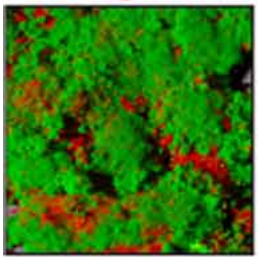
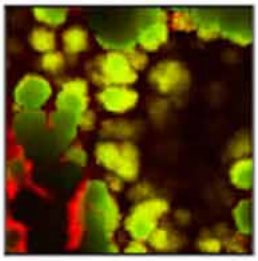


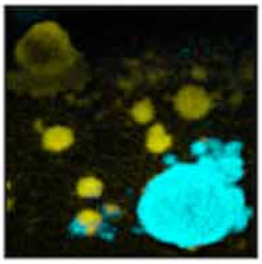
PROCEEDINGS

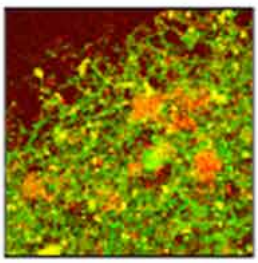
Technical Advisory Conference

July 24-26, 2007 • Bozeman, MT



CENTER FOR BIOFILM ENGINEERING





MONTANA STATE UNIVERSITY

GENERAL INFORMATION

CBE LEADERSHIP

***Phil Stewart**, CBE Director and Professor, Chemical & Biological Engineering*
***Anne Camper**, Associate Dean for Research, College of Engineering and Professor, Civil Engineering*
***Al Cunningham**, Professor, Civil Engineering*
***Brent Peyton,** Associate Professor, Chemical & Biological Engineering*
***Paul Sturman**, CBE Industrial Coordinator*

A BRIEF HISTORY OF THE CBE

The CBE was established in 1990 with 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, Montana State University 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.

The CBE's Technical Advisory Conferences provide exposure to new biofilm information and opportunities for interaction.

The summer 2007 TAC featured a poster session held in the laboratories and hallways of the CBE. Adie Phillips, above, discusses her work with conference attendees.

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.

Education is at the heart of the CBE's success. The CBE's third goal is to sustain productive interdisciplinary undergraduate and graduate education programs involving team research on industrially relevant projects.

Industrial Associates Program Benefits

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

CBE Technical Advisory Conferences

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

Education and Training Workshops

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

Research/Testing Projects

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

Product/IP Development Consulting

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

Regulatory Interactions

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

For More Information

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

Presentation and Poster Abstracts
Technical Advisory Conference: July 24–26, 2007

Session 1: Wound Biofilms

Session 2: Special Presentations

Session 3: Biofilm Behavior and Control

Session 4: Biofilm Methods

Special Presentation

Session 5: Biofilms in Water Venues

Session 6: Microscopy

Session 7: Fungal Biofilms

Special Presentation

Poster Abstracts

SESSION 1: Wound Biofilms

S07-S02
Biology of wound healing

***John Olerud, MD**, Professor, Department of Medicine, Head, Division of Dermatology, University of Washington, Seattle, WA*

Cutaneous wound healing is essential for survival. It occurs through a series of highly conserved and highly coordinated steps which result in hemostasis, host defense from microbial challenges, reestablishing the barrier to fluid loss and eventually restoring much of the strength and functions of the original integument. An overview of normal wound healing will be discussed and illustrative examples of abnormal wound healing will be described. Chronic ulcers and percutaneous devices will be discussed and the role of bacterial infection and biofilm will be considered for these two types of cutaneous wounds.

S07-S03
Advances in wound microbiology

***Phil Bowler**, Director, Anti-Infectives & Microbiology, Wound Therapeutics Global Development Centre, ConvaTec, Flintshire, UK*

It is a given that micro-organisms, particularly bacteria, are responsible for wound infection and consequently the pathogens involved will delay healing. However, most wounds are not infected, yet they don't necessarily heal and are colonised by a wide variety of both aerobic and anaerobic micro-organisms that co-exist as polymicrobial populations (1). Consequently, the role of micro-organisms in non-healing wounds that do not display clinical signs of infection is still a matter of debate. Many experts have suggested that the microbial load (bioburden) in a wound is critical in determining whether or not healing will occur. Others have not been able to demonstrate a correlation between bioburden and wound healing (2).

From a qualitative perspective, it is widely perceived that specific pathogens, notably *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*, are responsible for delayed wound healing. However, it is important to consider the fact that wound colonisation occurs primarily from endogenous sources such as the gut, the mouth and the genito-urinary tract, where anaerobic bacteria outnumber aerobic bacteria by as much as 1000:1. Consequently, the likelihood of wounds being colonised by anaerobic bacteria is high, and when investigated in detail, anaerobic bacteria have been shown to constitute approximately 33% of the total microbial population in non-clinically infected wounds; this figure is higher in infected wounds (3). This indicates that micro-organisms usually exist as mixed aerobic-anaerobic polymicrobial populations in wounds and form a unique collection of possible pathogens that are a potential cause of wound infection and delayed healing.

The role of micro-organisms in wounds should therefore be viewed from the perspective of polymicrobial populations, rather than addressing the effects of one specific potential pathogen. Bacteria in mixed populations are known to be capable of interacting (e.g., synergy & quorum sensing) to induce effects that may be more detrimental to a wound. Bacterial synergy has been observed in an infected leg ulcer and involved *S. aureus* promoting the growth and pathogenicity of a pigmenting anaerobic bacterium (1).

In recent years, it has also become evident that chronic wound bacteria exist within a biofilm micro-environment that significantly increases their chance of survival within a potentially hostile environment. (4). Biofilm exopolymeric matrix provides increased protection to bacteria against the host immune system and antimicrobial agents and also provides an optimal environment for communication and synergistic interactions between populations of bacteria.

It is therefore probable that the bioburden, biofilm, and the types of pathogens and interactions between wound micro-organisms are factors which, in combination, are likely to contribute to delayed healing. It should be emphasised that there are many more host-associated factors that may affect healing (e.g., impaired immune response, age, vascular disease) and no single factor should be considered in isolation. However, from a microbiological perspective, it is important to note that topical antimicrobial formulations such as those containing iodine and silver have been shown to stimulate healing in non-infected, indolent wounds, indicating that colonisation (i.e., without clinical signs of infection) may delay healing in certain situations. From a wound management perspective, a key objective is to maintain a situation where the host is in control of the wound microflora. Any disruption to this balance may warrant additional therapies to supplement the host response.

(1) Bowler PG, Duerden BI, Armstrong DG. Wound Microbiology and Associated Approaches to Wound Management. *Clin. Micro. Rev*. 2001, **14**:244-269.
(2) Bowler PG. The 10^5 Bacterial Growth Guideline: Reassessing its Clinical Relevance in Wound Healing. *Ostomy Wound Management*. 2003, **49**:44-53.
(3) Bowler PG, Davies BJ. The Microbiology of Infected and Non-infected Leg Ulcers. *Int. J.* 1999, **38**:101-106.
(4) Percival SL, Bowler PG. Biofilms and Their Potential Role in Wound Healing, *WOUNDS*. 2004. **16**:234-240.

S07-S04
Overview of the Center for Wound Biofilm Research

***Phil Stewart**, Director, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Researchers at the CBE have embarked on a quest to find new ways to heal chronic wounds, thanks to a $2.9 million grant awarded in the fall of 2006 by the National Institutes of Health. The four-year project is a partnership between the CBE, the Division of Dermatology at the University of Washington, and the Southwest Regional Wound Care Center in Lubbock, Texas. Chronic wounds share characteristics with known biofilm infections and this observation led to the hypothesis that bacterial biofilm forms in chronic wounds and poses a major barrier to wound healing. Specific aims of the project are: 1) to demonstrate the presence of microbial biofilms in chronic wounds and characterize these communities, 2) to develop and characterize in vitro and in vivo biofilm wound infection models, and 3) to evaluate alternative strategies for managing chronic wound infections based on targeting the biofilm. This project has a practical drive toward new technologies for controlling biofilms in the context of wound care and the project team seeks to work with industry in pursuing Aim 3 in particular.

S07-S05
In vitro models of wound biofilms

***Garth James**, Medical Projects Manager, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Chronic wounds are a global healthcare problem and a major source of morbidity, mortality and healthcare expenditure. Biofilms have been implicated in delayed wound healing and biofilm-targeted therapies may provide a new approach for treating chronic wounds. The Center for Biofilm Engineering (CBE)—aided by collaborators at the Southwest Regional Wound Care Center and University of Washington (UW)—is developing models to evaluate biofilm-targeted therapies for treating wounds. The models include in vitro models with biofilms formed by bacteria isolated from chronic wounds, as well as biofilm/cell culture models. These test systems will enable the evaluation of candidate treatments prior to more extensive and expensive pre-clinical testing.

Wounds present a complex environment with a variety of attachment surfaces for biofilm growth, including necrotic tissue as well as matrix proteins, such as fibrin and collagen. Blood and exudate bathe the wound, bringing cells involved in the healing and immune responses. The microbial flora of wounds is also complex and a wide variety of bacteria have been identified from acute wounds as well as chronic wounds that may or may not show clinical signs of infection. Bowler *et al*. have stressed the potential importance of polymicrobial microbial communities, particularly as biofilms, in delayed wound healing. Our analysis of approximately 17 human wound specimens has revealed 16 different species of bacteria including 6 species of strictly anaerobic bacteria. The most prevalent bacteria were species of *Staphylococcus* and *Pseudomonas*. The majority of wounds contained multiple species of bacteria.

In vitro models provide a less complex environment to study the impact of new therapies on wound biofilms. The CBE has developed biofilm-only models utilizing two previously established models for evaluating antimicrobial efficacy against biofilms, the colony model (CM) the drip flow reactor (DFR), as well as a combined CM-DFR model. Bacterial species have included wound

isolates of *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*. The results have indicated that all of these species formed repeatable biofilms in the model systems. In addition, repeatable mixed-species biofilms of *S. aureus* and *E. faecalis* as well as *P. aeruginosa* and *E. faecali* have been developed. The response of these models to traditional and novel wound therapies is currently being evaluated.

A biofilm/cell culture model is also being developed. This model is based on the "scratch wound" model of keratinocyte behavior. Human kerotinocytes, provided by UW, were used to establish scratch wounds with a healing time of approximately 72 hours. We are currently evaluating the impact of biofilms on closure of these "wounds." Combined biofilm-cell culture and biofilm-tissue culture models will allow the investigation of treatment effects on both bacterial and host cells.

In addition to incorporating host cells, we are striving to develop biofilm models composed of polymicrobial communities that include strictly anaerobic bacteria isolated from chronic wounds. The further development of in vitro test systems promises to provide a relatively inexpensive test-bed for biofilm-targeted wound therapies.

S07-S06
Preclinical modeling: Studies on wound healing

***Stephen C. Davis**, Associate Professor, University of Miami Miller School of Medicine, Department of Dermatology & Cutaneous Surgery*

The purpose of this presentation is to discuss in vivo wound healing and infection studies using various porcine models. Pigs are used for our research model because their skin is similar to that of humans. Over the past 30 years, these models have been used to determine the efficacy of various dressings, devices and topical therapies. Our models include partial- and full-thickness wounds, second-degree burns, ischemic wounds, laser injuries, bacterial proliferation and barrier evaluations. The role of moist wound healing and its influence on bacteria will be discussed. Some of the studies that will be briefly reviewed are electrical stimulation, liquid bandage and the effect of topical antimicrobials.

S07-S07
Biofilm models in acute wounds

***Patricia M. Mertz**, Research Professor, University of Miami, Miller School of Medicine, Department of Dermatology & Cutaneous Surgery, Miami Dermatology Research Institute, LLC*

Objective: In this presentation a biofilm infection model using acute porcine wounds will be discussed. The model that our laboratory developed can separate both planktonic and biofilm phenotypes from bacterial wound infections. We present three studies: one which examines the effect of two topical antimicrobial agents against *Staphylococcus aureus* (SA), one that adds an enzyme before treatment, and one that evaluates silver-containing dressings against *Pseudomonas aeruginosa* (PA). We also discuss the effects of pre-treatment with enzymes and, with a "look to the future," suggest that the role of a dispersing factor and a bacteriophage engineered as enzymatic producer may aid in antimicrobial effectiveness.

Background: Antimicrobial therapies are traditionally evaluated with *in-vitro* assays, which usually demonstrate the antimicrobial efficacy against free-floating bacteria. Our in-vivo porcine wound model allows the study of both planktonic and biofilm-associated bacteria.

Experimental Design: A total of nine animals were used—six for partial thickness wounds and three for second- degree burns. We established two bacterial groups: one side of the animal had wounds that were inoculated and treated within 20 minutes (planktonic bacteria) and the other half of the wounds were allowed to develop a 48 hr biofilm under a film dressing before treatment. Wounds were divided into the following treatment groups: for *S. aureus*, inoculated partial thickness wounds received either 1) triple antibiotic ointment, 2) mupirocin cream or 3) untreated control. Wounds were treated twice daily; wounds were cultured for bacterial quantitation at 24, 48, 72, 96, and 120 hrs. Three other animals were inoculated with *S. aureus* and covered with film dressings for 48 hrs to create biofilms that were then treated with either 1) Pectinex Ultra SPL supplied by Novozymes BioTec, Inc., 2) mupirocin, 3) mupirocin & water vehicle, 4) Pectinex solution or 5) no treatment. *Pseudomonas aeruginosa*-treated burn wounds were treated with either 1) Nanocrystalline Silver, 2) Hydrocolloid Silver or 3) untreated and cultured at 24, 48, and 72 hours post treatment.

Results: For the *S. aureus* study we observed at least a 5.5 log CFU/ml reduction in planktonic *S. aureus* with topical treatments and complete eradication by 48 hours. In contrast, it took about five days to observe a 2 log CFU/ml reduction in the biofilm *S. aureus*. The addition of an enzyme (Pectinex) increased the efficacy of topical mupirocin. With the *P. aeruginosa* study, the hydrocolloid dressing significantly reduced planktonic bacteria counts as compared to untreated and nanocrystalline silver dressing (only by ~2 Log CFU/ml). However, biofilm bacteria counts for both dressings were similar to untreated control at all sample points.

Conclusion: Our studies demonstrate that when bacterial biofilms are established in wounds there is a longer response time for topical antimicrobial activity, suggesting bacterial resistance and the possibility that enzyme treatment can alter that response time.

S07-S08
Biofilm based wound care

***Randy Wolcott, MD**, Physician, Southwest Regional Wound Care Center*

Biofilm is an immense medical problem. Medical biofilm affects over ten million patients each year—costing billions of dollars—and the problem continues to rise at a rapid rate. This makes medical biofilm one of the most important medical problems facing the world today, rivaling cancer, heart disease, and diabetes. All chronic wounds have a similar biochemistry, which can only be explained by a shared pathology. Biofilm on the surface of the wound is the best explanation for excessive neutrophils, elevated proinflammatory cytokines, elevated MMPs, reduced TIMPs, and degraded growth factors common to all wounds. A biofilm model is the best explanation of why wounds demonstrate resistance to antibiotics, resistance to nonselective biocides, resistance to host defenses, variable culture results, and many other unexplained clinical phenomena. James *et al*. has demonstrated that biofilm is predominant in chronic wounds yet rarely seen in acute wounds, which has led to a wound biofilm model. Even though biofilm is present on the wound's surface, more evidence has been needed to demonstrate its detrimental impact on healing. By targeting the biofilm on the surface of chronic wounds, a statistically significant improvement in clinical outcomes has been demonstrated. This finding suggests that wound biofilm is indeed an important barrier to wound healing.

SESSION 2: Special Presentations

S07-S09
State of the CBE

***Phil Stewart**, Director, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Abstract not available.

S07-S10
Regulatory methods: Recommendations for a suitable method for biofilm disinfectants

***Stephen Tomasino**, Senior Scientist, US EPA-OPP Microbiology Laboratory, Environmental Science Center*

Abstract not available.

S07-S11
Caserna, nanowires, signal vesicles, and flying buttresses: Baroque architecture in biofilms

***Bill Costerton**, Director, Center for Biofilms, School of Dentistry, University of Southern California, Los Angeles, CA*

We will report on a program of research that has, as its central premise, the concept that prokaryotic cells are capable of building and using structures whose complexity and sophistication have heretofore been thought to have limited them to eukaryotic organisms. When bacteria are cultivated in multi-cellular communities, which are their actual mode-of-growth in virtually all natural and pathogenic ecosystems, we see structures and processes that have never been seen in liquid cultures of laboratory-adapted strains.

Bacteria that accomplish remarkable social feats, like coordinated swarming through soil and complex fruiting body formation, have been shown to communicate by means of signals that are enclosed in membrane vesicles. This mechanism allows the signals to be "addressed," by means of specific proteins in the membrane vesicles and the delivery of the signal can be delayed until it is "opened" by the recipient. The electrically conductive nanowires that have been discovered to conduct electrical "power" in *Shewanella* communities, have now been seen to connect bacterial cells in many other communities, including multi-species biofilms.

These nanowires are composed of pilin surrounded by spirally arranged cytochrome molecules, and we suggest that they may function in cell-cell communication, as well as in the distribution of metabolically generated energy throughout the community.

For more than three decades we have gradually realized that bacteria can form communities of remarkable complexity and the basic "mushroom, tower, and water channel" architecture has been described in pure culture and mixed-species natural biofilms. More recently large amounts of DNA have been found in the matrix in which biofilm bacteria are enclosed and specific instances of controlled cell lysis have been shown to be pivotal in the development of complex community structures like fruiting bodies and the tent-like communities formed by some marine organisms. But the most sophisticated community structures are probably the caserna formed by cells of certain strains of *Staphylococcus epidermidis*, by the controlled coalescence of the cellular contents of individual cells to form the walls and partitions that eventually fuse into macroscopic "honeycomb" structures. The control of this fusion process, by a prokaryotic genome of unremarkable proportions, may form the basis of new examinations of the control of community architecture by specific gene products.

S07-S12
Industry Presentation
Biocide industry: Past, present, and future challenges for biocontrol

***Michael V. Enzien**, Senior Research & Development Specialist, DOW Biocides, The DOW Chemical Company, Buffalo Grove, IL 60089*

Biocides have been used for control of microorganisms, knowingly or unknowingly, for over two thousand years. Many ancient applications of biocides for product preservation, wound care, and odor control are still practiced today. Three major changes have influenced the modern era of biocides: 1) synthetic chemistry spawned from the chemical refining industry, 2) EPA formation, and 3) European Biological Products Directive (BPD). Synthetic chemistry generated many new organic biocide molecules with specific and known chemical modes of action. The formation of the EPA, and subsequently the Federal Insecticide Fungicide Rodenticide Agency (FIFRA), placed limits on use, disposal, and toxicology of industrial biocides. Elimination of certain very useful but extremely toxic biocides was enforced by the EPA, ex. chromates and most heavy metals. The strict and extensive environmental and toxicology data required by the BPD will greatly limit the number of biocides that will be used in the European Union countries. The large cost to register new biocide actives and the relatively small market sizes will greatly limit the number of new biocides registered and developed in the future. The challenge for microbiologists will be to use the remaining biocide actives and combination of actives to meet the many challenging applications for microbial control. Examples of these applications will be discussed, amongst which biofilm control is a leading unmet need.

SESSION 3: Biofilm Behavior & Control

S07-S13
Examination of $scCO_2$ effects against *Bacillus mojavensis* biofilms

***Adie Phillips**, Research Engineer, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Supercritical CO_2 ($scCO_2$) has been shown to act as a disinfectant against microorganisms. While $scCO_2$ has been tested against vegetative cells and bacterial spores, no information seems to be available regarding the effect of $scCO_2$ on biofilms (Zhang *et al.*, 2006). We grew *Bacillus mojavensis* biofilms for 6 days on 1–2 mm screened sand support matrix. Three biofilm replicates and three suspended growth replicates were exposed to $scCO_2$ in a SFX 220 Supercritical Fluid Extractor. The treatment involved immediate pressurization to 2000 psi (138 bar), allowing the $scCO_2$ to equilibrate in the treatment chamber for 1 minute, and 19 minutes of $scCO_2$ flow at a flowrate of ~1 mL/min. The temperature of the extractor and restrictor was set to 35° C. After 20 minutes, a step-wise depressurization process occurred. Every 15 seconds the pressure was dropped 250 psi until 1000 psi, at which point the remaining 1000 psi was allowed to slowly vent over 4–5 minutes. Control samples were exposed to 35° C modified PBS solution flowing at 1 ml/min for 20 minutes. Samples were analyzed using direct counts and plate counts. Suspended growth samples revealed a 3-log reduction while biofilms showed a 1-log reduction in colony forming units (CFU). Direct count observations showed no statistically significant difference between control and $scCO_2$ treated samples.

S07-S14

Influence of EPS and natural organic matter on biofouling of microfiltration membranes coupled with powdered activated carbon

Mohiuddin Md. Taimur Khan, *Assistant Research Professor, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Larger pore membranes, including ultrafiltration (UF) and microfiltration (MF), are utilized for the separation of macromolecules and discrete particles from water. Activated carbon has been used increasingly as a polishing step for taste and odor compounds, for disinfection byproduct precursors, and as a support for biomass in the removal of biodegradable organic material. In water with a high organic load fouling of these membranes can occur, necessitating cleaning or even replacement of the membrane. Pretreatment of feed waters with activated carbon can reduce this load, but often results in higher microorganism counts in the feed to the high-pressure system with little net improvement in performance. Conversely, pretreatment coupled with UF or MF can reduce the microorganism load, but may not significantly impact the organic load. Activated carbon beds have also been used to remove organic and inorganic impurities from product streams in the food, pharmaceutical and petrochemical industries. Powdered activated carbon (PAC) can be combined with MF or UF to remove organic compounds as well as microorganisms. Previous researchers have found that natural organic matter (NOM) adsorbs both inside pores and on the membrane surface and forms a gel layer. The membrane surface properties, the type of membrane process and resultant NOM rejection, the nature of the NOM, and water chemistry all influence fouling behavior. There can also be biofouling. The biofouling process initially starts with the deposition of substrates such as extracellular polymeric substances (EPS) which form a highly hydrated nanogel layer on the membrane surface. EPS, also referred to as soluble microbial products (SMP) in the literature, are large molecular weight compounds that are excreted by bacteria. These compounds play a significant role in bacterial adhesion onto solid surfaces by altering the physicochemical characteristics (charge, hydrophobicity, etc.).

Bench-scale experiments on PAC-MF system were carried out using settled surface water (Tama River, Tokyo, Japan) either before or after treatment by a biofilter system during the rainy season in Japan. Each reactor consisted of a hollow-fiber MF membrane module operated in suction mode. The membrane module was submersed in a reactor made of 5 mm polyvinyl chloride plates with an effective volume of 5 liters. During this study, reactors 1, 2, and 3 received settled river water, while reactors 4 and 5 received effluent from the biofilter system. The biofilter consisted of a column packed with polyethylene cylinders. Reactors 2 through 5 were initially dosed with 40 g/L of PAC and all reactors were operated for 45 days at different residence times and aeration was used to disturb fouling. All systems were equipped with a backwash mechanism, providing two minutes of backwash after every 20 minutes of filtration. During this research we compared the amount of five different carbohydrates: (D(+) glucose and D(+) mannose, D(+) galactose, N-acetyl-D-galactosamine and D-galactose, oligosaccharides and L(-) fucose), proteins, and polysaccharides—which are the major components of EPS—among foulants of different reactors. At lower HRT, the amount of different types of carbohydrates on the membranes is higher even in the presence of PAC but the reactors containing PAC gave the longest operation. The amount of protein inside the foulants on different membranes was eight- to ten-times higher than the source waters. However, the amount of polysaccharide was lower inside the foulants compared to the source waters, which indicates that protein fouling is more prominent than polysaccharide fouling during the treatment of this surface water. Similar results were also observed regarding the influence of HRT, presence of PAC and use of biofilter-treated water.

The use of PAC in a membrane reactor (reactor 3) fed river water operated at half the loading rate and resulted in the same level of fouling (four cycles of cleaning in a set time period) as a parallel reactor without PAC (reactor 1). However, the addition of biofilter-treated water reduced the membrane fouling potentials. The distribution of organic matter was determined in both foulants and also bulk phase samples. Excitation-emission-matrix (EEM) is one of the fastest methods to measure the type of organic matter, and even though it is not quantitative it provided interesting results about the nature of the fouling. The technique relies on the identification of certain peaks based on their specific location. All had a protein-like (Peak 1), marine fulvic/humic-like (Peak 5) and fulvic/humic-like matter (Peak 6), but Peak 7 (STP effluent-like) was absent in all cases.

S07-S15
Investigation of dormant cells in biofilms

***Brenda Grau**, Postdoctoral Researcher, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

It was hypothesized that *Pseudomonas aeruginosa* biofilms are protected from killing by antimicrobials due to the presence of dormant cells within mature biofilms. A *P. aeruginosa* strain containing a stable, inducible gene for green fluorescent protein (GFP) was used to visualize and characterize the metabolically dormant and active cell populations within the biofilm. In up-shift experiments, cells were labeled by introduction of the inducing agent to mature biofilms. Only about 30% of the cells within the entire biofilm turned bright, thus labeling the active cells with GFP. Colony biofilms were serially disaggregated by mechanically removing successive layers. Each layer was analyzed by flow cytometry (FCM), showing it was possible to separate and collect populations based on GFP expression; cells expressing GFP were characterized as bright (GFP+) while the non-GFP expressing cells were characterized as dark (GFP-). Based on GFP expression, the bright cells were considered to be metabolically active while the dark cells were considered to be dormant with respect to protein synthesis. Using this novel approach of labeling cells in intact biofilms by their metabolic state and physically separating the metabolically active, top layer of the colony biofilm from the dormant bottom-most layer, we harvested cells to investigate genome-wide differences in gene expression between the two populations. In up-shifted colony biofilm fractions analyzed by FCM, top layers that showed > 90% GFP+ events and bottom-most layers that showed > 80% GFP- events were retained for RNA extraction and microarray analysis. Using Affymetrix *Pseudomonas aeruginosa* Gene Chip arrays, we have identified several genes that are differentially expressed relative to cell location in the biofilm. We are currently analyzing these genes for any potential protective mechanisms that may be conferred on the cells expressing these genes.

SESSION 4: Biofilm Methods

S07-S16
Methods of assessing efficacy of antimicrobial surfaces: A review

***Diane K. Walker**, Research Engineer, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Industry has started to incorporate surface-bound antimicrobials into many products. Some of the more common examples are found in the textile industry, paints and coatings, treated filters, plastics that are used in cutting boards, counter tops and toys, and in both temporary and permanent medical devices. Although manufacturers would like to make a biofilm claim for their products, currently there is no standardized approach for determining the efficacy of surface-bound antimicrobials against biofilm bacteria. The result is that either a company is using a method developed for planktonic organisms (both dried and hydrated) and extrapolating the results to biofilm claims, or that each company is developing their own method, creating tremendous pressure on the regulatory agencies to determine the validity of the proposed method.

The CBE is proposing the development of a method specifically for testing the efficacy of surface-bound antimicrobials against biofilm bacteria. The method will be generic enough in nature that it will be useful for both EPA and FDA applications. The process will start by reviewing the literature for the methods that are currently in use. This presentation will cover that review. From there, the committee can discuss some of the key attributes they believe the method must possess. The goal would be to take the suggestions and perform some preliminary work on refining a method. Results from this process will be presented at the next Technical Advisory Conference in February 2008.

S07-S17
Transcriptomes and proteomes: Identification of physiological constraints related to biofilm growth

***Matthew W. Fields**, Assistant Professor, Microbiology and Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Virtual Institute for Microbial Stress and Survival (http://vimss.lbl.gov/)

There is an on-going debate that biofilm formation is a developmental process of programmed changes in gene expression that result in a unique physiological state, and recent studies have demonstrated that biofilms can be more metabolically active than previously thought. Sulfate-reducing bacterial biofilms are an interesting model to study due to both potentially advantageous (e.g., heavy metal reduction) and deleterious (e.g., metal corrosion) roles in the environment, but little is known about the cellular events that lead up to biofilm formation and development, including the cellular material and gene products used to promote and maintain cell adherence under sulfate-reducing conditions.

Biofilm formation was observed on glass slides submerged in a CDC reactor (BioSurface Tech.) that contained a defined medium and a dilution rate of approximately 0.09 h^{-1}; transcriptomes and proteomes were compared between planktonic growth phases and a mature biofilm. The transcriptomes from multiple growth phases were also analyzed via hierarchal cluster and principal components analysis. The different growth states altered gene expression in amino acid transport, cell envelope biogenesis, defense, inorganic transport, intracellular trafficking, lipid metabolism, secondary metabolites and translation. However, the individual genes that had altered expression within these COGs (Clusters of Orthologous Groups) were different between the various growth modes. The biofilms did not cluster with planktonic growth-phases and represented cells with a distinct expression profile. In particular, genes that encoded putative proteins for the pyruvate-acetyl-CoA-formate node showed altered expression relative to exponential- and stationary-phase cells, and proteomic data at this node also showed altered expression profiles. In relation to the extracellular matrix, physiological and biochemical methods showed that the *D. vulgaris* biofilm did not contain significant levels of an extracellular polysaccharide, but was dependent upon protein filaments similar to a flagellum. Transcriptomic and mutational analyses corroborated these observations.

The results indicated that *D. vulgaris* biofilms were a unique physiological state and not simply a consequence of slower-growing, aggregated cells. The results suggested that carbon and energy flow were altered to achieve balanced nutrient acquisition in combination with slower rates of biomass production. Future work is needed to discern protein interactions that control metabolic flux through particular nodes in order to elucidate the relationships between genotype and phenotype in the context of distinct cellular states. While the expression of other systems is certainly involved in biofilm physiology, the presented results suggested that biofilm cells had distinct energy flow different from exponential and stationary phases.
An improved understanding of the genetic control for anaerobic biofilms will have implications for biotechnology, the environment and human health.

S07-S18
Special Presentation
Predatory prokaryotes: Thinking outside the bug

***Daniel Kadouri**, Department of Oral Biology and the Center for Oral Infectious Diseases, University of Medicine and Dentistry of New Jersey, Newark, NJ*

One of the major difficulties in controlling surface-attached bacteria is their enhanced resistance to antimicrobial agents—biofilms can be up to one thousand times more resistant to antimicrobial agents than their planktonic counterparts. The problem of enhanced biofilm resistance has led researchers to examine other methods of biofilm control. Among these alternative techniques is the use of biological control agents such as protozoa and bacteriophages. In our work we are studying the unique biology of predatory prokaryotes from the genera *Bdellovibrio* and *Micavibrio* and evaluating their ability to destroy pathogenic biofilms. *Bdellovibrio* and *Micavibrio* are both gram-negative bacteria. However, unlike most bacteria, these organisms are obligatory parasites that can survive only by feeding on other gram-negative bacteria.

Bdellovibrio was first isolated from soil in the early 1960s. In order to find its host *Bdellovibrio* swims extremely rapidly in its environment. After colliding with its prey *Bdellovibrio* penetrates the prey's periplasm and starts multiplying in the periplasmic space, feeding on its host from within. Subsequently, it bursts the cell envelope and starts a new cycle of attack. We demonstrated that *B. bacteriovorus* had the ability to reduce an existing *E. coli* biofilm by

more than 99% in just a few hours. Since *Bdellovibrio* multiplies rapidly within its host, even low numbers of *Bdellovibrio* (1-10 cells/ml) are sufficient to initiate an attack. To mimic the development of more naturally occurring biofilms, we have grown *E. coli* and *P. fluorescens* biofilms under high flow conditions. *B. bacteriovorus* was able to successfully attack the thicker biofilms grown in flow cell systems, suggesting that the action of this predator is not restricted to the surface of the biofilm. Furthermore, our data suggest that the predator not only can survive in the biofilm but also can feed, proliferate, and escape in order to start a new cycle of predation.

Another predator we are currently studying is *Micavibrio*. Unlike *Bdellovibrio*, which feeds on its prey as an endoparasite, *Micavibrio* exhibits a "vampire" like lifestyle leeching to its host as it feeds. We have found that, like *Bdellovibrio*, *Micavibrio* has an ability to attack and destroy existing biofilms of major human pathogens such as *P. aeruginosa*, *B. cepacia,* and *K. pneumoniae,* including numerous clinical isolates from these species. A reduction in biofilm biomass was observed as early as three hours after exposure to the predator and an approximately 2-log reduction in biofilm cell viability was detected following a 24 hr exposure period. In flow cell experiments *M. aeruginosavorus* was able to modify the overall *P. aeruginosa* biofilm structure while decreasing the viability of *P. aeruginosa* cells. When live *M. aeruginosavorus* was added to planktonic *P. aeruginosa* in rich medium, an increase in biofilm formation was observed at early time points. Further investigation suggested that the increase in biofilm formation was likely caused by an increase in cell-cell interaction brought about by the presence of the predator or active predation.

With the increasing interest in developing improved methods for controlling biofilms, there are potential advantages to using predatory prokaryotes for biological control. For example, they are highly specific for infecting bacteria and thus are harmless to nonbacterial organisms. The initial dose of the predator can be low, since this organism multiplies rapidly as it feeds, and as we have observed, the predator's population is maintained in the biofilm even though a majority of the host bacteria have been destroyed. *Bdellovibrio* and *Micavibrio* are also effective against bacteria that have multiple resistances to antibiotics, which is the situation in many biofilm settings. Finally, a key difficulty encountered in the use of biological control agents in reducing biofilm population is the inability of the agent to access the cells within the biofilm.

Our data suggests that both *Bdellovibrio* and *Micavibrio* have the capability to access extremely thick biofilms and are not restricted to the surface of the biofilms.

SESSION 5: Biofilms in Water Venues

S07-S20
Disease outbreaks in recreational water

***Michael J. Beach**, Acting Associate Director for Healthy Water, National Center for Zoonotic, Vector-Borne and Enteric Diseases, Centers for Disease Control and Prevention*

Abstract not available.

S07-S21
Drinking water issues in developing countries

***Tim Ford**, Department Head, Microbiology, Montana State University, Bozeman, MT*

A recent report in the *Lancet* demonstrates that diarrheal diseases continue to plague the developing world and remain a major cause of mortality, particularly in children under five years of age.[1] The global burden of diarrheal disease is huge and much can be attributed to contaminated water. However, we can only begin to guess at the true mortality and morbidity rates, as "official data" underestimates burden of diseases by several hundred-fold. Questionnaire-based surveys are one mechanism to begin to look at the full burden of disease—an approach we have taken in both India and Russia. This talk will touch on previous work conducted in Russia and describe our current approach to examine mortality and morbidity from cholera and other enteric diseases in communities associated with the Ganges River in India.

Drinking water issues are complex, but a clearly emerging area of interest to the public health community is the risk of future disease outbreaks, epidemics, and pandemics. The focus of this talk is on developing countries, but it must be remembered that a newly emergent pathogen in Southeast Asia can reach the US in twenty-four hours or less. The major question then becomes: how do we predict future waterborne (and other) disease risks? Cholera was unanticipated in South America in 1991, and a new strain of cholera with pandemic potential

(*V. cholerae* 0139) was unanticipated in Northern India in 1992. With changing climate and other changing environmental variables, we need a better way to predict and potentially mitigate future disease events.

The final part of this presentation will discuss the importance of a better understanding of pathogen ecology, including the biofilm mode of growth coupled with host ecology and susceptibility, and use of remote sensing technologies to provide better prediction of future disease events (shown schematically in Figure 1).

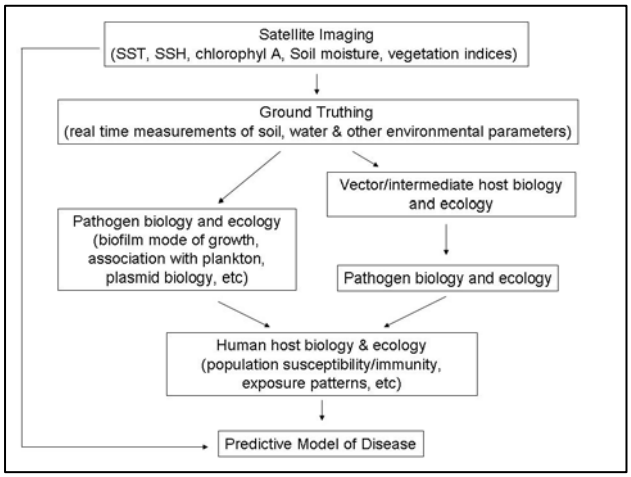


Figure 1.

S07-S22
Drinking water treatment strategies and regulations

***Anne Camper**, Professor of Civil Engineering, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Around the world sources for drinking water are becoming more limited in quantity and are declining in quality. Coupled with increased knowledge of potential health effects from contaminants in water, there is greater demand for more sophisticated water treatment technologies. These technologies are also being driven by economics and the regulations that set the requirements for finished water quality. In addition to high quality treatment, there is increasing need to protect this water as it travels from the treatment plant through complex distribution systems to the consumer.

Trends in drinking water treatment—including the use of alternative disinfectants, granular filtration procedures, and the use of membranes—will be discussed. The focus will be on how microorganisms are involved in the treatment and distribution of drinking water, including the beneficial and detrimental impacts of biofilms. Topics will include nitrification, degradation of organic matter and disinfectant by-products, association with corrosion, biofouling of surfaces, and pathogen/indicator organism persistence. There will also be an overview on how maintenance of distribution system integrity is critical for preserving water quality. All of this will be couched in terms of relevant regulations, including the Lead and Copper Rule, potential revisions of the Total Coliform Rule, the Disinfection/Disinfectant By-Product Rule(s), and the possibility for a distribution system rule/regulation.

S07-S23
Biofilms in recreational water

***Darla Goeres**, Senior Research Engineer, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

In treated recreational water venues such as swimming pools, hot tubs, spas, water parks, and splash pads, the water quality is typically maintained through filtration and the addition of chemicals to balance and disinfect the water. Similar to drinking water standards, it was never intended that treated recreational water be completely free of microorganisms, but, rather, the goal was to control the level of microbial contamination to lower the risk of infection for an immunocompetent adult. Because of the potential risk to human health, in the United States the Environmental Protection Agency (EPA) is responsible for registering swimming pool and hot tub disinfectants used to control microbial contamination in the water. The agency requires that the disinfectant pass a suspension test against both *Escherichia coli* and *Streptococcus faecalis,* as well as a field test. Testing the efficacy of the disinfectant against biofilm is not part of the current registration requirements. Although chemical hot tub disinfectants are regulated at the federal level, compliance rules for bacterial contamination in recreational water and enforcement of those rules is the responsibility of state and local health departments. Currently states are not required to coordinate their activities in this area. Therefore, there are three different entities governing treated recreational water venue design, use and water quality: the recreational water industry itself, a federal regulatory agency, and state and local health departments. In addition, a fourth and separate entity, the Centers for Disease Control and Prevention (CDC), is responsible for tracking all

waterborne disease outbreaks associated with recreational water use. This presentation will discuss the importance of considering biofilm growth in recreational water from the perspective of human health concerns, new treatment strategies and the need for changes in the current set of regulations governing this industry.

SESSION 6: Microscopy

S07-S25
Examination of tissue specimens for biofilms

***Alessandra Agostinho**, Research Associate, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Biofilms are now known to be the cause of many diseases—and are the reason that many infections are so difficult to treat. Positive diagnosis of a biofilm-related condition depends upon evidence and, ideally, this depends upon visualization of the suspected biofilm. We routinely examine tissue for evidence of bacterial biofilms; the sample types include human lung tissue biopsies and excised chronic wound tissues, rat or mouse tissue, and excised human joint tissue. In all of these cases, the objective is to acquire confirmation of biofilm presence via confocal or epifluorescence microscopy. At present there are very few fluorescent stains that are truly biofilm-specific; most also stain the supporting tissue intensely, making visualization almost impossible. BacLight Live/Dead is routinely used, because it dependably stains bacteria that are present. Live/Dead also stains surrounding tissue, so is useful only in cases where the stained tissue fluorescence does not overwhelm the target fluorescence. There are numerous sample-associated protocols that interfere with staining, including the fact that human tissue is generally formalin-fixed upon retrieval, so subsequent activity stains are not possible. We are investigating fluorescence methods for elucidating biofilms in tissue samples and are applying new fluorescent stains and immuno-fluorescence methods. We tested multiple stains for possible bacterial specificity. FM 1-43 (Molecular Probes/Invitrogen) is a green fluorescent stain new to biofilm research that does appear to have high bacterial specificity, although only on unfixed samples. Sytox Green (Molecular Probes) stains gram-negative bacteria as well as tissue, but the bacterial fluorescence is so strong that bacteria are easily identified. We also tested fluorescent polyclonal antibodies to *P. aeruginosa* and *S. aureus* on tissue samples positive for the bacteria and again observed specific and reliable staining only on unfixed tissue samples. We are currently examining fixatives other than formalin and staining protocols that might be applied prior to fixation.

S07-S26
A new method: Laser dissection microscopy of cryosectioned biofilms

***Ailyn Lenz**, PhD Candidate, Microbiology, Montana State University, Bozeman, MT*

Biofilms often display enhanced resistances to antibiotics and host defensive responses, in part due to the difference in the physiological status of the surface-associated bacteria compared to planktonic cells. Since biofilms contain gradients of oxygen and nutrients, the cells within biofilms are not physiologically uniform, and gene expression of individual cells varies spatially throughout biofilms. In the current study, we describe a technique that combines recent advances in laser capture microdissection microscopy (LCMM) with quantitative real time polymerase chain reaction (qRT-PCR), allowing us to quantify gene expression of cell subpopulations from within biofilms. Initially we used *Pseudomonas aeruginosa* containing an inducible green fluorescent protein (gfp) gene as a tracer to validate the methodology. Following induction, gfp fluorescence was observed along a thin band (~30 µm) at the top of the biofilms. qRT-PCR from laser microdissected samples demonstrated that gfp expression was greatest within this same 30 µm zone at the top of the biofilm ($P < 0.005$). In the absence of induction little fluorescence or gfp expression was observed in any part of the biofilm. In addition, we have obtained qualitative and quantitative information on gene expression patterns of individual genes from within layers of wild type *P. aeruginosa* biofilms. We determined that 16s rRNA amounts throughout the biofilm strata were relatively constant, indicating an even distribution of live cells and serving as an internal control candidate for future studies of similar nature. In addition, we measured mRNA levels of a housekeeping gene acpP and the quorum sensing regulated genes aprA and phzA1 in *P. aeruginosa* biofilms. Maximum expression of these genes was found in the outermost 30 µm segment of the biofilm with no detectable expression in the middle or base of the biofilm. Such patterns of expression indicate that bacterial cell growth occurs primarily within this small area of the biofilm and reflect the heterogeneous nature of these structured communities of cells. These results demonstrate that

LCMM and qRT-PCR may be used to detect localized levels of gene expression from within biofilms. This technology may now be used to characterize additional genes that may be expressed at spatially distinct sites within biofilms, such as genes involved in antibiotic tolerance, anaerobiosis, or interference with host defensive processes.

S07-S27
Extended lifetime of unstable GFP in *Escherichia coli* colony biofilms

***Audrey Corbin**, Research Associate, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Cells within a biofilm have different access to oxygen, nutrients and metabolic wastes. Due to these microenvironments, anabolic activity is heterogeneously distributed in biofilms. In the course of applying methods to determine spatial patterns of activity in *Escherichia coli* K12 biofilm, it was discovered that a green fluorescent protein (GFP), considered to be relatively unstable, was in fact persisting for periods exceeding 24 hours. Understanding spatial variation of cell activity within biofilm will facilitate design of new control strategies. Labeling regions of active DNA synthesis revealed two zones of replication. One was at the air interface (21 microns wide on average) and a thinner zone (7 µm) was located along the membrane interface. Much of the interior of the biofilm exhibited no DNA synthetic activity. Regions of active protein synthetic activity were identified using an inducible unstable GFP. A 4-hour induction with IPTG revealed a pattern of activity similar to the one observed for DNA synthesis: proteins were synthesized at the air interface (43 µm wide) and in a thinner zone adjacent to the membrane (14 µm). An alternative technique for probing anabolic patterns is to use an unstable GFP fusion to a growth-rate dependent promoter. The fis promoter is highly expressed during the early exponential phase; cells in that state of growth appear green. During the 48 hours of growth, colony biofilms have to be transferred to fresh media plates to renew access to nutrients. Unexpected patterns of growth activity were observed, as they depended on the number of these transfers. No fluorescence was observed in absence of transfer; one thin zone of activity was observed in the middle of the biofilm after one transfer at the 24th hour; two zones appeared after a 24 and 44-hour transfer; three zones after 12, 24, and 36-hour transfers. However when the same strain (fis-gfp[LVA]) was grown in a capillary flow reactor system, the biofilm presented a single green stripe at the nutrient interface. In colony biofilms, stripes observed on the fis strain patterns are the result of transfers to fresh media. GFP[LVA] fluorescence persists in biofilms, though it is an unstable variant of GFP that decays within a few hours in planktonic cultures. Unstable GFP variants have been developed as a way of monitoring recent gene expression, thanks to their relatively fast turn-over. However in colony biofilms, unstable GFP reporter fusions may not reflect the recent gene expression only, but also expression which has occurred during the whole experiment.

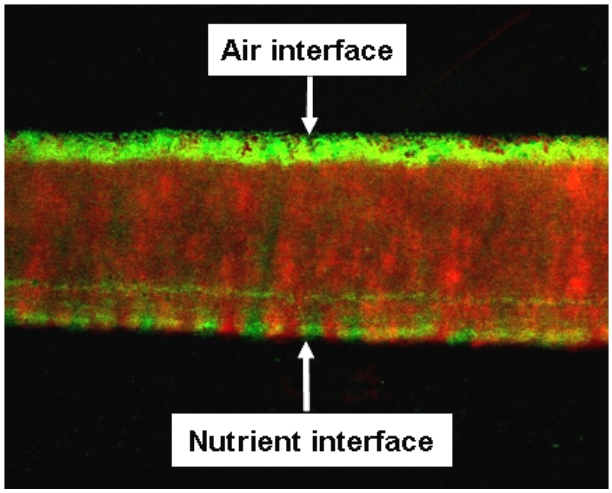


Figure 1.

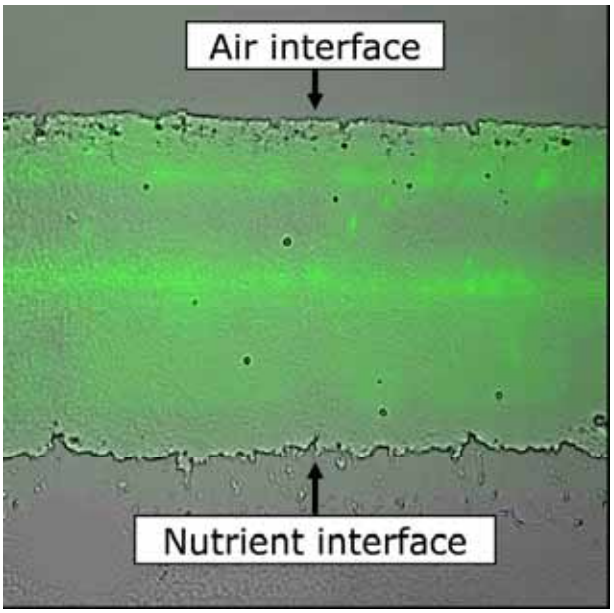


Figure 2.

S07-S28
Film Fest: Movies of biofilm destruction

***Willy Davison**, PhD Candidate, Chemical Engineering, Montana State University, Bozeman, MT*

Bacterial biofilms can pose serious problems in many settings—including the medical, food, water treatment, and pharmaceutical industries. One of the main problems associated with biofilms is that they are extremely difficult to completely remove from the surfaces to which they are attached. Bacterial biofilms exhibit great resiliency in a variety of changing environmental conditions while they continue to survive and thrive as communities. There are several ways to attack a biofilm's mechanical properties that may weaken its structure, thus making biofilm removal more feasible. Mechanical removal of biofilms can be achieved by increasing the shear stresses imposed on a biofilm by the fluid flowing around it. Under changing flow conditions (e.g., increased turbulence), biofilms have been shown to deform, migrate, or even detach from the surface. These changes in biofilms were visualized using time-lapse confocal scanning laser microscopy (CSLM).

Chemical removal of biofilms can be accomplished by the addition of certain chemicals or by altering the water chemistry surrounding a biofilm. Movies obtained with CSLM indicated that in the presence of urea or chlorine biofilms were loosened or liquefied and they lost structural integrity. Electrochemical removal can be achieved by applying an electric current to the surface of biofilm attachment. This water electrolysis alters the water chemistry by increasing pH and generates hydrogen gas that can scour the surface; both effects contribute to biofilm removal. Photochemical treatments have revealed a rapid dispersal of biofilm cells. *E. coli* biofilm clusters stained with Calcofluor white and then briefly exposed to UV light appeared to be instantaneously disaggregated. One explanation of the rapid dispersal of cells is that the fluorescence emission from the Calcofluor white generates reactive oxygen species within the biofilm clusters, creating a kind of Trojan horse attack of intact biofilms. Real-time movies of this phenomenon were captured using fluorescent light microscopy.

SESSION 7: Fungal Biofilms

S07-S29
Functional sub-populations in *Candida albicans* biofilms

***Peter Suci**, Assistant Research Professor, Microbiology, Montana State University, Bozeman, MT*

Fungal biofilms are composed of eukaryotic cells. In contrast to prokaryotic systems, in which examples of multicellular differentiation into distinct cell types is the exception, eukaryotic cells have a strong tendency to form "communities" that recruit specialized cell types to perform specific functions. This suggests that disruption of community interactions might be a more accessible target in fungal biofilms than in bacterial biofilms. *Candida albicans* is a fungus that is similar at the genomic level to the well characterized yeast, *Saccharomyces cerevisiae*, that is used in brewing and baking. However, unlike *S. cerevisiae*, *C. albicans* has developed the ability to transform itself into various morphological forms and also constructs copious biofilms. Filamentous forms are thought to be involved in pathogenesis, specifically invasion of tissue, and have been shown to play an essential structural role in biofilm development.
My *C. albicans* research began with the hypothesis that yeast forms may also play specialized roles in biofilms, namely in survival and dissemination. We had previously obtained evidence suggesting that yeast forms were responsible for resistance of *C. albicans* biofilms to antimicrobials that disrupt the plasma membrane. With funds from NIH we began exploring the hypothesis that yeast clusters that form on filaments at the medium interface of biofilms are specialized to play a role in detachment. The approach that we proposed was to use microarray analysis to determine if the transcriptome of these yeast was distinct from that of yeast grown in batch culture. The observation that the annotated strain of *C. albicans* undergoes a global detachment event in which the entire biofilm detaches from the surface has taken us on a tangent. We plan to exploit this phenomenon to identify transcription factors involved in regulating detachment and to test the hypothesis that detachment proceeds by recruitment of specialized subpopulations of cells that reside at the substratum interface. An intriguing possibility is that this detachment process is regulated at the community level. The take-home message is that viewing fungal biofilms as composed of functional subpopulations may open up new avenues for both eradication and control.

SPEAKER ABSTRACTS

S07-S30
Small molecules inhibit signaling pathways and hyphal formation in *Candida albicans*

***Kurt Toenjes**, Assistant Professor of Biology, Montana State University–Billings, MT*

C. albicans is the most common and, arguably, the most important causative agent of human fungal infections. Localized infections can lead to significant morbidity, while systemic infections in immunocompromised patients have a 35% mortality rate. During infection it is essential that *C. albicans* switch between different morphological states, including transitions between budded or yeast-like cells and hyphal forms. Understanding the multiple signaling pathways regulating the budded-to-hyphal growth transition will lead to tremendous insight into virulence mechanisms and may ultimately result in the discovery of new anti-fungal therapeutics. My research seeks to elucidate the signal transduction cascades that control the different morphological states and determine whether inhibition of the budded-to-hyphal growth transition by bioactive small molecules is an effective therapeutic strategy.
We have developed an assay to screen small molecule libraries for compounds that inhibit the budded-to-hyphal growth transition. Using this assay, five molecules have been identified from the Diverset E Chembridge small molecule collection and eleven molecules from the ICCB Bioactives collection of known cellular inhibitors.
The bioactive molecules all have activity as inhibitors of proteins involved in cell signaling. All these molecules inhibited the budded-to-hyphal transition and hyphal growth without affecting budded growth. Synergistic effects have been observed between several of the novel molecules and the molecules with known targets, suggesting that these molecules may be inhibiting related signaling pathways. HA14-1, a Bioactive inhibitor and Bcl-2p agonist, also inhibits the morphogenesis defect of cells lacking the putative ubiquitin ligase subunit Grr1p and disrupts the organization of intermediate filaments under hyphal inducing conditions. These data indicate that the use of small molecules to inhibit hyphal growth is a useful method for characterizing signaling pathways and for identifying potential new anti-fungal drugs.

S07-S31
Special Presentation: Highlights from the ASM Biofilms 2007 Conference

***Phil Stewart**, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

During the last week of March of this year, 620 scientists and engineers from around the globe met in Quebec City, Canada, to share their recent results and ideas about microbial biofilms. This was the fourth American Society for Microbiology special conference on the topic of biofilms. It was packed with 81 oral and 355 poster presentations. In this recap of the meeting, Dr. Stewart will briefly share highlights from a handful of the talks in an attempt to capture the excitement and diversity of the conference. Featured topics include phenotypic switching, mineral deposition, attack and counterattack in the interaction of bacterial biofilm with grazing protozoa or leukocytes, a mechanism that generates genetic diversity in biofilm populations, a probiotic strategy to prevent catheter infection, the engineering of wastewater treatment reactors to take advantage of recently tapped biodiversity, genetic clues about the enigmatic persister state that may contribute to biofilm recalcitrance to antimicrobials, and a strategy for targeting biofilms by interfering with iron metabolism.

S07-P361
A 3D computer model analysis of three hypothetical biofilm detachment mechanisms

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

Three hypothetical mechanisms of detachment were incorporated into a three-dimensional computer model of biofilm growth and development. The model integrated processes of substrate utilization, diffusion, growth, cell migration, and detachment in a cellular automata framework. The purpose of this investigation was to characterize each of the mechanisms with respect to four criteria: the resulting biofilm structure, the existence of a steady state, the propensity for sloughing events, and the dynamics during starvation. The three detachment mechanisms analyzed represented various physical and biological influences hypothesized to affect biofilm detachment. The first invoked the concept of fluid shear removing biomass that protrudes far above the surface and is therefore subjected to relatively large drag forces. The second pathway linked detachment to changes in the local availability of a nutrient. The third pathway simulated an erosive process in which individual cells are lost from the surface of a biofilm cell cluster. The detachment mechanisms demonstrated diverse behaviors with respect to the four analysis criteria. The height dependent mechanism produced flat, steady state biofilms that lacked sloughing events. Detachment based on substrate limitation produced significant sloughing events. The resulting biofilm structures included distinct, hollow clusters separated by channels. The erosion mechanism produced neither a non-zero steady state nor sloughing events. A mechanism combining all three detachment mechanisms produced mushroom-like structures. The dynamics of biofilm decay during starvation were distinct for each detachment mechanism. These results show that detachment is a critical determinant of biofilm structure and of the dynamics of biofilm accumulation and loss.

S07-P363
Anti-biofilm properties of chitosan-coated surfaces

R. Carlson, *R. Taffs, A. Hedegaard, W. Davison, and P. Stewart, Center for Biofilm Engineering and Department of Chemical and Biological Engineering, Montana State University, Bozeman, MT*

The surface associated process of microbial colonization and biofilm formation is a persistent and ubiquitous problem facing a broad range of disciplines. Examples include tenacious medical infections related to biofilm formation on implanted medical devices as well as microbial fouling of municipal water distribution systems. Common biofilm control strategies like the use of antibiotics or oxidizing chemicals are typically limited in their efficacy at inhibiting or removing biofilms.

We have investigated a chitosan coating system for retarding or preventing the formation of biofilms. The system does not utilize the common strategy of impregnating a polymer with an antimicrobial agent; instead it utilizes a biological polymer as both the coating material and the actual anti-biofilm agent. The thin, flexible coating is highly effective at retarding or preventing the formation of *Staphylococcus epidermidis*, *Klebsiella pneumoniae, Staphylococcus aureus,* and *Candida albicans* biofilms under medically relevant conditions. For instance, *S. epidermidis* surface counts (CFU/cm^2) are reduced on average 300,000-fold on the chitosan coating as compared to a control. More traditional coatings impregnated with antimicrobial agents like chlorhexidine were found to reduce *S. epidermidis* surface counts by less than 10-fold. The findings suggest this coating has potential for applications on surfaces, like implantable medical devices, that are susceptible to biofilm formation.

POSTER ABSTRACTS

S07-P393
To build a microbial factory: Investment cost and operating cost analysis of metabolic networks

***R. Carlson**, Center for Biofilm Engineering, Montana State University, Bozeman, MT*

Evolutionary success requires strategic allocation of scarce resources. Under conditions of nutrient sufficiency, metabolic networks are well known to be regulated for thermodynamic efficiency. However, efficient biochemical pathways are anabolically expensive to construct. A cost-benefit analysis of an *in silico Escherichia coli* network revealed the relationship between pathway proteome synthesis requirements, thermodynamic efficiency, substrate affinity and DNA coding sequence length. Pathway proteome synthesis requirements appear to have shaped metabolic network structure and regulation. Under conditions of nutrient scarcity and other general stresses, *E. coli* expresses pathways with relatively inexpensive proteome synthesis requirements instead of more efficient but also anabolically more expensive pathways. This evolutionary strategy provides a cellular function-based explanation for common network motifs like isozymes and parallel pathways and possibly explains 'overflow' metabolisms observed during nutrient scarcity. The study provides fundamental clues into evolutionary pressures and competitive metabolic designs.

S07-P405
Multispecies biofilm development on space station heat exchanger core material

***B.H. Pyle**[1], L.M. Vega[2], S.R. Roth[1], K.D. Pickering[2], P.J. Alvarez[3], M.C. Roman[4];*
[1]Montana State University, Bozeman, MT;
[2]NASA Johnson Space Center, Houston, TX;
[3]Rice University, Houston, TX;
[4]NASA Marshall Space Flight Center, Huntsville, AL

Background: Investigations of microbial contamination of the cooling system aboard the International Space Station (ISS) suggested that there may be a relationship between heat exchanger (HX) materials and the degree of microbial colonization and biofilm formation. Experiments were undertaken to test the hypothesis that biofilm formation is influenced by the type and previous exposure of HX surfaces.

Methods: *Acidovorax delafieldii, Comamonas acidovorans, Hydrogenophaga pseudoflava, Pseudomonas stutzeri, Sphingomonas paucimobilis, and Stenotrophomonas maltophilia*, originally isolated from ISS cooling system fluid, were cultured on R2A agar and suspended separately into fresh filter-sterilized ISS cooling fluid (borate buffer pH 8.3). Initial numbers in each suspension ranged from 10^6– $>10^7$ CFU/ml, and a mixture of all seven species contained $>10^7$ CFU/ml. Coupons of ISS HX material, previously used on orbit (HX Flight) or unused (HX Unused), polycarbonate (PC) and 316L polished stainless steel (SS) were autoclaved, covered with multispecies suspension in sterile tubes and incubated in the dark at ambient temperature (22–25 ^{o}C). Original HX material contained >90% Ni, 4.5% Si, and 3.2% B. For approximately ten weeks, samples of fluid were plated on R2A agar for colony counts and surface colonization in the biofilm was assessed by SYBR green or BacLight staining and microscopy.

Results: Suspension counts for the PC and SS samples remained steady at around 10^7 CFU/ml HX. Unused counts declined about 1 log in 21 d then remained steady, and HX Flight counts declined 2 logs in 28 d and stabilized at about 10^3 CFU/ml from 47–54 d. Yellow *S. paucimobilis* predominated on plates from HX Flight samples up to 26 d, then white or translucent colonies of other species appeared. All colony types were seen on plates from other samples throughout the trial. Epifluorescence microscopy indicated colonization of all surfaces by 21 d, followed by variable colonization. After 54 d, all but the HX Flight samples had well-distributed live and dead cells; the HX Flight samples had few cells and most were live as assessed by BacLight.

Conclusion: The results suggest that HX materials themselves inhibited surface colonization. The HX exposed on orbit to cooling system fluid inhibited growth of some species originally isolated from the system, whereas the unused HX material had a moderate effect compared to no inhibition with PC or SS controls. It is possible that, in addition to the original surface composition, the chemistry or microbiology of the ISS system caused deposition of inhibitory compounds on the HX Flight coupon surfaces; these may have inhibited inoculated species to differing degrees.

S07-P411
Biofilm enhanced geologic sequestration of supercritical CO_2

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Geologic sequestration of CO_2 is one strategy to reduce the emission of greenhouse gases generated through the combustion of fossil fuels. Geologic sequestration of CO_2 involves the injection of CO_2 into underground formations such as oil bearing formations, deep un-minable coal seams, and deep saline aquifers (White *et al*. 2003). There are several conditions which must be met for successful sequestration in these formations including: 1) temperature and pressure conditions must be such that CO_2 will be supercritical, 2) the aquifer must have a suitable aquitard trap, and 3) the receiving aquifer should have appropriate porosity and permeability. During operation of geologic CO_2 sequestration sites, supercritical CO_2 ($scCO_2$) would be injected into the receiving formation, resulting in elevated pressure in the region surrounding the point of injection. As a result an upward hydrodynamic pressure gradient may develop across the trapping aquitard. Upward "leakage" of CO_2 could occur due to the primary permeability of the aquitard, through fractures or near injection wells.

The research is part of the Zero Emission Research and Technology (ZERT) program, which is a collaborative research effort focused on understanding the basic science of underground carbon dioxide storage. The research hypothesis is that engineered microbial biofilms can be used to significantly decrease the permeability of high permeability areas in deep aquifers, such as near well casings or in fractures, and increase the mineralization (i.e., carbonate formation) after $scCO_2$ injection. This part of ZERT focuses on microbially based strategies and technologies for controlling leakage of supercritical CO_2 ($scCO_2$) during geologic sequestration.

We used a high pressure (~1300 psi), moderate temperature (≥ 32 °C) system to grow biofilms in 2.54 cm diameter, 5 and 11cm long, ~40 millidarcy Berea sandstone cores. Biofilm growth resulted in a two-order of magnitude reduction in permeability. Permeability increased slightly in response to starvation and $scCO_2$ challenges. The inoculum of *Shewanella fridgidimarina* was replaced by other species that were likely introduced with the sandstone core such as *Bacillus mojavensis* and a *Citrobacter* sp., both known biofilm- and EPS-forming subsurface organisms. Light, epifluorescence, and electron microscopy of the rock core revealed substantial biofilm accumulation in rock pore channels. Viable population assays of organisms in the effluent indicate survival of the bacteria after $scCO_2$ challenges. These observations are encouraging for the prospective use of engineered biofilm barriers for controlling leakage of geologically sequestered CO_2.

S07-P413
PCR/DGGE using "universal" 16S rDNA primers has limited resolution for soil bacterial community analysis

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Denaturing gradient gel electrophoresis has gained popularity for separating PCR products based on DNA sequence differences (PCR/DGGE). However, the resolution of DGGE may not be adequate for analyzing the great microbial diversity inherent in soils, especially when domain-specific or "universal" 16S rDNA primers are used. In theory, DGGE can be used to track shifts in soil microbial communities in response to a disturbance or a difference in treatment (fertilizer regime, crop rotation, tillage, etc.). However, it is not known how severe the disturbance must be in order for the resulting community shift to be detected using universal primers. In this study, DGGE was used first to profile the microbial communities in four surface soil samples taken at the corners of a 50cm square. Two of the samples came from a narrow footpath that was compacted and worn free of any vegetation, while the other two were in an adjacent undisturbed and relatively lush lawn. This site provided a ideal opportunity to investigate whether the lawn and path soil microbial communities would be similar due to their proximity, common soil type, climate, and previous history, or whether they would be markedly different due to the disturbance. DGGE profiles created using universal 16S rDNA primers were quite similar for both lawn and path soil microbial communities. These communities could, however, be distinguished on the basis of a few bands that were distinctly brighter in one DGGE profile compared to the other. The same analysis was performed on soil communities from two experimental cropping trials (in Montana and Kansas), in which the plots had a common history, but differed in recent management practices.

When universal primers were used, the profiles were indistinguishable, as expected. However, when either group-specific 16S rDNA primers or primers directed at selected functional genes were used, differences in DGGE profiles were detected. The results suggest that functional genes may be more informative targets for PCR/DGGE analysis than the phylogenetic 16S rRNA gene.

S07-P415
Deciphering cDCE degradation pathways in *Polaromonas* sp. strain JS666

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Polaromonas sp. strain JS666 is the first aerobic organism isolated capable of growth-coupled cis-dichloroethene (cDCE) degradation. Therefore, it is a promising bioaugmentation agent at cDCE-contaminated sites where the common groundwater contaminant and suspected carcinogen has migrated into an aerobic zone. Knowledge of the metabolic pathways involved in cDCE degradation in JS666 could provide insight into required nutrients and conditions for optimal bioaugmentation. However, the cDCE degradation pathways in JS666 have yet to be elucidated. We used a proteomic approach with 2D gel electrophoresis (2D-GE) to identify proteins involved in cDCE degradation. Potential reference substrates (i.e., ethanol and acetate) were screened to ensure that they did not induce cDCE degradation. Acetate cultures that were washed and suspended in medium containing cDCE as the sole carbon source exhibited a lag phase of 25–30 days before degradation began, indicating that acetate does not induce cDCE degradation. Conversely, ethanol-grown cultures that were washed and suspended in medium containing cDCE showed rapid degradation. The results indicated that ethanol could induce cDCE degradation and would not be an appropriate reference substrate for 2D-GE. Subsequently 2D gels of acetate- and DCE-grown cells were prepared in triplicate. Fifteen differentially expressed protein spots from the cDCE gels were excised and identified using LC/MS/MS and a MASCOT search. A comparison of the molecular weight (MW) and isoelectric point (pI) from the gels to the values predicted from MS verified the protein identifications. Experiments are currently underway to reconfirm the protein identifications from the 2D-GE experiments.

S07-P416
Visualization of antimicrobial action in *Staphylococcus epidermidis* biofilms

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The action of antimicrobial agents against bacterial biofilms of *Staphylococcus epidermidis* was visualized by a time-lapse microscopy technique that allowed spatial and temporal patterns to be discerned non-invasively. Biofilm was grown in a continuous flow glass capillary reactor for 24 h and then pre-stained with Calcein-AM. This fluorogenic esterase substrate loads cells with an unbound green fluorescent dye that remains trapped inside the cell as long as the cell membrane is intact. If membrane integrity is compromised, for example by an antimicrobial agent, the dye leaks out and the cell becomes dark. Using confocal scanning laser microscopy, the action of glutaraldehyde, chlorine, and a quaternary ammonium biocide were observed under flow conditions. Each antimicrobial exhibited a distinct spatio-temporal pattern of action in biofilm clusters.

During chlorine treatment, fluorescence loss occurred in a small (~10 micron) layer at the periphery of the biofilm which progressed toward the center, while the clusters were simultaneously eroded. This pattern could be attributed to limited penetration of chlorine due to a reaction-diffusion interaction. Treatment with the quaternary ammonium compound resulted in biphasic loss of fluorescence in biofilm clusters. A fraction of the cell population—mostly located in the interior of the clusters—remained bright for a longer time. This pattern suggests two populations within the biofilm: one that is rapidly permeabilized by the agent and a second that is much less susceptible. During glutaraldehyde treatment, biofilm clusters maintained most of the initial fluorescence. This retention of fluorescence suggests that this biocide does not cause cellular envelope permeabilization. Image analysis was applied to extract estimates of the penetration time and to quantify relative rates of action of the antimicrobial agents.

S07-P417
Investigations of dormant cells in *Pseudomonas aeruginosa* biofilms

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It was hypothesized that *Pseudomonas aeruginosa* biofilms are protected from killing by antimicrobials due to the presence of dormant cells within mature biofilms. A *P. aeruginosa* strain containing a stable, inducible green fluorescent protein (GFP) was used to visualize and characterize the metabolically dormant and active cell populations within the biofilm. In up-shift experiments, cells were labeled by introduction of the inducing agent to mature biofilms. Only about 30% of the cells within the biofilm turned bright, thus labeling the active cells with GFP. Dormant cells were labeled by growing biofilms to maturity in the continuous presence of inducer, then switching to media lacking inducer. This down-shift treatment produced a biofilm in which only the most dormant cells within the biofilm were bright after an extended period on media lacking the inducer. By disaggregating the biofilms and sorting each population by flow cytometry (FCM), it was possible to separate and collect populations based on GFP expression in which cells expressing GFP were characterized as bright events (GFP+) while the non-GFP expressing cells were characterized as dim (GFP-). When plating the GFP+ population of dormant cells for viability, 17% of bright events produced colony forming units (cfu). Similarly, when considering active cells, 21% of bright events were found to yield viable cells.

This novel approach of labeling cells in intact biofilms by their metabolic state was used in two ways. We explored the susceptibility of both active and dormant cells to antibiotic treatment, and we devised a method of physically separating the metabolically active, top layer of the colony biofilm from the dormant lower layers. In up-shifted colony biofilms, top layers that showed > 90% GFP+ events and bottom-most layers that showed > 80% GFP- events were retained for RNA extraction in preparation for determination of genome-wide differences in gene expression between the two populations. For the resistance studies, after FCM sorting into GFP+ and GFP- populations, each population of cells was plated for viability and their susceptibility to either ciprofloxacin or tobramycin was determined. In the down-shift experiments, active cells, compared to the dormant cells (GFP+), showed a 7-fold and 35-fold greater decrease in log cfu, respectively, after treatment with ciprofloxacin and tobramycin. In the up-shift experiments where the active cells were GFP+, the dormant cells experienced an actual increase (negative log reduction) in log cfu after treatment with the antibiotics, while the active cells again showed reductions in log cfu after exposure to the antibiotics. These data suggest that the antibiotic tolerance expressed by *P. aeruginosa* biofilms is affected by the presence of metabolically dormant cells within the biofilm.

S07-P418
Development of fluorescent reagent combinations specific to biofilm components

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Direct visualization of bacterial biofilms via microscopy can yield information about structure, community and chemical composition, and specific physiological activities. For examination of biofilms, a simple yet very informative combination of fluorescent stains would include one for all cells, one for activity, and one for extracellular polymeric substances (EPS). Stains do exist for each of these aspects alone (such as DAPI or concanavalin A), but existing combinations have serious weaknesses, such as overlap of emission signal, non-specific staining, and inadequate staining of the targets. We are developing new reagents and combinations of reagents that will allow simultaneous visualization of cells, cell activity, and EPS.

Pseudomonas aeruginosa ATCC 15442, *Staphylococcus epidermidis* ATCC 35984 and *Escherichia coli* ATCC 25922 biofilms were grown separately in CDC biofilm reactors. After 24 hours of continuous flow, coupons were removed from the reactors, stained, and imaged on both epifluorescent and confocal microscopes. Reagents were tested both for their ability to stain single aspects of a biofilm and also for their compatibility with the other tested reagents. Tested reagents are all commercially available, although none have a history in the literature of use on bacteria or biofilms. The reagents tested in this study were BODIPY® 630/650-X, SE, [6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid, succinimidyl ester], Calcein Green, Red and Violet

AM, and FM ® 1-43 [N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide]. BODIPY® 630/650-X, SE is an amine-reactive dye that is frequently conjugated to drugs, toxins, and nucleotides, and was expected to stain matrix components. The Calcein AMs are esterase substrates, which were evaluated for their ability to indicate cell activity. FM ® 1-43 is a lipophilic styryl dye with a history of use in eukaryotic cells for visualizing plasma membranes, studying neuronal activity and examining vesicle trafficking in fungi, but no documented use on bacteria. FM ® 1-43 stained all biofilms reliably and very specifically. The CAMs stained some biofilms reliably, some not at all, and some with variability. Bodipy stained *S. epidermidis* cells and the *P. aeruginosa* matrix, but did not stain either aspect of *E. coli* biofilms well.

S07-P419
Structural role for flagella in biofilm formation in *Desulfovibrio vulgaris* Hildenborough

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Studies on sulfate reducing bacteria (SRB) have been of interest due to reduction capabilities during metal corrosion and bioremediation. Sulfate-reducing bacteria are known to grow as biofilms on different surfaces; however, little is known about biofilm growth in SRBs. *Desulfovibrio vulgaris* Hildenborough has been a model organism for SRBs, but little research has been conducted on biofilm formation or maintenance. *D. vulgaris* ATCC 29579 (wild-type) and three mutants—Δ*fla*G, Δ*fli*A, and ΔMP (lacking the 200kb plasmid)—were grown in batch mode in a defined medium with lactate and sulfate; biofilms were allowed to form on glass slides. Wild-type cells were motile and formed a continuous monolayer of cells on the glass as observed by crystal violet staining and SEM. Initial results indicated that Δ*fla*G mutants were motile, while the ΔMP and Δ*fli*A mutants were less motile or not motile. Significant amounts of carbohydrate were not measured within wild-type biofilms (0.01 ug hexose sugar per ug protein), and biofilms stained with Calcofluor white, Concanavalin A, and congo red revealed little external carbohydrate (e.g., EPS). TEM analysis of wild-type biofilms grown on SiO_2 grids also showed little EPS, but the presence of 'filaments' were observed in both TEM and SEM images. The filaments, possibly a form of modified flagella, were present within wild-type biofilms but fewer were seen in Δ*fla*G and were almost completely lacking in the Δ*fli*A and ΔMP mutants. Crystal violet staining revealed that Δ*fla*G, Δ*fli*A, and ΔMP mutants produced 5-fold, 2-fold, and 3-fold less biofilm compared to the wild-type, respectively. As observed with wild-type biofilms, negligible amounts of carbohydrate were detected within the mutant biofilms. Filtrate samples of the wild-type biofilms were also analyzed and a 1D protein gel indicated that the biofilm matrix was enriched for certain polypeptides. These results indicated that *D. vulgaris* appears to rely on a proteinacous material to form and maintain its biofilm matrix and that flagella, or a modified form of flagella, play an important role, not only in initial formation of *D. vulgaris* biofilm, but also in biofilm stability.

S07-P420
Changes in microbial community structure during biostimulation for uranium reduction

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Former radionuclide waste ponds at the ERSP-Field Research Center in Oak Ridge, TN, pose several challenges for uranium bioremediation. The site is marked by acidic conditions, high concentrations of nitrate, chlorinated solvents, and heavy metals. Above-ground treatment of groundwater, including nitrate removal via a denitrifying fluidized bed reactor (FBR), pre-conditions the groundwater for subsurface uranium immobilization. A series of re-circulating wells serve to create a subsurface bioreactor to stimulate microbial growth for *in situ* U(VI) immobilization. Well FW-104 is the injection well for the electron donor (i.e., ethanol); well FW-026 is the extraction well for the recirculation loop; well FW-101 and FW-102 are the inner zones of

biostimulation; and FW-024 and FW-103 are upstream and downstream wells, respectively, which are the outer protective zones. Bacterial community composition and structure of groundwater from the wells were analyzed via clone libraries of partial SSU rRNA gene. Both qualitative and quantitative methods were used to analyze the changes in bacterial diversity and distribution. LIBSHUFF analysis was used for the comparison of bacterial community population between the different clone libraries.

Bacterial community from the denitrifying FBR was different from the groundwater bacterial community, which indicated that different bacterial communities were stimulated in the two separate systems. The clone libraries of the re-circulating wells showed that over each phase of manipulation for uranium immobilization, the bacterial communities of the inner zones of biostimulation were more similar to each other than to those of the outer protective zones. The outer protective zones were more similar to the injection well. Clone libraries from FW-104 (injection), FW-101 and FW-102 showed that bacterial communities of the three wells were initially similar but developed changes through time. FW-101 and FW-102 bacterial communities developed changes in parallel, while those of FW-104 showed gradual change. These results were further compared to data generated from Unifrac analysis. Preliminary results with Unifrac analyses showed that the bacterial community in each of the wells developed changes during the bioremediation process, and the changes could be attributed to the variations along temporal, spatial, and geochemical scales. Diversity indices showed that bacterial diversity tended to increase during the initial phase of uranium bioreduction and decreased toward the end of uranium bioreduction (i.e., low U(VI) levels). As uranium levels declined, increasing *Desulfovibrio* and *Geobacter*-like sequences were detected from the clone libraries; the *Desulfovibrio*-like sequences predominated over time. The results were further confirmed via qPCR and the results correlated with OTU distributions for *Desulfovibrio*. The results indicated that the bacterial community composition and structure changed upon stimulating for uranium bioreduction conditions and that sequences representative of sulfate-reducers and metal-reducers were detected in wells that displayed a decline in U(VI). Further analysis is underway to determine the relationships between different functional groups and site geochemistry.

S07-P421

Manipulating the mechanical properties of biofilm

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The goal of this project is to investigate the response of biofilm material properties when exposed to various chemical treatments, ultimately producing another method for controlling biofilm. Biofilm colonies may be strengthened using multivalent cations created when certain salts are put into solution. These positively charged cations will bind with negatively charged strands of the biofilm extracellular polymeric substances (EPS). This will crosslink the biofilm and tighten the EPS, causing an increase in biofilm material strength. Conversely, treatments can be applied to weaken biofilm. Urea, a compound which is known to disrupt non-covalent bonds, has proved to be a good chemical for weakening the biofilm.

The bacterium used in this experiment to create the sample biofilm colonies was FRD1, a mucoid, alginate-producing strain of *Pseudomonas aeruginosa*. The solutions tested were: NaCl, $FeCl_3$, $AlCl_3$, $MgCl_2$, $CaCl_2$, and urea. Creep tests were performed on a rheometer to determine material properties of the biofilm. Comparing the strain of the untreated samples to the strain of the treated samples shows how the treatment of the biofilm affects its material properties. The data seems to fit the Burger model quite well. Curve-fitting the data with the Burger model allows for the elastic and viscous parameters to be obtained. These data allow for calculations to be made that determine the constitutive properties of the biofilm and how treatments affect these values. The results from this project show a clear increase in the material strength of the multivalent cation-treated biofilm. Divalent cations $MgCl_2$ and $CaCl_2$ had a 2–6 fold increase in their elastic and viscous coefficients. The trivalent cation $FeCl_3$ had a 12–60 fold increase in its elastic coefficients. The samples treated with urea show a clear weakening of the biofilm, with a 1.3–2.5 fold decrease in it coefficients. This research concludes that it is possible to control the material properties of FRD1 biofilm colonies through the use of chemical treatments.

S07-P422
How susceptible to chlorine disinfection are detached biofilm particles?

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The disinfection susceptibilities of suspended planktonic cells have been well studied for a large variety of biocides. These biocides have been found to be much less effective against cells in biofilms, requiring biocide concentration orders of magnitude higher than those necessary to kill suspended planktonic cells.

Although the detachment of aggregated cells from biofilms is of fundamental importance to the dissemination of contamination and infection in both public health and clinical settings, the disinfection efficacies of commonly used biocides on detached particles have not yet been investigated. The question arises: Can cells in detached aggregates be killed with disinfectant concentrations sufficient to kill planktonic cells? We hypothesize that detached particles are less susceptible to biocides than planktonic cells.

For the purpose of testing and comparing biocide susceptibilities of planktonic cells, cells in biofilms, and detached cell aggregates, we designed experiments as follows: *Salmonella typhimurium*, as a model pathogen, is grown in standardized laboratory reactors for enhanced repeatability. Planktonic cultures are grown in a continuously stirred chemostat, while biofilm is obtained from the coupons of a CDC biofilm reactor (BioSurface Tech. Corp.). Detached aggregates can be sampled from the outflow of the CDC biofilm reactor. Disinfection experiments are performed with sodium hypochlorite concentrations from 1–40 ppm in order to calculate log reduction rates for each scenario.

For cells in detached particles, we anticipate an intermediate susceptibility to the biocide compared to the susceptibilities of planktonic cells and biofilm cells. Different factors, such as particle size, growth rates, and extracellular polymeric substances, may influence the biocide susceptibility of detached aggregates.

S07-P423
Molecular biology of mouse skin flora

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Central venous catheter (CVC) use has become a common practice in hospitals, particularly in intensive care units. According to the CDC, it is estimated that over 250,000 blood stream-associated infections occur annually due to CVC use. There is a growing need for the evaluation of methodologies that aim to decrease infection risk in intravascular catheters. The use of an animal model, particularly the mouse model, to evaluate efficacy before proceeding to expensive clinical trials is becoming a routine protocol. To improve understanding of the interactions between the mouse skin and the catheter, the diversity of mouse skin flora must be characterized. This project aims to understand which organisms are present in mouse skin and which of those may cause infections in intravenous catheter models. In addition, the characterization of normal mouse skin flora is crucial to evaluating complications when intravenous catheters are inoculated with known pathogenic strains of bacteria. The goal of this project was to determine the normal flora of mouse skin using molecular techniques, focusing primarily on *Staphylococcus* species. Preliminary examination of mouse skin flora by standard clinical microbiology techniques has shown that mouse skin flora varies from the normal skin flora of humans.

The molecular biology of mouse skin flora was analyzed by 16s DNA sequencing of cultured mouse skin isolates. Seventy three isolates were evaluated based on 16s DNA sequencing. The five most predominant species present were: *Staphylococcus xylosus, Staphylococcus nepalensis, Staphylococcus saprophyticus, Staphylococcus aureus, and Enterococcus faecalis.* In addition, mouse skin flora was analyzed by 16s DNA sequencing from amplified bacterial DNA of mouse skin samples. Denaturing gradient gel electrophoresis was used to evaluate the number of species present. Eighteen of the DGGE bands were cut, amplified, and sequenced. Sequence results confirmed the presence of *Staphylococcus xylosus, Staphylococcus nepalensis, Staphylococcus saprophyticus,*

Staphylococcus aureus, and *Enterococcus faecalis.* Also present in the mouse skin DNA samples, were several other bacteria not identified by culture techniques, including: *Burkholderia cepacia, Microbacteriaceae* sp., and *Porphyromonas gingivalis*.

S07-P424
Constructed wetland rhizosphere microbial community analysis using group-specific primers and denaturing gradient gel electrophoresis

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Molecular techniques were used to determine the microbial community structure and activity in the rhizosphere of constructed wetlands and to identify microbial community differences by using a variety of 16S group-specific primer combinations. Denaturing gradient gel electrophoresis (DGGE) fingerprints initially obtained from rhizosphere samples using universal bacterial primers revealed a poor resolution of differences largely because real differences were likely masked by the great complexity of the profiles. In order to visualize and compare between plant species and various regions of a root surface, a variety of group-specific primers were selected. The specific groups considered were *Betaproteobacteria*, *Deltaproteobacteria*, and *Bacillus*. Universal 16S primers were also used for each sample as a standard for comparison. Molecular analysis of rhizosphere communities was accomplished by PCR amplification of 16S rDNA with primers targeting variable regions of the rDNA gene and using a reverse primer containing a GC-rich "clamp" to facilitate gel separation of distinct genotypes. DGGE was performed using a 40% to 70% denaturation gradient and an 8% to 12% gradient in the polyacrylamide. Breaking down this complex community into smaller, more specific groups made further analysis and visualization by DGGE more informative. By creating community profiles with a more simplistic scope, a more complete microbial view of this highly diverse habitat was obtainable.

S07-P425
Optimizing flow cytometry to detect viable but non-culturable, viable-culturable, and membrane-damaged bacteria

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Traditional culture methods may underestimate bacterial numbers due to sublethal environmental injury, inability of target bacteria to take up nutrients in the medium, and other physiological factors. Approaches that reduce selectivity, decrease bias from sample storage and incubation, and reduce assay time are needed. Flow cytometry (FCM) is a sensitive technique that can rapidly monitor physiological changes of microorganisms, especially when related to membrane integrity.

The main objectives of this research were to establish the quickest, most accurate, and easiest ways to estimate the proportions of viable but not culturable (VBNC), viable and culturable (VC), and dead cells as indicated by membrane integrity using optimized flow cytometry. The technique involved selecting the dye that yielded the highest intensity—SYTO 9, STYO 13, SYTO 17, and SYTO 40, which stain all cells, paired with propidium iodide (PI), which stains cells with damaged membranes—for four bacterial species (*E. coli* O157:H7, *P. aeruginosa*, *P. syringae,* and *S. typhimurium*). Optimized instrument settings to minimize noise and signals from detritus were also determined. Two complete sets of data to enumerate the VBNC and VC stages of these bacteria in mid-log phase were obtained. In addition, cells were heat stressed and the methods were used to determine the fraction of live, dead (membrane-compromised), and culturable cells. By comparing results from pure cultures at mid-log phase, 1 to 64% of cells were VBNC, 40 to 98% were VC, and 0.7 to 4.5% had damaged cell membranes. When cells were heat treated, the method reliably determined the fraction of cells in each state. The specific methods most likely can be directly applied to other organisms, or modest modifications may be required for optimization.

Optimized FCM shows promise for rapid, unbiased detection of VBNC, VC, and those with compromised membranes. These methods for dye selection and noise reduction can be used to assess the response of organisms to environmental stressors.

S07-P426
Analysis of biofilm extracellular polymeric substance (EPS) diffusion by nuclear magnetic resonance (NMR)

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The primary objective of this research is to acquire Magnetic Resonance Microscopy (MRM) data for the predictive modeling of momentum and mass transport in biofilm systems and to determine structure-function relationships over a hierarchy of scales, from macroscale clusters to the molecular structure of the EPS hydrogel. MRM is a noninvasive, nondestructive tool able to access several observable quantities in biofilms, such as chemical composition [1, 2], diffusion [2, 3], and macroscale structure transport [4, 5]. The study presented here extends the work published earlier by Veeman *et al*. [2], where spectrally resolved diffusion was measured in biofilm. This study measures spatially resolved mass transfer in biomass, determines material content, and estimates the percentage of fast- and slow-diffusing components of specifically identified molecules. Using pulsed field gradient NMR techniques, the signal from free water is crushed in order to view the spectra of components such as carbohydrate, DNA and proteins. The diffusion data demonstrate that biofilm EPS contains both a fast and a slow diffusion component for the major constituents. The dependence of the diffusion on antimicrobial and environmental factors suggests that the polymer molecular dynamics measured by NMR are a sensitive indicator of the biofilm function.

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S07-P427
Effect of chlorite ion on nitrification

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Nitrification is the biologically mediated conversion of ammonia to nitrite and nitrate. It is a two-stage reaction. In the first stage ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite, which is the substrate for nitrite oxidizing bacteria (NOB). It is a major problem in the drinking water industry. In a recent survey two-thirds of the drinking water utilities that use chloramines for secondary disinfection in their distribution system report evidence of nitrification. Biological instability of finished water can arise from ammonia introduced via chloramine formation and released ammonia from chloramine decay. Nitrification can degrade water quality and can potentially impact compliance with the Safe Drinking Water Act (SDWA). Degradation in water quality can also affect the aesthetic quality of water and generate customer complaints due to unacceptable tastes, odors, and particulates in water.

A variety of techniques to control nitrification are in common use by utilities: raising the chloramine residual entering the distribution system, changing the chlorine to ammonia ratio, reducing chloramine demand by additional treatment, flushing, and breakpoint chlorination. Recent research has shown that the chlorite ion can control nitrification. In this project we investigated the effect of the chlorite ion on nitrification in a simulated household plumbing system (using both copper and PVC).

Four modified CDC reactors were used to simulate the household plumbing system. All reactors were fed with 0.71 ppm of NH_3. Water in these reactors was stagnant for eight hours and then flushed with new fresh water. Chlorite was introduced to this system at 0.2 ppm and gradually increased to 20 ppm. It was observed that chlorite is effective in inhibiting nitrification at high dosages only in a copper system, although the dose is far in excess of current regulations. There was little effect on the

parallel PVC system. The MPN method was also used to enumerate AOB and NOB. Chlorite was found to be more effective on NOB than on AOB.

S07-P428
A new method to study wound biofilms: The "drip flow reactor-colony biofilm model"

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Chronic wounds are an important world public health problem that has increased with the aging and obesity of the population. The presence of biofilms in wounds has been implicated in slow healing, which is related to suffering, disability, and a large burden on financial resources. Further biofilm research is needed to better understand its

impact on wound healing, so it has become important to develop models for growing relevant biofilms. The goal of this study was to develop a model that would mimic a wound biofilm, in which the biofilm collects nutrients for growth from below. This was accomplished using an association of two methods already in use for the study of chronic wounds biofilms: the colony model and the drip flow reactor (DFR).

The set-up consisted of polycarbonate porous membranes which were placed on Millipore absorbent pads inside a DFR, inoculated with a clinical wound isolate of *Staphylococcus aureus* and fed with 10%-strength tryptic soy broth. Several runs were performed to determine repeatability of results and to make any changes that were needed to further develop the model. Relatively thick biofilms (ca. 2 x 10^{13} CFU/membrane) were formed in this model system. Once the model was established, treatments were evaluated to determine their effect on the biofilm. These consisted of 0.25% Dakin's solution (5% hypochlorite in saline), a saline control solution, honey, and diclofenac sodium.

The new membrane-based model showed to be a reliable method by presenting repeatable results for control and treatment runs. As for the treatments tested, 0.25% Dakin's solution was the most effective, with approximately a 4 log reduction in viable bacteria.

S07-P430
Biofilm-enhanced deep subsurface sequestration of carbon dioxide

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Geologic sequestration of supercritical CO_2 into underground formations such as oil bearing formations, deep un-minable coal seams, and deep saline aquifers is one strategy to reduce the emission of greenhouse gases generated through the combustion of fossil fuels (1). Upward "leakage" of CO_2 could occur due to the primary permeability of the aquitard, through fractures, or near injection wells. In order to develop subsurface CO_2 storage as a viable engineered mechanism to reduce concentrations of atmospheric CO_2, it is imperative to develop methods to reduce CO_2 leakage and enhance carbonate mineral formation (2). We are investigating the utility of engineered subsurface biomineralizing biofilms as a mechanism to reduce the porosity and permeability of underground formations and thus CO_2 leakage. In the environment, natural cementation of geological formations occurs constantly over time due to physical, chemical and biological reactions (3). Biologically, the cementation or plugging process is often carried out by the precipitation of carbonate minerals by different bacterial strains. We intend to enhance and control such biomineralization processes by engineering subsurface biofilms that will precipitate solid phase calcium carbonate minerals ($CaCO_3$). Increasing the pH and production of HCO_3^- ions leads to $CaCO_3$ saturation and precipitation. This will be achieved by bacterial ammonification via ureolysis and will offer a controllable engineered strategy to (i) reduce the porosity and permeability of underground formations and (ii) provide a sacrificial mineral layer on well-bore concrete for protection from acidic groundwater (due to the dissolution of CO_2). In order to optimize the most effective single, binary or tertiary mixtures of ureolytic and non-ureolytic bacteria in specific media for engineering biomineralizing biofilms which will reduce CO_2 leakage, we are performing (i) static incubation experiments and (ii) pulsed flow experiments at atmospheric pressure to simulate flow through porous media as it would occur in the underground formations.

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S07-P431
Two PAS domain protein mutants suggest that both O_2 sensing and metabolism are important for biofilm formation in *Shewanella oneidensis* MR-1

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Shewanella oneidensis MR-1 is a versatile microorganism that utilizes a variety of electron donors and acceptors. It is important to understand the physiological responses of MR-1 in relation to the environmental stresses it may experience during bioremediation. The roles of two closely related sensory box proteins, SO3389 and SO0341, were assessed. Both ORFs contain PAS, PAC, GGDEF, and EAL domains, which have been implicated in multiple phenotypes; however, the physiological role(s) of proteins have not been fully established. Although SO3389 and SO0341 have similar domain architecture, both proteins appear to exhibit different physiological responses with respect to environmental stimulus. Initial experiments were conducted with LS4D, a minimal medium. Aerobic growth rates were similar for the two mutants and the wild type (WT). Motility assays showed impaired motility in SO3389, while SO0341 had similar motility as WT. Since these proteins contain PAS domains, experiments were performed to study the effect of oxygen on biofilm formation. Both SO3389 and SO0341 were affected in biofilm formation irrespective of rate of aeration. WT formed optimum biofilm at 150 rpm. Apart from biofilm production, pellicle formation was tested in minimal media. WT and SO0341 formed relatively the same amount of pellicle, while that in SO3389 appeared to be impaired. The redox indicator used in the minimal medium also indicated that SO3389 appeared to be metabolizing oxygen slower than WT and SO0341. The mutant SO3389 lagged for about 40 h when transferred from aerobic to anoxic medium, but its growth rate was similar to WT once growth was initiated. Interestingly, this result was not observed for SO0341 in that it appeared to be the same as WT. This is the first report to compare two proteins with similar domain architecture yet having different physiological responses. This is also the first report of a multi-domain PAS protein involved in biofilm formation. The data suggested that O_2 may be a major signal that is sensed by SO3389 and SO0341, but further work is needed to elucidate the respective signal(s) and the mechanism(s) of signal transduction.

S07-P432
Biofilm formation as a mycobacterial stress response

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Mycobacterium avium is an environmental organism and opportunistic pathogen with exceptionally well developed resistance to drugs, environmental stresses, and the host immune response. To adapt to these disparate conditions, *M. avium* must control its transcriptional response, reacting to various cues received in these situations. Since *M. avium* is also recognized to live in biofilm communities, we hypothesize that transcriptional analysis will provide insights into the adaptation of *M. avium* to biofilm growth. As a means of studying this, we have observed that the addition of AI-2 alters the phenotype of bacterial cultures from the planktonic to the biofilm mode of growth. To characterize the transcriptional adaptation associated with this phenotypic switch, we have used transcriptome analysis of *M. avium* in the presence or absence of AI-2, to identify informative changes in gene expression.

Planktonic *M. avium* cultures were incubated for three days with and without AI-2. The cells were harvested and total RNA was isolated for hybridization with whole-genome microarrays. For those genes that were significantly up- or downregulated after statistical analysis, primers were designed and qRT-PCR was used to investigate their regulation in biofilm RNA compared to planktonic RNA without AI-2.

Five genes were shown to be significantly upregulated in both biofilm RNA and in response to AI-2: the alkyl hydroperoxide reductases AhpC and AhpD, the trehalose synthase TreS, their transcriptional regulator OxyR and a conserved hypothetical protein. These genes are predicted to be involved in oxidative stress response and pathogenesis.

Mycobacteria react to a wide variety of stresses by expressing the same set of defense genes. All of the genes tested that are known to be involved in drug resistance, virulence and oxidative stress are upregulated in biofilms as well, which could indicate that biofilm formation is a mycobacterial stress response.

Standardized Biofilm Methods Laboratory projects

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The information was prepared by S. Goeres, B. Humphreys, and N. Beck will provide an update on a selection of projects currently in progress in the Standardized Biofilms Methods Laboratory (SBM). The intent is to provide examples of how the SBM modifies the *grow*, *treat*, *sample,* and *analyze* protocols for different applications. Specifically, Ms. Goeres will describe the experimental system and test methods she is using to determine the efficacy of treated piping material. Mr. Humphreys will review the methods he has used to determine the efficacy of a liquid germicide against a mixed consortium of bacteria grown in the CDC biofilm reactor, and Mr. Beck will provide an update on progress made in the standardization of using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to determine the number of active bacteria present in a *Pseudomonas aeruginosa* biofilm grown in the CDC biofilm reactor.

Biofilm-based wound care: A new hope

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Clinicians are failing to effectively treat the growing problem of chronic wounds. The planktonic paradigm has failed to provide us with effective treatments and it is time to examine new approaches to the management of chronic wounds.
The Southwest Regional Wound Care Center in Lubbock, Texas, has taken a biofilm-based wound care approach. The primary aim of biofilm-based wound care is to disrupt the biofilm in order to enable the host's immune system to heal the wound.

The basic strategies employed in biofilm-based wound care are as follows:

I) Alter the anatomy of the wound to favor the host response over the biofilm
II) Physically remove biofilm using frequent debridement
III) Consider antibiotic therapy
IV) Apply anti-biofilm and antimicrobial agents
V) Consider advanced wound management techniques
VI) Apply specific biocides

Using biofilm-based wound care strategies, the center recently achieved a rate of 67% healing in a group of patients meeting three core criteria for indicating surgical amputation in lower extremity wounds: critical ischaemia, diabetes mellitus, and osteomyelitis. Using biofilm-based wound care, there is new hope for healing "non-healable" wounds.