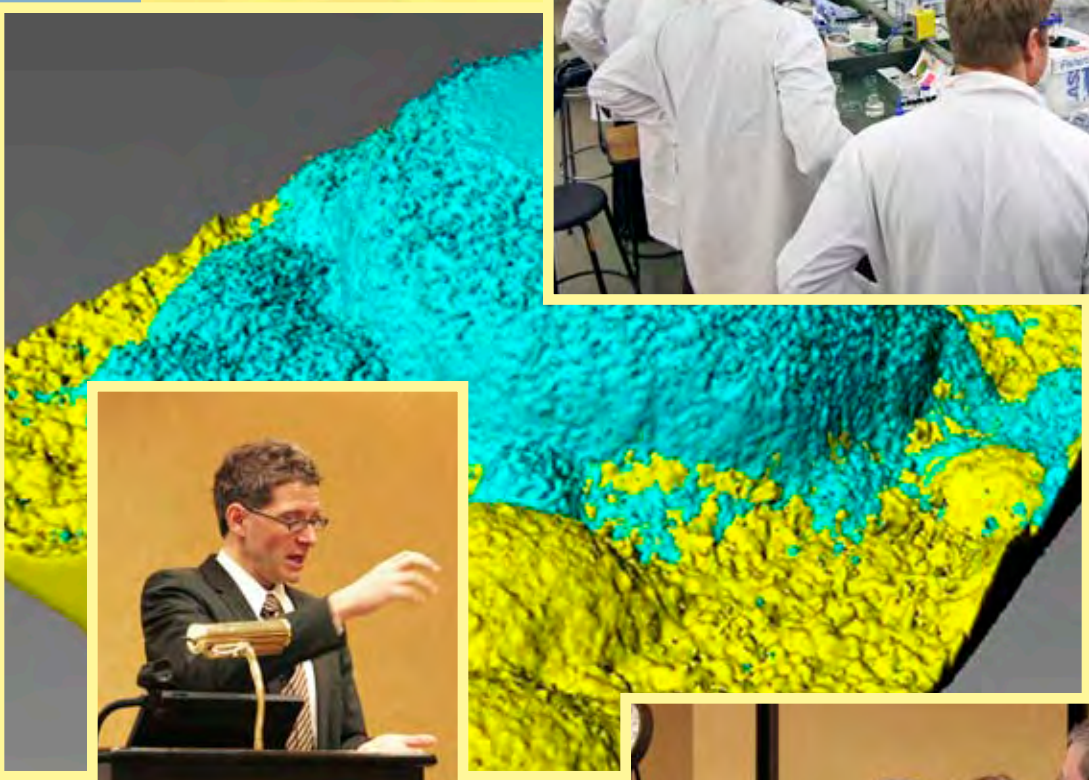
February
2008

PROCEEDINGS

Technical Advisory Conference



MONTANA STATE UNIVERSITY

■ Center for Biofilm Engineering

About the Center for Biofilm Engineering

CBE Leadership: Director and Executive Committee

***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.

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 primary 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: February 6–7, 2008

Keynote Presentation

Session 1: Industrial Biofilm Investigations

Session 2: Visualizing Biofilms

Session 3: Health Care-Related Biofilms

Poster Abstracts

CBE Technical Advisory Conference: February 6–7, 2008
Speaker Abstracts

Keynote Presentation

Biofilm dispersal: Molecular mechanisms to novel control strategies

Presenter: Jeremy S. Webb, BBSRC David Phillips Research Fellow
Affiliation: School of Biological Sciences, University of Southampton, UK

All organisms need to disperse to and colonize new environments. Studies of biofilm-forming bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa*, have revealed that microcolonies in mature biofilms undergo processes of cell lysis and dispersal. Characteristics of cells that disperse from biofilms include enhanced biofilm formation and phenotypic variability compared to pre-dispersal biofilm cells. We observed that accumulation of reactive oxygen and nitrogen species (RONS) in the interior of microcolonies triggers dispersal. The predominant RONS in *P. aeruginosa* microcolonies was peroxynitrite. Add-back of nitric oxide (the precursor of stable peroxynitrite) to *P. aeruginosa* biofilms induces a shift from biofilm to planktonic cell physiology and greatly increases the sensitivity to antimicrobials and dispersal of biofilm cells. These phenotypic changes appear to occur through NO-mediated interference with cyclic-di-GMP signaling pathways, which are known to regulate biofilm formation and dispersal in diverse bacteria. NO production, cell lysis, and dispersal are observed also in clinical cystic fibrosis *P. aeruginosa* isolates, and therefore NO-mediated biofilm dispersal may have implications for the control of CF biofilms. We have also shown that NO can induce detachment and dispersal of complex multi-species biofilms in industrial systems. Addition of NO chemical donors to biofilms formed in drinking water systems caused biofilm detachment from pipe materials. Thus, new understanding of the mechanisms of biofilm dispersal can reveal novel strategies for the manipulation of biofilms in medical and industrial settings.

SESSION 1: Industrial Biofilm Investigations

Overview of biofilm detachment mechanisms

Presenter: Brent Peyton, Professor, Chemical & Biological Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The removal of cells and cell products from an established biofilm is called biofilm detachment, and is one of the least understood processes affecting biofilm accumulation and activity. Biofilm detachment affects numerous industrial and natural systems and can result in the spread and subsequent re-establishment of problem biofilms by the seeding of clean downstream surfaces or products. Detachment processes that will be discussed include erosion, abrasion, and sloughing that result from a number of different mechanisms including growth, starvation, shear stress, cell signals, and response to environmental changes and biocides. Methods of observing and quantifying detachment rates have changed slightly over the years; however, quantitative models of detachment have evolved significantly from the "flat plate" models of the past. Future directions for detachment research will be suggested.

Biofilms in bioenergy and carbon sequestration

Presenter: Robin Gerlach, Associate Professor, Chemical & Biological Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Microbial biofilms have potential industrial applications in the conversion of renewable biomass into ethanol and other high value products, as well as in deep subsurface carbon dioxide sequestration. This presentation will highlight current efforts to develop biofilm reactors for lignocellulose conversion and deep subsurface biofilm barriers for the enhancement of carbon dioxide sequestration.

Bioenergy: The CBE and the MSU Thermal Biology Institute are pursuing the exploration and use of extremophilic microorganisms for the conversion of lignocellulosic materials (e.g., sawdust, woodchips, and switchgrass) into high value products such as biofuels and bio-plastics or precursors. Ethanol, butanol, organic acids, methane, and hydrogen are examples. Microbial enrichments from different areas in Yellowstone National Park have shown potential for cellulose and lignocellulose degradation. These enrichment communities have been characterized using molecular and culture-based tools and are currently being evaluated for their possible use in biobased-fuel and product development.

Carbon Sequestration: Biofilm barriers are being evaluated for their potential to assist in the geological sequestration of carbon dioxide (CO_2) in underground formations such as oil-bearing formations, deep un-minable coal seams, and deep saline aquifers. 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 to increase the mineralization (i.e., carbonate formation) after supercritical carbon dioxide ($scCO_2$) injection. We have evaluated the effect of $scCO_2$ on biofilm permeability and bacterial survival as well as strategies to enhance biologically induced carbonate mineral formation. $ScCO_2$ treatment has also been suggested as an alternative to other sterilization methods—such as steam, ethylene oxide, and gamma irradiation treatments—due to its potential compatibility with materials that are sensitive to heat, radiation, and strongly oxidizing agents. CO_2 is considered to be relatively non- toxic, chemically-inert, non flammable, and can be used in the absence of water.

Hence, the work to be presented is relevant to a wide range of research and development efforts in industry, academia, and national laboratories.

Biocontrol studies at Novozymes Biologicals

Presenter: Sarah C. McHatton, Senior Research Scientist, R&D
Authors: S.C. McHatton, I.M. Williams, E.D. Rhine, and K. Wood
Affiliation: Novozymes Biologicals, Inc., 5400 Corporate Circle, Salem, VA 24153

Bacteria in biofilm communities typically require higher concentrations of biocides for effective control than do planktonic species. These studies examine an alternative control strategy based on competitive inhibition by beneficial bacteria. *Bacillus* strains were investigated as potential biological control agents of planktonic and biofilm-associated *Pseudomonas* and *E. coli* using various methods optimized for selective enumeration of the target undesirable organism rather than the beneficial *Bacillus* candidate. Several *Bacillus* liquid cultures delivered to bored wells in agar demonstrated zones of clearing on *Pseudomonas* or *E. coli* lawns.

Another assay showed some *Bacilli* could inhibit quorum sensing pathways, as *Serratia* sp. pigmentation or *Vibrio* sp. luminescence was suppressed near wells with positive *Bacillus* candidates, even though the *Serratia* and *Vibrio* growth continued on the agar. A novel microtiter plate screen was developed and used to track fluorescence from a green fluorescent protein-producing *P. aeruginosa*, which diminished in the presence of various *Bacillus* isolates and consortia. Co-cultures of a specific *Bacillus* consortium and *P. aeruginosa* in test tubes with removable porcelain coupons showed a 4.3 log reduction in attached *P. aeruginosa* within 24 hours, compared to the axenic *P. aeruginosa* control. Furthermore, the degree of *Pseudomonas* control was dependent on

the magnitude of the *Bacillus* dose. Studies for up to 2 weeks in CDC biofilm reactors using another *Bacillus* strain vs. *Ps. aeruginosa* demonstrated greater than 1-log control of attached *Pseudomonas*. Results from these assays suggest that certain *Bacillus* spp. may be able to prevent or delay biofilm buildup by undesirable organisms. This environmentally friendly, non-chemical biocontrol solution could be applied in a variety of consumer and industrial settings where pathogen, slime, fouling, or corrosion control is needed.

Transcriptomics and molecular techniques for biofilm investigation in industrial systems

Presenter: Matthew W. Fields, Assistant Professor of Microbiology
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

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 at a systems level that constitute distinct physiological states. Transcriptomic and proteomic methods provide detailed analyses to probe the physiological states of a cellular population under specific growth conditions; these datasets can be compared to better understand the coordinated responses and possible linkages. The responses of a sulfate-reducing bacterium, *Desulfovibrio vulgaris*, to electron donor depletion, nitrite stress, chromium stress, and biofilm formation will be discussed.

SESSION 2: Visualizing Biofilms

A quantitative description at multiple scales of observation of accumulation and displacement patterns in single- and dual-species biofilms

Presenter: Anne Camper, Professor of Civil Engineering
Authors: Ben Klayman and Anne Camper
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

This presentation, based on data from Ben Klayman's recently completed PhD project, describes a novel approach: using confocal microscopy and image analysis for quantitatively assessing how bacterial microcolonies behave in a biofilm. One set of experiments used *Pseudomonas aeruginosa* labeled with a green fluorescent protein and *Escherichia coli* O157:H7 with a red fluorescent protein construct. The organisms were grown as single- and dual-species biofilms in 1mm glass capillary flow cells and were monitored over time using the confocal microscope. Colonization and biofilm development patterns were analyzed after 1) co-inoculation of the two organisms, and 2) sequential inoculation of the *E. coli* after *P. aeruginosa* biofilm development. Colonization and biofilm development patterns were associated with the fluid flow regime as evaluated using a finite element analysis (CFX™, ANSYS Europe, Ltd.). The shear stress varied from a minimum near the edges to a maximum in the center of the flow cell. Initial colonization by both species occurred at the outer edges of the flow cell (lowest shear). *P. aeruginosa* subsequently migrated toward the center of the flow path, but *E. coli* was never observed outside of the 200-micron outer edge. *E. coli* was not able to persist unless *P. aeruginosa* was present. Biovolumes of the two species were followed over time and analyzed using the Metamorph™ (Molecular Devices) image analysis program. *P. aeruginosa* reached a much higher final cell density along the entire surface (>99% of the total biovolume), while the 200-micron outer edges showed that *E. coli* occupied 50% of the total biovolume.

A second set of experiments utilized the same strain of *P. aeruginosa* labeled with either a yellow or cyan fluorescent protein. The two labeled organisms were added at the same concentration to the flow cell system. Accumulation and displacement behaviors of single clusters of each label were collected while the biofilm was developing (non-steady state). User script was written in Metamorph™ software to measure, over time, the volume and centroid locations of clusters and small pockets of cells of a different label within the clusters. From

these measurements the accumulation rates and displacement vectors were calculated. The distribution of cluster accumulation rates was bounded by the planktonic growth rate (upper bound) for small clusters and was frequently negative (indicating loss of biomass) for larger clusters. Expanding large clusters physically displaced neighboring cells and smaller clusters.

Recent advances in cellular automata modeling of multispecies biofilm systems

Presenter: Al Cunningham, Professor of Civil Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The CBE continues to conduct fundamental biofilm research in which observations of biofilm behavior made in flow cells (or batch reactors) are correlated with computational simulations using cellular automata (CA modeling). A cellular automata model is an array of computational "cells" programmed with a series of equations and rules (automata) that govern how the cells interact with one another. We present here a summary of the cellular automata modeling concept, followed by comparison of CA modeling results with experimental observations of a dual-species biofilm composed of *Pseudomonas aeruginosa* challenged by *Escherichia coli.* Model simulations are presented visually, using time-lapse videos, as well as quantitatively. The CA model considers biofilm and fluid processes at the "single cell" scale. For example, a typical CA modeling exercise will simulate biofilm processes occurring over a surface area of 300 by 300 microns. Therefore, additional simulation of hydrodynamics and mass transport must be added in order to bring the effects of system geometry (i.e., pipe flow or aquifer flow) into the simulation. An example of this type of scale-up will also be presented.

The session will conclude with a discussion of possible applications of CA modeling to industrially relevant biofilm systems.

Fluorescence hyperspectral imaging of biofilms*

Presenter: Howland D.T. Jones, Member of Technical Staff
Affiliation: Sandia National Laboratories, Albuquerque, New Mexico, USA
*****Sandia is a multi-program laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy under Contract DE-ACO4-94AL85000.

Hyperspectral imaging combined with multivariate analysis techniques, such as multivariate curve resolution (MCR), has proven to be a powerful imaging tool for imaging and attaining quantitative data on many biological samples, including biofilms. Whereas overlapping fluorophores are problematic for many traditional fluorescence microscopy techniques, this technique allows us to separate many overlapping fluorophores and create interpretable quantitative images from both known and unknown biological samples. MCR provides a relative quantitative analysis of the hyperspectral image data without the need for standards, and it discovers all the emitting species present in an image, even those in which we have no *a priori* information. We have designed and built two hyperspectral imagers for the support of many biological investigations at Sandia. The first imager was originally built to scan and quantify multiple fluorophores on DNA microarray slides; however this imager is useful for many applications in which large areas need to be scanned and imaged, e.g., biofilms on water-treatment membranes. The other imager is a 3D confocal hyperspectral fluorescent microscope that was developed to enhance our research into a variety of biological systems (e.g., host-pathogen interactions in innate immune cells, investigating photosynthesis in cyanobacteria, and understanding plant tissue systems for biofuel applications). In this presentation, I will discuss the methods we use to generate relative quantitative images from our hyperspectral image data sets and I will illustrate the benefits of hyperspectral imaging by showing several examples of biological images we have collected. I will also discuss our efforts to reduce biofouling on reverse osmosis membranes in order to improve industrial water treatment procedures for clean drinking water. One challenge with imaging fouled membranes is the high degree of auto-fluorescence emanating from the membrane

itself. The auto-fluorescence is similar in intensity to the labeled biofilm components and spectrally overlaps the fluorophores used to selectively stain the biofilm microorganisms. This auto-fluorescence complicates traditional filter-based fluorescent microscopes from separating features due to membrane from those resulting from the biofilm itself. Combining our hyperspectral imager with our fast and efficient MCR algorithms allows us to separate these overlapping fluorophores and create interpretable relative quantitative images, thereby providing an increased understanding of the membrane fouling process itself.

Direct visualization of antimicrobial action within model oral biofilms

Presenter: Phil Stewart, Center for Biofilm Engineering
Authors: Shoji Takenaka[1], Harsh Trivedi[2], Betsey Pitts[3], Audrey Corbin[3], and Phil Stewart[3]
Affiliations: [1]Niigata University, Japan; [2]Colgate-Palmolive; [3]Center for Biofilm Engineering, Montana State University, Bozeman, MT

A microscopic method for non-invasively visualizing the action of an antimicrobial agent inside a biofilm was developed and applied to describe spatial and temporal patterns of mouth rinse activity on model oral biofilms. Three-species biofilms of *Streptococcus oralis*, *Streptococcus gordonii*, and *Actinomyces naeslundii* were grown in glass capillary flow cells. Bacterial cells were stained with the fluorogenic esterase substrate Calcien AM (CAM). Loss of green fluorescence upon exposure to an antimicrobial formulation was subsequently imaged by time-lapse confocal laser scanning microscopy. When an antimicrobial mouth rinse containing chlorhexidine digluconate was administered, a gradual loss of green fluorescence was observed that began at the periphery of cell clusters where they adjoined the flowing bulk fluid and progressed inward over a time period of several minutes. Image analysis was performed to quantify a penetration velocity of 4 microns per minute. An enzyme-based antimicrobial formulation lead to a gradual, continually slowing loss of fluorescence in a pattern that was qualitatively different from the behavior observed with chlorhexidine. Ethanol at 11.6% had little effect on the biofilm. None of these treatments resulted in removal of biomass from the biofilm.

Most methods to measure or visualize antimicrobial action in biofilms are destructive. Spatial information is important because biofilms are known for their structural and physiological heterogeneity. The CAM staining technique has the potential to provide information about the rate of antimicrobial penetration, the presence of tolerant subpopulations, and the extent of biomass removal effected by a treatment.

SESSION 3: Health Care-Related Biofilms

Bacterial biofilms in chronic rhinosinusitis (CRS)

Presenter: Martin Desrosiers, MD, FRCSC, Associate Clinical Professor, Department of Otolaryngology – Head and Neck Surgery
Affiliation: Université de Montréal and McGill University, Montréal, Québec, Canada

Chronic rhinosinusitis (CRS) is among the three most common chronic diseases in North America, affecting approximately 31 million people in the United States each year. Over 400,000 surgeries for this condition are performed annually in the US, making it one of the most common surgical procedures. Of particular concern to us are the 10-50% of patients who fail to obtain long-lasting relief with surgery. The management of these patients remains difficult because the pathophysiology and the relative contribution of each of the various factors involved in this disorder have not been clearly established. The role of bacteria in this disorder has been difficult to understand as bacteria have been cultured in only 50% of patients undergoing primary endoscopic sinus surgery (ESS). Additionally, the flora recovered is different from that in acute bacterial sinusitis, with high recovery rates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Bacterial biofilms may provide some answers to this perplexing clinical problem. Our group has previously demonstrated a poor outcome in post-ESS patients

harboring *S. aureus* or *P. aeruginosa* with the capacity to form a biofilm in-vitro. As part of this presentation, I present our experience with in-vitro assessment of a number of topical therapy modalities targeting bacterial biofilms grown from these clinical isolates taken from individuals with hard-to-treat sinus disease. As expected, bacterial biofilms are resistant to antibiotics at concentrations found after oral administration. However, antibiotics at concentrations 1000x minimally inhibitory concentration (MIC) are capable of killing bacteria in biofilm form. Of interest, however, is that other strategies targeting the biofilm matrix by chemical or mechanical means can also be effective in reducing bacterial biofilms in these in-vitro models.

Summary: Bacterial biofilms represent a probable contributing factor to the development and persistence of CRS and as such represent an interesting target for further investigation and therapy. Topical therapies represent an interesting treatment modality for bacterial biofilms involved in CRS, thus strategies involving enhanced bacterial killing and mechanical or chemical disruption of biofilms are likely to play a role in this developing area.

Disclosure: Financing of these projects was obtained from Foundation Antoine Turmel, a non-profit philanthropic foundation supporting medical research and from MedtronicXomed.

Consultant: MedtronicXomed, Bayer Global, ALLUX Medical

Molecular characterization of wound biofilm communities

Presenter: Patrick Secor, PhD Candidate, Cell Biology
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Our current understanding of the bacterial flora of chronic wounds is primarily based on results from traditional culture-based characterization methods. These methods may be biased towards bacteria that readily grow under laboratory conditions. We characterized wound samples by amplification, using several different primers and sequencing of bacterial 16S ribosomal RNA genes from DNA extractions of chronic wound debridement specimens. DNA from 31 individual wound specimens was amplified and sequenced. Of the 31 specimens analyzed, 12 were found to harbor *Pseudomonas aeruginosa*, while *Staphylococcus aureus* was found in 10 of the specimens. Additionally, 19 different strictly anaerobic species of bacteria were found in these specimens. In a separate study, DNA extractions from 10 specimens of each of three types of chronic wounds (venous leg ulcers (VLU), diabetic foot ulcers (DFU), and pressure ulcers (PU)) were pooled and analyzed using three different methods of molecular analyses. These methods included cloning and shotgun sequencing, denaturing gradient gel electrophoresis (DGGE), and pyrosequencing. Overall, the results indicated VLU harbored primarily Gram-negative rods, DFU contained aerobic and anaerobic Gram-positive cocci, and PU contained primarily Gram-positive anaerobic cocci. Generally, results with the three different analysis methods agreed. Further development of molecular tools promises to improve our understanding of wound biofilms as well as aid in the diagnosis and treatment of wound infections.

Biofilm wound infection in a diabetic (db/db) mouse

Presenter: Phillip Hochwalt
Authors: Phillip Hochwalt[1], Ge Zhao[1], Robert Underwood[1], Marcia Usui[1], Pradeep Singh[2], Garth James[3], Philip Stewart[3], John Olerud[1] and Philip Fleckman[1]
Affiliations: Departments of [1]Medicine (Dermatology) and [2]Microbiology, University of Washington, Seattle, WA; and [3]Center for Biofilm Engineering, Montana State University, Bozeman, MT

Chronic wounds are a major source of morbidity and mortality in patients with diabetes, immobility, and venous stasis. Microbial biofilm is present in the chronic wounds of many of these patients. We hypothesize that the presence of biofilm may be a key factor in impairing wound healing. The microorganisms present in biofilm are

protected from topical antiseptics, systemic antibiotics, and host defenses. For this reason, biofilm infections persist and are extremely difficult to treat. Systematic study of chronic ulcers is challenged by the lack of standard chronic wound models.

Our goal is to create a reproducible chronic wound by application of biofilm to an already well-characterized diabetic (db/db) mouse wound. Bacterial biofilm was developed by incubating planktonic *Pseudomonas aeruginosa* (PAO-1) and transferring to polycarbonate membrane filters placed on LB agar plates. The biofilm (~10^8 CFU) was transferred from the filters to 6mm wounds created on the dorsal skin of diabetic mice and the wounds were covered with Tegaderm® dressing. The biofilm was transferred to the wounds at different time points following wounding. If biofilm was transferred soon after wounding, the mice died. However, mice that were allowed to recover from the surgery before infection with biofilm developed purulent wounds that persisted for several days. In addition, the timing of Tegaderm® application proved to be critical to the formation of purulence and wound persistence. Ultimately, the development of a reproducible chronic wound infection in a diabetic mouse will allow *in vivo* testing of potential anti-biofilm therapies.

Staphylococcus aureus* biofilms prevent scratch wound closure *in vitro

Presenter: Kelly Kirker, Research Scientist, Medical Biofilm Laboratory
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Chronic wounds are characterized by prolonged inflammation, an altered wound matrix, and the failure to re-epithelialize. Chronic wounds are also characterized as supporting a diverse microbial flora. A literature review by Bowler examined culture data from 62 published studies dating between 1969 and 1997[1]. The most predominant wound isolate was *Staphylococcus aureus* (reported in 63% of the studies), followed by coliforms (45%), *Bacteroides* spp. (39%), *Peptostreptococcus* spp. (36%), *Pseudomonas aeruginosa* (29%), *Enterococcus* spp. (26%), and *Streptococcus pyogenes* (13%)[1]. It has been speculated that bacteria colonizing chronic wounds exist as biofilm communities[2-4]; however, there few data illustrating the role of biofilms in chronic wound pathogenesis. This study establishes a novel method for studying the effect of biofilms on cultured cells. Furthermore, it specifically examines the influence of *S. aureus* biofilms on keratinocyte migration.

Co-cultures of *S. aureus* biofilms and primary human keratinocytes (HKs) were created by initially growing *S. aureus* biofilms on tissue culture inserts (with a 0.2 µm membrane), then transferring the inserts with fully formed biofilms to existing HK cultures. This method allowed diffusible factors produced by the biofilm to pass into the cell culture medium while excluding the bacteria themselves. A wound model was developed by initially scratching the confluent HK culture with a plastic pipette tip prior to the biofilm application. At various time-points HK cultures were imaged and analyzed to monitor wound closure. Control HK cultures contained no biofilm inserts.

The effect of biofilm exposure was evident after 24 hours. Wounds in control cultures were 68.7±5.1% closed while biofilm-exposed cultures were 7.3±4.7% closed. These differences were significant ($P<0.001$). Differences were even more pronounced as the assay time continued. By 72 hours, wounds in control cultures had closed, while wounds in biofilm-exposed cultures had expanded (100% vs. -14.6±16.3% closed, $P<0.001$). This study demonstrates that biofilms inhibit keratinocyte wound closure *in vitro*. Furthermore, the model developed may prove useful for the evaluation of anti-biofilm therapies.

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Production of cell-cell signaling molecules by chronic wound bacteria

Presenter: Alex Rickard, Assistant Professor of Biological Sciences
Authors: Katelyn R. Colacino[1], Katherine Zander[1], Elinor deLancey Pulcini[2], Daniel Rhoads[3], Randall Wolcott[3], Garth A. James[2] and Alexander H. Rickard[1]
Affiliation: [1]Department of Biological Sciences, Binghamton University, Binghamton, NY
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[3]Southwest Regional Wound Care Center, Lubbock, TX

Chronic wounds such as diabetic foot ulcers and decubitis ulcers (bed sores) support multi-species biofilms. Species within wound biofilms likely interact with one another to maintain their niche and limit wound healing. How the species interact and minimize wound healing has yet to be determined. One possibility is that autoinducer molecules, which mediate cell-cell signaling between bacteria, coordinate the activity of species within wound biofilms. Two types of autoinducers include autoininducer-1 (AI-1, a N-(D-3-hydroxybutanoyl)-homoserine lactone) and autoinducer-2 (AI-2, 4,5-dihydroxy-2,3-pentanedione). These molecules can help coordinate group behavior, mediate cellular responses to environmental stress, and also allow bacterial cells to negate host defense mechanisms. Thus, is it is the aim of the work reported here to determine if cell-cell signal molecules are produced by bacteria isolated from human chronic wounds. Based upon differences in colony morphology, 21 strains were isolated from chronic wound debridements. Strains were identified to the genus or species level by biochemical identification at a CLIA-certified microbiology laboratory and complete 16S rRNA gene sequencing. To determine if the strains produce autoinducer molecules, 96-well bioluminescence assays were performed that utilized the AI-1 detecting strain *V. harveyi* BB886 and the AI-2 detecting strain *V. harveyi* BB170. Amounts of AI-1 and AI-2 produced by the strains were determined by comparing exponential and stationary phase spent media to filtered unused media (media control) and filtered spent media from *V. harveyi* BB120 and *V. harveyi* BB152 (autoinducer positive controls). All 21 wound strains were identified, and these belonged to seven genera. Based upon the *V. harveyi* bioluminescence assays, none of the 21 chronic wound strains produced AI-1. All produced AI-2, and inter- and intra-species differences in the amounts of AI-2 produced were detected. Furthermore, AI-2 concentrations were growth-phase dependent. *Escherichia coli* CWI269 and *Pseudomonas* sp. CWI174 produced the highest concentrations of AI-2 (>125-fold induction) while *Acinetobacter baumannii* CWI177 and *P. mirabilis* CWI266 produced the least (approx. 50-fold induction). In conclusion, biofilm bacteria from chronic wounds produce AI-2. Implications from our findings will be discussed.

Poster Abstracts

CBE Poster #363

Date: 07/2007
Title: **Anti-biofilm properties of chitosan-coated surfaces**
Authors: R. Carlson, R. Taffs, A. Hedegaard, W. Davison, and P. Stewart
Affiliation: Center for Biofilm Engineering and the Department of Chemical & 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.

CBE Poster #380

Date: 06/2006
Title: **Direct visualization of spatial and temporal patterns of antimicrobial action inside model oral biofilms using two-photon laser microscopy**
Authors: S. Takenaka[1], B. Pitts[2], W. Davison[2], B. Unterreiner[2], and P. Stewart[2]
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Sponsor: Colgate-Palmolive

A microscopic method for non-invasively visualizing the action of an antimicrobial agent inside a biofilm was developed and applied to describe spatial and temporal patterns of mouth rinse activity on model oral biofilms. Three-species biofilms of *Streptococcus oralis*, *Streptococcus gordonii*, and *Actinomyces naeslundii* were grown in glass capillary flow cells. Bacterial cells were stained with the fluorogenic esterase substrate Calcien AM (CAM). Loss of green fluorescence upon exposure to an antimicrobial formulation was subsequently imaged by time-lapse confocal laser scanning microscopy. When an antimicrobial mouth rinse containing chlorhexidine digluconate was administered, a gradual loss of green fluorescence was observed that began at the periphery of cell clusters where they adjoined the flowing bulk fluid and progressed inward over a time period of several minutes. Image analysis was performed to quantify a penetration velocity of 4 microns per minute. An enzyme-based antimicrobial formulation lead to a gradual, continually slowing loss of fluorescence in a pattern that was qualitatively different from the behavior observed with chlorhexidine. Ethanol at 11.6% had little effect on the biofilm. None of these treatments resulted in removal of biomass from the biofilm.

Most methods to measure or visualize antimicrobial action in biofilms are destructive. Spatial information is important because biofilms are known for their structural and physiological heterogeneity. The CAM staining technique has the potential to provide information about the rate of antimicrobial penetration, the presence of tolerant subpopulations, and the extent of biomass removal effected by a treatment.

CBE Poster #393

Date: 07/2007
Title: **To build a microbial factory: Investment cost and operating cost analysis of metabolic networks**
Authors: R. Carlson
Affiliation: Center for Biofilm Engineering and the Department of Chemical & Biological Engineering, Montana State University, Bozeman, MT
Sponsor: NSF EPSCoR

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.

CBE Poster #397

Date: 02/2007
Title: **Development of a rapid biofilm analysis kit**
Authors: A. Hilyard, S. Goeres, N. Beck, A. Cunningham, D. Walker, B. Warwood, and D. Goeres
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsor: Montana Board of Research and Commercialization Technology

The Center for Biofilm Engineering at Montana State University and the Montana-based company BioSurface Technologies, Inc. (BST) have collaborated to develop a rapid biofilm analysis kit. In certain field applications—for instance a distribution system—biofilm needs to be monitored and treated with anti-biofilm products. Viable plate counts (VPCs) are commonly used to monitor bacteria in the field. However, VPCs have drawbacks: they require at least 24 hours to produce results, they require a basic knowledge of microbiology and aseptic techniques, and they are known to underestimate the actual number of active bacteria in industrial and environmental systems. A biofilm analysis kit that is inexpensive, simple, rapid, repeatable, accurate, and field applicable would fill a gap in the current market.

The biofilm analysis kit in development uses the tetrazolium salt CTC (5-cyano-2,3-ditolyl tetrazolium chloride). Active bacteria take up CTC and reduce it to a fluorescent, insoluble crystal. Epifluorescent microscopy or flow cytometry are used to count the total number of bacteria with crystals. A second approach is to elute the crystals into solution using ethanol and read the color intensity on a spectrophotometer. This kit will employ the CTC elution technology to determine numbers of active bacteria present in a sample. The development of the kit is a two-phase project. During the first phase (completed), a CTC protocol was optimized for analyzing the number of

active bacteria in a *Pseudomonas aeruginosa* ATCC 700888 biofilm, grown according to ASTM method E2562-07 in the CDC reactor. The second phase includes making the technology field compatible.

The four main questions asked during phase one were:

- Which CTC concentration and time result in the lowest standard deviation?
- What is the smallest biofilm density CTC can detect?
- What materials are most compatible with CTC?
- How does the estimated percent kill in biofilm treated with chlorine compare for the CTC elution method and VPC?

The data showed that an increase in time or CTC concentration increased absorbance readings, resulting in a positive linear correlation. However, it is unknown if data will remain linear beyond the observed times and concentrations. There was not a convincing argument that one combination of time and concentration produced a smaller standard deviation than any of the other combinations. The CTC elution method was not able to detect a six $\log_{10}$ (cfu/cm^2) *Pseudomonas aeruginosa* biofilm grown with 3 mg TSB/l during continuous flow, but was able to detect a seven $\log_{10}$ (cfu/cm^2) biofilm grown with 30 mg TSB/l. CTC stained Teflon and stainless steel the least. For the efficacy testing, the CTC elution method estimated a 97% kill compared to the 99.99% kill found using the viable plate count method. This difference in percent kill is attributed to the CTC method's high limit of detection. The remainder of the project will focus on developing the kit's hardware.

CBE Poster #411

Date: 03/2007
Title: **Biofilm enhanced geologic sequestration of supercritical CO_2**
Authors: A. Cunningham, R. Gerlach, A. Phillips, G. James, R. Hiebert, and L. Spangler
Affiliation: Center for Biofilm Engineering and Montana State University, Bozeman, MT
Sponsor: US Department of Energy, Award No. DE-FC26-04NT42262

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 that 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 to 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 ($\geq$ 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.

CBE Poster #414

Date: 05/2007

Title: **PCR/DGGE using "universal" 16S rDNA primers has limited resolution for soil bacterial community analysis**

Authors: M. Burr, J. Faulwetter, A. Camara, A. Nocker, S. Clark, and A. Camper

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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 50 cm square. Two 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 an 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. The profiles were indistinguishable when universal primers were used, 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.

CBE Poster #415

Date: 07/2007

Title: **Deciphering cDCE degradation pathways in *Polaromonas* sp. strain JS666**

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Sponsor: Department of Defense, ESTCP, NSF Graduate Research Fellowship

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, 2-D 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 proteins identifications from the 2D-GE experiments.

Center Poster #416

Date: 07/2007
Title: **Visualization of antimicrobial action in biofilms**
Authors: W. Davison and P. Stewart
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

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 actions 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 and then 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.

CBE Poster #418

Date: 07/2007
Title: **Development of fluorescent reagent combinations specific to biofilm components**
Authors: B. Pitts[1], D. Gray[2], and P. Stewart[1]
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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.

CBE Poster #419

Date: 07/2007

Title: **Structural role for flagella in biofilm formation and stability in *Desulfovibrio vulgaris* Hildenborough**

Authors: M. Clark[1,2], Z. He[3], M. Duley[1], G. Zane[4], R. Edelmann[1], J. Zhou[3∫], J. Wall[4∫], and M. Fields[2,5∫]

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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 and biofilms were allowed to form on glass slides. Wild-type cells were motile and formed a continuous mono-layer of cells on the glass as observed via crystal violet staining and SEM. Initial results indicate that Δ*fla*G mutants are motile, while ΔMP and Δ*fli*A mutants are less motile or not motile. Significant amounts of carbohydrate were not measured within the biofilm (0.01μg hexose sugar per μg protein) and biofilms stained with calcofluor white, Concanavalin A, and congo red revealed little external carbohydrate (e.g., EPS) within the wild-type *D. vulgaris* biofilm. TEM analysis of wild-type biofilms grown on SiO_2 grids also showed little EPS, but '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. Negligible amounts of carbohydrates were present 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 more on a proteinacous material to form and maintain its biofilm matrix and that flagella, or a modified form of flagella, may play an important role in not only initial formation of *D. vulgaris* biofilm but also in biofilm stability.

CBE Poster #420

Date: 07/2007

Title: **Changes in microbial community structure during biostimulation for uranium reduction at different levels of resolution**

Authors: C. Hwang[1,7], W.-M.Wu[2], T. Gentry[3], J. Carley[4], S. Carroll[4], D. Watson[4], P. Jardine[4], J. Zhou[5∫], C. Criddle[2], and M. Fields[6,7∫]

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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 and than 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 to decrease 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, and 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.

CBE Poster #421

Date: 07/2007
Title: **Manipulating the mechanical properties of biofilm**
Authors: M. Sutton and P. Stewart
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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- to 6-fold increase in their elastic and viscous coefficients. The trivalent cation $FeCl_3$ had a 12- to 60-fold increase in its elastic coefficients. The samples treated with urea show a clear weakening of the biofilm, with a 1.3- to 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.

CBE Poster #422

Date: 07/2007
Title: **How susceptible to chlorine disinfection are detached biofilm particles?**
Authors: S. Behnke and A. Camper
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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 concentrations that are 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.

CBE Poster #424

Date: 07/2007
Title: **Constructed wetland rhizosphere microbial community analysis using group-specific primers and denaturing gradient gel electrophoresis**
Authors: J.L. Faulwetter, M.D. Burr, O.R. Stein, and A.K. Camper
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsor: USDA

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 is obtainable.

CBE Poster #426

Date: 09/2007
Title: **Magnetic resonance microscopy diffusion study of biofilm EPS**
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From water service utilities to pharmaceutical processing, biofilms occur and create havoc in almost every water-based industrial process. Thus, there is an urgent need to better understand the internal behavior, transport, and activities of these biofilms. 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 and nondestructive tool able to access several observable quantities in biofilms, such as chemical composition[1,2], diffusion[2,3], and macroscale structure and transport[4-7]. 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 specific spectral peaks. 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 slow-diffusion component for the major constituents. The dependence of the diffusion on antimicrobial and environmental factors suggests the polymer molecular dynamics measured by NMR are a sensitive indicator of the biofilm function.

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CBE Poster #430

Date: 07/2007
Title: **Biofilm enhanced deep subsurface sequestration of carbon dioxide**
Authors: L. Wheeler, S. Biebel, L. Schultz, and R. Gerlach
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsor: Zero Emissions Research and Technology (ZERT)

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 by 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, (ii) pulsed flow experiments at atmospheric pressure to simulate flow through porous media as it would occur in the underground formations, and (iii) continuous flow experiments at atmospheric pressure to monitor the effect of continuous flow on crystal formation.

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CBE Poster #431

Date: 07/2007

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

Authors: A. Sundararajan[1], L. Creighton[2], T. Yan[3], D.M. Klingman[3], J. Zhou[4], and M.W. Fields[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 and have homologs in other *Shewanella* species, 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 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, and ORP data also suggested that △SO3389 utilized oxygen at a slower rate. 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. 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.

CBE Poster #433

Date: 07/2007
Title: **Just say NO (Nitric Oxide) to biofilms**
Authors: C.C. Miller[1], L.L. Boegli[2], B.B. McMullin[1], L.J. Bickle[2], and G.A. James[2]
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Sponsor: Nitric Bio Therapeutics

Biofilms consist of microbial communities attached to surfaces and have been implicated in a variety of chronic infections. Biofilm bacteria are more tolerant to antimicrobial agents than free-floating bacteria. *In vitro* experiments have demonstrated that exogenous gaseous nitric oxide (gNO) was an effective antimicrobial agent against a wide range of free-floating bacteria. However, it was unknown whether gNO would be as effective against bacterial biofilms. This study evaluated the efficacy of gNO treatment against *Staphyloccocus aureus* biofilms. The biofilms were grown with a wound isolate of *S. aureus* using a CDC Biofilm Reactor. Treatments of 200 parts per million (ppm) or 1,600 ppm gNO were applied to the biofilms for periods of 24 and 2 hours, respectively. Culturable bacteria were enumerated using plate count techniques. Treatments with 200 ppm resulted in a mean log reduction of 4.6 after 24 hours, which was significantly greater ($p=0.012$) than control treatments. Treatments with 1600 ppm resulted in a mean log reduction of 6.9 after 2 hours. Confocal scanning laser microscopy of biofilms prepared using the LIVE/DEAD® BacLight™ bacterial viability kit indicated increasing proportions of red (dead) cells with treatment time and dosage. Scanning electron microscopy of treated biofilms suggested cell envelope damage had occurred to the bacteria. Overall, the results indicated that gNO was an effective treatment for killing *S. aureus* biofilms. Coupled with recent successful case studies using gNO for the treatment of refractory wounds, these data suggest further efficacy and safety trials are warranted for this novel antimicrobial treatment for wound therapy.

CBE Poster #435

Date: 03/2007
Title: **Molecular analysis of chronic wound biofilms**
Authors: P. Secor[1], E. deLancey Pulcini[1], R. Wolcott[2], G. James[1], and P. Stewart[1]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT;
[2]Southwest Regional Wound Care Center, Lubbock, TX
Sponsors: National Institutes of Health, Southwest Regional Wound Care Center, INBRE

Chronic wounds are a frequently encountered problem in elderly and bedfast patients and are produced by trauma or pathologic insult. Chronic wounds include a loss of skin or underlying tissue and do not heal with conventional treatment. The main focus of this research was the identification of the different bacterial species that form biofilms in chronic wounds using several different molecular techniques. Research has shown that not all bacterial species can be cultured in the laboratory; so the vast majority of bacteria in chronic wounds may not be detected by simple culture tests. Initial molecular analysis revealed chronic wounds harbored diverse bacterial communities including strictly anaerobic genera that were not detected using culture methods. Fresh debridement samples were provided from different areas of the wound bed to analyze the spatial heterogeneity of wound biofilms. DNA was extracted from the debridement samples, and primers that targeted the conserved regions of the bacterial 16S ribosomal subunit gene were used to amplify segments of the 16S genes using the polymerase chain reaction (PCR). DNA extracted from the edge of the wound bed was compared to the DNA extracted from the center of the wound bed. DNA extracted from debrided tissue from the same wound at different times during the course of treatment was also evaluated. The amplified 16S DNA population was then analyzed using denaturing gradient gel electrophoresis (DGGE). This method provided the means to estimate the diversity of the microbial populations inhabiting these chronic infections. The PCR products were also cloned and sequenced to determine the species present in the wound ($n=97$). The specificity of different primer sets was also taken into consideration. Some primer sets gave poor results (34.7% specificity towards the 16S gene) while others were

much more effective (79.2% specificity). The genes that were amplified with the less stringent primers included human, mouse, canine, insect, and plant genes, while the genes pulled out with the more specific primers described species present in the wound samples. These results indicate that the use of molecular based methods may prove to be a useful diagnostic tool to assess wounds for the presence of bacterial species not usually detected using traditional culture based methods. Overall, this study confirmed the presence of complex and diverse microbial communities in chronic wound biofilms.

CBE Poster #437

Date: 01/2008
Title: **A study of inhibition and biosorption of select metals with a sulfur-oxidizing, moderately thermophilic acidophile**
Authors: J. Aston[1] and B. Peyton[1], B. Apel[2], and B. Lee[2]
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Sponsors: MSU IGERT, INL (Idaho National Laboratory) and Montana EPSCoR

Background: *Acidithiobacillus caldus* is an acidophilic bacterium that oxidizes reduced sulfur compounds as an energy source. *At. caldus* is ubiquitous to bioleaching environments, where it leaches metals by oxidizing metal sulfides. To date, few studies have studied interactions between *At. caldus* and its environment. Organic acids are often produced by microorganisms and may cause de-coupling in low pH environments where *At. caldus* thrives. In addition, metals are known to interfere in enzyme and DNA function.

Methods: The inhibitory effects of several organic acids were examined. In addition, effects of the metals lead, zinc, and copper were determined. Cultures were grown in triplicate at 45 °C, pH 2.5, and shaken at 150 rpm. Sodium tetrathionate was added as an electron donor (1 g/L) in an aerobic, defined medium. Organic acids or metals were added in varying concentrations. Inhibition was characterized by changes in specific growth rates, determined via direct cell counts. The concentrations at which 50% inhibition was observed were calculated (IC50). Concentrations of organic acids were measured using capillary electrophoresis. ICP-MS was used to quantify metal concentrations during growth. Acid, metal, and cell-free controls were used as appropriate.

Results: Oxaloacetate had the lowest IC50 at 28 ±1.5 μM. Malate had the highest IC50 at 84 ±7.2 μM. IC50's of 39 ± 2.5μM, 180 ± 3.7 μM, and 2370 ± 130 μM were observed for lead, zinc, and copper respectively.

Conclusion: All organic acids and metals tested exhibited an inhibitory effect. Oxaloacetate exhibited the highest level of inhibition of the organics tested. Of the metals tested, it appeared that *At. caldus* is relatively tolerant to high copper concentrations. The results of this research lead to a better understanding of microbial processes in biomining environments, and may help to increase the efficiency of metal leaching.

CBE Poster #438

Date: 01/2008
Title: **Transcriptional response of *P. aeruginosa* biofilms to antibiotic treatment**
Authors: J. Folsom and P. Stewart
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsor: National Institutes of Health

Very little information is available on the effect of antibiotic treatment on the gene expression of *Pseudomonas aeruginosa* biofilms. This information is useful for identifying possible targets for the prevention or elimination

of these biofilms. We have performed microarray analysis on *P. aeruginosa* drip flow biofilms in an effort to identify potential targets for control efforts. Biofilms were grown in drip flow reactors using minimal media for 3 days prior to exposure to ciprofloxacin or tobramycin. After 12 hours of exposure, biofilms were harvested by scraping and RNA was extracted. Affymetrix *P. aeruginosa* microarrays were used to measure the response to these antibiotics. Ciprofloxacin significantly ($\alpha<0.02$) affected the transcription of 181 genes, with tobramycin affecting ($\alpha<0.02$) 296 genes. The two antibiotics shared an effect on 103 genes, with only one gene (PA0085) being expressed in an opposing manner. There were 78 genes affected only by ciprofloxacin exposure and 183 genes that were only affected by exposure to tobramycin. Comparison of our results to other published microarray literature indicates that planktonic *P. aeruginosa* respond differently to ciprofloxacin, having in common only the induction of pyocins by ciprofloxacin. Ciprofloxacin exposure of biofilms results in the induction of more LexA regulated genes than found with planktonic cells. The response of biofilms to tobramycin shares 103 genes with the response to ciprofloxacin. The results of this work will be used as a guide for further study of biofilm adaptive responses to these antibiotics. A better understanding of how biofilms respond to antibiotics may lead to better biofilm interventions.

CBE Poster #439

Date: 01/2008

Title: **Zinc speciation: Implications for improved biocides in industrial applications**

Authors: J. Moberly[1,2], A. Staven[1], R. Sani[2,3], and B. Peyton[1]

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Sponsor: National Science Foundation under Award # EAR-0628258

Due to its high solubility under a wide range of pH conditions, aqueous zinc (Zn^{2+}) is found in many systems. This high solubility under a variety of conditions may be used to advantage when using zinc as a biocide in industrial processes. However the speciation of zinc (and other metals) is critical in determining its toxicity to microorganisms. It is known that a variety of ligands can decrease metal toxicity to microbes in aqueous medium by chelation (e.g., EDTA, citrate, NTA) and precipitation (e.g., PO_4, H_2S, CO_3). Current models hold that the activity of the free metal ion in aqueous solution dictates the toxicity to microorganisms and biota. However, our work using cultures of *Arthrobacter* sp. JM018 isolated from a metal-contaminated site implies that this may not be the case. Combining thermodynamic modeling using Visual MINTEQ (v 2.52) and batch culture studies under several conditions suggests that the toxic species is not the free ion but a hydrolysis product ($ZnOH^+$ or $ZnOH_2^o$) that is the toxic species of zinc. Optimizing conditions to favor increase of these metal species may lead to improved antimicrobial solutions in industrial processes.

CBE Poster #440

Date: 01/2008

Title: **The effect of growth substrate on U(VI) and Cr(VI) toxicity**

Authors: M. VanEngelen[1], E. Field[1], B. Peyton[1], R. Gerlach[1], W. Apel[2], B. Lee[2], W. Smith[2], and A. Miller[2]

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Sponsor: U.S. Department of Energy, Office of Science, Environmental Remediation Science Program (ERSP) contract DE-FG02-03ER63577, DE-FG02-03ER63582

Background: Low-level waste (LLW) sites contaminated with heavy metals, including chromium (Cr) and uranium (U), commonly contain cellulosic waste in the form of paper towels, cardboard, wood, and other materials. As this cellulosic waste is broken down, it can provide a variety of substrates capable of promoting

bacterial growth, which can in turn strongly affect metal mobility through reduction or bioaccumulation. While both Cr(VI) and U(VI) have been shown to inhibit bacterial growth, it was hypothesized that metal toxicity could vary with growth substrates. Investigating this possibility is therefore necessary if successful remediation strategies are to be implemented.

Methods: A *Pseudomonas* isolate was used in a series of aerobic growth experiments in which a variety of compounds, including butyrate, ethanol, lactate, and glucose, were used as substrate. Substrate was added to a concentration of 15 mM as carbon to chemically defined liquid media buffered with 10mM PIPES. Parallel experiments were conducted in the presence of U(VI) with concentrations ranging from 5–200 µM UO_2Cl_2. The growth rate under each condition was calculated to assess the IC_{50} of U(VI) when different growth substrates were used. In addition, cells were grown in the presence of 10 µM Cr(VI) to assess the effect of substrate on growth in the presence of Cr(VI) and Cr(VI) reduction rates.

Results: As hypothesized, the IC_{50} of U(VI) varied with growth substrate. When grown on ethanol, the isolate was severely inhibited at relatively low U(VI) concentrations, exhibiting an IC_{50} of ~25 µM U(VI). When grown on lactate and butyrate, the IC_{50} of U(VI) was ~100 µM and ~125 µM, respectively. The U(VI) was more toxic when dextrose served as substrate with an IC_{50} value of ~50 µM U(VI). Growth in the presence of Cr(VI) and Cr(VI) reduction rates was also affected by substrate. When ethanol or butyrate was used, both growth and reduction rates were lower compared to growth and reduction rates when either lactate or glucose was used.

Conclusions: The effect of growth substrate on U(VI) and Cr(VI) toxicity was demonstrated, providing information that can aid in bioremediation efforts.

CBE Poster #441

Date: 10/2008

Title: **Undergraduate research in the Standardized Biofilm Methods laboratory enhances education**

Authors: L. Lorenz, J. Hilyard, D. Walker, K. Buckingham-Meyer, D. Goeres, and P. Sturman

Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Undergraduate students have a unique opportunity to gain laboratory research experience at the Center for Biofilm Engineering. The Standardized Biofilm Methods (SBM) Laboratory is distinctive in that it provides internship opportunities only to students who are working toward their undergraduate degrees. Originally the SBM was designed to perform short-term industry testing projects, which best accommodated undergraduate students' schedules. This mission has since evolved to include the development of standardized biofilm testing methods based on biofilm growth reactors. The SBM's current goal is to develop, standardize, validate, and publish methods for quantifying biofilm and assessing anti-biofilm treatments for the benefit of academia, regulatory agencies, and industry. Interns play an integral part in attaining success in all areas of this goal.

SBM interns benefit greatly from their experience working in a research laboratory. In addition to laboratory work, interns are given the opportunity to help with workshops, to make posters highlighting their research, and to give presentations to their peers as well as the CBE's industrial associates at Technical Advisory Conferences (TAC). The interns are not required or expected to have any laboratory experience, so much of this is provided to them during their time in the SBM. Their experience is meant to be a mentoring internship that enhances their education and is made available by SBM staff researchers and more experienced interns. Former undergraduate interns have gone on to graduate school, medical school, and industrial employment. The work they have performed at the CBE has been funded by sponsoring companies, the Environmental Protection Agency and the Montana Board of Research and Commercialization Technology, as well as the Industrial Associates of the CBE. Many projects have focused on methods development and have led to standardized biofilm methods published by the American Society of Testing and Materials—two of which have been approved and two that are currently

under review. This poster gives an overview of undergraduate research and collaborations in the CBE and specifically in the SBM laboratory. It also highlights two current SBM projects currently being conducted by undergraduate interns.

CBE Poster #442

Date: 01/2008
Title: **Alkaline hydrolysis and biotransformation of TNT by thermoalkaliphiles from Yellowstone National Park**
Authors: C. Albaugh-VanEngelen, B. Peyton, and R. Gerlach
Affiliation: Thermal Biology Institute (TBI), Montana State University, Bozeman, MT
Sponsor: TBI and Inland Northwest Research Alliance (INRA)

The combination of abiotic alkaline hydrolysis and biodegradation by thermoalkaliphiles from Yellowstone National Park has shown potential for remediation applications of certain hazardous organic compounds including 2,4,6-trinitrotoluene (TNT). There is a demand for safe removal of TNT from soil and groundwater at munitions facilities. At high pH and high temperature, TNT was completely hydrolyzed to a number of products—one being 1,3,5-trinitrobenzene (TNB), which is also a hazardous compound. A bacterial culture belonging to the *Anoxybacillus* genus was recently isolated at pH 9 and 60°C from an alkaline hot spring in the Heart Lake Geyser Basin. This culture was enriched on glucose in the presence of up to 40mg/L TNT. As TNT was abiotically hydrolyzed, the TNB produced from the reaction was subsequently consumed by the bacterium in a co-metabolic process. In concert, the processes of alkaline hydrolysis and biological degradation have been observed to successfully remove TNT and TNB. Further analysis is required to identify any other major TNT hydrolysis products and their fates in the presence of thermoalkaliphilic cultures.

CBE Poster #443

Date: 01/2008
Title: **Isolation and characterization of a heterotrophic nitrifying bacterium from a drinking water distribution system**
Authors: Gem D. Encarnacion[1], Lynne H. Leach[1], Xueju Lin[2], and Anne K. Camper[1]
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[2]Stroud Water Center, Philadelphia, PA
Sponsors: American Water Works Association Research Foundation and Stroud Water Center

Chlorination in drinking water systems has been instrumental in decreasing water-borne diseases; however, chlorine can interact with organics in the water to form toxigenic disinfection byproducts (DBPs). Therefore, many water utilities are switching to the use of chloramines to reduce the levels of DBPs. The degradation of chloramines introduces ammonia into the distribution system. During a nitrification episode, the ammonia is biologically converted to nitrite then nitrate. Nitrification episodes are associated with a decrease in the disinfectant residual and an increase in the heterotrophic plate counts (HPC).

It is well established that autotrophic nitrifying bacteria are involved in distribution system nitrification. However, we propose that heterotrophic bacteria may also be contributing to nitrification episodes. In our study, a heterotrophic nitrifying Pseudomonad was isolated from a water distribution system by the serial dilution to extinction method (10^{-1} to 10^{-5}). An attempt to PCR-amplify the gene for ammonia monooxygenase (AMO) was not successful.

As analyzed by denaturing gel electrophoresis (DGGE), community profiles of the less dilute cultures were more complex as compared to those of the more dilute cultures. Nitrite was initially produced in all cultures; however, over time, nitrite was subsequently lost in all the dilutions except for the 10^{-5} culture. A denitrification gene for

the reduction of nitrite was present in all of the less dilute cultures, but was absent in the 10^{-5} culture where nitrite was retained.

Our study has successfully isolated a heterotrophic nitrifying bacterium from a water distribution system. This isolate will enable the development of a molecular tracking technique that may be used to predict and possibly prevent the impact of nitrification events. With the physiological role of heterotrophic nitrification being obscure, isolates such as this one can be used to further understand the nitrification process.

CBE Poster #444

Date: 10/2007

Title: **Autoinducer-2 triggers the oxidative stress response in *Mycobacterium avium* leading to increased biofilm formation**

Authors: H. Geier[1], S. Mostowy[2], G.A. Cangelosi[2], M.A. Behr[3], and T.E. Ford[1]

Affiliation: [1]Department of Microbiology, Montana State University Bozeman, MT;
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[3]Seattle Biomedical Research Institute, Seattle, WA

Sponsor: NIH Grant Number P20 RR-16455-06 from the INBRE Program of the National Center for Research Resources

Background: Autoinducer-2 (AI-2) is a quorum sensing signal involved in regulating processes such as bioluminescence and biofilm formation. As a metabolic byproduct, AI-2 can also exert effects on bacteria that are independent of quorum sensing pathways. This study demonstrates the role of AI-2 on biofilm formation by *Mycobacterium avium*, an opportunistic pathogen that forms biofilms in drinking water pipes and potable water reservoirs. Addition of AI-2 to *M. avium* biofilm cultures resulted in increased biofilm formation, despite the apparent absence of AI-2 sensing genes in the *M. avium* genome. Microarray studies of treated biofilm cultures revealed upregulation of oxidative stress response genes in response to AI-2 exposure. In order to test the specificity of AI-2 as a signal in this process, we added hydrogen peroxide and cell-free supernatants from AI-2-producing and non-producing bacteria to *M. avium* cultures and assessed the effects on biofilm formation.

Methods: *M. avium* biofilms were grown in 96-well microtiter plates in the presence of 0.25 μM, 2.5 μM, 25 μM, 250 μM and 2500 μM of the chemically synthesized AI-2 precursor DPD. Hydrogen peroxide was added at concentrations of 0.05 mM, 0.5 mM, 5 mM and 50 mM. Furthermore, cell-free supernatants of *Vibrio harveyi* BB170 and MM32, *Escherichia coli* and *Pseudomonas aeruginosa* were added according to their ability to produce the borated form of AI-2 and its knockout, the enteric form of AI-2, and no AI-2, respectively. After 12 days of incubation at 37μC, biofilm formation was evaluated using the crystal violet staining method.

Results: The addition of 25 μM AI-2 resulted in a 1.7-fold increase in *M. avium* biofilm mass, and the same effect was obtained by adding 50 mM hydrogen peroxide. Cell-free supernatants of *V. harveyi* increased biofilm formation up to 1.8-fold, whereas the addition of *E. coli* supernatant resulted in a slight decrease of biofilm formation compared to the negative control. *P. aeruginosa* supernatants did not affect biofilm formation. The supernatant of the AI-2-knockout mutant *V. harveyi* MM32 induced biofilm formation up to 1.5-fold, similar to the wild type *V. harveyi* strain.

Conclusions: Although the genome of *M. avium* lacks sequences resembling common AI-2 biosynthetic or sensing genes, the bacterium nonetheless reacts to AI-2 by forming biofilms. Its chemical structure suggests that AI-2 could act as a reactive oxygen species that triggers the mycobacterial oxidative stress response leading to increased biofilm formation. This model was supported by the finding that hydrogen peroxide also induces increased biofilm formation in *M. avium* cultures. Thus, it can be concluded that AI-2 acts as an environmental cue or stress factor, rather than as a classical quorum sensing signal in this bacterium. This observation illustrates

how AI-2-based signaling can have multiple mechanisms. The differential response of *M. avium* to factors from different bacteria, regardless of their ability to produce AI-2, suggests that *M. avium* may respond to a variety of environmental cues, and may be able to differentiate between between bacterial species that produce them.

CBE Poster #445

Date: 12/2007

Title: **Biofilm enhanced subsurface sequestration of supercritical CO_2**

Authors: Andrew C. Mitchell[1], Adrienne Phillips[1], Randy Hiebert[1], Robin Gerlach[1], John Kaszuba[2], Kirk Hollis[2], and Al Cunningham[1]

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Sponsor: Zero Emissions Research & Technology (ZERT)

In order to develop subsurface CO_2 storage as a viable engineered mechanism to reduce concentrations of atmospheric CO_2, any potential "leakage" of injected supercritical CO_2 ($scCO_2$) from the ground to the atmosphere must be reduced. Here, we investigate the utility of biofilms, assemblages of microorganisms firmly attached to a surface, as a means of reducing $scCO_2$ leakage.

First, experiments were performed to test whether biofilms were more resilient than planktonic cells to $scCO_2$. *Bacillus mojavensis* biofilms were grown on a sand support matrix in $scCO_2$ extractor cartridges at 30°C. *B. mojavensis* was also grown under suspended planktonic conditions in the same media overnight and aliquots were decanted into $scCO_2$ extractor cartridges. Biofilm and suspended *B. mojavensis* samples were processed on a Supercritical Fluid Extractor with pressurization to 2000 psi at 35°C, and a 20-minute flow of $scCO_2$. Suspended growth samples revealed a 3-log reduction in cell viability, while biofilm showed only a 1-log reduction, demonstrating that biofilms are more resilient than planktonic cells to $scCO_2$. Protective extracellular polymeric substances make up the biofilm matrix and likely provide a protective barrier against $scCO_2$.

Second, the ability of biofilms to grow under high pressure and reduce the porosity of porous geological matrices was investigated using a unique high pressure (8.9MPa), moderate temperature ($\geq$ 32°C) flow reactor containing 40 millidarcy Berea sandstone cores. The flow reactor containing the sandstone core was inoculated with the biofilm forming organism *Shewanella fridgidimarina.* Electron microscopy of the rock core revealed substantial biofilm accumulation in rock pores resulting in <99% reduction in core permeability—which did not increase in response to starvation and $scCO_2$ challenges. Viable population assays of organisms in the effluent indicated survival of the bacteria following $scCO_2$ challenges of <71h and starvation for <363h.

Biofilms are more resilient to $scCO_2$ than planktonic cells, they display continued viability under high pressure, and they are able to significantly reduce porous media porosity under high pressure. This is extremely encouraging for the prospective use of engineered biofilm barriers for controlling leakage of geologically sequestered CO_2.

Poster

Date: 02/2008

Title: **Biofilms and resistant bacterial infections**

Authors: Irshad Mohammad, Mathew L. Moy, Zack A. Moy, Aaron Bush, and Irving M. Bush

Affiliation: All Care Medical Center, West Dundee, Illinois

There is now an established role of bacterial biofilm formation in many medical problems like catheter-associated infection, prostatitis and struvite (infected stone) calculogenesis. This role has been extended to sinusitis, respiratory infections in patients with cystic fibrosis and in infections involving orthopedic implants. With

reference to this, we ask the question, why do patients with seminal vesiculitis and prostatitis have negative semen cultures (in samples obtained by ejaculation and seminal vesicle massage)? Seminal vesiculitis over the past 100 years has been difficult to diagnose, and our purpose is to find ways to identify and delineate the bacteria trapped in the biofilms and develop methods to eradicate the bacteria causing the infection.

Biofilm growth is hypothesized as an important mechanism by which bacteria are able to persist in vivo and cause infection. Although epidemiological evidence indicates that microbial biofilms play a role in indwelling device-associated bloodstream and urinary tract infections, direct evidence supporting this claim is lacking in most cases. For instance the Gram-positive opportunistic pathogen *Enterococcus faecalis* is frequently implicated as a causative agent of urinary tract infections, bacteremia, and bacterial endocarditis and is often isolated from biofilms on various indwelling devices. *Pseudomonas aeruginosa* is the causative organism of nosocomial infections and is notorious for persistence, which is now thought to be due to its switching from planktonic form to a biofilm phenotype.

Antibiotic resistance can be increased 1000-fold in bacteria which form biofilms. This is attributed to various factors: a weak electric charge over the biofilm which repels the antibiotic molecule, improved interactions and communication between the bacteria within the biofilm, a reduced metabolic rate, and genetics (as is now suggested by research from three universities). Any success with antibiotics is soon lost with the emergence of persistor cells, which are dormant bacteria living within the biofilm and resurrect the ‘colony’ after a time interval.

We are also investigating the effects of urine on biofilm-forming bacteria in the urinary tract. The occurrence of Tamm-Horsfall protein/Uromodulin and other protective substances in human urine is intriguing. It has been postulated that Uromodulin modulates cell adhesion and signal transduction by interacting with cytokines and, more specifically, inhibits the aggregation of calcium oxalate crystals and provides a defense against urinary tract infection. In this context, the behavior of biofilm producing bacteria in the environment of urine is being studied.

We postulate that biofilms are a significant factor in chronic infections, and research on methods to increase penetration of antibiotics into the biofilms is being pursued and is a more productive avenue than newer antibiotics. Electric current (bioelectric effect), magnetic field (biomagnetic effect), and chemicals (proteolytic enzymes, mucolytic agents) can be areas of high yield in our quest. This will contribute to a better clinical management of chronically infected patients, and should prompt development of strategies to better support anti-infective treatment.