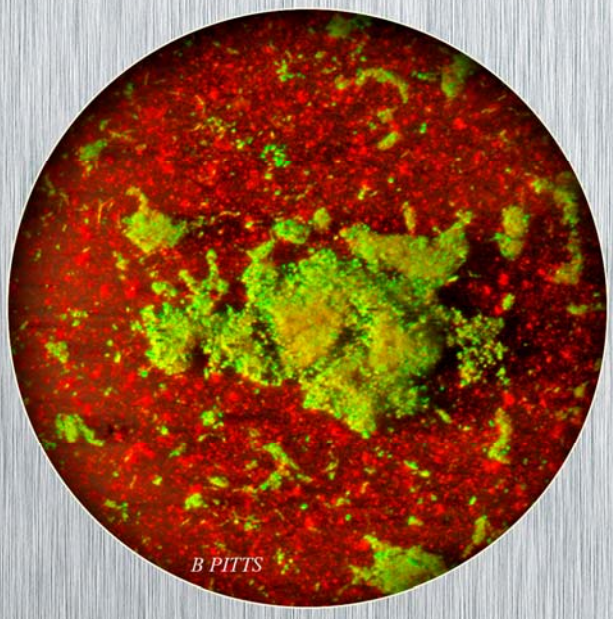
MONTANA STATE UNIVERSITY

Center for Biofilm Engineering

montana biofilm SCIENCE & TECHNOLOGY meeting

JULY 2010



PROCEEDINGS

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Speaker Abstracts

SESSION 1: Ophthalmologic Biofilms

Biofilms and ocular infections

Presenter: Phil Stewart, CBE Director
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The potential role of biofilm formation in the etiology of ocular infections stemming from contact lens wear is examined. The presentation begins with some microscope images of worn contact lenses and used contact lens cases, underscoring the common occurrence of biofilms on these surfaces. A literature review suggests these points: 1) storage cases are frequently contaminated, 2) biofilm accumulates on storage cases more readily than on a contact lens in the eye, 3) biofilms on storage cases or contact lenses are hard to kill with disinfectants (relative to suspended cells), and 4) data attempting to correlate case contamination with hygiene practices are mixed. Some relevant phenomena from the literature on biofilm physiology and control will be recapped, namely: that biofilms can be oxygen sinks, that many disinfectants fail to remove biofilm even if they do result in some killing, and that biofilms can accumulate in the near continuous presence of an antimicrobial agent. These observations and phenomena will be synthesized in a discussion of how biofilm formation may contribute to ocular infections.

Microbial biofilms, contact lenses, and contact lens care products

Presenter: Tim Morris, Senior Principal Scientist, Global Development R&D Microbiology
Affiliation: Bausch & Lomb, Rochester, NY

Abstract not available.

Contact lens-associated biofilms: Model development and recent advances

Presenter: Pranab K. Mukherjee, Assistant Professor, Center for Medical Mycology
Affiliation: University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, OH

Fungal/bacterial keratitis is a devastating ocular disease and an important cause of morbidity and blindness. Fungal keratitis has been reported in different parts of the world, particularly in tropical areas, where it may account for more than 50% of all ocular mycoses. Both fungal and bacterial keratitis has been associated with the ability of microbes to attach to contact lenses and form biofilms. We developed an *in vitro* model for testing the ability of fungi (*Fusarium*, *Candida*) to form biofilms on contact lenses (etafilcon A, galyfilcon A, lotrafilcon A, balafilcon A, alphafilcon A, and polymacon). We also developed and used a bacterial biofilm-contact lens model to determine whether three different bacteria commonly associated with contact lens keratitis and inflammation (*Pseudomonas aeruginosa*, PA; *Serratia marcescens*, SM; and *Staphylococcus aureus*, SA), can form biofilm on silicone hydrogel contact lenses. Biofilm formation was quantified by metabolic activity (XTT assay, for fungal biofilms) or quantitative culturing (colony-forming units, CFUs; for bacterial biofilms). Gross morphology and architecture of the formed biofilms were evaluated using scanning electron microscopy (SEM) and confocal microscopy (CSLM). Susceptibilities of the formed biofilms to commonly used contact lens care solutions were also assessed. To validate our *in vitro* results, we developed a murine model of contact lens associated *Fusarium keratitis*, in which a 2 mm

diameter punch of contact lens-associated *F. oxysporum* biofilms was placed on abraded corneal epithelium of either untreated or cyclophosphamide-treated C57BL/6 mice, or on IL-1R-/-, MyD88-/- TLR2-/- or TLR4-/- mice. After 2h, the lens was removed, and corneal opacification, colony forming units and histopathology were evaluated. Our results showed that clinical isolates of *Fusarium* and *C. albicans* formed biofilms on all types of lenses tested, and that biofilm architecture varied with the lens type. Moreover, differences in hyphal content and architecture were found between the biofilms formed by these fungi. We also found that two recently isolated keratitis-associated fusaria formed robust biofilms, while the reference ATCC 36031 strain (recommended by the ISO guidelines to test disinfectants) failed to form biofilms. Furthermore, using the developed *in vitro* biofilm model, we showed that *Fusarium* biofilms exhibited reduced susceptibility against these solutions in a species- and time-dependent manner. The tested reference and clinical bacterial (PM, SM, SA) strains formed biofilms on lotrafilcon A silicone hydrogel contact lenses as dense networks of cells arranged in multiple layers with visible extracellular matrix. These biofilms were resistant to commonly used biguanide-preserved multipurpose care solutions. While PA and SA biofilms were susceptible to a hydrogen peroxide and a polyquaternium-preserved care solution, SM biofilms were resistant to a polyquaternium-preserved care solution but susceptible to hydrogen peroxide disinfection. In contrast, the planktonic forms were always susceptible. In the developed *in vivo* mode, C57BL/6 mice developed severe corneal opacification within 24h, which remained elevated for 72h, and resolved spontaneously. In contrast, corneal opacification progressed in cyclophosphamide-treated mice due to unimpaired fungal growth in the cornea, with hyphae penetrating the corneal stroma. The phenotype of MyD88-/- and IL-1R-/- mice was similar to that of cyclophosphamide-treated animals, with significantly less cellular infiltration and higher CFU. We also found that although TLR4-/- mice had a pronounced cellular infiltrate and developed corneal opacification, CFU were significantly higher than C57BL/6 mice. These results demonstrate that both fungi and bacteria form biofilms on contact lenses, and that these biofilms are resistant to commonly used lens care solutions. Molecular level studies are currently being performed to determine the mechanisms regulating biofilm formation and the role of specific proteins/genes in biofilm-associated infections.

SESSION 2: Biofilm Fundamentals

Physiological roles of a *Shewanella oneidensis* MR-1 PAS protein involved in oxygen consumption, transfer to anoxia, and biofilm formation

Presenter: Anitha Sundararajan, PhD Candidate, Microbiology
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Shewanella oneidensis MR-1 is a facultative anaerobic bacterium that can utilize a wide assortment of electron acceptors for energy; however, little is known how energetically versatile organisms such as *S. oneidensis* sense and process environmental stimuli to optimize electron flow and metabolism. In order to elucidate function for putative sensory proteins that help control metabolism, the physiological role of a conserved hypothetical protein (SO3389) in *S. oneidensis* MR-1 was characterized. The predicted ORF encodes a putative sensory box protein that has PAS, GGDEF, and EAL domains. An in-frame, deletion mutant was constructed (△SO3389) and approximately 95% of the ORF was deleted (i.e., intact domains were not present). Under aerobic conditions, the mutant had a similar growth rate but consumed oxygen at slower rates, formed less biofilm, and had decreased motility. When transferred to anoxic conditions, the mutant displayed an extended lag period with different electron acceptors. Transcriptomic studies suggested that the growth deficiency when transferred to anoxic media was a consequence of the inability to up-express and down-express particular *c*-type cytochromes. In addition, fumarate reductase activity was decreased. The complemented strain did not lag when transferred from aerobic to anoxic growth

conditions with different electron acceptors. Despite low peptide sequence identity (31% to 42%), the modeled structure for the SO3389 PAS domains was highly similar to the crystal structures of FAD-binding MmoS and NifL PAS domains. Based on physiological, genomic, and bioinformatic results, we demonstrate that the conserved hypothetical protein, SO3389, is an O_2/redox-sensor that is involved in the regulation of O_2 consumption, biofilm formation, and transitions to anoxia.

Factors affecting biofilm formation by *Desulfovibrio vulgaris* Hildenborough

Presenter: Andrew Sabalowsky, Postdoctoral Research Associate
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Desulfovibrio vulgaris is a ubiquitous marine and groundwater sulfate-reducing anaerobic bacterium. It is associated with both iron pipe corrosion and heavy metal groundwater remediation, such as reduction of chromium and uranium *in situ*. To gain an understanding of the roles of *D. vulgaris* in these processes, we are investigating factors affecting biofilm formation, electron transfer, extracellular physical features, and their interrelatedness. Electron donor and acceptor sources and ratios, as well as *D. vulgaris* mutants are being tested in suspension, as well as in modified CDC reactors with biofilm growth on glass slides. Mutants deficient in the pilA, flaG, or echA gene have been compared to growth by the *D. vulgaris* Hildenborough wild type strain (WT), as well as a *D. vulgaris* mutant deficient in the megaplasmid (ΔP). Suspended growth in test-tubes reveals similar growth rates and final culture densities when comparing the pilA, flaG, and echA mutants to WT for both lactate and pyruvate as electron donors with non-limiting sulfate (50 mM). The plasmidless mutant (ΔP) grows at approximately 80% the rate of the wild type, though to similar final densities. While *D. vulgaris* WT forms dense biofilms on glass in the CDC reactors, none of the mutants tested form true biofilms, with only sparse colonization, as determined by Field Emission Scanning Electron Microscopy (FE-SEM). Although different concentrations of lactate and sulfate in CDC reactor operation generally only affect total biofilm biomass, operations with sulfate limiting conditions (60 mM lactate and 15 mM sulfate) resulted in biofilm detachment after 72–96 hours of operation, with no re-colonization during the next 72 hours, despite a dense planktonic biomass. Current and future work also includes growth of *D. vulgaris* on anodes in microbial fuel cells, investigating the roles of extracellular features such as pili and flagella in extracellular electron transport, and investigating alternative electron donor sources, and electron donor/acceptor ratios.

SESSION 3: Biofilms in the Built Environment

The house as a system: How it impacts moisture in buildings

Presenter: David Bell, Director of Building Science
Affiliation: Masco Contractor Services, Daytona Beach, FL

The house is a system, and by incorporating basic construction principles of building science, we can build homes that are healthy and safe, durable, comfortable, and energy efficient. By incorporating the following seven elements into home construction—tight construction, pressure balancing, combustion safety, interior moisture management, improved thermal systems, fresh air ventilation, and right sized HVAC—we can control the flow of moisture, and significantly reduce the potential for moisture related issues (mold) in the home.

The use of various building materials can have a dramatic impact on building performance; in most instances these decisions are made without a basic understanding of their impact on the house as a system, and in particular their impact on moisture in both liquid and vapor forms. An understanding of how air,

heat, and moisture travel through various building components is the first step in learning how to build high performance homes.

To be able to deal with these issues in production building today requires that an effective process be established, one that can be duplicated by a variety of sub-contractor personnel. For this to be successful the process must be simple, easily trained, and leave little margin for error. The process to be able to establish these objectives already exists but has not been applied to the home building industry. The Environments for Living program provides the industry with this process.

Development of filamentous fungal biofilms

Presenter: Julia Kerrigan, Assistant Professor, Mycology
Co-authors: Jordon Gruber, Virginia Waldrop, Kirthi Kiran Yadagiri
Affiliation: Department of Entomology, Soils, and Plant Sciences, Clemson University, Clemson, SC

The importance of fungal biofilms has been known for years, with the majority of research focused on yeasts for medical applications and a few disparate studies on industrial and municipal systems. Biofilms formed by filamentous fungi are ubiquitous and of great concern because of their persistence in industrial, medical, and household environments. We are interested in the basic biology of filamentous fungal biofilm formation and applications of prevention and removal. The Drip Flow Reactor (BioSurface Technologies) is being used to develop a relevant method for engineering biofilms for hard surface industrial applications. This system involves growing biofilms in a controlled reactor with constant stresses that include nutrient limitations, shearing forces, and harmful chemicals. Biofilms are grown on a coupon surface, which allows for additional examinations such as biocidal tests and microscopic observations. *Aspergillus niger* has been used because it is commonly found in biofilms, is widely used in industrial applications, and has a characteristic appearance that allows for easy detection of a contaminant. The first stage of biofilm formation involves spore attachment and germination. Hyphal tip growth produces an extracellular polysaccharide that helps the fungus grow and allows for attachment within the liquid environment. Hyphae form an interconnected network that helps it cling to itself and other surfaces. As hyphal proliferation continues, spores are produced, germinate, and give rise to additional biofilm mass. A mature *A. niger* biofilm is composed of a confluent mucilaginous hyphal matrix covered with conidiophores, vesicles, and phialides bearing chains of conidia. Having an understanding of the phenotypic steps in biofilm formation provides important baseline information for additional studies, including the mechanistic actions of biocidal activities, live/dead cell visualization, and related applications. Additional common filamentous fungi will be used to determine the adaptability of the system to morphologically different species and provide protocols for further studies.

Fungal biofilms associated with commercial carpet tile: Aspects of preservation, sanitization and recycling contaminants

Presenter: Daniel Price, Director, Microbiology
Co-author: Brandi M. Eason
Affiliation: InterfaceFLOR Research and Development, LaGrange, GA

Our laboratory studied development of fungal growth on commercial carpet. The carpet studied was carpet tile that measured 50 cm on a side. The face construction had low-height loop pile nylon fiber with a polyvinyl plastisol backing. Fungal growth and the formation of fungal biofilms typically were associated with the polymer backing that typically rests on concrete subfloor. In most cases, the concrete subfloors had vapor emissions that were out of specification with recommended installation conditions. In other cases a moisture event such as a flood or plumbing leak proved to be the trigger event that facilitated mold growth and subsequent odor. In these events, the polymer backing supported genera, including *Cladosporium, Aspergillus*, and *Penicillium,* that may survive extended periods of desiccation.

Strategies to prevent mold colonization of carpet include control of relative humidity coupled with maintenance, i.e., routine vacuuming with HEPA filtered vacuum machines and periodic vacuum extraction. Other preemptive strategies include the incorporation of antimicrobial preservatives either at the face fiber or at the backing layer at the base of the carpet fiber. Incorporated preservative treatments tend to have better durability after multiple shampoos. Commercial carpet manufacturers and the EPA prefer incorporated or bound treatments because of durability and safety factors. Here we report on the incorporation of a phosphated amine complex (Intersept®) and its efficacy and durability in carpet backing. New standard methods for rapid screening carpet with incorporated preservatives were used to determine (qualitatively) fungal inhibitory activity prior to and after sanitizing cleaning. Carpet tile preserved with Intersept and used for eight years in a southwestern US hospital neo-natal ICU unit retained antifungal activity after multiple cleanings. Expression of antifungal activity with soiled, used carpet tiles was usually enhanced following the cleaning process.

The US Green Building Council's (USGBC) Leadership in Energy and Environmental Design (LEED) rating system has tremendously impacted building design and interior furnishings. LEED credits for both pre-consumer industrial and post-consumer recycled content may have inadvertently increased the level of viable biocontaminants (inherent bioburden) in certain building and interior finishes. The path of reclaimed and recycled carpet tile was examined for viable fungal contaminants at each step in the recycling and remanufacturing process. Our preliminary data indicate a stepwise reduction in viable fungal contaminants due both to separation/filtration of dirt and particulates during fiber and backing separation process and exposure to heat during polymer agglomeration and melt-fusion of polyvinyl crumb. The impact of fungal biocontaminants on interior finishes with recycled content (especially carpet) will necessitate good process control during reclamation and remanufacturing. Moisture control, efficient maintenance procedures, and incorporated broad spectrum preservatives are proven strategies which can prevent or delay the development of fungal biofilms on commercial carpet tile.

Biofilms on exterior painted surfaces: What grows and why

Presenter: Gary Horacek, Director, Technical Microbiology Services
Affiliation: Troy Corporation, Florham Park, NJ

Fungal and algal growth on coated (painted) surfaces will always occur upon environmental exposure. Coating structure and design can slow the progress of microbial growth, but ultimately cannot prevent it when environmental conditions are conducive. Because growth is inevitable, dry film antimicrobials are routinely included in all exterior coatings world-wide. The same is true of many other building products such as caulks, sealants, grouts, roof coatings, moisture barriers, EIFS, stuccos, plasters, adhesives, OSB, etc. The dry film preservatives are added to prevent or slow disfigurement and deterioration of the protective or decorative coating. Coating destruction exposes the underlying substrate to the environment and subsequently to biological and environmental attack and eventual failure.

Fungi (mildew, mold) are ubiquitous in nature as prime movers in the recycling of organic materials. They will destroy or disfigure natural and man-made building products over time. Coatings were developed to cover 'open' areas so that a smooth appearance was presented to block moisture intrusion and to provide a reduced footprint for mildew penetration into the underlying substrate.

The goal of this presentation is to illustrate the extent of fungal colonization of coatings in the environment, to explore the factors that influence fungal growth, and to touch on what can be done to prevent fungal growth. A key finding is that biological attack of coatings is mediated through niche environments that feature fungal successional dynamics. Furthermore, algae often play a significant role in enabling such biological attack in addition to being disfiguring agents in their own right. Basic to understanding these niche environments is to map and define the collection and movement of moisture within both the macro- and the micro-environment of the coating, including within the coating and substrate itself.

Effective anti-fungal dry film chemicals are well known to industry; but they alone are not sufficient for adequate control of destructive fungal growth. We now know that algal growth (normally regarded as a cosmetic issue) has a strong influence on fungal growth. Even though fungal-algal interactions are highly variable in timing, order of succession, and the extent of the interaction, it can easily be demonstrated that a combination fungicide/algaecide placed within the coating provides superior protection compared to even very high concentrations of a fungicide alone. This holds true even when the overwhelming growth within the colonized coating is fungi.

When working with the prevention of dry film colonization on a practical level, laboratory work is of limited value, as it does not model the natural fungal succession process (which is highly variable and non-predictable). Nor is it possible to artificially integrate fungal growth with algal growth. While such testing can help in ranking products, it is known that scientifically designed exterior exposure studies must be performed in multiple climatic regions to define truly effective methods of fouling control. Enough is known to demonstrate that the next frontier in improved control of dry film growth on exterior coatings is to find the right mixture of algaecides to blend with known effective fungicides.

Microbial colonization of nonporous surfaces in a workplace and residential environment

Presenter: Jayne Morrow, Environmental Engineer, Biochemical Science Division
Co-Authors: Autumn Downey and Sandra Da Silva
Affiliation: NIST, Gaithersburg, MD

Reliable and precise methods for characterization of microbial contamination of surfaces is critical to developing cleaning and decontamination plans to ensure public health and public safety as well as developing an understanding of what constitutes a healthy indoor microbiome. Determination of parameters that affect the adhesion and subsequent removal efficiency of microorganisms associated with surfaces is of fundamental importance to understanding and improving upon surface sampling methodologies used to characterize indoor environments. The research community has demonstrated that sampling efficiency is highly dependent on experimental conditions including variation in surfaces tested (glass, polymer, stainless steel, porous and sealed concrete), recovery materials (polyester, cotton, microfoam, etc.), wetting agents (sterile water, buffer, surfactants), and organisms (*Bacillus anthracis, B. subtilis, Escherchia coli, Listeria monocytogenes*); however, little work has focused on sampling surfaces when characterizing unknown microbial populations. We are beginning to look at sampling efficiency and surface colonization in the indoor environment. Microbial community populations native to inhabited office and residential spaces were recovered from coupons (Teflon, aluminum, copper, stainless steel) after 1, 5, and 7 weeks exposure to normal operational environments. For pre-wipe samples, coupons were vortexed and sonicated in 25 ml phosphate buffered saline plus 0.04% Tween 80 (PBST) to remove microbial growth. Additional 7-week samples were processed by polyester-rayon wipe sampling versus direct extraction by vortexing and sonication in PBST. For all samples, 10 ml of eluant was filtered for plating on each media type (i.e., TSA, R2A). Distinct colony morphologies were further characterized by 16S rRNA sequence analysis (nearly 100 samples sequenced) and fluorescent *in situ* hybridization for 3-D structural relationship. Colonization varied based on surface type and location; however, colonization surface coverage increased over time for all surfaces in a residential kitchen (average values ranging from 0.5 to 2.5 CFU/cm^2) and on steel and Teflon in an office environment (< 1.0 CFU/cm^2). On 7-week wipe samples, wipe sampling efficiency varied based on surface and the extent of colonization. This work is an initial effort to understand the surface colonization of the indoor environment and the fundamentals critical to the successful sampling of microbes relevant to a range of environmental, clinical, and public safety sector stakeholders.

The lungs of our built environments: The hunt for biofilms and beyond

Presenter: W. Curtis White, President
Affiliation: White IEQ Consultants, LLC

Built environments—from buildings to transit vehicles—are filled with varied microhabitats. These habitats change with the seasons, the habits and practices of the occupants, the function of the built space, and the life of the facility. Complex communities of microorganisms exist throughout these facilities. These "communities" change through time and challenge our view of biofilms and their impact on deterioration, staining, odors, and human morbidity and mortality. The impact on the life and utility of building materials, operating systems, furnishings, and occupants is staggering, and the economic and human tragedy represents a burden to owners and the overall economy. This interplay of environment and microflora shows up in the air handling systems from the air intakes, conditioning equipment, supply air systems, the occupied space, and return air systems.

This presentation will show the diversity of these environments and the nature and impact on the buildings, the processes and products, and the occupants of the diverse and ever-changing microflora definable as biofilms and beyond.

Biofilms in premise plumbing

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

Premise plumbing systems providing potable water have several characteristics that make them susceptible to biofilm growth and release, including a very high surface-area to volume ratio, potentially long stagnation times, decay of disinfectants, warm to hot temperatures, and abrupt high flow rates. Although general heterotrophic bacteria have not been shown to be associated with health risks, the potential for the growth of opportunistic pathogens exists. Also, the use of some point of entry or point of use devices may give a false sense of security regarding the microbiological quality of potable water in the built environment. This presentation will cover some of the aspects related to the prevalence of opportunistic pathogens proliferating in biofilms in these environments. The presentation will also cover some of the pros and cons of point of use/point of entry devices and their related biofilms.

Legionella and other microorganisms in biofilms from a hot water system

Presenter: Barry Pyle, Research Professor, Microbiology
Affiliation: Montana State University, Bozeman, MT

Stainless steel coupon stacks were inserted in cartridges in a recirculating by-pass loop in a laboratory building hot water system (ca. 45˚C). After several weeks, a fluorescent antibody stain showed that presumptive *Legionella pneumophila* had colonized coupons; more *L. pneumophila* antibody stained cells were seen on scraped coupons. This suggests that the Legionellae were attached closer to the surface than some other organisms. Presumptive Legionellae were detected on only a few of many plate cultures, and PCR results were also variable. Coupons removed after several years in the system suggested the presence of *L. pneumophila* by antibody staining, with some possibly associated with larger cells stained with SYBR Green. It is possible that these larger cells may be amoebae. The results suggest that Legionellae may colonize biofilms in hot water systems where they may also associate with other organisms. The study also demonstrates the use of a recirculating by-pass loop to study microbial colonization of water distribution systems.

SESSION 4: Biofilm Methods

Anti-fouling betaine modifications for medical devices and industrial application

Presenter: Christopher Loose, Chief Technology Officer
Affiliation: Semprus Biosciences, Cambridge, MA

Biofilms are key contributors to problems in a variety of industries including medical devices (infections), water filtration (contamination), food/agriculture (contamination), ventilation (odor), and piping (occlusion). In the clinical setting, leaching antimicrobial coatings have had limited success in >30 day applications and suffer from potential toxicity and generation of drug-resistant strains. Existing non-leaching technology is quickly deactivated by blood products. We examined a potentially superior approach by using highly water-coordinating, non-fouling materials to prevent bacterial attachment and subsequent biofilm formation in a challenging blood environment. We have previously demonstrated the ability of these structures to inhibit thrombus formation in blood flow loop studies, even after long term exposure to human plasma. In the current study, the viability of this anti-thrombogenic surface to protect chronic (>30 day) vascular devices against biofilm formation was assessed by exposing modified devices to human plasma for 56 days, refreshing the plasma weekly, followed by a biofilm challenge.

Polyurethane catheter substrates (10-French rods) were modified using proprietary, non-fouling betaine polymers. *Staphylococcus epidermidis* ATCC 35984 was used in a modified CDC system to assess antibiofilm performance following preconditioning. In order to mimic the clinical setting, samples were stored in 100% citrated human plasma (CHP) for one day or 56 days prior to bacterial challenge. Articles were then exposed to a bacterial suspension of 10^6 CFU/ml in 1xPBS in batch mode for 2 hours at 37°C with agitation. Thereafter, the rods were transferred to a fresh reactor containing 1:10 Tryptic soy broth (TSB) + 0.25 wt% glucose, and flow of sterile media (8 ml/min) was initiated. Biofilm growth was monitored by enumerating viable CFUs via sonication and plate counting and by macroscopic visualization of biofilm surface coverage after 24 hours. Log reduction (LR) differences were calculated from adherent bacterial densities recovered from surface-modified rods and polyurethane controls. Betaine surface-modified rods demonstrated a mean LR of 1.49 (SE 0.38, $p<0.0001$) after 1 day in plasma and a mean LR of 1.49 (SE 0.63, $p<0.0001$) after 56 days of storage in plasma, demonstrating consistent performance over chronic exposure to a clinically relevant environment. This betaine modification has demonstrated dual anti-microbial and anti-thrombotic characteristics after 56 days of human plasma exposure, potentially enabling a range of chronic medical device—as well as industrial—applications.

Use of laser capture microdissection microscopy and qRT-PCR to characterize localized gene expression in biofilms

Presenter: Mike Franklin, Associate Professor, Microbiology
Co-Authors: Kerry S. Williamson, Kathleen McInnerney, and Ailyn C. Pérez-Osorio
Affiliation: Montana State University, Bozeman, MT

Bacteria in biofilms are physiologically heterogeneous. The heterogeneity may be due to a variety of processes, including: adaptive variability, where cells respond to their local microenvironment; genetic variability, due to mutation and recombination events of biofilm subpopulations; and stochastic gene expression events. Physiological heterogeneity in biofilms influences infectious disease processes, since cell subpopulations in biofilms show differential sensitivities to antibiotics. To characterize this heterogeneity, we combined laser capture microdissection (LCM) and quantitative reverse transcription PCR (qRT-PCR) to study variability in gene expression patterns in *Pseudomonas aeruginosa* biofilm vertical transects. Initial experiments were performed to validate the approaches by correlating expression of an inducible gene for the green fluorescent protein (*gfp*) with GFP fluorescence. We then analyzed expression of a number of genes from wild-type *P. aeruginosa* biofilms. The genes included: (i) the housekeeping gene,

acpP, (ii) genes regulated by quorum sensing, *aprA*, *phzA*, (iii) a quorum sensing regulator, *rhlR*, and (iv) the stationary phase sigma factor, *rpoS*. Each of these genes had the highest mRNA abundance at the top 30 µm of *P. aeruginosa* biofilms. For each of these genes, less than one mRNA transcript per cell was found in the middle or bottom of the biofilms. In contrast, the abundance of the ribosomal RNA (16S rRNA) had relatively uniform amounts throughout the biofilms. The rRNA results and the results demonstrating that *rpoS* and *rhlR* had the highest mRNA abundances at the top of the biofilms suggested that cells near the air/biofilm interface were in a transition phase between exponential and stationary phase. Cells in the deeper regions of the biofilm were in a late stationary phase and are possibly dormant. Finally, we combined LCM with microarray analyses on cells isolated from the top and bottom of the *P. aeruginosa* biofilms. The microarray results confirmed the qRT-PCR findings regarding localized expression of *acpP*, *aprA*, *phzA*, *rhlR*, *rpoS*. The results also confirmed the relatively uniform abundance of 16S rRNA throughout the biofilm regions. In addition, the global transcriptomics results provide information on expression processes that may be unique to certain regions within the biofilms.

FISHing in biofilm: Advanced *in situ* molecular techniques and 3D image analysis

Presenter: Kristen Brileya, PhD Candidate, Microbiology
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Fluorescence *in situ* hybridization (FISH) is a powerful tool for studying microorganisms in their native position in a sample. This approach closes the gap in the rRNA cycle whereby probes can be designed based on knowledge of sequences alone, and pure cultures or isolates are not needed. Using multiple probes allows for detection of up to seven different organisms per sample, which can be useful for examining mixed cultures and multi-species biofilms. FISH can be done on intact biofilm (3D FISH) or on thin sections using various methods for embedding whole biofilm samples. Current work using FISH focuses on determining the function of individual community members using a suite of methods. rRNA, DNA, and mRNA synthesis can be assayed using probes targeting 16S-23S intergenic spacer regions, immunocytochemical detection of the thymidine analog BrdU with FISH, and immunocytochemical detection of digoxygenin labeled probes with Catalyzed Reporter Deposition (CARD) FISH respectively. Substrate uptake and utilization can be determined and potentially quantified for individuals in a mixed population, including thin sections of biofilm, using FISH combined with microautoradiography (MAR). The newest techniques for determining substrate utilization allow single cells in a sample, located with FISH, to be analyzed by Raman microscopy or nanometer secondary ion mass spectrometry (nano-SIMS). Since all of the mentioned FISH methods require a visual inspection by microscopy to determine the results, analysis of images collected is an essential component of each experiment. *daime* is a free program designed specifically to handle images collected from FISH and biofilm FISH samples. *daime* can be used to determine abundance of specific cell types in a biofilm sample where direct counts are not feasible. It can also be used to perform statistical analyses of spatial localization, such as whether certain cell types coaggregate, are repulsed by each other, or are distributed randomly. Finally *daime* can be used to reconstruct 3D images and videos of high quality using hardware technology from modern video game display hardware. These advanced molecular techniques, in concert with powerful image analysis software, offer exciting opportunities for biofilm research across its disciplines from health sciences to the environment.

Healthcare-related pathogen disinfection and survival in a four-species potable water biofilm

Presenter: Margaret Williams, Research Microbiologist
Affiliation: Centers for Disease Control and Prevention, Atlanta, GA

Background: Biofilms in potable water distribution systems (PWDS) frequently contain opportunistic pathogens such as *Burkholderia cepacia, Pseudomonas aeruginosa*, and nontuberculous mycobacteria (NTM), which can cause infections in susceptible individuals, especially in healthcare settings. Several cases

of healthcare-associated infections have been traced to potable water from healthcare premise plumbing.[1,2] Although exposure to potable water for healthy individuals rarely results in infection, people with compromised immune systems, open wounds, or recent surgery are at risk of infection. To determine risk of exposure to opportunistic pathogens, and to develop improved infection prevention methods, laboratory studies are required to characterize pathogen interactions with biofilms and the response of pathogens to disinfection. The purpose of this study was to develop a reproducible model system that could be used to investigate the interaction and disinfection of pathogenic bacteria in PWDS biofilms.

Methods: The native drinking water bacteria comprising the biofilm were *Bradyrhizobium japonicum, Delftia acidovorans, Mycobacterium mucogenicum,* and *Sphingomonas paucimobilis*. Comparisons of 72 hour dual- and single-species biofilms were made in 96-well plates containing autoclaved tap water. Model WDS biofilm was developed with a mix of all four species in a CDC Biofilm Reactor (CBR) on polyvinylchloride (PVC) coupons in a 16-day period. Biofilm bacteria were quantified after removal from the PVC surface by dilution and plating on R2A medium, which was incubated for 14 days at 22˚C. Species were differentiated by colony morphology and time of colony appearance.

Results: Paired-growth assays indicated that *B. japonicum* and *M. mucogenicum* produced more biofilm when incubated in the presence of *D. acidovorans* or *S. paucimobilis* than when incubated alone. No synergistic or inhibitory effects occurred with other species combinations. All four species grew together in the CBR and remained in the biofilm during the entire 16 days (n=4). Mean total biofilm counts for each sampling day for replicate experiments ranged from 5.35–6.57 Log_{10} CFU/cm^2. Results indicate that the model system provides reproducible biofilm counts on PVC surfaces (log standard deviations of the mean ranged from 0.071–0.245).

Conclusions: A reproducible PWMS biofilm model was developed that can be used to investigate the interaction of pathogenic bacteria with biofilms and determine the efficacy of potable water disinfectants.

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Biofilm efficacy test: Validation of the single tube protocol

Presenter: Kelli Buckingham-Meyer, Research Assistant
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The Standardized Biofilm Methods Laboratory is collaborating with the EPA to develop a standard method for testing the efficacy of liquid disinfectants against biofilm bacteria. Research has shown that biofilm bacteria are more resistant to disinfection than suspended or dried bacteria. In efficacy testing, the goal is to use the most relevant test system possible to estimate antimicrobial performance under actual use conditions. Therefore, if a liquid antimicrobial is to be used to kill biofilm bacteria, it is important to use biofilm bacteria as the test subject.

The EPA has developed a draft method for Biofilm Disinfection Efficacy Evaluations known as the "single tube" method. The method tests a repeatable *Pseudomonas aeruginosa* biofilm grown in the CDC biofilm reactor (ASTM Method E2562). A rinsed coupon is placed in a 50 mL conical tube; then treatment is added to the tube followed by neutralizer. The tube is then sonicated and vortexed to remove the biofilm from the coupon and to disaggregate the clumps. The bacterial suspension is serially diluted, spread plated, and enumerated.

The presentation will discuss the advantages and disadvantages of the single tube method, as well as results of an initial two-laboratory collaborative study that suggests the single tube method shows promise. The reproducibility standard deviation of the log reduction (LR) was acceptable for two of the three disinfectants in the study. The untreated data were reproducible across the two labs studied, and repeatable across the multiple experiments within each lab.

Statistically assessing limits of detection and performance standards

Presenter: Al Parker, Biostatistician and Research Engineer
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Substitution rules necessitated by limits of detection yield biased estimates of the mean and variance of a response. For example, for viable plate counts of bacteria, a common substitution rule is to use a 1 at the highest dilution where no colony forming units are observed. Some EPA guidance documents recommend substituting 1/2. Either of these substitution rules yields mean viable plate counts that are not, on average, equal to the true number of organisms being studied. Furthermore, the true variability of the counts is underestimated. This talk will focus on how statistical modeling techniques can be used to help deal with these issues. The effects on performance standards, such as estimation of a log reduction or repeatability standard deviation with confidence, will also be considered.

Development of a biofilm coupon holder and sampling test kit

Presenter: Kevin Cook, Assistant Professor, Mechanical and Industrial Engineering
Affiliation: Center for Biofilm Engineering and Montana State University, Bozeman, MT

This presentation discusses the results of a two-year collaborative project that included researchers from Standardized Biofilm Methods Laboratory, students and a professor from the MSU Mechanical and Industrial Engineering Department, and the Bozeman-based biofilm reactor supply company BioSurface Technologies. These three entities combined their expertise in biofilm testing and methods development, product development and fabrication, and product marketing to design and fabricate laboratory tools that enable researchers to more efficiently conduct biofilm efficacy testing. The goal of this project, which was sponsored by the Montana Board of Research and Commercialization Technology, was to design, manufacture, and test a comprehensive biofilm efficacy test system resulting in products that BioSurface Technologies could potentially market and sell as a complement to their existing product line. Mechanical engineering (ME) and mechanical engineering technology (MET) students collaborated with researchers in three phases to accomplish this goal. Phase I was implemented in the first year as an ME and MET senior capstone project. Phase II consisted of two MET summer interns working jointly as lab technicians and engineers. Phase III was implemented in the second year as an MET senior capstone project. Each phase resulted in lessons learned and recommendations for improvement. Ultimately, success was accomplished only when the engineering students were trained in lab research techniques and were able to learn the "vocabulary" required to communicate with the research scientists. With this new skill, engineers were able to more effectively discuss product needs and requirements with researchers and transform those requirements into product specifications more efficiently. The final products developed met the needs of the researchers, as well as the marketing desires of the biofilm reactor supply company, and researchers acquired a better understanding of the skills and abilities engineers trained in the mechanical design process can bring to the research experience. In addition, the learning experience gained by these undergraduate engineering students was greatly enhanced through these "real-world" experiences. Finally, this project resulted in formalization of the lessons learned into an interdisciplinary internship process that can be implemented into any research lab.

The goal of this presentation is to convey that value can be added to research activities through design and development of laboratory tools and equipment by mechanical engineering and mechanical engineering

technology undergraduate students. Specifically, project development history, goals of the project, and improvement activities implemented as a result of the project will be presented, along with the format developed to encourage this type of collaborative internship or undergraduate research activity in the future.

SESSION 5: Wound Biofilms

Recent advances in the characterization of human chronic wound biofilm

Presenter: Anne Han, Wound Fellow, Department of Dermatology
Affiliation: Johns Hopkins Medical Institutions, Baltimore, MD

Chronic wounds contain persistent bacterial populations that may function as benign colonizers or pathogenic factors that impede healing. Until recently, the complexity of wound microflora was underappreciated because standard culture techniques did not identify the full inventory of bacteria or their spatial and metabolic relationships with each other. Our metagenomic data demonstrated a great diversity of organisms in the wound flora, especially previously unrecognized anaerobes. Fluorescent in-situ hybridization localized different bacteria, and epifluorescence microscopy visualized the relationship between various species in highly organized biofilms. Quorum sensing is a density-dependent interspecies signaling system that has been linked to the formation of biofilm and regulation of virulence factors. We found high levels of quorum sensing molecules in chronic wounds. These data provide new information on the identity, organization, and behavior of microorganisms within the chronic wound, and pave the way for targeted therapeutic interventions to promote wound healing.

Chronic wound healing in biofilm-challenged diabetic mice

Presenter: Ge Alice Zhao, Senior Fellow, Division of Dermatology, Department of Medicine
Affiliation: University of Washington, Seattle, WA

Chronic wounds are a major clinical problem in modern health care system. They lead to considerable medical costs, lost productivity, morbidity and mortality. A major difficulty in studying chronic wounds is the lack of a satisfactory animal model in which interventions can be systematically evaluated. Our previous work demonstrated that challenging wounds created in diabetic (db/db) mice with *Pseudomonas aeruginosa* (PAO-1) biofilm significantly delayed wound healing at 4 weeks post-wounding. The scabs from the biofilm-challenged wounds contained aggregates of bacteria, forming organized structures embedded in extracellular matrix. The goal of this study is to improve consistency of biofilm-challenged wounds and to determine time-to-closure of biofilm-challenged mouse wounds. PAO-1 bacteria, cultured on polycarbonate membrane filters for 72h to form biofilms, were transferred onto 2-day wounds (6 mm) created on the dorsal surface of db/db mice (biofilm-challenged). Both biofilm-challenged and control wounds without biofilm challenge were covered with an occlusive dressing for 12 days. Wounds were harvested at 4, 6, and 8 weeks post-wounding. Control mice lost 5% weight at 4 weeks and biofilm-challenged db/db mice lost 10% weight from 4 weeks to 8 weeks. At 4 weeks, 5 of 6 (83%) control non-challenged wounds healed; 0 of 5 (0%) biofilm-challenged wounds healed; by 6 weeks, 4 of 5 (80%) of the biofilm-challenged wounds healed; and by 8 weeks, 4 of 4 (100%) of the biofilm-challenged wounds healed. Colony forming units (CFU) on biofilm-challenged wounds only contained 10^3–10^4 *P. aeruginosa* after 4 weeks. In contrast, scabs from the same wounds had a bacterial load of 10^7 CFU. Our results indicate that typical 6 mm *P. aeruginosa* challenged wounds healed by 6 weeks post-wounding. Quantification of bacteria in the wounds and scabs confirmed our previous finding that a majority of bacteria localized within the scabs instead of the wounds. This db/db mouse biofilm wound model will be a useful test bed

for exploring new strategies to control biofilm *in vivo* as well as studying the effects of bacterial biofilm on host wound healing molecular pathways.

Defining the impact of microbes in chronic wounds: A biofilm reactor

Presenter: Hamed Motlagh
Co-Authors: John G. Thomas, Steven Percival
Affiliation: West Virginia University, Health Sciences Center, Biofilm Research Laboratory for Translational Studies, Morgantown, WV and Advanced Medical Solutions (AMS), Cheshire, England

Introduction:
We previously reported on the design, development and evaluation of a Triphasic Wound Model (TWM) to mimic the biphasic environment of a chronic wound. Our 3-tiered design strategy was to measure the impact of A) simple or complex biofilms (prokaryote) on a B) eukaryotic monolayer, utilizing a poloxamer interface to stimulate biofilm development, C) separated by a pore size specific support.

Most recently, we reported on the success of an oral "replacement therapy" *Lactobacillus reuteri* (Lr), to limit the biofilm consequences in our model. Here, we wanted to expand the TWM, utilizing a different device recognizing the platform could be multi-phasic.

Materials and Methods:
Mono-, co-culture, and complex biofilms were made using one through four species (*Pseudomonas aeruginosa* (Pa), *Staphylococcus aureus* (Sa), *Candida albicans* (Ca), and *E. coli* (Ec)) suspended in 30% poloxamer F127 (BASF, Germany). Mouse fibroblast cells (NIH 3T3) were maintained using TC media with 10% fetal bovine serum with no antibiotics on a 6-well plate. The biofilms were separated by the insert with 0.3 or 4.0 mm pore size to separate diffusible products between the biofilm and fibroblasts (Corning plates and Transferable Permeable Support, 24mm Inserts). Four strains of *Lactobacillus* were obtained from AMS, England: 1) *L. plantarum* (Lp), 2) *L. rhamnosus* (Lr), 3) *L. salivarius* (Ls), and 4) *L. casei* (Lc). The assembled TWM was incubated at 37°C for 24 and 48 hours in 5% CO_2 at pH 7.0. Eukaryotic monolayers were trypsinized, stained with Trypan blue and counted, a measure of cell death versus an un-inoculated control. Prokaryotic biofilms were placed at 4°C, and stained with Live/Dead stain (Molecular Probes, Syto 9 and PI) for Flow Cytometry using BD FACs Scan to assess consequences of Lr strains on community viability.

Results:
The TWM PLUS optimized individual growth of the eukaryotic and prokaryotic cells. The impact upon fibroblasts (NIH 3T3) and positive Trypan blue dead cells increased with the number of biofilm prokaryotes (1–4) ranging from $2x10^4$ to $9x10^4$ after 24 hours. Interestingly, the most impact was observed with monospecies of Sa and least with Pa. A 24 to 72 hour biofilm of *Lactobacillus* species (1–4) decreased the toxicity for each of the pathogenic combinations (1–4), species specific: 1) Lr, 2) Lp, 3) Ls, and 4) Lc. Flow cytometry (FC) indicated for each species a complex interaction paralleling Tryptan Blue results.

Conclusion:
"Restorative Microbiology" remains a viable, non-antibiotic option for reestablishing a beneficial barrier in chronic wound management, recognizing the anti-Koch nature of biofilms. The TWM PLUS is a flexible platform that will allow for analysis of biofilm diffusible products, the impact of organism community, and neutralization by unique prokaryotic/eukaryotic interface placement, including silver dressings.

Wound biofilm research at the CBE

Presenter: Garth James, Medical Projects Manager
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

The CBE has conducted a number of *in vitro* studies related to biofilms in chronic non-healing wounds. This investigation has incorporated a variety of laboratory model systems including the drip-flow reactor (DFR), the colony/DFR (C/DFR), as well as co-culture of biofilms and human cells (keratinocytes and fibroblasts). These studies have also involved various species of bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Clostridium perfringens*. The DFR was used to evaluate the relationship between biofilm age and antibiotic tolerance using both *S. aureus* and *P. aeruginosa*. Under identical conditions *P. aeruginosa* biofilms achieved tolerance to gentamicin after 24 hours of growth, whereas it took 96 hours for *S. aureus* biofilms to become tolerant. Partial removal of these biofilms did not reduce antimicrobial tolerance. We also combined the colony biofilm model with the DFR to form the C/DFR in an effort to simulate a wound environment. We have used this model to evaluate topical wound treatments and dressings for efficacy against *S. aureus* and *P. aeruginosa* biofilms, as well as biofilms containing both of these species. We have also formed biofilms in the C/DFR that contain a strictly anaerobic species, *Clostridium perfringens*, as well as *S. aureus* and *P. aeruginosa*. These biofilms have a distinctly layered and highly channelized structure with particular species occupying each layer. Although the presence of biofilms in chronic wounds has been reasonably well established, the effects of these biofilms on wound healing are poorly understood. We have used biofilm co-culture with human keratinocytes (HK) and fibroblasts (HF) as well as medium conditioned by the growth of planktonic (PCM) and biofilm (BCM) bacteria to investigate the effects of secreted factors on human cells. Treatment with BCM resulted in significantly more reduction in the viability of HK than PCM. Furthermore, BCM treatment and not PCM treatment induced apoptosis in HK. Microarray analysis revealed significant differences in the expression of a number of chemokine and cytokine genes. These results were confirmed at the protein level using enzyme-linked immunoabsorbant assays. We then used specific inhibitors to investigate cell signaling pathways involved in chemokine and cytokine production. Preliminary results indicate the effects of BCM on HK are mediated via different pathways than PCM. These results suggest that biofilms have specific effects on wound healing cells that are not elicited by planktonic bacteria.

SESSION 6: Systems Analysis of Biofilms

Molecular level *in silico* analysis of mass and energy flows in microbial communities

Presenter: Ross Carlson, Assistant Professor, Chemical and Biological Engineering
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT

Complex microbial communities drive the earth's biogeochemical cycles. In spite of their importance, the biochemical interactions within these communities are not yet well understood, nor are many *in silico* methodologies available for studying them. Three *in silico* methodologies based on stoichiometric network analysis were developed for studying mass and energy flows in microbial communities on the molecular level. Each approach has distinct advantages and disadvantages suitable for analyzing systems with different degrees of complexity and different levels *a priori* knowledge. These methodologies were tested and compared using the extensive data from the phototrophic, thermophilic mat communities at Octopus and Mushroom Springs in Yellowstone National Park. The models included three community guild members: cyanobacteria, filamentous anoxygenic phototrophs, and sulfate reducing bacteria. The *in silico* models were used to explore fundamental microbial ecology questions including the prediction and explanation for measured relative abundances of the oxygenic phototrophic primary producer cyanobacteria and the filamentous anoxygenic phototrophic bacteria. The three approaches represent a flexible toolbox which can be rapidly adapted to study other microbial systems with a variety of electron

donors and acceptors on scales ranging from individual cells in a pure culture to entire ecosystems represented by metagenomic data.

Characterization, classification, and analysis of undefined biofilm communities using molecular techniques

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

Biofouling costs US industry billions of dollars each year. The microbial biofilm communities that result are complex mixtures of interactive organisms that often resist treatment and control. While a significant number of studies have investigated this topic in the past, few have used modern molecular biology techniques that can reveal molecular-level interactions. These techniques, developed for complex natural environmental systems, can provide valuable insights into industrial microbial ecology and our ability to monitor and control biofilms in those systems.

System complexity and heterogeneity in industrial systems is similar to natural systems. This presentation describes our analysis of natural systems and highlights potential industrial applications of those same techniques. These applications include determination and quantification of microbial contamination without culturing, effects of changing operating conditions on resulting biofilm communities, targeted applications of biocides and organism specific testing of biocide efficacy. These techniques can help lead to improved organism-specific treatment and control strategies. An improved understanding of the detailed microbial community and its organism-specific response to treatments will allow for the development of better management strategies and treatment trains involving biocides and chemical/physical treatment.

Microbial community dynamics associated with different environments and processes

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

In general, it is assumed that bacterial diversity will depend upon the degree of perturbations and will change in structure and composition in response to geochemical changes (e.g., nutrients, pH, E_h, etc.). Our recent results with subsurface bacterial communities have indicated that populations exhibited distributions at the landscape scale in agreement with predictable geochemical factors, and that population distributions depended on the particular conditions of the local environment. In addition, bacterial communities displayed idiosyncratic responses during transitions with respect to time. With respect to microbial ecology, an idiosyncratic theory assumes that the relationship between species richness and ecosystem function does not follow a consistent pattern. The notion of niche development through the application of selective pressures provides a possible framework for use of molecular ecology to improve process performance in a variety of environments. Questions remain about how diversity and stability are related in terms of biochemical function at the population and community level, whether all engineered systems would behave similarly, and whether the niche exclusion and selection concepts can be applied to improve models with respect to biotic variables. Several examples will be discussed from different systems that relate bacterial communities, geochemical parameters, and spatial distribution.

Poster Abstracts

Industry posters

Date: 07/2010
Title: **Biofouling-resistant polymeric materials incorporating covalently bonded anti-biofilm small molecules**
Authors: ***Eva Garland***[1], Lingling Peng[2], Joseph DeSousa[2], Bruce Novak[2], Christian Melander[1,2]
Affiliation: Agile Sciences, Inc., 840 Main Campus Dr., Raleigh, NC 27606
North Carolina State University, Dept. of Chemistry, Raleigh, NC 27695

Agile Sciences' library of small molecules is based on a marine natural product with known anti-fouling properties. These molecules inhibit and disperse biofilms of gram-positive and gram-negative bacteria as well as fungi, and they have been shown to be non-toxic in *in vitro* and *in vivo* model systems. When Agile's molecules are covalently bonded to a methacrylate polymer, the polymer is resistant to biofilm formation from pathogenic bacteria as well as from heterogeneous bacteria sources present in an industrial water sample. Applications of Agile's anti-fouling polymeric material include filtration membranes, medical devices, and surface coatings.

Date: 07/2010
Title: **Broad spectrum antimicrobial activity of dual anti-biofilm/anti-thrombotic betaine surfaces for medical device application**
Authors: ***Clinton Dawson***, Victoria E. Wagner, Raisa Fabre, Paul Stoodley, Christopher Loose
Affiliation: Semprus BioSciences, One Kendall Square, Building 1400, 1st Floor, Cambridge, MA 02139

Patients with blood-contacting medical devices may suffer from complications such as thrombosis and infection, resulting in significant morbidity and mortality. Current prevention strategies include incorporation of leaching antimicrobials and immobilized agents (e.g., heparin). These approaches typically attempt to avert either thrombosis or infection independently and usually lack longevity. In addition, the use of antimicrobials may promote resistance. Our approach has been to develop a highly non-fouling surface modification with dual functionality that simultaneously inhibits thrombus formation and microbial biofilm development. We have previously demonstrated that a betaine polymer surface reduces thrombus formation in a blood-flow loop model. The current study investigates the antibiofilm activity of this surface modification to two common medical device pathogens: *Staphylococcus epidermidis* and *Escherichia coli*.

A modified CDC system (mCDC) was used to investigate antibiofilm activity of polyurethane catheter substrates modified with proprietary, non-fouling betaine polymers. Samples were preconditioned in 50% FBS for one day prior to the bacterial challenge to better mimic a clinical environment. The bioreactor system consisted of a 100 ml working volume glass reactor containing a stainless steel sample holder, stir bar, and inlet and outlet ports for media exchange. Initially, test articles were exposed to a bacterial suspension of 10^6 CFU/ml in 1X PBS in batch mode for 2 hr to allow attachment of planktonic bacteria. After a 2 hr incubation period, test articles were transferred to a new reactor and flow of sterile media was started (*S. epidermidis*: 1:10 (TSB w/ .25% Glc); *E. coli*: M63, supplemented with 1mM $MgSO_4$, 0.2% glucose, and 0.5% casamino acids). Media and flow conditions were optimized to minimize planktonic growth while providing nutrients to promote surface growth of attached bacteria. Following an additional 24 hr period, test articles were assessed for macroscopic biofilm using a qualitative scoring system based on visually observed surface coverage of the biofilm (e.g., 1=0–20%; 2=21–40%; 3=41–60%; 4=61–80%; 81–100%). Test articles were then sonicated to remove adherent bacteria and dilution plated. Colony forming units per milliliter (CFU/ml) were enumerated after 24 hr incubation at 37°C.

To prepare test articles, carbothane with barium sulfate (Carb+$BaSO_4$) 10 French rods were modified with a betaine polymer to create a highly non-fouling surface. Betaine presence was confirmed by ATR-FTIR and SEM. Samples were sterilized prior to testing by a combination of ethanol (EtOH 70%) and UV light. Control (Carb+$BaSO_4$) and active (betaine-modified) samples were then tested in the mCDC reactor system as described above. Viable adherent bacterial concentrations (CFU/ml) were determined for both control and active samples. Log reductions (LR) were calculated as the difference of the averages of adherent bacteria on control surfaces minus active surfaces. LR values were also converted to percent reductions. Statistically significant differences were determined using Student's T-test. Replicate experiments (n=10 for *S. epidermidis* and n=11 for *E. coli*) were performed.

Center for Biofilm Engineering posters

CBE Poster #487

Date: 07/2009

Title: **Application of culturing and molecular techniques to elucidate the influence of cellulosic waste on microbial community structure at an INL simulated waste site**

Authors: ***Erin K Field***[1], S D'Imperio[1], M VanEngelen[1], BM Peyton[1], R Gerlach[1], BD Lee[2], A Miller[2], WA Apel[2]

Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT; [2]Idaho National Laboratory, Idaho Falls, ID

Sponsored by: Department of Energy, Environmental Remediation Sciences Program

Low-level radioactive waste sites frequently contain cellulosic waste in the form of paper towels, cardboard boxes, or wood contaminated with heavy metals and radionuclides such as chromium and uranium. To understand how the soil microbial community is influenced by the presence of cellulosic waste products, multiple soil samples were obtained from a non-radioactive model low-level waste test pit at the Idaho National Laboratory. Samples were analyzed using 16S rDNA clone libraries and 16S rRNA gene microarray (PhyloChip) analyses. Both the clone library and PhyloChip results revealed changes in the bacterial community structure with depth. In all samples the PhyloChip detected significantly more unique Operational Taxonomic Units (OTUs), and therefore more relative diversity, than the clone libraries. Calculated diversity indices suggest that diversity is lowest in the Fill and Fill Waste layers and greater in the Wood Waste and Waste Clay layers. Principal coordinates analysis and lineage specific analysis determined that Bacteroidetes and Actinobacteria phyla account for most of the significant differences observed between the layers. Overall, these results suggest that the presence of the cellulosic material significantly influences the bacterial community structure in a stratified soil system. This study demonstrates the value of using PhyloChip and clone library analyses to complement each other to gain more information about the microbial community. Current and future studies include flow-through column studies in which the influence of metal mobility on the microbial community as metal contaminated cellulosic waste is broken down will be assessed through the use of PhyloChip and GeoChip (a functional gene microarray) analyses.

CBE Poster #493

Date: 04/2009
Title: **Molecular level *in silico* analysis of mass and energy flows in microbial communities**
Authors: ***Ross P Carlson***[1,2], R Taffs[1], JE Aston[1], K Brileya[1], Z Jay[2], CG Klatt[2], S McGlynn[2], N Mallette[1], S Montross[2], R Gerlach[1], WP Inskeep[2], DM Ward[2]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT; [2]Thermal Biology Institute, Montana State University, Bozeman, MT
Sponsored by: National Science Foundation

Three methods were developed for the application of stoichiometry-based network analysis approaches to the study of mass and energy flows in microbial communities. Each has distinct advantages and disadvantages suitable for analyzing systems with different degrees of complexity and *a priori* knowledge. These approaches were tested and compared using data from the thermophilic, phototrophic mat communities from Octopus and Mushroom Springs in Yellowstone National Park (USA). The models were based on three distinct microbial guilds: oxygenic phototrophs, filamentous anoxygenicphototrophs (FAP), and sulfate-reducing bacteria (SRB). Two phases, day and night, were modeled to account for differences in the mass and energy sources and the routes available for their exchange.

The *in silico* models were used to explore fundamental questions in ecology including the prediction of, and explanation for, measured relative abundances of primary producers in the mat, theoretical tradeoffs between overall productivity, and the generation of toxic by-products, and the relative robustness of various guild interactions.

The three modeling approaches represent a flexible toolbox for creating cellular metabolic networks to study microbial communities on scales ranging from cells to ecosystems.

CBE Poster #503

Date: 11/2009
Title: **Visualizing the biofilm matrix using new and classic fluorescent stains**
Authors: ***Betsey Pitts***[1], D Gray[2]
Affiliation: [1]Center for Biofilm Engineering, Montana State University, Bozeman, MT; [2]Life Technologies, Molecular Probes, Eugene, OR

Biofilms are complex, adherent micro-ecosystems, potentially containing many types of organisms, architectural features, and structural building blocks. Direct visualization of biofilms via microscopy has demonstrated the presence of two dominant constituents of interest: cell bodies and extracellular polymeric substances (EPS), of which the EPS has been more challenging to visualize by fluorescence microscopy. Researchers have successfully used several fluorescent stains for cell bodies such as LIVE/DEAD BacLight Bacteria, acridine orange, and DAPI, but stains labeling the EPS have generally been limited to calcofluor white and specific fluorescently labeled plant lectins such as wheat germ agglutinin and concanavalin A. To identify new stains for labeling the EPS, we screened a variety of fluorescent stains on *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Escherichia coli* biofilms and observed the labeling patterns. The biofilms were grown on glass coupons in a CDC reactor and labeled with stains diluted in water. Each sample was imaged on either an epifluorescence or confocal microscope with water immersion objectives. We found a number of fluorescent stains, originally developed for other applications, capable of illuminating aspects of biofilm extracellular matrices in these organisms. These stains vary in chemical structure, excitation and emission spectra, and include reagents such as SYPRO® Ruby, BODIPY® 630/655-X, SE, Texas Red® C2-dichlorotriazine, m-dansylaminophenylboronic acid, and CellMask™ Orange plasma membrane stain. The variety of stains that labeled the EPS may allow for simple reagent

multiplexing, and combinations are being further optimized to allow robust multifactoral analysis of biofilms within a single sample.

CBE Poster #504

Date: 08/2009
Title: **Analysis of methane producing communities within underground coal beds**
Authors: ***Elliot Barnhart***[1], J Wheaton, A Cunningham, M Fields
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: US Department of Energy

We have conducted initial phylogenetic diversity studies using inoculated coal from methane producing wells in the Powder River Basin (PRB) of southeastern Montana and Wyoming. Methane generating enrichments were grown with coal as the only energy source and compared to enrichments with acetate. Preliminary data revealed an extremely diverse bacterial community established in coal cultures compared to enrichments without coal. DNA sequences indicative of methanogens (methane-producing archaea) were detected in both enrichments. These findings offer a compelling motive for further investigations of the biogeochemical processes controlling coal bed methane (CBM) production. The research is aimed at enhancing the fundamental understanding of the ecology and physiology of methane producing communities with the intent of identifying strategies for enhancement of *in situ* CBM production.

CBE Poster #506

Date: 11/2009
Title: **Bacterially induced calcite precipitation and strontium co-precipitation under flow conditions in a porous media system**
Authors: ***Robin Gerlach,*** AC Mitchell, L Schultz, AB Cunningham
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: US Department of Energy and the National Science Foundation

The process of *in situ* carbonate mineral formation has implications in many environmental applications including, but not limited to, aquifer decontamination, enhancement of soil stability, and carbon capture and storage (CCS). The high stability of carbonates and the potential for co-precipitation of contaminants within carbonates are attractive attributes for several potential engineering applications.

Ureolytic precipitation of calcium and strontium carbonates by *Sporosarcina pasteurii* was examined in two-dimensional flat plate porous media reactors. Complete reactor plugging due to biofilm formation and calcium carbonate precipitation was achieved in Sr-free systems after 14 hours and in Sr-inclusive systems after 15 hours. Comparison of the reactor influent and effluent after 11 hours indicated that Ca^{2+} concentrations in the Sr-free reactor effluent were reduced to approximately 0.48% of the influent concentration, while the Ca^{2+} and Sr^{2+} concentrations of the Sr-inclusive effluent were reduced to 0.64% and 2.34% of the influent concentration, indicating a slight inhibitory effect of strontium on calcium carbonate precipitation. Despite this slight inhibition, more than 98% of the Ca^{2+} entering the reactors was precipitated. Calcite was identified as the main mineral formed and larger mean crystal size and density were observed near the reactor influent. Homogenous partition coefficients calculated from extracted precipitates suggest higher Sr^{2+} partitioning near the inlet region, where higher precipitation kinetics exist. Results confirm the possibility of effective calcite-based co-precipitation of Sr^{2+} under flow conditions and contribute toward the development of field-scale calcium carbonate mineral-based immobilization strategies.

CBE Poster #508

Date: 10/2009
Title: **Media pH, nitrate utilization, and accumulation of TAG: Two members of the Chlorophyta**
Authors: ***Rob Gardner***, P Peters, K Cooksey, B Peyton
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: US DoD-AFOSR; US DOE-Office of Biomass Production; NSF-IGERT

One important subtask during the former DOE Aquatic Species Program—researching algae capable of biofuel production—was finding a so-called "lipid trigger." While no individual molecule has been identified, circumstances that influence accumulation of triacylglyceride (TAG) have been investigated. Our group and others have shown that nitrogen limitation and—for a single species of *Chlorella*—a rise in culture medium pH, triggers TAG accumulation.

Due to the potential commercial use of pH and nitrogen levels to influence lipid production, the interplay between these two "environmental factors" was further investigated on *Chlorophyceae scenedesmus* sp. and *Coelastrella* sp. Growth was monitored optically and TAG accumulation was routinely monitored by Nile Red fluorescence and confirmed by Gas Chromatography. Both organisms grew in all medium treatments and TAG accumulation was observed by two distinct control points, medium pH and nitrogen limitation.

CBE Poster #509

Date: 11/2009
Title: **Counterintuitive effects of quorum sensing on biofilm antibiotic tolerance**
Authors: ***Trevor Zuroff***, J Lloyd-Randolfi, H Bernstein, L Jimenez Taracido, R Carlson
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: NIH-National Center for Research Resources (NCRR)

Biofilms plague both medical and industrial surfaces and have inspired intensive antifouling efforts using technologies like antibiotic impregnated coatings and quorum sensing inhibitors. This study systematically examines the effect of AI-2 quorum sensing, nutritional environment, temperature, and culture growth stage on *Escherichia coli* biofilm antibiotic tolerance. These fundamental parameters were found to strongly influence biofilm antibiotic tolerance, causing up to ten-million fold differences in viable cell counts over a twenty-four hour treatment period. For instance, interrupting AI-2 quorum sensing via gene knock outs in either ΔluxS, ΔlsrR, or ΔlsrF at 37°C on LB medium supplemented with glucose resulted in approximately 10 million more viable cells than wild-type or ΔlsrK gene knock out cultures when treated with the antibiotic ampicillin. However, this effect was negated when the cultures were grown on LB medium in the absence of glucose or grown at room temperature (21°C). The findings were tested at different culturing phases and also compared with planktonic culture behavior. Both had significant effects on cell viability after antibiotic treatment. This study highlights the dynamic nature of biofilm antibiotic tolerance. The results are critical for rationally designing and testing antibiofilm strategies.

Acknowledgments: Grant Number P20 RR16455-08 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH) and by NIH Grant Number EB006532

CBE Poster #514

Date: 01/2010
Title: **Analysis of morphological switch associated with viable but not culturable (VBNC) state in *Helicobacter pylori***
Authors: ***Crystal Richards***, K Williamson, AK Camper
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: US EPA under the Science to Achieve Results (STAR), Center for Native Health Partnerships

Introduction: The gastrointestinal pathogen *Helicobacter pylori* infects approximately 50% of the world-wide population; however the exact route of transmission remains poorly understood. *Helicobacter pylori* is known to become viable but not culturable in most environments outside the mammalian stomach. It has been observed that the loss in culturability coincides with a change in cell morphology from a spiral, flagellated form to a coccoid, aflagellated form. The purpose of this research is to characterize the mechanism of cell morphology switching that is associated with loss of culturability in *H. pylori*.

Methods: The VBNC state was generated in *H. pylori* by prolonged exposure to atmospheric oxygen and by nutrient deprivation. *H. pylori* cells were grown on tryptic soy blood agar for 48 hours then transferred to a tryptic soy blood agar biphasic slant that contained a pool of tryptic soy broth with 5% fetal calf serum. Biphasic slants were incubated in a microaerophilic atmosphere for 24 hours and then were allowed to age naturally or were given one of two experimental treatments. *H. pylori* cells subjected to the experimental treatments were removed from the microaerophilic atmosphere and either incubated in the slant at 37˚C with atmospheric oxygen or the cells were transferred to ultra-pure milliQ water and then incubated at 37˚C. All three treatments were sampled regularly for viable and total cell counts. Additionally RNA was extracted from healthy and stressed *H. pylori* cells at 24 hours and after 7 days, purified and assessed for quality on the agilent bioanalyzer 2100.

Results: *Helicobacter pylori* converts to a non-culturable form within 24 hours of exposure to both experimental treatments. Healthy cells that were allowed to age in a microaerophilic atmosphere for 21 days showed a decrease in culturability accompanied by a concomitant increase in coccoid cells. After exposure to atmospheric oxygen, *H. pylori* switched from a helical form to a smaller, coccoid morphology (>95% of total cells were coccoid after 21 days). Nutrient deprivation also caused a complete loss of culturability; however the morphological conversion was significantly different, with approximately 40% converting to coccoid morphology after 21 days. Analysis of RNA quality showed intact 16s and 23s rRNA extracted from healthy cells and cells early in the oxygen exposure treatment. However, nutrient deprivation appeared to cause non-random fragmentation of the rRNA as early as 4 hours after exposure.

Discussion: The conversion of *H. pylori* to a coccoid form is thought to be indicative of the switch to a VBNC state. Indeed, healthy cultures that age in a microaerophilic atmosphere seem to lose culturability in relation to an increase in coccoid cells. This morphological switch is also seen under exposure to atmospheric oxygen. However, this research has shown that *H. pylori* can become non-culturable without a majority of cells switching their morphology (seen in nutrient deprivation treatment). Nutrient deprivation also caused *H. pylori* to break down rRNAs, producing a reproducible fragmentation pattern. This fragmentation may be responsible for the inability of *H. pylori* to switch its morphology and may be indicative of an active mechanism for coping with environmental stresses.

CBE Poster #519

Date: 04/2009
Title: **Microbially enhanced solubility and mineral trapping of sequestered supercritical CO_2**
Authors: ***Alfred B. Cunningham***, L Schultz, R Gerlach, S Parks, L Spangler, AC Mitchell
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Sponsored by: US Department of Energy: EPSCor and ZERT

Two major concerns challenging the deployment of geologic CO_2 sequestration are potential migration out of the storage reservoir by preferential pathways (e.g., leaky well bores or faults), and the long term liability for stored CO_2. Engineering of biogeochemistry has the potential for addressing both these concerns by plugging apertures with reaction product, or by accelerating trapping mechanisms. Previous research has determined that the ureolytic biomineralization process, when properly engineered, can precipate copious quantities of calcium carbonate into aquifer pores and fractures, thus providing a potential technology for plugging leakage pathways available to injected CO_2 (Cunningham *et al*. 2008; Mitchell *et al*. 2008). More recent research has also discovered that ureolytic biomineralization is capable of sequestering anthropogenic CO_2 from the gas phase into the mineral phase as $CaCO_3$—thereby facilitating a method for enhanced mineral-trapping of injected CO_2 in the subsurface as well as from waste streams above ground. Of equal importance we have found that ureolytic biomineralization enhances the capacity of the brine for CO_2(g) and dissolved carbonate ions, thus increasing the potential for solubility-trapping of injected CO_2.

This paper summarizes our experimental program, in which biofilms capable of performing ureolysis are grown in porous media and stimulated to deposit calcium carbonate uniformly along the path of flow. Additionally we report results from the bacterial hydrolysis of urea (ureolysis) in microcosms containing synthetic brine with variable headspace pressures [p(CO_2)] of $13CO_2$. These experiments demonstrate a net flux of head space $13CO_2$ into the brine and precipitated mineral phases. Therefore ureolytic biomineralization may lead to technologies which simultaneously reduce CO_2 leakage from geologic formations, and can trap CO_2 in non-labile mineral and aqueous phases.

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CBE Poster #520

Date: 07/2010
Title: **Investigation of coal-derived sorbents for contaminant removal**
Authors: ***Chris Durgan***, P Sturman, D Walker
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: Montana Board of Research and Commercialization Technology

Industries of all types (petrochemical, microfabrication, mining, and more) require cheap and efficient sorbents to clean effluent streams (liquid or gas), to remediate hazardous waste sites, and for emergency chemical spill control. Activated carbon is commonly used and effective, but large quantities can be expensive. Coal is a cheaply obtained substrate and already exhibits many desirable characteristics of a sorptive material—complex micropore structure to increase surface area, nonpolar carbon sites for

sorption of nonpolar molecules such as benzene, and functionalized (polar) sites for sorption or chelation of ionic (metals) or polar contaminants. In this set of experiments, the sorptive capabilities of several untested materials were compared to proven media. Simulating conditions found in common applications of groundwater remediation and drinking water treatment, a variety of metal and organic contaminants were tested.

CBE Poster #521

Date: 07/2010
Title: ***In situ* microbial reduction of selenium as source control in phosphate mine waste**
Authors: ***Lisa Bithell Kirk***[1], B Peyton[1], S Childers[2], R Gerlach[1]
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Sponsored by: Inland Northwest Research Alliance, EPA Science to Achieve Results, Montana Water Center, and Idaho Mining Association

This study of subsurface microbial ecology investigates selenate reduction by indigenous micro-organisms, using naturally available carbon in backfilled phosphate mine waste at sites in southeast Idaho, with an ultimate goal of defining how backfilled mine pits can be ecologically engineered to reduce toxic and mobile selenate to insoluble and non-toxic elemental selenium. Several *Dechloromonas*-like, indigenous facultative ß-proteobacteria rapidly reduce selenate within a consortium of cold-tolerant hydrocarbon-degrading microbes. Temperature, lithology, and oxygen availability influence extent and rate of selenate reduction. More selenate-reducing organisms live in anaerobic shale than chert or mudstone, and almost no selenate reduction occurs when oxygen is present. Microbial reduction is distinguished from abiotic processes by evidence of biotic stable isotope fractionation and comparison with killed controls. Operational waste management strategies that promote Se(VI)-reduction by indigenous organisms using native carbon offer a sustainable, design-based approach to natural attenuation of selenium in mined rock.

CBE Poster #522

Date: 08/2009
Title: **Detection of uranium oxidation and solubility using NMR**
Authors: ***Sarah J. Vogt***, JD Seymour, BD Stewart, BM Peyton, SL Codd
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: US DOE, NSF, Murdock Trust

The conversion of soluble uranyl ions (UO_2^{2+}) by bacterial reduction to insoluble uraninite (UO_2) is being studied as a way of immobilizing spent uranium waste [1-3]. Under anaerobic conditions, several known iron-reducing types of bacteria have been shown to also use the uranyl ion as an electron acceptor. Preliminary tests using a suspension of uraninite (UO_2) particles produced by *Shewanella putrefaciens* CN-32 bacteria show a dependence of the T_1 and T_2 on the oxidation state and solubility of the uranium. Gradient echo and spin echo images were compared to quantify the T_2* effect caused by the magnetic field fluctuations of the uraninite particles and soluble uranyl ions. Since the precipitate studied is suspended in liquid water, the effects of concentration and particle aggregation are also being explored. A suspension of uranium particles was injected into a polysaccharide gel, which simulates the precipitation of uraninite in the extracellular biofilm matrix. A reduction in the T2 of the gel surrounding the particles was seen [4]. Therefore it may be possible to detect the presence of uraninite precipitate within a biofilm during the bacterial reduction reaction.

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CBE Poster #523

Date: 04/2010
Title: **Magnetic resonance analysis of physically crosslinked biopolymer gels**
Authors: ***Hilary T. Fabich***[1], JE Maneval[2], D Bernin[3], JD Seymour[1], SL Codd[1]
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[3] Dept. of Chemical and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden
Sponsored by: NIH, INBRE

Using the non-invasive properties of nuclear magnetic resonance (NMR), the mobility of water in a biopolymer gel can be examined by measuring diffusion and magnetic relaxation. Understanding the molecular role of water in physical gelation and water distribution on gel material properties has great potential to increase understanding of biological function [1]. Alginate and the impact of a divalent cation on gelation has been extensively studied using NMR [2,3]. During formation of the gel under certain conditions, small capillaries are formed inside the gelled structure [4]. These may provide molecular transport pathways through the entangled biopolymer network and can control how water and ions are transported through the gel. Another point of interest in this system stems from the role of acetylated and deacetylated alginates in biofilm formation [1]; NMR data on the relaxation and diffusion of water in model alginates provide baseline data for exploring the role of water in biofilm formation.

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CBE Poster #524

Date: 07/2010
Title: **Colocalization of syntrophs in a methanogenic biofilm**
Authors: ***Kristen A. Brileya***[1], R Hatzenpichler[2], APArkin[3], TC Hazen, MW Fields[1]
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Sponsored by: NIH, INBRE

Transfer of reduced carbon and electrons between microbial community members is of interest in methanogenic systems which represent natural mediators of atmospheric carbon flux. The current work uses a dual-culture approach to examine the structure of syntrophic biofilm formed by the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough and the methanogenic archaeon *Methanococcus maripaludis*. We hypothesized that biofilm structure would reflect the energetic benefits of living in close association; the aim of the study is to visualize intact 3-dimensional biofilm structure to make testable predictions of structure-function relationships. Biofilm was grown in a continuously stirred biofilm reactor

where cells could attach to a silica surface or remain suspended. Intact biofilm was fixed for fluorescence in-situ hybridization (FISH) and embedded in agarose to maintain 3-dimensional structure. FISH revealed a framework of *D. vulgaris* with both single cells and large micro-colonies of *M. maripaludis* interspersed within the biofilm. FISH also confirmed steady-state biofilm irregularity, with ridge, valley, and spire macro-architecture. SybrGreen counterstaining confirmed the presence of extracellular material. Colorimetric assays indicated cell-associated carbohydrate was composed of .035 μg hexose/μg protein, .017 μg pentose/μg protein and .011 μg uronic acid/μg protein, similar to *D. vulgaris* mono-culture biofilm and approximately 5 times less than *M. maripaludis* biofilm. Filaments presumed to be protein have been observed in dual-culture biofilm matrix with electron and atomic force microscopy, and matrix was sensitive to proteinase K treatment during preliminary work with Catalyzed Reporter Deposition FISH. Syntrophic biofilm 3-D structure appears to be driven by *D. vulgaris* while providing an advantageous situation for *M. maripaludis* to establish presumably active micro-colonies throughout the *D. vulgaris* scaffold.

CBE Poster #525

Date: 07/2010
Title: **Impact of biofilm mechanical properties on secondary velocities**
Authors: ***Garret Dan Vo***, J Heys
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: N/A

Biofilms are complex mixtures of microorganisms and they play a critical role in engineering and medicine. The focus of this mathematical modeling work is on the physical interaction between a biofilm and a moving fluid. It was inspired by magnetic resonance microscopy work at Montana State University that measured surprisingly high secondary flow velocities induced by a biofilm. The mathematical model used here is based on the Immersed Boundary Method, which has been validated previously to show good agreement with experimental measurements. The simulation results show that the biofilm is often lifted upward into the flow by the moving fluid, mostly due to a recirculation that forms behind the biofilm. The uplift is maximized for mechanical properties found in typical biofilms. This implies that a stiffer or softer biofilm would have less uplift and, consequently, lower secondary velocities. The model suggests that biofilms are optimized to increase secondary velocities and mixing in their immediate environment.

CBE Poster #526

Date: 07/2010
Title: **Comparing the disinfection of planktonic cells, biofilms, and detached biofilm particles in single species and dual species cultures**
Authors: ***Sabrina Behnke***, AK Camper
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Sponsored by: Unilever U.K. Central Resources Limited

Abstract not available.

CBE Poster #527

Date: 07/2010
Title: **Microbial diversity in a humic-free environment on the Cotton Glacier, Antarctica**
Authors: ***Heidi Smith***[1], C Foreman[1], B Sattler[2], Y-P Chin[3], D McKnight[4]
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Sponsored by: NSF OPP-0838970

A supraglacial stream forms annually on the Cotton Glacier, Antarctica. Analysis of dissolved organic matter (DOM) from this stream in 2004-05 and again in 2009-10 showed that the concentration was low (44–48 μM C), and lacked humic signatures, unlike typical DOM of microbially based ecosystems. Our results indicate that DOM in this system is seasonally formed from soluble microbial products and that a reservoir of recalcitrant humified DOM does not pre-exist. In most aquatic ecosystems, humic DOM acts as a natural sunscreen and the absence of humics may thus represent an additional stressor influencing the microbial community. Nonetheless, the stream contained an active microbial assemblage with bacterial cell abundances from 2.94×10^4–4.97×10^5 cells ml^{-1}, and bacterial production ranging from 58.8–293.2 ng C l^{-1} d^{-1}. Chlorophyll-a concentrations ranged from 0.3 to 0.53 μg l^{-1} indicating that algal phototrophs were the probable source of the DOM. Microbial isolates produced a rainbow of pigment colors, suggesting adaptation to UV stress, and were similar to those from other cryogenic systems (Cytophagales and β-Proteobacteria lineages). Clone library analysis of the microbial assemblages from the stream water, ice, sediments and aeolian communities were significantly different, but still related to organisms from other cold temperature environments. Taken together, these results suggest that the occurrence of related phylotypes from diverse glacial environs is due to similar survival strategies and that UV stress due to the absence of humics is important in supraglacial streams. Supraglacial streams provide an example of contemporary microbial processes on the glacier surface and a natural laboratory for studying the microbial adaptation to the absence of humics, as well as chemical processes controlling the eventual genesis of humic DOM.

CBE Poster #528

Date: 06/2010
Title: **Development of an NMR- and MS-based metabolomics research core at Montana State University**
Authors: ***Laura Jennings***[1], V Copié[1], B Bothner[1], J Hilmer[1], E Dratz[1], G James[2], P Secor[2], P Stewart[2]
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Sponsored by: NIH, NCRR, COBRE

The NMR/MS Metabolomics Core at Montana State University was recently established with funds provided by an NIH NCRR Administrative Supplement to CoBRE Grant RR024237. Metabolomics seeks to analyze small-molecule metabolites or metabolite fluxes in biological systems including urine, serum, tissue extracts, and microbial cells exposed to different physiological conditions. An inherent difficulty with measurements of metabolites (in contrast to gene or protein expression) is that metabolites have diverse chemical and physical properties, so that no one analytical platform is capable of measuring them all. Our goal is to develop a metabolomics research infrastructure that integrates NMR with LC/MS and GC/MS techniques to cover the measurement of as many metabolites as is feasible. The focus of our current metabolomics research is to better understand and contribute to the modeling of the biochemical processes that are altered in diseased states (in our case, primarily infectious diseases and host immune responses). MSU's Metabolomics Core, in collaboration with researchers at the Center for Biofilm

Engineering, is currently investigating host-pathogen interactions in chronic wounds using NMR and MS metabolite profiling approaches. *Staphylococcus aureus* is an important human pathogen and a predominant organism found in chronic-wound micro-communities. Metabolite profiling of *S. aureus* excretions has revealed metabolites unique to biofilms and may provide insight into the mechanism of pathogenesis and the persistence of infection in chronic wounds.

CBE Poster #529

Date: 04/2010
Title: **Research support for a comprehensive biofilm test system**
Authors: S Anderson, S Adam, ***Kevin Cook***, ***Darla Goeres***
Affiliation: Center for Biofilm Engineering, Montana State University, Bozeman, MT
Sponsored by: Montana Board of Research and Commercialization Technology

Biofilm forms when bacteria adhere to surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can help anchor them to all kinds of material such as metals, plastics, soil particles, medical implant materials, and tissue. At the Center for Biofilm Engineering (CBE), researchers grow biofilm under shear to perform efficacy tests. Researchers in the CBE's Standardized Biofilm Methods Laboratory (SBML) conduct efficacy testing on biofilm grown in the CDC (Center for Disease Control) reactor according to ASTM standard E2562 developed by the SBML at CBE. The goal of the project is to develop tools, including a coupon manipulation device and product(s), which will comprise a biofilm efficacy testing kit. The developed products will be rugged, improve the repeatability of the experiment, reduce the amount of strain on laboratory technicians, and be easily manufactured by Biosurface Technologies (BST), of Bozeman, Montana. This tool must allow efficient manipulation of multiple coupons without biasing test results. Such a set of tools has been recognized as comprehensive biofilm efficacy test system (CBET). So far, the requirements of the project are undergoing completion and will be tested after completion.

CBE Poster #530

Date: 07/2010
Title: **A modified CDC biofilm reactor to produce mature biofilms on the surface of PEEK membranes for an *in vivo* animal model application**
Authors: ***Dustin L. Williams***[1,2], KL Woodbury[1], AE Parker[3], RD Bloebaum[1,2,4]
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Sponsored by: US Department of Veterans Affairs, National Institutes of Health

Background: Animal models of infection have typically consisted of implantation of a device in conjunction with inoculation near the device of planktonic (free-floating) bacteria [1,2]. In contrast, there are few *in vivo* studies involving the use of well-established, mature biofilms. This study provides a novel method of growing mature biofilms on the surface of polymeric membranes that can be used for *in vivo* applications.

Methods: The CDC biofilm reactor was modified to hold eight 1 cm^2 polyetheretherketone (PEEK) membranes using guillotine-like holders. A clinically relevant strain of *Staphylococcus aureus* containing the icaADBC gene was collected and confirmed to be a biofilm producer by Congo Red agar growth. Five hundred mL of brain heart infusion broth (BHI) were inoculated with ~1.5 x 10^8 cells and grown for 24 hours at 28.5°C. A 10% BHI broth solution was then flowed through the system at ~5.8mL/min and similarly incubated for an additional 24 hours. Bacteria on the membranes were imaged by scanning

electron microscopy (SEM), quantified by traditional growth methods and imaged by confocal laser scanning microscopy (CLSM) with the Filmtracter™ LIVE/DEAD® stain to confirm viability.

Results: SEM images of the biofilms indicated that they produced significant three-dimensional mushroom- or pillar-like structures and significant extracellular polymeric substance (EPS) that appeared to serve as a scaffold for bacterial growth. LIVE/DEAD® stain images indicated that the biofilms were viable. The average number of bacteria on n=20 membranes was 7.03×10^9 (SD=1.67×10^9). Statistical analysis indicated that there was no significant difference in the number of bacteria per membrane after three separate runs of the reactor ($p>0.05$).

Discussion: It is estimated that 99.9% of bacteria in nature reside in a biofilm structure. As such, wounds such as open fractures have significant potential to become contaminated with bacteria that reside in a biofilm [3-5]. This study suggests that biofilms of *S. aureus* can be grown on the surface of PEEK membranes that can be used to develop an *in vivo* animal model for orthopaedic implant-related infections.

Acknowledgments: Financial support received from the VA RR&D and the NIH (1 R01 AR057185-01).

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CBE Poster #531

Date: 03/2010

Title: **EFRI-HyBi: Fungal processes for direct bioconversion of cellulose to hydrocarbons**

Authors: ***Natasha Mallette***[1,2], B Peyton[1,2], R Carlson[1,2], G Strobel[3], K Hunt[1,2], S Strobel[4], M Smooke[4]

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Sponsored by: NSF Emerging Frontiers in Research & Innovation (EFRI)

While considerable national effort has been focused on ethanol production, very little research— beyond characterization of cellulolytic fungal enzymes—has examined the potential role of fungi in renewable fuel production. *Gliocladium roseum* (NRRL 50072) is an endophytic fungus recently isolated from Northern Patagonia by Gary Strobel (MSU). *G. roseum* produces and excretes "mycodiesel," an extensive series of straight chained and branched medium chain-length hydrocarbons including heptane, octane, undecane, dodecane, and hexadecane (Strobel et al., 2008). This organism has the potential to produce petroleum directly using a cellulose fermentation process that is essentially carbon neutral. The goal of this research is to determine kinetic parameters of optimal fungal growth and hydrocarbon production through fermentation experiments. Experimental results from shake flask and 5 L reactor runs have verified hydrocarbon compound production under many different growth conditions. Biomass yields have improved from 0.05 g/L to 4.8 g/L. The pH tolerance of *G. roseum* is in the acidic range, and optimal

temperature is between 16–23°C. These preliminary results confirm the ability of *G. roseum* to produce valuable fuel compounds. Future research will focus on product chemistry and yields, and completing the mass balance for the system.