation than in batch, and measurement of cell numbers in the effluent revealed biofilm-to-planktonic yield even at the early stages of biofilm development, before a steady-state biofilm biomass was reached. This yield was maintained after biofilm development reached steady state. We propose that the rapid growth of cells at the attachment surface during early biofilm formation is matched at the outer regions of older, multi-layered biofilms, while cells closer to the attachment surface reduce their metabolism to ensure survival. The growth at the outer regions is independent of the overall biofilm activity, but necessarily more sensitive to environmental perturbations due to high metabolic activity. Indeed, exposing mixed culture biofilms grown at different nutrient conditions to a biocide showed significant differences in the biofilm-to-planktonic yield. These results suggest that cell yield from biofilms is a continuous process not restricted to discrete physiological stages or physical and chemical conditions that cause dispersion, such as the introduction of high shear or a dispersant. These results imply that biofilms may serve as an important source of planktonic cells, including pathogens, in flow environments, and

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

therefore should be considered during the design of microbial control strategies.

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Physiological and phylogenetic characterization of the dispersed and loosely attached fraction of activated sludge flocs

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High turbidity and biomass discharge problems in activated sludge plants are associated with a relatively small fraction of solids ($<10\%$) that tends to remain in suspension or to detach easily from average flocs. Sludge deflocculation is commonly caused by stresses and often appears to be linked to specific substrate metabolism and electron donor conditions. Despite its large impact on treatment performance, the dispersed and loosely attached floc fraction has been poorly characterized, particularly in respect of microbial composition and activity. A better phylogenetic and physiological characterization of activated sludge bacteria in the unsettled sludge fraction can help to clarify the microbial roles in bioflocculation, specifically concerning bacterial identity and flocculating mechanisms.

This study focussed on the characterization of easily detachable bacteria in activated sludge flocs from a nutrient-removing treatment plant. Bacteria were characterized in supernatants from settled activated sludge before and after applying low shear ($G \approx 600\ s^{-1}$). Both free-swimming and pin-point floc-associated cells were quantified by 4′,6-diamidino-2-phenylindole (DAPI) staining and their phylogeny was determined by fluorescence *in situ* hybridization. Furthermore, the levels of potentially active bacterial cells were investigated on the basis of the ratio of the number of cells fluorescing with the EUBmix gene probe targeting most bacteria to the number of DAPI-stained cells (ratio = EUBmix : DAPI).

The results indicate that 60–70% of the cells in undisturbed supernatants from settled sludge were associated with microcolonies or small flocs and the remaining were free-swimming, single cells. About 99% of the particles exhibited a cross-sectional area $\leq 5\ \mu m^2$, based on quantification of particle distributions. The shear used ($G \approx 600\ s^{-1}$) induced sludge deflocculation and consistently higher cell and particle concentrations in the supernatants and reflects turbulence values above those inducing reflocculation and experienced by sludge in full-scale plants. Total cell concentrations in supernatants after settling ranged from 2×10^7 to 9×10^7 cells/ml and after shear from 10×10^7 to 70×10^7 cells/ml. Relatively lower bacterial EUBmix : DAPI ratios in the supernatants suggested that unsettleable cells were less active than those in settled sludge, for which EUBmix : DAPI ratios were close to 80%. Supernatants from sludge subjected to shear contained significantly fewer potentially active cells than did the settled sludge, since the EUBmix : DAPI ratios in the supernatants after shear averaged only 50%. Quantification of different phylogenetic groups showed differences in the population structure between the supernatants and the settled sludge. *Betaproteobacteria* and *Alphaproteobacteria* were the most dominant bacterial groups in settled sludge whereas *Deltaproteobacteria* and *Betaproteobacteria* were the most abundant groups in the supernatant from undisturbed sludge.

This study showed that cells in the dispersed, non-settleable fraction of activated sludge are potentially less active than cells constituting the average settled sludge, which evidences the primordial role that microbial metabolism

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

plays in maintaining flocculating properties in complex systems. Furthermore, the dispersed biomass in activated sludge has a phylogenetic structure relatively different from that of settled sludge, which may render this biomass prone to deflocculate and/or remain in suspension. The physical characterization of the dispersed biomass indicated that the main contributors to the total volume of dispersed, non-settleable biomass in activated sludge are not free-swimming cells but a few small, pin-point flocs (approximately 1%) containing 60–70% of the total cells.

Towards a deterministic model of biofilm detachment: an experimental study

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Progress in the understanding of biofilm detachment has been hampered by a general ambiguity over the interrelationship between biological and physical cues that mediate detachment, by poor experimental design in the study of detachment phenomena and by the use of semi-empirical parameters in biofilm models. We believe that an improved deterministic model of both erosion rate and sloughing frequency and magnitude can be achieved, provided the spatial and temporal physiological state in the biofilm is comprehensively accounted for. While some promising modelling frameworks have been proposed, there is, however, a paucity of experimental data available to corroborate such modelling approaches.

The membrane-aerated biofilm reactor (MABR) in which oxygen is supplied to the biofilm solely from the substratum has many advantages in the study of biofilms. The unique features of the system include the ability to manipulate the region and thickness of zones of microbial activity [1], the ability to eliminate planktonic growth independent of the liquid residence time [1], the ability to determine instantaneous values of the biofilm effective diffusion coefficient [2], and the ability to determine instantaneous values of average respiration rate [1] and the ease of biofilm thickness measurement [1]. If the intra-membrane oxygen pressure is controlled, the location and thickness of the region of maximum respiration activity can be manipulated and may be located uniquely adjacent to the biofilm–solid interface whilst growth near the biofilm–liquid interface is minimized. Alternatively, the membrane can be pressurized with pure nitrogen and the biofilm aerated through the liquid phase as in a conventional biofilm. Thus the MABR approach facilitates study of the link between detachment rate and a range of growth conditions and locations that would not be feasible in a conventional biofilm system.

We presented interim results of a study in which the MABR is employed to investigate detachment from established biofilms of *Vibrio natriegens*, under controlled environmental and hydrodynamic conditions, with the primary objective of establishing the relationship between growth rate and the erosion mode of detachment. Detachment was quantified by periodic dry weight measurements and by time-lapse photography of the biofilm itself. Preliminary experiments have focussed on quantifying erosion and sloughing as separate indicators of overall detachment rate. The relationship between changes in localized biofilm thickness and sloughing events was established and average biofilm thickness correlated well with overall detachment rate. A decrease in detachment rate was observed under nutrient-starved conditions, along with a slight increase in thickness, which was unexpected; this may have been due to the metabolism of residual carbon in the biofilm. This exemplifies the difficulty of attempting to achieve a pseudo steady state in

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

such experiments. In general, the overall detachment rate correlated with the thickness of the respiring layer within the stratified biofilm, although further experimentation will be required to fully establish the relationship between the thickness and location of the respiring layer and biofilm detachment.

REFERENCES

1. Casey, E., Glennon, B. & Hamer, G. (1999) Oxygen mass transfer characteristics in a membrane aerated biofilm reactor. *Biotechnology and Bioengineering* **62**, 183–192
2. Casey, E., Glennon, B. & Hamer, G. (2000) Biofilm development in a membrane aerated biofilm reactor: effect of flow velocity on performance. *Biotechnology and Bioengineering* **67**, 476–486

Effect of backwash on the characteristics of biofilm in a biological activated filter reactor using elemental sulfur particles

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Autotrophic denitrification using elemental-sulfur packed-bed reactors has been successfully applied in treating nitrified groundwater, leachate and synthetic waste water. Owing to the lower denitrification rate, autotrophic denitrification has a relatively small biomass growth as compared with heterotrophic denitrification. Clogging in sulfur packed-bed reactors has therefore been rarely observed, even after they have been operating for long periods. In contrast, heterotrophic reactors require frequent backwash to remove excess biomass and thus prevent deterioration of performance. There are, however, few studies about the effect of backwash on sulfur packed-bed reactors. In this research, a pilot-scale sulfur-packed biological activated filter reactor (BAFR) was operated with a backwash facility to study the effect of backwash on the performance of the reactor as well as on the characteristics of the biofilm along the height of the reactor.

The BAFR with a mixed culture of autotrophic denitrifying bacteria was operated in the upflow mode to treat synthetic nitrified waste water with a concentration of 220 mg nitrate-N/l. After formation of a biofilm on the sulfur media, the BAFR achieved 100% nitrate removal efficiency within 3 months, at a flow rate of 9.5 l/day, equivalent to a hydraulic retention time (HRT) of 5.5 h. When a quasi steady state was obtained, a backwash was performed on the BAF to remove the excess sludge as well as to study the effect of backwash on the characteristics of the biofilm. Several sulfur granules were extracted from different heights in the BAFR before and after backwash for analysis. It was found that, before the backwash, the biomass concentration of the BAFR at the lower region was about five times higher than at the top. The backwash process caused detachment of biofilm from the sulfur media and relocation of the media. As a consequence, the biomass density of the BAFR after backwash was more evenly distributed over the height of the reactor, but much less than before the backwash. Whereas before backwash the biofilm thickness on the media varied greatly along the height of the reactor, it became more uniform and much thinner after the backwash. Accumulation of nitrite in the BAFR after backwash implied not only a deterioration of removal efficiency due to less biomass, but also an alteration in the composition of the microbial community. We suggest that certain groups of bacteria, which were responsible

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

for the reduction of nitrite to nitrogen gas, were most probably located at the outer layer of the biofilm and were largely removed during the backwash process. Therefore the performance of the BAFR after backwash could not be fully recovered after operating the reactor for another 3 months, despite using the same synthetic waste water and an HRT four times higher than the initial one. Detailed data on microbial community analysis of the biofilm were presented to support the suggested hypothesis. Implications of the effect of backwash on the characteristics of the biofilm as well as on the performance of the BAF were also discussed.

Process performance and biomass properties in membrane-aerated bioreactors

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Membrane-aerated bioreactors (MABRs) present a unique technological innovation where oxygen and other substrates (i.e. carbon and nitrogen) diffuse from different sides of a membrane-supported biofilm. MABRs offer advantages related to energy and oxygen requirements, as well as to biomass handling, that may reduce operating costs in biological waste-water treatment. The biofilms formed may possess distinct properties, including the potential to couple aerobic and anaerobic processes thus combining chemical oxygen demand (COD) and nitrogen and phosphorus removal. The objectives of this study were to examine the properties of biofilms in MABRs carrying out simultaneous carbon and nitrogen removal, and to determine what effect oxygen partial pressure has on process performance and biomass properties. Two laboratory-scale MABRs were established and operated continuously for a total of 243 days. Initially, reactors were run at 21% oxygen with a substrate loading of 11.34 kg biological oxygen demand/1000 m^2 per day and 2.27 kg N/1000 m^2 per day, based on a synthetic feed. Hydrodynamic conditions were minimized with an average fluid velocity at the membrane surface of 22 cm/min. Under these conditions the removal efficiency for COD and nitrogen removal was 85% and 75%, respectively. Cryosectioned and stained biofilm samples from these reactors, analysed by confocal laser scanning microscopy (CLSM), were found to be less dense at the membrane interface (i.e. the bottom), where there is high oxygen availability. Fluorescence *in situ* hybridization was applied for the identification of nitrifiers and denitrifiers in the biomass formed. The membrane-supported biofilms appeared to have a layered structure with nitrifiers and higher organisms associated with the centre of the biofilm. To study the influence of oxygen partial pressure, one reactor was shifted to oxygen-enriched air at 75% O_2, 50% O_2 and 35% O_2, in three separate experiments in this sequence. The second reactor was held at 21% O_2 (control). All other conditions remained the same. Nitrification and denitrification were found to occur under all oxygen-enriched conditions. Total nitrogen removal was the highest at 50% O_2, while COD removal efficiencies remained the same. The biofilm was found to detach following a shift to higher oxygen levels and did not form at 75% and 50% oxygen. Biofilms were found to form at 35% and 21% oxygen. Using lectin-conjugated fluorescent stains for carbohydrate analysis of the polysaccharides formed and SYPRO Orange staining for proteins, coupled with CSLM, revealed differences in extracellular polymeric substances of the suspended biomass formed under oxygen-enriched conditions and the biofilm at 21% and 35% O_2. Surface characterization of the biomass revealed that, at higher oxygen partial pressures, biomass was less hydrophobic and possessed a higher net negative surface charge.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

Bioaugmentation via conjugation in biofilms treating 3-chloroaniline: effects of selective pressure

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Biological treatment is an effective strategy for treating waste waters contaminated with xenobiotic compounds. We investigated horizontal gene transfer via conjugation as a means of transferring catabolic genes to biofilm treatment systems. Our aim was to determine the effect of selective pressure on the outcome of conjugative gene transfer of pWDL7::*rfp*, encoding genes for 3-chloroaniline degradation, and subsequent 3-chloroaniline (3-CA) removal.

Conjugation experiments were performed in a four-channel flow cell. Biofilms of *Ralstonia eutropha* JMP228n*gfp* were grown and, after an acclimation period, the donor strain *Pseudomonas putida* UWC3 (pWDL7::*rfp*) was added to the biofilm. Control channels were left without added donors. Flow cell influent was switched to media with varying concentrations of 3-CA. Confocal laser scanning microscopy was used to detect bacterial cells expressing fluorescent proteins: donor and recipient strains were differentiated on the basis of fluorescence; transconjugants were detected on the basis of dual expression of red fluorescent protein and green fluorescent protein; and biofilm architecture was quantified using the PHLIP image analysis package. 3-CA concentrations were measured in the effluent using high-performance liquid chromatography. The long-term effects of variations in selective conditions upon fitness of transconjugants was examined.

Transfer of pWDL7::*rfp* from the *P. putida* donor strain to recipients and its effect on 3-CA degradation were first tested in batch planktonic systems. Batch experiments indicated that unaugmented cultures of the donor strain alone are incapable of achieving 3-CA degradation. In contrast, after addition of donor cells, transfer of pWDL7::*rfp* occurred and 3-CA degradation was observed. Microradiography using ^{14}C-labelled 3-CA showed that removal of 3-CA is performed by individual cells containing pWDL7::*rfp*, as evidenced by microautoradiography-positive single cells in a culture that degraded 3-CA. The flow cell experiment showed that donors and transconjugants were maintained only under initially highly selective conditions, with 3-CA as sole carbon source. Those transconjugants were maintained over 6 weeks, even when the carbon source was switched to be non-selective.

The study confirmed that it is possible, and necessary, to introduce genes into a microbial system to obtain 3-CA removal, and that removal is performed by uptake of 3-CA into individual plasmid-containing cells. We have shown the maintenance of transconjugants when exposed to fluctuating levels of 3-CA.

Effect of phosphorus on biofilm growth in a completely mixed biofilm reactor

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More and more studies show that bacterial growth in drinking water is not always regulated by carbon but rather by phosphorus concentration. However, the role of phosphorus in biofilm formation in distribution networks is not completely understood. In our study the effect of microbiologically available phosphorus (MAP) on biofilm growth in drinking water was investigated

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

in full-scale experiments over 3 years. Completely mixed biofilm reactors Propella® were supplied with drinking water taken from five locations with different MAP concentrations ranging from 0.23 to 10.22 μg P/l. Inlet water contained no active chlorine and assimilable organic carbon (AOC) concentration varied from 114 to 273 μg/l.

Reactors were supplied with drinking water for about 1 month (to reach semi steady state) at controlled conditions (temperature 15 °C, flow rate 0.25 m/s, dilution rate 0.084 h^{-1}) before polyvinylchloride coupons were removed and examined. MAP concentrations in the effluent from reactors were always lower than in the influent, indicating that phosphorus was limiting bacterial growth in the reactors. Total bacteria counts in the biofilm ranged from 5.1E + 06 to 2.8E + 07 cells/cm^2. MAP correlated positively with total amount of cells and heterotrophic plate counts in biofilm. The correlation slope was similar to that obtained in other studies where plug flow reactors were used [1], although the absolute values of bacteria in the biofilm at a similar level of MAP were significantly higher in this study. This phenomenon should be investigated further. Specific biofilm growth rate (assuming no bacterial growth in bulk water) was in the range 0.005 to 0.02 day^{-1}. These values are lower than those determined for carbon-limited drinking waters [2, 3]. We observed that the biofilm growth rate decreased with the increase of MAP concentration in water, which implies that biofilm growth in phosphorus-limited water does not always obey Monod kinetics. This observation should be taken into account in the development of biofilm formation models.

In summary, this study showed that phosphorus may be limiting bacterial growth in drinking water biofilms; with increase of phosphorus in the water, the biofilm formation rate decreases whereas bacterial numbers in the biofilm increase. Removal of phosphorus below 1 μg/l did not prevent biofilm formation in completely mixed reactors.

The work was undertaken as a part of the research project SAFER ("Surveillance and control of microbiological stability in drinking water distribution networks"), which is supported by the European Commission within the Fifth Framework Programme, "Energy, Environment and sustainable development programme" (contract no. EVK1-2002-0018).

REFERENCES

1. Lehtola, M. J., Juhna, T., Miettinen, I. T., Vartiainen, T. & Martikainen, P. J. (2004) Formation of biofilms in drinking water distribution networks, a case study in two cities in Finland and Latvia. *Journal of Industrial Microbiology and Biotechnology* **31**, 489–494
2. Block, J. C., Haudidier, K., Paquin, J. L., Miaza, J. & Levi, Y. (1993) Biofilm accumulation in drinking water distribution systems. *Biofouling* **6**, 333–343
3. Boe-Hansen, R., Albrechtsen, H.-J., Arvin, E. & Jorgensen, C. (2002) Bulk water phases and biofilm growth in drinking water at low nutrient conditions. *Water Research* **36**, 4477–4486

Impacts of biofilm development on reactive transport in porous media under variable flow regimens

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The study of biofilm/porous media interactions is important in secondary oil recovery, groundwater and soil bioremediation, and the long-term storage of supercritical carbon dioxide. The majority of work on studying the impact

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

of biofilm growth on the hydraulic and transport characteristics of porous media has been done using constant flow systems. We investigated flow characteristics of biofilm/porous media systems under constant head conditions that are more indicative of natural environments such as intrinsic bioremediation and long-term bioclogging applications (biological hydraulic barriers). Meso-scale porous media, flat-plate reactor systems were used to study bioreactive flow characteristics in biofilm/porous media systems under both constant head and constant flow conditions. Biofilms developed using the naturally bioluminescent bacteria *Vibrio fischeri* were grown in the porous media reactors. The impact of varying degrees of biofilm growth and accumulation on the hydraulic characteristics of the porous media were studied under constant flow and constant head conditions using breakthrough curves analysis and continuous digital photographic imaging. Image analysis of the bioluminescence produced by the *V. fischeri* biofilm was used to monitor, locate and quantify bacterial activity within the porous media systems. A non-toxic, non-adsorptive conservative bulk fluid dye was used to capture images of flow channels in the biofilm/porous media matrix. These images were combined with the bioluminescent images of biofilm activity to characterize reactive flow in the porous media under both constant flow and constant head hydraulic conditions.

Growth and distribution of biofilm in the porous media reactors was evaluated periodically using a combination of bioluminescent imaging, microbial vital stains, and microscopy. Gray-scale analysis of the dye tracer images provided quantitative information on the transient distribution and density of biofilm within the reactor and the dynamic relationship between biofilm development and porous media hydrodynamics. The images also provided information on the network of flow channels that developed within the porous media under both constant head and constant flow. Analysis of the fluorescein breakthrough curves provided information on the flow hydraulics within the reactors and the effects of biofilm development and flow regimen. Finally, analysis of the bioluminescent images indicated the location and relative degree of biological activity within the biofilm/porous media systems, and the impact of flow regimen and biofilm development on reactive transport.

Previous work on constant flow systems showed the development of a well-defined flow channel in the biofilm/porous media matrix resulting in higher flow velocities and shorter breakthrough times. These results also showed that the majority of biological activity in the system took place along the dynamic flow channels, which varied in size, number and location with time. Under constant head conditions, no primary flow channels were developed and the flow through the biofilm/porous media system was more diffuse and well distributed across the system. We included results from a number of hydraulic and biofilm activity studies aimed at better understanding the impacts of varying degrees of biofilm growth on advective and reactive transport in porous media under constant head conditions.

Influence of biofilms on colloid mobility in the subsurface

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Transport processes in subsurface environments are determined by complex interactions between the soil matrix and dissolved as well as particulate substances. During rain water infiltration, pollutants can be transported not only in solution but also in colloidal or colloid-bound form. In nature, the transport of such colloids does not take place under well-defined conditions

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

and is influenced by many parameters and interactions. For example, variations in ionic strength, presence of different ions and the involvement of biofilms have to be taken into account. In this study, it was shown that all these parameters have a great impact on the sorption and retention of colloids as well as on their remobilization. Indeed the presence of biofilms led to enhanced colloid retention. However, the extent of this retention was related to the ionic strength and the presence of monovalent or divalent cations. On the basis of the results obtained, three examples of transport scenarios for the interaction of colloids with biofilms in the subsurface were derived:

1. *Indirect hydrodynamic influences*
   If colloids are infiltrated into the subsurface under high ionic strength conditions they tend to aggregate and cause pore clogging in the soil matrix. Thus the flow velocity is enhanced in the remaining pores and this can lead to the detachment of adhered cells and previously sorbed pollutants or colloids.
2. *Direct interactions (sorption and coelution)*
   If colloids are infiltrated under conditions where monovalent cations are predominant, two effects can be observed: (a) there is enhanced sorption of colloids to the biofilm matrix, which leads to the retention of colloids; and (b) if a decrease in ionic strength takes place, the biofilm matrix is destabilized and a co-elution of sorbed colloids together with detaching biofilms compartments can occur.
3. *Direct interaction (sorption)*
   If divalent cations are predominant during the infiltration of colloids, a strong sorption of the colloids to the biofilm matrix is observed. The colloids are completely retained and will be remobilized only to a low extent even under changes in ionic strength. From these results, it could be predicted qualitatively that, under high ionic strength conditions, retention of colloids within the soil matrix is generally favored. The biofilms act as a sorption surface and sink. But if ionic strength decreases under predominating monovalent cations or a substitution of divalent by monovalent cations takes place, a remobilization of colloids can be observed. In this case, biofilms will be a source for colloidal or colloid-bound pollutants. Environmental influences that cause significant changes in ionic strength and/or in the type of predominant ions (mono/divalent) will drastically change the mobility of colloids and thus potentially also the mobility of pollutants. This could, for example, occur in the case of intense rain events, infiltration of thawing salt during snowmelt, sea water infiltration or intertidal processes at estuaries.

This work forms part of the cooperative research project KORESI ("Kolloidaler Stofftransport bei der Regenwasserversickerung") financed by the DFG. We thank Professors U. Förstner, F. H. Frimmel and P. A. Wilderer and their staff, for constructive input and discussion.

Biofilms in amendable *in situ* microcosms indicate relevant electron acceptor processes at a BTEX-contaminated aquifer

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To assess microbial communities in an anaerobic BTEX (benzene, toluene, ethylbenzene and xzylene)-contaminated aquifer we used specifically designed *in situ* microcosms (BACTRAPS) facilitating development of biofilms at

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

specific electron acceptor processes (TEAPs). The microcosms contained polymeric beads consisting of Nomex and powdered activated carbon. The beads explore high internal surface areas and have capabilities of trapping organic contaminants. The modification of beads with ^{13}C-labeled benzene or toluene and detection of ^{13}C in microbial-lipid-derived fatty acids after *in situ* exposure of the system has already shown biodegradation of toluene but also of benzene under *in situ* conditions at the edge of the aquifer plume [1].

In our study, we amended the microcosm with toluene and additionally with potential electron acceptors (e.g. sulfate, nitrate or Fe(III) salts) and exposed them together with non-amended controls in a monitoring well at the center of the plume (zZ12A/00, 16 m depth). During *in situ* exposure (approximately 4 weeks) biofilms developed from the viable autochthonous microbial communities based on the respective contaminant and TEAP. Biofilms were visualized and quantified by laser scanning microscopy. Efficiency of biomass formation and structural differences of developed microbial communities were characterized by profiling the membrane lipids. Polar lipid fatty acid profiles were compared with intact phospholipid profiles, analyzed by newly developed high performance liquid chromatography–tandem mass spectroscopy methods. The latter method promises higher sensitivity and increased specificity for signature biomarker quantification. Microbial respiratory quinones profiles pointed to the different redox states of the three TEAPs and gave further information about the phylogenetic grouping of the microorganism grown under *in situ* conditions. Fatty acid biomarkers were comparable to respective biomarkers obtained from sediments recovered at the site during the original drilling of the wells. This *in situ* microcosm approach enabled the monitoring and identification of the relevant metabolic processes, metabolites and potential key players.

REFERENCE

1. Geyer, R., Peacock, A. D., Miltner, A., Richnow, H.-H., White, D. C., Sublette, K. L. & Kästner, M. (2005) *In situ* assessment of biodegradation potential using biotraps amended with ^{13}C-labeled benzene or toluene. *Environmental Science and Technology* **39**, 4983–4989

Functional biodiversity of complex biofilms grown on polychlorinated biphenyl oil

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Polychlorinated biphenyls (PCBs) were used in many applications but were eventually determined to be environmental toxins. Worldwide 1.5 million tonnes of PCBs were produced, from which a substantial amount has been released into the environment since the early 1930s. No natural products are known to be similar to PCBs, which consist of a biphenyl core substituted with 1–10 chlorine atoms, permitting the formation of up to 209 different compounds, called congeners. Their chemical stability and toxicity make PCBs serious and persistent environmental pollutants. The performance and composition, as well the distribution of microbial communities able to degrade PCBs, are not well known but many strains have been isolated that can degrade lower chlorinated PCBs. Microbial communities organized in biofilms show a multitude of interactions, including carbon sharing, interspecies communication and steep physicochemical gradients and we assumed that

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

biofilm communities could be good candidates for PCB degradation. To find out how widely distributed the ability of microbial communities is to degrade these compounds, soil samples from 10 sites in Germany showing distinct characteristics were used to grow biofilms on PCBs (PCB oil). All of them formed multi-species biofilms as revealed by single-strand conformation polymorphism (SSCP) analyses of the 16 S rRNA gene polymerase chain reaction products and degradation of several PCB congeners was confirmed by gas chromatography analysis. The identification of the main SSCP bands by 16 S rRNA gene sequence analysis revealed distinct biofilm communities depending on the origin of the samples.

Regarding the PCB degradation, communities from PCB-contaminated sites showed a better efficacy for degrading PCBs. Nevertheless, the communities from non-polluted sites degraded up to 45% of monochlorinated biphenyls and up to 17% of a pentachlorinated congener. Confocal laser scanning microscopy revealed the dynamics of biofilm formation and maturation on the PCB oil, which was mirrored in distinct sequences of biodiversity changes and congener degradations. The results imply that the potential for the bioremediation of the xenobiotic PCBs is common in different soils and that different biofilm communities could form functional units to degrade PCB congeners that are not attacked by any of the isolated members of the biofilm.

Identification and characterization of biofilm formation phenotypes of several clinically relevant *Streptococcus pyogenes* serotype strains

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Streptococcus pyogenes (group A streptococci, GAS) is an important bacterial pathogen and exclusively infects humans. One preferential GAS entry portal and initial colonization site is the oral mucus membrane. At this site, GAS encounter the residential biofilm flora that serves as a natural barrier to physically exclude and repel pathogens. Nothing is known about the GAS interaction with the residential biofilms prior to establishment of a successful infection and whether GAS can organize themselves in such biofilm structures. Consequently, in an ongoing study we are elucidating the molecular mechanisms of the interaction between GAS and selected viridans streptococci as the key players of the physiological biofilm.

As a first step, we identified *in vitro* GAS biofilm formation phenotypes. Using batch cultures, a newly set up continuous flow system and various detection methods, including safranin staining, scanning electron microscopy, and confocal laser scanning microscopy (CLSM), we were able to demonstrate biofilm formation of GAS serotype strains M2, M6 and M18 comparable in form and structure to those formed by *Staphylococcus epidermidis* positive control. Whereas GAS serotype M6 built large biofilms on uncoated plastic surfaces, serotypes M2 and M18 preferred fibronectin- and collagen type I-coated surfaces for biofilm growth, respectively. Using CLSM, we measured GAS biofilm layers with heights between 10 and 30 μm, a typical range also reported for other pathogenic microorganisms that preferentially live in biofilms. Furthermore, we used LIVE/DEAD stain for fluorescence microscopy and CLSM to show that live and dead cells were equally distributed in all layers of the GAS biofilm. In a second step, the influence of nutrient components

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

in culture medium on GAS biofilm formation was analyzed. Maximum yield was achieved with brain heart infusion (BHI) medium. Supplementation of BHI with different concentrations of various carbohydrates revealed structural differences of serotypes M6 and M18 GAS biofilm architecture as demonstrated by safranine stain–light microscopy and reflection electron microscopy. Both serotype strains formed biofilms with modified and looser cell structures in the presence of higher carbohydrate concentrations. Generally, GAS biofilms showed narrower layers in the absence of carbohydrate supplements in the medium.

In a third step, mixed biofilms with GAS and *Streptococcus oralis* or *Streptococcus salivarius* were generated and analyzed for structure, cell numbers, viability and production of GAS virulence factors. Currently, the data are complemented by whole genome DNA array hybridization experiments. Obviously, GAS are well able to integrate themselves into foreign biofilms, but show altered virulence factor production depending on their biofilm partners. As a potential future supplementation of antibiotic therapies, the techniques established in this study are now also used to select viridans streptococci as probiotic candidates that prevent or cure GAS integration into oral biofilms.

Selected probiotic bacteria disrupt biofilm development of vancomycin-resistant *Enterococcus faecium*

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Enterococci are natural inhabitants of the gastrointestinal tract, but they are also a major cause of nosocomial infections. Many hospital-acquired infections are caused by biofilm-forming microorganisms. These are difficult to treat, as bacteria in biofilms are 10–1000 times more resistant to antibiotics. Commensal bacteria or probiotics have the potential to inhibit pathogenic biofilms due to the production of antimicrobials and biosurfactants, or by means of quorum quenching. The objective of the present study was to determine the effect of probiotic bacteria on biofilm formation of vancomycin-resistant *Enterococcus faecium* (VREF).

Thirty probiotic strains of the genera *Lactobacillus* ($n = 18$), *Bifidobacterium* ($n = 8$), *Lactococcus* ($n = 2$), *Enterococcus* ($n = 1$), and *Propionibacterium* ($n = 1$) were tested for their ability to inhibit biofilm formation of VREF in a microtiter plate biofilm assay. In short, VREF was grown in a microtiter plate and after 3, 6 and 24 h cell-free culture supernatants (CFCSs) of the probiotic strains, adjusted to pH 7, were added. After overnight incubation with the CFCSs, growth and biofilm formation were measured. The effect of CFCSs on the vitality of VREF, and the influence of nutrient limitation on growth and biofilm formation, were analysed. To identify the biofilm-inhibiting component, CFCSs were tested for heat stability, biosurfactants, proteinaceous and lipid substances. The component's fragment size was determined by filtration at 3, 10 and 30 kDa.

VREF growth and biofilm formation were strain-specifically modulated. Therefore, the probiotic strains were divided into three groups: (1) 10–30% increase in growth, less than 40% reduction in biofilm development; (2) 10–30% increase in growth, 50–85% reduction in biofilm development and (3) 5–25% reduction in growth, 35–75% reduction in biofilm development.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

The CFCSs that inhibited biofilm formation did not kill VREF, as evidenced by propidium iodide staining. This inhibition could not be attributed solely to the presence of biosurfactants or a lack of nutrients. Preliminary data showed that the major fraction of the inhibiting components is heat stable, smaller than 3 kDa, and non-proteinaceous. The inhibiting effect of CFCSs in group 2 was reduced by 20–25% after lipase treatment, which suggests that a part of the inhibiting fraction contains a lipid moiety. Inhibition of biofilm formation in group 3 was not affected by lipase treatment.

Probiotic CFCSs are able to disrupt biofilm development of VREF. The CFCSs can be divided into three groups, due to their effect on growth and biofilm development of VREF. It appears that group-specific mixtures of metabolites are responsible for the effects observed.

Comparison of the extracellular polymeric substances of *Candida albicans* and *Candida dubliniensis* biofilms

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Candida albicans and *Candida dubliniensis* live as benign commensal organisms in the oral cavity of both healthy and unhealthy individuals, behaving, under certain conditions, as opportunistic pathogens causing candidiasis. These two *Candida* species have been mismatched for years, but recently *C. dubliniensis* was recovered from the mouth of immunosupressed patients and identified as a different species. Candidiasis is usually related to the *Candida* capacity of forming biofilms on inert or biological surfaces, being the phenotype associated with infections. Biofilms are complex structures of microbial communities attached to a surface, in which microorganisms are embedded in a matrix of extracellular polymeric substances (EPS) composed mainly of proteins and polysaccharides. The biofilm matrix has the potential for determining possible mechanisms of resistance of *Candida* biofilms. Several factors are known to affect the production of EPS, namely growth medium, growth phase and substratum. This study focussed on the influence of artificial saliva growth medium on the composition of EPS of biofilms formed by both *C. albicans* and *C. dubliniensis* strains.

Biofilms of one strain of *C. albicans* and two strains of *C. dubliniensis* were formed in an artificial saliva growth medium (ASGM) and compared with those formed in Sabouraud dextrose broth (SDB) and analysed after 48 h.The differences between the EPS of the biofilms were evaluated (after sonication) in terms of proteins (quantified using the BCA (bicinchoninic acid) protein assay kit) and polysaccharides (quantified using the phenol–sulphuric method). Proteins were also analysed by sodium deodecyl sulphate–polyacrylamide electrophoresis (SDS-PAGE).

In SDB the amount of proteins and polysaccharides in the EPS of biofilms formed by *C. albicans* was lower than in the EPS of biofilms formed by *C. dubliniensis* strains. In the presence of ASGM, the amount of proteins and polysaccharides was similar among the EPS of biofilms of *C. albicans* and one of the *C. dubliniensis* strains and was lower in biofilms of *C. albicans* than in biofilms of the other *C. dubliniensis* strain. Analysis of protein profiles obtained by SDS–PAGE showed that all strains present similar patterns independently of the medium of biofilm formation.

Biofilms formed in ASGM originated different amounts of EPS, in terms of either polysaccharides or proteins, as compared with those formed in SDB.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

Differences were also found in the profile of extracellular proteins of each strain, depending on the medium.

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Influence of quorum-sensing regulated production of an antimicrobial component by *Serratia plymuthica* on establishment of dual species biofilms with *Escherichia coli*

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Mixed-species biofilms on surfaces of food processing equipment, water distribution systems, medical implants, catheters, etc. are a potential reservoir of bacterial pathogens that may constitute a health hazard. We previously isolated from a food-processing environment [1] a biofilm-forming strain that was identified as *Serratia plymuthica* RVH1 [2]. We showed that the strain contains a quorum-sensing system consisting of the *N*-acyl-L-homoserine lacton synthase SplI, homologous to LuxI, and a response regulator SplR, homologous to LuxR. Recently it was shown that this system regulates the production of an extracellular protease, chitinase and nuclease and the production of an antimicrobial component (AC) (R. Van Houdt *et al.*, unpublished work).

To demonstrate that the production of AC is quorum-sensing regulated, *S. plymuthica* and knockout strains in *splI*, *splR* and an unknown gene necessary for AC production were spot inoculated on LB soft agar seeded with *Escherichia coli*. The wild-type strain caused a significant inhibition zone, whereas the knockout in AC production had none. The *splI*, *splR* and double knockouts induced respectively a smaller, slightly larger and similar inhibition zone as compared with the wild type. Addition of synthetic signal molecules complemented the *splI*$^-$ phenotype. Together these results are an indication of a quorum-sensing regulation of AC component production with SplR acting as a repressor.

We then investigated the effect of AC production on establishment of a dual species biofilm in a flow cell system inoculated with *S. plymuthica* and *E. coli* in a 1 : 100 ratio. When the bacterial populations were recovered from the flow cell after 3 days of continuous growth and plated on selective media, the density of *E. coli* (colony-forming units (c.f.u.)/cm^2) in the biofilm was clearly inversely correlated to AC production by its *S. plymuthica* partner. The density (log c.f.u./cm^2) of *E. coli* reached 2 when grown in combination with wild-type *S. plymuthica* RVH1, but 7 and 5 in combination with either the AC production knockout strain or the *splI* knockout, respectively. Exogenous addition of signal molecules in the last case resulted in an almost complete absence of *E. coli* from the biofilm. Loss of repression of AC production by SplR even led to biofilms from which no *E. coli* could be recovered. Confocal scanning laser microscopy with green fluorescent protein-labeled *S. plymuthica* strains and red fluorescent protein-labeled *E. coli* confirmed these differences in *E. coli* numbers. These results show that quorum sensing fine tuning of AC production plays an important role in the possible incorporation of *E. coli* MG1655 in dual species biofilms containing *S. plymuthica* RVH1.

This work was supported by a scholarship from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen).

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

REFERENCES

1. Van Houdt, R., Aertsen, A., Jansen, A., Quintana, A. L. & Michiels, C. W. (2004) Biofilm formation and cell-to-cell signalling in Gram-negative bacteria isolated from a food processing environment. *Journal of Applied Microbiology* **96**, 177–184
2. Van Houdt, R., Moons, P., Jansen, A., Vanoirbeek, K. & Michiels, C. W. (2005) Genotypic and phenotypic characterization of a biofilm-forming *Serratia plymuthica* isolate from a raw vegetable processing line. *FEMS Microbiology Letters* **246**, 265–272

Biofilm formation by the thermophilic and cellulolytic actinomycete *Thermobifida fusca*

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Cellulose is the most abundant biopolymer and renewable energy source on Earth, and its decomposition, which is carried out almost exclusively by microorganisms, is a key step in the cycling of carbon in the biosphere. It has long been known that cellulolytic bacteria may adhere to their insoluble substrate as it is degraded, although surprisingly little is known about microbial growth, colonization and community development on insoluble cellulosic substrates. Previous investigations indicated that two Gram-positive cellulolytic soil bacteria – *Cellulomonas uda*, a facultative aerobe, and *Clostridium phytofermentans*, an obligate anaerobe – specifically adhered to nutritive surfaces forming biofilms, but cells did not colonize non-nutritive surfaces. We hypothesized that biofilm formation is a general strategy used by microbes in the degradation of insoluble substrates and that it may serve as a means for microbes to secure a nutrient and persist in their environments. The objective of this study was to characterize biofilms produced by *Thermobifida fusca*, a Gram-positive cellulolytic actinomycete isolated from compost, which rapidly degrades cellulose by means of a well-characterized extracellular cellulase system and is a causative agent of "farmer's lung", the most common type of hypersensitivity pneumonitis.

Thermobifida fusca was cultured with dialysis tubing (regenerated cellulose) as a nutritive surface or with non-nutritive surfaces such as glass, plastic, metal or Teflon. Cells of *T. fusca* grew, colonized and formed biofilms tightly attached to both nutritive and non-nutritive surfaces. Dialysis tubing membrane disintegrated and dissolved as a result of biofilm growth on this cellulosic substrate. Microscopical analyses of biofilms on dialysis tubing revealed cells embedded in the fibrous material, suggestive of a matrix of extracellular polymeric substances (EPS). Biofilms that formed on glass were mucoid in appearance and they stained with calcofluor white, indicating the presence of β-(1–4)- and/or β-(1–3)-carbohydrates. Carbohydrate was quantified using a sulfuric-acid phenol method. The carbohydrate contents of both biofilm growth and non-attached mycelial cell pellets increased during growth. Cells were stained to examine carbohydrate composition using several different lectins conjugated with a fluorescent chromophore. Concanavalin-A lectin was found to bind to *T. fusca* biofilms and, in the early stages of growth, to non-attached mycelial pellets. This observation suggested that the polymeric components of EPS in *T. fusca* biofilms and mycelial pellets included α-linked D-mannosyl and/or α-linked D-glucosyl residues.

The results of this study demonstrated that *T. fusca*, a thermophilic cellulolytic actinomycete, colonizes both nutritive and non-nutritive surfaces and forms biofilms that are encased in a carbohydrate-containing EPS matrix, a hallmark of biofilm production. Furthermore, the results indicate that non-attached mycelial pellets also accumulate carbohydrate, and thus, may possess some of the characteristics of biofilms.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

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Biomonitoring of bacterial contamination on different surfaces of food-processing machines

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Formation of biofilms in plant of the food and pharmaceutical industries causes product contamination and concomitant large-scale economic damage. To avoid such contamination, companies have to make great efforts to optimize cleaning procedures, which are time and cost intensive. One way to reduce the cleaning periods, and thus plant downtime, is to look at alternative materials and surfaces with lower affinities for contamination.

As a first stage in developing methods for characterizing the materials according to their anti-adhesive properties, we identified the following steps:

Define contamination of the surfaces with microorganisms.
Detect cell adhesion with fluorescence microscopic methods.
Detect non-microbial contamination.
Define cleaning.
Verify the cleaning result.

We designed a protocol that allowed controlled microbial contamination of coupons consisting of various materials.The samples of different materials and surfaces were contaminated with *Escherichia coli* cells. Subsequently the cells were counted after staining with the DNA dye 4′,6-diamidino-2-phenylindole. As the adhesion of microorganisms to surfaces is greatly influenced by previous adhesion of macromolecules such as proteins or polysaccharides, we looked for a method of detecting this non-microbial contamination. We therefore contaminated the samples with whey protein containing β-lactoglobulin, which could be detected by binding to a specific fluorescence-labeled antibody. In initial experiments, samples of different materials and surfaces with different roughnesses and coatings were tested.

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Role of the flagella during the adhesion of *Listeria monocytogenes* EGD-e to inert surfaces after cultivation at different pHs and temperatures

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Listeria monocytogenes is a foodborne pathogen responsible for listeriosis, with an overall mortality rate around 20–30%. This pathogen can multiply at refrigeration temperatures, high salt concentration and low pH. Moreover, *L. monocytogenes* can adhere to inert surfaces and evolve into a well-structured cell cluster organization: a biofilm. The biofilms of *L. monocytogenes* were found to be less sensitive to cleaning procedures and they constitute a reservoir for cross-contamination in the food industry. The adhesion is therefore crucial in the contamination process.

Adhesion assays of *L. monocytogenes* EGD-e were conducted in polystyrene microtiter plates (initial cell concentration: about 8×10^8 cells/well) for 4 h at 20 °C in physiological medium supplemented with chloramphenicol at a

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

sub-bactericidal concentration. The adhesion was assessed after cell cultivation in TSBYE (trypticase soy broth with yeast extract) buffered with phosphate at pH 5, 6, 6.5, 7 and 7.5 and at temperatures of 8, 20 and 37 °C. In parallel, flagella were observed after cultivation at the various pH and temperature conditions using optical microscopy at 1250× after Leifson staining.

The results indicated that the percentage of the attached cells was significantly higher (7.79%) at pH 6 and above and at 8 °C (7.19%) and 20 °C (9.52%), than at pH 5 (0.56%) or at 37 °C (0.19%). In correlation with this, flagella were detected only at pH 6 and above and at 8 and 20 °C.

To verify the implication of flagella in the initial adhesion step of *L. monocytogenes* EGD-e to inert surfaces, an in-frame deletion was introduced into the gene *fla*A encoding the flagellin, which is the major component of the bacterial flagella. Adhesion assays conducted with EGD-e Δ*fla* indicated that the percentage of attached cells decreased significantly at pH 6 and above (1.54%) and at 8 °C (0.40%) and 20 °C (0.33%) as compared with the wild type.

In conclusion, the conditions of food conservation could have an impact on the adhesion capability of *L. monocytogenes* EGD-e and this is linked to the presence of the flagella, whose synthesis is controlled by these conditions. The environmental conditions inhibiting the flagellum synthesis would certainly delay the biofilm formation on inert surfaces during food processing.

Adhesion of *Saccharomyces cerevisiae* to stainless steel: influence of surface properties

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In contrast to the extensive literature describing bacterial biofilms, little attention has been paid to fungal biofilms, except for medically relevant yeasts such as *Candida albicans*. However, studying *Saccharomyces cerevisiae* is of great interest, since this microorganism is considered to be a pertinent model for eukaryotes because it is non-pathogenic and its genome has been sequenced entirely. It is also widely used in numerous industries, especially that of food processing. The present study focussed on the adhesion of *S. cerevisiae* to a 316L stainless steel surface, with a view to determining the influence of the yeast surface properties and the characteristics of the solid surface, respectively. To reach this aim, a dedicated set-up equipped with a shear stress flow chamber was used to characterize the adhesion under various operating conditions. The role of the material was studied by testing other supports, covering a broad range of surface properties such as surface free energy and roughness: polypropylene (hydrophobic), polystyrene (mildly hydrophobic, similar to stainless steel) and glass (hydrophilic). All materials were smooth with respect to the size of yeast, with roughness average values (R_a) ranging from 5 to 90 nm. The surface properties (charge, hydrophobicity, electron-donor and electron-acceptor components) of different baker's yeast cells (industrial and laboratory strains) were also determined and compared.

For glass and polymers, it was shown that the adhesion of yeast cells was substratum dependent and driven by the balance of the Lifshitz–van der Waals and Lewis acid/base interactions. Even though 316L stainless steel and polystyrene exhibited nearly identical surface free energies, stainless steel promoted a totally distinct behaviour that was characterized by a strong adhesion, whatever the yeast strain. However, for industrial cells, a significantly higher variability in the results was observed, which is worth investigating.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

All these results stressed the necessity to elucidate the specificity of stainless steel by notably identifying the molecular mechanisms governing *S. cerevisiae*/stainless steel interactions. First, X-ray photoelectron spectroscopy analysis of the metal surface was performed in order to determine the characteristics of the passivated layer (composition, homogeneity, etc.) and to detect the possible adsorption of organic species present in the liquid or released by the cells. Secondly, the role of the molybdenum content was estimated by comparing the adhesion of yeast cells on 316L and 304L stainless steel surfaces. The effect of an ageing procedure was also examined, by submitting 316L stainless steel to several cycles of soiling and cleaning steps. From a biological point of view, the role of the yeast adhesins was investigated by using different cell wall glycoprotein mutants.

Investigating the mechanical strength of biofilms with fluid dynamic gauging

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The ability to measure the mechanical strength of biofilms is a necessary step in the understanding of biofilm systems as a whole in their environment. Mechanical properties of biofilms are determined by the growth conditions and in turn influence detachment processes and the stability of the biofilm against external mechanical stresses.

In industrial applications it is helpful to know the mechanical properties of biofilms so that biofouling can be prevented by the application of mechanical stresses to remove unwanted biofilms. Moreover, when constructing a mathematical model of biofilm development we need to know the mechanical strength of the biofilm matrix with respect to applied shear stresses so that we can predict the detachment processes.

The method of fluid dynamic gauging (FDG) has been developed to measure the mechanical strength of organic and inorganic fouling layers on plane surfaces. A defined shear stress parallel to the substratum surface is applied and the point of detachment can be recorded. In our study this method was applied to measure the mechanical strength of biofilms. Therefore multi-species biofilms inoculated with activated sludge were cultivated at varying substrate loadings in a modified rotating disc reactor (RDR). This novel reactor type provides the possibility of cultivating biofilms under equal substrate conditions but subjected to a gradient of shear stress along the radius of the discs. Furthermore, confocal laser scanning microscopy was used together with established staining techniques and digital image analysis to investigate the structure of the intact biofilm as compared with other parts of the biofilms after they were exposed to a defined shear force with FDG.

Our recent investigations suggested the following results:

1. The mechanical strength of the biofilm is strongly determined by its structure.
2. In terms of stability, a weaker heterogeneous layer on top and a more stable bottom layer could be distinguished.
3. This bottom layer of biofilm with a thickness of about 100 μm could not be removed by using shear forces of up to 7.3 Pa.
4. Furthermore, the bottom layer of the biofilm contains a high portion of extracellular polymeric substances, which seem to provide a higher stability in the biofilm matrix.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

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Three-dimensional biofilm model with individual cells and continuum extracellular polymeric substances matrix

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This work presents a completely new and innovative type of biofilm model by combining an individual description of microbial particles with a continuum representation of the biofilm matrix. This hybrid model retains the advantages of each approach, while providing a more realistic description of the temporal development of biofilm structure in two or three spatial dimensions. The general model derivation takes into account any possible number of soluble components. These may be substrates and metabolic products that diffuse and react in the biofilm with the individual microbial cells. The cells grow, divide and produce extracellular polymeric substances (EPS) in a multi-species model setting. The EPS matrix is described by a continuum representation as an incompressible viscous fluid, which can expand and retract due to generation and consumption processes. The cells are modeled to move as a result of a pushing mechanism between cells in colonies and by an advective mechanism supported by the EPS dynamics. Detachment of both cells and EPS matrix follows a continuum approach, whereas cells attach in discrete events.

Three case studies are presented for model illustration and investigation: heterogeneous biofilm structure development, biofilm consolidation, and biofilm microcolony formation. Overall heterogeneity of the biofilm interface is very much dependent on the internal pressure governed by growth and decay of the different biomass components. The biofilm consolidation is explained by biofilm shrinking due to EPS and cell degradation processes. This mechanism describes formation of a denser layer of cells in the biofilm depth. Occurrence of other known structural characteristics, such as formation of an irregularly shaped biofilm surface under nutrient-limited conditions, was also apparent from the simulations. Microcolony formation is investigated by growth of autotrophic microbial colonies in an EPS matrix produced by heterotrophic cells. Size and shape of colonies of ammonia-oxidizing and nitrite-oxidizing bacteria were studied comparatively in a standard biofilm and in a biofilm growing on a membrane with aeration from the membrane side.

Modeling results suggested that the governing process behind biofilm consolidation is the presence of a negative pressure in the lower region of the biofilm, generated by EPS and cell degradation processes. The pressure gradients may lead to biofilm volume shrinking, with cell transport towards the substratum. This mechanism can explain the formation of a denser layer of cells in the biofilm depth. Two- and three-dimensional model simulations showed the same trend. The modeling results also suggested that the shape and size of autotrophic colonies embedded in the EPS develop as a function of nutrient availability.

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A three-dimensional computer model analysis of four hypothetical biofilm detachment mechanisms

J. D. Chambless and P. S. Stewart*

Four hypothetical mechanisms of detachment were incorporated into a three-dimensional computer model of biofilm growth and development. The model integrated processes of substrate utilization, diffusion, growth, cell migration and detachment in a cellular automata framework. The purpose of this investigation was to characterize each of the mechanisms with respect to four

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

criteria: the resulting biofilm structure, the existence of a steady state, the propensity for sloughing events and the behavior during starvation. The four detachment mechanisms analyzed represented various physical and biological influences hypothesized to affect biofilm detachment. The first detachment mechanism was based on the height of the biofilm. The probability of a cell detaching was made proportional to the square of its height above the substratum. This mechanism reflects, in an empirical fashion, the influence of fluid shear. The second mechanism made biofilm cell detachment a result of substrate starvation. When the local concentration of substrate around a biofilm cell dropped below a particular level for an extended period, the cell detached from the biofilm. The third mechanism addressed erosion. The detachment probability of a cell was made inversely proportional to the number of neighboring cells. Only cells that were located at the biofilm–bulk fluid interface were able to detach. The final mechanism combined the height and neighbor mechanisms. The different detachment mechanisms demonstrated diverse characteristics regarding structure, sloughing, and the existence of a steady state. The height-dependent mechanism produced flat biofilms that lacked sloughing events. A clear steady state was achieved for every case run with this mechanism. Detachment based on substrate starvation produced significant sloughing events in almost every case. The resulting biofilm structures included distinct, hollow clusters separated by channels. This mechanism, like the height mechanism, also produced clear steady states of biofilm growth. The neighbor mechanism did not produce either a non-zero steady state or sloughing events. The behavior of this mechanism was highly dependent on the specified detachment constant. If the constant was too high the biofilm decayed completely and if it was too low the biofilm achieved unrestricted growth. The structures of the biofilms produced by this mechanism were similar to those of the substrate starvation mechanism but did not include cluster hollowing. The final mechanism combined the neighbors and height mechanisms to create an amalgamation of their individual behaviors. This scenario created streaming biofilm structures that were unlike those of any other mechanism. Only experiments that were dominated by height detachment reached a steady state. The behaviors generated by these detachment mechanisms under conditions of nutrient downshift (starvation) are currently being simulated.

Modelling biofilm growth, detachment and fluid flow in a cross-section of tube reactors

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Recent applications of magnetic resonance imaging (MRI) to biofilms grown in tubular reactors has revealed the potential of this tool for investigation of transport phenomena and structure development in biofilm systems. The ability of the MRI technique to provide a high resolution, non-invasive determination of flow velocities and biofilm structure renders this an ideal source of data to further improve our understanding of biofilm dynamics. Here, we propose a two-dimensional model of biofilm development in a cross-section of a tubular reactor. The model uses IbM (individual-based modeling) to describe the growth and spread of biomass, coupled with diffusion–reaction equations for determination of the two-dimensional substrate distribution. The model computes the fluid velocity along the axial direction in the tube and, consequently, the tangential shear stress acting on the biofilm. The model was created by extending a previously developed framework for biofilm modeling. New routines were added to describe the circular geometry of a section of the tube reactor and for the solution of the axial fluid

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

velocity profile, also computed previously for squared section of honeycomb monolith packing. The model illustrates the development of the biofilm in the inner walls of a tube reactor. Therefore the model is geared to the study of biofouling under laminar flow conditions. The computational volume is a two-dimensional representation of the circular cross-section of the tube reactor with the computational domain consisting of three subdomains: (1) solid carrier, (2) biofilm, and (3) liquid phase. At each time iteration of the simulated biofilm development, the field of axial velocities is determined by solving a simplified Navier–Stokes equation, which includes only the velocity component in the axial direction. At the same time, radial solute concentration fields are computed by solving the diffusion–reaction mass balances for the relevant soluble substrates in pseudo steady state. Monod kinetic equations are used for biomass growth and for the consumption of a growth limiting soluble substrate. The location of a concentration boundary layer is determined from the two-dimensional field of liquid flow velocity using empirical correlations obtained from experimental studies of biofilm growth in tube reactors. Model simulations showed how the dynamics of biofilm development can be followed in time, with structural heterogeneity deriving from the reactor operating conditions. The pressure drop that results from biofilm growth on the inner tube walls can be also determined if constant liquid flow rate is applied. Likewise, combined pressure and volumetric flow loss can be computed from the Poiseuille flow. An illustrated version of this abstract is available at <http://biomath.itqb.unl.pt/~jxavier/jxavier-biofilms2006-abstract.pdf>.

Biofilm games

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The evolution of cooperative behaviour is one of the fundamental problems of evolutionary theory because cheaters that benefit from cooperating individuals, but do not invest in cooperation, have a higher fitness and it is difficult to explain how cooperation can evolve under such circumstances.

A primitive form of cooperative behaviour in bacteria is based on a trade-off between growth rate and yield: a high growth yield means that the substrate is used economically, and this economy benefits all individuals using this resource but comes at the cost of a reduced growth rate for the cooperating individual. In contrast, a cheater with a higher growth rate benefits from the economy of the others without paying the cost of a reduced growth rate.

Game theory has been one of the few approaches that have been successful in explaining the evolution of cooperation, almost exclusively using the Prisoner's Dilemma as the framework for investigating the conditions under which cooperation might arise. The Prisoner's Dilemma is characterized by the following ranking of the payoffs for the two players: Temptation to defect > Reward for mutual cooperation > Punishment for mutual defection > Sucker's payoff for the cooperator playing with a defector ($T > R > P > S$). This means that defection is always the winning and cooperation the losing strategy.

However, it is unclear to which extent the Prisoner's Dilemma game is played in nature. Here we asked which games the two trade-off-based growth strategies play in the contrasting environmental setting of chemostats and biofilms.

In chemostats, fitness is determined by the specific growth rate, usually described by the Monod model (using maximal growth rate and substrate affinity as the two growth parameters). The payoffs for the two players can be derived from evaluating the Monod function at the respective steady-state substrate concentrations. The resulting ranking of payoffs is $T > R = P > S$, placing this game between the Prisoner's Dilemma ($T > R > P > S$) and

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press

Deadlock games (T > P > R > S), defection is the best (dominant) strategy in either case.

In biofilms, the fitness of the players depends on the spatial structure, which changes with time. An analysis of payoffs in a simplified, flat biofilm with horizontally separated players is therefore more instructive. In this case, the fitness of the players is given by (growth yield) × (influx of substrate per area). At a low frequency of invaders, the ranking of payoffs is (R > T > S > P), which is known as the Game of Harmony. At a high frequency of invaders, the ranking is (R > S > T > P); this game has not been awarded with a name because it is of no theoretical interest. In either game, cooperation is the best (dominant) strategy.

In conclusion, the determinants of fitness in chemostats are maximal specific growth rate and substrate affinity, making defection the dominant strategy in a game similar to the Prisoner's Dilemma, while, in simplified biofilms, fitness is determined solely by the yield, making cooperation the dominant strategy in a game similar to the Game of Harmony.

https://doi.org/10.1017/S1479050506001992 Published online by Cambridge University Press