

# HYDROPHOBINS – Using hydrophobins to prevent microbial biofilm growth on mineral surfaces

**Rieder A. (1), Schwartz T. (1), Obst U. (1), Bollschweiler C. (2), Gutt B. (3), Zoller J. (3), Fischer R. (3)\***

(1) Department of Microbiology of Natural and Technical Surfaces, Institute of Functional Interfaces (IFG), Karlsruhe Institute of Technology (KIT), e-mail: thomas.schwartz@kit.edu

(2) BASF SE, e-mail: claus.bollschweiler@basf.com

(3) Department of Microbiology, Institute for Applied Biosciences, Karlsruhe Institute of Technology (KIT)  
e-mail: reinhard.fischer@kit.edu

\*Coordinator of the project

## Introduction

Microbial biofilms are an extremely successful way of life. Bacteria and fungi benefit in this symbiotic life form of metabolic exchange, protection and genetic flexibility. They produce a matrix of organic molecules in which they are embedded and which offers new habitats to other organisms, such as other bacteria or fungi. Biofilms cannot be avoided to colonize surfaces in unsterile habitats. So, they can be found everywhere in nature and in technical systems, but they play an ambivalent role. On the one hand biofilms are essential to degrade and transform water contaminations, but on the other hand they can diminish product qualities and damage capital equipment. Biofilms can cover medical equipment such as catheters and pathogenic bacteria, which may be living in the biofilms, are a continuous source of infection of the patients. In addition, the metabolism of the biofilm microorganisms may change the composition of the fluids or contaminate them with their products. As biofilms are all-round, the understanding of the biofilm formation and its manipulation are of prime importance in microbiology and material sciences.

The choice of a material and the corresponding surface properties like mechanical properties, structure, polarity, and chemistry influence the binding of various molecules and cells. The surface properties affect the biocompatibility of

a material and consequently also bacterial adhesion, and biofilm growth. In this project hydrophobins are used as a novel modification of surfaces to change surface properties like hydrophobicity and thus might have an effect on biofilm formation.

Hydrophobins are fungal proteins, which self-assemble on hydrophobic as well as hydrophilic surfaces into extremely stable monolayers. Recombinant hydrophobins provide the opportunity to use these highly surface-active proteins for large-scale surface coatings. Hydrophobins are non-toxic and can be used for surface modification and functionalization (with e.g. enzymes) of industrial relevant materials like steel, plastics, and ceramics.

In this project hydrophobin coated surfaces and their properties are studied with respect to bacterial cell adhesion, cell differentiation, and cellular growth with the aim to influence biofilm formation.

In the first part of the project recombinant hydrophobins were produced and purified. Different surfaces were coated with hydrophobins and characterized, since the coating efficiency is the basis for subsequent biofilm formation studies. Biofilms were grown on natural as well as hydrophobin coated surfaces and different methods were established to analyse biofilm formation.

Since the hydrophobin coated surfaces did not reduce microbial growth, we designed modified fusion hydrophobins and attached cationic antimicrobial peptides (AMPs) to the hydrophobins.

## 1. Hydrophobins

Hydrophobins are small proteins with about 100 amino acids, which are produced by filamentous fungi. In nature they coat the surface of fungal spores to ease the growth out of the ground and to protect the spore itself. It is recently known that hydrophobins also prevent the human immune recognition of airborne fungal spores.

There are two classes of hydrophobins, which only differ in the arrangement of eight highly conserved cysteine residues. Self-assembled monolayers formed by class I hydrophobins are more stable than those formed by class II hydrophobins. For our research we are using DewA, which is produced by *Aspergillus nidulans* and belongs to the class I hydrophobins. Since hydrophobins are highly surface-active they can easily be used for surface modification and functionalization. Until recently, the use of hydrophobins for large-scale surface coatings has been prevented by the fact that they had to be isolated from fungal cultures in a lengthy process. Recently the BASF SE achieved a breakthrough and established an expression system for the large-scale production of hydrophobins.

### 1.1 Fusion hydrophobins

Based on the large-scale industrial production, it is now possible to obtain large amounts of hydrophobins in a comparatively short time, but due to their characteristics, the hydrophobins

had to be produced as so called fusion hydrophobins. The BASF SE provides two fusion hydrophobins for surface coating. The available fusion hydrophobins are composed of the class I hydrophobin DewA of *Aspergillus nidulans* and a fusion partner. The fusion partner is the synthase yaaD of *Bacillus subtilis* in complete respectively shortened form (Figure 1). The synthase is used to lead the hydrophobin into the inclusion bodies in *Escherichia coli* for the purification. H\*Protein B has the shortest version of yaaD which still works as a leader. The fusion hydrophobins have a mass of 47 kDa respectively 19 kDa, whereas the hydrophobin part itself has a mass of only 10 kDa. In fusion hydrophobin H\*Protein A the hydrophobin part forms 20%, in H\*Protein B 50% of the total protein mass.

### 1.2 Modification of the fusion hydrophobins

Because of the lack of being antimicrobial by itself, we modified the fusion hydrophobins. We used cationic antimicrobial peptides (AMPs), which are only 9 to 50 amino acids in size and are proven to be active against bacteria and even against yeasts and filamentous fungi. They are an alternative to antibiotics and do not affect human cells. Until now there is no resistance known. The AMPs were fused to the N-terminus of the long and short version of the yaaD in the expression vector pQE60 (Figure 2). The modified plasmids (pHHC-011, pHHC-012, pF1-011 and pF1-012) were transformed into *E. coli* BL21. The induction of the expression was performed with 0.5mM IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside) at OD<sub>600</sub> 0.6 to 1.0; we let the culture grow over night. The purification of the modified fusion hydrophobins

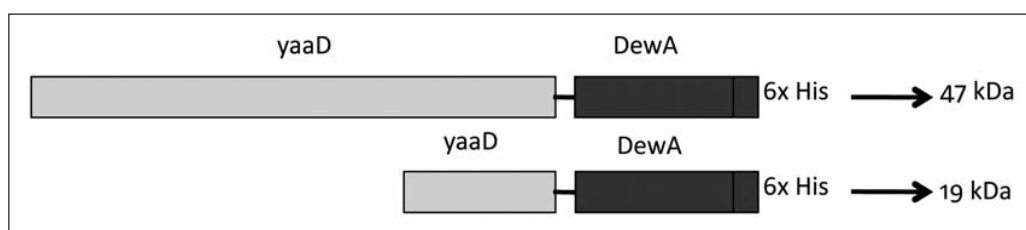


Figure 1: The fusion hydrophobins consist of the synthase yaaD in complete (H\*Protein A) respectively shortened (H\*Protein B) form, the class I hydrophobin DewA and a C-terminal His-tag.

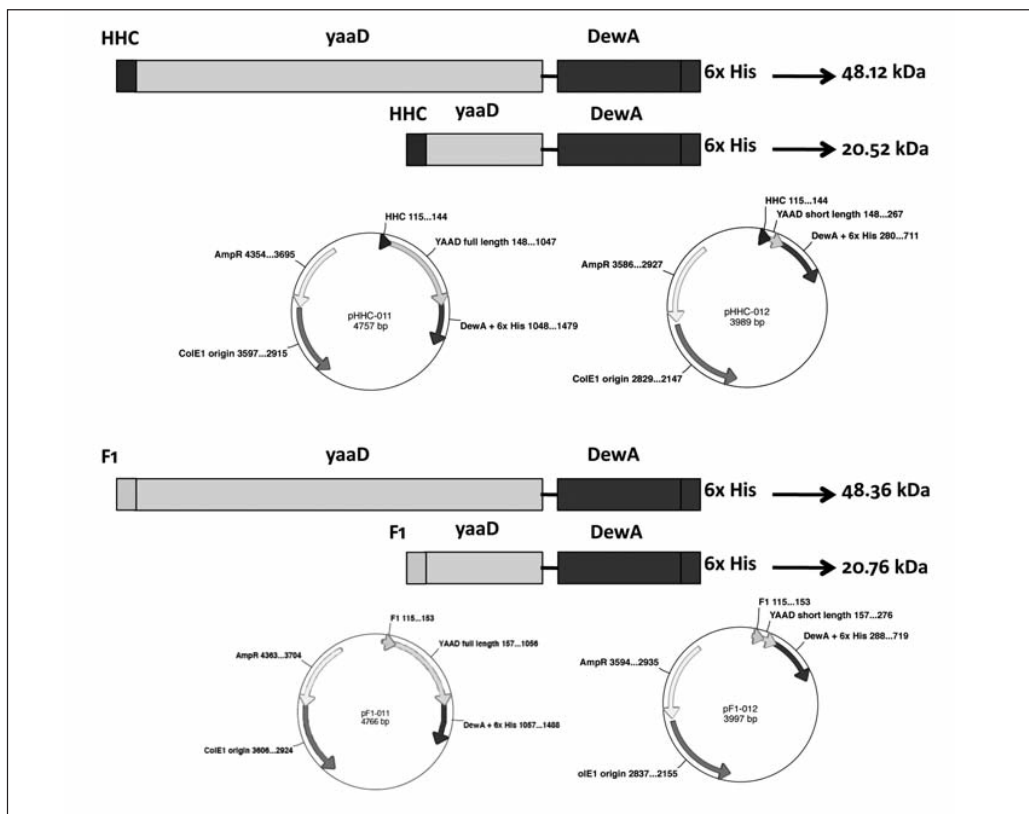


Figure 2: Fusion hydrophobins with cationic antimicrobial peptides. We fused the AMP to the N-terminal site of the yaaD. After biochemical calculations the AMPs are on the top of the coated surfaces and the contact point for biofilms.

was done by an approved protocol of the BASF SE, where a pH-shift from 12 to 9 dissolves the modified fusion hydrophobins out of the IBs. After centrifugation the dissolved proteins remain in the supernatant, which was lyophilized afterwards. The molecular masses of the modified fusion proteins are only a little larger, because of the small size of the AMPs. The modified fusion hydrophobins have a mass of about 48 kDa and 20 kDa, respectively (Figure 3).

### 1.2.1. Testing the functionality of the modified fusion hydrophobins

We used different organisms, gram-positive and gram-negative bacteria and eukaryotes (*S. pneumoniae*, *B. subtilis*, *E. coli*, *P. putida* and *A. nidulans*) to show the functionality of the modified fusion hydrophobins. We performed killing curves (*A. nidulans*, *B. subtilis*, *S. pneumoniae*) and peptide activity tests (*A. nidulans*, *B. subtilis*, *S. pneumoniae*, *P. putida*) to show this. First we tested if only the fusion hydrophobins

have an effect on the growth of microorganisms. We used the strain *B. subtilis* TMB488, which is a reporter strain for the two-component system LiaFSR and is inducible by cell envelope stress by antibiotics, which have an effect on the lipid II cycle (Jordan et al., 2007). Using different concentrations of the fusion hydrophobin we could not detect a growth inhibition (Figure 4). We found the same results with the other organisms. Killing curves were performed using the modified fusion hydrophobins, but there was no difference in the growth recognizable.

Because of these results we decided to perform peptide activity assays in microtiter plates. Peptide activity assays were performed earlier to check the antimicrobial activity of the AMPs F1 and HHC in *A. nidulans* (Mania et al., 2010). We found MICs (Minimal Inhibitory Concentrations) of about 1 µg/ml respectively about 5 µg/ml, so we decided to use these AMPs for modification.

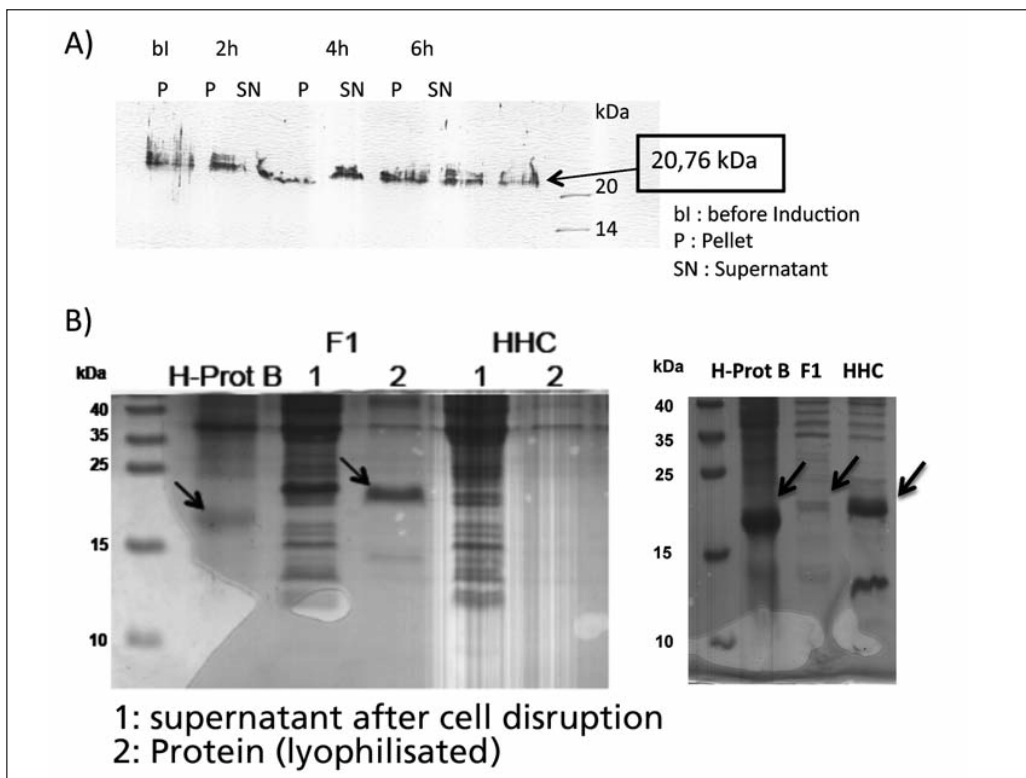


Figure 3: Over expression analysis. A) Western blot: The modified fusion hydrophobin F1-012 serves as an example for a western blot. The modified fusion hydrophobins were transformed with *E. coli* BL21 an expression strain. With induction by 0.5mM IPTG the modified fusion proteins were expressed and could be detected by the 6x-His tag. There is a clear band at about 20kDa, which corresponds to the mass of the modified fusion hydrophobin. B) SDS-Gels: By over expression the modified fusion proteins were enriched and applied on 15-15% SDS Gels. Of the lyophilized protein powder we applied 10µg/ml in each column. As comparison serve the fusion hydrophobins we got from the BASF SE.

In each column of the plate, one peptide is diluted from top to bottom to roughly identify the MIC. This is done in Minimal Medium (MM) with glucose as sole carbon source and resazurin as indicator of viability. Resazurin is a blue dye that turns pink when it is reduced, for example by respiration of living microorganisms in the medium. Each well is inoculated with a defined amount of spores (105 spores) of an *A. nidulans* wild type strain (RMS011). First we tested all the AMPs we have in our stock (F1, HHC, #15, #29, Bac2a, C6, D5, E6, Indolicidin and Kai13) and then we tested the modified fusion hydrophobins. There was no growth inhibition of the F1 and HHC fusion hydrophobin in any tested organism (Figure 5, and data not shown), although the peptides alone were highly active. This suggested an inhibitory effect of the fused hydrophobin.

### 1.2.2. Modification of fusion hydrophobins with a surface-tethered peptide

Meanwhile Hilpert et al. (2009) found some AMPs, which work better while they are tethered to surfaces. One of these peptides, KaiH, consists of 12 amino acids (WIVVIWRRKRRR) and has a mass of 1.7 kDa. We attached this peptide to the N-terminus of the shortened and full-length version of the yaaD and cloned it also in the pQE60 vector (Figure 6). As a negative control we chose a peptide slightly changed peptide, hW (WIVVIWRKRRR), and attached it also to the shortened and full-length versions of the yaaD. The over expression and purification will be done according to the same protocol as before.

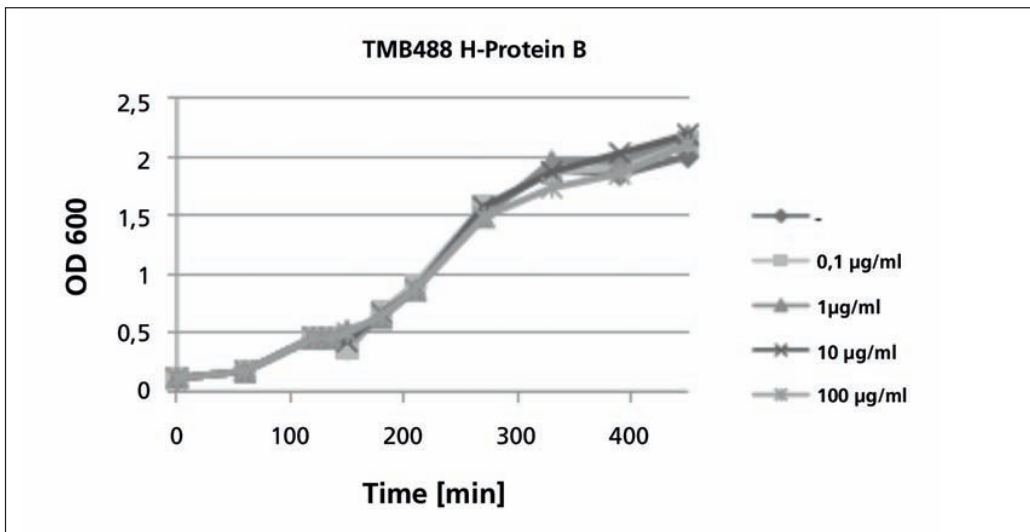


Figure 4: Growth curve of *B. subtilis* TMB488. The strain grew in LB-Media (lysogenic broth) with 5 µg/ml chloramphenicol. A 100ml culture was inoculated at OD<sub>600</sub> 0.1 with a preculture of *B. subtilis* TMB488. Then let it grow until OD<sub>600</sub> 0.6 when it was split into five 10ml cultures. To each of these cultures we gave a different concentration of fusion hydrophobins (0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml). One of these grew without any add-on.

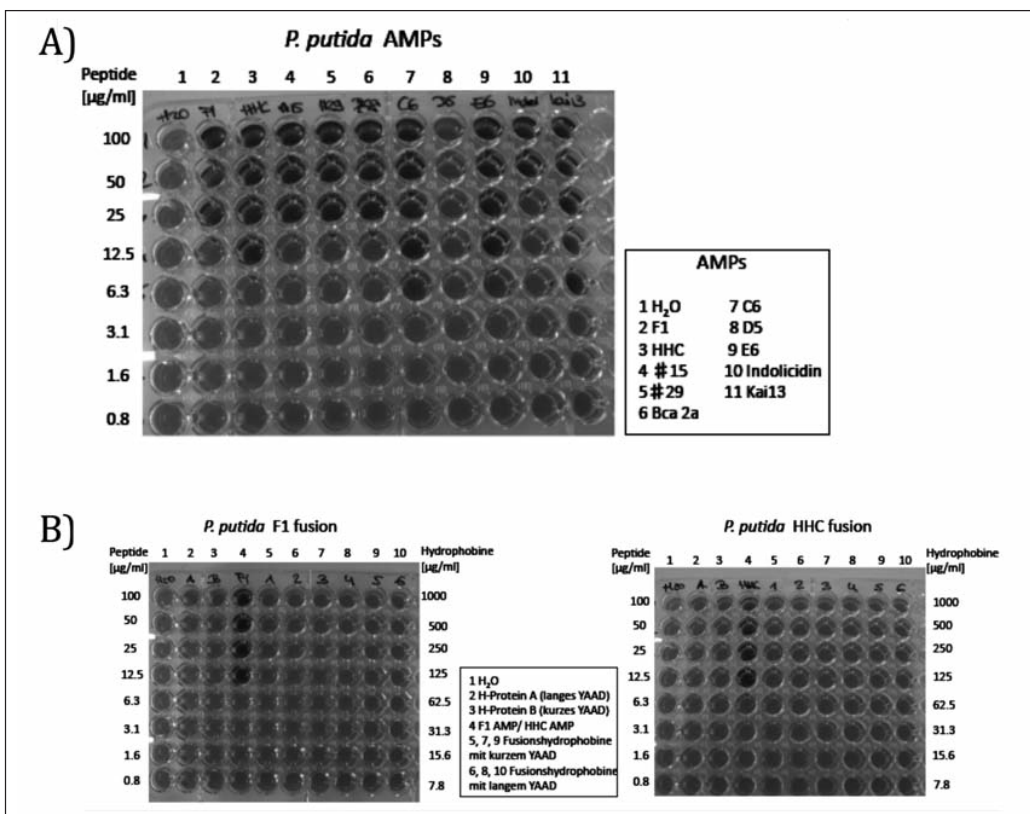


Figure 5: Peptide activity assays with *P. putida*. A) Testing 10 different AMPs for antimicrobial activity. As control serves aqua dest. In each column there is another peptide with decreasing concentration. B) In a second step the modified fusion proteins (5-10) were tested. In each column there is another modified fusion protein with decreasing concentration. As control serve the AMP (F1 or HHC), aqua dest. and the fusion hydrophobins from the BASF SE.

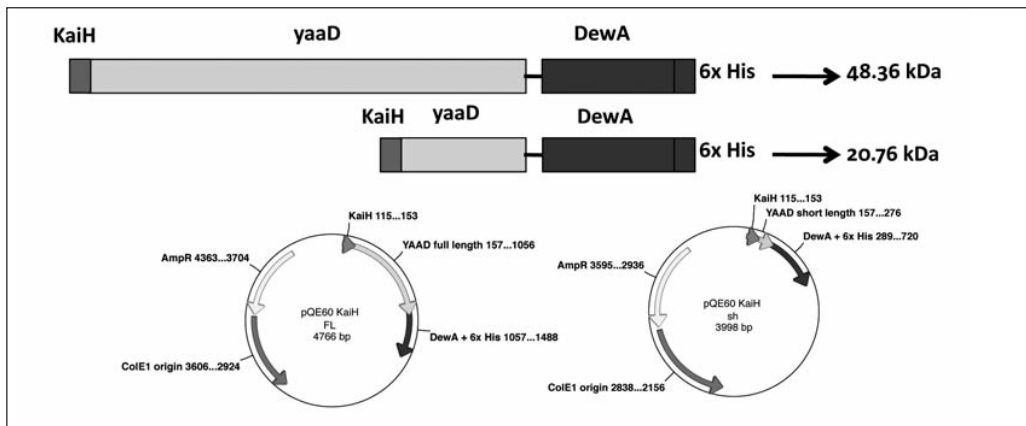


Figure 6: Modified fusion hydrophobins with the cationic antimicrobial peptide KaiH. Fusion of the AMP to the N-terminal side of yaaD and cloning in the pQE60 vector.

## 2. Characterization of hydrophobin coated surfaces

In this project hydrophobin coated surfaces and their properties are studied with respect to bacterial cell adhesion, cell differentiation, and cellular growth with the aim to influence biofilm formation.

In a first step unmodified recombinant fusion hydrophobins were used and a coating protocol was developed. The hydrophobin coated surfaces were characterized under various aspects.

### 2.1 Surface coating with hydrophobins

Glass surfaces were used as reference material for coating experiments. Glass is a very hydrophilic material and thus comparable to ceramic materials. The glass surfaces were coated with the fusion hydrophobins H\*Protein A and H\*Protein B. The used concentration of the hydrophobin solution was 10  $\mu$ M. The glass surfaces were coated according to *Janssen et al. (2004)*. The hydrophobins were solved in buffer (50mM Tris-HCl, 1mM CaCl<sub>2</sub>, pH 8). Subsequently, the surfaces were cleaned with ethanol and incubated for 1-16 hours at 23-80 °C in the hydrophobin solution. During the incubation the hydrophobins self-assembled on the surface in  $\alpha$ -helical conformation. The surfaces were rinsed with distilled water and dried at room temperature. Half of the surfaces was further treated for the induction of a  $\beta$ -sheet shift. The  $\beta$ -sheet conformation of the hydrophobin coating is said to be more

stable than the  $\alpha$ -helical conformation. For this the surfaces were incubated for 10 minutes at 80 °C in 2 % SDS-solution, subsequently rinsed with distilled water and dried.

Different parameters like incubation temperature and incubation time might influence the self-assembly behavior and the postulated monolayer of hydrophobins. These parameters were changed to analyze the fundamental characteristics of fusion hydrophobins on surfaces.

### 2.2 Characterization of hydrophobin coated surfaces

Different surface analysis methods were applied for the characterization of hydrophobin-coated surfaces and the clarification of the influence of the variable coating parameters. The hydrophobin-coated surfaces were analyzed in matters of change of surface hydrophobicity and homogeneity of the coating. In addition also the adsorption characteristics of hydrophobins were studied.

#### Change of surface hydrophobicity

Contact angle measurement (CA) is a simple-to-adopt method for surface analysis. It is used to detect the presence of films or coatings and to determine their properties. The contact angle describes the shape of a liquid droplet resting on a solid surface and thus gives information about the wettability of the surface. The more a liquid droplet spreads, the smaller is the contact angle and the more hydrophilic

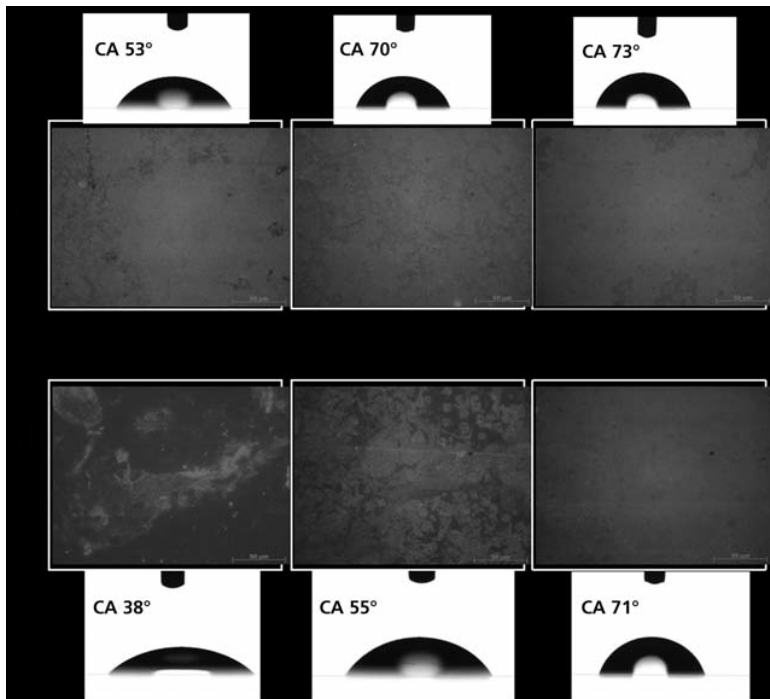


Figure 7: Contact angles and immunofluorescence microscopy are applied to monitor the change of surface hydrophobicity and coating homogeneity of hydrophobin coated surface. The fusion hydrophobins H\*Protein A and H\*Protein B adhere in a temperature and time dependent manner. The longer the incubation time the more homogenous is the coating in alpha-helical and beta-sheet conformation. Less surface hydrophobicity is lost with the induction of the beta-sheet shift at long incubation times.

is the surface. Since hydrophobins are amphiphilic proteins, which self-assemble on surfaces the contact angle of uncoated vs. coated surfaces is changed and used as a parameter for the efficiency of the coating.

For contact angle measurements the *Contact Angle System OCA20* and the software *SCA20* (Dataphysics, Filderstadt, Germany) were used. The contact angle of a 5  $\mu$ l water droplet (sessile drop technique) was calculated with the Laplace-Young equation. The contact angle was determined on at least five different spots on the surface.

The hydrophobin coating changed the wettability of the glass surface. Glass is a hydrophilic material (contact angle  $11^\circ \pm 2^\circ$ ). With the self-assembly of the hydrophobins on the glass surface it became more hydrophobic. The change of the surface wettability was dependent on the incubation time, the incubation temperature and the hydrophobin conformation.

H\*Protein A and H\*Protein B changed the wettability of the glass surface in a very similar way. Longer incubation times and an increased temperature resulted in significant hydrophobicity changes of about  $60^\circ$  (Figure 7). The induction of the  $\beta$ -sheet shift caused losses of

up to  $20^\circ$  of primarily achieved surface hydrophobicity. Here a big influence of incubation temperature and incubation time was noticed. Coatings prepared at room temperature lose much more hydrophobicity compared to coatings at  $80^\circ\text{C}$ . The longer the incubation time the more stable was the coating and the less was lost with the induction of the  $\beta$ -sheet shift.

These results indicated that the longer the incubation time and the higher the incubation temperature the more efficient and stable was the hydrophobin coating. A **long incubation time** (16h) and **high incubation temperature** ( $80^\circ\text{C}$ ) **increased** the **surface hydrophobicity** of hydrophobin coated surfaces significantly.

In addition to the surface hydrophobicity also the stability of hydrophobin coatings was determined with contact angle measurements. The surface hydrophobicity of freshly coated surfaces was compared to the surface hydrophobicity of surfaces stored at room temperature for up to five weeks. The coating was stable and no change was detected in surface hydrophobicity over time.

#### *Homogeneity of hydrophobin coating*

To determine the homogeneity of the hydrophobin coating immunofluorescence microscopy was applied. Immunofluorescence microscopy is often used in biology and medicine to visualize proteins and their distribution. The proteins act as antigens and can be detected with fluorescent labeled antibodies. They are studied using a fluorescence or confocal microscope.

For the detection of the hydrophobin layer on the surface a primary Anti-his antibody was used which specifically binds to the His-tag of the proteins. A secondary fluorescent labeled antibody binds to the Fc region of the primary antibody and could be detected with the fluorescence microscope.

The characteristics of H\*Protein A and H\*Protein B on glass surfaces were very similar. Surface coatings prepared at room temperature in  $\alpha$ -helical protein conformation were homogenous with just a few holes and cracks. The longer the incubation time the more homogenous was the coating, but the differences were insignificant. After the induction of the  $\beta$ -sheet shift the surface coating was very disordered. On glass surfaces coated for one respectively six hours at room temperature no hydrophobin coating was detectable with immunofluore-

scence microscopy after the  $\beta$ -sheet shift. These results were in accord with the contact angle data, which showed that a lot of hydrophobicity was lost on these surfaces.

The surfaces which were coated at 80 °C were much more homogenous compared to the surfaces coated at room temperature. There were slight differences between the various incubation times, but all coatings in  $\alpha$ -helical conformation were homogenous (Figure 7). After the induction of the  $\beta$ -sheet shift big differences occurred. The longer the incubation time the more homogenous was the coating. These results confirmed the contact angle data. The longer the incubation time and the higher the incubation temperature the more homogenous was the coating in the  $\alpha$ -helical and the  $\beta$ -sheet conformation. It was essential to incubate the materials for **16 hours at 80 °C** in the protein-solution to form a **homogenous hydrophobin-layer in  $\alpha$ -helical and  $\beta$ -sheet conformation**.

To confirm the results of the immunofluorescence microscopy AFM (atomic force microscope) measurements were applied. The measurement were performed in the measurement cell of an MFP-3D BioAFM (Asylum, Mannheim) having a commercial  $\text{Si}_3\text{N}_4$  cantilever of

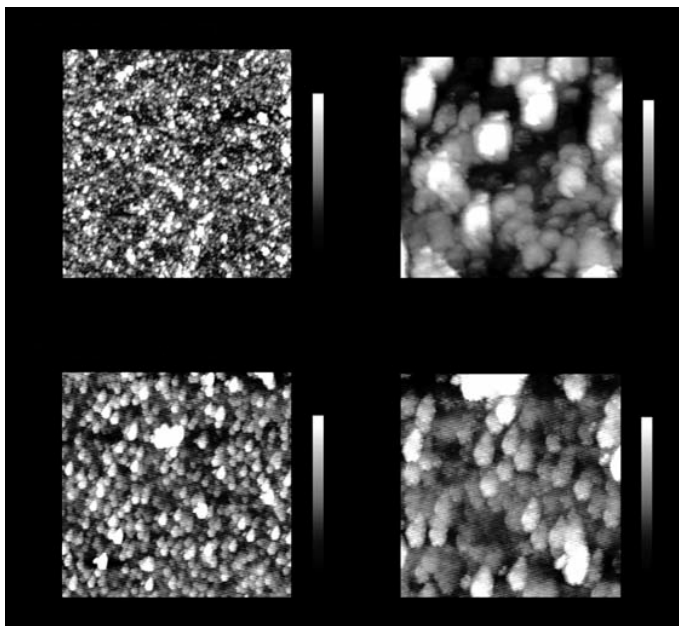


Figure 8: AFM height images of glass surfaces coated with H\*Protein A and H\*Protein B in alpha helical conformation for 16 hours at 80 °C. The proteins self assemble on the surfaces in ordered globular structures.



a normal spring constant of 0,56 N/m (nano and more) in air. The microscope was operating in an AC-mode, where the tip was scanned back and forth at 0° along the horizontal line in a scan range of 10 µm. The AFM was used with a 5 µm z-range and 150 µm x- and y-range scanner (type J Digital instruments). Topographic images were evaluated with the Scanning Probe Image Processor (SPIP).

On glass surfaces hydrophobins adhered in ordered protein layers. Surface coatings performed at 80 °C with H\*Protein A and H\*Protein B in alpha-helical and beta-sheet conformation showed a globular protein adsorption pattern (Figure 8). These results were in accordance with the immunofluorescence microscopy. Also at the **nano level** hydrophobin coatings performed for **16 hours at 80 °C** were **homogenous**.

#### *Adsorption characteristics of hydrophobins*

The adsorption characteristics of fusion hydrophobins were monitored with QCM-D (quartz crystal microbalance with dissipation) measurements. QCM is a very sensitive tool to detect changes in weight and thus a helpful method to sense adsorption processes on surfaces. The quartz crystal microbalance determines a mass per unit area by measuring the change in frequency of a quartz crystal resonator. The resonance is disturbed by the addition or removal of a small mass due to, for example, protein deposition at the surface of the acoustic resonator. It can be used under vacuum, in gas phase and in liquid environments. In addition to measuring the frequency, the dissipation is measured. The dissipation is a parameter quantifying the damping in the system, and is related to the sample's viscoelastic properties.

The adsorption of H\*Protein A and H\*Protein B (10µM in 50mM Tris-HCl, 1mM CaCl<sub>2</sub>, pH 8) were analyzed on a SiO<sub>2</sub> coated quartz crystal. The protein layer thickness and the absorbed mass were calculated with the Voigt viscoelastic model. The protein adsorption was monitored for up to 16 hours at 20 °C and a flow rate of 50 µl/min. The stability of the for-

med protein layer was determined by rinsing with 2% SDS solution.

H\*Protein A formed a layer of 17 ± 3 nm and H\*Protein B of 14 ± 2 nm on the SiO<sub>2</sub> surface. After 1 hour of incubation the maximum layer thickness was already reached. No more changes were observed during further incubation. These results were in accordance with the data of the contact angle measurements and the immunofluorescence microscopy. To achieve a homogenous hydrophobin coating and a significant change of the surface hydrophobicity short incubation times were sufficient. The formed coating thickness of 17 ± 3 nm respectively 14 ± 2 nm matched the expected thickness of hydrated monolayers of fusion hydrophobins. The fact that even with a longer incubation time no changes of the larger thickness were monitored, pleads for the formation of a stable monolayer.

The adhered protein mass was 17 ± 3 mg/m<sup>2</sup> for H\*Protein A and 14 ± 2 mg/m<sup>2</sup> for H\*Protein B (Figure 9). Unfortunately the formed protein layer was not stable under these conditions. After rinsing with 2% SDS solution the hydrophobin layer was detached completely. As already mentioned, the temperature plays an important role in the formation of a stable hydrophobin layer. Contact angle measurement and immunofluorescence microscopy have shown that higher temperatures (80 °C) were necessary to form stable monolayers. The QCM experiments were performed at 20 °C. This might result in this non-stable hydrophobin layer.

Various surface analysis methods were established and applied for the characterization of hydrophobin coated surfaces. At a later stage of this project these techniques will also be used for the characterization of surface coatings with modified fusion hydrophobins. The influence of the modification on the surface performance of the fusion hydrophobins will be determined.

### **3. Analysis of microbial biofilm formation on different surfaces**

Biofilms were grown on natural as well as

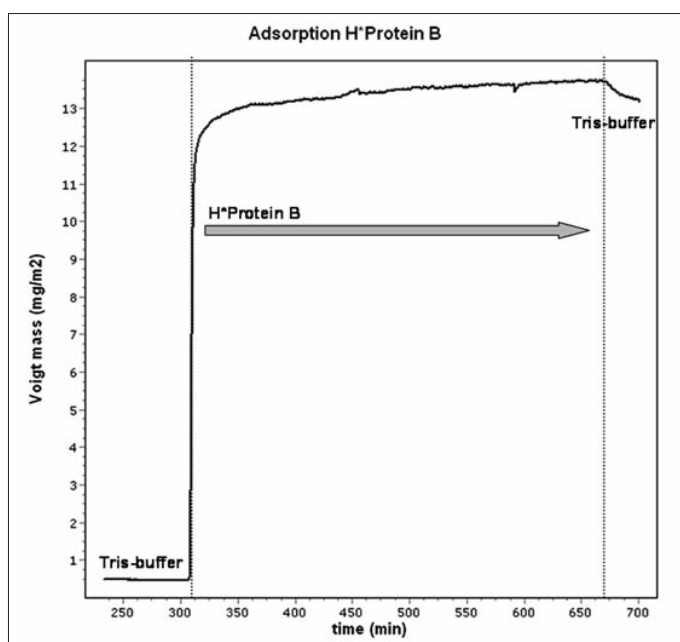


Figure 9: Adsorption of hydrophobin H\*Protein B (10 $\mu$ M) in Tris buffer (50mM Tris, 1mM CaCl<sub>2</sub>, pH 8.0) on SiO<sub>2</sub> surface monitored with QCM-D. The adsorped mass was quantified with Voigt viscoelastic model.

hydrophobin coated surfaces with single species and natural mixed populations. Different methods were applied and established to analyze the bacterial and fungal population in terms of population composition, microbial density and spatial distribution. Additionally the interaction of bacteria and fungi in biofilms was monitored.

### 3.1 Bacterial biofilm formation

Microbial biofilms represent a special, very successful life form of bacteria. The microorganisms benefit in this symbiotic life form from metabolic exchange, genetic flexibility and protection, which a biofilm offers in comparison to planktonic growth. Nearly all bacterial species are able to exhibit this type of growth firmly adhering to a substrate, in addition to the planktonic freely floating growth variety. A special characteristic of all biofilms, besides firm adhesion to one site, is their highly structured character (Costerton *et al.* 1999, Costerton *et al.* 2001.) The development of a biofilm proceeds in various chronological steps. These are dependent on the involved bacterial species external factors and also surface characteristics. The first step, the initial adhesion is reversible. With the production of a matrix of organic molecules, the extracellular matrix, the adhesion gets irreversible. The bacteria are

embedded in the extracellular matrix, which also offers new habitats to other organism such as other bacteria or fungi. The biofilm starts to mature and to form three-dimensional structures. A mature biofilm releases single cells, which again adhere to the substrate and start the biofilm life cycle again.

In order to characterize the influence of hydrophobin coated surfaces on bacterial biofilm formation the primary adhesion as well as mature biofilms grown on uncoated and coated surfaces were analyzed.

#### Primary adhesion

The primary adhesion is the first and consequently the crucial step at the change from planktonic to sessile growth.

#### Primary adhesion of *Escherichia coli* on hydrophobin coated and uncoated glass slides

To analyze the primary bacterial adhesion a GFP-tagged *E. coli* strain (*E. coli* BW3110, pJOE 4056.2 His e-GFP) was used. After induction of the promoter with 0.2% Rhamnose the »green fluorescent protein« (GFP) is stable expressed intracellular. The bacteria can be detected with the epifluorescence microscope without further labeling steps.

*E. coli* was grown in biofilm reactor on hydrophobin coated and uncoated glass slides and

the bacterial adhesion was monitored at different time points for **up to 24 hours**. The biofilm was washed in 0.89 % NaCl to remove loosely attached microorganisms and subsequently analyzed with epifluorescence microscopy.

More bacteria adhered on hydrophobin coated glass slides compared to uncoated glass slides.

#### Quantification of biofilm growth

For the quantification of the first steps of the biofilm formation a crystal violet assay was applied. Individual cavities of a 96-well microtiter plate (polystyrol) were coated with H\*Protein A and H\*Protein B according to the established protocol. The **GFP-tagged *E. coli*** (*E. coli* BW3110, pJOE 4056.2 His e-GFP) was grown overnight and diluted in sterile growth medium (OD<sub>600nm</sub> 0.25). 100 µl bacterial suspension were portioned in each cavity and incubated at 37 °C for **up to 6.5 hours**. The adhered bacteria were stained with crystal violet and the biofilm formation could be quantified with the determination of the optical density at 590nm.

At most time points a higher adhesion of *E. coli* was detected on hydrophobin coated surfaces compared to uncoated surfaces. After just 0.5 hours a significant higher bacterial adhesion on the various hydrophobin coated surfaces was monitored. This effect diminished and even inverted after 6.5 hours. Now more bacteria adhered on hydrophobin coated surfaces compared uncoated polystyrol (Figure 10).

#### Mature biofilm

The mature **bacterial biofilms** were grown in **wastewater effluent** on hydrophobin coated and uncoated glass slides for up to **four weeks** and subsequently analyzed in terms of population composition, microbial density and spatial distribution.

#### Population analyses

Molecular biological population analyses like PCR-DGGE (polymerase chain reaction with subsequent denaturing gradient gel electrophoresis) allow studying the diverse population of bacteria in wastewater biofilms. Population analyses were used to detect variations in the composition of the bacterial population on hydrophobin coated and uncoated surfaces. For the analysis of the bacterial population the biofilm DNA was isolated and amplified in a PCR-reaction with the primer set 27f and 517r targeting the eubacterial 16S rDNA. The 16S rDNA of prokaryotes is the most conserved (least variable) gene and for this reason used to identify bacteria. The initial PCR-reaction resulted in a mixture of PCR amplicons which all have the same length (526 bp). Sequence variations like differences in GC content and distribution were used to separate these amplicons in a denaturing gradient gel electrophoresis. Here each band represents one bacterial species. DGGE banding patterns can be used to visualize variations in microbial diversity and provide a rough estimate of the richness

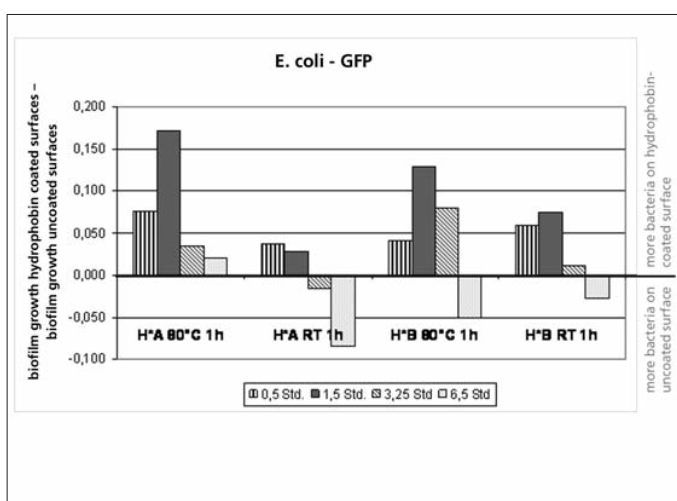


Figure 10: The primary adhesion of *E. coli* was monitored at different time points and quantified with crystal violet staining.

The differences in the biofilm growth on hydrophobin coated and uncoated surfaces are shown. At early time points (0.5 h) significantly more bacteria adhere on hydrophobin coated surfaces.

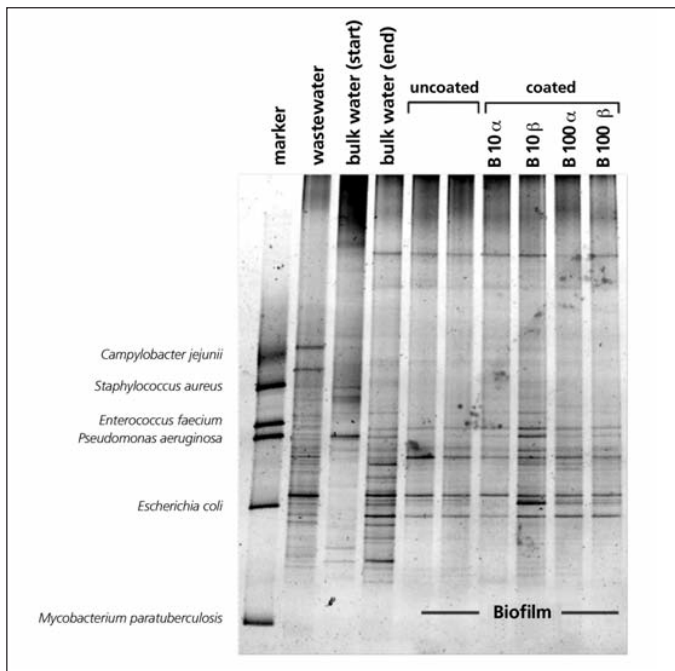


Figure 11: DGGE profiles of amplified partial 16S rRNA genes of planktonic wastewater bacteria (right) and wastewater biofilms (left). The bands obtained from different pure cultures of hygienically relevant bacteria serve as reference marker (left): *Campylobacter jejunii*, *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *E. coli*, *Salmonella enterica*, *Mycobacterium paratuberculosis* (top to bottom). The banding pattern of wastewater biofilms on uncoated and coated materials showed no significant differences.

and abundance of predominant microbial community members. The bacterial population was analyzed with PCR-DGGE (Figure 11). The planktonic bacterial population (left) differed clearly from the biofilm population (right). No significant differences were visible in the banding pattern of biofilms grown on different surfaces. The bacteria adhered with the same preference to natural and hydrophobin coated surfaces.

#### Microbial density

A further characteristic of biofilm growth is besides the population composition the number of bacteria growing in a biofilm. Colony forming units (CFU) were used to estimate the growth density. The biofilm was washed in 0,89% NaCl to remove loosely attached microorganisms, scraped of the surface with a sterile cell scraper, resuspended in sterile PBS and serially diluted. Suitable dilution steps were plated in triplicates on DEV-agar. They were incubated for up to 7 days at 37 °C. The bacterial colonies were counted and the number of bacteria per square cm was calculated. A lot of bacteria grew on the uncoated and hydrophobin coated surfaces, but there was no significant difference in the cell numbers on various materials.

#### Spatial distribution of biofilm growth

Biofilms were stained directly on the surface with the DNA intercalating fluorescent dye Syto9 to scan the spatial distribution of biofilm growth. Prior to fluorescent staining the biofilm was washed in 0.89% NaCl to remove loosely attached microorganisms. Syto9 solution was dropped on the biofilm sample to stain all present microorganisms. The sample was incubated for 20 minutes in the dark at room temperature and subsequently washed carefully with water. The slides were prepared with the mounting media Citifluor and a coverslip for fluorescence microscopy. No significant differences were observed in the spatial distribution of the biofilm growth on uncoated and hydrophobin coated surfaces.

Different steps of biofilm development were monitored on hydrophobin coated and uncoated surfaces. Hydrophobin coated surfaces influence the primary bacterial adhesion. The bacteria are attracted by the hydrophobin coated surfaces and adhere in higher numbers as on uncoated surfaces. But the impact of hydrophobin coated surfaces on biofilm formation seems to be time dependent. In mature biofilms (4 weeks) no differences in biofilms

established on natural and hydrophobin coated surfaces could be detected.

The established techniques for the biofilm characterization will further be applied to characterize the influence of modified hydrophobins on biofilm formation. The hydrophobins will be modified with antimicrobial peptides to enhance their effect on biofilm formation.

### 3.2 Fungal biofilm formation

In addition to bacteria there are also fungi inside of a biofilm. When the mature biofilm is formed, fungi joined the biofilm. To analyze the fungal populations in biofilms we use native biofilms. A native biofilm is a biofilm, which can be found in the natural environment and harbors more than one microorganism species. In a native biofilm, protozoan, algae, bacteria and fungi can be found.

### Design of a clone library

To analyze and identify the fungal population of a biofilm a molecular biological approach was used and a clone library was designed. A clone library offers the advantage that also fungi, which are not able to grow on agar plates, can be detected and identified. With a clone library it is possible to estimate the abundance of single fungal species in biofilms. For the generation of the clone library the complete biofilm DNA was isolated and amplified in a PCR-reaction with the primer set ITS1 and ITS4. These primers are specific for the ITS-region of eukaryotes. The ITS-region is well suited for the identification of fungi since it has a higher variability than other regions of the rDNA. The PCR products were cloned in *Escherichia coli* and tested with a colony PCR (primer set M13/T7) and the restriction enzyme

Table 1: Isolated fungi and protozoa from native biofilms. A clone library was designed for the isolation of the fungi with the amplified ITS-region in *E. coli*. The sequences were analyzed by a M13/T7 PCR, a digestion and by the online-tool BLAST. Plant 1 and 2 are the different places in the purification plant of Karlsruhe/Neureut. Plant 1 is the place in the mechanical treatment direct after the rack and the second place was in the biological treatment of the plant. Influent biofilm is a biofilm, which was incubated in influent wastewater for one month. The isolates from the biofilm of the lake are also listed in the table.

Species	Order	Season	Origin
<i>Achlya</i> sp.	Saprolegniales	Winter	Plant 1
<i>Anguillospora longissima</i>		Winter	Lake
uncultured Blastocladiomycota		Spring	Plant 2
<i>Candida boidinii</i>	Saccharomycetales		Influent biofilm
<i>Candida pelliculosa</i>	Saccharomycetales	Winter	Plant 1
<i>Candida tropicalis</i>	Saccharomycetales	Winter, Spring	Plant 1
<i>Cladosporium cladosporioides</i>	Capnodiales	Winter	Lake
<i>Fusarium merismoides</i> var. <i>merismoides</i>	Hypocreales	Fall	Plant 1
<i>Galactomyces geotrichum</i>	Saccharomycetales	Spring	Plant 1
<i>Geotrichum</i> sp. ( <i>Galactomyces</i> sp.)	Saccharomycetales	Spring, Fall	Plant 1
<i>Mortierella</i> sp.	Mortierellales	Winter	Plant 1
<i>Mucor racemosus</i>	Mucorales		Influent biofilm
<i>Nectria lugdunensis</i>	Hypocreales	Winter	Lake
<i>Penicillium commune</i>	Eurotiales		Influent biofilm
<i>Pichia anomala</i>	Saccharomycetales	Winter	Plant 1
<i>Phoma herbarum</i>		Winter	Lake
<i>Phythium</i> sp.	Pythiales	Winter	Plant 1
<i>Protoventuria alpina</i>	Helotiales	Summer	Plant 1
<i>Rhinoctadiella atrovirens</i>	Chaetothyriales	Spring	Plant 1
<i>Rhodotorula mucilaginosa</i>	Sporidiobolales	Spring	Plant 2
<i>Saccharomyces cerevisiae</i>	Saccharomycetales	Spring	Plant 1
<i>Sporobolomyces lactosus</i>	Erythrobasidiales	Fall	Plant 1
<i>Sporobolomyces symmetricus</i>	Erythrobasidiales	Spring	Plant 1
uncultured Tremellomycetes		Winter	Plant 1
<i>Trichosporon</i> sp.	Tremellales	Winter, Spring	Plant 1
<i>Trichosporon cutaneum</i>	Tremellales	Winter	Plant 1
<i>Trichosporon domesticum</i>	Tremellales	Summer	Plant 1
<i>Trichosporon montevideense</i>	Tremellales	Winter	Plant 1
<i>Cyclidium glaucoma</i>	Pleuronematida	Spring	Plant 2
<i>Entamoeba</i> sp.	Entamoebida	Spring	Plant 1
<i>Oxytricha</i> sp.	Stichotrichida	Spring	Plant 2
<i>Paramecium tetraurelia</i>	Peniculida	Spring	Plant 2
<i>Zoothamnium</i> sp.		Winter, Spring	Plant 1, 2
<i>Zoothamnium plumula</i>		Winter	Plant 1

MspI. Clones, which showed different banding patterns on an agarose gel after the restriction digest, were sequenced.

The isolated and identified fungi and protozoa from wastewater biofilms and biofilm from a lake in Karlsruhe-Leopoldshafen are listed in table 1. In addition to these isolates, 111 clones were sequenced which were not described so far. All isolates of the biofilm from the plant in autumn, the Alb, the effluent and the Rhein were species, which are not described so far. Most of the undescribed clones belong to the genus *Candida* and *Trichosporon*, which can be shown in a genealogical tree. Until now it is in general not much known about fungi in biofilms. These clones are very interesting since they might be fungi preferably growing in biofilms (Table 1). There was no significant difference in the fungal population on uncoated and hydrophobin coated materials.

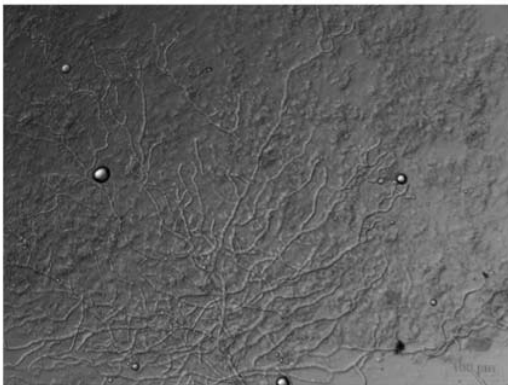
### ***Spatial distribution of biofilm growth***

Biofilms were stained with Calcofluor directly on the surface of glass slides. Calcofluor binds specifically to chitin and cellulose and stains fungi and algae. Prior to fluorescent staining the biofilm was washed in 0.85% NaCl to remove loosely attached microorganisms. For the staining of fungi and algae a small volume of 0.1% Calcofluor and 15 % KOH were dropped on the biofilm and subsequently washed with 70 % ethanol and water. The samples were analyzed with the fluorescence microscope (Figure 12).

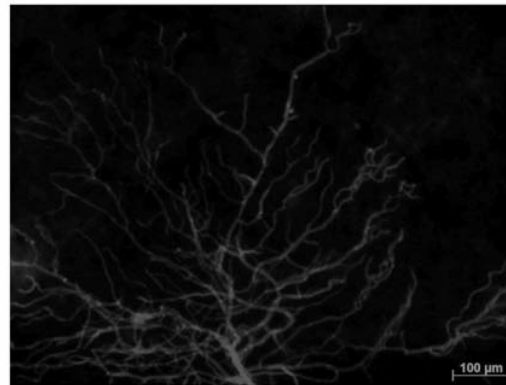
### ***3.3 Interaction of bacteria and fungi in biofilms***

Fluorescence *in situ* hybridization (FISH) was applied to analyze the distribution and interaction of bacteria and fungi in biofilms. FISH is a technique used to detect and localize the presence of specific DNA sequences in situ. FISH uses specific fluorescent-labelled probes that bind to only those parts of the genome with

**DIC**



**Calcofluor**



**Overlay**

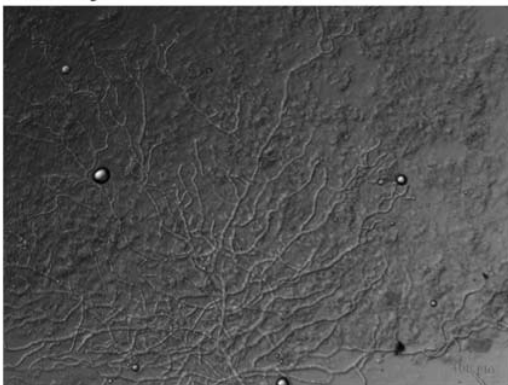


Figure 12: Spatial distribution of biofilms on uncoated surfaces stained with Calcofluor. The glass slide was incubated for four weeks in the influent of the purification plant in Karlsruhe/Neureut. After the incubation the slide was washed with 0.85 % NaCl and stained with 0.1 % Calcofluor and 15 % KOH and washed again with 70 % ethanol and water.

which they show a high degree of sequence similarity. With this technique different micro-organism groups can be stained in situ with diverse fluorescent dyes.

To analyze the interaction of bacteria and fungi two different probes were applied. EuUni was labeled with Fluorescein and bound to a highly conserved region of the eukaryotic 18S rRNA. The prokaryotic 16S rRNA was detected with the Cy3 labeled probe Eub338. The FISH was done as described *Baschien et al. 2008*. The biofilms were fixed, dehydrated and afterwards hybridized with the probes. The samples were analyzed with the fluorescence microscope. For our investigations we used glass slides, which were directly incubated in the influent of the plant Karlsruhe/Neureut and in the Alb (effluent of the plant). Figure 13 shows a FISH of a biofilm, which was incubated in the

influent of the plant. The slide was incubated for four weeks, washed with 0,85 % NaCl to remove loosely attached microorganisms and hybridized with both probes.

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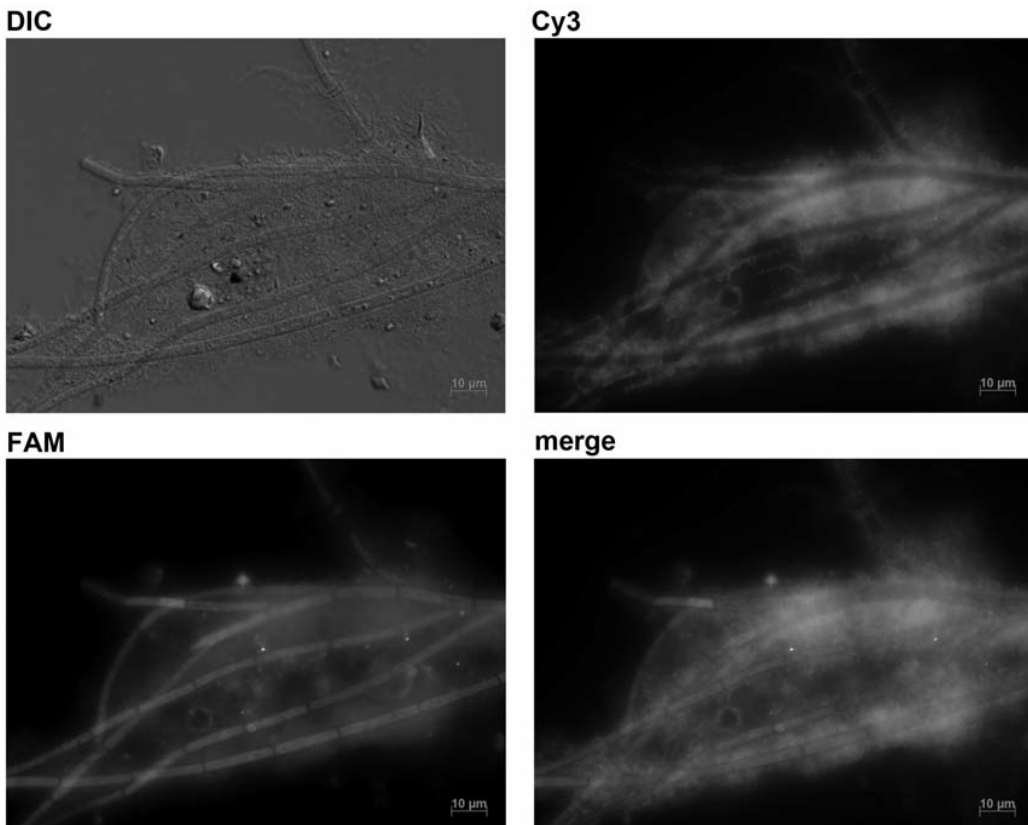


Figure 13: Fluorescence in situ hybridization with a biofilm of the purification plant. The glass slide was incubated for four weeks in the influent of the purification plant Karlsruhe/Neureut. Afterwards FISH was carried out with the specific probes for fungi and bacteria. The biofilms were fixed, dehydrated and hybridized with the probes. Shown in red is the bacteria specific probe (Cy3), in green the fungi specific probe (FAM).

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