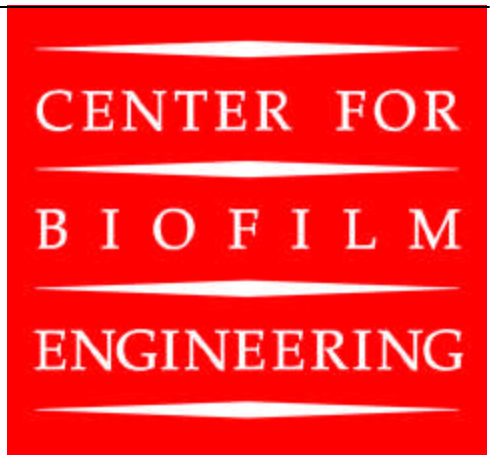
PROCEEDINGS



Summer 2002 CBE Technical Advisory Conference

July 22–25, 2002
Montana State University–Bozeman
Bozeman, Montana

Sponsored by the
Center for Biofilm Engineering
a National Science Foundation
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 Engineering*
***Anne Camper**, Associate Professor, Civil Engineering & Associate Dean for Research, COE*
***Al Cunningham**, Professor, Civil Engineering*
***Marty Hamilton**, Professor, 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 a close. The CBE's continued success is built on the foundation of many years of productive government-university-industry 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 the mechanisms at work in bacterial biofilms. The CBE has been a leader in defining the structure and function of biofilms on surfaces, in understanding the 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 useful. Industrial partnerships keep the CBE from being a traditional university "ivory tower" department, collecting information that has no practical application. 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 the following:

- **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 – July 22–25, 2002

Posters

SPEAKER ABSTRACTS

SESSION 1: Biomedical

S02-S01
Session Introduction

Jeff Leid, *CBE Immunology Projects Director*

S02-S02
Biofilms and host defense systems

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

The remarkable resistance of biofilms to antibacterial agents, and the consequent recalcitrance of biofilm infections to antibiotic therapy, have (rightly) occupied much of the attention of the biofilm community over the past two decades. Much less attention has been paid to the equally clinically important phenomenon of the failure of normal host defense mechanisms in clearing these biofilm infections by the activity of specific antibodies and accelerated phagocytosis. In chronic bacterial infections like cystic fibrosis pneumonia, the host defense mechanisms are not notably compromised, but the infection routinely persists through four to five decades, and the host response not only fails to clear the infection but actually exacerbates the host's condition.

The failure of specific bactericidal antibodies to kill sessile bacteria growing in biofilms has been noted, both *in vitro* and *in vivo*, and this phenomenon has been attributed to the interference of the biofilm matrix, "filed" under "failed treatment strategies," and largely forgotten. We don't use either active or passive antibody therapy against biofilm infections. Early experiments in killing biofilms by attacking them with phagocytes in the presence of opsonizing antibodies led to the equally depressing conclusion that few bacteria were killed, and this failure of a major host defense mechanism was again attributed to the biofilm matrix. We simply noted that phagocytes were attracted towards biofilms, and they were activated as they responded chemotactically to these sessile populations, but that they were not successful in engulfing and killing any significant number of sessile organisms. These experiments were conducted before the discovery of the complex web of cytokine responses that control effective phagocytosis, so no details of the failure of the phagocyte responses could be elucidated. The CBE conducted an analysis of the effects of the oxidative burst of phagocytes, in particular, the release of peroxides, and noted that sessile bacteria were protected to a very large extent by the matrix itself and by enzymes (e.g. catalase) arrayed within the matrix material. On the clinical side, immunizations of chronically infected patients led to the deaths of some CF patients, and we came to understand that "frustrated phagocytosis" can wreak havoc in affected tissues—so we put immune strategies even further back on the shelf. We routinely immune-suppress patients with chronic bacterial infections, and the paradox of the failure of normal host defenses in one whole category of bacterial infections remains unexplained.

The CBE was lucky to find three young people with awesome skills in modern immunology (Jeff Leid, Mark Shirtliff, and Luanne Hall-Stoodley), and to team them with our fabulous biofilm dynamics team (led by Paul Stoodley). We have now re-examined the role of host defenses in biofilm infections. The response of activated phagocytes to the presence of biofilms is being examined with full monitoring of the cytokine responses of the phagocytic cells as they sense and respond to the sessile cells, and almost all of the 15-year-old dogmas are being found to be false. Sam Silverstein (Columbia University) has collaborated with the CBE in designing unequivocal experiments, in which all possible reinforcements will be made to favor phagocytes in their attack on fairly weak young biofilms, and the question will be asked "can immune mechanisms kill biofilms under any circumstances?" This new initiative at the CBE will serve to re-open the question of whether host defense mechanisms can be manipulated to benefit patients with chronic biofilm infections. Perhaps more important, it will also address the long-standing and important question of how these sessile communities have evolved mechanisms to avoid engulfment and killing by phagocytic cells, including the free-living amoebae that they face in natural and engineered environments.

S02-S03
Localization of immune system components in biofilms

***Jeff Leid**, Immunology Projects Director, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Biofilms are highly resistant to biocides—including a variety of antibiotics—and clearance by the human immune system. One of the mechanisms behind this resistance has been attributed to the inability of leukocytes to engulf and kill the bacteria within the biofilm. Two important components in this process are biofilm-specific antibodies and complement components that are present in the blood stream. These two types of proteins are responsible for antibody-dependent cell mediated killing and a specialized form of phagocytosis called opsinization. For example, one of the pathways of complement-mediated killing of foreign pathogens involves coating of the bacteria with complement proteins that leads to an increased ability of neutrophils and macrophages to phagocytose and kill the invading pathogens. Here, we have studied the localization of complement component C3 and *S. epidermidis* specific IgG at various ages of biofilm growth. Our data demonstrate that one of the potential mechanisms that hinders the ability of leukocytes to attack biofilms may be a decreased ability of C3 and biofilm-specific IgG to penetrate and bind to the bacteria within the biofilm.

S02-S04
Leukocyte interactions with *Staphylococcus epidermidis* biofilms

***Jeremy Mitchell,** Undergraduate Researcher, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

A classical concept of the mechanism behind biofilm resistance to antimicrobial agents has been the idea of limited or decreased penetration of both antibiotics and cells of the human immune system. Recently we have demonstrated that under conditions mimicking physiological shear found in the blood stream, human leukocytes penetrate and respond to fully mature *S. aureus* biofilms, but not to those grown under static conditions. Here, we have continued these studies with a closely related strain—*S. epidermidis*. Interestingly, we observed good leukocyte penetration in statically grown *S. epidermidis* biofilms for up to 5 days of growth, at which time there was a dramatic decrease in the ability of leukocytes to penetrate the biofilm. Leukocytes interacting with maturing and fully mature *S. epidermidis* biofilms under shear conditions were also investigated. The differences seen between *S. aureus* and *S. epidermidis* highlight the important differences between biofilms not only of different species but also of species that are closely related, and should lead to caution in generalized statements about research results. Our results demonstrate that even in closely related species, the human immune system may respond differently to the respective biofilms.

S02-S05
Proteomic evaluation of *Staphylococcus aureus* biofilms

***Mark Shirtliff,** Postdoctoral Researcher, Environmental Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Staphylococcus aureus has been shown to attach to surfaces, producing a multilayered biofilm embedded within a glycocalyx. This glycocalyx, or slime layer, protects the bacteria from the host immune system and antimicrobial removal strategies. The initial attachment and subsequent formation of the biofilm phenotype have long been implicated in the development of persistent infections including osteomyelitis, endocarditis, and contamination of indwelling medical devices. In addition, *S. aureus* is responsible for the *in situ* bacterial fouling of a number of surfaces, including saliva ejectors and rinse applicators in dental clinics, flexible endoscopes, the internal surfaces of dialysis machines and peritoneal catheters in dialysis patients, surfaces in dairies, and plastic materials and machinery used for industrial food packaging.

The goal of this proposal was to evaluate the structural characteristics (through microscopy) and protein production (through 2D-gel electrophoresis and subsequent matrix-assisted laser desorption/ionization mass spectrometry peptide analysis—MALDI-TOF MS) of various aged biofilms produced by *S. aureus* (either in pure culture or in mixed species biofilms

with *P. aeruginosa*). Microcolonies existed in discrete structures (30–80 µm high), and these structures had an intricate channel network between them that presumably provided access to environmental nutrients. While not previously reported for *S. aureus*, these are much like the structures previously reported in mature *P. aeruginosa* biofilms. The mixed species biofilms utilized *S. aureus*^{GFP} (strain RN4220 with constitutive green fluorescent protein expression) and *P. aeruginosa* (strain PAO1) (~10^7 CFU's/species) that were grown alone or together. When the biofilm was observed using confocal microscopy, not only was the three-dimensional structure of biofilms maintained in the mixed species growth conditions, but the two bacterial species also seemed to exist as a homogenous mixture within the mixed species biofilms.

In the monospecies cultures of *S. aureus*, a number of proteins appear throughout all growth conditions, appear during early growth then disappear during later phases, or do not get produced until the later phases of growth. We found the de novo expression of over 40 proteins in a biofilm mode of growth when compared to planktonic. In addition, approximately 1/3 of the total proteome demonstrated differential production between the various stages of biofilm development (i.e. initial adhesion, immature, maturing, and fully mature). The detected classes of proteins will be discussed. Protein profiles were also evaluated for mixed species cultures with *S. aureus* and *P. aeruginosa*. While the *P. aeruginosa* 2D-gel proteins are spread out in the pH 5–7 onlinear portion of the pH gradient, the *S. aureus* proteins are concentrated in the pH 3–5 acidic range of the gel.

By fully understanding *S. aureus* biofilm formation and maturation, one may be able to engineer novel materials, surfaces, production line approaches, and/or antimicrobial strategies that resist or eliminate staphylococcal fouling and biofilm formation. In addition, biofilm-specific proteins may be used as vaccine candidates to prevent the formation of *S. aureus* persistent infections. The results obtained in the evaluation of *S. aureus* biofilm formation may be used as a model for the biofilm formation by other closely related gram-positive bacterial species, including *Streptococcus spp*., *Listeria spp*., *Clostridium spp*. and *Bacillus spp.* Lastly, by evaluating the effects that mixed biofilm formation has on *S. aureus* biofilm development and protein expression profiles, bacterial species-to-species interaction during biofilm formation may be elucidated for the first time.

S02-S06
Aseptic versus septic failures in total joint arthroplasties: Difficulties in the detection of biofilm on prosthesis by standard means

***Gerhard Maale**, M.D., Orthopedic Surgeon, Presbyterian Hospital, Dallas, TX*

The determination of the cause of prosthetic failures in total joint arthroplasties can be difficult. Pre-operative studies, including differential imaging, such as routine radiographs, tri-phase bone scans, computerized axial tomography, and magnetic resonance imaging, have been unable to discern septic sources of failures from aseptic causation. White blood cell scans, once thought specific for events associated with acute inflammation, have recently demonstrated false positivity in "pseudo-tumors of prosthetic debris." Labs show such white blood cell counts, sed rates, and CRP's can be elevated in septic, as well as, non-septic patients. Frozen sections, however, if showing acute inflammation on H&E preparations, are diagnostic of infection. Chronic active inflammation—which can be seen in chronic septic failures—is seen in both septic and aseptic failures. Culturing at the time of surgical revision has a rate of retrieval of 80%, including false positives.

We studied 5 cases of suspected aseptic failures of total hip arthroplasties, secondary to an inflammatory response to an oil residual on the prosthetic device at the time of implantation. This mechanism has been carefully investigated as a cause of failure over the last year by a prosthetic manufacturing company on hundreds of hip implants. The patients with these failures all had pain on weight bearing, a radiolucent zone around an acetabular prosthesis, and increased uptake on bone scan around the acetabular prosthesis. At the time of revision, frozen sectioning revealed chronic inflammation which was culture-negative. The acetabular prosthesis were processed in 80% Etoh and Tris buffer immediately upon retrieval. Confocal microscopy with green & red stains, FISH probes for Staphylococcus, and scanning electron microscopy were performed on the acetabulums or their associated parts. All were positive for Staphylococcus-induced biofilms.

This study demonstrates our inability to discern septic from aseptic failures in orthopedic procedures, involving implants, by pre-operative and intra-operative means. Better mechanisms for this discrimination are suggested.

S02-07
Ultrasound interactions with biofilms

Bill Pitt, *Visiting Scientist and Professor of Chemical Engineering, Brigham Young University*

Low frequency ultrasound has several applications in the medical field, which has traditionally used high frequency ultrasound for diagnostic and therapeutic applications. For example, low frequency ultrasound is efficient in creating cavitation bubbles which can interact beneficially or detrimentally with local cells. An oscillating or cavitation gas bubble creates fluid convection called microstreaming that greatly enhances transport of small molecules. However, vigorous cavitation can kill cells by generating strong shear forces or free radicals. Low frequency ultrasound has been shown to significantly enhance the transport of molecules through membranes and biofilms. Using the colony biofilm technique, we have shown that 70 kHz ultrasound significantly increases the transport of gentamicin through *E. coli* and *P. aeruginosa* biofilms. It also enhances the permeability of *P. aeruginosa* cell membranes to aminoglycoside and b-lactam antibiotics. Certain intensities of low frequency ultrasound enhance the growth rate of biofilms, most probably by increasing the transport of limiting nutrients or oxygen into the biofilm. Another significant application of ultrasound to biofilm research is in using ultrasound to remove a biofilm from a surface, and then to disperse the biofilm in preparation for enumeration of the bacterial cell density. Care must be employed in such techniques because not all ultrasonic baths are sufficiently powerful to remove and disperse biofilms, particularly with some species.

SESSION 2: Cell Signaling

S02-S08
Session Introduction

Bill Costerton, *CBE Director*

S02-S09
Acyl-HSL-based signaling in bacterial biofilms: What is a quorum?

Matt Parsek, *Professor of Civil and Environmental Engineering, Northwestern University,*

It has been over thirty years since acyl-HSL-based quorum sensing was first described in the marine luminescent bacterium *Vibrio fischeri*. Since that discovery, researchers have shed light on both the molecular mechanism of quorum sensing and the wide number of organisms that use it to regulate various processes. Unfortunately, most of what is known about quorum sensing to date is based on shaken liquid culture. This has lead to misconceptions as to how quorum sensing might function in naturally occurring populations. In this presentation, I will discuss important parameters that may influence the quorum sensing mechanism in biofilm populations. Transcriptional fusions of quorum sensing regulated promoters to the green fluorescent protein were used to monitor gene expression in heterogeneous biofilm populations. I will provide evidence that a variety of parameters influence the size of a quorum in a biofilm. I will also discuss phenotypic variants isolated from *Pseudomonas aeruginosa* biofilms and their relationship to quorum sensing. Finally, I will discuss how these different parameters that affect quorum sensing in biofilms may also affect potential control strategies targeting quorum sensing.

S02-S10
Regulation of *Staphylococcus aureus* pathogenesis via quorum sensing mechanisms

Naomi Balaban, *Assistant Professor of Pathology, University of California, Davis*

Infectious diseases associated with indwelling medical devices are considered a major problem in health care. One of the most common and virulent organisms involved in biofilm formation is *Stapylococcus aureus*, which comprises many of the infective strains that have become resistant to available antibiotics.

Two distinct mechanisms are considered important pathogenic steps in foreign body infections caused by staphylococci. Those are adhesion to polymer surfaces

or to host cells and subsequent accumulation of toxin-producing bacterial cells. *S. aureus* pathogenesis is regulated by the exchange of chemical signals between cells in a process known as quorum sensing**.** When the bacteria exist in low numbers, they express adhesion molecules that allow them to colonize. Once their numbers increase, they produce toxins in response to a protein termed RAP that they themselves produce. RAP then phosphorylates its target molecule TRAP, to further activate regulatory genetic loci such as *agr* to express or suppress the expression of adhesion molecules and toxins.

Our studies are aimed at interfering with *trap* and *agr* function using anti RAP antibodies and a peptide termed RIP, which interferes with staphylococcus quorum sensing. In the presence of RIP, TRAP phosphorylation is inhibited, resulting in inhibition of both biofilm formation and toxin production. RIP has already been shown to prevent S. *aureus* infections in vivo including: cellulitis, keratitis, mastitis, arthritis, osteomyelitis and sepsis. In addition, RIP has been shown to prevent Dacron-graft associated staphylococcal infections, including drug resistant strains of both *S. aureus* and *S. epidermidis.* We therefore suggest that RIP can be used to coat devices known to be subject to colonization by staphylococci and thereby prevent consequent staphylococcal infections.

S02-S11

The role of hydrodynamics in expression of quorum sensing in *Pseudomonas aeruginosa* biofilm

Laura Purevdorj, *Ph.D. Candidate, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Quorum Sensing (QS) and hydrodynamic conditions have been independently shown to be important in determining the structure of bacterial biofilms. However, hydrodynamics may also influence the concentration of signaling molecules in the biofilm and therefore, also QS mechanisms. The objective of our study was to investigate the role of hydrodynamics in shaping the nature of QS expression during biofilm growth and differentiation. Biofilms were grown from *Pseudomonas aeruginosa* PMH 509, a reporter strain containing a plasmid encoded gene fusion between green fluorescent protein (GFP) and *lasB*. *lasB* is required for the synthesis of elastase, a known virulence factor in *Pseudomonas aeruginosa* and is up-regulated through QS. Biofilms were grown for 5 days in square (1 x1 mm) glass flow cells at different flow rates (0.01ml/min, 0.1ml/min and 1ml/min). Confocal microscopy was used to determine the onset of GFP expression in the biofilm as well as the pattern and localization of GFP and, therefore, *lasB* expression in the biofilm. Scion image analysis software and bright field microscopy were used to measure biofilm development by surface area coverage and biofilm thickness. The effluent concentration of the cell signal oxo-dodecanoyl homoserine lactone (OdDHL) and viable cell count measurements were also monitored. After 3-4 days of growth the biofilms formed a dense layer of cells with a thickness of 22 ±13 μm interspersed with hemispherical cell clusters 30 ± 8 μm thick. No GFP expression was observed throughout the experimental time period. However, after the flow was turned off GFP expression in these biofilms was evident within 12 ± 4 hrs. For a positive control OdDHL was exogenously added at inducible concentrations, directly into one of the flow cells. Within about 20 minutes GFP expression in the biofilm was observed. These preliminary results suggest that flow conditions can play an important role in influencing Quorum Sensing mechanisms in *Pseudomonas aeruginosa* biofilms.

SESSION 3: Industry Forum

Presentations by:

S02-S12
Max Sherman, *DePuy*

S02-S13
Mel Czechowski, *Church & Dwight*

S02-S14
Tim Opperman, *Genome Therapeutics*

SESSION 4: Environmental Biofilms

S02-S15
Session Introduction

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

S02-S16
Field scale bioremediation: Unexpected solutions

Craig Criddle, *Associate Professor of Environmental Engineering and Science, Stanford University*

In the scale-up of laboratory research for field application, two critical issues are the delivery of substrates and the presence of inhibitory agents in complex mixtures. Strategies for addressing these issues will be discussed with reference to two case histories: the first is a large bioaugmentation project at Schoolcraft, Michigan, and the second a biostimulation project at Oak Ridge, Tennessee. At the Schoolcraft site, a full-scale biocurtain was established by bioaugmentation with *Pseudomonas stutzeri* KC at the advancing edge of a plume of carbon tetrachloride contamination. The biocurtain was created using 15 delivery wells spaced one meter apart, screened from 30'–80' below ground surface, and connected to an above-ground mixing and inoculation system. Base-amended groundwater was injected to adjust pH to the optimal level for strain KC (7.8-8.2), and cells grown in large volumes of filter-sterilized groundwater were injected. The resulting biocurtain was maintained by weekly acetate addition. Analysis of terminal restriction fragments of PCR-amplified 16S rDNA indicated a shift in microbial community structure following inoculation. Nitrate levels declined rapidly due to denitrification, and carbon tetrachloride levels fell to low levels that were subsequently maintained for over a four-year period.

At the Oak Ridge site, wastes from the manufacturing of atomic bombs were dumped into an unlined lagoon over a 31-year period. This practice resulted in the mobilization of highly acidic groundwater containing high levels of uranium, nitrate, aluminum, sulfate, heavy metals, and chlorinated solvents. We are preparing a field assessment of *in situ* reductive biomineralization of uranium with a companion ex-situ remediation effort. The *in situ* uranium immobilization experiment will be performed within a novel nested recirculation cell. An *ex situ* system will remove nitrate in a fluidized bed bioreactor; other contaminants will be removed by pH adjustment and precipitation. For full-scale applications, we anticipate that the developed *in situ* process will be of value for remediation of the plume fringe, where contaminant concentrations are reduced, while the *ex situ* process will be of greatest value in remediating highly contaminated source zones.

S02-S17
Bacterial transport through porous media

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

The successful bioaugmentation of contaminated subsurface environments relies on the effective transport and distribution of bacteria through porous media. Starved bacterial cells have been shown to transport significantly better through porous media than their vegetative counterparts. Starved cells have therefore been successfully utilized for manipulating the hydraulic conductivity of porous media for enhanced secondary oil recovery and the establishment of hydraulic subsurface biobarriers.

The research presented will provide a quantitative assessment of the extent of transport enhancement of the starved dissimilatory metal-reducing bacterium *Shewanella algae* BrY. Intermediate scale column studies (30 cm and 3 m length) were conducted to compare the transport of starved and freshly cultivated (vegetative) cells of *S. algae* BrY through quartz sand columns.

The fractional recovery (number of cells recovered in the effluent compared to number of cells injected) and the normalized breakthrough concentration were statistically significantly greater for starved cells in both 30 cm and 3 m long columns. The distribution of starved cells sorbed to the sand along the flowpath was, in addition, more homogeneous for starved cells. The advection-dispersion equation combined with the colloid filtration theory was sufficient to approximate the breakthrough of starved *S. algae* BrY cells but failed to describe the transport of vegetative cells appropriately.

The enhanced transport of long term nutrient starved bacteria has often been attributed to the decrease in average cell size during starvation. However, according to the filtration theory, cell size alone is not sufficient to explain the difference in transportability between starved and vegetative cells of *Shewanella algae* BrY. The second part of the presentation will document changes in a number of transport related cell properties of *S. algae* BrY during carbon and nitrogen starvation. The cell buoyant density decreased during starvation, the apparent diffusion coefficient and the cell surface charge (measured as zeta potential) increased but no change in hydrophobicity was observed. According to the filtration model applied, the cell size, buoyant density, and diffusion coefficient of starved cells could partly explain the improved transport behavior of starved cells through porous media, while the transport of vegetative cells was drastically overpredicted. The observed slight changes in cell surface charge and hydrophobicity were not sufficient to explain the difference between starved and vegetative cells. It was concluded that parameters beyond the ones investigated appear to be responsible for the differences in porous media transport of starved and vegetative *S. algae* BrY cells and that a better understanding of these parameters must be obtained before an appropriate mathematical model can be developed.

S02-18

Biodegradation studies of PAH and TNT *at the molecular level* by synchrotron radiation-based infrared spectromicroscopy

Thomas Borch, *Ph.D. Candidate, Environmental Sciences, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

This presentation will showcase the use of Synchrotron Radiation-Based Infrared Spectromicroscopy to investigate the biodegradation of polyaromatic hydrocarbons (PAH) and trinitrotoluene (TNT) at the molecular level. This presentation will include an introduction to SR-FTIR Spectromicroscopy and SR-FTIR biodegradation of pyrene and TNT. This novel analytical approach offers insight into several biodegradation-related issues associated with these compounds, including the impact of different chemistries on compound desorption and the impact of humic acid on compound bioavailability.

S02-S19

Spectroscopic evaluation of chromate reduction by metal-reducing bacteria

Andrew Neal, *Assistant Research Professor, Microbiology, Montana State University–Bozeman, 59717*

Chromium solubility increases with valence state, thus many remediation technologies aim to reduce chromate to the trivalent ion. Not only is chromate soluble and thus highly mobile, but it is also a known carcinogen and the pentavalent ion, although unstable, has been demonstrated to mediate DNA cleavage. Using *Cellulomonas* sp. isolated from the DoE Hanford site, WA and *Shewanella oneidensis*, this talk aims to demonstrate the utility of various spectroscopic techniques, including electron (Electron Energy Loss and X-ray Photoelectron Spectroscopies), X-ray (X-ray Absorption Spectroscopy) and vibrational (Raman) spectroscopies in identifying the valence state of Cr-species associated with bacterial cells of interest to bioremediation. The presented spectra demonstrate that Cr rarely exists in one valence state and reduced phases associated with cells commonly are a mixture of Cr(III) and Cr(IV).

SESSION 5: Regulatory Issues

S02-S20

Recent developments in regulatory policy

Marty Hamilton, *Professor of Statistics, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

In the past few months, the CBE has interacted with regulatory agencies and standard setting organizations. This is a report on interactions with the EPA, FDA, ASTM, AOAC, OSAP, and OECD. All of these agencies and organizations are now aware of the importance of biofilms and most are taking action to develop or approve antibiofilm test methods.

S02-S21
What's new in biofilm research at the Centers for Disease Control

Rod Donlan, *Biofilm Team Leader, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA*

The CDC Biofilm Laboratory has developed a new biofilm reactor that is designed to provide 24 reproducible biofilms on selected materials under dynamic, open system conditions, and can be sampled without interruption of flow and with minimal risk of contamination of the system. This system was validated with three different, clinically relevant organisms: *Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Streptococcus pneumoniae.* We have also used this system to investigate other biofilms, including those of organisms important in otitis media. By modifying the system operating parameters, it was possible to optimize conditions for biofilm formation by *S. pneumoniae*. Interface of the biofilm reactor with an Attenuated Total Reflectance Cell provided additional information on biofilm formation that was corroborated with viable and total count measurements.

We also investigated sampling issues that arise with the conventional microscopy method. We performed experiments to determine whether random sampling of fields on the filter provided more reliable estimates than the conventional systematic sampling. We evaluated the variability between filters and assessed the potential advantage of counting five fields on each of three filters rather than counting 20 fields on a single filter.

REVIEW PAPER

S02-S22
Surface characteristics that really matter

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

As we set out to engineer systems in which biofilm formation will be minimized, our instincts lead us to visualize materials whose surface characteristics will preclude bacterial adhesion, and the subsequent development of sessile microbial communities. Because of the importance of this process for many member companies, I will present a compendium of CBE research, some of which dates back before the beginning of the Center (1990), as a practical guide for biofilm control in a wide variety of systems.

The DLVO theory that describes the association of colloidal particles with surfaces has been used to predict the rates at which bacteria will adhere to surfaces, on the assumption that bacterial cells are charged particles that will obey these physical rules. People have concluded that certain bacteria are hydrophobic and will not adhere to hydrophilic surfaces, and vice versa, and some laboratory experiments have confirmed these predictions, but these materials have failed in practical tests. The twin problems are that bacteria have many protrusions from their complex surfaces (e.g. slime and pili), and are not smooth charged spheres, and that cells of lab strains of bacteria are much smoother than "wild" cells and tend to obey the DLVO rules and mislead the unwary. The second instinctive thought is that smooth surfaces might be much less susceptible to bacterial colonization, particularly in high shear systems, and polished surfaces have shown favorable results with the "bald" cells of lab-grown strains. These very smooth surfaces have also failed in practical tests where organic molecules coat the surfaces, wild bacteria adhere via their "hairy" processes, and turbulence has been show to actually impinge the bacteria onto the surface and increase adhesion and colonization. We can now state that the millions of dollars spent in corporate and university research to find materials that resist biofilm formation, by virtue of their inherent surface characteristics, have failed to discover even one single useful coating or material. The most important single factor governing the rates of bacterial colonization and biofilm formation on a surface is the cleanliness of that surface, in terms of accreted organic polymers and pre-formed biofilm matrices. Many experiments have shown that bacteria adhere to pre-fouled surfaces +/- 10 times as fast as they adhere to clean surfaces, and we have shown that bacteria can "reoccupy" dead biofilm matrices at an alarming rate. This factor is critical for medical devices because it gives bacteria in the area a much better chance of forming biofilms, before they are attacked by phagocytes and/or antibiotics, and clinical data confirm much higher rates of infection of "dirty" devices.

Conventional antibacterial agents can kill incoming planktonic cells, and signal blockers like the brominated furanones and the RIP biofilm blocker discussed by Dr. Balaban can inhibit biofilm formation, and coatings containing these molecules show great promise. A new cooperative project with Buddy Ratner (UWEB) shows even greater promise, in that these agents may be incorporated into coatings with an ultrasonic-responsive cellulose "skin" that can be made permeable to release these agents "on demand." Biofilm-resistant coatings may be useful in once-through systems, and in systems with short-term biofilm risks, but long-term biofilm control is best achieved by promoting the fouling of cleanable surfaces at the inlet, and minimizing biofilm formation in the system itself.

BIOFILM ACROSS CAMPUS

S02-S23
Water and public health: Domestic and international perspectives

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

Drawing on epidemiological research in India and Russia, this talk will focus on the global burden of infectious waterborne disease. The health burden of disinfection resistant pathogens such as *Cryptosporidium parvum* is essentially unknown in developing countries, where the public health focus is on dysentery, cholera and other diseases with high associated mortality. Our recent seroprevalence studies, however, suggest that exposure to pathogens such as *C. parvum* is widespread. Within developed nations, the focus is more on pathogens that remain unrecognized in developing countries and the poorly characterized risks from toxicity of disinfection by-products. For example, in our U.S. work, we focus on the survival of *Mycobacterium avium* in drinking water biofilms. We also examine the different routes of exposure to this pathogen in the home. Although considerable advances have been made in pathogen detection in biofilms, there is still much to be done to evaluate human health risks from drinking water biofilms – in terms of pathogen infectivity, the emergence of new pathogens, or emergence of more virulent strains or strains with increased antibiotic resistance. Using examples from both our international and US research programs, this presentation will focus on both what is known and what still remains to be understood.

S02-S24
Thermophiles and biofilms: Complexity, simplicity, and novelty

Timothy R. McDermott, *Co-Director, Thermal Biology Institute, Montana State University–Bozeman, 59717*

Yellowstone National Park is home to an estimated 10,000 distinct geothermal features. The YNP geothermal system is known and appreciated world-wide for its relatively pristine condition, but also because of the wide range in temperatures and chemistries that occur throughout. Virtually any combination of temperature and chemistry (i.e. pH, metals, etc.) can be found in both aquatic and terrestrial habitats, with such environmental variability setting the stage for the occurrence of significant diversity within the microbial communities that call these extreme environments home. This presentation will provide a few snapshots of work conducted within the Montana State University Thermal Biology Institute that involves both naturally occurring and cultivated biofilms. Examples of complex and simple microbial communities associated with geothermally heated soils and in aquatic systems will be discussed, along with results of biofilm cultivation work. Finally, interesting properties of the novel thermophilic bacteria, fungi, and viruses that are being discovered and characterized by TBI faculty will be briefly described.

SESSION 6: Biofilm Control

S02-S25
Diffusion in biofilms

Phil Stewart, *Professor of Chemical Engineering and CBE Deputy Director and Research Coordinator, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Many of the apparent differences between biofilm and planktonic systems can be explained by accounting for diffusive transport limitation of solutes into or out of microbial aggregates. Some of the biofilm phenomena that can be traced, at least in part, to diffusion limitation include coexistence of diverse microorganisms, spatially heterogeneous patterns of growth, distinct patterns of gene expression, microbially influenced corrosion, and the reduced susceptibility of biofilms to antimicrobial agents. This presentation summarizes literature data on effective diffusion coefficients in biofilms and outlines simple quantitative approaches to the analysis of diffusive transport phenomena in biofilm. Equations are presented for calculating the diffusive penetration time of a non-interacting solute and for analyzing the penetration of reacting solutes. The application of these formulae is illustrated with example calculations such as the time for an antimicrobial mouthwash to penetrate a patch of dental plaque or the depth of penetration of a reactive antimicrobial agent into a biofilm. The issue of whether water channels in heterogeneous biofilm structures alleviate mass transport limitations is discussed.

S02-S26
Alginate biosynthesis: Understanding the molecular mechanisms

Stephanie Douthit, *Ph.D. Candidate, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Chronic *Pseudomonas aeruginosa* infections in patients with cystic fibrosis (CF) are characterized by bacterial isolates that overproduce the exopolysaccharide alginate, which confers a mucoid phenotype to the isolates. This bacterial phenotype correlates with a poor prognosis for the patients, since these bacteria are difficult to eradicate with antibiotics. Alginate is a long chain polysaccharide consisting of b1-4 linked D-mannuronate (M) with its C-5 epimer, L-guluronate (G), randomly dispersed throughout the polymer, and with O-acetyl groups linked to M residues. Alginate is polymerized as polymannuronate, and then modified to its final form. These modifications give *P. aeruginosa* alginate its specific properties including: (i) resistance to host immune defenses such as opsonic and non-opsonic phagocytosis, (ii) ability to form thick three-dimensional biofilms, and (iii) possibly increased resistance to antibiotic. Of particular interest is the modifying enzyme AlgG. This enzyme has dual functions as a C-5 epimerase that converts D-mannuronate into L-guluronate and may also be important for transport of the alginate polymer through the periplasm. In this study, bioinformatics and site-directed mutagenesis approaches were used to identify functional domains within this enzyme. Sequence alignments with C-5 epimerases and alginate lyases from other *Pseudomonas sp.* and from *Azotobacter vinelandii* revealed two conserved amino acid motifs (the DPHD motif and NNRSY motif). Site-directed mutations of specific amino acids in these motifs were made. *In vivo* enzyme activities were tested following introduction of the mutant genes into *P. aeruginosa* FRD462, a strain derived from the CF isolate FRD1, that has a point mutation in *algG*, and *P. aeruginosa* FRD1200, an *algG* deletion mutant, which only secretes uronic acid residues. In FRD1200, mutations in D324, P325, H326, and D327 disrupted epimerase function but complemented the secretion defect, suggesting that this site is a catalytic domain for epimerase activity. In the NNRSY motif, amino acids S364, Y365, and Y385 had only little effect on either epimerase activity or secretion activity. However, N362 failed to complement the secretion defect seen in FRD1200, suggesting that this amino acid motif plays a role in the alginate transport. Interestingly, mutations in R316 and N361 do not complement epimerase activity in FRD462, yet complement epimerase and secretion defect in FRD1200. This indicates that the point mutation in the FRD462 *algG4* allele is dominant. Mutations in R316 and N361 may create weak binding sites for protein/protein interactions in the proposed complex and are out-competed by the *algG4* allele. It has been suggested that AlgG and AlgK provide important function in alginate transport by protecting the polymer from AlgL an alginate lyase located in the periplasm. Identifying domains within these proteins

that are protective to the polymer may become important target sites in order to disrupt alginate production and biofilm formation.

S02-S27
Modeling nutrient-limited killing of biofilm bacteria

Phil Stewart, *Professor of Chemical Engineering and CBE Deputy Director and Research Coordinator, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

A computer model of biofilm accumulation was applied to investigate one mechanism of slow killing of bacteria in biofilms by antimicrobial agents. The model simulates the situation of diffusion limitation of a nutrient, leading to zones of slow or no growth in the depths of the biofilm. The rate of killing by an antimicrobial agent is assumed to be directly proportional to the local growth rate of the bacteria. The model predicted nutrient concentration gradients and stratified patterns of growth in biofilms thicker than about 50 microns. This non-uniform growth resulted in reduced rates of killing of biofilm cells. The rate of killing was reduced as the biofilm thickness increased or when the bulk concentration of nutrient was decreased. A further refinement of the model introduced a hypothetical cell state intermediate between live and dead. Cells in this "damaged" state were assumed to continue to consume nutrient even though their inability to reproduce means that they would be scored as dead in a viability assay. Live cells were transformed to damaged cells and damaged cells to dead cells in the presence of the antimicrobial agents. The existence of the damaged cell state was shown to enhance the protection afforded by growth in a biofilm. These computer experiments demonstrate that nutrient limitation leading to slow growth is a plausible mechanism of biofilm tolerance to killing by antimicrobial agents.

SESSION 7: Biofilm Methods

S02-S28
Estimating the ruggedness of a laboratory method: Experimental design

Marty Hamilton, *Professor of Statistics, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

A thorough investigation of a new laboratory biofilm growth method will include experiments to assess the method's ruggedness. The goal of ruggedness assessment is to describe the extent to which the biofilm is affected when there is some departure from the protocol; e.g., when the laboratory temperature is a few degrees below the protocol's specified temperature. Because there are so many physical, chemical, and biological specifications to consider when assessing ruggedness, many experimental runs may be required. For the CDC Biofilm Reactor growth protocol being developed at the CBE, we plan to investigate the ruggedness for five parameters. A conventional factorial design is impractical because it requires ~170 runs of the growth protocol. Our approach is to conduct the experimentation in two phases. The first phase utilizes a fractional factorial design and requires 10 runs. The second phase uses a one-at-a-time design and requires at most 15 runs. Putting results from the two phases together, we can assess the ruggedness via statistical regression analysis. Although this talk focuses on ruggedness testing, the principles involved are applicable to other contexts.

S02-S29
Dual staining of bacterial biofilms

Jessica Janzen, *Undergraduate Researcher, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Microscopic imaging has been crucial to biofilm research. Imaging studies have shown that flow and nutrient conditions, as well as concentrations of cell signaling molecules, play key roles in biofilm development. The depth-resolving technology of confocal microscopy, which allows *in situ* visualization, has also been essential in biofilm research. High-resolution confocal images provided

the first evidence for the structural three-dimensional complexity of biofilms and changed the paradigm of how biofilms on surfaces were perceived. These studies have been fundamental to a better understanding of the complex nature of biofilms, their relationship to the surface substratum, microbial physiology, and species composition.

While multiple staining of eukaryotic cells is routine for defining eukaryotic structure, multiple staining of biofilm structure has been limited to a few stains. Visualization of bacteria can provide important information about the spatial distribution of bacterial cells within the biofilm. Moreover, it is impossible to evaluate bacterial biofilms on surfaces in unknown samples where biofouling is suspected without bacterial morphology. Previous stains used for this have included nucleic acid stains and the protein stains fluorocein or FITC. However, these stains are not specific to bacteria. Therefore we were interested in evaluating carbohydrate- and lipophilic-specific stains in conjunction with a nucleic acid dye to enhance the study of the spatial distribution of bacteria within extracellular matrix. Fluorescently labeled lectin and lipophilic dyes were used to: 1) enhance the visualization of three-dimensional structural heterogeneity within a biofilm; 2) enhance visualization of bacterial morphology and spatial distribution within a biofilm; and 3) assess these biochemical markers for specific staining of extrapolymeric substances (EPS). Results were obtained for biofilms of both gram-positive and gram-negative bacteria. These results should help us to better evaluate biofilms on surfaces of unknown etiology and help characterize a biofilm "signature".

S02-S30
Combination of biofilms and solid state electronics: A potential sensing device

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

Biofilms are microbial communities that respond sensitively to environmental perturbations in complex ways that have yet to be completely determined. Thus they offer the potential to act as the primary component in "cognitive" sensing devices. The first step in exploiting this possibility is to develop methods to transduce the environmental response into a measurable signal. Collaboration between a bioengineer, a microbiologist and a physicist has resulted in a transduction scheme that would allow biofilm sensing elements to be incorporated into solid state electronics devices. The transduction scheme involves the combination of magnetoelectronics and biogeochemistry.

S02-S31
Measuring the spatial distribution of *Leptothrix discophora* in a 3-species consortium biofilm

Scott Campbell, *MS Candidate, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The monospecies biofilm of *L. discophora* ennobled 316L stainless steel to an E_{corr} of +323 mV_{SCE} in 5 days due to the deposition of manganese oxides on the metal surface. However, in a 3-species consortium biofilm where *L. discophora* was present, only a partial ennoblement of +143 mV_{SCE} was noted after 5 days. Shortly after achieving this E_{corr}, the potential fell to +122 mV_{SCE}. Fluorescent *in situ* hybridization using 16s rRNA probes in the *L. discophora* monospecies biofilm, we detected *L. discophora* heterogeneously distributed throughout the entire biofilm including at the biofilm-metal interface. However, in the consortium biofilm, we detected *L. discophora* in the top 40% of the biofilm with 49.2% of this bacterium located in the top 10 to 30 ? m. In another sample of the biofilm, where the thickness was approximately 120? m, we once again detected 72.7% of *L. discophora* in the top 40% of the biofilm with 58.9% of the bacterium located in the top 20–40 ? m. In two representative samples of a 3-species biofilm, only 0.09% and 0.99% of *L. discophora* was located at the substratum, respectively. Oxygen microelectrode studies revealed that in the *L. discophora* monospecies biofilm, there does exist oxygen at the substratum in 2 of the 4 profiles taken in concentrations ranging from 3.85 to 4.35 mg/L. In the 3-species consortium biofilm, the biofilm became anoxic approximately 200 ? m from the substratum. These experimental results indicate that *L. discophora* was part of the initial colonization of the 316L stainless steel coupon, but either moved away from the surface of the stainless steel because of microenvironmental changes, i.e. oxygen depletion, or was displaced by the developing biofilm ultimately resulting in only a partially ennobled steel surface.

SPEAKER ABSTRACTS

S02-S32
Long-term biofilm structure reproducibility

Deanne Stookey, *Undergraduate Researcher, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Due to biofilm structural heterogeneity, it is hard to reproduce biofilms, therefore hindering research. Though many studies have sought to quantify the physical parameters of biofilms, there is still no set definition of reproducibility in biofilms. Some studies have been done on the short-term reproducibility of biofilms, but none have been done on their long-term reproducibility.

The purpose of this study is to test reproducibility in long-term biofilms and to define reproducible parameters. To test reproducibility, the image analysis techniques developed by Biofilm Structure and Function Research Group will be used.

A three-species biofilm made up of *P. aeruginosa* (ATCC#700829), *P. fluorescens* (ATCC# 700830), and *K. pneumoniae* (ATCC#700831) will be grown in two identical flat plate flow reactors located on a inverted microscope. Approximately 30–50 images will be acquired from each reactor. These images will be used to calculate are areal porosity, fractal dimension, diffusion differences, textural entropy, energy, and run lengths for each reactor. Once the average values of the parameters and standard deviations are calculated for each reactor, a two-tailed t-test will be run to test the statistical acceptability of both the individual parameters and biofilm structural reproducibility as a whole.

S02-P286
Detachment distribution of single cells and cell clusters from *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms

***Suzanne Wilson**, Ryan Cargo, Chayne Piscitelli, Paul Stoodley, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The detachment of cell clusters from biofilms is important for the dissemination of bacteria in both clinical and industrial environments. In this study detached biomass from biofilms grown under a constant flow rate were filtered from the effluent and microscopically examined to determine the size distribution (number of cells per particulate) and detachment frequency from biofilms composed of gram-positive and gram-negative bacteria. Biofilms were grown from individual strains of *Pseudomonas aeruginosa*. The non-mucoid PAO1 wild type strain was used for base comparisons. FRD1, a mucoid cystic fibrosis pulmonary isolate, was used to assess the influence of alginate production on detachment, and the ?lasI::tet quorum sensing mutant PAO-JP1 was used to assess the influence of cell signaling on detachment. Additionally, we investigated detachment from *Staphylococcus aureus* (a gram-positive non-motile species that grows in clusters) biofilms. Biofilms were grown in glass flow cells for 5 to 11 days under laminar flow. Effluent samples were taken on various days during the course of the experiment; data from day 5 were selected for statistical analysis. The detachment distribution from each of the Pseudomonas strains was similar (PAO1 averaged 3.5 cells/cluster, JP1, 3.1 and FRD, 2.9 respectively). Interestingly, most of the detached particulates from the Pseudomonas biofilms occurred predominantly as single cells (c.a. 75%), ranging from 55% for PAO1 to 90% for FRD1. However, only 20% for PAO1 and 50% for FRD1 of the detached biomass occurred as individual cells. A large proportion of the detached biomass occurred as large aggregates. Alginate production by strain FRD1 did not result in significantly larger aggregates of detached biomass. Detaching particulates from the *S. aureus* biofilm were larger (11.2 cells/cluster) and more evenly distributed among cluster sizes. While the probability of the detachment of large cell clusters appears to be much smaller than for single cells, the impact of the relocation of detached particulates containing large numbers of cells may provide a major health risk.

S02-P287
Effects of initial adhesion events on the physiology of *Pseudomonas aeruginosa*

***Elinor Pulcini**, Anne Camper, Center for Biofilm Engineering at Montana State University—Bozeman, 59717*

Background: Bacteria in biofilms have been shown to be metabolically and physiologically different from planktonic bacteria. Most studies have been conducted on mature biofilms that can be days or more old. Previous experiments suggest that wholesale changes in protein expression do occur during the first few hours of attachment, indicating a general change in physiology. An understanding of the physiologic changes that occur in a bacterial cell during initial biofilm development is crucial for the eventual control of biofilm formation.

Methods: The goal of this research project was to elucidate the changes in physiology and metabolism that occur in *Pseudomonas aeruginosa* during biofilm formation, with particular interest paid to the processes of initial adhesion (T $\geq$3 hours). Chemostat grown cultures were used to inoculate Teflon™ mesh for 1, 2, and 3 hours. In addition, a comparison of differential protein expression during stress conditions and during attachment was made in order to assess the induction of global regulatory mechanisms during biofilm development. Variations in protein expression were visualized using two dimensional (2-D) gel electrophoresis. Proteins were subsequently selected for identification using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

Results: A total of 55 proteins were found to be differentially expressed throughout the 3-hour experimental time period. Eight proteins not visualized in planktonic samples were up-expressed in as little as 10 minutes from time of attachment. Twenty five proteins up-regulated during initial adhesion were identified and were found to be involved in LPS and alginate production, virulence factor expression, and antibiotic resistance. Twelve proteins differentially expressed during stress and attachment were analyzed. Results suggest that various signal transduction pathways are up-regulated.

Conclusion: These variations in protein expression during initial attachment indicate the complexity of biofilm formation. Changes in protein expression occur rapidly and continue during the initial attachment phase of biofilm development.

S02-P288
Field study: Biofilm accumulation in hot tubs

***Darla Goeres**, Linda Loetterle, Marty Hamilton, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

A literature survey showed that hot tub users may be at risk for respiratory, eye, ear, skin, gastrointestinal and urinary tract infections. Although little research exists that directly links biofilm in hot tubs to disease, data suggests that biofilm may serve as a reservoir. To document the presence of biofilm in hot tubs, a field study of four Bozeman-area hot tubs was conducted to test the accumulation of planktonic and biofilm bacteria over a four-week period. Sampling included bulk fluid, coupons attached to the wall, aerosolized bacteria, filters and general water chemistry (pH, temperature, alkalinity, hardness and disinfectant). Coupon biofilm accumulation ranged from 0.65 to 3.19 $\log_{10}$ cfu/cm^2 after 4 weeks. New filters were installed in two tubs and after 4 weeks both had an approximate 2 $\log_{10}$ cfu/cm^2 accumulation of biofilm. Bulk fluid concentrations ranged from non-detectable to 3.85 $\log_{10}$ cfu/mL. These results document the need to include biofilm as part of a standard protocol for testing the efficacy of hot tub disinfectants.

S02-P289
CDC biofilm reactor: Moving research tools into the open market

***Darla Goeres,** Marion Osterud, Wesley Bauman, Marty Hamilton, Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717*

The fields of product claim support and education are in need of biofilm growth methods. Therefore, a niche market for the production and sale of standardized biofilm reactors has emerged. The Montana Board of Research and Commercialization Technology funded a two-year grant to the CBE to develop a standardized biofilm reactor system which the Montana company BioSurface Technologies Corp. (BST) will market. For this development project, the CBE selected the reactor designed by Rod Donlan and Ricardo Murga of the US Centers for Disease Control and Prevention (CDC). The CDC initial data showed that a repeatable biofilm could be grown in this reactor. The CDC Biofilm Reactor also has many promising features including: eight easily removable rods that enable a researcher to remove one rod with out disturbing the rest of the system; 24 coupons which allow for more sampling opportunities; flexibility in the operating conditions (aerobic versus anaerobic, continuous flow versus intermittent flow); baffled stir bar to allow for continuous shear; and control and treated coupons are harvested from the same reactor. The Standardized Biofilm Methods Group at the CBE is now conducting ruggedness testing on the reactor system in two phases using a central composite response surface experimental design.

S02-P290
Use of GFP (Green Fluorescence Protein) to map patterns of growth in *Pseudomonas aeruginosa* biofilms

***Amandine Bugnicourt,** Phil Stewart, Mike Franklin, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The *gfp* gene codes for a fluorescent protein. This widely used reporter system allows identifying a specific gene expression by targeting the *gfp* gene into a DNA sequence of interest since the expression of this gene will be coupled with the expression of the gfp protein. One of its biggest advantages is its non-invasive character in living cells; that is why it is especially used in the CBE to study biofilm development.

The aim of this study was to map the spatial patterns of growth in biofilms. An (IPTG)-inducible GFP construct allowed us to map patterns of growth in biofilms since the active growth zones were the ones where GFP was highly expressed. The organisms chosen for this study were *P. aeruginosa* PAO1 and FRD1. They were grown in colony biofilms.

Colony biofilms were grown under different conditions: 52h with no IPTG, 48h without IPTG and 4h with IPTG(1mM), 52h with IPTG. Colony biofilms frozen sections were examined by epifluorescent microscopy and the following pictures were taken.

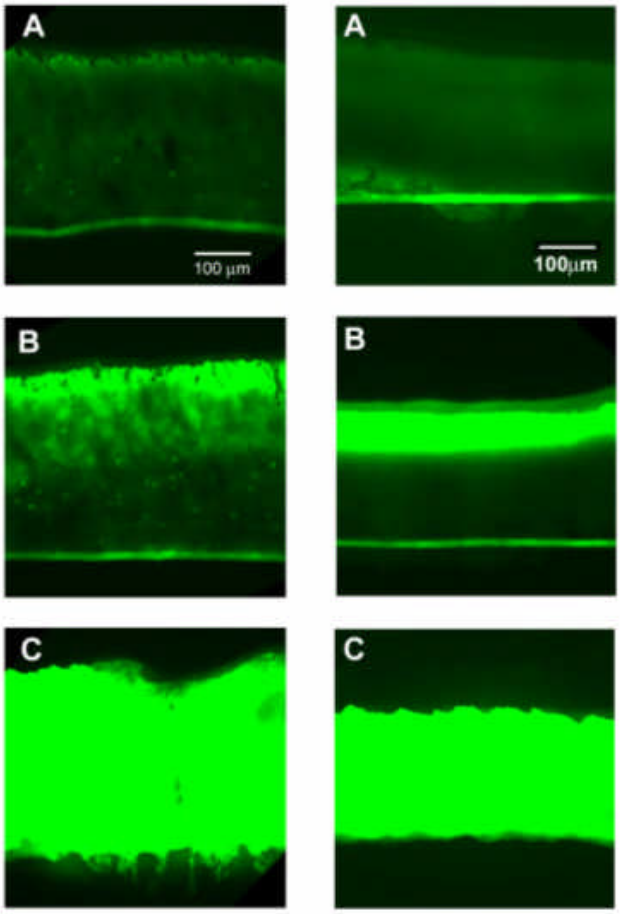


Figure 1. *Visualization of pattern of growth in Pseudomonas aeruginosa FRD1(pAB1) and PAO1(pAB1) frozen sections of colony biofilms.*
FRD1 : A 0h induction PAO1 : A 0h induction
B 4h induction B 4h induction
C 48h induction C 48h induction

As visualized on the Figure 1B, both FRD1(pAB1) and PAO1(pAB1) presented a band of bright fluorescence along the air interface of the biofilm when grown for 48h without IPTG and 4h with IPTG(1mM). When grown for 52h without IPTG (non-induced control), no such band was visible (figure 1A). When grown for 52h with IPTG (positive control), bright fluorescence was present throughout the biofilm (figure 1C).

The pattern of growth in *Pseudomonas aeruginosa* biofilms seems stratified, especially in PAO1, with a region of highly active growth along the air interface followed by a region of slow growth in the interior. The region of active growth is limited to the first (52±5) µm of the colony biofilm for FRD1 and (72±10) µm for PAO1.

S02-P291
Leukocyte interactions with *Staphylococcus epidermidis* biofilms

Jeremy Mitchell, *Mark Shirtliff, Jeff Leid, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

A classical concept of the mechanism behind biofilm resistance to antimicrobial agents has been the idea of limited or decreased penetration of both antibiotics and cells of the human immune system. Recently, we have demonstrated that under conditions that mimic physiological shear found in the blood stream, human leukocytes penetrate and respond to fully mature *S. aureus* biofilms but not to those grown under static conditions. Here, we have continued these studies with a closely related strain, *S. epidermidis.* Interestingly, we observed good leukocyte penetration in statically grown *S. epidermidis* biofilms up to 5 days of growth, at which time there was a dramatic decrease in the ability of leukocytes to penetrate the biofilm. Leukocytes interacting with maturing and fully mature *S. epidermidis* biofilms under shear conditions were also investigated. The differences seen between *S. aureus* and *S. epidermidis* highlight the important differences between biofilms not only of different species but also of species that are closely related and should lead to caution in generalized statements about research results. Our results demonstrate that even in closely related species, the human immune system may respond differently to the respective biofilms.

S02-P292
PNAG localization in biofilms

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Although it is known that poly-N-acetyl glucosamine (PNAG) is required for *Staphylococcus aureus* biofilm growth, its localization within the biofilm is relatively unknown. PNAG is responsible for the cell-cell adhesion and the virulence of *S. aureus* biofilms. Here it is demonstrated that PNAG is concentrated among structures created by the *S. aureus* biofilm during formation, such as the towers and elevations. Additionally, PNAG localization changes during the

maturation of the biofilm. In early and maturing biofilms, PNAG is localized primarily throughout the towers, and in mature biofilms it is predominantly found at the base of the towers. This regulation may play a role in the ability of *S. aureus* biofilms to form 3-dimensional structures.

S02-P293
The role of hydrodynamics in expression of quorum sensing in *P. aeruginosa* biofilm

Laura Purevdorj, *Ph.D. Candidate, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Quorum Sensing (QS) and hydrodynamic conditions have been independently shown to be important in determining the structure of bacterial biofilms. However, hydrodynamics may also influence the concentration of signaling molecules in the biofilm and therefore, also QS mechanisms. The objective of our study was to investigate the role of hydrodynamics in shaping the nature of QS expression during biofilm growth and differentiation. Biofilms were grown from *Pseudomonas aeruginosa* PMH 509 a reporter strain containing a plasmid encoded gene fusion between green fluorescent protein (GFP) and *lasB*. *lasB* is required for the synthesis of elastase, a known virulence factor in *Pseudomonas aeruginosa* and is up-regulated through QS. Biofilms were grown for 5 days in square (1 x1 mm)) glass flow cells at different flow rates (0.01ml/min , 0.1ml/min and 1ml/min). Confocal microscopy was used to determine the onset of GFP expression in the biofilm as well as the pattern and localization of GFP and, therefore, *lasB* expression in the biofilm. Scion image analysis software and bright field mic roscopy were used to measure biofilm development by surface area coverage and biofilm thickness. The effluent concentration of the cell signal oxo-dodecanoyl homoserine lactone (OdDHL) and viable cell count measurements were also monitored.

After 3-4 days of growth the biofilms formed a dense layer of cells with a thickness of 22 ±13 μm interspersed with hemispherical cell clusters 30 ± 8 μm thick. No GFP expression was observed throughout the experimental time period. However, after the flow was turned off GFP expression in these biofilms was evident within 12 ± 4 hrs. For a positive control OdDHL was exogenously added at inducible concentrations, directly into one of the flow cells. Within about 20 minutes GFP expression in the biofilm was observed. These preliminary results suggest that the flow conditions can play an important role in influencing Quorum Sensing mechanisms in *Pseudomonas aeruginosa* biofilms.

S02-P294
Leukocyte response to maturing and fully mature *Staphylococcus aureus* biofilms

Mark Shirtliff*, Anne Camper, Paul Stoodley, Bill Costerton, Jeff Leid , Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Bacterial biofilms are a common cause of persistent disease in humans. They are responsible for a number of illnesses including cystic fibrosis, endocarditis, osteomyelitis, and various nosocomial diseases related to central venous catheters, urinary catheters, prosthetic heart valves, and orthopedic devices. *Staphylococcus aureus* is a common pathogen responsible for nosocomial as well as community infections. This pathogen readily colonizes indwelling catheters and forms microbiotic communities termed biofilms. *S. aureus* bacteria in biofilms are more resistant to clearance from antibiotics and attack from the body's immune system than their respective planktonic counterparts. For years, the mechanism behind biofilm resistance to attack from the immune system's sentinel leukocytes has been conceptualized as a lack in the ability of the leukocytes to penetrate the biofilm. We demonstrate that leukocytes attach to and penetrate a fully mature (7-day-old) *S. aureus* biofilm. We also demonstrate that a maturing (2-day-old) biofilm elicits a cytokine response after 2 hrs post incubation with human leukocytes. Specifically, cytokines IL-1b, IL-12 and IFN-g were induced, suggesting an attempt to mount a Th1-type response. These data represent the first demonstration of leukocytes penetrating a mature biofilm and also represent the first characterization of a cytokine response to a *S. aureus* biofilm.

S02-P295

NH_3 effect on methanogenic activity of a biofilm enriched with methylaminotrophic methanogenic *Archaea*

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Methylamine is used by metanogens but not by sulfate reducing bacteria (SRB) as a carbon source. Methylaminotrophic methane-producing archaea (mMPA) enriched biofilm has been developed to improve the methanogenesis during sulfate enriched effluents treatment in fixed bed biomass reactors. The inhibiting effect of ammonia on planktonic methanogenic archaea has been recognized, but its concrete effect on the activity of these same cells in sessile state (biofilm) has been little studied.
The aim of this work was to study NH_3 concentration effect on the methanogenic activity of an mMPA enriched biofilm. Discontinuous systems (200 ml vials) containing ceramic rings colonized with the experimental mMPA enriched biofilms in presence of 0, 25, 50, 100, 200, 400, or 800 mg NH_3 l^{-1} (initial concentration) was incubated during 10 days at 37 degrees Celsius. The daily produced methane was determined by gas chromatography and the methanogenic activity was measured, calculating the slope between the methane production and time.
The result shows that the greater methanogenic activity reaches 2,543 g QOD methane $g^{-1}VSS^{-1}day^{-1}$ under 25 mg NH_3 l^{-1}, and a significant reduction of methanogenic activity occurs with increasing ammonia concentration. The results suggest that the ammonia effect of the methanogenic activity on the experimental mAPM enriched biofilm is less than that described for the planktonic methanogenic consortia.

S02-P296

NH_3 effect on the growth kinetics of a biofilm enriched with methylaminotrophic methanogenic *Archaea*

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The inhibiting effect of ammonia on activity and growth of planktonic methanogenic archaea has been recognized; however, its concrete effect on the growth of methanogens in sessile state (biofilm) has not been studied. This is particularly true in anaerobic fixed biomass reactors colonized by methylaminotrophic methane producing archaea (mMPA) enriched biofilm that has been developed to improve methanogenesis during sulfate enriched effluents treatment, in fixed bed biomass reactors. The aim of this work was to study NH_3 concentration effect on ceramic colonization by mMPA.

Series of discontinuous systems (50 ml vials) containing from 0 to 800 mg NH_3 l^{-1} were used, that were inoculated under anaerobic conditions with 10^{5-} ^{cell} ml^{-1}, obtained from stock sludge, in the presence of ceramics rings, and incubated to 37 ºC. The biofilm structure was observed using Scanning Electronic Microscopy, and adhered cell counts were done each 48 h. using Epifluorescence microscopy (microbial total count), standard MPN technique and 16S rRNA (dot-blot) determination for general methane producing archaea (gMPA) and mMPA. The biofilm growth speed was calculated using the Gompertz model. The results show that a decrease of the biofilm growth speed occurs while ammonia concentration increases. The greater growth speed under 25 mg NH_3 l^{-1} (1,32 d^{-1} for tMPA and 1,25 d^{-1} for mMPA). Fimbria inhibition is observed under 100 mg NH_3 l^{-1}; a strong decrease of the gMPA growth speed was observed under 400; and under 800 mg NH_3 l^{-1}, deformation of the cells occurs (0,67 d^{-1} for total MPA y 0,5 d^{-1} for mMPA).

S02-P297
Bacterial signaling: Effects of environment on signaling intensity

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Research done at the Center for Biofilm Engineering and other research facilities has indicated a relationship between bacterial signaling and biofilm formation. The specifics of the relationship, however, are not fully understood despite a number of publications on the subject. One of the main difficulties in elucidating the signaling–biofilm relationship is that much of the data in the current literature reflects a number of conflicting ideas and hypotheses. Different researchers are finding very different results.

The hypothesis for this work is that signaling levels are greatly affected by local environments and conditions, and that data discrepancies are due to the different setups of various researchers. To explore this hypothesis, this work examines the signaling levels of *Pseudomonas aeruginosa* in the presence of systematically varying environmental conditions. *Pseudomonas aeruginosa* 509 (provided by Dr. Parsek) is a strain of bacteria with plasmid based *gfp* reporter cloned into *lasB*. This reporter indicates when the genes downstream of the *las* promoter are up-regulated by signaling. The reporter was incubated in a 96-well plate versus varying environments with measurements take every 30 minutes. The kinetic curves from the experiments indicate a 5- to 6-hour lag to fluorescence production, with the fluorescence reaching a plateau between 10 and 14 hours. Data taken from the plateau regions of the curves indicate the highest fluorescence levels (thus highest communication levels) occurred at: High [Mn], [Succinate] from ~2.5 x 10-6 – 2.5 x 10-10 g/L together with [Fe] ~1.0 x 10-5 – 1.0 x 10-7 g/L, and [TSB] of ~3.0 x 10-2 – 3.0 x 10-6 g/L with [Fe] from 1.0 x 10-5 – 1.0 x 10-7 g/L. High fluorescence readings obtained from the well containing high iron concentration may be inaccurate due to precipitation.