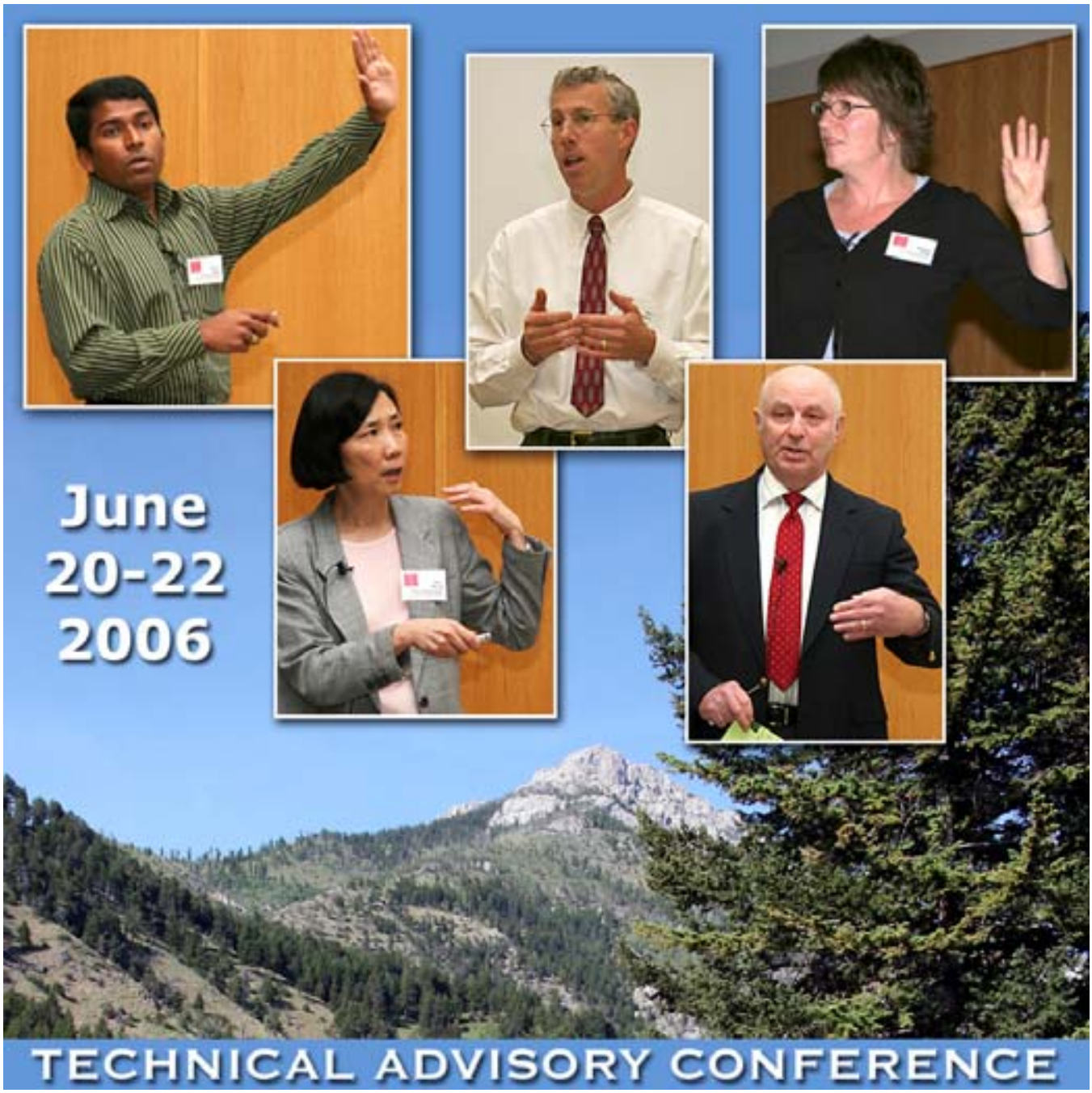
MONTANA STATE UNIVERSITY

Center for Biofilm Engineering

Bozeman, Montana

PROCEEDINGS



GENERAL INFORMATION

CBE LEADERSHIP

***Phil Stewart**, CBE Director and Professor, Chemical & Biological Engineering*
***Anne Camper**, Professor, Civil Engineering & Associate Dean for Research, COE*
***Al Cunningham**, Professor, Civil Engineering*
***Brent Peyton,** Associate Professor, Chemical & Biological Engineering*
***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.

Workshops are offered in conjunction with each Technical Advisory Conference.

Both Andreas Nocker-Einsiedler and Mark Burr received the CBE's 2006 Outstanding Researcher Award during the June Technical Advisory Conference.

MISSION AND GOALS OF THE CBE

The mission of the Center for Biofilm Engineering is to advance the basic knowledge, technology and education required to understand, control and exploit biofilm processes.

The CBE has identified goals in three areas of activity.

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

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

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

Industrial Associates Program Benefits

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

CBE Technical Advisory Conferences

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

Education and Training Workshops

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

Research/Testing Projects

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

Product/IP Development Consulting

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

Regulatory Interactions

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

For More Information

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

Presentation and Poster Abstracts
Technical Advisory Conference: June 20-22, 2006

Posters

SPEAKER ABSTRACTS

SESSION 1: Antimicrobial Surfaces

S06-S01
Antimicrobial surfaces in perspective—a review

Ross Carlson, *Assistant Professor of Chemical and Biological Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

From honey to crocodile excrement to nanoparticles, different approaches have been used over the millennia to control or to prevent biofilms on medical, communal and industrial surfaces. An overview of ancient and modern approaches to control microbial growth on surfaces will be discussed. While these strategies have met differing levels of success, there is still a need for new, effective antimicrobial surfaces. In a classic case of 'what's old is new,' the lessons learned by organisms over billions of years of evolution may provide insight into new strategies to counter ever emerging microbial challenges.

S06-S02
Synthesis and testing of new N-halamine biocidal materials

Dave Worley*, Professor and Interim Chair, Department of Chemistry, Auburn University, Auburn, Alabama*

The presentation will begin with a brief discussion of N-halamine monomer structures and N-halamine chemistry to include why the compounds are useful biocides. The majority of the presentation will be focused upon N-halamine biocidal polymeric materials which have been developed at Auburn University during the last decade. Preparation methods and test results will be presented for N-halogenated poly(styrenehydantoin) beads to be used in water disinfection applications, for biocidal textile materials, and for biocidal surface coating materials (polyurethane paints and siloxanes). Potential applications of the chemistry and materials will be discussed.

S06-S03
Control of biofilms accumulation on chitosan-coated surfaces

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

The main purpose of this research was to observe how chitosan may prevent the growth of biofilm on glass beads in a porous media reactor and its ability to reduce suspended bacterial counts. Chitosan (poly b-(1→4) N-acetyl-D-glucosamine) is a deacetylated product of chitin obtained from crab or krill and possesses a wide safety margin compared to the other positively-charged emulsifying agents. A 1% chitosan solution was made by dissolving it in glacial acetic acid. The chitosan solution was coated onto 3 mm diameter borosilicate glass beads. Rhoplex emulsion was first fixed on the beads and the chitosan solution was immobilized on this emulsion. Reactor C-1 contained glass beads without coating; Reactor C-2 had glass beads coated only by Rhoplex, and Reactor C-3 contained beads coated with Rhoplex emulsion and 1% chitosan. The volume of each reactor was 1072.27 ml, and the hydraulic retention time (HRT) was 5 hrs. The effluent from biologically activated carbon (BAC) columns was used as the influent for these three reactors. The average CFU/ml in the BAC effluent was 2.65×10^4 and the flow rate was maintained at 3.57 ml/min. The reactors were operated for nearly one month.

The chitosan-coated bead reactor (C-3) reduced 99~99.9% (2~3 log) of the influent cells for nearly four weeks, but the C-2 and C-1 reactors were ineffective after one week. These results show that the beads can capture cells at a significant rate for a very specific time period, after which their efficacy declines. From the stereoscope images, the thickest biofilm was formed on the chitosan-coated beads. The influent and effluents of the reactors were collected for different kinds of analyses. The effluent Total Organic Carbon (TOC) of the C-3 reactor was the lowest prior to the increase in bacterial growth; there was no change or a slight increase across the other two reactors. During the first few days, it appears that there was a release of material from the chitosan coating leading to the spike in turbidity, which then decreased to the lowest value of the three reactors. Interestingly, this increase in turbidity did not correlate with detection of chitosan or chitosan fragments by HPLC. The average Oxidation-Reduction Potential (ORP) values of the influent and

effluents of the C-2 & C-1 reactors were 215±20 mV, but the initial ORP of the effluent of the C-3 reactor was very high (285mV). This value decreased sharply with time, and at the end of operation the ORP was 245 mV. The probable reason for this decrease is a decline in the number of chitosan-reactive sites that influenced the potential for oxidation and reduction.

To assay for the release of chitosan or its breakdown products, the influent and effluents were assayed by HPLC. The molecular weight of constituents in the influent varied from 7,003,594 D to 10,711D, but that of the effluents of all three reactors were below the detection limit. The oligosaccharides from the BAC, C1 and C2 reactors varied from 15 D to 100 D (lower than the MW of a chitosan monomer) and those from the C3 reactor were below detection initially and then rose slightly to 12 D, which is still smaller than a chitosan monomer. These results suggest that the bead column system is good at capturing higher molecular weight dissolved molecules. Biofilm on the beads was extracted in three different layers (bottom, middle and top layers) of each reactor. The heterotrophic plate counts (HPC) of the C-1 and C-3 reactors were the highest. The extracted DNA was also quantified and the C-3 reactor values were the highest in each layer compared to layers in the C-2 and C-1 reactors. To obtain an initial understanding of the community structure in the three reactors in the three layers, the extracted biofilm DNA was used to load denaturing gradient gel electrophoresis gels (DGGE). The community structure varied between reactors, but did not vary within a single reactor. Cloning of samples gave a 56-clone library of each sample.

The efficacy of batch solutions of 1% chitosan (the same amount added to the beads) against the organisms in the BAC water and different gram-negative bacteria (*E. coli* O157:H7, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Pseudomonas syringae*) has been tested. The disinfection kinetics followed the first order decay equation and fit the Delayed Chick-Watson model. In the absence of bacteria, the chitosan was stable but declined in contact with cells. Disinfection rate constants, *'k' and 'CT'* for 90% inactivation of cells, were determined. We are now continuing the batch experiments with three different gram-positive and two yeast cells at the same disinfection conditions. The most important parameter is the ratio between cell and available chitosan. The possible future extension of this research will be (1) the modification of chitosan by Cu, arginine, & Ag, (2) development of the immobilized and modified chitosan coated beads, and (3) a disinfection study with specific gram-positive/ gram-negative bacteria and yeast cells.

S06-S06
Testing antimicrobial surfaces

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

Surfaces that prevent microbial attachment and/or subsequent growth and biofilm formation have tremendous potential in industry and medicine. A variety of products claiming antimicrobial surface properties are available on the market, and the development of new products is an intensely studied research area. However, there is no internationally recognized performance standard for evaluating antimicrobial surfaces. A variety of in-vitro test systems have been developed and used at the CBE for evaluating bacterial attachment and biofilm growth. These include flow cells, the rotating disc reactor, the CDC biofilm reactor, and a variety of specialized systems for particular applications. Quantifying the number and physiological state of bacteria on test surfaces has also been accomplished using a variety of methods. Some methods attempt to remove and disaggregate attached cells from the surface and then to quantify the re-suspended biofilm by methods such as viable plate counts or total protein and other assays. Another approach has been to evaluate the intact biofilm by direct microscopic observation or dye binding assays. This presentation will discuss the various approaches for growing and quantifying biofilm, with a focus on medical devices.

S06-S07
Textiles and antimicrobial testing

***Mark Fornalik,** Eastman Kodak Company*

Eastman Kodak Company has launched an effort in its Specialty Materials group, with a focus on silver antimicrobials. One of the first products from this effort is a silver antimicrobial formulation that is applied topically to polyester textiles. The term "antimicrobial," however, is vague and open to misinterpretation. For example, most industry standard tests in antimicrobial textiles examine the impact of topically treated antimicrobials on planktonic organisms, not on sessile organisms. Efficacy is measured by percent reduction over a control, rather than log reduction. The chemical and process "history" of a treated fabric greatly influences the selection of microbiology testing conditions as well as the outcome of the testing. Finally, "antimicrobial" in the textile industry typically means antibacterial, not antifungal, because the majority of testing is directed toward bacteria.

This talk will present our current understanding of each of these topics, and identify some of the gaps in antimicrobial textile testing.

SESSION 2: Biofilms in Food and Food Processing

S06-S08
Biofilms in the food industry

***Stewart Clark**, PhD Candidate, Microbiology, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Biofilms in the food industry have long been associated with pipe fouling and long-term storage concerns. Indirectly biofilms have been implicated in product spoilage and the resistance of pathogens to disinfection regimes. Common food-associated pathogens such as *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteridis* have been isolated from biofilms in dairy, beef and poultry processing plants. The effectiveness of sanitizers in the reduction of bacteria and their adherence has been shown to be dependent upon the bacteria present and the processing materials used. The use of sanitizer cocktails has been adopted as a control strategy in many areas of the food industry in order to combat biofilm persistence. However not all biofilms are regarded as objectionable, and in the aquaculture industry in particular, specific biofilm growth is actually encouraged to enhance settlement and metamorphosis of larval abalone, polychaetes and other benthic invertebrates.

S06-S09
Controlling undesirable bacteria in floor drains with competitive bacteria

***Mike Doyle**, Director, Center for Food Safety, University of Georgia, Athens, Georgia*

Reducing pathogen contamination with practical, effective, and environmentally friendly food safety interventions at critical sites within the food chain can be an insuperable challenge. An example of such a challenge is controlling the persistence of *Listeria monocytogenes* in floor drains of food processing facilities. After screening thousands of microbes, we have isolated and validated the efficacy of a small number of site-specific bacterial isolates that can substantially reduce the harborage of Listeria in floor drains. Critical parameters to obtaining successful competitive microorganism treatments include: (1) competitive microbes must colonize critical sites where target pathogens localize, (2) competitive microbes should survive well and persist in primary sites of colonization, (3) competitive microbes should produce antimicrobial activity to target pathogens, (4) competitive microbes should not possess virulence or promiscuous antibiotic resistance genes, (5) competitive microbes must survive at specified cell numbers in commercial package throughout the product's predetermined shelf life, and (6) competitive microbes must survive at effective cell. Beneficial microbes that can out-compete pathogens in the niches where they frequently reside in food processing facilities can be a productive approach to reducing the transmission of foodborne pathogens in difficult to treat locations in the food continuum.

S06-S10
Biofilms on produce and household surfaces: Microscopic imaging and microbial community analysis

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

There are quite possibly no surfaces within the biosphere on Earth that are not—or cannot be—colonized by microbial biofilms. Thus, human food items as well as normal household surfaces represent potential substrata for microbial attachment and growth, as well as a sink for potentially pathogenic microbial species. In 1999, a study was undertaken at the CBE to survey the presence of microbial biofilms on a number of regular food items removed from grocery shelves, as well as on a number of common household surfaces. Surveyed produce items included carrots, mushrooms, lettuce, and tomatoes; household surfaces included AC fins and ducts, kitchen sponges, cutting boards, walls, floors, and sills, water filters, and towels and socks. Selected samples of the above items were imaged for the presence of microbial biofilms using cryostage SEM, CSLM with Live/Dead staining, epifluorescence microscopy with acridine orange staining, light microscopy with alcian blue staining, and phase contrast and differential interference contrast microscopy. In addition, selected isolates from tomatoes and socks were subjected to Gram staining, fluorescent in situ hybridization (FISH) targeting somewhat general bacterial groups, and to the API and BBL identification systems. At the time this biofilm survey was conducted, it was thought that existing biofilms on food and household surfaces, if present, could provide a natural reservoir for the

survival of potentially pathogenic bacteria—an idea certainly supported by the ubiquitous presence of biofilms observed on all surfaces included in this study.

S06-S11
Biofilms in the food processing environments

***Amy Wong**, Professor of Food Microbiology, University of Wisconsin-Madison*

Microbial attachment and biofilm development in food processing environments are potential sources of contamination and may lead to food spoilage or disease transmission. Many foodborne pathogens and spoilage organisms can form biofilms on materials such as stainless steel, polystyrene, polyester, and rubber that are commonly present in food processing equipment. These organisms may survive for prolonged periods, depending on factors such as the attachment surface, the amount and nature of residual soil, temperature, and relative humidity. Areas that are more prone to biofilm development include dead ends, joints, valves, and gaskets. In addition, equipment surfaces can be corroded with age, developing pits and cracks in which soil and bacteria can collect. Biofilm organisms are more resistant than their planktonic counterparts to adverse conditions that may be encountered in a food-processing environment such as heat, desiccation, cleaners, and sanitizers. It has been shown that even with cleaning and sanitation procedures consistent with good manufacturing practices, microorganisms can remain on equipment and other food contact surfaces. This presents a challenge to food processors, especially in the control of ubiquitous foodborne pathogens such as *Listeria monocytogenes* or sporeformers such as *Bacillus cereus*. Much effort has been focused on developing alternative strategies (in addition to routine cleaning and sanitizing) to further minimize the potential for contamination of foods. One such strategy is the application of cold plasma technology for surface modification to impart antifouling characteristics and for decontamination of water, air, or surfaces. Some of our findings with the use of cold plasma processes will be presented.

SESSION 3: Biofilm Methods

S06-S12
Log reduction calculations

***Marty Hamilton**, Professor Emeritus, Statistics, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The log reduction is a quantitative measurement of disinfection efficacy. In concept, the log reduction is the difference on the log10 scale between the number of bacteria exposed to the disinfectant and the number of bacteria that survive that exposure. A log reduction of zero indicates a completely inefficacious disinfectant, and a large log reduction indicates good efficacy. When the disinfection efficacy test utilizes multiple samples, such as multiple control and treated coupons in a biofilm test, there are alternative formulas for calculating the log reduction, and the alternatives can produce different numerical answers. We recommend the so-called “mean of logs” approach for calculating the log reduction.

There are various alternatives to the log reduction; e.g., the percentage kill and the survival fraction. Even if we are planning to use one of the alternative measures when describing the results of a disinfection study, we first calculate the log reduction, do all of the statistical analyses for the log reduction, and, as the final step, convert the log reduction into the chosen alternative measure of efficacy. A heuristic justification for our recommendations will be presented along with some useful formulas.

For the conventional disinfectant test against biofilm bacteria, the log reduction measures the influence of the disinfectant in removing the biofilm as well as in killing the bacteria. If interest is solely on the ability of the disinfectant to kill the bacteria, the conventional log reduction is potentially biased because of (1) biofilm wash-off during the disinfect/neutralize step, (2) inefficient removal of the biofilm from its growth surface (substratum) for purposes of performing a viable cell count, or (3) poor disaggregation of the suspension that contains the removed biofilm prior to performing the viable cell counts. In this presentation, bias will be defined mathematically. For case (1), wash-off, formulas for the bias will be presented along with an example showing how the log reduction can be biased; that is, too large (positive bias) or too small (negative bias), on the average. This presentation provides the theoretical foundation for the subsequent two talks in this session which will describe laboratory biofilm experiments for evaluating each of the three potential sources of bias in specific disinfectant testing contexts.

S06-S13
Effects of cell wash-off on log reduction

***Alex Hilyard**, Standard Biofilm Methods (SBM) Intern, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

A laboratory disinfectant efficacy test against biofilm bacteria begins by growing a biofilm in a well controlled laboratory reactor that contains removable growth surfaces (coupons). Disinfectant efficacy is determined by comparing the number of viable cells remaining on a disinfectant-treated coupon to the number on an untreated (control) coupon. The typical testing protocol requires that each coupon is subjected to a variety of manipulations, e.g., treating, neutralizing, removing, and disaggregating. If, due to these manipulations, a significantly larger fraction of viable bacteria is lost from the treated coupon than from the control coupon, the viable bacteria count for the treated coupon would be artificially small, thereby biasing the efficacy measure. For this reason, the observed efficacy is a measure not only of the disinfectant's killing power, but also includes the disinfectant's propensity to remove viable bacteria from the coupon surface. The treatment liquid has always been assumed to kill the bacteria rinsed off of the coupon surface. The purpose of this investigation was to determine the extent to which cell wash-off biases log reduction calculations.

For these experiments, a *Pseudomonas aeruginosa* (ATCC 15442) biofilm was grown in the CDC reactor on glass coupons. Four experiments were performed in which six coupons were sampled. Three control coupons were rinsed, treated with synthetic hard water (pH = 7.2 ± 0.5) for 10 minutes, and neutralized. The three treated coupons were rinsed, treated with a 1000 mg/L free chlorine treatment prepared in synthetic hard water (pH = 7.2 ± 0.5) for 10 minutes, and neutralized with sodium thiosulfate. Biofilm was scraped from the coupon surface, homogenized, diluted, and plated. The control and treatment water were sonicated for 4 minutes, diluted, and plated.

These experiments demonstrate that a fraction of the biofilm washed off the coupon surface during treatment and neutralization remained viable. The mean log reduction in viable cells associated with a 1000 mg/L chlorine treatment for 10 minutes was equal to 1.412 ± 0.164 SEM, and this log reduction was not biased by cell wash-off.

S06-S14
EPA disaggregation study

***Kelli Buckingham-Meyer**, Research Assistant, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The log reduction (LR) value for an antibacterial agent is based on viable plate counts. Viable plate counts require that biofilm is first removed from a surface and then disaggregated into a suspension of single cells. If the removal and/or disaggregation efficiency varies between treated and control samples, then calculated LR values are not valid. Since the removal and disaggregation steps are imperfect, one should determine whether they potentially bias the LR. The objective of this study was to determine the feasibility of routine laboratory checks on the biofilm removal and disaggregation steps. Feasibility was established by demonstrating that there is at least one practical, inexpensive, and reliable method for checking each step.

The experiments for evaluating a check on biofilm removal were conducted using *Pseudomonas aeruginosa* biofilms grown in the Drip Flow and CDC reactors. Biofilm coupons were removed from the reactors and scraped or sonicated to remove biofilm. To evaluate the biofilm removal step, various check methods were tried, including methods based on the pour plate method, the agar blot method, the agar overlay method, the Multiple Removal Method (MRM), the dry weight assay method, the crystal violet colorimetric assay method, or the CTC assay method. Results for the removal studies demonstrated that the MRM assay proved to be useful for determining the removal efficiency of any step within a chosen removal method. The crystal violet assay, dry weight assay and CTC assay proved to be useful methods for differentiating biofilm covered coupons from coupons that were relatively clean due to a chosen removal method.

The experiments for evaluating a check on disaggregation were conducted using *P. aeruginosa* biofilms grown in the CDC reactor. Biofilm was scraped from the surface of a coupon into suspension. The suspension was homogenized with or without the addition of surfactants or high shear. A sample from the suspension was serially diluted and drop-plated to obtain viable plate counts. Another sample from the suspension was filtered onto a membrane and stained with Syto 9, then placed under the microscope. The particles were observed in each of 20 fields of view, where a particle could be either a discrete, isolated, multicellular clump or a single cell. The total number

of cells was calculated for each field of view. The counts were scaled up to produce a predicted viable cell count. For each predicted count there was an associated observed viable cell count. Disaggregation efficiency was evaluated by plotting the predicted versus observed as compared to the line of equality.

We conclude that it is feasible to devise protocols for checking the removal and disaggregation steps. In the future, we plan to document and thoroughly evaluate the most promising methods. Eventually we will nominate laboratory protocols to one of the organizations that review and approve standard laboratory methods.

S06-S15
A new standard method: The drip flow reactor with *Staphylococcus aureus*

***Jackie Whitaker**, Standard Biofilm Methods (SBM) Intern, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The Drip Flow Reactor (DFR), manufactured by BioSurface Technologies Inc. (BST), is useful for growing bacterial biofilms with a high cell density under low fluid shear conditions. Originally created as a research tool in Dr. Phil Stewart's Biofilm Control laboratory, the DFR demonstrated great potential to be standardized and commercialized. During the standardization process, a standard operating procedure (SOP) was developed for growing a repeatable *Pseudomonas aeruginosa* biofilm. Operating the DFR at the SOP resulted in a mean log density of 9.3 cfu/cm^2 with a repeatability standard deviation equal to 0.32. Laboratory testing also showed that the DFR was rugged with respect to four important operational factors—nutrient concentration, the angle at which the reactor was tilted to induce flow, the nutrient flow rate, and ambient temperature.

The CBE industrial associates have expressed a need for adapting the DFR SOP for other organisms. Because the DFR is often used for medical biofilm research, the SOP was modified for growing a *Staphylococcus aureus* biofilm. A set of preliminary experiments that altered nutrient concentration during batch and continuous flow, the batch time and the continuous flow time were performed to determine an initial SOP. All experiments were conducted at 37°C. A second set of experiments was then done to test the SOP's response to changes in reactor angle (10°), flow rate (0.8 mL/min/channel) and nutrient concentration (2,500 mg/L Tryptic Soy Broth (TSB)) during continuous flow phase. Preliminary data collected at SOP conditions indicates the protocol is repeatable.

Session 4: Industrial Water Treatment

S06-S17
Microbially influenced corrosion of copper in drinking water biofilms

***Mohammed Shahedur Rahman**, PhD Candidate, Civil Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The Lead and Copper Rule (LCR) sets the action level for copper in distribution systems at 1.3 mg/L. Copper corrosion can cause not only negative health effects, but also can damage the water supply infrastructure. It is known that water quality factors having the greatest effect on lead and copper corrosion are pH, alkalinity or dissolved inorganic carbonate (DIC), orthophosphate concentration, and buffer intensity. Also, because the microbial community in the distribution system is influenced by nutrients, the nutrient concentration in water may play a significant role in microbial copper corrosion. Because of the disinfection by-product (DBP) rule, many water utilities have switched to monochloramine. When monochloramine decays it forms ammonia, which may influence copper corrosion and cause nitrification in the distribution system and plumbing systems. The objective of this project is to investigate the effect of total organic carbon and ammonia on copper corrosion under stagnant flow conditions and to discover the diversity of the biofilm in a simulated plumbing system. A modified version of the commonly used CDC reactors was used in this project. In the first set of experiments, two types of copper coupons (new and old, i.e. pre-exposed to 0.1N NaOH solution) were used. These reactors were fed with water with different carbon (2~4ppm) and ammonia (0.36~0.71ppm) concentrations. Biologically treated tap water was used to supply the homogenous bacterial population. Water in the reactor was stagnant for eight hours and then flowed for five minutes. At the low carbon concentration for both old and new copper, total copper concentration is lower than that for high carbon reactors. A similar trend was also found in the case of the dissolved copper. Heterotrophic plate counts also showed higher numbers for high carbon reactors. After three months of operation the biofilm was sampled from the reactors and DNA was collected. Molecular techniques such as PCR and DGGE were used to analyze the microbial community profile of these samples. In the second set of experiments, pre-aged copper and PVC coupons were used with high carbon (4 ppm) and ammonia feed. After three months of operation, the PVC reactors showed evidence of nitrification, while the copper reactors also expressed

nitrification within five months. The nitrification in copper reactors may be delayed by copper toxicity. We are now investigating the population and processes of nitrification in these reactors. The microbial population in those reactors is being analyzed using PCR and DGGE. Also, a batch test is on going to estimate heterotrophic and autotrophic nitrification.

S06-S18
Bacterial transport in biofilms

***Wes Bauman**, M.S. Candidate, Civil Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The introduction of bacterial pathogens into drinking water systems from inadvertent and/or deliberate addition can lead to potential public health consequences. There is little information available on the entrainment of these organisms in biofilms on pipe surfaces and on filter media. In this study, a porous media biofilm reactor was used to investigate the ability of an established mixed-species drinking water biofilm to trap *E. coli* 0157:H7 as a model pathogen. This opportunistic human pathogen has the ability to survive in drinking water distribution systems by integrating into biofilms. Quantitative PCR targeting the Shiga-like toxin 1 (stx1) gene, plate counts, and direct microscopic counts are being used with a cyan-labeled *E. coli* O157:H7 strain to determine the fraction of organisms retained in a natural biofilm grown under different nutrient conditions in a porous media reactor. Threshold cycle (C_T) values from quantitative PCR were correlated with plate counts and direct counts. The results will be compared with those where inert fluorescent latex beads have been added to reactors as a surrogate for bacteria.

To date, results have been collected from control reactors where the organism has been added in the absence of a biofilm. An inoculum of 10^9 cfu/ml was added to sterilized reactors fed with filter-sterilized, dechlorinated drinking water at 25 ml/min. Five effluent pore volumes were collected in half pore volume increments before draining the reactor and performing destructive sampling to enumerate the retained fraction. Plating was done immediately upon collecting samples. Direct counts and DNA extractions were done within twenty-four hours with the samples stored at 4˚C. As with the experiments performed with fluorescent latex beads, most of our inoculum passed through the reactor for control experiments. Mass balances for control reactors have demonstrated a degree of error with our methods, as our percent recovery exceeded 100% for every experiment. Other experiments currently underway will determine how the control data compare with bacterial capture in the same reactor system when there is a precolonized biofilm. This biofilm will be grown using a 0.5 mg/L carbon supplement to represent drinking water conditions.

S06-S19
Inorganic particle transport in biofilms

***Anne Camper**, Professor of Civil Engineering/ Associate Dean for Research and Graduate Studies, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Biofilms are known to capture and retain inorganic particles as well as other organisms, but this process is poorly characterized. This process is important in a variety of situations, but is of particular interest in process water for personal care products or the food industry, in drinking water, or other environments where the captured particles may be undesirable or pathogenic organisms. One of the difficulties in investigating particle capture using organisms is that they are influenced by the environment, which affects their behavior in the system as well as in recovery methods. Previous work has found that mass balances using organisms are typically poor, with recoveries often below 10%. Consequently, we have chosen to use fluorescent latex beads of a size (1 µm diameter) and charge (negative) similar to that of many bacteria in water systems.

The experiments used porous media reactors operated in an upflow mode. Tracer studies demonstrated that there was no preferential flow. In initial experiments, a 10^3 inoculum of beads was added to reactors with no biofilm, a biofilm developed on 0.5 mg/L carbon, and a biofilm grown on 1.5 mg/L carbon. These conditions were used to simulate situations with varying levels of pre-existing biofilm growth. Five pore volumes of water were collected at half-volume increments and the beads concentrated by filtration. The columns were then drained and destructively sampled to determine the number of beads retained on the porous media and reactor walls. The fluorescent beads were enumerated using a ChemScanRDI instrument that uses laser detection. Mass balances on the beads could be closed, demonstrating that the method was providing us good recoveries. Most of the beads passed through the porous media, but there was an increasing trend of retention with the thicker biofilms. Maximal retention in the biofilms was approximately 7% in the control and low-nutrient biofilm, and 11% in the thicker biofilm.

In subsequent experiments, either 10^5 or 10^6 beads were added to the same porous media reactors precolonized with a biofilm grown on 0.5 mg/L carbon. Sampling protocols were identical to those used earlier. The method was more prone to error with higher particle numbers, and total recoveries tended to be up to 130%. Interestingly, the percent captured seemed to be approximately the same for each situation. Seven percent were retained in the biofilm when 10^5 beads were added, and 10% were retained when ten-fold more were used.

These results suggest that the biofilm surface is not saturated with the particles under any of the test conditions. However, the number captured appears to be proportional to the quantity that is added. These results will be compared with experiments using actual bacteria to determine if the beads can be used as a surrogate for their capture in biofilms.

S06-S20
Controlling localized corrosion in a complex cooling water system

***Mike Dorsey**, Senior Specialist, DuPont*

A DuPont plant has experienced severe localized corrosion from a complex corrosion phenomenon in its cooling water system. A large semi-closed cooling water system was created 10 years ago and since has had aggressive localized corrosion attack particularly on carbon steel surfaces. The problem has been exacerbated with microbiological influenced corrosion.

A new treatment program has been applied and is currently providing improvement in corrosion rates. The presentation will outline the background and the current status of the system.

Session 5: Microscopy

S06-S21
Scoping biofilm structure

***Betsey Pitts**, Research Associate/Facilities Manager, Microscopy, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Our idea of what a biofilm is and looks like changes with the tools we use to examine it. This presentation will focus on some of the Center's recently acquired imaging tools, which include new microscopes as well as new fluorescent stains, and what new perspectives on biofilm structure we obtain with them.

S06-S22
Using fluorescent proteins to assess biofilm growth

***Ben Klayman**, Ph.D. Candidate, Civil Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Many advancements have been made in labeling organisms with fluorescent tags since the first fluorescent protein gene was cloned from the jellyfish *Aequorea victoria.* This protein has been intentionally mutated to produce more rapidly maturing proteins, as well as those with shifted emission spectra, such as the cyan and yellow variants. More recently a new protein (DsRed) has been isolated from the coral *Discosoma striata.* The genes coding for these proteins can be inserted into the chromosome or onto plasmids that can be transformed into bacterial cells. The proteins are synthesized within the organisms and label the bacteria without the need for adding other stains or dyes. Consequently, individual cells can be observed non-destructively and in real time.

The use of these proteins has significantly advanced our ability to obtain information about the metabolic state and/or position of individual cells within a biofilm. The proteins can report on expression of a gene of interest or detection of a particular bacterial species. They can also be used in combination to report on more than one condition or several species at a time. Unstable proteins can be used to examine temporal changes in gene expression / local conditions. Fluorescent proteins can also be used in a more quantitative manner. Here at the CBE we are using fluorescent proteins and microscopy to generate data on the volume occupied by multiple species versus time in biofilm grown in a flow cell reactor. In addition, they can be used in single- species biofilms to examine growth rates at various locations within a biofilm cluster.

This presentation gives a review of the development of fluorescent protein applications for biofilm analysis, as well as current methods being used here at the CBE and in the literature at large.

S06-S23
AFM biofilm investigation

***Recep Avci**, Director, Image and Chemical Analysis Laboratory, Montana State University, Bozeman, MT*

*Zhiyong Suo[1], **Recep Avci**[1], Fernando Teran Arce[1], Laura Kellerman[1], Xinghong Yang[2], Rick Veeh[3], and Betsey Pitts[3]*

1) Imaging and Chemical Analysis Laboratory (ICAL), MSU Physics, Bozeman, MT 59717

2) Veterinary Molecular Biology Department, Montana State University, Bozeman, MT 59717

3) Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717

Atomic force microscopy (AFM) has been employed extensively to explore bacteria and other microorganisms because of its high-resolution imaging and sensitive force-determining capabilities in air and in the physiological environment of microorganisms. In ICAL we have successfully used AFM to study such microorganisms as *Salmonella typhimurium*, *Haemophilus influenzae* and diatoms to obtain information on their morphology and on their mechanical and adhesion properties. The imaging of a bacterium in an aqueous buffer and/or in air is achieved by immobilizing it using an appropriate technique on an appropriate surface. An example of such an image of *Salmonella typhimurium* is shown in Fig. 1.

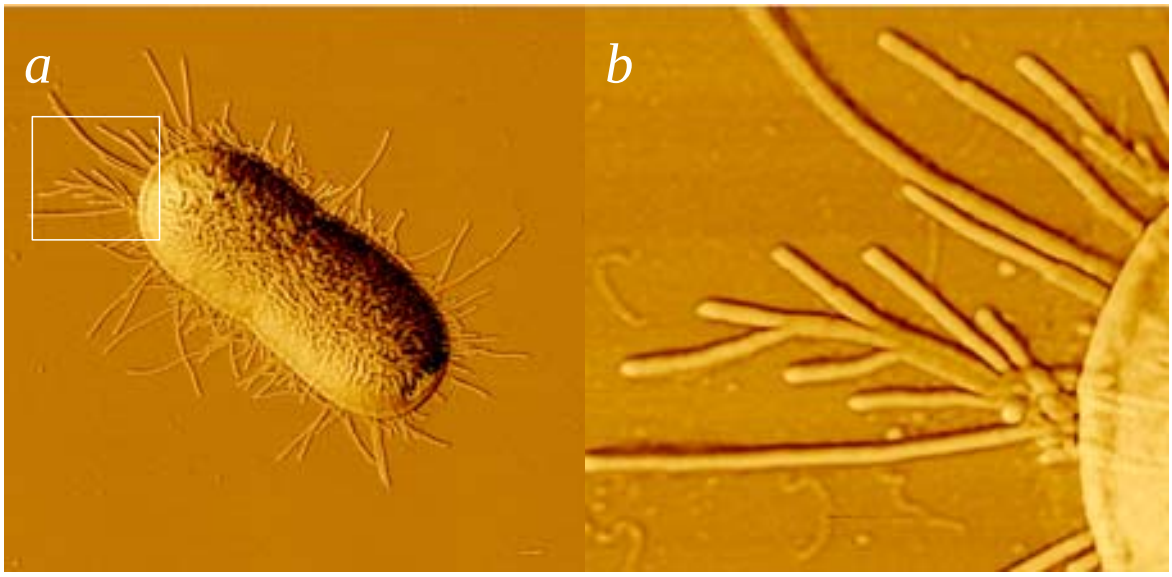


Fig. 1. AFM phase images obtained in ICAL showing *Salmonella typhimurium* expressing excess fimbriae at different magnifications: *(a)* Image of a dividing bacterial cell. A scan size of 4.5x4.5 μ m^2 was used. *(b)* Higher magnification of the white square marked in *(a)*, showing the fine details of the fimbriae. A scan size of 1.0x1.0 μm^2 was used. Both images were taken in air using AFM in tapping mode.

By using cleverly designed techniques to attach microorganism(s) to an AFM cantilever, one can construct a *bioprobe* with *live* microorganism(s), which can then be used to perform adhesion measurements on such surfaces as fouling release materials, catheters, epithelial cells and implant materials. By attaching proteins to sharp AFM tips via flexible tethers single-molecule receptor-ligand interactions can be determined. In our talk we plan to give a brief introduction to AFM and to show such examples as bacterial imaging, quantitative single-molecule receptor-ligand interactions, as well as adhesion and elasticity measurements on microorganisms in their physiological environments.

Regulatory Presentation

S06-S24
Regulatory methods: Recommendations for a suitable method for biofilm disinfectants

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

The U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs has responsibility for regulating antimicrobial products (pesticides) including those used to control pathogenic bacteria, viruses and other microorganisms on inanimate surfaces. The EPA recognizes biofilm as a pest. Therefore, any product claim to prevent, destroy, repel, or mitigate biofilm requires registration under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). As a component of registration, product efficacy must be demonstrated to support a specific claim; however, standard methods do not exist for determining the performance of biofilm disinfectants. Recommendations on desirable method attributes, testing of laboratory protocols, and suggestions for information to support the selection of a regulatory method will be provided. The applicability, relevance, standardization, and quality control aspects of potential methods will be emphasized.

Session 6: Environmental Biofilms

S06-S26
Transformation of pure-culture and mixed-population biofilms with genes of value for bioremediation

***Ron Crawford**, Professor of Microbiology, University of Idaho, Moscow, Idaho*

Bacterial transformation by naked DNA is thought to contribute to gene transfer and microbial evolution within natural environments, especially within biofilms where genetic exchange can be facilitated by the close proximity to one another of members of the biofilm microbial community. We tested the hypothesis that it is possible to take advantage of natural transformation processes to modify the phenotypes of biofilm communities by giving them specific and desirable functions. We demonstrated that biofilms composed of either pure cultures or mixed populations can be transformed with specific catabolic genes such that the communities acquire the ability to degrade a particular xenobiotic compound. Biofilms were transformed by plasmids bearing genes encoding green fluorescent protein (mut2) and/or atrazine chlorohydrolase (atzA). Confocal microscopy was used to quantify the number of transformants expressing mut2 in the biofilms. Degradation of atrazine by expressed atzA was quantified by tandem mass spectrometry. PCR analysis was performed to confirm the presence of atzA in transformed biofilms. These results indicate that it should be possible to use natural transformation to enhance bioremediation processes performed by biofilms.

S06-S27
Selective removal of DNA from dead bacteria and molecular monitoring of disinfection efficacy

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

A new method for distinguishing between live and dead bacteria has been developed. It consists of a fast and easy-to-perform treatment (around 10 min.) of a bacterial sample with a novel chemical (propidium monoazide, PMA) that limits the diagnostic analysis to the live portion of mixed communities. This distinction is essential in microbial diagnostics, as the potential risk from pathogens is normally limited to the live fraction of a bacterial community.

The method could have important implications for pathogen screening in water and food, sterility testing of drugs and personal care products, sanitization in the health care setting, as well as in the agricultural context. In contrast to another earlier reported method, the advantage of the new procedure is that it works with a wide spectrum of bacterial species. Furthermore, it was shown that disinfection efficacy can be monitored. The killing of the common pathogens *Salmonella typhimurium* and *Listeria monocytogenes*, using hypochlorite and benzalkonium chloride, respectively, serve as examples. Increasing killing of the pathogens could be directly detected using quantitative PCR.

The method consists in a simple treatment of bacterial samples prior to extracting and analyzing the genetic material. The principle is based on the intactness of bacterial cell membranes. Membrane integrity is a well-accepted criterion for distinguishing live from dead cells. Cells with

permeable membranes take up PMA—which does not, however, penetrate cells with intact membranes. Once inside dead cells, PMA attaches to the DNA. Exposure to strong visible light results in irreversible binding of this chemical to the DNA (at the same time, the light exposure renders any unbound chemical, which is still free in solution, inactive). As a result of the binding process, the DNA from these dead cells presumably becomes clumpy and insoluble and gets lost during the subsequent DNA extraction. Consequently, only the DNA from live cells with intact membranes does not take up the chemical remains. This relevant portion of the DNA can be further analyzed using different molecular diagnostic techniques.

S06-S28
Electron transport in engineered biofilms

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

Biological processes such as energy generation, compound transformation and growth are governed by electron transport processes. Hence, the development of a thorough understanding of reduction-oxidation (redox) reactions is one of the most important challenges in microbiology. The Office of Science at the Department of Energy is currently supporting a Grand Challenge in Biogeochemistry to reveal the mechanisms and processes that allow bacterial cells to transfer electrons to solid phase electron acceptors such as iron minerals. Similar to the challenges that such mineral reducing bacteria face, electron transport limitations can play an important role in biofilms. Thus, it is likely that bacteria and bacterial biofilms have developed specialized ways to ensure sufficient electron transport.

This presentation will summarize our current understanding of electron transport processes in biofilms. Some of the mechanisms and processes governing electron transport will be described, and examples of ways in which biofilms and attached bacteria overcome the electron transport challenge will be given. The presentation will close with conclusions about how the improved understanding of electron transport processes in biofilms can a) aid us in engineering more effective biofilm systems for beneficial purposes and b) potentially improve methods for biofilm removal.

S06-S28
Determinants of bacterial cell surface physicochemistry and architecture

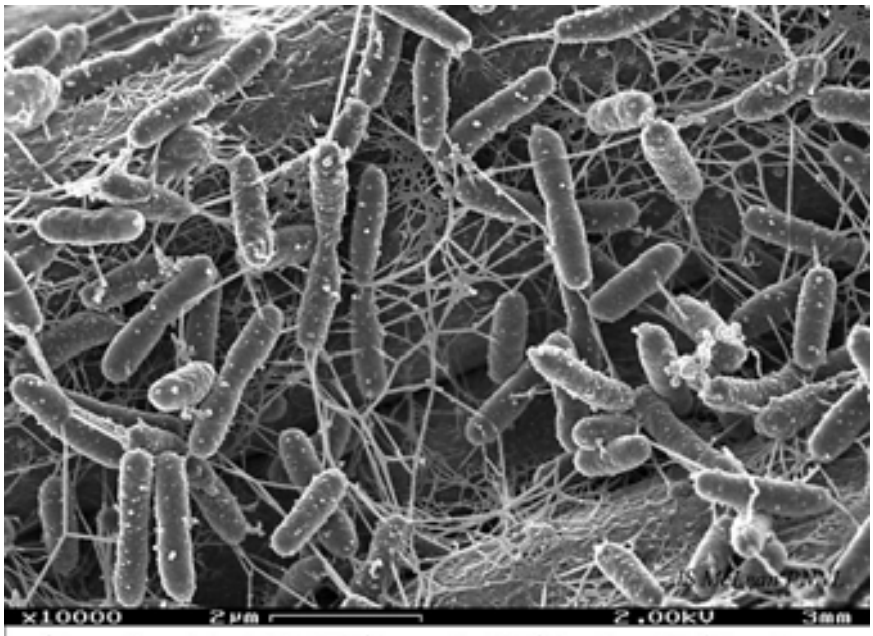
***Andrew Neal**, Savannah River Ecology Laboratory, Aiken, South Carolina*

Attachment of dissimilatory iron reducing bacteria to Fe(III)-oxide and oxyhydroxide mineral surfaces has important implications for environmental Fe-cycling and water quality in subsurface environments, including the mobility and availability of metal and radionuclide contaminants. Cell attachment at Fe(III) mineral surfaces should facilitate the direct transfer of electrons from cell to mineral, an important process in subsurface environments with low dissolved organic carbon, since extrinsic electron shuttling capacity is likely to be low. For cells capable of reduction of solid electron acceptors, the outer membrane (OM) is important as a site for iron-reductase expression. Mechanistically, there is a clear role for OM structures to exert significant influence upon cell-mineral interaction beyond electron transfer. Gaining greater insight into the biomolecular basis for adhesion to iron minerals is essential, not only in understanding predominant mechanisms for direct electron transfer, but also in predicting transport of cells through porous vadose and subsurface environments. The outer membrane architecture of *Shewanella* is dynamic: terminal electron acceptor availability significantly affects capsule development in both *S. oneidensis* and *S. putrefaciens* as determined by physicochemical properties (cell fixed charge density and electrophoretic softness) from microelectrophoresis and chemical characterisation (ATR-FTIR and XPS). Furthermore, genes associated with both Type II and Type V protein secretion also exert significant influence upon the outer membrane topology. The implications of these findings for electron transfer and cell attachment and biofilm formation will be discussed.

Bacterial nanowires: Electrically conductive filaments and their implications for energy transformation and distribution in natural and engineered systems

***Jeffrey McLean**, Biological Sciences Division, Pacific Northwest National Laboratory*

Shewanella oneidensis MR-1 produced electrically conductive pilus-like appendages called bacterial nanowires in direct response to electron acceptor limitation. Mutants deficient in genes for c-type decaheme cytochromes MtrC and OmcA, and those that lacked a functional Type II secretion pathway displayed nanowires that were poorly conductive. These mutants were also deficient their ability to reduce hydrous ferric oxide and in their ability to generate current in a microbial fuel cell. Nanowires produced by the oxygenic phototrophic cyanobacterium Synechocystis PCC6803 and the thermophilic, fermentative bacterium *Pelotomaculum thermopropionicum* reveal that electrically conductive appendages are not exclusive to dissimilatory metal reducing bacteria and may, in fact, represent a common bacterial strategy for efficient electron transfer and energy distribution.



Shewanella oneidensis MR-1 Biofilm on microbial fuel cell graphite felt anode.

S06-P371 (revised)
Evaluation of antimicrobial rinse solutions for urinary catheters

***L. Bickle**, E. Pulcini, G. James, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Urinary catheters are frequently used in medical practice, but routine catheter insertion can introduce bacteria, such as *Escherichia coli*, from the urethra into the bladder. Once introduced, the bacteria can attach and colonize the lumen of the catheter, resulting in a biofilm and urinary tract infection that responds poorly to antibiotic therapy. For long-term catheter patients, an infection may cause blockage in the catheter, even serious kidney or blood infections. Sterile saline is currently used to prevent biofilm formation; however, infection remains a problem and many infected catheters must be replaced. NovaCal Pharmaceuticals, Inc. (NovaCal) recently developed a promising alternative to sterile saline in the form of antimicrobial rinse solutions. An in vitro test system was developed to evaluate the efficacy of NovaCal's antimicrobial rinses as an alternative to sterile saline or catheter replacement.

The test system involves 5 catheters aseptically installed with a sterile Artificial Urine Media flow at 0.75 mls/minute. Each catheter is inoculated with a urease positive strain of *Escherichia coli* overnight culture and allowed an attachment period of 2 hours. The media flow is resumed and the biofilm is grown for 3 days. Four test solutions and a sterile saline control are pumped (2mls/minute) via valves from the bag-end of the catheter, through and out the bladder-end of the catheter. The total treatment time is 40 minutes, including 10 minutes to pump and 30 minutes of stationary disinfection. The catheters are then sampled by scraping sections of tubing and plating on agar for viable cell counts. The catheters are also imaged using Confocal and Scanning Electron Microscopy.

Using the in vitro system, NovaCal's test solutions showed a significant reduction in viable cell counts, which were verified visually by microscopy.

Plate count results:

Test solution	Average viable cell counts for 3 run	Average log reduction (compared to control)
Sterile saline	1.22E +09	
NVC-101 at 0.01%	6.84E+04	6.54
NVC-101 at 0.1%	3.07E+02	8.22
NVC-422 at 4mM	4.65E+04	5.53
NVC-422 at 40mM	5.86E+03	7.56

S06-P374
Contaminant degradation by zero valent iron

***D. Steppler** and R. Gerlach, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The contamination of soils and groundwater is a growing problem for many United States governmental agencies and industries. Specifically, the Departments of Defense and Energy, the Environmental Protection Agency, and NASA are seeking to develop efficient ways to control and detoxify 2,4,6-trinitrotoluene (TNT), chlorinated organics (e.g. carbon tetrachloride), and heavy metal contaminated sites. Several studies have been performed with the aim to detoxify each of these contaminants alone in a system, but there are many sites with more than one contaminant present. So it is important to understand the behavior of these contaminants in mixed waste streams. Our study was designed to determine the detoxification kinetics and product formation for each contaminant in zero-valent iron (ZVI) permeable subsurface barriers. We determined the influence of contaminant mixtures and anthroquinone-2,6-disulfonate (AQDS), a model for humic substance, on the detoxification

mechanisms. We found significant differences in transformation pathway and kinetics depending on the composition of the mixed waste stream. Our findings will contribute towards improved ZVI-based permeable reactive barrier design.

S06-P376
Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal active and inactive regions

***Suriani Abdul Rani**, Philip Stewart, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Biofilms harbor both active and inactive cells, and it is a challenge to characterize the spatial and population heterogeneity of specific activities within a biofilm. Spatial patterns of DNA replication and protein synthetic activity were imaged by techniques developed using staphylococcal systems. The first technique measures DNA synthetic activity by pulse-labeling with the thymidine analog 5-bromo-2-deoxyuridine (BrdU) followed by immuno-fluorescent detection of brominated DNA. The second technique makes use of an inducible green fluorescent protein construct that can be used to detect the capacity for de novo protein synthesis. These techniques were applied to biofilms grown in three different reactor systems. In all cases, measurements revealed that even in simple single-species biofilms, complex spatial distributions of anabolic activity occur. In a colony biofilm system, two distinct regions of DNA synthetic activity were observed—one close to the nutrient interface and another adjacent to the air interface. A similar pattern was measured by GFP induction. The dimensions of DNA synthetic activity ranged from 25 to 31 µm and the average protein synthetic activity ranged from 36 to 38 µm at the air interface.

When pure oxygen was introduced, a wider zone of active DNA replication (45 µm) and GFP synthesis (59 µm) was measured at the gas interface. Calculated oxygen penetration (26µm) corresponds with the zones of respiratory activity (19 to 38 µm), DNA synthetic activity and protein synthetic activity measured at the air interface. The dimensions of DNA synthetic activity and protein synthesis activity at the nutrient interface ranged from 13 µm to 19 µm. The addition of glucose to the media increased the zone of protein synthesis at the nutrient interface to 33 µm. Stratified patterns of activity were also observed in biofilms developed in two continuous-flow reactors. While biofilms harbor regions of active anabolism, the techniques also demonstrate that these biofilms contain regions of complete inactivity. Such inactive zones may contribute to the special ecology of biofilms and tolerance to antimicrobial agents. The techniques, particularly BrdU labeling, are generic and may find application to many microbial biofilm systems.

S06-P377
Technique to visualize extracellular polymeric substance in biofilms grown under different shear conditions

***K. Buckingham-Meyer**, D. Goeres, D. Walker, M. Hamilton, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The matrix that binds biofilm bacteria consists of extracellular polymeric substances (EPS), proteins, DNA and water. Many cellular fluorophores exist that allow microscopic visualization of bacteria within biofilms. However, matrix-specific stains are fewer in number. Stains such as lectins bind specific monosaccharide or polysaccharide components of EPS while others bind non-specifically. The goal of this study was to visualize EPS components of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms grown under three different shear conditions via confocal scanning laser microscopy (CSLM).

Biofilms consisting of *P. aeruginosa* or *S. aureus* biofilms were grown on glass coupons under different shear conditions in three laboratory reactor systems: CDC biofilm reactor (CDC), drip flow reactor (DF) and static biofilm reactor (SB). The CDC was the high-shear system; the DF, the low-shear system; and the SB, the no-shear system. The coupons were removed from the growth systems, rinsed and stained with viability stains, propidium iodide and/or Syto 9. The coupons were rinsed and then either calcofluor white (CW) (a non-specific EPS stain) or TRITC-conjugated concanavalin A (ConA) (a monosaccharide specific stain) was applied. Microscopic images were collected using an AOBS CSLM with one-photon and two-photon excitation. CSLM image stacks were analyzed using MetaMorph and Imaris software.

Biofilms grown in the CDC, DF and SB reactors contained varying levels of EPS. When CW-stained biofilms were analyzed via CSLM, the ability of this stain to bind non-specifically was confirmed by the presence of cloud-like EPS structures and varying fluorescence intensity measurements among the

biofilms grown under the different shear conditions. It was observed that the ConA bound to specific regions of the biofilm structures indicating that EPS components are heterogeneously distributed. The images collected in this study indicate that ConA and CW exhibit promising potential for differentiating specific and non-specific EPS components of biofilms grown under different shear conditions.

S06-P378
Magnetic resonance microscopy analysis of biofilms

***S.L. Codd**, R. Fell, J. Hornemann, A. Lysova, J.D. Seymour, and P.S. Stewart, Center for Biofilm Engineering, at Montana State University, Bozeman, MT*

Magnetic resonance microscopy (MRM) has been used to characterize the advective transport in a biofilm capillary reactor. The biofilm generates non-axial flows that are up to 20% of the maximum axial velocity. The presence of secondary velocities of this magnitude alters the mass transport in the bioreactor relative to non-biofilm fouled reactors. The characteristics of the transport indicate a scaling of the observed oscillatory flow with the capillary diameter. Ways of statistically evaluating these observations are being explored. MRM techniques are capable of spectrally and spatially resolved diffusion coefficients. We are applying these methods to the biofilm matrix and observing the impact of antibiotic activity on the diffusion of the various spectral peaks. By observing changes in the diffusion we are able to determine whether the antimicrobial is acting by changing the cell or EPS structure. Attempts are underway to extend these measurements to the spatial dimension to measure changes that occur in the biofilm structure as a function of depth.

S06-P379
Standard method to assess antimicrobial efficacy in dental unit waterlines

***Paul J. Sturman**, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Introduction: Microbial growth in dental unit waterlines can cause process water to exceed drinking water standards for bacterial loading, necessitating antimicrobial treatment. This has led to the development of many products designed to control both planktonic organisms and biofilm growth in dental units. However, no standard method yet exists to test the efficacy and performance of these products.

Objectives: ADA/ANSI Working Group 9.48 sought to develop an inexpensive and versatile laboratory method to assess DUWL antimicrobial efficacy under a wide range of conditions.

Methods: A defined, dual-species microbial consortium was used in this test method. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were grown in separate chemostats, diluted to a total concentration of approximately 500 CFU/ml with make-up water, and added intermittently to the DUWL apparatus on a schedule intended to mimic dental chair use. Effluent planktonic cell counts and biofilm were sampled on a daily basis to assess the onset of steady state conditions within the 1.5mm diameter, polyurethane water lines. The test apparatus contains 2 parallel water line systems such that one line is used to test additive products according to the manufacturers' instructions, and the other line receives only bacterially loaded water (control). Daily sampling of the test and control lines included viable counts of planktonic effluent cells and biofilm cells. Preliminary tests were performed with chlorine as a model biocide.

Results: Preliminary results indicate that a mature dual-species biofilm is grown in 10 days to a concentration of $5x10^3$ CFU/cm^2 (n=23). Test lines treated with chlorine (30 mg/l, 5 minutes/day) beginning at day 12 showed an immediate 2-log reduction in attached cells; however, treatment efficacy decreased over 14 days.

Conclusions: This apparatus can both effectively duplicate biofilm growth in a clinical setting and measure changes in attached viable cells as a result of treatment.

S06-P380

Direct visualization of spatial and temporal patterns of antimicrobial action inside model oral biofilms using two-photon laser microscopy

Shoji Takenaka, *Betsey Pitts, William Davison, Benjamin Unterreiner, Philip Stewart, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Many approaches to measure or visualize antimicrobial action in biofilms are reported. But most of them have limitations in that they are either destructive or provide single-time point information. In addition, most give no spatial or temporal patterns of antimicrobial action inside biofilms. The purpose of this study is to visualize the spatial and temporal patterns of antimicrobial action inside model oral biofilms directly with the chemicals using two-photon laser microscopy.

Streptococcus oralis, *Streptococcus gordonii*, *Actinomyces naeslundii*, which frequently initiate colonization and coaggregate with each other on the tooth surface, were allowed to develop for 20 h in the glass capillary biofilm reactor. Biofilm was stained with calcein-AM for 2 h statically, and antimicrobial action against biofilm was analyzed by confocal scanning microscopy. Calcein-AM causes intact bacterial cells to stain green, and treatments that damage the cell membrane cause the green fluorescence to leak out of the cell. The chemicals used in this study were alcohol-free chlorhexidine and ethanol.

The chlorhexidine reaction was effective. But chlorhexidine did not appear to affect micro-organisms at depths of 100 or 120μm even in continuous flow for 20 min; the biofilm was not killed completely even after 20 min. The patterns of calcein-AM intensity between the chemicals were quite different.

The CLSM approach allows the non-destructive and three-dimensional analysis of fully hydrated microbial communities. Furthermore, two-photon imaging is optimal for analyzing thick biofilms and for avoiding photobleaching. In this study, the two-photon was used to evaluate the change in bacterial viability deep in biofilm clusters using several different treatments. It was clearly demonstrated to be useful in assessing microbial activity over time after treatment.

S06-P381

Developing a model for the flow cytometric analysis of ice cores

Bryan Close, *Christine Foreman, John Priscu, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Methods have been developed to use a flow cytometer to analyze particles in glacial ice cores. These methods include: staining, sample preparation, and data analysis procedures. The system was tested for accuracy and precision using particle size standards and comparisons with microscopic particle counting methods. A data processing model was built in order to easily quantify the data output by the system. This system is capable of distinguishing between biotic and abiotic particles, and can not only quantify the particles but provide information on their size distribution. The data analysis routine developed can take these distributions, fit curves to them, and output many useful statistical parameters. Glacial ice contains an important reservoir of information on past climatic events. Information on the distribution of biotic and abiotic particles in ice will increase our understanding of biogeochemical processes that occurred during past glacial and interglacial periods on our planet.

S06-P382

Heterogeneity and distribution of biofilm on reverse osmosis and nanofilter membranes in rotating disk reactor system

M.M.T. Khan, *B. Mickols, D.J. Moll, J. Niu and A.K. Camper, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Membrane filtration in water treatment enables the production of safe potable water by removing microorganisms and inorganic and organic compounds. Biofouling is a major reason for flux decline in plant performance. The formation of biofilm on membranes within an RO/NF element raises the applied filtration pressure as well as causes a flux decline. To observe the biofilm formation potential on the surface of Reverse Osmosis (RO) and Nanofiltation (NF) membranes, ten different RO membranes and one NF membrane developed by the FilmTec Corporation, a wholly owned subsidiary of the Dow Chemical Company, were examined.

The biofilm formation study was carried out in three Rotating Disk Reactor (RDR) systems. Ten RO membranes including Dow's commercial membranes BW30XLE and BW30, eight experimental RO membranes, and one NF membrane were used. Each reactor was operated for 31 days at constant temperature. The rotation speed of the rotor in the RDRs was 50 rpm. The feed was biologically activated carbon-treated water (average HPC of 1.63 x 10^4 cfu/ml) supplemented with nutrients (C:N:P). The residence time for all RDRs was 3 h and final carbon dose was 1.5 mg/L. For analysis, the membranes were carefully prepared without disturbing the biofilm. Membranes were cryo-sectioned at a thickness of 5.0 μm. The LIVE/DEAD *BacLightTM Bacterial Viability Kit* was used for Live/Dead staining of cells on the surface of the membrane. The stained membranes were observed under an epifluorescence microscope. For AFM analysis the membrane samples were dried before taking the images. The image height and width were up to 1000 nm and 25 μm respectively. In addition to viewing the fouled surfaces, the membranes were also observed after cleaning with a rubber scrubber and nanopure water wash.

On the surface of RO #1, #5, #8 and #11 membranes, large numbers of cells were observed and the relative number of live cells (dyed green) was greater than dead cells (dyed red). On the surface of RO #3, #4, #6, #7, and #9 membranes, many cells were also observed, but the relative number of dead cells was greater than that of live cells. The lowest population was observed on the surface of the RO #2 membrane. Here, the relative number of live/dead cells was almost equal. Compared to all RO membranes, the NF membrane showed the lowest accumulation of cells. Based on this experiment, it may be concluded that differences in surface properties or treatments on the membranes contributed to variations in biofilm formation and the proportion of live/dead cells.

Atomic force microscopy (AFM) was used to determine the distribution of the biofilm after physical cleaning. This simple technique removed the material in the microporous and mesoporous spaces of the membranes. The uneven surface of the membranes gave more heterogeneity in the biofilm thickness, which may cause lower cake resistance from the biofilm during actual membrane operation. For each membrane sample, 15 cryo-sections were taken and from each slice, one line-scan image of green and red cells was taken. MetaMorph surface topography image analysis software was used to quantify the images and estimate the average and statistics that define the heterogeneity of the biofilm thickness. Each image gave different distributions of live and dead cells found in the biofilm. The skewness of the peak/distribution of the line-scan image depends on the distribution of live and dead cells along the scan. To calculate the thickness at which live and dead cells occur in each image, the width at the mid-height was taken. Based on optical analysis and the number of days of observation, it appears that the lowest maximum average growth of biofilm will be less than 1 μm/day on the NF, RO #2, #7, #9 and #11 membranes (which varied from 0.61 to 0.97 μm/day). The *standard error of the mean* was low and varied from 1.0 to 4.0 μm. In light of this caveat, relative comparisons between the membranes can be made, and the best "performance" was seen with RO #2 and #9. Based on actual filtration data, the A-value was the lowest (0.098 gfd/psi) with RO #9 and highest (0.37 gfd/psi) with NF. The P%SP was higher (1% $MgSO_4$) with NF than with Dow's commercial membranes BW30XLE (0.7% NaCl) and BW30 (0.4% NaCl). Membrane RO#2 and NF showed moderate and very high membrane permeability respectively. The fouling tendency was neither related to membrane permeability (A-Value) nor related to the different types of membrane (RO vs. NF), which illustrates that membrane biofilm formation is controlled by other surface characteristics. At present, membrane researchers consider membrane biofilm formation to be equivalent to biofouling and treat it as a "black box." A better understanding of the progress and growth of biofilms would decrease operational costs and membrane replacements and increase process performance. Although the analyses described here are somewhat simplistic, they provide one approach toward understanding biofilm formation and biofouling.

S06-P383
Factors influencing growth-coupled cDCE oxidation by *Polaromonas sp.* strain JS666

L.K. Jennings *and J.M. Gossett, Cornell University, Ithaca, NY, USA*

A novel bacterium—*Polaromonas sp.* strain JS666—recently isolated by Coleman et al., aerobically oxidizes *cis*-1,2-dichloroethene (cDCE) using this common groundwater contaminant and suspected carcinogen as its sole carbon and energy source. This was the first conclusive evidence of growth of an aerobic organism on cDCE. The discovery of growth-coupled cDCE degradation by

strain JS666 eliminates the need for the addition of a co-substrate, making strain JS666 a promising bioaugmentation agent at the numerous sites in which cDCE has accumulated, typically as a result of incomplete reductive dechlorination. The use of JS666 as a bioaugmentation agent requires a better understanding of the factors that influence cDCE degradation.

Laboratory experiments were designed to investigate the effect of pH, culturing technique, and biomass concentration on cDCE degradation. Results showed that cDCE degradation was inhibited at low pH (~6.3-6.4) and could be restored by readjusting the pH to neutral by the addition of NaOH. Two different culturing techniques for growing JS666 were investigated in this work, including (i) the use of succinate as a co-substrate with cDCE to grow JS666 to high biomass levels, followed by centrifugation, and a wash and re-suspension of the cells into fresh medium with cDCE as the sole carbon source, and (ii) the transfer of a small fraction of actively degrading culture to fresh medium with cDCE as the sole carbon source. Transfer cultures demonstrated growth coupled to cDCE degradation and allowed for sustained degradation of cDCE for extended periods of time. The lag phase prior to the onset of degradation was reduced with each transfer from approximately forty days to less than one day. Furthermore, the rate of disappearance of cDCE increased in transfer cultures as degradation proceeded. On the other hand, activity in cultures that were washed and re-suspended into fresh media at high biomass concentrations resembled cometabolic degradation in that degradation rates declined, biomass concentration decreased, and degradation was not sustained. Furthermore, the degradation rates decline more rapidly in cultures with high biomass concentration than in cultures with low biomass concentration. Biomass concentration in transfer cultures was an order of magnitude lower than in washed and re-suspended cultures. Experiments were carried out to access the significance of biomass concentration on cDCE degradation by strain JS666.

S06-P384
Evaluating the effect of biocides on the plant pathogen *Pseudomonas syringae*

K.E. Sossa, *C.E. Morris, D.C. Sands, A. Camper, J. Becerra and H. Urrutia, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Pseudomonas syringae is one of the most important bacterial phytopathogens in Chile and it can cause important losses in agricultural products. The biocides usually used to control bacterial pathogens of plants in Chile are copper-based compounds (CuOH, $CuSO_4$, CuO_2) and antibiotics (streptomycin sulfate, streptomycin/oxytetracycline), but they have limited efficiency. In the screening of biocide efficiency for plant pathogens, few studies have considered the in situ conditions of bacterial colonization of plants, including biofilm formation that might hinder biocide efficiency in the field. The objective of this study is to evaluate the effect of biocides with different mechanisms of action on physiological viability and infectivity in plants of *Pseudomonas syringae* pv *aptata* strain CC94 grown as colony biofilms.

Colony biofilms grown for 4 days on 10% TSA and 0.1% TSA were treated with different concentrations of hydrogen peroxide, copper sulfate and streptomycin sulfate. The analyses to evaluate the effect of the biocides were: 1) to visualize in cryosections the live, dead and actively respiring cells in the biofilms, 2) to count culturable, live, dead and actively respiring cells and 3) to determine infectivity in plants after biocide treatment. Colony biofilms from 10% TSA had a thickness of about 100 µm and had 1x 10^{10} cells/colony; biofilms from 0.1% TSA had a thickness of about 50 µm and contained 1x 10^8 cells/colony.

Pseudomonas syringae strain CC94 is highly resistant to H_2O_2, because it produces a high concentration of catalase. In the presence of a catalase inhibitor (KNO_2) a significant decrease in infectivity of plants and in cell viability is observed for cells exposed to 2% and 5% H_2O_2 from colony biofilms grown on 0.1% TSA and 10%, respectively. When the H_2O_2 is in the presence of iron, it was observed that H_2O_2 has effect only to a low concentration of 0.075% H_2O_2, causing bacterial death (4.4-log reduction in culturable cells and 3-log reduction on live and respiring cells) in biofilms from 10%TSA and 0.1%TSA. In *Pseudomonas syringae* we have observed a mode-one killing effect

of a low concentration of H_2O_2 and a mode-two killing effect of a high concentration, as has been described for *Escherichia coli*.

$CuSO_4$ at 1000 mg/L produces a 4.5-log reduction of culturable cells from 0.1%TSA, and reduces only by 2 log units the live and actively respiring cells. However, after $CuSO_4$ treatment, the cells maintain a level of infectivity nearly equal to non-treated cells. For cells from 10% TSA, $CuSO_4$ at 1000 mg/L causes a reduction of 2 logs of culturable, live and actively respiring cells but does not reduce infectivity.

Streptomycin sulfate at 1000 mg/L produces a 4-log unit reduction of culturable cells from 10%TSA and 0.1%TSA.

The effect of the mode-one killing of H_2O_2 and streptomycin seems to be independent of thickness and cell concentration (mass) in the biofilm, however the mode-two killing of H_2O_2 and copper depend on the thickness and on the cell concentration in the biofilm.

S06-P385
Influence of electron shuttling compounds on antibiotic efficacy

***H. Cicon**, R. Gerlach, P.S. Stewart, W.M. Davison, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Bacterial biofilms can cause perpetual infections in damaged human tissue and implanted medical devices. Infectious bacteria attach to the devices or tissue and resist antimicrobial chemotherapy by growing in biofilms which protect them. Recent studies have supported the hypothesis that slow or no growth of *S. epidermidis* is responsible for its reduced susceptibility to certain antibiotics. Thus, it is likely that stationary-phase-like cells harbored in nutrient and oxygen limited regions of biofilms can withstand antibiotic treatment to a better degree than actively growing cultures.

Electron shuttling compounds, such as AQDS (9, 10-anthraquinone-2, 6-disulfonic acid) or pyocyanin have been found to enhance electron transport, and thus, possibly energy generation and growth. The purpose of this study was to determine the effects that AQDS had on the efficacy of antibiotics on *S. epidermidis* and *P. aeruginosa*. It was hypothesized that AQDS reduces the Minimal Inhibitory Concentration (MIC) of antibiotics by increasing electron transport and thus metabolism in biofilms. Both antibiotic E-test strips and colony biofilms on polycarbonate filter membranes were used to test antibiotic susceptibility. E-test strips determined the Minimum Inhibitory Concentration (MIC) of different antimicrobial agents. Developed membrane colonies were exposed to treatment plates containing combinations of Tryptic Soy Agar (TSA), antibiotic, and AQDS. Results were obtained by counting CFUs after biofilm colonies were harvested off of the membranes and ran through a series of dilutions.

These experiments produced evidence which disproved the hypothesis that AQDS would reduce the MIC of antibiotics by increasing electron transport and metabolism in biofilms. The results produced by the biofilm colony experiments demonstrate that AQDS does not reduce the MIC of the antibiotic, tetracycline, on *S. epidermidis* or *P. aeruginosa* based on comparisons between surface area concentration of viable cells on treatment and control membranes.

S06-P386
Measuring viable cell loss resulting from coupon manipulation during disinfectant testing

***A. Hilyard**, K. Buckingham-Meyer, D. Goeres, M. Hamilton, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

A laboratory disinfectant efficacy test against biofilm bacteria begins by growing a biofilm in a well-controlled biofilm reactor that contains removable growth surfaces (coupons). Disinfectant efficacy is determined by comparing the number of viable cells remaining on a disinfectant-treated coupon to the number on an untreated (control) coupon. The typical testing protocol requires that each coupon is subjected to a variety of manipulations; e.g., treating, neutralizing, removing, and disaggregating. If, due to these manipulations, a significantly larger fraction of viable bacteria is lost from the treated coupon than from the control coupon, the viable bacteria count for the treated coupon would be artificially small, thereby biasing the efficacy measure. For this reason, the observed efficacy is a measure not only of the disinfectant's killing power, but also includes the disinfectant's propensity to remove viable bacteria from the coupon surface. The purpose of this investigation was to determine the extent to which viable bacteria are lost from control coupon surfaces due to laboratory manipulation for each of three different laboratory test systems.

Three different systems were tested to simulate high, low, and no-shear environments for glass coupons. The three systems used were the CDC reactor (high shear), Drip Flow reactor (low shear), and the Static Biofilm system (no shear). The bacterium used for these experiments was *Pseudomonas aeruginosa* (ATCC 15442). Five experiments were done in duplicate for each system. Six coupons were sampled during each experiment from all three reactors. Two coupons were scraped right away, two coupons were rinsed, treated, then scraped, and the final two coupons were rinsed, treated, neutralized, then scraped. For the coupons that were rinsed and treated, the rinse and treatment water were also sampled. For the coupons that were rinsed, treated, and neutralized, the rinse water and treatment/ neutralizer water were sampled. All samples were homogenized, diluted, and plated for viable cells lost.

These experiments showed that, for the Drip Flow reactor, greater than 75% of the viable cells remained on the coupon surface after the coupon was rinsed, treated, and neutralized. In contrast, fewer than 16% of the viable cells remained on the surface of coupons from the Static Biofilm reactor after being rinsed, treated, and neutralized. The percent of viable cells on the coupons from the CDC reactor were between these two values. For coupons from the Static Biofilm reactor, the greatest percent of wash-off cells was found in the treatment/ neutralizer water. These experiments demonstrate the importance of including wash-off measurements as well as surface counts when disinfectant testing.

S06-P387
Inhibition of sulfate reducing bacteria biofilm current research in soils and water

***Elisa Korenblum**, Gina Sebastian, Lucy Seldin & Brent Peyton, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Biocorrosion of mild steel pipelines occurs as a consequence of non-uniform biofilm development on the metal surface, which leads to the formation of oxygen concentration gradients and differential aeration cells near the metal surface. Participation of the sulfate reducing bacteria (SRB) in biocorrosion process of pipelines and other metal surfaces of oil industry has been suggested. SRB growth in biofilms promotes corrosion by hydrogen utilization and cathodic depolarization on the metal surface. Also SRB produce H_2S, which directly attacks the metal surface. Conventional methods of microbially influenced corrosion (MIC) protection, like application of biocides to inhibit the colonization of disadvantageous bacteria, after they are established, are expensive and require frequent maintenance. In addition, sessile mode bacterial growth can promote resistance mechanisms to those compounds, bringing about the application of high doses which, therefore may lead to environmental risks.

In a previous study we showed that two antimicrobial substances (AMS) produced by strains *Bacillus licheniformis* T6-5 and *Bacillus fimus* H_2O-1, isolated from a Brazilian oil reservoir, were able to inhibit planktonic SRB growth *in vitro.* The current project is focused on the action of two AMS (T6-5 and H_2O-1) produced by *Bacillus* strains against sessile SRB. The aim of this study is apply microscopy techniques to study the effectiveness of those AMS against sessile SRB. Microscopic examination of the entire biofilm will enable us to increase our understanding of the spatial organization that occurs within the biofilms before, during, and after AMS treatments, and on the surfaces supporting their development.

S06-P388
Bioremediation and the role of mixed pollution: TNT and Cr(VI)

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

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.

Reductive bio-transformation is gaining momentum as an option for the clean-up of polluted subsurface soils. This strategy serves to immobilize Cr(VI) and TNT. When Cr(VI) is reduced to Cr(III), its toxicity and solubility drop dramatically. The reduction of TNT leads to the formation of reduced metabolites that can bind strongly to soils. Additionally, some metabolites of TNT are more toxic than the parent compound; it is therefore necessary to understand pathways of TNT reduction.

To date, no studies have addressed the influence that *mixtures* of TNT and Cr(VI) have on the biore-mediation process. This lack of understanding is problematic because Cr(VI) and TNT are

encountered as co-contaminants at US Department of Defense and Superfund Sites. Work investigating the influence of TNT and Cr(VI) on their simultaneous bioreduction by a represen-tative 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 metabolites of TNT acted to enhance Cr(VI) reduction rates.

These findings may be used in the effective design of successful bioremediation strategies at mixed contaminant sites. It has been clearly demonstrated that a complex contaminant matrix can not be accurately modeled as a simple summation of the behavior observed for each of its respective components in isolation.

S06-P389

Toxicity of select organic acids to the moderately thermophilic acidophile *Acidithiobacillus caldus*

***B. Apel**, J. Aston, and B. Peyton, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Acidithiobacillus caldus is a moderately thermophilic acidophile found within both natural and industrial bioleaching environments. Previous work has indicated that *At. caldus* is a chemo-lithotrophic autotroph capable of oxidizing sulfur, tetrothionate, thiosulfate, and hydrogen under aerobic conditions. In addition, mixotrophic growth has been observed when reduced sulfur compounds and either glucose or yeast extract were available. Previous work has suggested a synergistic improvement in mineral oxidation and leaching efficiency when *At. caldus* is present within a bioleaching microbial community. Further understanding of *At. caldus* capabilities, particularly its metabolic interactions and mechanisms, could lead to a greater understanding of the community metabolics of bioleaching microbial communities, improved mineral leaching operations, and a better understanding of acid mine drainage remediation.

An important first step in understanding the metabolic capabilities of *At. caldus* is to determine its tolerance of key organic acids. Specifically, within the scope of this study, the effects of the following organic acids were examined: malate, acetate, pyruvate, fumarate, succinate, oxaloacetate, and α-ketoglutarate. Organic acids such as these have been observed to have toxic effects on the iron oxidizing *Acidithiobacillus ferrooxidans* and sulfur oxidizing *Acidithiobacillus thiooxidans*, both of which are important members of the bioleaching microbial community. To date, no study has outlined the effects of said acids on the growth kinetics of *At. caldus*. Such organic acids may be introduced to the community via the metabolic pathways of acidophilic heterotrophs, such as the archaea, *Ferroplasma acidiphilum*.

Within the preliminary work presented here, all organic acids tested exhibited some inhibitory effect towards the growth of *At. caldus* under chemolithotrophic growth conditions. With the exception of oxaloacetate, which was toxic at very low concentrations, *At. caldus* appears to have a higher tolerance for organic acids than does *At. thiooxidans* and comparable tolerances to those previously observed of *At. ferrooxidans*. Oxaloacetate was observed to inhibit growth at concentrations as low as 25 µM. *At. caldus* growth was found to be completely inhibited by concentrations of pyruvate and succinate at 1000 µM. *At. caldus* was the most tolerant of malate, acetate, fumarate, and α-ketoglutarate; where complete inhibition was not observed, even up to 5000 µM.