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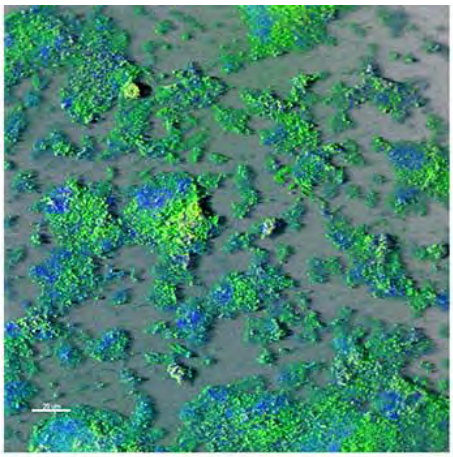

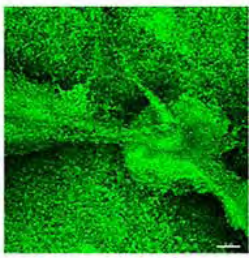

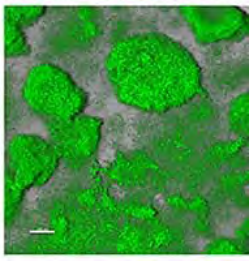

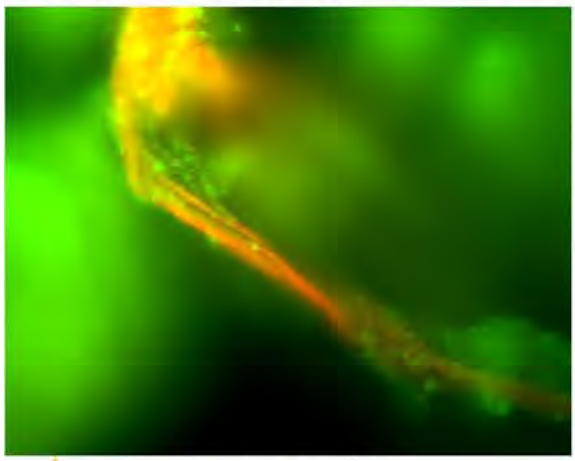

MONTANA STATE UNIVERSITY | College of ENGINEERING

Center for Biofilm Engineering

BOZEMAN, MONTANA

About the Center for Biofilm Engineering

CBE Leadership: Director and Executive Committee

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

A Brief History of the CBE

The CBE was established in 1990 with a grant awarded to Montana State University 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 firm foundation of many years of productive university-industry-government collaboration in pursuit of its vision to be a world leader in fundamental research, science and engineering education, and industrially relevant technology.

Mission and Goals of the CBE

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

The CBE has identified goals in three primary areas of activity.

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

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

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

Industrial Associates Program Benefits

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

CBE Technical Advisory Conferences

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

Education and Training Workshops

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

Research/Testing Projects

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

Product/IP Development Consulting

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

Regulatory Interactions

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

For More Information

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

CBE Technical Advisory Conference: July 15–17, 2008

Speaker Abstracts

Poster Abstracts

Speaker Abstracts

SESSION 1: Coatings for biofilm inhibition

Combating bacterial adhesion, biofilms, and implant-associated infection via nitric oxide release

Presenter: Mark Schoenfisch, Associate Professor, Department of Chemistry
Affiliation: University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Despite sterilization and aseptic procedures, infections associated with medical devices continue to present a serious ongoing threat in the biomedical arena. Such infections are difficult to treat because they are caused by bacteria that embed themselves within a protective biofilm matrix on the device surface. While the number of biofilm-associated infections and deaths continues to rise due to antibiotic resistance, the rate of discovery of new antibiotics has slowed significantly.

The endogenous roles of nitric oxide in human physiology, including the body's response to invading pathogens, are well established. Since the first reports describing NO as the endothelium-derived relaxation factor in the mid 1980s, much work has been devoted to elucidating the pathways of nitric oxide generation and activation in vivo, making it one of the most studied molecules in biomedical science. Cells of the immune system (e.g., macrophages) produce nitric oxide to facilitate killing of invading organisms such as bacteria and fungi. In concert with oxygen and superoxide, nitric oxide induces both oxidative and nitrosative stress on pathogens directly and through reactive intermediates.

A key issue in the development of nitric oxide-based therapeutics is delivery of nitric oxide to areas prone to infection. This requirement illustrates the demand for methods by which nitric oxide can be chemically harnessed for delivery at the site of interest (e.g., a catheter surface). Indeed, the astounding pace of discovery on the physiological roles of nitric oxide demands methods for generating nitric oxide in a controlled manner to facilitate both an improved understanding of its role in biological systems and the development of nitric oxide-based antimicrobials. My research group has focused on the synthesis and characterization of materials that spontaneously and controllably release nitric oxide for a variety of biomedical applications. We have demonstrated the ability of polymeric nitric oxide release to reduce bacterial adhesion, biofilm formation and infection both on the bench and in appropriate animal models.

This presentation will introduce: 1) the chemistry of nitric oxide; 2) the development of nitric oxide-releasing scaffolds; and, 3) biocompatibility testing of such materials with respect to bacterial adhesion, biofilm formation, and infection.

Related Publications:

1. Nablo BJ and Schoenfisch MH, "Antibacterial properties of nitric oxide releasing sol-gels," *J Biomed Mater Res*, 2003; 67A, 1276–1283.
2. Marxer SM, Rothrock AR, Nablo BJ, Robbins ME, and Schoenfisch MH, "Preparation of nitric oxide (NO)-releasing sol-gels for biomaterial applications," *Chem Mater*, 2003; 15, 4193–4199.
3. Nablo BJ and Schoenfisch MH, "Poly(vinyl chloride)-coated sol-gels for studying the effects of nitric oxide release on bacterial adhesion," *Biomacromolecules*, 2004; 5, 2034–2041.
4. Nablo BJ, Prichard H, Butler R, Klitzman B, and Schoenfisch MH, "Inhibition of implant-associated infections via nitric oxide release," *Biomaterials*, 2005; 26, 6984–6990.
5. Hetrick EM and Schoenfisch MH, "Reducing implant-related infections: Active release strategies," *Chem Soc Rev*, 2006; 35, 780–789.
6. Hetrick EM and Schoenfisch MH, "Antibacterial nitric oxide-releasing xerogels: Cell viability and parallel plate flow cell adhesion studies," *Biomaterials*, 2007; 28, 1948–1956.
7. Hetrick EM, Prichard H, Klitzman B, and Schoenfisch MH, "Reduced foreign body response at nitric oxide-releasing subcutaneous implants," *Biomaterials*, 2007; 28, 4571–4580.

8. Shin JH, Metzger SK, and Schoenfisch MH, "Synthesis of nitric oxide-releasing silica nanoparticles," *J Am Chem Soc*, 2007; 129, 4612–4619.
9. Shin JH and Schoenfisch MH, "Inorganic/organic hybrid silica nanoparticles as a nitric oxide delivery scaffold," *Chem Mater*, 2008; 20, 239–249.
10. Hetrick EM, Shin JH, Stasko NA, Johnson CB, Wespe DA, Holmuhamedov E, and Schoenfisch MH, "Bactericidal efficacy of nitric oxide-releasing silica nanoparticles," *ACS Nano*, 2008; in press (available online).

Methods of assessing biofilm inhibition on surfaces

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

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

The Standardized Biofilm Methods Laboratory proposes to develop a method specifically for testing the efficacy of surface-bound antimicrobials against biofilm bacteria. The method will be generic enough in nature to be useful for both EPA and FDA applications. The process was begun by reviewing the literature for the methods that are currently in use. Key attributes the new method must possess have been identified, and preliminary testing has begun to refine a method. Results from this process will be presented.

Biopassive and bioactive (biocidal) surfaces

Presenter: Andreas Mühlebach
Affiliation: Ciba Specialty Chemicals, Inc., Group Research, CH-4002 Basel, Switzerland

Introduction and Project Target

Higher demand of biocides due to increased customer awareness, new regulations that ban most classes of classical biocides, new application areas, and higher hurdles/costs for the registration of new biocides will lead to a shift away from toxic substances to modern, sophisticated organic surface chemistry: Biopassive surfaces (proteins, bacteria, viruses, fungi, algae, spores, etc., do not adhere and cannot form biofilms) and bioactive surfaces (bacteria, etc. are killed).

The project target was to develop—together with ETH-Zürich—new approaches for biopassive and bioactive surfaces and to develop test systems to investigate the adhesion of microbes under static and dynamic conditions. Are covalently attached biocides able to kill bacteria efficiently?

Experimental

Starting with basic investigations of the well-known PLL-g-PEG (poly-L-lysine-graft-polyethylene glycol) system developed at ETH-Z, telechelic PMOXA (poly-2-methyl-2-oxazoline) was synthesized by controlled/living cationic ring-opening polymerization (CROP) and grafted onto PLL. Purification was done by dialysis. Different analytical tools like NMR and MALDI-TOF-MS were used to characterize the PLL-g-PMOXA polymers and determine the grafting ratio. In parallel to these comb copolymer systems, the direct grafting of PEG hydrogels via photo-polymerization of PEG-(di)acrylate/PEG-photoinitiator mixtures onto polymeric substrates (PP foils and spin-coated polystyrene as model substrates) was investigated.

Protein adhesion from full human serum under static conditions was determined by optical waveguide light mode spectroscopy (OWLS) and quartz crystal microbalance (QCM). Direct imaging with an optical microscope was used to determine the number of attached bacteria (*E. coli*) on treated transparent substrates. Live/dead staining with fluorescent dyes was used to distinguish living from dead bacteria. New functional ammonium- and pyrimidine-type biocides were synthesized and attached to PEG and PMOXA chains.

Results
Plasma/corona treatment of plastic foils followed by photo-polymerization of a mixture of hydrophilic monomers and photoinitiator forms an inexpensive, stable, transparent biopassive layer (d>100 nm). This system is as efficient as the PLL-g-PEG system.

The covalent attachment of an ammonium-type biocide onto PLL-g-PEG gave a transparent bioactive layer as seen by the increased number of dead bacteria. This system was further expanded, using other biocides and attachment reactions and PMOXA as biopassive polymer.

A new simple test system for biopassive/bioactive surfaces was developed: Bacteria were stained with dyes (or genetically modified to produce a green fluorescent dye), followed by direct microscopic imaging of the surfaces. Counting the number of attached bacteria on treated vs. untreated surfaces is a good measure for "biopassivity," whereas "bioactivity" is related to the relative amount of living and dead bacteria.

Discussion and Conclusion
The collaboration with ETH-Z, with its know-how in material characterization and evaluation (including access to modern instruments), and synergies with current Ciba activities (in particular Ciba's "Bioprotection" team) led to the discovery and development of an inexpensive, generally applicable, and stable biopassive coating system. Bioactive surfaces (layers with covalently attached biocides) and biopassive/bioactive combinations are currently made and tested.

Acknowledgements
The author would like to thank the following people for their contributions: R. Konradi, N. Graf, J. Moeller and M. Chabria, ETH-Z; E. Nyfeler, W. Hoelzl, C. Hendricks-Guy and T. Deisenroth, Ciba.

SESSION 2: Environmental biofilms

Biologically induced mineralization: Fundamentals and possible applications

Presenter: Robin Gerlach, Associate Professor, Chemical and Biological Engineering
Co-authors: Al Cunningham, Andrew C. Mitchell, Stacy Biebel, Logan Schultz, Laura Wheeler, Lee Spangler
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The microbially mediated formation of minerals is a process that has long been known to occur in nature and the biologically induced formation of iron and carbonate minerals has been studied intensively. Recently, biologically induced carbonate mineral formation has been evaluated for a number of environmental and industrial uses. These include the sequestration of carbon dioxide (CO_2) in the form of calcium carbonate (e.g., calcite) minerals, the co-precipitation of heavy metals and radionuclides such as strontium (Sr) or uranium (U), the stabilization of soils in construction projects, dust suppression, and self-healing materials. The combination of carbonate mineral precipitation with biofilm- and carbonate-mineral induced reduction of permeability—if successfully developed—has the potential to reduce or control flow in the subsurface while removing compounds of interest (e.g., CO_2 or radionuclides).

This presentation will highlight the principles of biologically induced carbonate mineral precipitation, highlight our current efforts in the development of possible applications in the area of carbon sequestration and

environmental remediation. Results from deep subsurface (i.e., high pressure) and shallow subsurface (i.e., low pressure systems) will be presented and followed by an outlook of future research and development efforts being pursued.

Biological ice nucleators in snow and rain

Presenter: Christine Foreman, Assistant Research Professor, Land Resources and Environmental Sciences
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Before a cloud can produce rain or snow, rain drops or ice particles must be formed, which requires nuclei for condensation. Biological ice nucleators, a.k.a. rain-making bacteria, function as freeze catalysts at relatively warm temperatures, and if aerosolized and transported into clouds, these particles may impact meteorological processes by inducing precipitation. The concentration and nature of ice nucleators in precipitation collected from Montana and Louisiana (USA), the French Alps and Pyrenees (France), Ross Island (Antarctica), and in the vicinity of Whitehorse, Yukon (Canada) were examined. The temperature of detectable ice nucleating activity for the majority of the samples was $\geq$ -5 °C, which is the warmest of all naturally occurring ice nuclei in the atmosphere. Digestion of the samples with lysozyme (i.e., to hydrolyze bacterial cell walls) led to reductions in the frequency of freezing; heat treatment greatly reduced or completely eliminated ice nucleation in every sample, consistent with the activity being of proteinaceous origin. Multiple regression was used to construct models to predict the concentration of microbial cells and biological IN in snow based on the concentration of non-purgeable organic carbon (NPOC), NH_4^+, and Ca^{2+} or Cl^-, NO_3^-, Ca^{2+}, and NPOC, respectively. Our results indicate that biological ice nucleators are omnipresent in the atmosphere and that, for some geographic locations, the concentration and activity of these particles is related to the season and precipitation chemistry.

SESSION 3: Water/Industrial Biofilms

Water quality changes in premise plumbing

Presenter: Anne Camper, Professor, Civil Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Water produced by a municipal water treatment plant or on-site treatment process may be of very high quality, only to reach the end-user in less than optimal condition. An often unappreciated source of these changes in water quality is premise plumbing. For the purposes of this presentation, premise plumbing is defined as the water conveyance system that extends beyond the water meter or service line into a building. Premise plumbing can consist of copper, plastics, or galvanized iron piping (residential, healthcare facilities, office buildings, industry), as well as stainless steel, plastics or glass (industry). In addition, there may be lead components in service lines, solders, or brass fixtures. Water quality changes may be the result of the leaching of metals or the growth of organisms and biofilms. Biofilm growth is favored due to dead ends, stagnant water conditions (especially overnight), decreased disinfectant residuals, etc. These biofilms may cause problems when released; this could lead to product contamination or human exposure. The latter is of particular concern if there are opportunistic pathogens growing in the biofilms that can be released and ingested or inhaled. An overview of water quality changes in premise plumbing, with an emphasis on biofilm growth, will be presented.

Legionella—(re-)awakening to the amoeba-based pathogens of distribution system biofilms

Presenter: Nicholas Ashbolt, Senior Research Microbiologist
Affiliation: National Exposure Research Laboratory, ORD, US EPA, Cincinnati, OH 45268

Fecal pathogens have long been the focus of concern in the distribution of drinking waters. Yet today, with distribution system 'failures' accounting for the majority of waterborne outbreaks in the USA, there is growing realization that pathogens endemic to aquatic biofilms may also be of concern, particularly to susceptible sub-populations. These concerns have focused on strains of *Legionella* spp., *Mycobacterium avium* complex and *Helicobacter pylori*, as raised in EPA's contaminant candidate lists (CCL-1, 2 & 3). Such pathogens, however, may only be the tip of the 'iceberg'. Turning to biofilm ecology, it is clear from studies on *Legionella* that biofilm amoeba play an important 'Trojan Horse' role by introducing, amplifying, and possibly up-regulating virulence in *Legionella* and other pathogens. However, there are very few studies of *Legionella* spp. pathogenesis aimed at associating the role of biofilm colonization, parasitization of biofilm microbiota and release of virulent bacterial cell/vacuoles into drinking water distribution systems. Moreover, the implications of these environmental niches for drinking and reuse water exposures to pathogenic legionellae are poorly understood. On-going research at EPA's Cincinnati laboratories into the putative role of biofilms and amoeba in the proliferation, development, and dissemination of potentially pathogenic *Legionella* and other intracellular pathogens will be discussed. The goal of this research is to aid in identifying control strategies and to provide key data to enable quantitative microbial risk assessments of biofilm-associated pathogens.

Effect of chlorite on microbial nitrification in a simulated premise plumbing system

Presenter: Shahed Rahman, PhD candidate, Civil Engineering
Lynne Leach, Postdoctoral Research Associate
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The problem of nitrification in drinking water supplies in United States was identified as early as 1935. A 1996 American Water Works Association Research Foundation (AWWARF) survey indicated that two-thirds of drinking water utilities that use chloramine as disinfectant experienced some degree of nitrification. Nitrification episodes in chloraminated drinking water and premise plumbing systems have been linked to an increase in heterotrophic bacterial abundance and, therefore, are associated with a risk of pathogen intrusion. Nitrification in drinking water distribution systems is nearly impossible to stop once it has begun, and there are currently no effective methods available, short of shock chlorination, to reverse a nitrification event. To mitigate nitrification events and to pinpoint appropriate predictive and remedial approaches, it is vital to understand the efficacy that disinfection techniques have on the microbial community.

This project investigated the effect of chlorite on nitrification in a simulated household plumbing system. Chlorite is a known inhibitor of autotrophic bacterial nitrification; however, the efficacy of chlorite inhibition on heterotrophic nitrification is unknown. Reactors were used to simulate a household plumbing system. Copper and PVC coupons were used, as they are the two most common household plumbing materials. These reactors were fed water with humics (4ppm) as the organic carbon source, ammonium sulphate (0.71ppm) as nitrogen source, and biologically treated tap water to supply the bacterial inoculums. Water in the reactors was stagnant for eight hours and then flowed for five minutes. The pH of the influent water was maintained at approximately 8.15 and alkalinity was approximately 35 mg/L as $CaCO_3$. Chlorite was introduced into one copper and one PVC reactor. Initially chlorite was added to both reactors at 0.2 ppm and gradually increased to 2.0 ppm; finally a shock load of 20 ppm was applied. The effect of chlorite on the PVC system was not significant. In the copper reactor, nitrification was only inhibited by chlorite during the shock treatment at 20 ppm. Because the addition of 20 ppm chlorite did not inhibit nitrification, and the autotrophic amoA gene was not detected via PCR, it was hypothesized that heterotrophic microbes could be contributing to active nitrification in the reactors. In fact, heterotrophic nitrifying pseudomonad, as well as a fungal strain, were isolated from the copper reactor.

Water treatment biocide applications and automated controls in industrial cooling water systems

Presenter: Michael Dorsey, Senior Materials Corrosion Technologist
Affiliation: DuPont Engineering Technology
Presenter: Doug McIlwaine, Senior Scientist
Affiliation: ChemTreat

Industrial cooling water systems have specific treatment needs in order to perform to their full potential. There are four target goals that most companies and water treatment suppliers use as key performance indicators: corrosion rate, scaling, deposition, and microbiological fouling. The indicators are related to each other and are used to gauge the overall performance of the water treatment program. This presentation will cover primarily the microbiological control applications and how they are normally applied and monitored.

From microbially influenced corrosion to microbial fuel cells

Presenter: Zbigniew Lewandowski, Professor, Civil Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Some mechanisms active in microbially influenced corrosion of metals can be used to generate electrical energy. Devices that perform this operation are known as microbial fuel cells (MFCs). Thus far, the energy generated this way is modest, but there is a growing interest in using MFCs to power electronic devices in special applications, such as powering telemetry systems deployed at inaccessible locations where exchanging batteries is costly or, sometimes, impossible.

There are many unresolved technical problems that need to be addressed before power sources based on MFCs can be implemented, of which the low cell potential, low power and the fluctuating environmental factors are the most obvious. I will discuss the mechanisms of microbially influenced corrosion that we consider promising in the development of microbial fuel cells, and among them the corrosion of stainless steels by manganese oxidizing bacteria, and our attempts to use microbially deposited manganese oxides as cathodic reactants in microbial fuel cells.

The goal of our recent studies is to use the mechanisms of microbially influenced corrosion to construct power supplies based on MFCs, equipped with power management circuitry able to decide how much energy needs to be accumulated and how much energy needs to be used to power the attached sensors and to transmit the results to remote receivers using radio or hydrophones, in the case of underwater operations. It is required that all electrical circuits of such devices are powered by the energy generated by MFCs.

In addition to MFCs that we study in our laboratories, we have MFCs deployed in the ocean in Newport, Oregon, and in San Diego, California. The research is sponsored by the US Office of Naval Research (ONR).

Consumer product biofilm related problems

Presenter: Alex Blanchard, Senior Scientist in R&D Microbiology
Affiliation: Procter & Gamble Technical Centres, Egham, Surrey, United Kingdom

Biofilm research is one of the key capabilities of the Microbiology organisation within P&G. Of particular interest is the impact biofilms have on general consumer product manufacturing. The majority of products manufactured by consumer goods companies are not regulated as foods and drugs, and so no strict manufacturing guidelines exist. It is important to note that manufacturing systems for most consumer products are not intended to be sterile, but there are often requirements on the appropriate microbiology standards for the finished products. To achieve finished product specifications can be a challenge because of the type of products manufactured; cosmetic products, for example, may include many ingredients, including unrefined plant extracts and perfumes, that are composed of multiple compounds.

The ways P&G minimizes biofilm problems that occur during manufacturing will be discussed, and consideration will be given to general consumer product manufacturing problems.

SESSION 4: Biofilm methods and microscopy

Standardizing a method for rapid biofilm analysis

Presenter: Darla Goeres, Assistant Research Professor
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

In biofilm efficacy testing, the goal is to grow a biofilm that is relevant to the environment where a disinfectant will be applied and then test the disinfectant under real use conditions. The Standardized Biofilm Methods Laboratory (SBML) has developed and promotes a strategy for biofilm methods development that is based upon partitioning biofilm methods into four discreet steps, then standardizing each step separately. The steps required for testing the efficacy of an anti-biofilm treatment are: 1) grow a relevant and repeatable biofilm using a biofilm reactor, 2) treat a mature biofilm with biocide or antibiotics, 3) remove a representative biofilm sample, and 4) analyze the sample for a quantitative and/or qualitative estimate of kill and/or removal as a result of the treatment. This approach results in less methods development, yet yields hundreds of potential combinations of potential methods. Researchers interested in biofilm efficacy testing choose the most relevant combination of grow, treat, sample, and analyze methods to best serve their particular needs. All of the current standardized biofilm methods use the viable plate count method to analyze for quantitative estimates of kill and/or removal.

In July 2006, the SBML, in collaboration with BioSurface Technologies, Inc. (BST), received funding from the Montana Board of Research and Commercialization Technology to develop a rapid biofilm analysis method that utilizes the tetrazolium salt CTC (5-cyano-2,3-ditolyl tetrazolium chloride). Active bacteria take up CTC and reduce it to a fluorescent, insoluble crystal. At this point in the process, epiflourescent microscopy or flow cytometry could be used to count the total number of bacteria with crystals. A second approach is to elute the crystals into solution using ethanol and read the color intensity on a spectrophotometer. The SBML chose to standardize the elution technology because of its simplicity with regard to time, skills, and equipment requirements. This presentation describes the process used to standardize the CTC elution technology and the results of that process. Finally, I will present a method that relates the repeatability standard deviation found for the CTC elution method to the repeatability standard deviation found for the viable plate count data.

Laser microscopy in biofilm research—current options and future potential

Presenter: Thomas R. Neu, Senior Scientist, Department of River Ecology
Affiliation: Helmholtz Centre for Environmental Research–UFZ, Magdeburg, Germany

Confocal laser scanning microscopy (CLSM) by means of one-photon excitation is now a routine technique for examination of three-dimensional microbial communities. CLSM is one of the approaches that resulted in major progress in biofilm understanding. The key advantage of CLSM is based on its 3-dimensional sectioning capability of living, fully hydrated microbial biofilms. CLSM has been employed in many variations in order to study structural features of biofilms and bioaggregates. Prominent examples are: nucleic acid staining, lectin-binding analysis of the polymer matrix, fluorescence in situ hybridisation (FISH), measurement of cell/enzyme activity, green fluorescent protein (GFP) technique, and probing the microhabitat. Ten years ago instruments equipped with pulsed infrared lasers suitable for two-photon excitation (2PLSM) became available. A literature survey showed that this new technique has been employed in only few microbiological studies. Nevertheless new fluorescence techniques compatible with CLSM and 2PLSM are available, some of which have been rarely used in biofilm research. One of the techniques is fluorescence lifetime imaging (FLIM). The lifetime of an excited fluorochrome contains a valuable piece of information of the microhabitat which is mostly neglected in laser microscopy. Another fluorescence technique suitable for biofilm research is fluorescence correlation spectroscopy / microscopy (FCS). The technique measures the movement of a fluorochrome in a tiny volume and may be used

to calculate diffusion. New technical developments allow for improved usability of CLSM instruments. For example, super continuum light sources are now available for free selection of the excitation line. Another feature called controlled light exposure microscopy (CLEM) will help to overcome extreme differences in fluorescence intensities within one section. Because environmental biofilms are often large in area and thickness, optical coherence tomography (OCT) can be a useful technique for imaging biofilms. Selective plane illumination microscopy (SPIM) may be another option for imaging of large samples. With respect to high resolution, two new techniques are now commercially available. Stimulated emission depletion (STED) microscopy overcomes the limitations of classical light microscopy and allows imaging at 100 nm resolution. The problem of unequal resolution in axial versus lateral direction can now be solved by using 4Pi microscopy. However both techniques are hampered by some constraints which will be discussed. A further new approach to increase the resolution is achieved by structured illumination microscopy (SIM) although it is not available yet. In conclusion, there are several fluorescence techniques with a large potential that have not been taken advantage of for biofilm examination. Other new technologies improve the practicability of the microscope, although with some instruments only. There are also laser microscopy approaches for large samples. Major improvements have been made in terms of resolution. What is still needed for practical laser microscopy of biofilm samples? The wish list includes new fluorescent probes, more flexibility in terms of sample mounting, and improved digital image analysis procedures.

Salmonella typhimurium biofilms at the gene level

Presenter: Stewart Clark, PhD candidate, Microbiology
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Salmonella enterica serovar Typhimurium is an important water- and foodborne pathogen with a diverse ecological niche. In addition to colonizing the small intestine of the human host, invading through enterocytes, and triggering fluid secretion leading to the typical diarrhoeal symptoms, *Salmonella* have also been shown to survive and replicate inside host cells. Outside of the human host, *Salmonella* has been shown to survive as part of dynamic biofilms in aquatic environments, in nematodes and amoeba, and on plant surfaces. The aim of this study was to compare some key pathways in *Salmonella typhimurium* through transcriptional profiling during planktonic and biofilm growth and subsequently to propose some niche-related adaptations. Biofilms of an environmental isolate of *S.typhimurium* were grown under nutrient-limiting conditions using a CDC Reactor. Whole-genome microarrays were used to identify significantly regulated gene expression. Confirming existing literature, the putative transcriptional regulator csgD of the LuxR superfamily, was consistently up-regulated in the biofilm mode of growth, confirming its importance as a control unit for biofilm formation. Interestingly, Salmonella Pathogenicity Island-1 (SPI-1) genes as well as the majority of the pSLT Virulence Plasmid, usually associated with enterocyte invasion, were up-regulated in the planktonic phase of growth, whereas SPI-2 genes, usually associated with intracellular replication and systemic infection, were up-regulated during biofilm growth. Two other pathways exhibiting similar expression patterns between the biofilm mode of growth and observed virulence are the oxidoreductases, hydrogenase 3 complex (hyp, hyd and hyc) and NADH dehydrogenase (nuo). Both enzyme pathways have previously been demonstrated to be required for colonization and virulence in the natural environment and the human host. Perhaps the most interesting observation was the strong co-regulated expression of the Propanediol Utilization (pdu) operon and Cobalamin Biosynthetic (cob) operon, together carrying genes necessary for propanediol utilization and de novo synthesis of vitamin B12 respectively. This enteropathogen is regularly likely to encounter 1,2-propanediol, a breakdown product of the common plant sugars rhamnose and fucose, giving it a competitive advantage in a natural biofilm community. Furthermore, the formation of polyhedral bodies (similar to carboxysomes) involved in 1,2-propanediol degradation may be important in sequestering toxic aldehydes, providing protection during high-density biofilm living. Further evidence of stress-related gene expression under biofilm growth conditions came from expression of rpoE and pspAE (extracytoplasmic stress response) and dnaK (stress-related molecular chaperone).

Protein expression in biofilm models

Presenter: Elinor deLancey Pulcini, Research Scientist, Medical Biofilm Laboratory
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

In vitro model systems have become a standard procedure for biofilm research. It is well accepted that different model systems produce different biofilms, which can vary due to such parameters as shear force, flow rate, temperature, nutrient availability or residence time. This can make comparisons between biofilm models somewhat problematic. For example, the effects of a treatment regimen on a biofilm can vary by the model in which it is tested. The goal of this work was to examine protein expression patterns in three different model systems compared with planktonic cultures. *Staphylococcus aureus* biofilms grown in the drip flow model, colony model, and the 96-well microtiter plate model were used for this study. Proteins were extracted from samples collected from each model, as well as planktonic cultures, and subjected to proteomic analysis by 2D gel electrophoresis. Protein expression patterns were most similar between the colony model and the drip flow reactor model, and between planktonic culture and the 96-well microtiter plate model. Proteins were selected from each sample for analysis by MALDI-TOF/TOF. Results enabled ranking of the models tested for the relative difference from planktonic sample cultures.

The impacts of *S. aureus* biofilm on keratinocyte morphology

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

Chronic wounds are characterized by prolonged inflammation and failure to re-epithelialize and do not respond well to conventional treatment. Many factors have been implicated in the delayed healing of these wounds, including microbial infection. It has been speculated for several years that chronic wound infection may be biofilm related. *Staphylococcus aureus* has been implicated in several infectious diseases, including acute and chronic skin infections. An in vitro model was developed to study host/pathogen interactions, along with the role biofilm formation plays in pathogenesis. *S. aureus* biofilms were grown on 0.2 µm culture inserts and placed on top of a monolayer of human keratinocytes. Use of the culture inserts allowed for the removal of the biofilm from the keratinocytes with minimal disruption of the biofilm, allowing for a convenient method for the study of host/pathogen interactions. *S. aureus* biofilm secretions induced a significant disruption of the cytoskeleton in the keratinocytes followed by induction of widespread apoptosis. The disruption of cytoskeletal proteins and induction of apoptosis in keratinocytes may impact the natural healing process by inhibiting the re-epithelialization of the wound bed leading to the chronic state of the wound. Planktonic *S. aureus* studied in the same manner were not found to induce these effects. Here we demonstrate that *S. aureus* biofilm formation is critical for the disruption of the keratinocyte cytoskeleton and induction of apoptosis in vitro.

Proteomic analysis for the determination of biodegradation pathways in *Polaromonas* sp. JS666

Presenter: Laura Jennings, visiting PhD candidate, Environmental Engineering, Cornell University
Authors: LK Jennings[1,2], SF Nishino[3], RB Payne[3], JC Spain[3], JM Gossett[1]
Affiliation: [1]Cornell University, Ithaca, NY, [2]Center for Biofilm Engineering, Montana State University, Bozeman, MT, [3]Georgia Institute of Technology, Atlanta, GA
Sponsors: SERDP, ESTCP, NSF Graduate Research Fellowship

Polaromonas sp. JS666 is the first bacterium isolated that is capable of growth-coupled *cis*-dichloroethene (cDCE) oxidation. Therefore, JS666 is a promising bioaugmentation agent for cDCE-contaminated sites where this common groundwater contaminant and suspected carcinogen has migrated into aerobic zones. The cDCE metabolic pathways in JS666 have yet to be elucidated, and knowledge of them could provide insight into required nutrients and conditions for optimal bioaugmentation. We used a proteomic approach to identify proteins up-regulated by cDCE compared to the reference substrate glycolate. 2D gel electrophoresis used in conjunction with LC/MS/MS and MALDI-TOF/TOF mass spectrometry revealed the up-regulation of a glutathione S-transferase (GST), cyclohexanone monooxygenase, and haloacid dehalogenase (HAD). The up-regulation of these proteins, which was confirmed by transcriptomic experiments, indicates that there may be two possible cDCE

degradation pathways including (i) the monooxygenase-catalyzed formation of a DCE epoxide, which is transformed by a GST, and/or (ii) the GST-catalyzed dehalogenation of cDCE, forming a glutathione conjugate that can be sequentially oxidized by a chloroacetaldehyde dehydrogenase (CAD) and HAD to glycolate. Enzyme activity for a CAD, HAD, and GST was detected in cDCE crude extracts of JS666.

Experiments are currently underway to confirm the functional activity of these up-regulated enzymes and to solidify their roles in the cDCE degradation pathways in JS666.

Helpful insights: How can the FDA Antimicrobial Guidance apply to biofilm claims in medical devices

Presenter: Chiu S. Lin, PhD; Director, Division of Anesthesiology, General Hospital, Infection Control, and Dental Devices
Affiliation: Office of Device Evaluation, Center for Devices and Radiological Health, U.S. Food and Drug Administration

It is well known that biofilm formed on the surfaces of indwelling medical devices, such as implants and indwelling catheters, plays an important role in device-induced infection. In order to control or eradicate biofilm formation on these devices, manufacturers have created a strategy of coating or impregnating their devices with an antimicrobial agent. It is uncertain whether the antimicrobial treatment on device surfaces is clinically effective in controlling biofilm formation. FDA has increasingly received premarket applications for devices that contain antimicrobial agents. Because of this interest, the FDA has recently published a draft guidance document entitled: "Draft Guidance for Industry and FDA Staff: Premarket Notification [510(k)] Submissions for Medical Devices that Include Antimicrobial Agents," which was published on July 19, 2007.

In this presentation, I will provide a brief overview of this draft guidance, as it relates to FDA's current review approach to medical devices with antimicrobial agents. Although this guidance does not specifically address the issue of biofilm, I will attempt to provide a "helpful insight" on how this guidance can be applied to biofilm issues in medical devices. Currently, there is no standardized and scientifically sound validation test method and criteria that can be used to assess the clinical effectiveness of the antimicrobial coating on the surface of medical devices in controlling biofilm formation. For this reason, FDA encourages an open discussion and offers an opportunity for collaboration to arrive at clinically meaningful evidence to support clearance or approvals of device with biofilm claim.

Update on *Biofilms: The Hypertextbook*

Presenter: Al Cunningham, Professor, Civil Engineering and
Rocky Ross, Professor, Computer Science
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Biofilms: The Hypertextbook is a unique, Web-based teaching and learning resource on the subject of microbial biofilms that is currently being developed with support from the National Science Foundation and CBE Industrial Associates. Topics of potential interest include the Introduction to Biofilms, Properties of Biofilms, Biofilm Modeling, Industrial and Environmental Biofilms, and Biofilms in Health and Medicine. The hypertextbook will be an educational resource for teachers, students, and practitioners interested in microbial biofilms. **We are particularly interested in providing content that is useful to the CBE Industrial Associates**. Please visit our display during the poster session and provide suggestions for material content and presentation. This project is directed by Al Cunningham and Rocky Ross.

SESSION 5: Nitrogen compounds and their biofilm impact

Gaseous nitric oxide (gNO): A novel antimicrobial agent

Presenter: Chris Miller
Authors: *†Chris Miller PhD., *Bevin McMullin BSc
Affiliation: *University of British Columbia, Division of Infectious Diseases, Vancouver, Canada.
†Consultant to NitricBio Therapeutics and Cardinal Health

Background

Nitric oxide (NO) is part of the innate immune system that is produced within the nasopharynx, wounds, and specifically by the phagocytic cells as a chemically reactive molecule. Bacteria, viruses, fungi and parasites are all susceptible to NO. NO is a gas molecule that passes unhindered through cell membranes, targeting a wide range of macromolecules. NO production slows the infection process until the more specific acquired immune system takes over. Some microbes have been able to turn off the NO producing machinery of the host cells by having a limited amount of detoxifying compounds (thiols) to inactivate NO, but ultimately all are susceptible to NO killing (1). Because of NO's broad spectrum antimicrobial activity, it may be difficult to develop resistance. We have discovered that the same concentration of NO that is cytotoxic to microbes is not cytotoxic for mammalian cells (2,3).

When the immune system's NO generating ability is overwhelmed or compromised, infection and disease occurs. Increasing the body's ability to produce more NO through gene therapy or synthetic NO donor drugs is of interest in medicine. The ability to deliver sufficient amounts of NO through these two approaches to infected regions has remained elusive. We suggest a novel approach, which is to directly deliver gaseous nitric oxide (gNO) to fight infection by using nature's best weapon, NO, against microbes in large short-lifetime doses delivered locally to overwhelm the pathogen (4). This approach takes advantage of three characteristics of NO. First, NO is a broad-spectrum antimicrobial. Second, human cells tolerate high concentrations of NO. Third, NO is able to overwhelm the defense mechanisms pathogens employ to neutralize NO activity.

Because of its short half-life, the action of NO is localized when delivered topically. In wound care studies in humans, side-effects have not been apparent. The use of exogenous gNO at effective physiological levels also completely avoids the mechanisms deployed in antibiotics. If this approach can be safely delivered, the therapy will be inexpensive, technologically achievable, and easily adaptable for use in both inhaled pulmonary and topical skin infections. Ideally, therapeutic use of gNO could replace the use of antibiotics as frontline therapy by mimicking the body's natural first line method of defense against infections and thereby directly reduce drug resistance.

Our initial work has identified the effective dose and time required to rapidly eliminate all bacterial strains tested within hours (5). We have characterized the antimicrobial effects of gNO by delivering it from a pressurized cylinder in minute part-per-million dose flows (6). The data confirms that gNO is a broad spectrum antimicrobial agent covering a wide range of organisms, including multi-drug resistant bacteria. Multiple phase II studies are currently underway in evaluating the safety and efficacy of gNO as an antimicrobial therapy for cystic fibrosis, chronic sinusitis, tuberculosis, leishmaniasis, tinea pedis and influenza.

ABSTRACT

Introduction:

It is unknown whether gNO is effective against drug resistant bacteria such as Methicillin-Resistant *S. aureus* (MRSA) in an in vivo setting. We report the effect of gNO against five planktonic species of MRSA, a biofilm in vitro MRSA model compared to Vancomycin and a case study wherein gNO was used to successfully treat graft rejection, secondary to MRSA colonization refractory to Vancomycin.

Methods:

1. Using previously validated techniques, MRSA strains ATCC 33592, 700698, 700699, 49775 (PVL positive), 49774 (PVL positive) were challenged for 8 hours with 200 ppm gNO (1,600ppm*hours) (5).

2. A drip flow reactor (7,8) was used to grow a MRSA biofilm which was exposed to R ingers lactate, gNO 1,600ppm, air or Vancomycin (100xMIC).
3. A 17 year-old male with a purulent free gracilis muscle flap with STSG cover with heavy MRSA growth despite Vancomycin 1gm IV q8h for 14 days received 200ppm gNO at 1 Lmin-1 for 8 hours nightly with a device previously reported.3

Results:

1. A 6 log10 cfu/mL reduction in all five MRSA strains ensued after exposure to 1,600ppm*hours.
2. gNO reduced MRSA in the biofilm model by 6.6 log10 cfu/cm2, relative to the air treatment, whereas Vancomycin had little reduction (0.08 log10 cfu/cm2) relative to lactated Ringers solution.
3. Three treatments of 200 ppm for approximately 9 hours reduced MRSA growth from heavy to moderate, the wound bed was cleaner and more granulated. The lesion was regrafted. gNO was administered for another 27 nights without the need for antibiotics resulting in a 100% graft take and complete healing.

Conclusions:

These results suggest that gNO therapy is effective at reducing MRSA complicated skins grafts that are refractory to Vancomycin treatment.

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Genetic and metabolic clues for the treatment of anaerobic *Pseudomonas aeruginosa* biofilm infections

Presenter: Dan Hassett, Professor, Molecular Genetics
Affiliation: Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati,OH 45267

A hallmark of chronic late-stage cystic fibrosis (CF) airway disease is the formation of antibiotic/phagocyte-resistant biofilms by the important opportunistic pathogen *Pseudomonas aeruginosa*. In this setting the organism is known to grow under reduced oxygen tension and is even capable of respiring anaerobically within the thick, slightly acidic airway mucus. Thus, proteins involved in anaerobic metabolism represent potentially important targets for therapeutic intervention. In CF, there is also reduced production of antimicrobial nitric oxide (NO) by airway epithelia. Remarkably, we have found that some of the most formidable *P. aeruginosa* mutant CF isolates are remarkably sensitive to NO and/or acidified nitrite ($A\text{-}NO_2^-$). First, strains lacking the rhl quorum sensing circuit that are often encountered in CF sputum commit a metabolic suicide in biofilms by anaerobic overproduction of endogenous NO. A dissection of the molecular basis underlying this interesting and clinically

important finding specifically involves a "dysregulation" of particular genes involved in anaerobic respiration. Second, sputum isolates from chronic late-stage CF are often mucoid, resulting from overproduction of a viscous exopolysaccharide called alginate. The vast majority (87%) of mucoid strains harbor mutations in mucA, encoding an anti-sigma factor. Surprisingly, mucoid bacteria perish during anaerobic exposure to A-NO_2^- in a pH-dependent fashion. More importantly, A-NO_2^- kills mucoid bacteria (a) in anaerobic biofilms; (b) in vitro in ultrasupernatants of airway secretions derived from explanted CF patient lungs; and (c) in mouse lungs in vivo in a pH-dependent fashion. Finally, *P. aeruginosa* protects itself from endogenous anaerobic NO gas by NO reductase (NOR). Paradoxically, a NOR- mutant can survive anaerobic growth because it possesses a "circuit breaker"-like switch governed by the global transcriptional activator, ANR. Endogenous NO gas can compromise the $[4Fe\text{-}4S]^{2+}$ cluster of ANR, resulting in dramatically reduced transcription of genes encoding nitrate and nitrite reductases, the enzymes responsible for NO production. Collectively, the ultimate goal of this line of research is to offer metabolic clues as to novel means by which to control or eradicate this formidable pathogen from the CF airways.

Consequences of nitrate and nitrite stress in *Desulfovibrio vulgaris*

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

Sulfate-reducing bacterial biofilms are an interesting model to study due to both potentially advantageous (e.g., heavy metal reduction) and deleterious (e.g., metal corrosion, sulfide production) roles in the environment, but little is known about the cellular events that constitute physiological responses to nitrogenous compounds, namely nitrate and nitrite. Previous physiological studies have demonstrated that *D. vulgaris* growth was inhibited by nitrite and nitrate and that reduction was observed to be primarily a mechanism of detoxification for planktonic cells. Cells exposed to either nitrate or nitrite up-expressed genes involved in energy metabolism, oxidative stress, and iron homeostasis. However, some genes were regulated in a unique manner respective to nitrate or nitrite and these results suggested that sets of different proteins were used to deal with nitrate or nitrite exposure. Recent work with *D. vulgaris* suggested that surface-adhered cells had modified energy metabolism compared to planktonic cells and these results suggested that biofilm cells may respond differently compared to planktonic cells. Bench-scale flow reactors are being developed for the characterization of biofilm responses to nitrate, nitrite, oxygen, and chromate.

Biofilm viscoelastic properties—measurements & modifications

Presenter: Warren Jones, Associate Professor, Civil Engineering
Authors: Warren Jones and Michael Sutton, MS, Environmental Engineering, Department of Civil Engineering, MSU
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Biofilms are viscoelastic materials, exhibiting characteristics of both liquids and solids. Improving our understanding of the viscoelastic properties of biofilms will allow superior design and implementation of strategies for managing biofilms. In this project, a parallel plate viscometer was used to make direct measurements of these physical properties of biofilms. This device allows a constant shear stress to be applied between two rotating plates, and measures the resulting movement. The relationship between these two properties during the application of the stress, and the subsequent relaxation or "rebound" following the removal of the stress, were used to calibrate a model for the properties of the biofilm. Next, a variety of treatments, from biocides to chelators to cations, were applied to biofilms of *Pseudomonas aeruginosa* (FRD1) and *Staphylococcus epidermidis* that had been grown as colony biofilms. Not only were the two original biofilms quite different in mechanical properties, but their responses to the same treatment were different, and sometimes even opposite.

This research illustrated that it is possible to alter the mechanical properties of biofilm through chemical addition. Further, there are significant differences between the ways that the material properties of biofilms of different species of bacteria will be affected by a chemical treatment. Finally, it was observed that the 4-parameter Burger

model for constitutive mechanical properties of biofilms fit the vast majority of the collected data, so that this model proves useful in comparing properties of biofilms grown or treated under various conditions.

Penetration and efficacy of daptomycin against staphylococcal biofilms

Presenter: Phil Stewart, Professor, Chemical and Biological Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The penetration and biofilm killing activity of the lipopeptide antibiotic daptomycin (DAP) were investigated in vitro using staphylococcal biofilms. DAP penetration was evaluated by time lapse confocal scanning laser microscopy and image analysis using *Staphylococcus epidermidis* biofilms grown in flow cells. Biofilms were treated with a flowing solution of fluorescently-labeled DAP and the relative fluorescence in the center of cell clusters was compared to the surrounding fluid. DAP freely accessed all regions of large, dense biofilm cell clusters. The time required for fluorescently-labeled DAP to penetrate to the center of biofilm cell clusters with radii of one or two hundred microns and attain half of the plateau intensity was 91 ± 71 seconds. The effective diffusion coefficient of DAP in the biofilm was estimated to be approximately 28% of the diffusion coefficient of the drug in pure water. Bacterial killing was evaluated using methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms formed in a drip flow reactor. Established MRSA biofilms were treated with antibiotics including DAP (40 µg/mL), linezolid (40 µg/mL), vancomycin (20 µg/mL), or minocycline 150 (µg/mL) for periods of 6, 12, 24, and 48 hours. Log reductions in colony forming units were calculated relative to a control exposed only to lactated Ringers solution within each experiment. DAP was the most effective agent, resulting in a mean log reduction of 2.4 ± 0.4 after 6 hours and 3.7 ± 0.2 after 48 hours. Vancomycin and linezolid were ineffective for all treatment periods with log reductions less than 0.5. Minocycline had moderate efficacy with mean log reductions of 1.2 ± 0.4 and 1.1 ± 0.3 after 24 and 48 hours, respectively. The results suggest that DAP activity against staphylococcal biofilms is unlikely to be limited by penetration, and DAP was more effective against MRSA biofilms than other antibiotics commonly used to treat serious staphylococcal infections.

Modeling material aspects of biofilms

Presenter: Isaac Klapper, Professor, Mathematics
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Biofilms are not merely microbial ecosystems, but they are also large enough in scale so that macroscale physical processes can be important, including continuum mechanics and advective-diffusive transport. Viewed as materials, biofilms are quite interesting: they are living, growing, viscoelastic fluids with a surprising ability to respond to and defend themselves against their environments. They must be able to withstand variable external mechanical fluid shear stress without being too pliable or too brittle. They must be able to transport needed nutrients and substrates on coarse grained scales while excluding, to the extent possible, antagonistic substances.

These are of course complicated issues; hence it can be useful to develop theories and to test those theories in model forms where key theory elements can be isolated. This talk will present a quick discussion of basic principles and review of modeling strategies and results.

Topics to be considered include the role of conservation laws and constitutive relations, interplay of diffusion and reaction, physical properties of heterogeneous materials, and interaction of viscoelastic materials with imposed fluid shear.

Poster Abstracts

CBE Poster #438

Date: 07 / 2008
Title: **Transcriptional response of *P. aeruginosa* biofilms to antibiotic treatment**
Authors: ***James Folsom*** and P. Stewart
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsor: National Institutes of Health

Very little information is available regarding the effect of antibiotic treatment on the gene expression of *Pseudomonas aeruginosa* biofilms. This information is useful for identifying possible molecular targets for the prevention or elimination of these biofilms. In order to identify potential targets for treatment efforts, we have performed microarray analysis on *P. aeruginosa* biofilms grown in drip flow reactors. Biofilms were grown in drip flow reactors using minimal media for 3 days prior to exposure to ciprofloxacin or tobramycin. After 12 hours of exposure, biofilms were harvested by scraping and RNA was extracted. Affymetrix *P. aeruginosa* microarrays were used to measure the response to these antibiotics. Ciprofloxacin significantly ($\alpha<0.02$) affected the transcription of 181 genes, with Tobramycin affecting ($\alpha<0.02$) 296 genes. The two antibiotics shared an effect on 103 genes. There were 78 genes affected only by Ciprofloxacin exposure and 183 genes that were only affected by exposure to Tobramycin. Comparison of our results to other published microarray literature indicates that planktonic *P. aeruginosa* respond differently to Ciprofloxacin, having in common only the induction of pyocins by ciprofloxacin. Ciprofloxacin exposure of biofilms results in the induction of more LexA regulated genes than found with planktonic cells. The response of biofilms to Tobramycin shares 103 genes with the response to Ciprofloxacin. Thirteen candidates were chosen from genes affected by antibiotic exposure, and transposon mutations of these genes were obtained from the University of Washington collection. Ten of those were confirmed as having the correct insertion location, and these strains were tested for decreased resistance to Ciprofloxacin and Tobramycin. *Biofilms of two strains were greater than 1 log less tolerant of Ciprofloxacin (bold in table below).

Genes tested for their effect on biofilm antibiotic resistance showing latest results*

Locus / Gene Name	Description	Tobramycin Kill (Log)	Ciprofloxacin Kill (Log)
MPAO1	Wild type	1.27	1.54
PA0805	Hypothetical protein	0.7	2.32
PA0807	Conserved Hypothetical protein	1.66	2.09
PA0910	Hypothetical protein	0.63	**2.62**
PA0911	Hypothetical protein	0.61	2.41
PA1041	Probable outer membrane protein precursor	0.78	2.4
PA1343	Hypothetical protein	0.99	1.98
PA3718	Probable MFS transporter	0.96	**2.92**
PA3720	Hypothetical protein	0.42	2.16
PA5446	Hypothetical protein	0.72	1.63
PA5470 / prfH	Probable peptide chain release factor	1.33	1.23

*Information is a snapshot of the results through June 20th, and may differ from the final results that will appear on the poster.

CBE Poster #443

Date: 02 / 2008

Title: **Isolation and characterization of a heterotrophic nitrifying bacterium from a drinking water distribution system**

Authors: ***Gem D. Encarnacion***[1], L.H. Leach[1], X. Lin[2], and A.K. Camper[1]

Affiliation: [1] Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2] Stroud Water Research Center, Avondale, PA

Sponsors: American Water Works Research Association Foundation and Stroud Water Center

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

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

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

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

CBE Poster #445

Date: 05 / 2008

Title: **Microbially enhanced geologic containment of sequestered supercritical CO_2**

Authors: ***Andrew C. Mitchell***[1], Adrienne Phillips[1], Laura Wheeler[1], Logan Schultz[1], Randy Hiebert[1], Robin Gerlach[1], Al Cunningham[1], Lee Spangler[2]

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Sponsor: Funded by the Zero Emissions Research Technology (ZERT) fund, from the U.S. Department of Energy (DOE), Award No. DE-FC26-04NT42262.

Geologic sequestration of CO_2 involves injection into underground formations including oil beds, deep un-minable coal seams, and deep saline aquifers with temperature and pressure conditions such that CO_2 will likely be in the supercritical state. It is also important that the receiving aquifer have sufficient porosity and permeability and be overlain by a suitable aquitard trap. Supercritical CO_2 will be injected into the receiving formation resulting in elevated pressure in the region surrounding the point of injection. As a result, an upward hydrodynamic pressure gradient may be developed across the trapping aquitard. Upward "leakage" of CO_2 can subsequently occur due to the primary permeability of the aquitard through fractures or near injection wells.

This paper will focus on microbially based strategies and technologies for controlling leakage of supercritical CO_2 during geologic sequestration. We will examine the concept of using engineered microbial barriers which are

capable of precipitating large amounts of crystalline mineral (e.g., calcium carbonate) resulting in significant reduction of formation porosity and permeability. These "biomineralization" barriers, if shown to be stable over time, will provide a method for plugging preferential flow pathways in the vicinity of CO_2 injection, thereby reducing the potential for unwanted upward migration of CO_2. A summary of biofilm and biomineral formation observed in porous media will be presented, along with corresponding observations of reduced porosity and permeability.

CBE Poster #446

Date: 04 / 2008

Title: **Characterization of biofoulant and its effect on reverse osmosis and nanofiltration membrane surfaces**

Authors: ***Sara E. Nelson*** [1,2], M.Md. Taimur Khan[2], and A.K. Camper[2]

Affiliation: [1] Department of Cell Biology and Neuroscience, Montana State University, Bozeman, MT
[2] Center for Biofilm Engineering, Montana State University, Bozeman, MT

Sponsor: The Dow Chemical Company

Biofouling is a major reason for flux decline in the performance of membrane-based water treatment plants. To observe the biofilm formation and its effects on the reverse osmosis (RO) and the nanofiltration (NF) membranes, two commercially available membrane surfaces (RO-BW30 and NF-270, respectively) developed by the FilmTec Corporation, were examined. The biofilm formation study was carried out in rotating disk reactor (RDR) systems without filtration, and the feed was biologically activated carbon-treated tap water supplemented with nutrients. Staining and epifluorescence microscopy revealed more cells on RO than NF surfaces. Based on image analyses of 5-μm thick cryosections, the accumulation of hydrated biofoulants on RO and NF surfaces were more than 0.74 and 0.64 μm/day, respectively. The biofoulants increased hydrophobicity—as determined by contact angle—of these surfaces up to 30^o and 4^o for RO and NF surfaces, respectively. Furthermore, biofouling also increased the RO and NF surface roughness up to 60 nm and 50 nm, respectively, and a wide range of changes of the chemical elements on the membrane surfaces was observed with AFM and XPS. Good agreement between the biofilm morphology and its impact on surface properties was found, suggesting the validity of this new scientific concept for membrane assays.

CBE Poster #447

Date: 04 / 2008

Title: **Transcriptomic and proteomic analysis of *Desulfovibrio vulgaris* ATCC 29579 biofilms under conditions conducive to metal reduction**

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Sponsor: U.S. Department of Energy

Desulfovibrio vulgaris ATCC 29579 is a sulfate-reducing bacterium (SRB) that is commonly studied in the context of metal corrosion and heavy-metal bioremediation. SRB populations are frequently observed in pipe and subsurface environments as surface-associated populations. In order to compare the physiological states of *D. vulgaris* biofilm and planktonic cells, transcriptomic and proteomic analyses were done on biofilms formed upon glass slides within a CDC reactor under sulfate-reducing conditions. Transcript and protein expression profiles were unique, but sets of genes had similar trends in transcript and protein levels, namely genes associated with energy conservation. An annotated *ech* hydrogenase, formate dehydrogenase, and pyruvate ferredoxin oxidoreductase had increased transcripts and protein in the biofilm samples. Several other hydrogenases and formate dehydrogenases also showed an increased protein level for biofilm cells, and proteins for each step in the

reduction of sulfate to sulfide were increased. Decreased transcript and protein levels were observed within the biofilm for putative *coo* hydrogenases, as well as a lactate permease and *hyp* hydrogenases. Genes annotated for amino acid synthesis and nitrogen utilization were also among the most dominant changers within the biofilm state. There was an increase in putative proteins for the synthesis of amino acids arginine, cysteine, methionine, and histidine. Alternatively, enzymes annotated for the synthesis of aromatic amino acids showed decreased protein levels in the biofilm samples. Both transcript and protein levels for ribosomal proteins were notably decreased within the biofilm cells. The results indicated that biofilm cells may have an alternate flux of carbon and energy which may influence metal-reducing and metal-interacting capacity. In addition, fluxes within amino acid production suggested nutrient limitations within biofilm cells. Results from this study will provide insight to better control the growth of sulfate-reducing biofilms.

CBE Poster #448

Date: 04 / 2008

Title: **Chromium(VI) reduction capacity within *Desulfovibrio vulgaris* planktonic and biofilm cells**

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Sponsor: U.S. Department of Energy

Desulfovibrio vulgaris Hildenborough is a model organism for sulfate-reducers that can reduce heavy metals and radionuclides [e.g., Cr(VI) and U(VI)] and has shown great potential for the bioremediation of contaminated soils. *D. vulgaris* planktonic and biofilm cells were characterized for both reduction capabilities, physiological response, and survival during Cr(VI) exposure. Planktonic cells were washed to remove residual sulfide and inoculated into fresh medium to an OD_{600} of 0.1. A concentration of 0.05 mM Cr(VI) caused planktonic cells to lag for approximately 25 h even though Cr(VI) was not detectable 5 h post-exposure. During this period, a small amount of lactate was utilized even though there was no accumulation of acetate, and sulfate was not reduced. Viability decreased 4-fold and 40-fold during the first 10 and 20 h post-Cr(VI) exposure, respectively. Cell growth effects were not dependent upon increased ORP, but could be restored upon a non-dose dependent addition of ascorbate at the time of Cr(VI) exposure. Transcriptomic analysis of the planktonic cells indicated the majority of differentially expressed genes included annotated genes involved in phage synthesis, particular reductases / dehydrogenases, heavy metal resistance, and protein turnover. Biofilm cells grown upon glass slides within a CDC reactor were placed into a separate reactor with freshly prepared LS4D medium to remove planktonic counterparts and exposed to 0.l mM Cr(VI). Additional Cr(VI) was flowed through the reactor at approximately 0.25µmol / min and the dilution rate was approximately 0.38 h^{-1} in order to minimize planktonic growth. Cr(VI) levels declined steadily over the first 10 h while protein levels remained fairly constant throughout Cr(VI) exposure. Interestingly, cell viability did not decrease within the first 20 h post-Cr(VI) exposure. Initial results suggested that planktonic cells may have a higher rate of reduction per mg of protein compared to biofilm cells, but biofilm cells retained greater viability during Cr(VI) exposure.

CBE Poster #449

Date: 03 / 2008

Title: **Biodiversity and spatial concordance of an *in situ* system for uranium bioreduction**

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The elucidation of how populations of interest interact in a given community and how the community responds to stress and perturbations can help infer the interplay between stress pathways and gene networks that help optimize bacterial biochemistry. A goal of VIMSS is to characterize the responses of bacterial communities at multiple levels of resolution in order to understand biochemical capacity at DOE waste sites. The current work uses a series of re-circulating wells that create a subsurface bioreactor to stimulate microbial growth for *in situ* U(VI) immobilization (Wu et al., ES&T 41:5716-5723). Bacterial community dynamics were investigated in a series of re-circulating wells that created a subsurface "bio-reduction zone" to stimulate bacterial growth with ethanol for *in situ* bioremediation of U(VI) at the Field Research Center of the U.S. Department of Energy, Oak Ridge, TN. Different experiments were conducted to alter the subsurface environment to better understand strategies that would improve the remediation process. Within this framework, the interrelationships between the biogeochemistry were studied in order to characterize the community and ecosystem ecology with respect to microbiology of an engineered system. Bacterial community composition and structure of groundwater samples were analyzed via clone libraries of partial SSU rRNA genes. UniFrac analyses showed that the bacterial community in each of the wells developed changes during the bioremediation process, and the changes could be attributed to the variations along temporal and spatial scales. Relationships between community diversity and ecosystem function were idiosyncratic, and these results suggested the population distributions depended on the particular conditions under which the local landscape was investigated. Principal component analysis showed that nitrate, uranium, sulfide, sulfate, and COD were strongly associated with particular bacterial populations. Sequences closely related to nitrate-reducing bacteria were predominant during the initial phase of the remediation process, but sequences representative of sulfate-reducers (*Desulfovibrio* and *Desulfosporosinus* spp.) and metal-reducers (*Geobacter* spp.) were detected at higher levels as uranium levels declined. Ultimately, sequences associated with sulfate-reducing populations predominated. Uranium levels declined below EPA drinking water standards, and community composition and structure were similar in both treatment wells after approximately 1.5 y despite going through different transitions. In addition, when engineering controls were compared to the community structure and composition via canonical ordinations, population distributions could be related with dissolved oxygen control and the presence of bio-stimulant. During the bio-stimulation, population distributions followed geochemical parameters; these results indicated that bacteria exhibited distributions at the landscape scale in concordance with predictable geochemical factors. The data indicated that relationships between community structure and ecosystem function were idiosyncratic, but temporal and spatial concordance were eventually observed for the two bio-stimulated wells. The strong associations between particular environmental variables and certain population distributions will provide insights into establishing practical and successful remediation strategies in radionuclide-contaminated environments with respect to engineering controls and ecosystem function.

CBE Poster #450

Date: 05 / 2008

Title: **Genomic and physiological characterization of *Anaeromyxobacter* fw109-5, a metal- and nitrate-reducing bacterium isolated from uranium-contaminated sediment**

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Sponsor: ESPP2 (MDCASE) is part of the Virtual Institute for Microbial Stress and Survival (VIMSS) supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics: GTL Program through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U.S. Department of Energy. These sequence data were produced by the U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/).

Anaeromyxobacter fw109-5 is a mesophilic, iron-reducing bacterium that was recently isolated from subsurface sediments at the ERSP-FRC in Oak Ridge, TN. The groundwater at the sampling location had a pH of 6.1 and contained approximately 1.4 mM nitrate and 0.9 μM hexavalent uranium. *Anaeromyxobacter* spp. are high G+C (73.5%) delta-*Proteobacteria* related to the genus *Myxococcus*. Based upon SSU rRNA gene sequences, the closest cultivable relative is *Anaeromyxobacter dehalogenans* 2CP-C with 96.5% sequence identity. The strain fw109-5 grows in the pH range of 4.0 to 9.0, but optimal growth was observed from pH 7.0 to 8.0. To date, known electron donors include acetate, lactate, ethanol, and pyruvate, and electron acceptors include nitrate, nitrite and iron(III) but not AQDS. Yeast extract and peptone do not support growth, and the organism requires low substrate concentrations for growth (i.e., oligotrophic conditions). Optimal growth occurs under anaerobic conditions, and microaerophilic conditions can be tolerated. The *Anaeromyxobacter* fw109-5 genome is 5.3 Mb in size with 4,336 candidate protein-coding genes. The slow-growing bacterium is predicted to have two *rrn* operons, and almost 30% of the predicted ORFs are classified as conserved hypothetical proteins. A large percentage of estimated ORFs are predicted to be part of a signal transduction pathway with enrichment in serine/theronine kinase putative proteins. In comparison, fw109-5 had similar numbers of putative two-component and one-component signal transduction proteins as other sulfate- and metal-reducing delta-*Proteobacteria*, but fewer compared to *Myxococcus xanthus*. In addition, preliminary data suggest social behavior and sporulation. The genome is predicted to encode a full glycolytic and tricarboxylic acid cycle as well as a pyruvate dehydrogenase complex. Approximately 105 putative proteins are predicted to contain heme-binding sites, with almost half being multi-heme proteins.

CBE Poster #451

Date: 06 / 2008
Title: **Transcriptomic characterization of a sensory-box mutant during transitions between aerobic and anoxic growth conditions**
Authors: ***Anitha Sundararajan***[1], Z. He[2], M. Joachiamik[3], J. Zhou[2], and M.W. Fields[1]
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Sponsor: U.S. Department of Energy

Shewanella oneidensis MR-1 is known to exhibit metabolic versatility with respect to electron acceptors, and it is hypothesized that a varied assortment of signaling pathways is required to sense extracellular stimuli and optimize metabolism and physiology. The possible roles of a sensory box protein, SO3389, were assessed in wild-type, mutant, and suppressor strains via transcriptomic profiles. Mutant cells were impaired in the ability to carry out anaerobic metabolism when transferred from aerobic medium. Interestingly, the mutant eventually grew anoxically, and results indicated that a low frequency suppressor population had overcome the growth defect. Multiple transcriptomes were compared in order to determine possible differences between the strains under aerobic and anoxic conditions (lactate and fumarate). The wild-type and mutant cells grew in a similar fashion in aerobic shake flasks, and the two strains displayed few differences in aerobic transcriptomic profiles. When wild-type cells were transferred from aerobic to anoxic growth conditions, cells up-regulated a variety of genes compared to aerobic cells. Transcript levels did not change significantly for a majority of putative fumarate reductase genes; however, presumptive genes involved in the conversion of succinate to α-ketoglutarate were up-expressed. The mutant cells did not up-express these genes when transferred to anoxic conditions. Wild-type cells also had higher expression levels for a putative decaheam cytochrome c gene and a putative flavoprotein, and the mutant had low expression levels for these genes. At 10 h post-transfer to anoxia, the mutant displayed elevated expression levels for an operon involved in arginine biosynthesis compared to wild-type cells. For the most significantly changing genes and operons identified by pairwise comparisons between growth conditions, we performed Pearson correlation profile searches to identify genes and operons with similar expression patterns. The data will help identify underlying genes that are up-expressed during transitions between aerobic and anoxic conditions in a metabolically diverse facultative bacterium.

CBE Poster #452

Date: 07 / 2008
Title: **Confocal laser microscopy on biofilms: Successes and limitations**
Authors: ***Betsey Pitts*** and P. Stewart
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsor: N/A

This poster presents a shorter version of an article by the authors from *Microscopy Today*, July 2008.

Imaging of bacterial biofilms with microscopes has been an essential and transformative method in biofilm research. Fluorescence microscopy can elucidate specific biofilm components and cellular activities that cannot be separated otherwise. In particular, confocal fluorescence microscopy extends that examination through the thickness of a fully hydrated, in situ biofilm, affording the potential for 3D, non-invasive, time-lapse imaging. This article discusses some striking examples of the insight provided by confocal fluorescence microscopy into biofilm structure, composition, and heterogeneity, and it will also enumerate some limitations of this imaging process.

CBE Poster #453

Date: 06 / 2008
Title: **Proteomic and transcriptomic analyses reveal genes up-regulated by *cis*-dichloroethene in *Polaromonas* sp. JS666**
Authors: ***Laura K. Jennings***[1,2], S.F. Nishino[3], R.B. Payne[3], J.C. Spain[3], J.M. Gossett[1]
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Sponsors: SERDP, ESTCP, NSF Graduate Research Fellowship

Polaromonas sp. JS666 is the first bacterium isolated that is capable of growth-coupled *cis*-dichloroethene (cDCE) oxidation. Therefore, JS666 is a promising bioaugmentation agent for cDCE-contaminated sites where the common groundwater contaminant and suspected carcinogen has migrated into aerobic zones. The cDCE metabolic pathways in JS666 have yet to be elucidated, and knowledge of them could provide insight into required nutrients and conditions for optimal bioaugmentation. We designed experiments using proteomics and transcriptomics to identify genes up-regulated by cDCE compared to the reference substrate glycolate. 2D gel electrophoresis used in conjunction with LC / MS / MS and MALDI-TOF / TOF mass spectrometry revealed the up-regulation of a glutathione *S*-transferase (GST), cyclohexanone monooxygenase, and haloacid dehalogenase (HAD). Microarray experiments confirmed the proteomics findings that these genes were among the most highly up-regulated of the 217 identified genes that were at least 1.5-fold up-regulated by cDCE. Two possible cDCE degradation pathways are consistent with these results including (i) the monooxygenase-catalyzed formation of a DCE epoxide, which is transformed by a GST, and/or (ii) the dehalogenation of cDCE by direct conjugation with a GST, forming a glutathione conjugate that can be sequentially oxidized by chloroacetaldehyde dehydrogenase (CAD) and HAD to glycolate. CAD enzyme activity was constitutively expressed while HAD enzyme activity was inducible by cDCE in crude extracts of JS666. Collectively these data indicate that proteomics and transcriptomics were effective at revealing genes up-regulated by cDCE using two independent and complementary techniques. Experiments are underway to confirm the functional activity of these up-regulated enzymes and to solidify their roles in the cDCE degradation pathways in JS666.

CBE Poster #454

Date: 04 / 2008
Title: **Metabolic network modeling: Applications to extreme environments—the Sulfolobales**
Authors: ***Reed Taffs*** and R. Carlson
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Sponsor: National Science Foundation Integrative Graduate Education and Research Training (NSF IGERT)

The Sulfolobales are an order of sulfur-metabolizing crenarchaea found in terrestrial hot springs, thermal soils, and volcanic pools at low pH and high temperature. First isolated in Yellowstone National Park in the early 1970's, they have since become useful as a model organism for crenarchaeal biology. Additionally, they are a potentially valuable source of thermostable and acid-tolerant enzymes of interest to industry (e.g. α-amylase).

The goal of this project is to consolidate available information from public genomes and scientific literature, creating mathematical models for the metabolic network of each species. The modeling approach used, known as elementary mode analysis, provides all of the steady state flow patterns through an input network. In our case, this is equivalent to the set of chemical transformations that could be performed continuously by the organism of interest.

These metabolic network models are useful for several purposes. They highlight connections as well as missing pieces in the knowledge base regarding these model organisms and allow prediction of the effects of certain genetic manipulations (specifically knockouts and the addition of foreign enzymes). This enables hypothesis-driven research into techniques for maximizing growth yields and over-expressing valuable enzymes in the native

host. This is important because attempts to grow and harvest these heat- and acid-tolerant enzymes in mesophiles at neutral pH have met with limited success due to improper folding of the proteins. Additionally, for some applications (e.g., bioleaching) it is necessary to grow the organisms in situ, where conditions may be inhospitable to many other microbes.

CBE Poster #455

Date: 04 / 2008

Title: ***Staphylococcus aureus* biofilms prevent scratch wound closure *in vitro***

Authors: ***Kelly R. Kirker***[1], P.Secor[1], G.A. James[1], P. Fleckman[2], J.E. Olerud[2], and P.S. Stewart[2]

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Sponsor: National Institute of General Medical Sciences (NIGMS) grant number 1P20GM078445-01

Chronic wounds are characterized by prolonged inflammation, an altered wound matrix, and the failure to re-epithelialize. Chronic wounds are also characterized as supporting a diverse microbial flora. A literature review by Bowler examined culture data from 62 published studies and described a complex wound microflora with *Staphylococcus aureus* as the most predominant species isolated (reported in 63% of the studies)[1]. It has been speculated that bacteria colonizing chronic wounds exist as biofilm communities[2-4]; however, there are few data illustrating the role of biofilms in chronic wound pathogenesis. This study establishes a novel method for studying the effect of biofilms on cultured cells. Furthermore, it specifically examines the influence of *S. aureus* biofilms on keratinocyte migration.

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

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

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CBE Poster #456

Date: 5 / 2008

Title: **Material and energy flow in a hot spring microbial mat**

Authors: S. Montross[2], J. Aston[1,3], ***Natasha Mallette***[1,3], R. Taffs[1,3], K. Brileya[1,4], S. McGlynn[5], C. Klatt[6], Z. Jay[6], R. Carlson[1,3], R. Gerlach[1,3] M. Fields[1,4], D. Ward.[6], and W. Inskeep[6].

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Sponsor: National Science Foundation (NSF) Integrative Graduate Education and Research Traineeship (IGERT)

The microbial mat community of a Yellowstone National Park alkaline-siliceous hot spring (Octopus Spring) was used as a model system for developing a material and energy flux analysis methodology based on key microbe activities. Central metabolic network models were developed for three distinct community members using genome sequence information from NCBI and the KEGG database. The Synechococcus sp. OS-B' and Roseiflexus sp. RS-1 genomes were selected to represent phototrophic and heterotrophic activities. In addition, a metabolic model consistent with typical sulfate-reducing bacteria (SRB) was included to account for biotic sulfate reduction observed within the mat. Elementary flux mode analysis was employed to investigate community material and energy flows using simulations representing three different size-scales and three distinct diel phases. These modeling approaches provide tools for evaluating theoretical possibilities regarding material and energy transfer among microbial populations, and a context for testing hypotheses about functional guilds, syntropy, and mutualism within microbial communities.

CBE Poster #457

Date: 07 / 2008

Title: **Chlorine susceptibility of *Salmonella typhimurium* and biofilm detachment characteristics**

Authors: ***Sabrina Behnke*** and A.K. Camper

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Sponsors: Army Research Office, Unilever U.K.

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

For the purpose of testing and comparing biocide susceptibilities of planktonic cells, cells in biofilms, and detached cell aggregates, we designed experiments as follows: *Salmonella typhimurium*, as a model pathogen, is grown in standardized laboratory reactors for enhanced repeatability. Planktonic cultures are grown in a continuously stirred chemostats, while biofilm is obtained from a tube reactor. Detached aggregates can be sampled from the outflow of the tube reactor.

Log reductions have been assessed for planktonic cultures for the range of 1–4 ppm of sodium hypochlorite. Cell concentrations in the chemostat were on average 7.00E+07 CFU/ml. However, fluorescent microscopy revealed that only ~ 35% of the biomass was present as single cells. The majority of biomass appeared in small clusters of up to 10 cells.

Detachment in the tube reactor at standard conditions has been observed and compared to detachment characteristics after induced nutrient starvation (removal of nutrients from the feed) and also after stopped-flow

conditions to allow for accumulation of molecules that may trigger detachment. The size distribution of clusters and cells during regular detachment is similar to that occurring in the chemostat. After removing nutrients from the feed to mimic starvation conditions, more single cells and fewer cell clusters leave the outflow of the biofilm tube reactor. Detachment of more clusters and less single cells is induced by stagnation of the feed in the tube reactor for 20 minutes, likely due to the accumulation of molecules that trigger detachment in *S. typhimurium* biofilms.

CBE Poster #458

Date: 06 / 2008
Title: **Nitrification control in household systems**
Authors: ***M. Shahed Rahman*** and A.K. Camper
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Sponsor: National Science Foundation (NSF)

Nitrification is a major problem in drinking water supplies in United States. According to a 1996 American Water Works Association Research Foundation (AWWARF) survey, two-thirds of drinking water utilities that chloraminate have experienced some degree of nitrification (Wilczak et al., 1996). In this project, the effect of copper ion, chlorite and chloramine on nitrification in a simulated household plumbing system was investigated.

Modified CDC reactors were used to simulate a household plumbing system. Two sets of reactors were used; each set consists of four reactors containing either PVC as a coupon material or copper. These reactors were fed water with humics as the organic carbon (4ppm) source, ammonium sulphate (0.71ppm) as nitrogen source and biologically treated tap water to supply the bacterial population. Water in the reactors was stagnant for eight hours and then flowed for five minutes. pH of the influent water was maintained at around 8.15 and alkalinity was around 35 mg / L as $CaCO_3$. All of these reactors were operated for more than a year until all of them nitrified completely.

In one nitrifying PVC reactor, copper was gradually introduced with the influent water as copper sulphate, 15 ppb, and gradually it was increased to 1.3 ppm, allowing a two-week exposure time for every concentration. To ensure that most of the copper was in the ionic form, the pH of the influent was then gradually lowered to 6.6. No significant change in nitrification was observed under any of these conditions. Heterotrophic plate count and autotrophic nitrifying bacteria were enumerated using R2A plates and an MPN method. No significant effect of copper on those populations was observed. In the second stage of the investigation, chlorite, an autotrophic nitrification inhibitor, was introduced in a nitrifying copper and a PVC reactor. Initially chlorite was added at 0.2 ppm and gradually increased to 2.0 ppm, and finally a shock load of 20 ppm was applied. The effect of chlorite on the PVC system was not significant, but in the copper system it inhibited nitrification only at 20 ppm. It took about two months after chlorite was discontinued for the copper reactor to restart nitrification. Nitrifiers, especially nitrite oxidizers, are more affected by presence of chlorite. In the final stage of the project, monochloramine was introduced to both PVC and copper reactors. Monochloramine was initially applied at 0.5:1 chlorine to ammonia ratio. Gradually this ratio was increased to 5:1. Nitrification activity was impacted at a 1.5: 1 ratio of chlorine to ammonia but was most effectively controlled at a 5:1 ratio. After eight weeks of exposure at the 5:1 ratio, monochloramine was discontinued. The copper reactor regained full nitrification ability within two months, but the PVC reactor required three months.

CBE Poster #459

Date: 05 / 2008
Title: **Penetration and efficacy of daptomycin in staphylococcal biofilms**
Authors: G.A. James[1], J.N. Steenbergen[2], W.M. Davison[1], ***Laura L. Boegli***[1], and P.S. Stewart[1]
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Sponsor: National Institutes of Health

This study examined the penetration and relative potency of daptomycin (DAP) for the treatment of staphylococcal biofilms. DAP penetration was evaluated by confocal scanning laser microscopy and image analysis using *Staphylococcus epidermidis* biofilms grown in flow cells. The biofilms were treated with fluorescently labeled DAP and the relative fluorescence in the center of cell clusters was compared to the surrounding fluid. DAP freely accessed all regions of large, dense biofilm cell clusters. The time required for fluorescently labeled DAP to penetrate to the center of biofilm cell clusters with radii of one or two hundred microns and attain half of the plateau intensity was 91 ± 71 seconds. The effective diffusion coefficient of DAP in the biofilm was estimated to be approximately 28% of the diffusion coefficient of the drug in pure water. In methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms formed in a Drip Flow Reactor, DAP [minimum inhibitory concentration (MIC) 1 µg / mL] potency was evaluated relative to three other antibiotics: vancomycin (VAN, MIC 2 µg / mL), linezolid (LZD, MIC 4 µg / mL), and minocycline (MIN, MIC 15µg / mL). All antibiotics were applied in lactated Ringers solution (LRS), which was also used as the control treatment. Established MRSA biofilms were treated with antibiotics at concentrations of 40 (DAP and LZD), 20 (VAN), and 150 (MIN) µg/mL for periods of 6, 12, 24, and 48 hours. Log reduction in colony forming units was calculated relative to the LRS control within each experiment and a mean log reduction (MLR) was determined. Treatment with DAP resulted in an MLR of 2.4±0.4 after 6 hours and 3.7±0.2 after 48 hours. VAN and LZD were ineffective for all treatment periods with MLRs of <0.5. Increasing the concentration of VAN to 200 and 400 µg/mL did not improve the potency over 24 hours. MIN had moderate efficacy with MLRs of 1.2±0.4 and 1.1±0.3 after 24 and 48 hours, respectively. The results suggest that DAP activity against staphylococcal biofilms is unlikely to be limited by penetration and DAP was more effective against MRSA biofilms than other antibiotics commonly used to treat serious staphylococcal infections.

CBE Poster #460

Date: 06 / 2008
Title: **The impacts of *S. aureus* biofilm on keratinocyte morphology**
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Sponsors: National Institute for General Medical Sciences (NIGMS), Montana INBRE

Chronic wounds are characterized by prolonged inflammation and failure to re-epithelialize, and do not respond well to conventional treatment. Many factors have been implicated in the delayed healing of these wounds, including microbial infection. It has been speculated for several years that chronic wound infection may be biofilm related. *Staphylococcus aureus* has been implicated in several infectious diseases including acute and chronic skin infections. An *in vitro* model was developed to study host/pathogen interactions along with the role biofilm formation plays in pathogenesis. *S. aureus* biofilms were grown on 0.2 µm culture inserts and placed on top of a monolayer of human keratinocytes. Use of the culture inserts allowed for the removal of the biofilm from the keratinocytes with minimal disruption of the biofilm, allowing for a convenient method for the study of host/pathogen interactions. *S. aureus* biofilm secretions induced a significant disruption of the cytoskeleton in the keratinocytes followed by induction of widespread apoptosis. The disruption of cytoskeletal proteins and induction of apoptosis in keratinocytes may impact the natural healing process by inhibiting the re-epithelialization of the wound bed, leading to the chronic state of the wound. Planktonic *S. aureus* studied in the same manner were not found to induce these effects. Here we demonstrate that *S. aureus* biofilm formation is critical for the disruption of the keratinocyte cytoskeleton and induction of apoptosis *in vitro.*

CBE Poster #461

Date: 05 / 2007
Title: **Biofilms as biobarriers**
Authors: J. Lennox[1] and J. Ashe[2] ***(Rocky Ross[2])***
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Sponsor: National Science Foundation, Grant Number 0618744

There is a growing recognition of the importance of biofilms in the teaching of undergraduate microbiology. Much of the emphasis to date has been on the clinical implications of biofilms. This emphasis, while important, does not begin to detail the significance of biofilms overall. The exercise described here—"Biofilms and Biobarriers"—enables students to simulate, in the laboratory, an important tool in the hands of environmentalists attempting to control the spread of hazardous materials in groundwater. This exercise was developed as an undergraduate student project at Montana State University under NSF Grant NSF DUE0618744.

This poster describes a laboratory exercise that demonstrates both biobarrier and bioremediation technology. Columns packed with glass beads or sand are inoculated with *Pseudomonas fluorescens*, a prodigious EPS producer. The column is "fed" and the reduction in rate of flow through the column due to EPS accumulation is measured over time. The disappearance of nitrate ion, a common contaminant in U.S. ground water supplies, is also followed in the column effluent.

This exercise represents a safe and inexpensive method for introducing these two environmentally important strategies into the classroom. These exercises are part of a growing collection of biofilm exercises to become components of *Biofilms: The Hypertextbook*, a project being developed under NSF Grant #DUE0618744.

Biofilms: The Hypertextbook is an ongoing effort aimed at producing a comprehensive, high-quality, active-learning, web-based, dynamic teaching and learning resource for education in biofilms. The project involves researchers from the Center for Biofilm Engineering and the Department of Computer Science at Montana State University as well as numerous collaborators and evaluators from around the world.

CBE Poster #462

Date: 06 / 2008
Title: **Spaceflight effects on virulence of *Pseudomonas aeruginosa***
Authors: ***Susan Broadaway***[1], T. Goins[1], C. Crandell[1], C. Richards[1], M. Patel[1], T. Anderson[1] and B. Pyle[1,2]
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Sponsor: NASA Ames Research Center

Pseudomonas aeruginosa is an opportunistic pathogen found in the environment. It is known to infect the immunocompromised. The organism has about 25 virulence genes that play different roles in disease processes. Several exotoxin proteins may be produced, including ExoA, ExoS, ExoT and ExoY, and other virulence factors. In spaceflight, possible increased expression of *P. aeruginosa* virulence proteins could increase health risks for spaceflight crews who experience decreased immunity. Cultures of *P. aeruginosa* strains PA01 and PA103 grown on orbit on Shuttle Endeavour flight STS-123 vs. static ground controls were used for analysis. The production of ETA was quantitated using an ELISA procedure. Results showed that while flight cultures of PA103 produced slightly more ETA than corresponding ground controls, the opposite was found for PA01. While it appears that spaceflight has little effect on ETA, stimulation of other virulence factors could cause increased virulence of this organism in space flight. Similar increased virulence in spaceflight has been observed for other bacteria. This is important because astronauts may be more susceptible to opportunistic pathogens including *P. aeruginosa*.

CBE Poster #463

Date: 07 / 2008
Title: **Tools necessary to institute a biofilm testing division in a microbiological facility**
Authors: ***Karen Moll***, K. Buckingham-Meyer, J. Gruber, L. Lorenz, M. Perkins, M. Radons, T. Ready, D.K. Walker, and D. Goeres
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Sponsors: CBE Industrial Associates

Many established microbiological laboratories are contemplating expanding their facilities to include biofilm testing. Biofilm efficacy testing involves four primary steps: (1) grow a relevant biofilm in the laboratory, (2) treat the biofilm with disinfectant, (3) collect a biofilm sample that includes both removal from the surface and disaggregation, and (4) analyze the sample for viable cells. This poster elucidates the details necessary to establish a biofilm testing laboratory by outlining the required training, equipment, standards, and incurred costs. The estimates will be based upon: standardized biofilm methods for growing biofilm using either the CDC Biofilm Reactor (ASTM Method # E2562-07), the Drip Flow Reactor (ASTM Work Item # 17813), or the Rotating Disk Reactor (ASTM Method # E2196-07); the Liquid Microbicide Method (Work Item # 17314) for treating the biofilm; then scraping, homogenizing and viable plating for the sample and analysis steps. The estimate will assume that the biofilm laboratory is being incorporated into a fully functional microbiology laboratory. Finally, two projects performed by Standard Biofilm Methods Laboratory (SBML) are described to provide examples of biofilm tests that may be performed in an established biofilm laboratory meeting the criteria described in this poster.

CBE Poster #464

Date: 06 / 2008
Title: **The effect of quorum-sensing knockouts on *Escherichia coli* K-12 biofilm formation, antibiotic resistance, and architecture**
Authors: ***Trevor R. Zuroff***, H. Bernstein, and R.P. Carlson
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsors: Montana INBRE-BRIN who received funding from NIH. The project described was supported by Grant Number P20 RR16455-08 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH).

Bacteria often communicate through the secretion of small hormone-like molecules known as autoinducers. This type of cell-cell signaling, based on population density, is termed quorum sensing. Quorum sensing is of particular interest in the study of surface-attached bacteria, or biofilms, because of the role it plays in the regulation of many gene functions, including those which regulate virulence factors. This project focuses on four genes (*lsr* F, *lsr* K, *lsr* R, and *lux* S) known to play integral roles in the mechanism of autoinducer-2 production, internalization, and processing in the *lux* S regulated quorum sensing system of *Escherichia coli* K-12. Knockout mutants for each gene are being explored in three different shear environments: colony biofilm reactors, drip flow biofilm reactors, and CDC biofilm reactors. The mutants are subjected to three different temperatures (37°C, 29°C, and 25°C) and two different antibiotics (kanamycin and ampicillin) in each reactor system. The goal is to gain a better understanding of how the quorum sensing system is involved in biofilm formation and antibiotic resistance, potentially leading to breakthroughs in the control of problematic biofilms.

CBE Poster #465

Date: 06 / 2008
Title: **Isolation of a sulfate-reducing bacterium from groundwater stimulated for uranium(VI) bioreduction**
Authors: ***Brad D. Ramsay***[1], S. Carroll[2], and M.W. Fields[1]
Affiliation: [1] Montana State University Center for Biofilm Engineering
[2] Oak Ridge National Laboratory
Sponsor: U.S. Department of Energy

The Field Research Center (FRC) is located within the Y-12 Security Complex near Oak Ridge, Tennessee; the site includes 243 acres of a previously disturbed contaminated area. The FRC consists of four, unlined surface impoundments that received nitric acid/uranium bearing wastes for approximately 30 years. The subsurface at the FRC contains one of the highest concentration plumes of mobile uranium located in the United States and also contains various levels of nitrate, heavy metals, and organic contamination (http: //www.esd.ornl.gov/nabirfrc/). Recently, biostimulation with ethanol was conducted for uranium(VI) bioreduction, and the experiment successfully reduced uranium(VI) levels to drinking water standards in monitored wells. During the biostimulation, sequences indicative of sulfate-reducing bacteria predominated; therefore, sulfate-reducing enrichments were established with groundwater and ethanol. Several positive enrichments were achieved with ethanol as the carbon and energy source, and cultures from well FW101-2B were selected for microbial isolations. Well FW101-2B was in the bio-stimulated zone down-stream of the injection well. The isolate could utilize both lactate and ethanol under sulfate-reducing conditions, and growth was inhibited in the presence of nitrate. Based upon the SSU rRNA gene sequence, the closest relative is *Desulfovibrio carbinophilus*.

CBE Poster #466

Date: 07 / 2008
Title: **Biofilms on ice: "Unveiling" a new matrix stain?**
Authors: ***Christine M. Foreman***[1,2], M. Dieser[1,2] and B. Pitts[1]
Affiliation: [1] Center for Biofilm Engineering, Montana State University, Bozeman, MT
[2] Land Resources and Environmental Sciences, Montana State University, MT
Sponsor: National Science Foundation

Organisms that exist in icy environments possess mechanisms to protect themselves from extremes of thermal and radiative conditions that would cause severe damage to non-adapted organisms. While evaluating the potential of bacterial pigments to serve as cryo- or solar radiation protectants in our Antarctic bacterial culture collection, we came across an interesting phenomenon involving a control organism, *Escherichia coli* K12. Broth cultures of *E. coli* were subjected to a series (0, 1, 2, 6, 10, 15, 20, 30, 40, 50, 70, and 100) of 12-hour freeze-thaw cycles, rotating between -20°C and 6°C. After 2 freeze-thaw cycles viability of *E. coli* decreased significantly (CFUs dropped three orders of magnitude), and by 40 cycles there was 100% mortality (as determined by culturability). Over the course of the freeze-thaw cycles the organisms produced an enormous amount of what appears to be extracellular polymeric substances (EPS), presumably as a protective mechanism to avoid desiccation and intracellular ice nucleation. Using the confocal microscope in combination with several fluorescent stains, we were able to visualize the exquisite architecture of the biofilm matrix.

CBE Poster #467

Date: 02 / 2007
Title: **Development of immobilized chitosan coated beads for biogrowth control**
Authors: ***Sara E. Nelson***, M. Md. Taimur Khan and A.K. Camper
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsor: The Eastman Kodak Company

The main purpose of this research was to observe how chitosan may prevent the growth of biofilm in a porous media reactor and to investigate its ability to reduce suspended bacterial counts and improve filtrate quality.

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. Reactor C-1 contained 3 mm 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 ml, and the hydraulic retention time (HRT) was 5 hrs.

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 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 the there was release of material from the chitosan coating which led to a spike in turbidity, and then decreased to the lowest value of the three reactors. Interestingly, this increase in turbidity did not correlate with a detection of chitosan or chitosan fragments by HPLC. The average 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 influences 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 was 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. We are now doing the DNA sequencing to obtain information about the respective species.

Parallel to the community analysis, batch experiments using indigenous organisms in BAC treated water and different gram-negative bacteria (*E. coli* O157:H7, *P. aeruginosa*, *S. typhimurium*, and *P. syringae*), gram-positive bacteria (*S. epidermidis* and *S. mutans*) and yeasts (*C. albicans* and *S. cerevisiae*) were done to obtain the disinfection kinetics for three different doses (51.5 ppm, 103.0 ppm and 154.5 ppm) of chitosan. The selection of these doses was based on the amount of chitosan added on the beads in C-3. The initial concentration of cells was 10^4 cfu/ml. 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. The disinfection rate constants, *'k' and 'CT'* for 90% inactivation of cells were determined. The most important parameter is the ratio between cell and available chitosan. The chitosan coated porous bead column can be used not only for polishing purposes, but also can be added with other filtration and adsorption devices to improve influent quality to drinking water quality standards. For example, if the influent has high organic carbon, a granular activated carbon (GAC) filter can be used upstream of the chitosan coated porous bead column to remove organic carbon, giving lower load on this column, and downstream a microfilter (MF) can be added to retain trace amount sof bacteria or suspended/colloidal particles.

CBE Poster #468

Date: 06 / 2008

Title: **Use of qRT-PCR to determine the number of cells and 16s rRNA levels collected from biofilms by laser capture microdissection**

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Sponsor: Robert D. Watkins Graduate Research Fellowship, ASM.

Advances in quantitative reverse transcriptase PCR (qRT-PCR) and qPCR allow sensitive detection of bacterial gene expression from low numbers of cells. We have used qRT-PCR in combination with laser capture microdissection microscopy (LCMM) to quantify mRNA amounts of individual genes from cells at defined locations within microbial biofilms. For accurate quantification of gene expression, it is necessary to estimate the number of cells per sample. In addition, mRNA levels of specific genes should be normalized to an internal RNA control. Culturing techniques are not sufficient for estimating cell numbers from the LCMM-generated samples. Therefore, we used qPCR to estimate cell numbers by determining the number of chromosomal DNA molecules per sample. We also used qRT-PCR to determine the number of 16s rRNA molecules per cell as a potential normalizing factor. Both DNA and RNA were extracted from small numbers of *Pseudomonas aeruginosa* cells growing in logarithmic and stationary phase, using the TriReagent method of nucleic acid extraction. The *P. aeruginosa* PAO1 genome has four copies of 16s ribosomal DNA. Using qPCR with dual-labeled probes, we obtained 5 to 11 copies of the 16s rDNA per cell (n=12), in good agreement with predicted values for actively dividing cells. The number of ribosomes per cell was also analyzed by using qRT-PCR of the16s rRNA using dual-labeled probes. Samples from cells growing in logarithmic phase had between 62,000 and 159,000 copies of 16s rRNA (n=12). Stationary phase cultures had between 11,000 and 19,000 copies of 16s rRNA per cell (n=12). Dual-labeled probes allow multiplex qRT-PCR of several genes simultaneously. Therefore, these results demonstrate that, by using multiple probe sets, it is possible to quantify mRNA transcript abundances, rRNA levels, and the number of bacterial cells obtained from individual LCMM-generated biofilm samples.

CBE Poster #469

Date: 7/2008

Title: **The impact of direct current on *S. epidermidis* biofilms in the presence of ciprofloxacin**

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Sponsor(s): Montana State University College of Engineering, Peter Ewing Capital Management LLC, The Allegheny-Singer Research Institute, The Montana Board of Research and Commercialization Technology Grant Agreement No. 08-03

While we are continually seeing advances in medicine, the treatment of implant associated infections remains challenging. A 2004 article in *The New England Journal of Medicine* reported that of 600,000 joint prostheses implanted in the U.S. annually, approximately 12,000 will develop infections[1]—the majority of which are due to staphylococcal species (specifically *Staphylococcus epidermidis* and *Staphylococcus aureus*[2,3]). While long-term antimicrobial therapy with multiple antibiotics can be effective in some cases[3,4], the consequence of failure of this therapy may require the removal and replacement of the device in a one or two stage surgical process accompanied with an extensive course of antibiotics[3] and an estimated average cost of medical and surgical treatment of $30,000[1]. With these challenges, the development of novel strategies for treatment is desirable.

Treatment of bacterial biofilms with low levels of direct current (DC) was first reported by Blenkinsopp et al in 1992. The study showed that, while a biocide or direct current alone had little to no effect on bacterial survival, the combination of direct current and the biocide was shown to significantly increase killing efficacy[5]. It was thought that this synergistic phenomenon, termed the bioelectric effect, could be optimized to enhance antibiotic

treatment of orthopedic device related infections without the time, pain, and cost of surgery. The purpose of the current research was to investigate the application of varying low levels of direct current on *S. epidermidis* biofilms in a dilute nutrient solution and a salt concentration of normal saline thought to approximate the conditions in an infected artificial joint.

S. epidermidis biofilms were grown at 37ºC on 1.27 cm diameter polycarbonate coupons in a CDC biofilm reactor using a standard protocol operating for 24 hours in batch mode with full strength tryptic soy broth (TSB) and 16 hours of continuous flow with $1/10^{th}$ strength TSB. The biofilm coated coupons were then aseptically moved from the CDC reactor and placed in polycarbonate wells for treatment. Each well with current had its own circuit consisting of an inline ammeter and a current controller plugged into a DC power supply connected to platinum electrodes inserted through the lid at the far ends of each treatment well. A treatment solution of $1/10^{th}$ strength TSB with 9 g/L NaCl and 2.5 mg/L ciprofloxacin when applicable was added to each well. Each well held three coupons and current was applied along the long axis of the well for 24 hours at 37ºC. Coupons were sampled by scrapping and plating on tryptic soy agar plates.

A significant decrease in the log cell density was observed with the application of direct current at 2, 3, 4, and 5 mA of direct current both in the presence and absence of ciprofloxacin. It is thought that the observed killing was mainly due to electrolysis products that produced gradients across the well of both free chlorine and pH. Past work in this area has often used a media with minimal salt to minimize electrolysis products. However, the interest in working with a salt concentration of normal saline (0.9% NaCl) made electrolysis reactions far more prevalent in this system. Wells that contained ciprofloxacin in addition to the current typically saw an additional 0.5–1.0 log cell density decrease when compared to the wells at the same current level but no antibiotic. While a synergistic effect between ciprofloxacin and the current (the bioelectric effect) may have been present, it is thought that the larger electrolysis impact masked any observation of that effect.

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