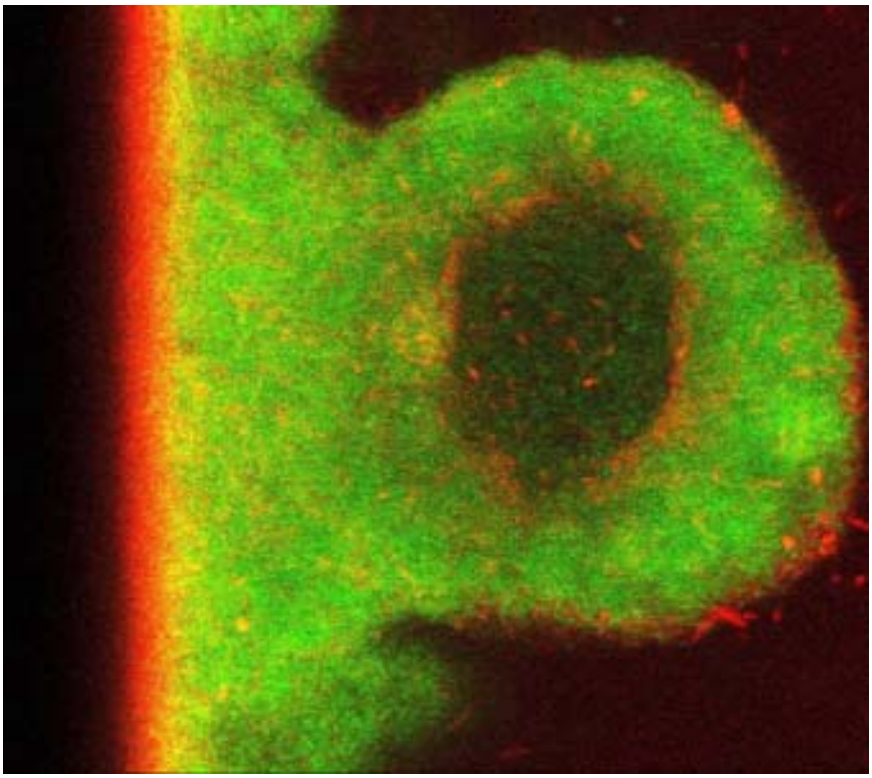
CENTER FOR BIOFILM ENGINEERING

PROCEEDINGS



Winter 2004 CBE Technical Advisory Conference

February 5–6, 2004
Montana State University–Bozeman
Bozeman, Montana

Sponsored by the
Center for Biofilm Engineering
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Engineering Research Center
at Montana State University–Bozeman

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 Stoodley,** Assistant Research Professor, Microbiology and Civil Engineering*
***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 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.

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 an interdisciplinary undergraduate and graduate education program involving team research on industrially relevant projects.

THE INDUSTRIAL ASSOCIATES PROGRAM

In addition to governmental funding sources, the CBE is funded through its diverse group of Industrial Associate members.

Benefits of membership include:

- **Attendance at Industrial Meetings**. The semi-annual meetings are exclusive to Industrial Associate members and CBE research collaborators (non-member companies may visit once to preview the Industrial Associates program). At each meeting, exclusive workshops are provided to give Industrial Associates hands-on training on the latest biofilm analytical techniques.
- **One vote on the CBE Technical Advisory Committee** to guide CBE research and policy.
- **Two days of consultation**.
- **Long-term visits** to conduct collaborative research.
- **Research sponsored by one company or a consortium of companies**.
- **Specialized workshops**.
- **Access to students trained in interdisciplinary, team research**.
- **Early access to publications**.
- **Access to the CBE's Biofilm Systems Training Laboratory (BSTL)**.

CBE WEB SITE

More information about the Center for Biofilm Engineering is available at its website:
http://www.erc.montana.edu/

Presentation and Poster Abstracts
Technical Advisory Conference: Feb. 5–6, 2004

Posters

SPEAKER ABSTRACTS

Keynote Speaker:

W04-S01
Battling Bacteria by Jamming Their Command Language

Michael Givskov, *Director, Centre for Biomedical Microbiology, Danish Technical University, Lyngby, Denmark*

Control of growth of unwanted bacteria is one of the most important technical achievements of mankind. In medicine and agriculture, treatment scenarios are based on antibacterial compounds such as antibiotics with toxic and growth inhibitory properties. Opportunistic pathogens often cause persistent (chronic) infections which lead to various tissue interferences and inflammation. One important difference to the pathogens is that the opportunists live as biofilm communities in the host. Biofilms are ubiquitous in nature, and more than 60% of all microbial infections are now believed to involve biofilms. In this state the bacteria tolerate the highest deliverable doses of antibiotics which make them impossible to eradicate. Biofilm infections are getting more and more common due to the widespread application of medical implants (catheters, artificial heart valves, etc). Such artificial surfaces create ideal environments for the development of biofilm infections. Taking the development in age distribution into account, this will become a major medical problem of the 21st century that urgently needs attention. *The solution might very well lie in the design of a new generation of target specific, anti-pathogenic (in contrast to anti-bacterial) drugs.*

Several bacteria are capable of working in flock and show organized behavior when they establish themselves and express their arsenal of tissue damaging virulence factors in the eukaryotic host. For this purpose, the bacteria have developed a primitive, chemically based command language denoted quorum sensing (QS). QS enables bacteria to keep track of their numbers and is considered to afford bacteria a mechanism to minimize host responses by delaying the production of virulence factors until sufficient bacteria have been amassed to overwhelm host defense mechanisms and enables the bacterium to strategically cause disease. But QS is also a mechanism by which bacteria expose part of their genetic repertoire for other organisms, prokaryotes as well as eukaryotes. The central role of QS systems in expression of host-associated phenotypes, including virulence factor production and biofilm development and the fact that they function by means of low molecular weight external signals make them ideal drug targets. Compounds able to paralyze the command language do not affect any vital function of the bacterium and thus will not interfere with its growth. When growth is not affected, there is no selection pressure for the development of resistant bacteria, and they are not expected to eliminate communities of helpful and beneficial bacteria present in the host.

In my presentation, I will outline a strategy for the isolation and identification of new anti-pathogenic drugs and deliver a proof of concept. My presentation also demonstrates that such anti-pathogenic compounds are in fact present in certain foods and suggests that the diet may offer a natural prophylaxis and treatment against chronic infections.

SESSION 1: Fate of Undesirable Organisms in Clean Water Systems

W04-S02
Session Introduction

Anne Camper, *Associate Professor, Civil & Environmental Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

W04-S03
Detection of Pathogens in Biofilms

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

Biofilms are ubiquitous on surfaces in drinking water treatment and distribution systems. Pathogenic bacteria in the water may become trapped by these biofilms, making the biofilm a “record” of the water that has passed over and through it. Detection of pathogens in biofilms at points along a water distribution network has been proposed as a strategy for tracking the source of a bioterrorist attack on a water facility.

Two parallel laboratory column studies were conducted to test our ability to detect the enteric pathogen *E. coli* O157:H7 in drinking water effluents and biofilms. In the first study, biofilm was grown in columns containing either glass beads or lightweight plastic, using dechlorinated drinking water as the inoculum. Biofilm was sampled using an air-water backwash that scoured biofilm cells from the

substrata. Heterotrophic agar plate counts were about one log higher in the backwash sample than in pre-backwash column effluent, indicating that the backwash was effective at removing biofilm. Columns containing mature biofilm were seeded with *E. coli* O157:H7, and the backwash and effluent were sampled periodically. Detection was accomplished by culturing on selective agar and by direct microscopy using fluorescent antibodies specific for the O157:H7 serotype. *E. coli* O157:H7 was detected in biofilm (backwash) samples collected at least 10 days following seeding, suggesting that the biofilm retained evidence of a simulated bioterrorist event for that length of time.

The second column experiment was conducted to evaluate the ability of *E. coli* O157:H7 to persist in granular anthracite medium used in biological filters at drinking water treatment facilities. The anthracite supports biofilm that removes organic carbon from the waterstream and, therefore, reduces the nutrient supply for biofilm growth in the distribution system. Columns were packed with anthracite from the local water treatment plant, conditioned on a feed of dechlorinated tap water for 30 days, then seeded with *E. coli* O157:H7. Following seeding, persistence of *E. coli* O157:H7 in the effluent and on the anthracite was determined. Biofilm was sampled from the anthracite by sonication and filtration, and effluent was concentrated by filtration. Detection was achieved by the culturing and antibody protocols used in the first experiment, as well as by polymerase chain reaction (PCR) detection of a specific gene. Neither culturing nor PCR detected the pathogen for more than a few days following seeding, but cells were still detected by microscopy for up to 20 days. There was no evidence of *E. coli* O157:H7 persistence after 30 days. After 60 days, anthracite was removed from the columns and used to inoculate different broths to try to resuscitate viable *E. coli* O157:H7 cells, but without success. In addition, genetic fingerprints of the effluent and biofilm microbial communities over the course of the experiment were produced using denaturing gradient gel electrophoresis (DGGE). The band corresponding to *E. coli* O157:H7 was evident in the effluent community for about two days following seeding. Effluent and biofilm communities showed similar composition.

W04-S04

Attachment and Persistence of Pathogens in Drinking Water Biofilms

***Garth James**, Staff Microbiologist, MSE Technology Applications, Inc., Butte, MT*

The ability of biofilms to capture and retain particulate matter from passing fluids has been documented in a wide variety of environments. However, interactions between allochthonous pathogens and indigenous biofilms have not been well characterized. The behavior of pathogenic biological agents in water distribution systems is important both for preventing waterborne disease and for remediation of contaminated systems. Furthermore, the capture and retention of pathogens by biofilms may be useful for improving sensing and monitoring strategies for intentionally or unintentionally introduced contaminants in water distribution systems. This research evaluated the attachment and persistence of pathogens including *Salmonella typhimurium* cells, *Cryptosporidium parvum* oocysts, and *Bacillus cereus* spores in biofilms formed by drinking water microorganisms. Experiments were conducted with biofilms grown on polystyrene surfaces in rotating disk reactors supplied with treated drinking water. *S. typhimurium* cells and *C. parvum* oocysts attached rapidly to the biofilms, reaching peak attached populations within one hour of exposure. Spores of *B. cereus* reached peak attached populations more slowly, with maximum attachment after approximately four hours of exposure. Nonetheless, the proportion of introduced spores captured by the biofilms was higher than for cells of *S. typhimurium*. The highest proportion of introduced pathogens that attached to the biofilms was demonstrated in preliminary experiments with *C. parvum* oocysts.

For all three pathogens evaluated, the number of organisms captured by the biofilms was proportional to the number added to the fluid phase of the system. Evaluation of the persistence of *S. typhimurium* cells attached to drinking water biofilms using immuno-assay techniques indicated the pathogen could be detected within the biofilms for at least 35 days after exposure, although culturability of the pathogen on a selective differential medium declined considerably over this time period. Overall, the results indicate that pathogens are readily captured and retained by biofilms, which may influence strategies for maintaining safe drinking water supplies and lead to improved monitoring methods for pathogens in water distribution systems. This material is based on work

supported by the Defense Advanced Research Projects Agency (DARPA) under Contract No. DAAH01-03-C-R037. Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the DARPA.

W04-S05
Public Health Implications of Mycobacterial Survival in Municipal Water Systems

Tim Ford, *Department Head, Microbiology, Montana State University–Bozeman, 59717*

The burden of environmental mycobacterial disease has declined in recent years with the advent of antiretroviral therapy for AIDS sufferers. However, disseminated infections, primarily with *Mycobacterium avium* complex (MAC), occurred in up to 50% of this population in the past, with high associated mortality. In the non-AIDs population, the rate of infection is estimated at 1.8 per 100,000 individuals, with disease outcomes that include pulmonary disease, cervical lymphadenitis, cutaneous infections and hypersensitivity pneumonitis. It is likely that this disease burden is underestimated. Early research implicated drinking water as a source of infection, particularly by *Mycobacterium avium* complex (MAC), with identical strains isolated from a hospital hot water system and AIDS patients. Our research has shown that MAC are common contaminants of water distribution systems around the Boston area and that biofilms are a likely site for both survival and growth of these opportunistic pathogens. Laboratory research suggests that clinical isolates of MAC can survive and proliferate in mixed biofilms at the biofilm/substratum interface, presumably protected from the overlying water by other species. Our work also indicates that biofilm-associated MAC are the more virulent, "white" morphotype of the organism. This paper will present the evidence that MAC are common contaminants of municipal water biofilms and that water, used both as a source of drinking and hot water, presents a significant route of exposure to this organism. Public health implications for dissemination of other biofilm pathogens will also be discussed.

W04-S06
Biofilms, Biodegradation and Surfaces

Anne Camper, *Associate Professor, Civil & Environmental Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Biofilms that form in clean water systems exist in an environment that would not seem to be conducive to growth. Growth substrates are at low concentration, and quite often, disinfectants are present. In spite of this, biofilm formation does occur. The research discussed in this presentation will show that these biofilms can grow at the expense of natural organic matter, particularly humic substances immobilized in the biofilm and on the substratum. The presence of a corroding surface such as carbon steel or ductile iron greatly enhances biofilm growth. Results have also shown that low levels of the common oxidizing disinfectants chlorine and monochloramine may actually enhance biofilm growth rates and/or numbers. Although the interactions between organics, biofilm, disinfectants and the surfaces are unknown, the evidence suggests that there may be effects that extend beyond simple heterotrophic carbon utilization on these corroding surfaces. These observations may provide insight on how biofilms survive in these hostile conditions, and may also help us understand how pathogenic bacteria are able to persist.

SESSION 2: Advances in Biofilm Modeling

W04-S07
Session Introduction

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

W04-S08
Modeling Transport and Adsorption of Undesirable Bacteria to an Established Drinking Water Biofilm

Jace Harwood, *MS Candidate, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Biofilms in drinking water systems have been shown to play an important role in the survival and growth of

pathogenic bacteria. Water distribution systems have the ability to disseminate infectious agents or toxins widely among the population. The developed world is faced with many terrorist challenges, but one of the most troublesome involves the addition of chemical or biological agents to water distribution systems. Drinking water distribution systems could be potential targets for terrorist operations to quickly infect a large population with a chemical or biological agent. Little is known concerning how chemical or biological agents interact with and persist in natural biofilms, or how quickly these agents could travel through water distribution systems.

The goal of this project is to detect, predict, and remove pathogenic agents from water distribution systems. Laboratory experimentation coupled with mathematical model formulation will allow for accurate determination of how these agents will propagate in a biologically active, aqueous environment.

The simulation software AQUASIM was chosen to model biofilm growth due to its specialized ability to fit parameters in aqueous environments and biofilm systems. Application models were created and logistically altered to determine the interaction of hypothetical agents with a simulated biofilm. Results of our current work suggest that the AQUASIM model could be an efficient tool for quantifying the transport and fate of pathogens in water distribution systems.

W04-S09
Modeling Persistence of Bacteria in Biofilms Using Cellular Automata

Steven Hunt, *PhD Candidate, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Understanding the ability of an established drinking water biofilm to retain and slowly liberate "undesirable" bacteria is critical to identifying the long term effects of a sudden slug dose of harmful bacteria in a water distribution system. The mode by which these bacteria adsorb, persist, and disperse from established biofilms is currently poorly understood. A three-dimensional stochastic computer model of biofilm development (hereafter referred to as BacLAB) was initially created to explore different modes by which biofilms detach. BacLAB is currently being modified to investigate a variety of other biofilm processes including the persistence of undesirable bacteria in an established biofilm. BacLAB simulates the life cycle of a bacterial biofilm from the initial colonization of a surface to the development of a mature biofilm by mimicking the physical and biological behavior of a system with a simple set of experimentally determined "rules" applied locally to the basic biofilm unit (the cell). These local "rules," however, lead to patterns on a larger scale. Much as bacterial cells organize themselves in a biofilm, the resulting biofilm model structure is produced through a process of self-organization. Because the rules are stochastic, the model adequately captures the inherent variability observed in laboratory biofilms. Previous results demonstrated that the typical simulated biofilms eventually attain a steady state where biofilm growth was counterbalanced by detachment, and they produced cell areal densities comparable to those in laboratory biofilms. Also, the oft-observed 'mushroom-shaped' structure occurred in the model biofilm because detachment events created voids, leaving the remaining attached cells in hollowed or loosely attached clusters and towers.

By extending the BacLAB model to include laboratory data pertaining to the persistence of undesirable bacteria, we hope to develop a predictive model for use in risk assessment and scale-up. BacLAB will also provide a means by which to quantify and visualize the fate of these bacteria after entering an established biofilm.

W04-S10
Finite Element Modeling: A Tool for Predicting Hydrodynamic Forces on Biofilm Structures

Brett Towler*, PhD Candidate, 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) identify areas of high detachment susceptibility based on stress distributions; and 3) determine linear viscosity material coefficients from experimental data.

Special Presentation:

W04-S11
Recap of ASM Biofilms 2003 Themes

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

The American Society for Microbiology meeting "Biofilms 2003," held in Victoria, British Columbia, in early November 2003, was the largest biofilm meeting ever held. Seventy-six oral presentations and 369 posters were presented over five days. In this selective summary of the meeting, I will focus on five themes that stood out: 1) the expanding world of biofilm research and technology, 2) advances in characterizing microbial ecology in biofilms, 3) the occurrence of phenotypic variants in biofilms, 4) dispersal (detachment) from biofilms, and 5) interactions between microbial biofilms and higher organisms. These themes will be illustrated with data and images kindly provided by several of the meeting presenters.

SESSION 3: Metal-Microbe Interactions

W04-S12
Session Introduction

Zbigniew Lewandowski, *Professor, Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

W04-S13
The Role of Controlled Cultivation in Biogeochemistry and Systems Microbiology Research

Yuri Gorby, *Microbiologist, Pacific Northwest National Laboratory, Richland, Washington, 99352*

Technologies for determining a complete genomic sequence of virtually any biological entity have developed over the last two decades. Amazingly, high-throughput approaches can be used to completely sequence relatively small genomes, such as those from prokaryotes, in less than a day. Complete genome sequence information indexes the biological "potential" of an organism, since all of the information necessary to function as an independent entity is contained within the genome. In order to understand, however, how this information is expressed, regulated, and practically applied in natural systems, such as biofilms, it is necessary to determine how the genome is expressed at the level of transcription of RNA, translation and activity of proteins, and the concentration and reaction rates of intracellular and extracellular metabolites. Integration of controlled cultivation with high-throughput technologies to understand metabolic and regulatory networks constitutes the essence of "systems biology." Systems biology has arisen in the post-genomic era as a comprehensive, holistic approach for investigating and interpreting the complexity of biology. This developing discipline integrates results obtained from advanced technologies, such as microarrays and high-throughput mass spectrometry, with those from more traditional methods (enzyme assays, gel electrophoresis, etc.). This presentation will discuss the cultivation and analytical technologies and approaches used by researchers to investigate systems biology in microorganisms with particular emphasis on the metal reducing bacterium *Shewanella oneidensis* strain MR-1.

W04-S14
Determination of Uranium Concentration in Wastewater from Sulfate Reducing Biofilm Reactors Using Voltammetry

Enrico Marsili, *Visiting Scientist, University of Rome, Italy*

This presentation describes an improvement to the voltammetric method of uranium analysis in natural waters and in wastewater. Searching for a suitable technique to determine uranium concentration from microbial reactors operated under sulfate reducing conditions, we have tried the existing protocols of adsorptive stripping voltammetry and found that the analysis was not sensitive enough to determine uranium concentration in waste waters containing organics. To improve the performance of the method, which used cupferron as a ligand adsorbing uranium on the hanging mercury drop electrode (HMDE), we applied acid digestion method to our samples. Using samples from simulated sulfate reducing conditions, we tested the method and defined the optimum conditions for uranium analysis. We decided to use the uranium stripping peak at -1.1 ± 0.01 V (versus saturated AgCl reference electrode) of the anodic stripping potential (measured vs. saturated AgCl reference electrode). The other conditions are as follows: conditioning potential -0.1 V (versus saturated Ag/AgCl reference electrode), conditioning time -180 s, scan rate - 20 mV/s, peak height 50 mV, using sodium acetate 0.01 M at pH 4.5 as supporting electrolyte and 80 μM of cupferron. The detection limit (determined for the ratio signal/noise > 3) was in pure water 15 ng/L, and in wastewater 0.6 μg/L. Our method offers an alternative to other techniques for uranium analysis, such as Kinetic Phosphorescence Analysis (KPA) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Although all these techniques offer similar detection limits, voltammetry is portable and can be easily applied in the field.

W04-S15
Microbial Fuel Cells

Allison Rhoads, *MS Candidate, Environmental Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The goal of this study was to design a microbial fuel cell (MFC) that utilizes microorganisms on both the anodic and cathodic compartments. We designed a microbial fuel cell in which the anodic compartment carries out anaerobic fermentation, and the cathodic compartment uses *Leptothrix discophora* SP-6 to microbially deposit manganese oxide onto an electrode. In the cathodic compartment, microorganisms oxidize manganese, which is then reduced on the electrode by using electrons from the anode, thus causing manganese to act as a redox mediator. Through anaerobic fermentation, glucose is oxidized and an electron is donated to the redox mediator, 2-hydroxy-1,4-napthoquinone (HNQ), which then brings the electron directly to the electrode. Our fuel cell operated for over 500 hours with average stable anodic and cathodic potentials of -479 ± 22 mV_{SCE} and $+300 \pm 50$ mV_{SCE} respectively. The MFC produced 10 mA/m^2 short circuit current via a 510 Ω resistor.

W04-S16
Telemetry Systems for Microbial Fuel Cells

Avinash Shantaram, *MS Candidate, Environmental Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The current environment-monitoring telemetry systems are powered by batteries that have a limited life. These systems have to be checked on a timely basis to ensure sufficient energy is available in the battery to power the electronics needed for a telemetry link. A self-sustained power supply is needed to bypass the onboard battery of a telemetry system in order to make the system completely autonomous. The object of our research was to design a thermocouple-based temperature-monitoring telemetry system that can be powered entirely by a microbial fuel cell, thus eliminating the need for replacing/recharging batteries. Low power, high efficiency electronic circuitry was implemented in conjunction with the microbial fuel cell to provide a stable power source for wireless data transmission.

SESSION 4: Biofilm Methods

W04-S17
Session Introduction

Darla Goeres, *Research Engineer, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

SPEAKER ABSTRACTS

W04-S18
Viable Plate Count Methods Comparison: Spread Plate vs. Drop Streak

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

Pour-plate, spread-plate and membrane filter techniques are the currently accepted standards for enumerating viable bacteria in environmental samples. All three of these methods are labor- and cost-intensive. The drop streak method, consisting of three 20µl sample volumes per plate, decreases cost and labor in that it requires fewer supplies and spreading of the sample is not necessary.

The goal of this research project was to determine if the drop streak method was statistically comparable to the standard spread plate method in terms of repeatability. Environmental planktonic and biofilm samples received over the course of seven months were plated using the two methods. The samples were enumerated and analyzed using a fixed-effects, two-factor analysis of variance (ANOVA) model.

According to the ANOVA results for all experiments, the difference between the two plating methods was highly statistically significant (p-value < 0.001). For the planktonic samples, the mean difference of $\log_{10}(CFU/cm^2)$ between the two methods was 0.407 (95% confidence interval: 0.346 to 0.468). For the biofilm samples, the mean difference $\log_{10}(CFU/ml)$ between the two methods was 0.588 (95% confidence interval: 0.516 to 0.660).

Conclusions based on this data would indicate that the drop streak method is not appropriate for enumeration of environmental samples with an undefined population. This study also emphasizes the importance of determining the statistical relevance of new plating techniques compared to standardized plating methods.

W04-S19
Analysis of Laboratory Biofilm from Three Growth Reactors

Linda Loetterle, *Research Specialist, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Biofilm research requires reactor systems engineered to repeatedly grow a relevant biofilm. The researcher must choose a reactor system that will create a biofilm relevant to the research focus. Important parameters that influence biofilm growth in reactor systems include shear force, residence time, flow velocity, growth surface, nutrient concentration, temperature and organism(s) grown. Biofilm grown in three different growth reactors are compared visually, microscopically and in viable cell numbers. The reactors have different shear properties that contribute to the differences in the biofilm grown.

W04-S20
Assessing Biofilm Growth in Remote Areas Using the Mobile Biofilm Unit

Peter Suci, *Assistant Research Professor, Microbiology, Center for Biofilm Engineering, Montana State University–Bozeman, 59717*

A Mobile Biofilm Unit (MBU) has been designed and built, enabling biofilms that develop under natural conditions to be characterized both microscopically and spectroscopically in remote locations. An integral component of the MBU is a portable flow cell system coupled to a microscope and an image processing unit. Using this system, development of biofilms inoculated and fed from water channeled into the flow cell from a natural stream fed from a municipal reservoir was followed in real time. A prototype microscope-coupled imaging spectrometer has been laboratory tested in preparation for field testing. Reflectance spectra were collected in the visible range (0.5 to 0.9 µm) from an algal biofilm. Spectral classification analysis of images indicated that discrimination on the basis of spectral shapes could be used to identify regions of the biofilm altered by exposure to a toxin. This suggests that spectral data acquired using the MBU can be correlated with data acquired remotely using hyperspectral imaging. The first-stage MBU, powered by batteries coupled to solar panels, is ready for field testing. In the next year we plan to have the portable unit completed.

W04-S21
Phylogenetic Analysis of Clinical Biofilms: A Study of Two Infected Brain Shunts

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

Bacterial colonization of implanted tubing devices such as catheters and shunts can lead to serious secondary infections. Because culturing methods traditionally used to identify bacterial agents responsible for these infections are selective, less

discriminatory molecular approaches based on PCR amplification of extracted 16S rDNA are preferable for assessing bacterial diversity. This study was conducted to assess the diversity of bacteria colonizing two ventriculo-peritoneal brain shunts removed from patients who developed chronic infections subsequent to implantation. DNA was extracted from 1.5-cm segments of brain shunt tubing using a beadbeater system. The extracted 16S rDNA was then PCR-amplified using two different primer sets. PCR products were then cloned and/or re-amplified to add a 40-bp GC-clamp for separation by DGGE analysis. DGGE bands were sampled and re-amplified using their respective primer sets; and clones and/or re-amplified DGGE bands were sequenced. Reported sequences were subjected to a BLAST search for bacterial identification by comparison to the NCBI GenBank. Results of this study clearly identified the presence of a number of potentially pathogenic bacterial genera. A comparison of the different results from clinical culturing versus the use of molecular methods will be discussed. This study demonstrates that molecular techniques traditionally applied to microbial communities in environmental ecosystems also have applicability in studying microbial diversity in human-associated communities, and may help to fill the need for identifying potential pathogens as causative agents of infection and disease.

SESSION 5: Biofilm Control

W04-S22
Session Introduction

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

W04-S23
Role of Oxygen in Biofilm Susceptibility to Antibiotics

Lee Richards, *PhD Candidate, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 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 re-suspending bacteria from a biofilm into anaerobic medium should preserve the low level of susceptibility measured in the biofilm. The susceptibility of re-suspended biofilm bacteria was, in fact, intermediate between the biofilm state and planktonic state. If oxygen availability were the primary 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.

W04-S24
Electrochemical Control of *Staphylococcus epidermidis* Biofilms

Christy Rabinovitch, *Research Associate, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The feasibility of using an electrochemical approach to remove or kill biofilm was investigated. Sodium chloride (NaCl) was used as an electrolyte in this study. When an electrical potential was applied, a potent biocide, (chlorine as HOCl) was produced at the cathode, and hydroxyl ions (which increase the pH) were produced at the anode. *Staphylococcus epidermidis* biofilms were grown on stainless steel coupons in a drip-flow reactor for 48 hours at 37ºC, and exposed to electrolysis treatment via a 6-volt battery and small jumper cables. The *S. epidermidis* biofilms were treated for time periods varying from 10 seconds to 5 minutes, with the biofilm-covered coupon being either the cathode or anode. A metal wire or a second, clean steel coupon was used as the other electrode. Results suggest that when the biofilm-

covered coupon acts as the cathode, biofilm is killed by generation of chlorine at the cathode. When the biofilm-covered coupon acts as the anode, biofilm is efficiently removed by the high pH at the anode, which may dissolve the biofilm matrix, and by the generation of hydrogen bubbles, which assist in biofilm removal. Such electrochemical treatment would alleviate the need for copious amounts of biocide; however practical application of this approach would require surfaces that are not subject to electrochemical corrosion.

W04-S25
A Genetic Basis for *Pseudomonas aeruginosa* Biofilm Antibiotic Resistance

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A gene has been discovered that contributes to the reduced susceptibility of *P. aeruginosa* bacteria when growing as a biofilm. When the gene is knocked out, the bacteria still form a structurally normal biofilm, but they lose much of their resistance to the antibiotic tobramycin. A mutation in this gene has no effect on the susceptibility of planktonic cells. The locus identified, *ndvB*, is required for the synthesis of periplasmic glucans. These are cyclic polysaccharides that may interact with antibiotics and prevent or retard entry of the antimicrobial agent into the cell. These results indicate that the biofilm is not simply a diffusion barrier to the antibiotic, but rather the bacteria within the biofilm employ distinct mechanisms to resist the action of antimicrobial agents. As specific genes involved in protecting biofilms from killing by antibiotics and biocides emerge, new strategies for controlling unwanted biofilms can be developed.

W04-S26
The Limitations of Laboratory Strain Genomes in the Study of "Real-World" Pathogenesis

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Bacterial growth conditions *in vitro* are strikingly different from those in natural ecosystems such as the human host. In spite of this contrast, microbial research is still largely based on laboratory-adapted strains with reduced *in vivo* virulence. Laboratory reference strains such as K12 of *Escherichia coli,* PAO1 of *Pseudomonas aeruginosa* and COL of *Staphylococcus aureus* have been sub-cultured and passed among laboratories for decades since their first isolation. Notably, all these strains are impaired in biofilm formation as compared to their clinical counterparts.

The history of the Bacille Calmette-Guerin (BCG) vaccine strain provides an illustrative example for the evolution of bacterial strains *in vitro*: repetitive passage has transformed virulent mycobacteria into an attenuated live vaccine. Literature provides strong evidence that genetic alterations occurring in laboratory strains over time are not random, but are the result of *in vitro* growth conditions; bacteria increase in fitness at the expense of lost adhesion and virulence factors. In consequence, laboratory reference strains vary significantly from clinical strains. Any research based on their genome sequences may therefore miss important pathophysiological mechanisms, such as biofilm regulation, that are present only in clinical strains. This limitation affects DNA microarray studies, but it can also affect proteomic studies which reconstruct protein sequences based on the sequenced genome of a laboratory reference strain.

Furthermore, there is growing evidence for extensive genomic variety within a bacterial species. A species-specific backbone genome is complemented by individually acquired, strain-specific pathogenicity islands. Atypical nucleotide composition and codon usage patterns within these islands suggest the inserts to be the result of horizontal gene transfer in a multispecies *in vivo* environment. Because biofilm formation enhances horizontal gene transfer, it has a major impact on this genetic heterogeneity.
Even an unpassaged clinical strain will therefore never be representative of its species. Only the summation of strain-specific "communal gene pools" in a virtual supragenome can fully characterize the genome of an individual species. Such supragenomes have been extrapolated to double the size of the reference strain genome. In the future, gene expression analysis should be based upon supragenomes rather than on individual clinical or even laboratory reference strains in order to objectively document "real-world" pathogenesis.

W04-P316
Analysis of Laboratory Biofilm from Three Growth Reactors

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Biofilm research requires reactor systems engineered to repeatedly grow a relevant biofilm. The researcher chooses a reactor system that will create a biofilm relevant to the research focus. Important parameters that influence biofilm growth in reactor systems include shear force, residence time, flow velocity, growth surface, nutrient concentration, temperature and organism(s) grown. Biofilms grown in three different growth reactors are compared visually, microscopically and in viable cell numbers. The reactors have different shear properties that contribute to the differences in the biofilm grown.

W04-P318
Detachment and Antimicrobial Resistance of Single Cells and Cell Clusters from *Pseudomonas aeruginosa* and *Staphylococcus aureus* Biofilms

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The detachment of cell clusters from biofilm enables them to disseminate, flow downstream, and reattach, establishing another biofilm event. In this study, detached biomass emboli from *S. aureus* biofilms grown under a constant flow rate were filtered from the effluent and microscopically examined to determine their size distribution (number of cells per particulate) and detachment frequency. Detached emboli were also collected and exposed to various concentrations of antibiotic. The resistance of the detached particles appears to be directly related to their starvation state. The emboli were then washed and viability determined. By comparing the number of the various aggregate sizes with resulting total volume of each size category, it becomes evident that the impact of a large detachment event could be greater than the numbers of events would indicate. The resistance of the emboli to antibiotics emphasizes the negative impact they would have in clinical situations.

W04-P322
Spatial Patterns of Green Fluorescent Protein Expression in Biofilms

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Spatial patterns of green fluorescent protein (GFP) expression in *Pseudomonas aeruginosa* biofilms were determined. *Pseudomonas aeruginosa* with the plasmid *pAB1*, carrying an inducible, stable GFP, was used in this study. In the presence of the inducer isopropylthio-beta-D-galactoside (IPTG) and oxygen, bright green fluorescence develops in those cells that are actively synthesizing protein. The more active the growth is; the greater the GFP expression. Biofilms were grown in a glass capillary tube under continuous laminar flow of minimal glycerol-glutamate medium at ambient temperature (23^{o}C). Air was pumped at the same flow rate as the medium, 20mL/hr, to make the biofilms stronger and provide more oxygen for green fluorescence activation. This resulted in slug flow of medium and air bubbles through the capillary tube. After five days of growth, mature biofilms were ready for confocal microscope observation. The inducible GFP was proved suitable for the visualization of spatial growth patterns within biofilms. Time lapse images revealed the transient increase in fluorescence after IPTG addition to the medium. Biofilms emitted negligible green fluorescence without the IPTG induction, while the green fluorescence became more and more intense along the time course after IPTG addition. Greater GFP activity was evident at the surface of cell clusters than in the center. Activity appeared more uniform in smaller clusters and less uniform in larger clusters. These preliminary measurements illustrate the physiological heterogeneity that is present in these biofilms. Such variation in the metabolic activity probably contributes to the reduced susceptibility of these biofilms to antimicrobial agents.

W04-P324
Computer Model of Persister Cell Protection Mechanism of Biofilms Against Antimicrobial Agents

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Hypothetical mechanisms protecting microorganisms in biofilms from killing by antimicrobial agents were incorporated into a three-dimensional model of

biofilm development. The model, named BacLAB, simulates the growth of 28 seeded biofilm colonies over a period of 500 hours. The model space is a 513 micron square substratum. Oxygen diffusion is simulated through the biofilm with the Crank-Nicholson solution to the Fickian diffusion equation. The model is an attempt to gain insight into the resistance and detachment mechanisms of biofilms. Antibiotic action is simulated by a random chance of death for each cell during specified time intervals. Also included is the differentiation of some cells into dormant persister cells, along with a mechanism for the persister cell resuscitation. The results of the model show that the addition of antibiotics leads to an increase in nutrient availability in the interior of the biofilm. This is due to the fact that cells compromised by antibiotics stop consuming nutrients. When the antibiotic action is relaxed, cells throughout the biofilm begin growing at a higher rate, cutting off the nutrients that were previously diffusing to the innermost layers of the biofilm. This creates a starved environment for the inner layers resulting in the escape of the inner, living cells from the biofilm. A mass escape such as this, in many cases, causes a catastrophic detachment of the biofilm, leaving mostly dead and "persister" cells on the substratum, along with a small percentage of living cells. Another round of antibiotics may or may not eradicate the remaining living and persister cells. The ultimate goals of the model are to assist in the investigation of antibiotic dosing protocols and the identification of novel treatment strategies.

W04-P325
Artifacts in 1-Photon Confocal Imaging of Biofilms as Shown Using Fluorescent Beads and 2-photon Confocal Microscopy

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Confocal laser scanning microscopy (CLSM) is a standard tool for imaging bacterial biofilms. In thick or very dense biofilms, however, laser penetration into the sample can be low, resulting in poorly resolved images. Fluorescent beads were used in this study to elucidate potential artifact in biofilm imaging, especially in 3-dimensional reconstructions. Beads of varying diameter (1 to 15 micrometers) were imaged individually, in densely packed arrangements, and immobilized in biofilms using both 1-photon and 2-photon confocal microscopy. Resolution as measured by increase in apparent bead diameter was measured and compared. Biofilms were then imaged using 1- and 2-photon excitation, and apparent thickness was measured from 3-D reconstructions. 2-photon excitation showed much greater depth penetration and resolution, and biofilms were shown to be 2 to 3 times thicker than a 1-photon image of the same area indicated. Microcolonies that appeared to be 40 micrometers thick and hollow were shown with 2-photon excitation to be solid, and at least 80 micrometers thick. The bead studies and 2-photon imaging suggest that 1-photon CSLM can introduce serious structural and thickness artifacts to biofilm images.

W04-P326
Controlled Ultrasonic Antibiotic Release from Hydrogel Coatings for Biofilm Prevention

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Medical devices, such as intravascular and urinary catheters, are routinely employed in healthcare settings since they provide clinicians with a useful means of administering nutrients, drawing blood samples and drug delivery. In spite of these advantages, local and systemic infections are frequently associated with their use. In fact, implanted devices often provide a highly suitable surface for bacterial adhesion and colonization resulting in the formation of complex, differentiated and highly structured microbial communities known as biofilms. Once a biofilm infection is established, conventional treatments frequently fail, since bacteria growing in biofilms are much more resistant to antibiotics than their planktonic counterparts. As a result, a variety of implantable drug-delivery systems have been developed. However, drug release tends to decay over time, and these systems are prone to uncontrollable leaching.

To overcome this problem the University of Washington Engineered Biomaterials (UWEB) group developed a novel drug-delivery polymer matrix consisting of a poly 2-hydroxyethyl methacrylate hydrogel coated with ordered methylene chains forming an ultrasound-responsive coating. This system is able to keep the drug inside the polymer in the absence of ultrasound, but will show a significant drug release when low intensity ultrasound is applied. The drug embedded within the polymer is ciprofloxacin, an antibiotic well known for its action against Gram-negative bacteria. In collaboration with UWEB we have designed a flow cell system

incorporating the hydrogel coatings that allows simultaneous real time digital or confocal time-lapse microscopy and the application of ultrasound.

Pseudomonas aeruginosa biofilms were grown on the hydrogel surfaces in the flow cells with a bulk fluid flow of 1 ml/min. The development of cell clusters could clearly be resolved on the hydrogels using transmitted light, and single GFP-expressing cells could be observed using epi-fluorescence microscopy. Propidium iodide was used to assess the combined and single effect of antibiotic and ultrasound on viability. The uptake of PI correlated well with plate count reductions of cultures exposed to ciprofloxacin. Further testing will include the measurement of biofilm cell viability after exposure to ultrasound at different frequencies. The results of our studies may ultimately facilitate future development of medical devices sensitive to external impulses (ultrasound) capable of treating or preventing biofilm growth via "on demand" drug release.

W04-P327
Cell Movement in Biofilms

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The goal of this research project is to quantify individual cell and cell cluster movement within a mature Pseudomonas aeruginosa PAO1 biofilm. This research provides a fundamental understanding of cell dynamics within mature PAO1 biofilms, and is of interest to many biofilm researchers. This work builds largely on top of previous research done at the Center for Biofilm Engineering (CBE), and Soren Molin's group in Denmark. Previous CBE work quantified replication, emigration, and movement events in young (3-10 µm thick) PAO1 biofilms. Soren Molin's group has used fluorescent PAO1 constructs to make qualitative observations on biofilm formation, but there are no quantitative data on cell or cell cluster movement within the biofilm. For this project, time-lapse images of biofilm formation are captured using Scanning Confocal Laser Microscopy (SCLM) equipped with an Acousto-Optical Beam Splitter (AOBS), so that individual cells and cell clusters can be visualized with the aid of fluorescent constructs of PAO1. These constructs are each recorded to separate channels, allowing for spatial differentiation of various clusters and cells. Tracking volume and shape factor measurements for individual clusters over time leads to information regarding cell displacement and colony formation within the biofilm as a whole. Growth patterns at various locations within a biofilm are quantified, allowing for visualization of stationary regions within a biofilm, of interest to those studying antibiotic application and detachment phenomena.

W04-P328
Host Factors Required for *S. pneumoniae* Biofilm Formation

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Background: *S. pneumoniae* is the most common cause of respiratory tract infections. Colonization is the first necessary step for invasion. Although the biofilm mode of growth may be important for colonization and has been associated with chronic otitis media, biofilm formation has not been studied with pneumococci.

Methods: Pneumococcal strains 4, R6 (unencapsulated) and the opaque and transparent forms of serotype 6A were studied. We examined mucin binding and biofilm formation under static and flow conditions. Various host factors were added to the growth medium. Biofilms were analyzed by bacterial counts and confocal laser scanning microscopy. We visualized upper respiratory tract colonization and lung infection in a mouse model.

Results: Whereas R6 formed biofilms, encapsulated pneumococci expressed insufficient surface adhesion and intercellular cohesion to allow biofilm formation under flow conditions. Correspondingly, encapsulated pneumococci did not specifically bind bovine submaxillary mucin. Static conditions allowed the growth of dense pneumococcal aggregates on cellulose-acetate filters. Bacterial numbers thereby significantly increased in presence of hemin, acetylcholine, catalase, serum or whole erythrocytes. Mucin did not support biofilm formation. Biofilm formation between the transparent and the opaque phenotype did not differ. Microscopy revealed that encapsulated pneumococci colonize the upper respiratory tract in the form of single cells rather than as a biofilm. However, bacterial clusters occurred occasionally.

Conclusion: Our findings suggest that pneumococcal proliferation and biofilm formation depend on host tissue destruction. The intense host immune response to pneumococcal infections may promote bacterial

growth to high density and biofilm formation. *In vitro*, biofilm formation is facilitated by host-specific components that likely prevent or delay autolysis of the pneumococci.

W04-P329
Flow Cytometry: A Complement to the Biofilm Imaging Facility

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In the fall of 2003 the Center for Biofilm Engineering purchased a Becton Dickinson FACSAria sorting flow cytometer with the help of the Murdock Charitable trust and the Army Research Office. The new flow cytometer is an excellent addition to the biofilm image facility. Flow cytometry is a useful tool because of the ability to examine heterogeneous bacterial populations as well as to quantitate microorganisms within a biofilm. This will prove to be an excellent complement to confocal imaging data.

W04-P330
Nuclear Magnetic Resonance Imaging of the Fluid Dynamics around Biofilms

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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 one-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 congruent 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 revealed easily. In addition to imaging the biofilms, velocity distributions have been mapped for the one-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 to explore how traditional NMR spectroscopy can be used to study biofilms.

W04-P331
NMR Microscopy of Microbial Transport in Porous Media

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Biofilms are heterogeneous microbial structures that form on solid surfaces exposed to aqueous environments under physiological conditions. Biofilms consist of bacterial cell clusters held together in an extracellular polysaccharide (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.

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W04-P332
Anti-Biofilm Efficacy of DePuy 1 PMMA Bone Cement with Gentamicin

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The objective of this study was to determine the anti-biofilm efficacy of a gentamicin impregnated bone cement (DePuy1 with gentamicin). Experimentation involved the visualization and enumeration of bacterial colonization of PMMA bone cement samples. The cement was continuously exposed to greater than 10^4 concentrations of clinically relevant strains *Staphylococcus aureus* or *Staphylococcus epidermidis* obtained from infected orthopaedic prostheses. These experiments provided important information on the ability of the antibiotic bone cement to significantly reduce bacterial colonization. Standard, non-antibiotic containing, DePuy 1 bone cement was used as an untreated control. Modified microscope flow-cells, were used as growth reactors to allow for direct microscopy (bright field, epifluorescence with confocal) of the bone cement surfaces. The flow-cells were set up for continuous flow and conditions that most closely simulate the *in situ* dynamic environment occurring within the bone/bone cement/tissue interfaces. A recirculation loop was included into the apparatus setup to better model an *in situ* system in which, despite the moderate shears, has lower losses of antibiotics and removal of cells than would be present in a once-through reactor setup. The recirculation loop was implemented using a mixing chamber (~20 ml) to provide a homogenous solution, which is then passed through the flow cell and returned to the chamber. Bacteria and free nutrients were continually replenished while the effluent stream was removed to reduce the build up of waste and maintain the antibiotic levels similar to *in situ* conditions. The bone cement coupons were evaluated using the scrape-and-plate method and confocal image analysis of *Bac*Light (Molecular Probes) stained cells. The gentamicin impregnated bone cements showed significant reductions in surface colonization. The DePuy1™ Gentamicin bone cement demonstrating a 10^3 (3.0 logs) reduction in *Staphylococcus aureus* biofilm and $1.3x10^5$ (5.1 logs) reduction in *Staphylococcus epidermidis* biofilm, versus untreated controls. The scrape and plate results are corroborated by the confocal microscopy experiments, in which percent viability was reduced by between 24 and 55%, respectively.

W04-P333
Biofilm Control Using Natural Products

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Many plants have to deal with bacterial colonization, especially those is moist and aquatic environments. The Center for Biofilm Engineering and Sequoia Sciences formed a collaboration on the hypothesis that some of these organisms must have developed defense mechanism to deal with these colonizations and some

must therefore have compounds capable of inhibiting and/or removing biofilms. In an initial study 12,000 extracts were taken and partially purified. These extracts were screened for biofilm inhibitory effects using a crystal violet staining assay in 96 well microtitre plates. The screening technique determined 269 potential extracts with anti-biofilm activity. Some of these extracts have been purified to individual compounds and reexamined for biofilm inhibition. Some individual and groups of compounds are now being elucidated. Some of the compounds have indicated efficacy in continuous flow reactor system as well as the potential for biofilm removal. The reactor system suggests biofilm reduction at approximately the same level as a Rifampin impregnated material (approximately 1.5 log cell/cm^2). These results are backed up by the fact that some of the compounds are currently found in the Pharmacia as treatments for bacterial infections. This project is continuing to screen additional extract and analyze successful extracts for biofilm inhibitor compounds. Current results suggest a number of potential industrial and/or medically relevant biofilm inhibitors.