CENTER FOR BIOFILM ENGINEERING

PROCEEDINGS
Summer 2005 CBE Technical Advisory Conference

July 6–8, 2005
Montana State University
Bozeman, Montana

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Center for Biofilm Engineering
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at Montana State University

GENERAL INFORMATION

CBE LEADERSHIP

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

A BRIEF HISTORY OF THE CBE

The CBE was established in 1990 by 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 areas of activity. In the area of research, the CBE's goal is to do leading-edge fundamental research to elucidate mechanisms at work in bacterial biofilms. The CBE has been a leader in defining the structure and function of biofilms on surfaces, in understanding antimicrobial resistance mechanisms of biofilm, and in identifying the role of signal molecules in controlling bacterial behavior. Researchers at the CBE have demonstrated that biofilms are multicellular attached communities with primitive circulatory systems and a measure of cellular specialization. Understanding these "biofilm basics" presents opportunities for developing more effective strategies to control biofilms in industrial settings.

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

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

Industrial Associates Program Benefits

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

CBE Technical Advisory Conferences

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

Education and Training Workshops

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

Research/Testing Projects

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

Product/IP Development Consulting

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

Regulatory Interactions

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

For More Information

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

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Keynote Speaker:

S05-S01
Gallium, a Potential New Anti-Biofilm Therapeutic

Pradeep Singh, *Assistant Professor, Department of Internal Medicine, University of Iowa*

Bacteria living in biofilms cause many chronic infections and extensive industrial contamination. Unfortunately, no treatments exist that target biofilms, and novel approaches to combat biofilm infections are urgently needed. Recent data indicate that iron is a key signal that promotes biofilm development. This work suggests that interfering with bacterial iron acquisition and metabolism could have therapeutic effects against biofilm infections.

Gallium (Ga), a group IIIA transition metal, can substitute for Fe in biomolecular processes. Gallium enters bacteria via the same mechanism that cells utilize to acquire Fe. However, substitution of gallium for Fe renders some enzymes inactive, since gallium is not able to undergo redox cycling required for activity. Importantly, gallium binds to the siderophores of *Pseudomonas* sp. and other microbes.

The importance of iron in *P. aeruginosa* biofilm development and the ability of gallium to disrupt iron metabolism led us to investigate the effect of gallium on biofilm formation. We have found that low concentrations of gallium have a marked inhibitory effect on *P. aeruginosa* biofilm formation. Furthermore, in vitro, gallium did not interfere with either the growth inhibitory or anti-biofilm action of human lactoferrin (an iron binding protein with host defense functions). Gallium is a promising therapeutic for patients with biofilm infections because $Ga(NO^3)^3$ is already FDA approved for intravenous administration, and an oral formulation is under development. This lecture will describe our current work testing the anti-biofilm activity of gallium.

SESSION 1: Medical Biofilms

S05-S02
Overview of Research in the Medical Biofilm Laboratory

Garth James, *Medical Biofilm Laboratory Manager, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

The CBE's Medical Biofilm Laboratory (MBL) is involved in a wide variety of industrially funded projects related to biofilms in medical and dental systems. This work includes model development for in vitro oral care product testing, evaluation of dental unit waterline treatments, evaluation of biofilm inhibitors, testing of biofilm sensors, testing of biofilm removal technologies, and evaluation of biofilm formation on medical devices and tissue surfaces. The MBL is also involved in research to elucidate the role of biofilms in chronic wound infections.

A recently completed project in the MBL involved testing of treatments for removing biofilms from hemodialysis unit waterlines and provides an example of the type of work done in the MBL. This research was funded by Biomedical Development Corporation, Inc. (BDC), which supplied three disinfectant formulations for testing. The model system was adapted from a model previously developed at CBE. This model involved the use of a continuous flow stirred tank reactor (CSTR) as a simulated dialysis machine. Bacteria derived from dialysate make-up water, along with dialysate and nutrients, were supplied to the CSTR. The solution from the CSTR was continuously recycled through 0.5 meters of silicone tubing for seven days. The tubing was then divided into sections, treated with disinfectants, and assays were performed to determine populations of bacteria and amount of endotoxin remaining on the tubing. Control treatments had dense biofilms (4.9×10^5 to 2.3×10^8, mean 5.4×10^7 CFU/cm^2) and relatively high levels of endotoxin (3100 to 6200, mean 4300 EU/cm^2). Treatment with disinfectants, provided by BDC, resulted in log reductions of bacterial populations of up to 6.2 and endotoxin reductions of up to 94%. These results suggested that formulations provided by BDC were very effective against biofilms developed in the hemodialysis waterline model system.

S05-S03
The Results of a One-Stage Joint Revision for Infected Joints Using Radical Debridement and Antibiotic Cement

Gary Maale, M.D., *Orthopedic Surgeon, Dallas-Ft. Worth Sarcoma Group*

Introduction: The treatment of infected total joint replacements varies, ranging from amputation, resection arthroplasty to more functional total joint salvage. Salvage operations yield from 50%–80% published success at 2-year follow-up. Popular in the United States is a 2-stage debridement, with placement of an intermediate antibiotic-loaded cement spacer followed by definitive reconstruction at different timed intervals. This interval usually ranges from six weeks to six months. The cement antibiotic loads differ, but require a heat-stable powder form of the antibiotic for efficacy with methyl methracrylate cement. Although the antibiotic cement is thought to help prevent reattachment of planktonic forms of the bacteria, it also provides for a high dose of antibiotic locally for treatment of the residual bacteria in the surgical bed. While the antibiotic cement allows for wound closure, the key to successful treatment is the surgical excision of devitalized tissue and prosthetic devices, which are associated with biofilm formation. Described in this study are the results of a one-stage (immediate) revision utilizing antibiotic cement after radical debridement for infected joints.

Methods: Thirty-five patients were analyzed. Each had 2 minimal year follow-up treated by radical debridement removal of the joint prosthesis irrigation, changing of drapes and set-ups, re-irrigation and definitive exchange. All patients were pre-operatively imaged and staged by the MSIS staging as submitted by McPherson. Radical debridement was defined as removal of all dysvascular hard and soft tissue and removal of all prosthesis or related debris, which could serve as mediator for biofilm formation.

Results: All patients had local or systemic compromising factors. Two patients had a relapse of their infection—one treated with an above-knee amputation for a total knee, and one left with a draining sinus about total hip. 94% remain clinically free of infection. The most common complications were subluxation or dislocation of the patella in total knees secondary to soft tissue resection, and dislocation of total hip secondary to the need of constrained acetalubar prosthesis. Of interest is the difficulty in identifying the infection cases from other known failure etiologies for joints. Culture retrieval was under 50%, and 15 out of 35 patients had no acute inflammation.

Conclusion: The results for this one-stage treatment of infected joints are better than other published treatment protocols. The success of this strategy compared to others is thought to be the more radical debridement. Unfortunately, this is associated with more bone and soft tissue loss, and causes more dysfunction to the patient.

Difficulties in diagnosis of infection is clearly evident, with 15 of 35 showing only chronic inflammation at the time of surgery, and less than a 50% culture retrieval. Clearly, better technologies need to be made available for diagnosis and treatment of infected total joints.

S05-S04
Evaluation of Biofilms in Chronic Wounds

Ellen Swogger*, MSU-CBE Undergraduate, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

Biofilms may play a role in chronic infections, since they exhibit resistance to antibiotics and host immune response. In the case of wounds, chronic infections often occur and can be very painful and expensive to treat. These chronic wounds include diabetic foot ulcers, which are often considered non-healable and can result in amputation of the infected limb. The inability to heal these wounds may be due, in part, to biofilm formation in the wound. Little research has been done to investigate the role of biofilms in wound infections. Increased understanding may lead to improved treatment methods.

In this research, both chronic and acute wounds were examined for the presence of bacterial biofilms. Wound samples were supplied to the Center for Biofilm Engineering by the Southwest Regional Wound Care Center in Lubbock, Texas, which treats over one hundred of these infections every day. The aim of this research was to determine whether biofilms exist in any—or all—persistent wounds, and if they exist in acute wounds.

The project objective was accomplished by light microscopy of Gram-stained tissue sections and scanning electron microscopy of tissue surfaces. The combination of these methods determined biofilm presence in each wound. A statistical analysis of the results has shown that biofilms are prevalent in chronic wounds, but not in acute wounds. The results of this study not only elucidate why some treatments are ineffective in healing chronic wounds; they may be utilized in formulating new treatment methods for the victims of these wounds.

SPEAKER ABSTRACTS

S05-S05
Biofilm-Based Wound Care

Randy Wolcott, M.D., *Director, Southwest Regional Wound Care Center, Lubbock, TX*

Wounds that just one year ago were thought to be non-healable can now be healed much easier using a biofilm-based wound model. The discovery that the soft, slimy film often found on chronic wounds was not a disorganized mass of bacteria and white blood cells but was rather biofilm locked in mortal combat with host defenses was quite a surprise. This led to a unifying theory of wounds that suggests that the main reason why chronic wounds fail to heal is the presence of biofilm. Chronic wounds have been shown to be stuck in the inflammatory phase and have identical impairments of elevated proinflammatory cytokines, elevated matrix metalloproteases, low levels of tissue inhibitors of metalloproteases, and diminished growth factors, no matter what the cause of the wound. Biofilms, and their ability to evade host defenses while launching offensive attacks to keep the wound open, are the best explanation for this shared biochemistry of all chronic wounds. Many biofilm defenses have been identified over the last few years, but in vivo, the main defense mechanism that makes biofilms so recalcitrant to treatment is their ability to reconstitute quickly after insults such as application of biocides, or especially debridement. A 50-wound study demonstrated that the surface of most chronic wounds contains mature, organized biofilm.

Using biofilm-based treatment methods such as anti-bacterial biofilm agents, sharp debridement, dressings that resist or inhibit biofilm, antibiotics effective against biofilm, and bacteriophages and other anti-biofilm strategies has resulted in an awe-inspiring improvement in healing. This has caused us to expect to heal non-healable wounds. The amazing response of wounds to an anti-biofilm approach is excellent indirect evidence that biofilm is a major, if not the major, barrier to wound healing. The surprise was when we realized that something we took for granted, overlooked and felt was as harmless as a small bunny in a garden, is actually a very formidable foe. We need to work very hard at the research clinical level to learn more about biofilm if we are ever going to defeat this foe.

S05-S06
State of the CBE and Vision for the Future

Phil Stewart, *CBE Director, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

This presentation reviews research advances at the Center and in my own area of biofilm control, and touches on a number of the “hot topics” in biofilm research from outside the CBE as well. I also present my vision for the future of the Center for Biofilm Engineering (CBE) at Montana State University. There are four elements to this vision: 1) focusing on the CBE research niche, 2) embracing what I am calling a “synthesis” function, 3) building the CBE as an international hub for biofilm research, education, and technology transfer, and 4) working with companies to translate the biofilm concept and the exciting new science emerging from it into new products and processes in industry and medicine. These four elements build on the historically successful CBE areas of activity: Research, Education, Technology Transfer and Outreach.

S05-S07
Biofilms in the Hospitality, Food and Healthcare Industries

Scott L. Burnett, *Microbiologist, Ecolab, Mendota Heights, MN*

With business interests in a broad and diverse range of industries, Ecolab maintains an approach to research and product development that focuses on solving customers’ problems. In some instances, problems experienced by Ecolab customers are shared across industries. Biofilms and related issues are encountered across our business base. We are very pleased to become an industrial member of the Center for Biofilm Engineering and look forward to a beneficial relationship with its researchers, as well as with representatives of our fellow supporting companies. The aim of this presentation is to introduce the faculty, staff, and students of the Center and our colleagues throughout industry to Ecolab and its business interests, and to provide some examples of where we have helped our customers manage biofilm-related problems.

SESSION 2: Biofilm Structure and Function

S05-S08
Three-Dimensional Biofilm Structure and Activity

***Raaja Raajan Angathevar Veluchamy**, MSU-CBE MS Candidate, Environmental Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

For modeling purposes, the most useful experimental data are produced when they are measured in three dimensions. We have developed a method using artificial neural networks to predict some of the biofilm structure parameters in three dimensions. We found that when the data is in two-dimension artificial neural networks, predictions do not differ from any available methods such as cubic spline. However, when we used multi-dimension data, ANN was always able to predict biofilm structures more successfully.

S05-S09
Hollow Fiber Membrane Biofilm Reactor for Dye Wastewater Treatment

***Yeon Kyung-Min,** CBE Visiting Researcher, Institute of Environmental Protection and Safety, School of Chemical Engineering, Seoul National University, Korea*

Azo dyes are a major class of synthetic dyes extensively discharged in textile wastewater. Textile wastewater treatment is one of the most important environmental issues because of the xenobiotic nature of the synthetic dyes. Among the various available treatment technologies, biological processes have the advantage of being not only cost effective, but also environmentally friendly. However, this biological treatment system also requires a sequence of anaerobic/aerobic phases to improve efficiency because reductive cleavage of the azo bond takes place under anaerobic conditions, whereas the resultant aromatic amine can be metabolized under aerobic conditions. We developed the hollow fiber membrane reactor (HFMBR) to integrate these different oxygen conditions into one reactor. In this system, we used the biofilm formed on a membrane surface for dye wastewater treatment; with the combination of different operation modes it was possible to switch from the aerobic phase to the anoxic phase and vice versa. This system removed about 70% of the reactive azo dye. We proved this dye degradation activity by measuring the oxygen concentration profile in the biofilm using a dissolved oxygen microelectrode. All oxygen profiles indicated that there was a separation of aerobic and anaerobic zones, even in the same biofilm, according to depth. The anaerobic zone had a range of a few hundred micrometers, and this can be thought of as the main reason for the dye degradation activity in each biofilm.

S05-S10
Particle Capture in Biofilms

***Anne Camper**, CBE Professor, Civil Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

One of the many properties of biofilms is the prevalence of extracellular polymers that act as a bridge between the cells and the substratum. This matrix also has the ability to trap and retain chemicals, other organisms, and inert particles. Previous work has shown that biofilms are capable of physically trapping and retaining particles, and research at the CBE has been collected on the retention of fluorescent latex and polystyrene beads. Further work demonstrated that biofilms entrained other bacterial cells, including potential pathogenic organisms. This work was done using water of drinking-water quality, suggesting that even thin biofilms in nutrient-poor environments elicit this behavior. Because of the difficulty in recovering bacteria attached to biofilms and the problems in closing mass balances between cells added and cells recovered, experiments were designed to measure the trapping efficacy of fluorescent beads. The experimental system consisted of a well-mixed porous media column filled with glass beads upon which a biofilm was grown under three nutrient conditions (no added carbon, 0.5 mg/L and 1.5 mg/L). Bead recovery was excellent (>90%). Biofilms grown under more nutrient-rich conditions trapped a greater number of particles. The results are being compared with those collected using microscopy and fluorescent protein labeled cells. All of these data will provide insight on the fate and transport of pathogens in biofilm traps used to monitor the contamination of drinking water systems.

SPEAKER ABSTRACTS

Special Presentations

S05-S11
Bacteria Can Do Amazing Things: Tertiary Structure in Biofilms

Bill Costerton, *Director, Dental Biofilm Center, University of Southern California, Los Angeles*

The MH strain of *Staphylococcus epidermidis*, isolated from a canine lymphoma, was observed to produce a fine network of "aerials" throughout a liquid medium and a dense mass of white material at the bottom of a tube when cultures were retained for 8–10 days. Upon closer examination, the aerials were seen to consist of a solid strand of material, ca. 5 microns in diameter, with various numbers of bacterial cells associated with each strand, and the strands formed diffuse arches throughout the fluid phase of the medium. The material at the bottom of the tube was seen to consist of a mass of solid honeycomb-like structures devoid of bacteria in some locations, and associated with very large numbers of these organisms in areas in which the structures seemed to be "under construction."

The solid honeycomb material is notably elastic, seems to be made of protein, and shows a repeated pattern of deep individual "cells" that have mouth dimensions of 5 to 12 microns, by 2 to 6 microns. In areas in which the structures are beginning to form, individual cells are randomly distributed, but they appear to construct flat plate-like structures that fill much of the volume of the community. These structures and associations can best be seen by Scanning Electron Microscopy (SEM), but they can also be resolved in fully hydrated unfixed material examined by Confocal Scanning Laser Microscopy (CSLM). The bacterial cells form the plates by a process in which they associate with the nascent structures but do not seem to coalesce or to contribute their contents to the developing structures. After 1–2 days, the individual plates become arranged into "walls" that are approximately 40 nanometers thick, and are arranged in parallel arrays at an interval of 6 to 12 microns; these complex structures dominate areas of the macroscopic mass several hundreds of microns in extent. In the final stage of honeycomb formation, cells associated with the walls begin to form partitions between the parallel walls that now define the mouth of each honeycomb element. When the complex proteinaceous "apartment block-like" honeycomb structures are almost complete, the bacterial cells leave their surfaces and initiate the production of these amazing structures in other locations.

We suggest that bacteria are capable of making structures that are much more complex, and much larger, than any seen heretofore. These structures are much more complex than biofilms that are composed of bacterial cells and their associated extra-cellular matrix materials, because the organisms build and then abandon structures of remarkable size and complexity. Similar structures have been seen to be made by environmental strains of *Pseudomonas* and by *Chromobacter* strains isolated from soils. We believe that these "tertiary" structures may be made by many organisms, and that they have not been described previously because microbiologists have only studied planktonic cells in early growth stages.

S05-S12
The *Pseudomonas aeruginosa* Dispersion Autoinducer

David Davies, *Assistant Professor of Biology, University of Binghamton, NY*

In our laboratory we have observed that *Pseudomonas aeruginosa* strain PAO1 releases factors into culture medium that are able to induce the disaggregation of biofilm cell clusters, dispersing free swimming bacteria into the bulk liquid. This autoinduced dispersion response is a characteristic of late stage *P. aeruginosa* biofilms and has the potential to provide a novel avenue for the development of strategies to control and disrupt existing biofilms. We have developed extraction and separation methods by which this compound can be concentrated and purified from spent culture medium and, when added exogenously, will induce the premature dispersion of biofilms. The dispersion response is proportional to inducer concentration; the inducer compound retains activity over a period of at least two months and has activity against biofilms from one day of development through, to, and including steady-state biofilms. A modified microtiter plate assay was developed to evaluate the active fraction of spent culture medium on biofilm dispersion, following separation by HPLC. The dispersion inducer was shown to have activity in inhibiting the development of *P. aeruginosa* biofilms on glass and silicone substrata and has a range of activity in inducing the dispersion of mixed bacterial biofilms. We have also developed a transposon library for *P. aeruginosa* and have used this library to screen for genes activated in response to addition of the inducer compound. Using this method, we have shown that 43 clones are positive for activation of specific genes in the presence of the dispersion inducer compound. We are currently involved in testing these clones to characterize the genetic regulation associated with the dispersion response.

S05-S13
A Short Range Signaling System for Coordinated Dispersal in Biofilms

Paul Stoodley, *Associate Professor, Center for Genomic Sciences, Allegheny-Singer Research Institute, Pittsburgh, PA;* ***Laura Purevdorj-Gage,*** *Recent CBE PhD graduate, Microbiology, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

Abstract not available.

SESSION 3: Biofilm Methods

S05-S14
Drip Flow Biofilm Reactor: Design, Repeatability and Ruggedness

Nic Beck, Jackie Whitaker*, CBE Biofilm System Training Laboratory (BSTL) Interns, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

The CBE and BioSurface Technologies Corporation collaborated in designing a manufacturable version of the drip flow reactor and creating an associated standard operating procedure (SOP). In this reactor, there is gravity flow of liquid nutrient down a microscope slide positioned at a 10° angle, creating a low fluid shear environment for biofilm growth. In this presentation, we describe the reactor, SOP, and preliminary results from experiments conducted to assess the repeatability and ruggedness of biofilm growth in the drip flow system.

A biofilm growth system is said to be rugged if only small changes in viable cell log density (log10 cfu/cm^2) occur when there are slight departures from the SOP. It is said to be repeatable if there is little variation of log densities across independent experiments. To assess ruggedness, four operational factors (temperature, angle of coupon, flow rate, and nutrient concentration) were purposely varied around the SOP specifications. The experimental plan called for each of two technicians to conduct a few preliminary experiments, ten Phase I experiments, and eight Phase II experiments. In each experiment, the biofilm log density was determined for each of four coupons. The preliminary experiments demonstrated the repeatability of biofilm growth. The Phase I experiments allowed us to see if the log density was affected by specific combinations of operational factor settings. The Phase II experiments will provide additional data for constructing a reliable statistical model which will be the ultimate quantitative description of repeatability and ruggedness.

In the preliminary SOP experiments, the mean log density was 9.5, and the repeatability standard deviation was 0.23 (n = 28 coupons). There was negligible variation in log density values between technicians and among experiments. In Phase I experiments we found anomalous log densities at the highest observed nutrient concentration (330 mg/L). Consequently, for our Phase II experiments we used a lower range of nutrient concentrations. Phase II is partially completed. Available data (n = 80 coupons) show that the log density varied little over the observed range of nutrient concentrations (maximum of 300 mg/L) and the range of coupon angles. The log density was more sensitive to temperature and flow rate. At the SOP, the estimated mean log density was 9.4 and the repeatability standard deviation was 0.35, indicating good repeatability.

S05-S15
Parallel Testing to Determine the Influence of Biofilm Growth Conditions on Antimicrobial Log Reduction: The Rest of the Story

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

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

The growth systems, which are run in parallel, are inoculated with *Pseudomonas aeruginosa* ATCC 15442. Four coupons are removed from each system. Treatment is applied to half of the coupons and

control to the other half. The coupons are incubated for 10 minutes. The samples are neutralized at the end of the 10-minute treatment period. The following conclusions were made: Dried surface testing is not representative of biofilm disinfectant efficacy testing. The CDC biofilm test gives the most conservative estimate of efficacy. The three hydrated biofilm tests showed that as biofilm shear forces increase, the log reduction values decrease. The comparisons between dried biofilm and dried surface tests demonstrated that dried biofilm is more difficult to kill.

S05-S16
High Throughput Biofilm Screening

Elinor Pulcini, *CBE Research Associate, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

Conventional methods for biofilm testing can be labor-intensive, slow and inadequate for testing numerous compounds. High throughput (HTP) biofilm screening methods have been developed to address these issues. HTP biofilm screening methods utilize colorimetric assays based on the 96-well microtiter plate assay format. Biofilms can be grown either in microtiter plates or in 96-peg Minimal Biofilm Eradication Concentration (MBEC) plates. Quantitative spectrophotometric assays assess treatment effectiveness. The crystal violet assay quantifies biofilm removal as indicated by biomass. The respiratory indicator 5-cyano-2,3-ditoyl tetrazolium chloride (CTC) assay quantifies biofilm killing as a colorimetric assessment of the activity of bacterial cells within the biofilm. Overall, advantages and disadvantages of HTP biofilm screening methods as well as statistical considerations of the methods will be presented.

S05-S17
An Integrated Platform to Optimize Therapeutic Agents for Biofilm Activity

Tim Morris, *Senior Scientist, Cumbre, Inc., Dallas, TX*

Biofilm and device-related infections are typically recalcitrant to chemotherapy with current antibiotics, and there is a need for new effective agents. To develop improved therapies for these infections, we have implemented three complementary in vitro models and two in vivo rodent models for reproducibly measuring the biofilm killing activity of antibacterial drugs.

Evaluation of a series of experimentally attractive alternatives to viable cell counts, such as light or dye-based metabolic readouts, revealed both mechanism and model dependent biases in all surrogate endpoint methods tested. Thus, viable cell counts are the primary readout for all biofilm efficacy models. For in vitro testing, we modified previously described colony biofilms, polystyrene microplate biofilms, and drip flow biofilms to enable systematic comparisons of bacterial killing and spontaneous resistance development in antibiotic treated biofilms. Cumbre's colony biofilm method reproducibly rank-orders numerous reference antibiotics, requires only 25 µg test compound per biofilm, and is readily adapted for testing a variety of bacterial species. Key efficacy and drug resistance results from the colony biofilm method are confirmed by subsequent testing in polystyrene microplate and drip flow biofilms. For in vivo testing, a mouse infection model was adapted for standardized testing of antibacterials against biofilms formed on subcutaneous Teflon implants. The mouse model simulates peripheral tissue infections at poorly vascularized sites that are often associated with implanted medical devices. Additional in vivo tests are performed in a rat central venous catheter (CVC) infection model, which instead simulates infections of intravascular devices.

By integrating these biofilm-oriented models into a drug discovery program that also includes conventional planktonic cell oriented antibiotic testing, we have developed novel agents that demonstrate in vitro and in vivo biofilm killing activities superior to any currently approved systemic antibacterial drugs tested to date.

S05-S18
Distinction Between Viable and Dead Bacteria Using EMA-PCR

Andreas Nocker*, CBE Assistant Research Professor, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

The distinction between viable and dead bacterial cells poses a major challenge in microbial diagnostics. Due to the persistence of DNA in the environment after cells have lost viability, DNA-based quantification methods overestimate the number of viable cells in mixed populations or even lead to false-positive results in the absence of viable cells. RNA-based diagnostic methods, on the other hand, which circumvent this problem, are technically demanding and suffer from some drawbacks. A promising and easy-to-use alternative was published recently utilizing the DNA-intercalating dye ethidium monoazide bromide (EMA; Rudi et al. 2004, AEM

71:1018-1024). This chemical is known to penetrate only into 'dead' cells with compromised cell membrane integrity. Subsequent photo-induced cross-linking was reported to inhibit PCR amplification of DNA from dead cells. We provide evidence that, in addition to inhibition of amplification, most of the DNA from dead cells is actually lost during the DNA extraction procedure, probably due to being covalently linked to cell debris which goes into the pellet fraction during extraction. Exposure of bacteria to increasing stress and higher proportions of dead cells in defined populations lead to increasing loss of genomic DNA. Experiments were performed using *Escherichia coli* O157:H7 and a *Salmonella* sp. as model pathogens and using real-time PCR for their quantification. Results showed that EMA treatment of mixed populations of these two species provides a valuable tool for selectively removing DNA of non-viable cells using conventional extraction protocols. Furthermore, evidence is provided that EMA treatment of a mature mixed population drinking water biofilm containing a substantial proportion of dead cells can result in dramatically different community fingerprints compared with an untreated biofilm. The interpretation of such fingerprints can have important implications in the field of microbial ecology.

S05-S19
Analysis of Biofilm Communities Using DGGE

Mark Burr, *CBE Research Associate, Center for Biofilm Engineering at Montana State University, Bozeman, 59717,* ***Sabrina Behnke****, CBE Visiting Researcher, University of Duisberg-Essen, Germany*

Denaturing gradient gel electrophoresis (DGGE) is a useful molecular tool for displaying the species diversity in natural biofilms, with no requirement for culturing the bacteria in order to identify them. DGGE is able to separate 16S rDNA PCR products from different species based on their DNA sequences. The number of bands in a DGGE profile is a rough estimate of community diversity, and the intensity of bands is roughly related to the prominence of individual species, although any discrete band may originate from more than one species. There are significant disadvantages with the conventional DGGE protocol. First, it is often difficult to produce a well-resolved DGGE image, especially from samples low in DNA yield and/or contain PCR-inhibiting contaminants (e.g., mine tailings). Second, only about 400 base pairs (bp) of DNA sequence information are analyzed, which may limit our ability to identify species. Finally, retrieving DNA sequence information from gels is often difficult and sometimes requires cloning in order to obtain pure sequences.

In our modified protocol, a cloning step precedes DGGE analysis. Up to 1500 bp of 16S rDNA sequence is PCR-amplified and a clone library is made, in which each clone contains a single pure sequence from the community. Although these initial steps may be difficult with some DNA extracts, it is our experience that this is an easier pathway than the conventional protocol. Furthermore, the clone library is free of any contaminants in the original sample, provides unlimited DNA yield, and can be archived indefinitely. DGGE is used at this point to analyze the contents of the clone library—both individual clones, and pools of clones. When enough clones are pooled, the clone pool becomes a pseudo-community, and the clone pool DGGE profile begins to resemble the profile obtained using the conventional DGGE protocol. DNA sequence data are obtained from the clone library (up to 1500 bp), rather than from the gel as in the conventional protocol.

Since there is only semi-quantative information available from a DGGE pattern in terms of band location and band intensity, we also developed an approach that allows quantification of different genotypes in the community using DGGE. Initially we worked with a laboratory-grown biofilm in which each of the species or genotypes in the inoculum was known. Each of these genotypes was cloned and the mobilities were determined by DGGE to confirm that they were distinguishable from one another. Next, a clone library was created from the mature biofilm sample, and single randomly-picked clones were run in a gel parallel to a reference DGGE profile, obtained either by the conventional DGGE protocol or from a pool of clones. The final step was to count clones with identical mobilities, identity their genotype from their position compared to a reference lane, and express the prominence of each genotype as a percent of the total number of clones counted. Accuracy was improved by analyzing more clones, although this required running additional gels. Our method provided quantitative analysis of biofilm communities without culturing.

SESSION 4: Biofilm-Metals Interaction

S05-S20
Heavy Metal Reduction and Toxicity

Brent Peyton, *Associate Professor, Chemical & Biological Engineering, Washington State University*

Sulfate-reducing bacteria (SRB) play an important role in biotransformation of heavy metals in a variety of aqueous systems. Under anaerobic conditions, SRB

utilize the sulfate ion during oxidation of organic material, forming hydrogen sulfide that forms insoluble complexes with many heavy metals. In addition, SRB are known to directly reduce redox sensitive metals such as uranium and chromium.

Uranium (U) is a known carcinogen, and the high solubility of its hexavalent form can result in U transport to sensitive receptors. One potential method of treating U contamination is by using natural dissimilatory metal reducing bacteria, such as sulfate reducing bacteria (SRB), to reduce soluble U(VI) to insoluble U(IV) (as uraninite, UO2), which can lead to U immobilization and plume stabilization. The effects of U(VI) on SRB were studied using *Desulfovibrio desulfuricans* G20 in a metal toxicity medium containing bicarbonate or PIPES pH buffer (each at 30 mM, pH 7). In batch experiments, U(VI) toxicity was dependent on the medium buffer and was observed in terms of longer lag times and, in some cases, no measurable growth. The minimum inhibiting concentration (MIC), causing a complete inhibition of growth, was 140 µM U(VI) in PIPES buffered medium. This is 36 times lower than previously reported for *D. desulfuricans*. The solubility of U(VI) was significantly lower in PIPES buffer than in bicarbonate buffer; however, U(VI) in PIPES buffer was much more toxic than in bicarbonate buffer.

For SRB interactions with other heavy metals, there must be sufficient knowledge of the toxicity of various heavy metals to SRB populations. An SRB metal toxicity medium (MTM) that eliminates the formation of metal precipitates and minimizes metal complexation was developed to better understand the role of metal concentrations in SRB toxicity. At pH values from 6 to 8, with an increase in Pb(II) concentration, specific growth rates decreased and lag times increased. The minimum inhibiting concentration (MIC) of Pb(II) causing a complete inhibition in growth at pH 6 was 10 µM, as compared to 15 µM at pH 7.2 and 8. Using MTM, measured MIC values are 40 times lower than previously reported. Using MTM, the Cu(II) concentration causing 50% inhibition in final cell protein (IC50) was evaluated to be 16 µM, which is 100 times lower than previously reported. The results show that *D. desulfuricans* in the presence of Cu(II) follow a clearly different growth pattern than in the presence of Pb(II).

S05-S21
Mixed Waste Remediation: Influence of Biofilms, Minerals, and Organic Matter on the Fate of Explosives, Chlorinated Solvents, and Heavy Metals

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

Background: *Cellulomonas* strains from aquifer samples at the U.S. Department of Energy's Hanford site (WA) were shown to be capable of reducing solid and dissolved phase iron, natural organic matter, Cr(VI) and TNT under fermentative conditions. This presentation summarizes research on the reductive transformation of these compounds by strain ES6 in single and mixed component systems.

Methods: Batch and column studies were conducted in which the reduction of Fe(III)-citrate, hydrous ferric oxide (HFO), goethite, maghemite, magnetite, hematite, chromate, and TNT was investigated in the presence and absence of anthraquinone-2,6-disulfonate (AQDS) and humic substances.

Results: Fe(III) reduction by, and growth of, strain ES6 varied depending on the carbon source, iron mineral crystallinity, and presence of synthetic or natural organic matter, such as AQDS or humic substances. Cr(VI) reduction was significantly enhanced in the presence of AQDS and humic substances. The presence of Fe(III) minerals can result in an increase in Cr(VI) reduction rates. TNT transformation rates also increased in the presence of AQDS. The additional presence of HFO enhanced transformation rates of TNT to amino derivatives due to reactions of TNT with microbially produced surface-associated Fe(II). The presence of AQDS in the absence of HFO promoted fast transformations of TNT to unidentified polar products. When both microbially reduced HFO and AQDS were present simultaneously, the reduction of TNT was more rapid and complete via pathways that would have been difficult to infer solely from single component studies.

The simultaneous reduction of Cr(VI) and TNT by strain ES6 does not follow single component kinetics or pathways. TNT at micromolar concentrations substantially enhanced the kinetics of Cr(VI) reduction. The enhancement of Cr(VI) reduction by TNT was observed to be of the same magnitude as the electron shuttle AQDS. Hexavalent chromium was demonstrated to delay the initial reduction of TNT and promote azoxy compound formation.

Conclusions: This study demonstrates the complexity of transformation patterns in model systems where the interactions among bacteria, Fe minerals, contaminants, and organic matter have a pronounced effect on the degradation pathway of oxidized contaminants.

Special Presentations

S05-S22
Disinfecting Biofilm-Associated *Legionella pneumophila*: the Importance of Free-Living Protozoa

Rodney Donlan, *Centers for Disease Control and Prevention, Atlanta, GA*

Legionella pneumophila is an intracellular pathogen that will infect biofilm-associated free-living protozoa in the environment. The CDC Biofilm Lab, in collaboration with the CDC Respiratory Diseases Branch Lab, has performed studies over the last several years to explore the interaction of *L. pneumophila* with amoebae-containing biofilms in model potable water systems. Our goal has been to a) elucidate the importance of biofilm-associated protozoa in *L. pneumophila* survival in water systems, and b) determine whether, and to what extent, these free-living protozoa will protect *L. pneumophila* from commonly used disinfectants. We have shown that *L. pneumophila* will associate with potable water system biofilms, but actual growth in the biofilm is contingent upon the presence of associated protozoa, under the conditions provided by our model system. *Hartmannella vermiformis*, the protozoan used in our studies, also protects *L. pneumophila* from free residual chlorine and monochloramine, at concentrations normally found in drinking water distribution systems. There are a number of other pathogenic bacteria that have been shown to associate with free-living protozoa in the environment. We are interested in using our model system to examine the survival of these organisms in potable water systems.

SESSION 5: Alternative Control Strategies

S05-S23
Anti-Biofilm Surface Coatings

Ross Carlson, *CBE Assistant Research Professor, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

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 proprietary coating 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 pneumonia, 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 proprietary 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 susceptible to biofilm formation like implantable medical devices.

S05-S24
Cohesive Strength Measurement as a Tool for Evaluating Biofilm Removal Strategies

Ray Hozalski, *CBE Visiting Researcher, Associate Professor, Civil Engineering, University of Minnesota*

A critical step in learning how to attack the matrix of a biofilm is the development of techniques for measuring mechanical properties of biofilms, in particular the cohesive strength. Recently, a micro-mechanical technique was developed in our laboratory for measuring the cohesive strength of microbial

aggregates, including flocs and biofilms. The method is based on the observed deflection of a cantilevered glass micro-pipette as it separates a biofilm fragment from an intact biofilm, or separates a fragment from a microbial aggregate held by a second pipette via suction. The main advantage of our method is that it provides a direct quantifiable measure of the force of separation of an aggregate from a biofilm or floc. Furthermore, the size range of detached fragments (10 to 300 µm) is consistent with observations of particles detached from biofilms. Finally, the method does not require that the biofilm be grown on a special testing platform. This "micro-cantilever" method is now ready to be applied to improve our understanding of the mechanisms of biofilm cohesive strength and to develop alternative technologies for managing biofilm accumulation and fouling.

To date, we have measured the cohesive strength of biofilms grown in rotating disk reactors (median values ~ 3000 Pa) and in low shear drip-flow reactors (median values ~10–150 Pa). In addition to investigating the effects of shear conditions, we are also investigating variability of cohesive strength values among different species grown in the same manner. We also plan to investigate the effects of biofilm disruptor compounds and enzymes on cohesive strength in order to rate the effectiveness of biofilm removal approaches.

Biofilm cohesive strength and detachment are key factors in understanding issues as significant and diverse as the fouling of heat exchangers, the fouling of ship hulls, development of dental caries, treatment of cystic fibrosis, the treatment of infected medical implant devices, and the dissemination of bacterial plant pathogens. This research will lead to an improved understanding of cohesive strength and better approaches for facilitating biofilm detachment and mitigation of biofouled surfaces.

S05-S25
A 3D Model of Four Hypothetical Protective Mechanisms of Biofilms

Jason Chambless, *CBE PhD Candidate, Chemical & Biological Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

Four hypothetical mechanisms of biofilm protection against antimicrobials were incorporated into a three-dimensional model of biofilm growth and development. When compared to simulations of unprotected biofilms, each of the mechanisms provided some form of defense to the biofilm—either a direct protection against the antimicrobial, or the ability of the biofilm to reseed itself after the antimicrobial treatment subsided. When compared to each other, the behaviors of the four different protective mechanisms produced distinct shapes of killing curves and non-uniform spatial patterns of survival and cell type distribution. Analysis of the model leads to the conclusion that each of the mechanisms could be plausible avenues of biofilm protection, but if used in conjunction, could provide a robust defense to a myriad of various antimicrobials.

S05-S26
Arginine Enhances Antibiotic Action against *Pseudomonas aeruginosa* Biofilms

Phil Stewart, *CBE Director, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

Pseudomonas aeruginosa biofilms contain anoxic regions that harbor inactive bacteria. Chemical and physiological stratification of these biofilms has been demonstrated using oxygen microelectrodes and fluorescent probes to visualize patterns of protein synthesis. It is hypothesized that cells in the anaerobic niches of the biofilm are inactive and that these inactive cells survive antibiotic exposure. A prediction of this hypothesis is that the biofilm should be made more susceptible to killing by stimulating the metabolism of bacteria in the anoxic zones. This strategy was tested by providing the fermentable amino acid to biofilm during antibiotic exposure. Arginine enhanced the killing by ciprofloxacin and tobramycin under anaerobic, but not aerobic, growth conditions. Arginine also enhanced the killing of these antibiotics in mature biofilms. In the colony biofilm system, the log reduction achieved in the absence of arginine was 1.13 and 0.52 for 12 h treatment with ciprofloxacin and tobramycin, respectively. These log reductions rose to 3.23 and 1.63, respectively, when the medium was supplemented with arginine. Similar enhancement by arginine supplementation was measured in the drip-flow biofilm reactor system. These results show that arginine plus antibiotics reduced viable cell counts by a factor of 102 to 103 beyond that achieved by antibiotics alone.

S05-S27

Controlled Ultrasonic Release of Antibiotics from Hydrogel Surfaces

Patrick Norris, *Recent CBE MS Graduate, Mechanical Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, 59717*

Medical devices are routinely employed in healthcare settings since they provide clinicians with a useful means of administering nutrients, drawing blood samples and drug delivery. In spite of these advantages, local and systemic infections are frequently associated with their use. In fact, implanted devices often provide a highly suitable surface for bacterial adhesion and colonization resulting in the formation of complex, differentiated and highly structured communities known as biofilms. Once a biofilm infection is established, conventional treatments frequently fail as bacteria growing in biofilms are much more resistant to antibiotics than their planktonic counterparts. As a result, a variety of implantable drug-delivery systems have been developed. However, drug release tends to decay over time, and these systems are prone to uncontrollable leaching. To overcome this problem the University of Washington Engineered Biomaterials (UWEB) group has developed a novel drug-delivery polymer matrix consisting of a poly 2-hydroxyethyl methacrylate hydrogel coated with ordered methylene chains forming an ultrasound-responsive coating. The polymer hydrogel was loaded with ciprofloxacin, an antibiotic well known for its action against gram-negative bacteria. This system was able to retain the drug inside the polymer in the absence of ultrasound, but showed a significant drug release when low intensity ultrasound was applied. When ultrasound application was complete, the hydrogel returned to its original configuration and again exhibited minimal drug leaching. With the combination of this polymer system and ultrasonic energy, drug delivery can effectively be turned on or off as needed for treatment. We have incorporated these hydrogel coatings into a flow cell reactor in order to observe biofilm formation and growth over time using confocal microscopy. *Pseudomonas aeruginosa* biofilms were grown on hydrogel surfaces in the flow cells with a bulk fluid flow of 1 ml/min. Ultrasound was applied for twenty minutes every twenty-four hours for three days using a 43 kHz ultrasonic bath with a power density of 2–3 W/cm^2. Confocal images were taken both before and after ultrasound application. The confocal data was then analyzed quantitatively using the biofilm analysis software package COMSTAT. It was shown that over the course of three days, the average maximum thickness of the biofilm colonies decreased. Control experiments with the same hydrogel configuration without ultrasound application showed that the average maximum thickness of the biofilm colonies increased over time. Control experiments with hydrogels that were not loaded with ciprofloxacin also showed increased average maximum thickness, both with and without ultrasound application. The results of our studies may ultimately facilitate future development of medical devices sensitive to external ultrasonic impulses, capable of treating or preventing biofilm growth via “on demand” drug release.

S05-P312
Biofilm Growth Reactors

***Linda R. Loetterle**, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

This poster is a survey of various engineered reactors used to grow biofilm in the laboratory or field. The method of growth is presented as well as features of each system such as growth surface, flow characteristics and appropriate uses. Reactors are shown as either photographs or diagrams.

S05-P316
Analysis of Laboratory Biofilm from Three Growth Reactors

***Linda R. Loetterle**, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

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

S05-P343
Computer Model of Persister Cell Protection Mechanism of Biofilms Against Antimicrobial Agents

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

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

S05-P350
Drip Flow Biofilm Reactor: Commercializing a Research Tool

*Linda R. Loetterle, A. Anacker, N. Beck, **J. Whitaker**, D. Goeres, M. Hamilton, D. Walker, and B. Warwood, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Biofilm consists of bacteria attached to a surface. Bacteria in the biofilm secrete a plastic-like substance called extracellular polysaccharide, which forms the protective slime layer around the cells and binds them together. An important difference between individual, suspended bacteria and the organized community of biofilm bacteria is that biofilm bacteria can tolerate very high concentrations of disinfectants and antibiotics. New products are needed to control biofilm bacteria. Applied biofilm research directed at finding those new products requires laboratory methods and apparatus that incorporate the defining parameters for each environment where biofilm exists. The Center for Biofilm Engineering (CBE) at Montana State University and BioSurface Technologies Inc. (BST), a Montana company that manufactures and markets laboratory supplies, have a history of successful collaborative efforts in producing reliable, repeatable, and marketable laboratory reactors. This collaborative project will provide BST with a new product to manufacture and sell; namely, the Drip Flow Biofilm Reactor (DFR). In biofilm research there are four environments of particular interest: high fluid shear (high velocity fluid flow over the surface), moderate shear, low shear, and no shear. Currently, BST manufactures reactors that represent the high and moderate shear environments. The no-shear environment does not require a sophisticated reactor system. That leaves a void for a reactor that grows biofilm under low shear conditions. The DFR will fill that void. Funding has been provided by the Montana Board of Research and Commercialization Technologies to develop a laboratory apparatus (DFR) that simulates an environment different from the reactors in the current BST product line, to design the DFR to meet the BST manufacturing and marketing criteria, and to develop a standard operating procedure (SOP) for the DFR that is suitable for use as an official standard method.

S05-P352

Influence of Co-Contaminants and Natural Organic Matter on the Reduction of 2,4,6-Trinitrotoluene (TNT), Chromium(VI), and Carbon Tetrachloride by Zero-Valent Iron

*David Steppler, **R. Gerlach**, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

A large number of sites in our environment are contaminated with pollutants such as 2,4,6-trinitrotoluene (TNT), chlorinated organics (e.g. carbon tetrachloride), and heavy metals such as Cr(VI). It is in the interest of the DOD, DOE, NASA, and EPA to find effective ways to clean up these areas. Zero-valent iron can successfully reduce each of these contaminants. This study was designed to develop a better understanding of the effects of natural organic matter on the fate of each contaminant in ZVI systems and the effects of the contaminants on one another when present together in one system. It was found that natural organic matter slows the reduction of carbon tetrachloride and TNT, while it speeds up the reduction of Cr(VI). Moreover, Cr(VI) decreases the reduction rates of TNT and carbon tetrachloride, while TNT slows the reduction of carbon tetrachloride. Neither TNT nor carbon tetrachloride appear to have an effect on Cr(VI) reduction rates, and carbon tetrachloride does not seem to have an effect on the rate of reduction of TNT. This research will provide kinetic parameters describing mixed waste transformation by zero-valent iron in the presence of natural organic matter, and thus contribute to improved designs of permeable reactive subsurface barriers.

S05-P353

Bioremediation and the Role of Mixed Pollution: TNT and Cr(VI)

*Nicholas R. Ballor**, R. Gerlach**, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Contamination of soils with explosives such as 2,4,6-trinitrotoluene (TNT) and heavy metals such as hexavalent chromium (Cr(VI)) is a widespread environmental problem. Concerns stem from the highly toxic, mutagenic and carcinogenic properties of these substances. The widespread use and manufacture of these chemicals have led to their inevitable release into the environment. Reductive bio-transformation is gaining momentum as an option for the clean-up of polluted subsurface soils. This strategy can serve to immobilize Cr(VI) and TNT. When Cr(VI) is reduced to Cr(III), its toxicity and solubility decrease dramatically. The reduction of TNT can lead to the formation of reduced metabolites which bind strongly to soils. However, since some metabolites of TNT are more toxic than the parent compound it is necessary to understand the pathways of TNT reduction.

A number of studies have investigated factors influencing bioremediation of TNT and Cr(VI). The impact of carbon sources and the presence of various iron(III) minerals on the biological reduction of Cr(VI) and TNT has been investigated in some detail, as has the role of electron shuttles (often modeled by anthraquinone-2,6-disulfonate, AQDS). Past studies have primarily focused on bioremediation of single compounds and did not address the biological reduction of mixtures of TNT and Cr(VI). This lack of understanding is problematic because heavy metals and TNT can be encountered as co-contaminants at US Department of Defense and Superfund Sites. Work investigating the influence of TNT and Cr(VI) on their simultaneous reduction by a Gram positive subsurface bacterium (*Cellulomonas* sp. strain ES6) in the presence and absence of the electron shuttle AQDS will be presented. AQDS and Cr(VI) had distinct impacts upon observed pathways of TNT reduction. Additionally, reduced TNT metabolites appeared to enhance Cr(VI) reduction rates. These findings can potentially find application in the effective design of successful bioremediation strategies at mixed contaminant sites, since it has become evident that a complex contaminant matrix can not be modeled accurately as a simple summation of the behavior observed for each of its respective components in isolation.

S05-P354

GFP Expression in Dormant and Actively Growing *Pseudomonas aeruginosa* Biofilm Cells

***Lee Richards,** P.S. Stewart, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Pseudomonas aeruginosa biofilms have been implicated in a myriad of health-related problems including colonization of foreign bodies such as catheters and implants as well as chronic infections in

wounds and persistent colonization of the cystic fibrosis lung. As with many other biofilm infections, these infections can be extremely difficult to treat with traditional antibiotics. In the case of implants and catheters, it is often necessary to remove the device from the host, treat the infection and install a new device. This is obviously very costly and results in more hospitalization time for the patient.

The reasons theorized for the increased tolerance to antibiotics vary from mechanistic antibiotic penetration problems to cell-to-cell signaling mechanisms. One thought is that within the biofilm, cells are in many different stages of growth or dormancy. It is theorized that there are dormant cells within the biofilm that, when treated with antibiotics, have an innate tolerance and subsequently survive the treatment and allow the biofilm to reoccur after treatment has stopped. It is these dormant cells that are explored here.

Pseudomonas aeruginosa tagged with an inducible green fluorescent protein (GFP) was grown in colony biofilm systems and the pattern of GFP induction was observed with fluorescent microscopy. This, in turn, identified the actively growing cells (cells which had produced GFP within the induction period). Biofilms were also grown to maturity in the presence of the inducer and then shifted to media lacking the inducer, thus indicating the dormant cells (the cells which remained bright after being removed from the inducer). This phenomenon was also explored in a flow cytometer.

S05-P355
Patterns of DNA Synthesis in *Staphylococcus epidermidis* Biofilms

***Suriani Abdul Rani**, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717*

Growth activity and patterns of DNA synthesis in *Staphylococcus epidermidis* colony biofilms were visualized and quantified using 5-bromo-2-deoxyuridine (BrdU) labeling. Incorporation of the thymidine analog, BrdU, into DNA during DNA synthesis in place of the native nucleotide provides a marker for proliferating cells. Labeled DNA was subsequently illuminated with a fluorescently labeled monoclonal antibody that specifically recognizes brominated DNA. Distinct zones of DNA synthetic activity were observed close to the nutrient and air interfaces. Growth zones averaged between 16 and 30 μm. Slow-growing or non-growing cells were observed in the interior of the biofilms. Patterns of DNA synthetic activity changed in response to changes in the nutrient and oxygen conditions. When the biofilm was subjected to anaerobic conditions, the growth zone was only observed at the nutrient interface. When glucose was added to the medium, variability in patterns was observed, probably due to non-growing or dead cells. When pure oxygen was introduced, a thicker growth zone was observed at the air interface which averaged 46 μm. Capillary and drip flow biofilms displayed similarly stratified DNA synthesis patterns, suggesting the applicability of this protocol on other reactors. Overall, the patterns observed suggest significant spatial heterogeneity in biofilm growth. This study suggests that BrdU labeling can be used to map patterns of DNA synthesis in biofilms. The presence on non-growing cells can be an explanation for reduced susceptibility of biofilm towards antimicrobial agents.

S05-P357
Efficient Removal of THM Precursors Using MF Membrane Combined with High Concentration of PAC

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Powdered activated carbon (PAC) is an important tool for maintaining the safety and aesthetic quality of drinking water. The conventional method of in-line dosing limits the concentration and contact time of PAC resulting in lower removal efficiency and wastage of PAC before utilizing the full adsorption capacity of contaminants in raw water. A combination of MF and high dose (40 g/L) of PAC enables rapid adsorption of contaminants as well as more efficient use of PAC. Small-scale experimental units were constructed in Tamagawa Water Treatment Plant (TWTP) located in the southern part of Tokyo, Japan. Raw water from the Tamagawa River was used either directly or after biological filtration using tubular polypropylene pellets (5 mm in length, 3 mm I.D., and 4 mm O.D.) as the filter media. Without PAC in the membrane basin, the reduction rate of trihalomethane formation potential (THMFP) was less than 10%, whereas with PAC, the reduction rate was about 80%. The operational parameters such as backwashing and the cycle-time had little effects on THMFP removal rates. With the combination of high dose of PAC and microfiltration, the THMFP in the filtrate was less than 20 g/L even in the winter, which was higher than the average removal efficiency of THMFP in the

winter season. The removal rates of dissolved organic carbons (DOC) were almost similar to the THMFP reduction rates. Without PAC, the DOC removal rate was only 3%, whereas with 40g/L of PAC about 80% of DOC was removed. After different combinations of PAC dose and operational conditions, THMFP was tried to limit for any impending danger.

S05-P358
Bacterial and Viral Transport Mechanisms Through Microfiltration Membrane

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Microfiltration has been used in recent years extensively as a secondary or tertiary treatment process in wastewater treatment and as a finer treatment process in drinking water treatment. Microorganisms such as Qβ, MS2, T4 virus and several bacteria strains in *Pseudomonas, Alcaligenes* and *E. coli* groups were filtered through different pore sizes of *nuclepore* and *anopore* flat sheet membranes in dead end mode. The pore models neglecting diffusion terms were very useful in the investigation of microorganism removal in membrane filtration. The obtained rejection results were analyzed with an existing pore model in which the rejection was related to the ratio of solute size to pore size. The existing pore models (transport equations) were adjusted to accommodate the microfiltration range particles. The adjusted pore models showed good agreement with the obtained experimental rejection results for virus, while the bacteria rejection results showed a deviation. This deviation was partially explained by the wide size distribution of bacteria. The equivalent spherical diameter can be used to represent the virus size, while the oval diameter of the cells may be the best possible dimension for bacteria size in membrane filtration. This determination showed the possibility of bacteria cells approaching the membrane pores in their longitudinal direction.

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Control of Biofilms by Natural Products

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Highly resistant bacterial biofilms have become a significant challenge in industry and medicine. A biofilm infection may need to be treated with as much as a 1.5 thousand times higher dose of antibiotic than is required to treat a planktonic bacterial infection. In this project, 20,000 natural plant-derived compounds are being screened to determine their effects on biofilm formation. The compounds have come from a taxonomically diverse collection of plant samples from the United States and Gabon, Africa. The collection of plants includes those in aquatic environments, specifically those in stagnant water. The ability of these plants to live in wet, sometimes swampy environments, thick with biofilm growth, suggests that they may have some biofilm inhibition capabilities. Within the experiment, there are three specific objectives. The first of these is to screen the 20,000 plants extracts for biofilm inhibition using *Pseudomonas aeruginosa*. The plant extracts found to be successful in inhibiting biofilm growth will then be purified into individual compounds. Unfortunately, during this phase, experiments have not been found to be repeatable due to high variance in the data. The focus has been to decrease variance to ensure repeatability. In the final phase of the experiment, individual molecules will be screened for the dispersion of existing *Pseudomonas aeruginosa* biofilms.