CENTER FOR BIOFILM ENGINEERING

PROCEEDINGS

Summer 2004 CBE Technical Advisory Conference

June 29–July 1, 2004
Montana State University–Bozeman
Bozeman, Montana

Sponsored by the
Center for Biofilm Engineering
a National Science Foundation
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at Montana State University–Bozeman

a close. The CBE's continued success is built on the foundation of many years of productive university-industry-government collaboration in pursuit of its vision to be a world leader in fundamental research, science and engineering education, and industrially relevant technology.

GENERAL INFORMATION

CBE LEADERSHIP

***Bill Costerton**, CBE Director and Professor, Microbiology*
***Phil Stewart**, CBE Deputy Director and Professor, Chemical & Biological Engineering*
***Anne Camper**, Associate Professor, Civil Engineering & Associate Dean for Research, COE*
***Al Cunningham**, Professor, Civil Engineering*
***Marty Hamilton**, Professor Emeritus, Statistics*
***Paul Sturman**, CBE Coordinator of Industrial Development*

A BRIEF HISTORY OF THE CBE

The CBE was established in 1990 through a grant from the National Science Foundation's Engineering Research Centers program. The NSF-ERC program was created to increase U.S. industrial competitiveness and to re-invent science and engineering education in U.S. universities. Under the leadership of its founding director, Bill Characklis, the new engineering center also drew support from the state of Montana, MSU–Bozeman, and industrial partners gathered during its pre-1990 work as the Institute for Biological and Chemical Process Analysis. Since the beginning, CBE researchers have been recognized nationally and internationally for leading-edge biofilm research, and for taking an interdisciplinary approach to the study of microbial growth on surfaces. In the spring of 2001, the CBE's 11-year period of NSF-ERC program support drew to

MISSION AND GOALS OF THE CBE

The mission of the Center for Biofilm Engineering is to advance the basic knowledge, technology and education required to understand, control and exploit biofilm processes.

The CBE has identified goals in three areas of activity. In the area of research, the CBE's goal is to do leading-edge fundamental research to elucidate mechanisms at work in bacterial biofilms. The CBE has been a leader in defining the structure and function of biofilms on surfaces, in understanding antimicrobial resistance mechanisms of biofilm, and in identifying the role of signal molecules in controlling bacterial behavior. Researchers at the CBE have demonstrated that biofilms are multicellular attached communities with primitive circulatory systems and a measure of cellular specialization. Understanding these "biofilm basics" presents opportunities for developing more effective strategies to control biofilms in industrial settings.

The second goal of the CBE is to make its research relevant to real systems, where the information can be applied. Industrial concerns shape and focus the research efforts. Technology transfer at the CBE involves not only information, but methods and technology development.

Key to the center's success is the CBE's third goal: to develop and maintain interdisciplinary undergraduate and graduate education programs involving team research on industrially relevant projects.

Industrial Associates Program Benefits

The CBE Industrial Associates Program provides support to help fund industrially relevant research and allows close interaction between industry representatives and CBE researchers and students. Some specific benefits of membership in this program are detailed below.

CBE Technical Advisory Conferences

Twice each year, CBE members convene in Bozeman for a Technical Advisory Conference (TAC)—an exposition of what's new in CBE research and a review of what's happening around the world in biofilm science. The TAC is a great way to keep up on the science as well as to interact with other industry and government representatives and CBE researchers. Meetings are open only to CBE members and invited guests.

Education and Training Workshops

CBE members are entitled to attend either basic or advanced biofilm methods workshops free of charge. Workshops are held the day before TAC meetings in both summer and winter, and feature the latest techniques in growing and assessing biofilms. In addition, the CBE offers specialty workshops (either in our laboratory or at your facility), tailored to your individual company needs. These can range from covering the latest microscopy techniques for your R/D department to assisting the education of your sales force in general biofilm understanding.

Research/Testing Projects

CBE members can fund research and testing projects at a discounted rate. Advantages of directly funding project work at the CBE include complete confidentiality, negotiable intellectual property, and project direction by scientists and engineers at the top of biofilm investigation. In addition, the CBE offers Industrial Associate members no-cost participation in undergraduate research projects of their choosing. This is an opportunity to have a top-notch student work on a problem specific to your needs, at no additional cost.

Product/IP Development Consulting

CBE faculty and staff can assist members in evaluating commercial or product-related ideas as they relate to biofilms or biofilm control. Each member company is entitled to two free days of consulting annually. We can offer confidential feedback on R/D direction, marketing ideas or strategic decisions. Many of our members have found that this benefit alone is worth the annual membership fee.

Regulatory Interactions

Bringing a product to market today frequently requires registration with, or approval of, a regulatory agency. CBE staff maintain close ties with decision makers at FDA, EPA, and other US Government agencies concerned with biofilms. CBE scientists are on the forefront of biofilm methods development and assessment—areas of expertise frequently sought out by the regulatory community. In addition, it's our policy to feature regulatory agency speakers at our TAC meetings whenever possible. If you are seeking insight into the process of registration or approval, the CBE can help.

For More Information

To see if membership is a good fit for your company, please contact Paul Sturman, CBE Industrial Coordinator, at (406) 994-2102, or via email at paul_stu@erc.montana.edu. Or visit us on the web at www.erc.montana.edu.

Presentation and Poster Abstracts
Technical Advisory Conference: June 29–July 1, 2004

Posters

SPEAKER ABSTRACTS

Keynote Speaker:

S04-S01
Defining the *Vibrio harveyi* Quorum Sensing Network

***Bonnie Bassler**, Professor, Molecular Biology, Princeton University, New Jersey*

Many bacteria use a process of cell-cell communication called quorum sensing to distinguish times when they are alone from times when they are in a group.This process involves the production, release, detection, and response to small signal molecules called autoinducers. Presumably, quorum sensing evolved because the decision to transition from acting alone to participating in a group activity is a critical one for bacteria. In the marine bacterium *V. harveyi*, two autoinducers (AI-1 and AI-2) are detected and many genes are activated or repressed as cells make this transition. Sophisticated regulatory devices are located at different positions in the *V. harveyi* quorum sensing signal-transduction relay to ensure that the decision to transition from acting alone to acting as a member of a community occurs under the appropriate set of circumstances and with high fidelity. A coincidence detector regulates entry into high cell density mode, and an ultrasensitive switch involving multiple small regulatory RNAs exists to make the commitment step into "quorum sensing mode" definitive. For coincidence detection: the simultaneous presence of both autoinducers is required to initiate the transition from the individualistic to the group lifestyle. Thus, the detector differentiates the concurrent presence of AI-1 and AI-2 from all other conditions (no AI, AI-1 only, or AI-2 alone). Either autoinducer alone can minimally activate or repress target gene expression; however, significant regulation only occurs in the presence of both autoinducers. The coincidence-detection scheme likely protects the quorum sensing circuit from molecules in the environment that resemble the *bona fide* autoinducers, and provides *V. harveyi* a mechanism to ensure that entry into high cell density mode is highly accurate. The coincidence detector identified in *V. harveyi* is the first example of this type of sensory logic being used to control quorum sensing. For ultrasensitivity: a genetic screen to identify additional components of the *V. harveyi* and *V. cholerae* quorum sensing circuits revealed the protein Hfq. Hfq mediates interactions between small, regulatory RNAs (sRNAs) and specific mRNA targets. These interactions typically alter the stability of the target transcripts. In this particular case, Hfq mediates the destabilization of the mRNA encoding the quorum sensing master regulators LuxR (*V. harveyi*) and HapR (*V. cholerae*) implicating an sRNA in the circuit. Using a bioinformatics approach to identify putative sRNAs that could control *luxR*/*hapR* mRNA levels, four candidate sRNAs were identified in *V. cholerae*. The simultaneous deletion of all four sRNAs is required to stabilize *hapR* mRNA. Hfq, together with these sRNAs, creates an ultrasensitive regulatory switch that controls the critical transition into the high cell density, quorum sensing mode. The use of sRNAs results in a transition that is not graded, but rather an on-off switch, which turns off behaviors that are useful when carried out alone and turns on behaviors that are productive when carried out as a community. Thus, this ultrasensitive mechanism makes the commitment to the group lifestyle decisive.

S04-S02
State of the CBE Address

***Bill Costerton**, CBE Director, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The CBE has maintained, and even extended, its lead position in the exploration of new territory in the biofilm field. The approach that has enabled this sustained intellectual surge has been the inherent tendency of engineers to value direct observations over the process of extrapolation from *in vitro* data, that has been so dear to the hearts of microbiologists over the past 15 decades. Microbiologists have solemnly swabbed surfaces of tissues and devices, rubbed the swabs across agar plates, and counted the resultant colonies. Direct observations of the microbial populations of the human vagina and of failed orthopedic devices by FISH and PCR techniques have shown the swab data to be defective and have shown that traditional recovery techniques give poor results when applied to biofilms. Most microbiological techniques give "average" data over large surface areas, but even the simplest direct observations yield "maps" of bacterial distribution, and this spatial information is very valuable in both ecological and pathogenicity studies. Essentially we need to know where the bacteria are, how many there are, and what they are doing.

Two new forms of observation have become available for the study of microbial biofilms. NMR microscopy has come to the CBE through the work of Joe Seymour and Sarah Codd, and also through the PNL labs in Washington State. Basically, we can now image biofilms by a combination of NMR and confocal techniques, and we can then obtain

quantitative information concerning the organic molecules present in a defined zone within the specimen. This zone is cubic; it may be as small as 20 microns in the smallest dimension, and high-resolution data can be obtained without any detrimental effects on the specimen. At PNL they have fed glucose to a biofilm and then watched, over a five-day period, as the sessile community processed the sugar into simple organic acids and eventually to fatty acids and hydrocarbons. In an even more exact technique we are now able to resolve the matrix structure of biofilms using electron tomography, and we can resolve structures within this zone at the molecular level (< 30 nanometers). We have initiated a program to use electron tomography to identify molecules like DNA and pilus subunits in the "jungle" of the matrix, and to map their distribution in the intercellular spaces of the biofilm. As finer and finer tools become available, our resolve to base our biofilm research on direct observations will continue to be vindicated.

SESSION 1: Biofilm Methods

S04-S04
Standard Method Development: Efficacy Testing in Dental Unit Water Lines

***Shannon Goeres**, Biofilm Systems Training Laboratory Undergraduate Intern, Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Dental unit water line tubing has been shown to be a robust growth surface for biofilm. The intermittent use of dental units, which alternate between periods of stagnation and periods of very high flow (and shear) may release high bacterial counts at the outlet or hand-piece of the system. This can be attributed to three different factors: surface colonization, laminar flow, and surface area to volume ratio. Among the bacteria present in the system are Pseudomonas and Klebsiella species. When the hand piece is used, bacteria are aerosolized. Inhalation of the bacteria-laden aerosols can create health risks for both the patient and dental workers. The aim of this presentation is to illustrate how a working model of a dental unit water line system has been created. The method development will focus on a *P. aeruginosa* and *K. pneumoniae* dual-species biofilm. The system replicates a true dental unit water line by having three meters of 1.5 mm diameter tubing, and a fast (50 ml/min) flow rate which runs intermittently. The ultimate goals are to develop standard testing methods and to reduce overall biofilm accumulation within the system.

S04-S05
Parallel Testing to Determine the Influence of Biofilm Growth Conditions on Antimicrobial Log Reduction

***Kelli Buckingham-Meyer**, CBE Laboratory Specialist, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

In order to make product efficacy label claims, disinfectant manufacturers must demonstrate the efficacy of their products using standard test methods. These standard methods consist of treating planktonic bacteria either in suspension or applied to a surface and dried (hard surface carrier test). In some situations suspension or carrier tests may be sufficient. However, the question currently under consideration is: What testing will be required for companies wishing to make biofilm claims? We wanted to compare disinfectant efficacy on four systems exhibiting increasing shear forces with three classes of disinfectants at two concentrations. The systems include the quantitative carrier test (QCT), the colony biofilm reactor system (no shear), the drip flow reactor (DFR) system (low shear) and the CDC Biofilm reactor (CDC-BR) system (high shear), including hydrated and dehydrated biofilm. The three disinfectants to be tested are sodium hypochlorite, a quaternary ammonium compound (Quat-256) and a phenol-based compound (Grotanol Flussig).

The growth systems, which are run in parallel, are inoculated with *Pseudomonas aeruginosa* ATCC 15442. Four coupons are removed from each system. Treatment is applied to half of the coupons and control to the other half. The coupons are incubated for 10 minutes. The samples are neutralized at the end of the 10-minute treatment period.

Preliminary results for the sodium hypochlorite data set are as follows: The QCT test (dried bacteria) was the least stringent test, showing the greatest log reduction values at 100 mg/L and 500 mg/L sodium hypochlorite. The CDC-BR dehydrated biofilm test was the most variable test between the two treatment concentrations. A trendline fitted to the three hydrated biofilm tests showed that as shear forces of the biofilm increased, the log reduction values decreased for both treatment concentrations.

S04-S06
Long Term Monitoring of an Oilfield Bacterial Consortium

***Brandon Brooks**, Recent MSU B.S. Graduate, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Oilfield souring costs oil producers millions of dollars every year due to decreased oil value and health hazards to employees. It has been shown in previous work that nitrate-dosing of an oil well decreases H_2S production. It has been debated whether this is due to a chemical reaction between nitrate and hydrogen sulfide, or whether nitrate inhibits the activity of sulfate-reducing bacteria (SRB), which are responsible for sulfide production. Sulfide inhibition by nitrate may occur either as a result of the activity of other bacterial populations, such as nitrate-reducing bacteria (NRB), out-competing the SRB (since nitrate is a higher energy electron acceptor than sulfate) or because the presence of nitrate causes SRB to change their metabolism to utilize nitrate instead of sulfate. It has also been hypothesized that the addition of nitrate may increase the rate of biomass development in field situations because nitrate is a higher energy electron acceptor.

To test this hypothesis, a CDC Biofilm reactor was inoculated with a mixed consortium of bacteria from the Hawtah oilfield in Saudia Arabia. Hawtah-produced water samples were enriched for SRB in 3% salinity Postgate B medium and the enriched samples were used to inoculate the reactor. The reactor was run batch for 24 hrs after inoculation in Modified 3% Postgate C media. After the batch period the reactor was run continuously at approximately 1 ml/min of the same media. HACH kits were used to quantify the amount of hydrogen sulfide, total iron and sulfate in the effluent of the reactor. These values, along with the influent concentrations were used to develop mass balances on these substrates. The pH and the oxidation reduction potential were also measured in the reactor.

Once steady-state was reached, several coupons were harvested and the biofilm was scraped to quantify the pre-dosing bacterial community. Total cell counts were made using DAPI, total protein was assessed using the BCA method, total viable cell counts were made using spread plates, and genetic analyses were conducted using FISH and DGGE. After the pre-dosing coupon sampling, 100 mg/l nitrate was added to the influent media. Approximately 1.5 months after dosing began, coupons were again sampled and tested as described above to assess changes in the microbial community resulting from extended nitrate dosing.

SESSION 2: Environmental Biofilms

S04-S08
Influence of Siderophores on Metal Mobility and Transport

***Abbie Aiken**, Visiting PhD Student, Chemical Engineering, Washington State University*

Microorganisms can absorb, reduce, oxidize, solubilize or precipitate both toxic heavy metals and radionuclides, thus impacting the speciation, solubility and, ultimately, the mobility of these metals in the environment. In addition to the influence which whole cells can have on metal transport, microbes can also secrete compounds that facilitate or hinder metal mobility. It is the goal of this work to determine the influence that siderophores—extracellular metal-chelating compounds secreted by microorganisms—have on the mobility of both radionuclides and heavy metals. Many studies have focused on siderophores produced by organisms under neutral environmental conditions. This study focuses on the siderophores produced by organisms that thrive in high ionic strength and highly alkaline conditions.

Halomonas campisalis strain 4A has been identified as capable of producing siderophores under halo-alkaliphilic growth conditions. Siderophore production was confirmed through the use of the chrome azural S (CAS) agar plate method, which showed red-orange halos around the bacterial colonies indicative of siderophore production. These siderophores are produced under conditions of both high salinity and pH, with salt concentrations ranging from 0.4–1.8 M NaCl and a pH ranging from 8–11. The siderophores produced have been determined to be of the hydroxamate class via the Csáky assay method. The culture supernatant of *Halomonas campisalis* responded negatively to the Arnow assay, indicating that the siderophore produced does not contain any catechol moieties in its chemical structure. A purification scheme was developed that involved an initial extraction of the siderophore from the growth medium into benzyl alcohol followed by precipitation with diethyl ether. Additional purification was achieved via ion exchange chromatography and size exclusion chromatography. The structure of the purified siderophore was analyzed via LC/MS/MS equipped with an ESI source. The structure of the siderophore appears to closely resemble that of Desferrioxamine B (DEF).

S04-S09
Escherichia coli Capture by Established *Pseudomonas aeruginosa* Biofilm in a Capillary Flow Cell

***Ben Klayman**, PhD Candidate, Environmental Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Much research has been performed examining the ability of different organisms to form biofilms. Very little, however, has been done to examine the ability of an established biofilm to capture planktonic cells of another species. This project examines and characterizes the events following *Escherichia coli* capture by an established *Pseudomonas aeruginosa* biofilm in a continuous flow chamber using confocal microscopy.

In these experiments, *E. coli* was labeled with the DsRed protein, carried on a plasmid. *P. aeruginosa* was labeled with GFP, also carried on a plasmid. In this manner the two species could be recorded to separate channels and differentiated, even within dense clusters.

The *P. aeruginosa* base biofilm averaged about 50 microns in depth, with a spatially heterogeneous structure. *E. coli* cells were then added in static conditions to facilitate attachment before resuming a flow of sterile media. Microscopy verifies that 100% of the cells expressed the plasmid at the time of inoculation.

Confocal microscopy permitted 3-dimensional observations of *E. coli* attachment and subsequent interaction with the *P. aeruginosa* biofilm. Visual observations were then correlated to a quantitative analysis of the effluent using both plate counts and flow cytometry.

S04-S10
Biofilms in the Environment: Understanding Pathogens in Drinking Water Distribution Systems

***Stewart Clark**, PhD Candidate, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Biofilm dynamics in drinking water distribution systems are emerging as an important consideration for health- and safety-related research as the war on terror takes new shifts, and as governments examine more closely the potential threat of terrorist attacks on home soil. Emerging microbial pathogens such as *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Aeromonas hydrophila*, and recent incidences of drinking water-related outbreaks only serve to highlight the need for better understanding of the role of biofilms in distribution systems as potential safe-harbors of human pathogens. Furthermore, the increase of immuno-compromised individuals within the population necessitates more stringent health-related monitoring programs, policies and guidelines. The US EPA's Safe Drinking Water Act (SDWA) was amended in 1996 to ensure the quality of Americans' drinking water. Under SDWA, EPA sets standards for drinking water quality and oversees the states, localities, and water suppliers who implement those standards. Over the past few years, researchers at the CBE have been involved in various projects dealing with the potential of pathogens to become established in drinking water biofilms, to persist undetected in these low nutrient biofilms in a viable but not culturable (VBNC) state, and to be released long after initial contamination, resulting in outbreaks in susceptible sub-populations of the community. Some of the historical explanations for this scenario include the protective mechanisms offered by biofilm community-living, sharing of resources, metabolic adaptations and stress physiology. Current work in the Camper lab at the CBE is focused on utilizing the reductive power of molecular biology to hone in on the metabolic reasons for pathogen persistence in low nutrient environments and the role biofilms play in harboring these potential pathogens. These techniques include PCR, DGGE, cloning and sequencing, and the use of epifluorescent antibodies. Furthermore, we plan to move into cutting- edge molecular technology using real-time PCR and microarray analysis for viability and disease pathogenesis-related research.

S04-S11
Modeling Drinking Water System Biofilms Using Cellular Automata

***Al Cunningham**, Professor, Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The Cellular Automata Biofilm Model (CABM) has been developed by CBE researchers to investigate biofilm behavior computationally. Because model calculations are made at the 5 micrometer-length scale, the CABM can simulate (and visualize) complex biofilm processes such as cell growth, chemically mediated detachment, and multiple species interactions. This presentation will summarize the

features of the CABM and illustrate how the model is currently being modified to simulate the fate and transport of pathogens introduced into drinking water systems. Processes key to this simulation include steady state biofilm accumulation, attachment and persistence of pathogens in the biofilm, and subsequent detachment and re-entrainment of pathogens in the bulk fluid. Future modifications and applications will also be discussed.

S04-S12
Discovering the Microbial Diversity in Natural Biofilms

***Mark Burr**, Senior Research Associate, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

In studying natural biofilms, we often want to know the diversity of the bacterial species present and their relative abundance. In the past, two divergent approaches have typically been used to determine microbial diversity. One approach has been to create a 16S rDNA clone library and to sequence a representative number of clones. A second approach has been to produce a PCR-generated profile using denaturing gradient gel electrophoresis (DGGE) or similar molecular method. DGGE gives the big picture without providing specific information about individual members of the community. Cloning and sequencing gives precise information about individual members, but the composition of the whole community is only determined by screening and sequencing a large number of clones. In this study we developed a novel protocol in which we first cloned and then used DGGE in order to characterize an aquatic microbial community. First, a clone library was created from 16S rDNA PCR products. Next, clones were screened using PCR-DGGE that targeted the insert, such that each clone produced a single band in the gel. Finally, a simplified PCR-DGGE profile was reconstructed from the library by pooling an equal numbers of cells from two sets of 20 clones. Finally, we constructed a DGGE image in which individual clones were run in separate lanes in part of the gel, showing that we could successfully separate the clones by this method. The profiles of the two sets of pooled clones were also run in the same gel, allowing the alignment of individual clones with the bands in the profile. We believe this method will allow rapid determination of the distribution of bacterial species in naturally inoculated biofilms.

S04-S11
FDA Regulatory Outlook

***Janine Morris**, Branch Chief, Urology & Lithotripsy Devices Branch, US Food and Drug Administration*

Advances in biotechnology have lead to novel approaches to solve some of the most enduring challenges of ancient and modern medicine. Infection, and in particular device-related infection, has been a costly and enduring challenge that continues to plague the medical community and seems to have become only more complicated as we witness the emergence of bacterial resistant organisms and super infections. Because medical device-related infections are of such concern to the healthcare community, this has sparked an enthusiasm to develop and market new technologies—whether proven or unproven—to either prevent or treat such infections. The Center for Devices and Radiological Heath has the regulatory responsibility to assure the safe and effective use of medical devices. With the rapid development of new technologies that allow the attachment or incorporation of active agents such as biocides and antibiotics in the fabrication of many medical devices the Center is faced with new challenges from both a regulatory as well as scientific perspective in protecting public health.

From a regulatory perspective the addition of active agents in medical devices has been defined as a combination product that puts into question which jurisdiction and regulatory authority should be applied for these products. The emergence of such combination products has resulted in the formation of the Office of Combination Products (OCP) within the Food and Drug Administration. The speaker will review the goal and function of OCP as well as the different regulatory approaches and challenges faced with combination products, in particular those products with antimicrobial agents.

From a scientific perspective the development of medical devices with the addition of an antimicrobial agent raises a number of challenges with respect to assessing the safety and effectiveness of a new or modified product. The speaker will discuss some of the difficult scientific issues facing the agency in determining the adequacy of data to support a pre-market submission.

SESSION 3: Biofilm Structure-Function

S04-S15
Importance of Electron Shuttling Compounds in Biological Systems

***Robin Gerlach**, Assistant Research Professor, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Energy generation and growth in biological systems are governed by electron transport. Hence, reduction-oxidation (redox) reactions are critical considerations when controlling biological systems. It has become evident over the past decade that compounds capable of shuttling electrons can significantly influence the rate and extent of redox-reactions in the environment. Naturally occurring organic or inorganic electron shuttles such as humic substances or iron minerals are abundant in the environment. It has also been suggested that some bacterial strains produce electron shuttling compounds. Last, but not least, synthetic electron shuttling compounds such as anthraquinone-2,6-disulfonate (AQDS) have been used in research and have more recently been discussed to be used in environmental applications. This presentation will describe the potential importance of electron shuttling compounds in the environment and biofilms. A better understanding of the importance of electron transport processes in environmental systems will allow for the development of more efficient processes in biotechnology.

S04-S16
Bacterially Derived Environmentally Compatible Adhesives

***Gill Geesey**, Professor, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Wood adhesives play an essential role in the building materials, furniture, and construction industries. However, many of these adhesives contain significant amounts of toxic components and volatile organic compounds (VOCs). Also, most are produced from depleting petrochemical resources. We are currently investigating adhesives for the wood products market which are derived from microbial extracellular polysaccharides (EPS) that are generally non-toxic, biodegradable, and produced from renewable resources. This study focuses on a new EPS-based adhesive material with performance that represents a significant improvement over previously examined microbially derived adhesives. The new adhesive produced shear strengths on maple substrates of up to 3000 psi at 21 °C at 53 % relative humidity. Full strength was achieved in two days with half strength in 2 hours. Although shear strength was sensitive to moisture, improved results were achieved by partial acetylation of the EPS. This new product may compete with current commercial adhesives in some applications.

S04-S17
Modeling Interactions Between Fluid Forces and Biofilm Deformation

***Brett Towler**, Postdoctoral Researcher, Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Fluid-induced detachment and hydrodynamic drag are two problems associated with microbial fouling. Biofilm accumulation on wetted interfaces can impair the hydrodynamic performance of pipe networks and ship hulls. The costs associated with reductions in conveyance and increased fuel consumption can be substantial. Biofouling is also relevant in the medical field, where biofilm growth on medical devices can result in persistent infections and the detachment of dental biofilms has been linked to a range of systemic diseases.

The mechanics of this fluid-structure interaction are highly complex. Detachment and surface drag are due, in part, to the constitutive law governing biofilm. The flow regime is typically turbulent and, accordingly, solutions necessitate the use of a turbulence model. Finally, the equations governing the flow field and the attached biofilm must be coupled to reflect their interaction. Turbulence phenomena and geometric complexity prohibit the development of an analytical solution to this problem; however, using numerical methods to model the biofilm response to turbulent flow is feasible.

A computational model that describes the response of individual biofilm structures to turbulent flow has been developed at MSU's Center for Biofilm Engineering. The model employs a sequentially coupled finite element technique to resolve biofilm deformation and internal stress distributions due to changes in the surrounding flow field. Based on previous work, a linear viscoelastic constitutive law is employed to define the stress-strain relation. As a research tool, this model can be used to 1) elucidate the interplay between biofilm morphology and

hydrodynamics; 2) determine linear viscosity material coefficients from experimental data; and 3) identify areas of high detachment susceptibility based on stress distributions.

S04-S18
Predicting Biofilm Structural Parameters Using Artificial Neural Networks

***Raaja Raajan Angathevar Veluchamy**, MS Candidate, Environmental Engineering*

The goal of this study was to develop a modeling method to predict biofilm structures from limited data. To produce experimental data, we operated a flat plate flow reactor with a glass bottom for biofilm growth at glucose concentrations of 50, 100, and 150 mg/L and flow velocities of 3.2, 10, and 25 cm/s. The biofilms consisted of *Pseudomonas aeruginosa* (ATCC # 700829), *Pseudomonas fluorescens* (ATCC # 700830) and *Klebsiella pneumoniae* (ATCC # 700831). Thirty light microscopy images were acquired from the bottom of the reactor on a daily basis. The images were used to quantify biofilm structure parameters using ISA2D analysis. These structural parameters (areal porosity, fractal dimension, textural entropy, average diffusion distance, average horizontal run length and average vertical run length) were fed into the Artificial Neural Network (ANN). We ran a total of 9 sets of experiments under different flow velocities and glucose concentrations for 11 days and acquired 9 x 30 x 11 (= 2970) images for biofilm structure parameter quantification. These experiments were costly and required an extensive effort and a great deal of time to complete. It is therefore important to develop a method to predict biofilm structures using limited number of data. We used ANN to perform an inductive type of modeling of biofilm structure variation with respect to time. We used part of the experimental data to train ANN and successfully predicted the unused experimental data.

S04-S19
Magnetic Resonance Microscopy of the Impact of Biofilm Growth on Mass Transport in Bioreactors and Porous Media

***Joe Seymour**, Assistant Professor, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Magnetic resonance microscopy (MRM), or high spatial resolution magnetic resonance imaging (MRI) is developing as a biofilm imaging modality. The non-invasive nature of the method and ability to probe opaque systems provides for unique data complementary to other analytical techniques. In particular, the ability to measure spatially resolved dynamics in bioreactors and porous media provides the ability to quantify system transport. The current state-of-the-art in dynamics measurements by MRM for biofilm systems will be overviewed[1,2]. The velocity distributions in 1 mm-square duct bioreactors impacted with *Staphylococcus epidermidis* biofilm show non-axial secondary flows. The presence of secondary flows leads to complex mass transfer that can enhance or inhibit mixing, depending on the nature of the secondary flows. Discussion of current mass transfer coefficient models[3] and how the MRM data can allow for extension is presented. MRM data for the dynamics in porous media show a biofilm's growth-induced transition in system dynamics from normal to anomalous transport[4]. The transition indicates that the biofilms growth changes the porous media dynamics from those associated with homogeneous structure to heterogeneous structure. The ability to quantify the dynamics in this way provides new insight into the impact of biofilms on porous media transport.

1. Seymour, J. D., Codd, S. L., Gjersing, E. L. & Stewart, P. S. Magnetic resonance microscopy of biofilm structure and impact on transport in a capillary bioreactor. *Journal of Magnetic Resonance* **167** (2004).
2. Manz, B., Volke, F., Goll, D. & Horn, H. Measuring local flow velocities and biofilm structure in biofilm systems with magnetic resonance imaging (MRI). *Biotechnology and Bioengineering* **85**, 10.1002/10782 (2003).
3. Eberl, H. J., Picioreanu, C., Heijnen, J. J. & van Loosdrecht, M. C. M. A three-dimensional numerical study on the correlation of spatial structure, hydrodynamic conditions, and mass transfer and conversion in biofilms. *Chemical Engineering Science* **55**, 6209-6222 (2000).
4. Seymour, J. D., Gage, J. P., Codd, S. L. & Gerlach, R. Biofilm growth induced transformation of transport dynamics in porous media from normal to anomalous. *To be submitted* (2004).

SESSION 4: The CBE Imaging Facility

S04-S21
Flow Cytometry: A Complement to the Biofilm Imaging Facility

***Jennifer Sestrich**, CBE Research Assistant, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

With the support of the Murdock Charitable Trust and Army Research Office, the CBE was able to purchase a new Becton-Dickinson FACSAria flow cytometer. Flow cytometry is new to the CBE and fairly new to biofilms in general. With the use of this technology, one can measure physical as well as chemical properties of single cells in suspension. There are three basic components to a flow cytometer: fluidics, optics, and electronics. Hydrodynamic focusing, which is the major aspect of the fluidics component, allows for single cells to flow through an interrogation point. The lasers are focused at this interrogation point and illuminate the cells. Detectors then pick up the fluorescent signals and send them to the electronics component to be processed. A multitude of information can be obtained using the flow cytometer. Some of the types of data that may be obtained include staining intensity profiles, bacterial counts, statistics, and population percentages. Some current applications of flow cytometry at the CBE include bacterial counting, observing staining rates, sorting a known number of bacterial cells, and looking at mixed populations to obtain percentages. The sensitivity of the instrument is excellent, allowing researchers to observe subtle changes in bacterial populations. With this cutting-edge technology, the CBE will definitely benefit by having a novel way to examine biofilm communities.

S04-S22
Fluorescent Stains and Probes Used at the CBE

***Betsey Pitts**, CBE Research Associate and Facilities Manager, Microscopy, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Why choose a particular stain? Can planktonic staining protocols be used on biofilms? Is there a good live/dead probe? How accurate are conclusions drawn from staining?

At the CBE a wide variety of fluorescent stains are used to illuminate and probe all aspects of biofilms, including architecture and structure, metabolic processes and states, viability, and species composition. We use the term "probe" to refer to a fluorescent signal that is specific in origin, including fluorescent antibody staining, fluorescent *in situ* hybridization (FISH), fluorescent reporter genes, fluorescent constructs such as green or red fluorescent protein (gfp, rfp, yfp, cfp, etc.) and fluorescent enzyme assays. Stains include more generally applied fluorescence such as rhodamine, fluorescein, DAPI, and propidium iodide. The stains and probes used on biofilms at the CBE will be presented, with particular emphasis on discussing the above questions.

S04-S23
Imaging Biofilms on Alternative Surfaces

***Steve Fisher**, CBE Research Assistant, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Microscopes are designed for viewing flat, thin samples on glass slides. Most biofilms—especially those on alternative surfaces such as catheters, tissue or hot tub filters—present imaging challenges. These surfaces are often not flat, non-uniform in topography, and autofluorescent. Imaging bacterial biofilms on these surfaces presents a unique set of considerations. Recent additions to the CBE imaging facility have enabled us to work successfully with these surfaces. Specific examples of imaging biofilms on alternative surfaces will be discussed.

S04-S24
Image Analysis Using MetaMorph: Quantitative Techniques

***Willy Davison**, PhD Candidate, Chemical & Biological Engineering, Center for Biofilm Engineering, Montana State University–Bozeman, 59717*

MetaMorph® is a computer-based program used primarily for quantitative image analysis. Image analysis of pictures acquired from the confocal and light microscopes plays an important role in the overall understanding of biofilms. Multiple analysis techniques will be discussed, including intensity profiling and cell counting. An example of an intensity profiling application is the tracking of stain penetration into a biofilm over a given time period. Cell counting methods have been used to optimize the determination of cell numbers in many different situations. There will also be a brief demonstration of these techniques and their respective applications.

SPEAKER ABSTRACTS

S04-S25
Qualitative Image Analysis Using Imaris

***Patrick Norris**, MS Candidate, Mechanical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Qualitative analysis of microscope images has always played a major role in biofilm science. With the introduction of Confocal Scanning Laser Microscopy (CSLM), scientists can acquire stacks of images that allow three-dimensional observation. Stacks taken with a confocal microscope are then imported into image analysis software for further qualitative and quantitative analysis. Both the development of confocal microscopes and the development of the subsequent image analysis software packages have given scientists a further understanding of biofilm structure, species distribution, three-dimensional distributions of viable cells and other qualities of biofilms that are not necessarily obvious from two-dimensional images.

Imaris is an image analysis software package that was specifically developed for use with confocal image data. Imaris has emerged as the software of choice for scientists to analyze biofilm data qualitatively. The Imaris software allows the user to observe and display three dimensional data with perspective views and shadow projections to clearly present spatial depth and channel separation. With full animation capability, image data can be presented in movie format to give an audience clear understanding of the biofilm that is being presented. With three-dimensional controls such as rotating, panning and zooming, scientists will now be able to effectively communicate what was seen during image acquisition.

S04-S26
Education Initiative Overview

***Rocky Ross**, Professor, Computer Science, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

One of the missions of the CBE is biofilm education. In support of this mission, construction of a comprehensive teaching and learning resource has been initiated. Called ***Biofilms: The Hypertextbook***, this resource will make the study of biofilms accessible to students at all levels, from freshmen to graduate students, as well as to industrial employees seeking to understand more about biofilms. The hypertextbook is really a comprehensive web site on CD or DVD that incorporates hyperlinking to guide students at different academic levels through more or less challenging presentations of the material in the hypertextbook, according to their levels. Embedded throughout the hypertextbook are slide shows (e.g., showing instances of biofilms in the environment), video clips (e.g., showing how to carry out a particular biofilm experiment), and active learning models of biofilm processes (e.g., a biofilm accumulation model, a biocide model, an equilibrium partitioning model, and an image analysis model). A prototype of ***Biofilms: The Hypertextbook*** will be demonstrated.

Special Presentations:

S04-S27
Linking Identity and Function of Uncultured Bacteria in Biofilms

***Per Nielsen**, Professor, Environmental Engineering, University of Aalborg, Aalborg, Denmark*

Most procaryotes in natural and technical systems are still uncultured, mainly due to lack of suitable isolation procedures. Consequently, if we want more information about structure and function of complex biofilm systems, it is important to investigate the identity, the physiology and the biofilm forming properties of abundant microorganisms in systems of interest. By use of various culture-independent techniques, it is possible to conduct such studies. Molecular methods can be used for identification and quantification by the full cycle rRNA approach, and various *in situ* methods can link this identification with key physiological and biofilm-forming characteristics of individual bacteria. Some examples will be presented. In complex biofilms it was found that the strength of microcolonies critically depended on the different uncultured species present. Some were found to form very strong, others very weak, microcolonies and thus had a different importance for cohesive properties of the biofilm. The different species also responded differently to changes in pH, ionic composition, and presence of detergent. Information about the physiology of specific bacteria can be obtained by incorporation of radiotracers into active bacteria and visualized on a single-cell level by microautoradiography. The physiology can be linked to identity when oligonucleotide probes and fluorescence *in situ* hybridization are used in combination with microautoradiography. Such investigations of, e.g., denitrifying bacteria have shown that the physiology of several species under *in situ* conditions can differ significantly from the physiology of cultivated close relatives. This

emphasizes that it can be very difficult to predict how bacteria will behave in complex systems based only on studies of pure cultures. Examples of other *in situ* methods will also be given, as well as the use of the novel isotope array. This method is based on the incorporation of radiotracer into RNA in active cells, which can subsequently be detected on an RNA-array, thus allowing simultaneous detection of identity and activity.

S04-S28
Biomedical Applications of Viruses as Templates for Nanomaterials Synthesis

***Mark Young**, Associate Professor, Plant Sciences & Plant Pathology, MSU*

We have developed the use of viral protein cages as constrained reaction vessels for nanomaterials synthesis with applications in medicine and material sciences. This research is based on the concept that a viral protein cage devoid of its nucleic acid can serve as a precisely defined molecular surfaces for driving chemical reactions. This research focuses on the use of genetic and chemical modifications to impart desired functionality to viral protein cages by design. Modifications to the interior surface, the exterior surface and at the interface between the subunits that comprise the cage can all impart new functions to the virus cage. A number of animal and plant viral protein cages, in addition to non-virus protein cages are being utilized. Applications of this work include creation of cage-based targeted drug delivery systems, MRI bio-imaging agents, and magnetic materials with applications in advances in computer memory.

Session 5: Biofilm Control – Medical

S04-S29
Multicellular Nature of Biofilm Antimicrobial Tolerance

***Phil Stewart,** Professor, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Four proposed mechanisms of biofilm protection from antimicrobial agents are summarized, and the multicellular nature of each discussed. The four mechanisms are: slow antimicrobial penetration, slow growth in the biofilm, adaptive stress responses, and differentiated persister cells. These mechanisms are illustrated with experimental and theoretical results. The rapid penetration of antibiotic-sized fluorescent tracers into biofilms of *Staphylococcus epidermidis* has been directly visualized using confocal scanning laser microscopy. On the other hand, hydrogen peroxide fails to penetrate biofilms of *Pseudomonas aeruginosa* because it is deactivated by catalase in the surface layers of the biofilm faster than it diffuses in. Spatial heterogeneity in the pattern of protein synthesis in *P. aeruginosa* has been visualized using an inducible green fluorescent protein construct. Significant regions of the biofilm contain inactive cells that may be less susceptible to antimicrobial challenge. The adaptive response of a *P. aeruginosa* biofilm to hydrogen peroxide has been characterized using a reporter gene for an oxidative stress response. Persister cell formation has been investigated using a computer model of cell differentiation. Each of these hypothesized protective mechanisms depends on the aggregation of cells into multicellular clusters, the concerted metabolic or enzymatic activity of groups of microorganisms, or the differentiation of cells of the same species into a distinct, protected phenotypic state.

S04-S30
Virulence Factors Associated with *Pseudomonas aeruginosa* Biofilms

***Mike Franklin**, Assistant Professor, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Pseudomonas aeruginosa is an opportunistic pathogen that causes both acute and chronic biofilm-associated infections. Acute *P. aeruginosa* infections are found associated with implant devices and burn wounds. Patients with the genetic disorder cystic fibrosis (CF) often develop chronic *P. aeruginosa* infections, associated with pulmonary tissue. The nature of the extracellular matrix material required to maintain the structural integrity of these *P. aeruginosa* biofilm infections has been a subject of debate. Extracellular polysaccharides, proteins, and DNA have been implicated as extracellular matrix materials for *P. aeruginosa* biofilms. The differences observed in the various studies may reflect strain differences. Here, we used Fourier transform infrared spectroscopy and proteomic analyses to characterize the biofilm extracellular matrix material of two *P. aeruginosa* strains, a CF pulmonary isolate and a burn-wound isolate. Results for the CF isolate indicate that the extracellular polysaccharide alginate is the primary constituent of the biofilm matrix. Virulence factors, including proteases and the redox cycling compound pyocyanin are harbored in the extracellular matrix

material. The protein component of the matrix did not play a significant role in the biofilm structural integrity. For the burn-wound isolate, no alginate was observed in the biofilms, confirming recent results by Wozniak and coworkers. The results indicate that the nature of the biofilm matrix material depends on the bacterial strain, and also indicate that alginate is required for biofilm structure in chronic *P. aeruginosa* infections.

S04-S31
Is the Nasopharyngeal Colonization with *Streptococcus pneumoniae* a Biofilm-Related Process?

***Christoph Fux**, MD, Visiting Researcher, University of Bern, Bern, Switzerland*

Streptococcus pneumoniae (SP) are the leading cause of bacterial middle ear infections, community-acquired pneumonia and meningitis in industrialized countries. Most of the time, however, SP are benign colonizers of the upper respiratory tract, where they may persist for weeks to months and increase their virulence by horizontal genetic transfer. We hypothesized that nasopharyngeal colonization is related to a biofilm-mode of growth. The "golden biofilm rule" that metabolic inactivation mediates tolerance is challenged by the observation that SP undergo autolysis as soon as they reach the stationary phase of planktonic growth. This autolysis is controlled by 2-component sensor-regulator systems.

In contrast, SP grown in surface-adherent, multicellular conditions persist for at least one week and develop tolerance to penicillin. Inhibition of autolysin A, the major effector of physiological and antibiotic-induced autolysis, promoted pneumococcal biofilm formation in vitro. In a static model, the biomass of SP biofilms was increased by host factors representing key nutrients (e.g. choline, hem) or antioxidants (e.g. catalase). In the drip flow reactor, which imitates best the physiology of the upper respiratory tract, significant differences in the biofilm-forming capacity of individual serotypes became evident. Interestingly, serotypes known to be predominantly invasive formed poor biofilms, whereas the dominant colonizing serotypes formed good biofilms. The evaluation of serotype 6B suggested that biofilm-formation is strongly correlated to the expression of the transparent ("colonizing") or opaque ("invasive") phenotype. In serotype 23, biofilm formation in vitro was associated with stable nasopharyngeal colonization and the histological demonstration of SP biofilms in the mouse. The failure of serotype 4 to form biofilms both in vitro and in vivo correlated with only transient colonization of the mouse. In conclusion, biofilm formation of SP may be inversely correlated with invasiveness. More strains need to be tested to validate this hypothesis.

S04-S32
Growth Limitation of *Staphylococcus epidermidis* in Biofilms Contributes to Rifampin Tolerance

***Phil Stewart,** Professor, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Tolerance of *Staphylococcus epidermidis* in biofilms to killing by rifampin was correlated with limitation of bacterial growth in the biofilm state. Intact biofilm experienced a 0.62 log reduction when treated with 0.1 µg rifampin/ml for 4hours, whereas the same treatment of exponential-phase planktonic cells produced a log reduction of 4.48. Stationary-phase planktonic cells were nearly as tolerant as intact biofilm cells, experiencing a 1.11 log reduction. Biofilm bacteria grew at only ten percent of the maximum rate at which they grew on the same medium in planktonic culture. Killing was localized near the surface of the biofilm adjacent to the nutrient source, as revealed by staining with a respiratory dye. Increased nutrient concentration during antibiotic treatment enhanced killing of biofilm cells. Changing the oxygen tension in the gas phase above the biofilm during antibiotic treatment barely affected killing. It was hypothesized that the biofilm harbors significant numbers of stationary-phase-like cells in the nutrient-limited depths of the biofilm, and that these inactive cells are the ones that survive antibiotic challenge.

S04-P315
Role of Oxygen in Biofilm Susceptibility to Antibiotics

***Lee Richards**, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

The contribution of oxygen limitation to the reduced antibiotic susceptibility of *Pseudomonas aeruginosa* bacteria growing in biofilms was investigated. *P. aeruginosa* biofilms were grown in drip-flow reactors for three days, then treated with ciprofloxacin or tobramycin. Both antibiotics rapidly reduced viable cell numbers in an aerobic, planktonic culture. In contrast, neither antibiotic was effective against biofilms. An oxygen microelectrode was used to demonstrate the presence of oxygen concentration gradients in this system. The induction of a green fluorescent protein was localized in a band at the top of the biofilm adjacent to the medium source of nutrients and oxygen. These results provide evidence that oxygen limitation occurred in the lower stratum of the biofilm. If oxygen availability were the sole determinant of antibiotic susceptibility, then resuspending bacteria from a biofilm into anaerobic medium should preserve the low level of susceptibility measured in the biofilm. The susceptibility of resuspended biofilm bacteria was, in fact, intermediate between the biofilm state and planktonic state. If oxygen availability were the important determinant of antibiotic susceptibility, then one should be able to simulate the low susceptibility of biofilm cells by treating planktonic bacteria under strictly anaerobic conditions. Both antibiotics were able to kill bacteria under these conditions. The oxygen tension above the biofilm was changed during antibiotic treatment. This had little effect on biofilm susceptibility. We conclude that oxygen does modulate biofilm sensitivity to antibiotics, but oxygen limitation is only a partial explanation for the reduced susceptibility of biofilm bacteria.

S04-P317
Standardized Methods for the Biofilm Laboratory: Statistical Guidance

***Marty Hamilton**, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Five desirable characteristics of a reliable anti-biofilm test are repeatability, reproducibility, sensitivity, relevance, and ruggedness. For each of these characteristics, an example is provided to describe the experiment, method of statistical analysis, and the summary quantity suitable for assessing that characteristic.

S04-P330
Nuclear Magnetic Resonance Imaging of the Fluid Dynamics Around Biofilms

***E.L. Gjersing,** S.L. Codd, P.S. Stewart, and J.D. Seymour, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Nuclear Magnetic Resonance (NMR) Microscopy is an excellent method for investigating living systems, since it is innocuous and non-invasive. The technique has been used for years on the macroscopic scale in clinical applications as Magnetic Resonance Imaging (MRI). In addition to imaging internal structures of systems, NMR microscopy techniques can be used to obtain information about transport phenomena such as fluid velocities and diffusion. The current focus of this research is to map flow patterns and diffusion properties of biofilm-fouled capillaries. Characterizing how biofilms interact with bulk flow will contribute to better biofilm models and to the overall understanding of biofilm behavior. NMR experiments were used to image *Staphylococcus epidermidis* biofilms and the flow around them in 1 mm-square glass capillaries. Images of biofilm clusters show regions in the center that have “hollowed out” and contain fluid similar to that of the bulk. These results are consistent with confocal laser microscopy images. The advantage of using the NMR techniques to image biofilm structures is that there are no light or laser penetration barriers, and the innermost regions of the structures can be easily revealed. In addition to imaging the biofilms, velocity distributions have been mapped for the 1 mm capillary system. Laminar flows in clean, square capillaries display *z* direction velocities (those along the length of the capillary) that are both uniform and symmetrical while *x* or *y* (perpendicular to the walls) components of velocity are not present. In contrast, a biofilm-fouled capillary displays irregular flow patterns in the *z* direction along with distinct *x* and *y* flow perturbations. These results demonstrate that biofilms impact bulk flow in ways that cannot be ignored when modeling their behavior. Since the NMR system does not harm biofilms, images of the biofilm and its flow disturbances have been mapped over the course of growth. Future experiments will be used to create movies of biofilm development and begin exploring how traditional NMR spectroscopy can be used to study biofilms.

S04-P331

NMR Microscopy of Microbial Transport in Porous Media

Justin P. Gage, *Sarah L. Codd, Robin Gerlach and Joseph D. Seymour, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Biofilms are heterogeneous structures which form on solid surfaces exposed to aqueous environments under favorable conditions. Biofilms consist of bacterial cell clusters held together in an extracellular polymeric substance (EPS) matrix [1]. The presence of biofilms can cause biofouling, resulting in reduced heat exchanger and pump efficiencies, infections associated with the use of medical implant devices, and contamination in water distribution systems, as well as in the food processing industry. In addition, bacteria in the biofilm state are found in the subsurface and play an integral role in the biotransformation and transport of contaminant compounds. The study of the impacts of biofilms on transport properties of porous media systems is of interest in many fields such as packed-bed reactor design and operation, enhanced oil recovery, *in situ* bioremediation, and the modeling of contaminant transport in geological media. The rate of biofilm growth in porous media systems, and therefore the biotransformation rate, is strongly influenced by transport properties such as pore velocity distribution, dispersivity, and molecular diffusivity [2]. Therefore, the processes of subsurface mass transport, biotransformation and biofilm growth are all closely related. The use of nuclear magnetic resonance (NMR) imaging technology provides a non-invasive technique capable of resolving transport properties over different spatial and temporal scales under a variety of experimental conditions. Recent work in our group has demonstrated the ability of NMR imaging to image single biofilms in flow systems. A model porous media system comprised of 240 μm monodispersed polystyrene beads is used in this study. NMR imaging techniques combined with Pulsed Gradient Spin Echo (PGSE) methods were performed to gather diffusion, velocity and hydrodynamics dispersion data in the sterile porous media column [3]. In addition, similar experiments were conducted with biofilms present within the porous media column; transport properties were evaluated over large times to monitor for processes such as biofilm growth and decay.

References

[1] J. William Costerton, Zbigniew Lewandowski, Douglas E. Caldwell, Darren R. Korber, and Hilary M. Lappin-Scott. "Microbial Biofilms." *Annu. Rev. Microbiol*. **49**: 711–745 (1995).

[2] A..B. Cunningham, E.J. Bouwer, W.G. Characklis. *Biofilms*; W.G. Characklis, K.C. Marshall, Eds.; New York, 1990; Chapter 18, pp 697–732.

[3] J.D. Seymour and P.T. Callaghan, *AIChE J*. **43**: 2096–2111 (1997).

S04-P334

Influence of Solution Chemistry on the Reactivity of TNT with Zero-Valent Iron

Travis Nelson, *Robin Gerlach, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Recent studies suggest a growing trend concerning soil contamination near Department of Defense sites from such compounds as 2,4,6-trinitritoluene (TNT). These discoveries have prompted the research of *in situ* remediation strategies for such contaminants. Zero-valent iron is known to be an effective and cost-efficient reducer of TNT in permeable reactive subsurface barriers. The groundwater chemistry is known to influence reaction rates of reducible contaminants in zero-valent iron barriers. Using batch tests and HPLC-DAD analysis, an investigation was conducted concerning the impact of solution chemistry on zero-valent iron-mediated reduction of TNT. We used a Synthetic Ground Water (SGW) to control the variations in solution chemistry and evaluate their influence on TNT reduction. The most reduced TNT metabolite, 2,4,6-triaminotoluene (TAT), was produced in three of the six treatments. Information about the rate of TNT reduction in each treatment was also obtained. The results of this investigation show that both the reaction rate and product distribution of TNT reduction is affected by the solution chemistry. The lack of trace minerals such as lithium, copper, zinc, aluminum, nickel, cobalt, selenate, molybdate, and ferrous iron or the lack of a low concentration of complex organic compounds capable of electron shuttling appear to reduce the reaction rates.

S04-P335
Computer Model of Persister Cell Protection Mechanism of Biofilms Against Antimicrobial Agents

J. D. Chambless *and P. S. Stewart, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Hypothetical mechanisms based on the formation of persister cells protecting microorganisms in biofilms from killing by antimicrobial agents were incorporated into a three-dimensional model of biofilm growth. The results of two simulations: a base case with a non-resuscitating persister cell type, and a second case with a resuscitating persister cell type, show that the persister cells offered the population protection from antibiotics and a means for rapid recovery. The high numbers of persister cells that accumulated in the biofilm began to resuscitate and regrow once the antibiotics had been relaxed. Near the end of the simulation, the biofilm with resuscitating persisters had recovered to a more viable state than the biofilm without.

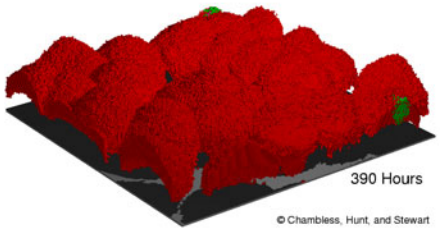

Figure 1. Model output at 390 hours for the simulation whose persisters do not resuscitate after antibiotics have been introduced (first simulation). Live cells are shown in green, persisters in purple, and dead cells in red. The biofilm is mostly dead, with a small pocket of regrowth in the lower right corner.

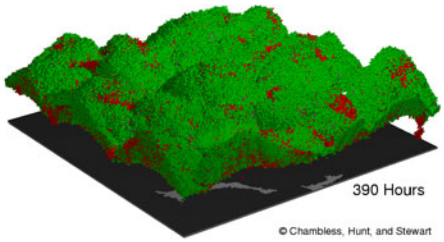

Figure 2. Model output at 390 hours for the simulation whose persisters do resuscitate after antibiotics have been introduced (second simulation). Live cells are shown in green, persisters in purple, and dead cells in red. The biofilm is much more active in its regrowth due to the resuscitation of the persisters.

S04-P336
Engineering a Mobile Biofilm Unit (MBU)

P. Gentile, *Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

The Mobile Biofilm Unit (MBU) is a prototype system engineered and designed to enable researchers to conduct extensive biofilm field studies, testing the feasibility of a remote environmental monitoring tool. Deliberate water contamination or various pollutants will result in alterations in the emitted spectrums of biofilm colonies natural to these waters. With continual advancements in hyperspectral imaging from aircraft, it may be possible to utilize the data-cubes of spectrums to monitor waterways without the need to deploy individual units. The MBU can then be used to "ground-truth a complete library of situations." However, on the microscale, the sensitivity of the organisms can provide us with a response time that far exceeds current methods. A hyperspectral camera mounted to an inverted microscope is used to record the emitted spectrums of these colonies at various microscopic levels. The biofilms are cultured in the field in four parallel capillary glass flow cells where the flow rates and pressure drops are monitored. Flow conditions inside the cells are set to replicate the natural stream shear stress levels on the biofilms. Preliminary hyperspectral imaging of biofilms shows that the emitted spectrum of similar biofilm colonies varies greatly at the different life stages that result from a deliberate poisoning. Whether the MBU is used as a portable ground-truthing unit or as a stand-alone environmental monitoring tool in the event of biological and chemical warfare, results can be collected quickly, allowing rapid notification of the public.

S04-P337
Predicted Structure of AlgG, the Alginate C-5 Epimerase of *Pseudomonas aeruginosa*, and Characterization of Its Functionally Important Amino Acids.

S. A. Douthit, *D. E. Ohman, M. J. Franklin, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Biosynthesis of the polysaccharide alginate is important for *Pseudomonas aeruginosa* to establish chronic pulmonary infections. Alginate is a linear polymer of b1-4 linked D-mannuronate (M) interspersed with its C-5 epimer, L- guluronate (G).

The G residues are introduced into the polymer by the periplasmic C-5 epimerase AlgG. Previous results showed that FRD1200, a deletion mutant of algG, secretes depolymerized alginate, suggesting that AlgG may protect alginate from degradation by the alginate lyase, AlgL. To characterize AlgG/alginate interactions, we characterized amino acid motifs important for enzyme activity. AlgG contains motifs with Gly-Iso repeats characteristic of proteins with CArbohydrate-binding and Sugar Hydrolases (CASH) domains. 3D-PSSM was used to provide structural predictions for AlgG, and the results suggested that AlgG forms a right-handed b–helix (RHbH) from amino acids 167-515. AlgG was threaded onto the crystallized CASH protein-pectate lyase C of *Erwinia carotovora*. The model predicts that AlgG has a long shallow groove that may accommodate alginate. This groove also contains the 324-DPHD motif. Site-directed mutations in 324-DPHD result in proteins that complement FRD1200 to mucoid phenotype, but do not complement epimerase activity. The D324A mutant protein was dominant negative over the wild-type AlgG, suggesting that this mutation lies in the epimerase catalytic domain. The 361-NNRSY motif, identified from amino acid sequence alignments with other epimerases, is predicted to be on the opposite side of RHbH. N362 and N367 stack with other Asn residues along the b-helix, characteristic of RHbH proteins. AlgG mutants of N362 or N367 do not complement FRD1200 to mucoid phenotype, suggesting that they disrupt Asn stacking and protein stability. The N-terminus of AlgG is not predicted to be part of the RHbH, and a deletion of residues 89–102 did not affect AlgG activity in FRD1200. The results suggest that AlgG forms a RHbH, with a groove containing the epimerase catalytic center. This structure may protect alginate from AlgL by channeling the polymer through the proposed alginate biosynthetic scaffold.

S04-P338

Calcium Enhances Biofilm Formation and Causes Changes in Protein Profiles of the Marine Bacterium *Pseudoalteromonas haloplanktis* During Surface-Associated Growth

M.A. Patrauchan, *S.A. Sarkisova, K. Sauer, M.J. Franklin, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Bacteria undergo a variety of physiological changes following changes in environmental conditions, including the conditions associated with growth in biofilms. Here, we show that the marine bacterium *Pseudoalteromonas haloplanktis*, forms biofilm clusters on both hydrophobic and hydrophilic surfaces, and that development of these biofilms is significantly affected by calcium concentrations. We used a proteomics approach (2-D and 1-D gel electrophoresis of cellular and secreted proteins), followed by cluster analysis of protein spots to characterize the physiological changes that occur in *P. haloplanktis* during biofilm formation. These data were correlated with confocal scanning laser (CSLM) electron microscopy (EM) analysis of the biofilms. The results demonstrate that a combination of environmental factors had a cumulative effect on the bacterial protein profiles, and that [Ca2+] had greater effect on the profiles of surface-associated cells than of planktonic cells. Following surface-associated growth at 0.25mM CaCl2, *P. haloplanktis* had 174 protein spot differences from planktonically grown cultures, representing approximately 22% of the total spots resolved by 2DGE. At 10mM [Ca2+] this percentage increased to 38%. Using cluster analysis of the protein spot signal intensities, we grouped the proteins affected by surface-growth and by [Ca2+] into five classes. Proteins from each class were characterized by N-terminal and/or MS/MS sequence analysis. Extracellular protein profiles also changed with surface-associated growth and with [Ca2+], with five proteins showing increased amounts and one showing decreased amount at 10 mM [Ca2+]. Characterization of the proteins by MsMS demonstrated that the Ca down-regulated protein was flagellin. Immunoblot and EM analysis confirmed that higher [Ca2+] and surface-growth repressed flagella formation. The results presented here demonstrate that [Ca2+] has a physiological effect on the marine bacterium during surface-associated growth. Its effect likely influences gene expression of biofilm-associated bacteria, in addition to its role in maintaining biofilm structure.

S04-P339
Cryoconites: Biofilms Entombed in Glacial Ice

Christine M. Foreman, *Brent C. Christner, Jill A. Mikucki, and John C. Priscu, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Cryoconites are typically small, cylindrical water-filled depressions with a bottom layer of sediment found in the ablation zones of glaciers worldwide. The holes are formed as lower albedo aeolian particles settle onto the glacier and melt to a depth of thermal equilibrium. The aeolian-deposited dust particles often carry microorganisms, inorganic elements, nutrients and organic matter, providing the essentials for life within these ice entombed micro-ecosystems. A phylogenetic study of a cryoconite from the Canada Glacier revealed the presence of bacterial and eukaryotic organisms; however it was unknown whether these organisms were capable of actively metabolizing within the holes. Thus, this study assessed the metabolic capabilities of organisms within cryoconites of the Taylor Valley, Antarctica.

S04-P340
Mycobacterium spp. In a Hot Water By-Pass System

A. Karmacharya, *E. Pulcini , S. Broadaway, B. Pyle, P. Prommasith, T. Ford, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Background: *Mycobacterium avium-intracellulare* complex (MAC) are ubiquitous in the environment, found in soils and surface waters. MAC is the causative agent for a number of opportunistic infections of immune-suppressed individuals, especially AIDS patients. The sources of MAC-related infections have not been elucidated; however drinking water, and drinking water biofilms, have been implicated. Higher concentrations of MAC have been found in hot water systems as compared to cold water. The goal of this study was to analyze for the presence of MAC in a hot water by-pass system established at Montana State University–Bozeman.

Methods: Stainless steel coupons were placed in pipes within the hot water by-pass system and were removed after four months. Coupons were sonicated in cold sterile-filtered Milli-Q water to remove attached bacteria. The samples were plated on Middlebrook medium. DNA was extracted from the samples and analyzed using PCR-RFLP (PRA) which involves the amplification of a 439-bp segment of the 65-kDa heat shock protein using TB11 (5'-ACCAACGATGGTGTGTCCAT) and TB12 (5'-CTTGTCGAACCGCATACCCT) primers and the restriction enzyme analysis of PCR products using *Bst*EII and *Hae*III.

Results: Plates were incubated at 37 °C for more than eight weeks with no detectable growth. However, PCR of the total DNA from both the coupons and from samples of overlying water revealed appropriately sized (439 bp) fragments for hsp65; PRA analysis of those fragments was inconclusive, possibly indicating the presence of more than one species of Mycobacteria.

Conclusion: Although we recognize that primers may not be completely Mycobacteria-specific, given the hot water environment (~50 °C), Mycobacteria are the most likely source of hsp65. MAC are extremely slow-growing, making their detection difficult using culture based methods. The use of molecular methods has provided a strong indication of the presence of Mycobacteria in biofilms grown on stainless steel coupons within a hot water by-pass system, even in the absence of detectable growth.

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Routes of Exposure of *Mycobacterium avium* Complex (MAC) in Drinking Water

P. Prommasith, *P. Crerevichient, S. Arsa, S. Phongern, T. Netpae, S .Khumklai, A. Patel, M. Rothermich, H. Chen, and T.E. Ford, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Background: Collectively referred to as MAC, both *Mycobacterium avium* and *M. intracellulare* are opportunistic pathogens that have been isolated from distribution systems. The USEPA has listed MAC on the Contaminant Candidate List (CCL), thus more information on their prevalence is needed. According to a three-month preliminary survey of tap water collected from distribution systems in Eastern Massachusetts, 13.18% (n = 258) were MAC-positive. A systematic survey was designed to further investigate the possible route of exposure to these bacteria.

Methods: Water and biofilm samples were taken from different sites within four distribution systems, and also from end-user homes. Cold water and hot water samples were taken from kitchen faucets and from shower heads of each participant household. Sampling was conducted quarterly over a one-year period. Water samples were membrane- filtered and cultured

on 7H10-OADC Middlebrook agar. Species identification was done by both PCR-restriction enzyme pattern analysis (PRA) of the *HSP65* gene and *in situ* hybridization with fluorescent oligo-nucleotide probes.

Results: 27.74% (n = 1370) of bulk water samples were positive for Mycobacteria. Of these, approximately 13.14% were MAC positive with numbers ranging from 2.4 to 4.0 x 10^4 cfu/L. No significant differences were detected in the presence of MAC in water from different faucets within a home, although numbers were slightly higher from shower head samples than from kitchen faucets. 100% of *M. intracellulare*-positive samples were found in cold water (8–17°C), whereas 66% of *M. avium*-positive samples were found in hot water (35–46°C). Throughout the one-year sampling period, water samples taken from within the home were consistently more MAC-positive (by an average of 10.4%) than water samples taken from distribution sites.

Conclusion: Distribution system sampling will routinely underestimate exposure to MAC in the home, particularly through hot water, which is preferentially colonized by *M. avium*. Tap water is a likely route of exposure to MAC and further studies are warranted on more effective monitoring and control.