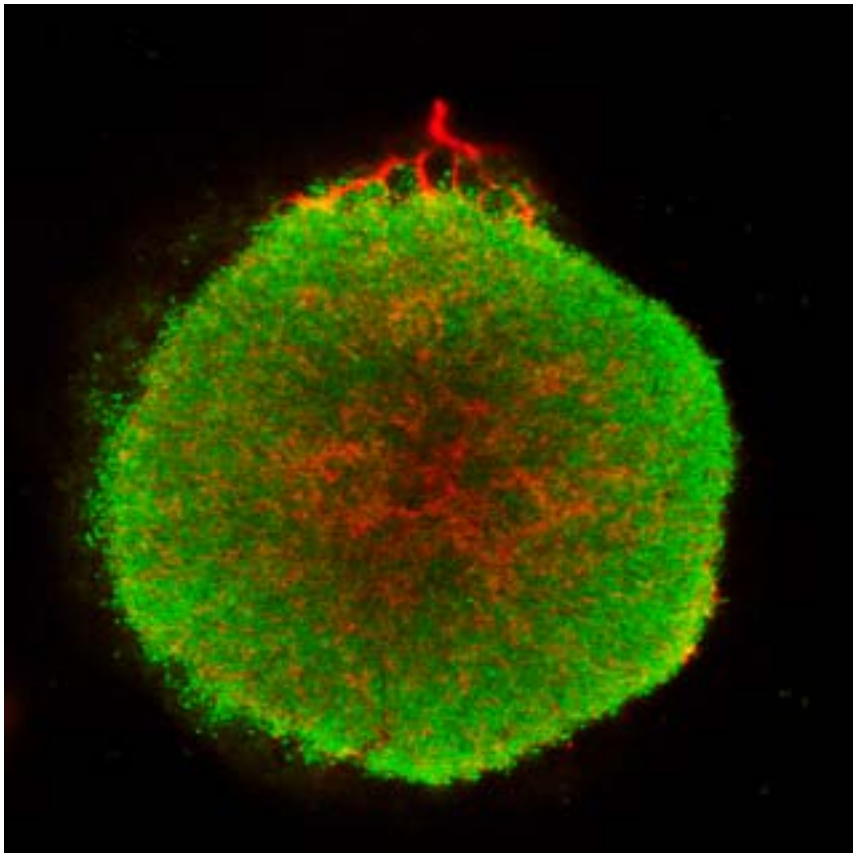
PROCEEDINGS



CBE Technical Advisory Conference

July 22–24, 2003
Montana State University–Bozeman
Bozeman, Montana

Sponsored by the
Center for Biofilm Engineering
a National Science Foundation
Engineering Research Center
at Montana State University–Bozeman

GENERAL INFORMATION

CBE LEADERSHIP

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

A BRIEF HISTORY OF THE CBE

The CBE was established in 1990 through a grant from the National Science Foundation's Engineering Research Centers program. The NSF-ERC program was created to increase U.S. industrial competitiveness and to re-invent science and engineering education in U.S. universities. Under the leadership of its founding director, Bill Characklis, the new engineering center also drew support from the state of Montana, MSU–Bozeman, and industrial partners gathered during its pre-1990 work as the Institute for Biological and Chemical Process Analysis. Since the beginning, CBE researchers have been recognized nationally and internationally for leading-edge biofilm research, and for taking an interdisciplinary approach to the study of microbial growth on surfaces.

In the spring of 2001, the CBE's 11-year period of NSF-ERC program support drew to a close. The CBE's continued success is built on the foundation of many years of productive university-industry-government collaboration in pursuit of its vision to be a world leader in fundamental research, science and engineering education, and industrially relevant technology.

MISSION AND GOALS OF THE CBE

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

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

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

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

THE INDUSTRIAL ASSOCIATES PROGRAM

In addition to governmental funding sources, the CBE is funded through its diverse group of Industrial Associate members.

Benefits of membership include:

- **Attendance at Industrial Meetings**. The semi-annual meetings are exclusive to Industrial Associate members and CBE research collaborators (non-member companies may visit once to preview the Industrial Associates program). At each meeting, exclusive workshops are provided to give Industrial Associates hands-on training on the latest biofilm analytical techniques.
- **One vote on the CBE Technical Advisory Committee** to guide CBE research and policy.
- **Two days of consultation**.
- **Long-term visits** to conduct collaborative research.
- **Research sponsored by one company or a consortium of companies**.
- **Specialized workshops**.
- **Access to students trained in interdisciplinary, team research**.
- **Early access to publications**.
- **Access to the CBE's Biofilm Systems Training Laboratory (BSTL)**.

CBE WEB SITE

More information about the Center for Biofilm Engineering is available at its website:
http://www.erc.montana.edu/

Presentation and Poster Abstracts
Technical Advisory Conference: July 22–24, 2003

Posters

SPEAKER ABSTRACTS

Keynote Speaker:
Pseudomonas aeruginosa biofilms: Identification of genes important for the biofilm phenotype

Barbara Iglewski, *Professor, Microbiology and Immunology, University of Rochester Medical Center*

Abstract not available.

SESSION 1: Keck Project

S03-S02
Session introduction

Phil Stewart, *CBE Deputy Director, Professor, Chemical Engineering*

S03-S03
Imaging biofilm structure and fluid flow with NMR microscopy

Erica Gjersing*, MS Candidate, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Nuclear Magnetic Resonance (NMR) microscopy is an excellent method for imaging living systems since it is innocuous and non-invasive. In addition to imaging, NMR microscopy techniques can be used to obtain information about transport phenomena such as fluid velocities and diffusion. The current focus of this research for the Keck Project is to gather images of biofilms and map the flow patterns through biofilm fouled capillaries. Insight about how biofilm structures affect bulk flow will contribute to better computer models of biofilm behavior. NMR microscopy techniques were used to image the biofilms and the flow around them in 1mm-square glass capillaries. NMR images through cross sections of the capillary in the longitudinal direction have shown "hollowed out" *Staphylococcus epidermidis* biofilm clusters. This phenomenon has been observed using many different techniques associated with the Keck Project. In addition to structural images, velocity profiles around biofilms have been collected in the x, y and z-directions. For laminar flows in a clean capillary, there are no x or y components of velocity, while the z direction is both uniform and symmetrical. In contrast, the biofilm fouled capillaries display irregular flow patterns in the z direction along with distinct x and y flow perturbations. These results show that biofilm fouling has a significant impact on bulk flow which should not be ignored in behavior models. Future experiments will examine how flow patterns change over the course of biofilm growth and under different environmental conditions, in addition to characterizing the diffusion in this system.

S03-S04
Diffusion in *Staphylococcus epidermidis* biofilms

Suriani Abdul Rani, *Undergraduate Researcher, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Time-lapse scanning confocal laser microscopy was used to visualize the diffusive penetration of an antibiotic-sized fluorescent dye into *Staphylococcus epidermidis* biofilms. Biofilms were grown in glass capillary tubes under continuous flow conditions for 24 hours at 37°C. A biofilm cell cluster was located using a 20X objective; then a solution of Rhodamine B was introduced and images were captured at the same spot every 5 seconds. Image analysis was performed using Metamorph software. Rhodamine B was selected because its size is similar to many antibiotics. Using the mathematical solution for diffusion in a sphere, the fluorescence intensity at the cluster center versus time data were fit. This fit yielded an estimate of the effective diffusion coefficient in the biofilm. The diffusion coefficient in the biofilm was 15 to 20% of its value in water. This is in good agreement with published measurements of diffusion coefficients of small solutes in biofilms. The time for the dye to permeate throughout a cell cluster a few hundred microns in diameter was a few minutes. Transient profiles of staining intensity within the cell cluster showed that probe intensity was initially greater closer to the outside of the cell cluster. As time progressed, the intensity became relatively uniform throughout the cluster. In future work, these experiments will be repeated, and cell clusters of different sizes will also be analyzed, as—theoretically—the diffusion coefficient should be similar. Results from these experiments suggest that molecules the size of biocides and antibiotics can permeate into biofilms by diffusion relatively quickly. The biofilm does not pose a generic physical barrier to the entrance of small solutes.

S03-S05

Spatial patterns of green fluorescent protein expression in biofilms

Ruifang (Grace) Xu, *PhD Candidate, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Spatial patterns of green fluorescent protein (GFP) expression in *Pseudomonas aeruginosa* biofilms were determined. *Pseudomonas aeruginosa* with the plasmid pAB1 was used in this study. In the presence of the inducer isopropylthio-beta-D-galactoside (IPTG) and oxygen, bright green fluorescence develops in those cells that are actively synthesizing protein. The more active the growth is; the greater the GFP expression. Biofilms were grown in a glass capillary tube under continuous laminar flow of minimal glycerol-glutamate medium at ambient temperature (23^{o}C). Air was pumped at the same flow rate as the medium to make the biofilms stronger and to provide more oxygen. This resulted in slug flow of medium and air bubbles through the capillary tube. After five days of growth, mature biofilms were ready for confocal microscope observation. Time-lapse images reveal the transient increase in fluorescence after IPTG addition to the medium. Biofilms emitted negligible green fluorescence without the IPTG induction, while the green fluorescence becomes more and more intense along the time course after adding IPTG. More GFP activity was evident at the surface of cell clusters than in the center. Activity appeared more uniform in smaller clusters and less uniform in larger clusters. These preliminary measurements illustrate the physiological heterogeneity that is present in these biofilms. Such variation in the metabolic activity probably contributes to the reduced susceptibility of these biofilms to antimicrobial agents.

S03-S06

A 3-D model of biofilm growth and detachment

Steve Hunt*, PhD Candidate, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

We have created a three-dimensional stochastic computer model of biofilm development in a negligible-shear environment (hereafter referred to as BacLAB). BacLAB simulates the life cycle of a bacterial biofilm, from the initial colonization of a surface to the development of a mature biofilm, by mimicking the physical behavior of a system with a simple set of experimentally determined "rules" applied locally to the smallest possible biofilm unit (the cell). These local "rules," however, lead to patterns on a larger scale. Much as bacterial cells organize themselves in a biofilm as a response to individual spatial conditions, the resulting model structure is produced in a process of self-organization rather than by some predetermined global plan for biofilm development. This self-organization facilitates the model's ability to adequately capture the inherent variability observed in laboratory biofilms. BacLAB is currently being used to theoretically explore the plausibility of different modes by which biofilms detach. Current results have demonstrated that the typical simulated biofilm eventually attains a steady state where biofilm growth was counterbalanced by detachment. Also, the oft-observed 'mushroom-shaped' structure occurred after detachment events created voids, leaving the remaining attached cells in towers and loosely attached clusters. The simulated cell areal densities were comparable to those in laboratory biofilms.

S03-S07

Dispersal mechanisms in *Pseudomonas aeruginosa* biofilms

Boloroo (Laura) Purevdorj, *PhD Candidate, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The colonization of clean surfaces by biofilms has been well documented *in vitro*, and combined microscopic and molecular techniques have revealed that biofilm formation is a multifactorial process regulated by both genetic and environmental factors. However, much less is known about detachment and dispersal in biofilms, their regulation, and the roles that these processes play in determining biofilm structure and dissemination in the life cycle of prokaryotes. Currently, biofilm detachment is usually described in terms of erosion (the continual detachment of single cells and small portions of the biofilm) and sloughing (the rapid, shear mediated massive loss of biofilm). More recently a novel mechanism of biofilm dispersal was described in the oral bacterium *Actinobacillus actinomycetem-comitans*. In this process the cells are released from inside of the mature cluster to the exterior portion of the biofilm thereby enabling the detached cells to explore more favorable conditions elsewhere. Although a similar cell dispersal phenomena has been

anecdotally noted in *P. aeruginosa* biofilms in various labs, the mechanism and factors that are involved in this process are still not clear. An understanding of this yet little-studied process would contribute to our current knowledge of biofilm dynamics particularly in the context of its contamination and dissemination commonly encountered in various industrial and medical settings. In this work we present visual characterization and further quantification of this detachment process (seeding dispersal) in a model *P. aeruginosa* biofilm grown in a once-through flow system by using the Digital Time-Lapse Imaging Microscope (DTLM) and Scanning Laser Confocal Microscope (SLCM). In order to determine the effects of different mutations on the seeding dispersal mechanism, we also utilized different isogenic mutants derived from the *P. aeruginosa* parent strain. Finally, the significance of flagellar motility and alginate overexpression in the seeding dispersal process and its implications are discussed in this work.

S03-S08
Cell movement in biofilms

Ben Klayman, *PhD Candidate, Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The goal of this research project is to quantify individual cell and cell cluster movement within a mature *Pseudomonas aeruginosa* PAO1 biofilm. This work builds largely on top of previous research done separately by Andy Rice here at the CBE, and Søren Molin's group in Denmark. Andy was able to quantify replication, emigration, and movement events in young (3-10 µm thick) PAO1 biofilms. Søren Molin's group has used fluorescent PAO1 constructs to make qualitative observations on biofilm formation, but there are no quantitative data on cell or cell cluster movement within the biofilm. For this project, time-lapse images of biofilm formation are captured using Scanning Confocal Laser Microscopy (SCLM) equipped with an Acousto-Optical Beam Splitter (AOBS), so that individual cells and cell clusters can be visualized with the aid of green, yellow, and cyan fluorescent constructs of PAO1. These constructs are each recorded to separate channels, allowing for spatial differentiation of various clusters and cells. Tracking volume and shape factor measurements for individual clusters over time will then potentially lead to a better understanding of cell displacement and colony formation within the biofilm as a whole. This information can then be used to improve biofilm cellular automata models by more accurately describing cell movement and displacement following cell division.

S03-09
State of the CBE address

Bill Costerton*, CBE Director, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Abstract not available.

S03-10
Assets of a CBE education

Erin Werner*, recent graduate, BS, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Abstract not available.

SESSION 2: Biofilm Methods

S03-11
Session introduction

Darla Goeres*, Research Engineer, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

S03-12
Antimicrobials regulation at EPA

Robert Brennis, *US EPA*

Abstract not available.

S03-13

Grow, analyze & disseminate: Progress in the standardized biofilm methods research area

Darla Goeres, *Research Engineer, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The goal of the Standardized Biofilm Methods (SBM) team is to develop and publish standard operating procedures for quantifying biofilm and assessing antibiofilm treatments. This talk will present a synopsis of three projects currently in progress: treating biofilm in hot tubs, alternate plating strategies and technology transfer efforts for the CDC Biofilm Reactor.

The SBM team is under contract with the US EPA Antimicrobials Division to develop a standard method for testing the efficacy of hot tub disinfectants. The reactor system design and associated operating procedure were finalized in March 2003. Since that time, three technicians completed the protocol 17 times. The standard deviations for the suspended and biofilm control data are less than + 0.5 logs. A dose-response curve demonstrates that the system is sensitive to treatment concentration. Final experimentation includes ruggedness testing, alternate treatments, alternate bacteria and field validation.

Plate counts are still accepted as a viable option for estimating the number of bacteria in an environmental sample. Traditional plating methods include the pour-and-spread plate techniques. Both methods are time and material intensive. Dr. Mark Shirtliff uses an alternate to these techniques called the three-drop method. This new method saves supplies by plating triplicates of a sample on one plate and saves time by requiring no spreading. The SBM team is investigating the three-drop method to determine if it yields results comparable in number and repeatability to the standard techniques.

The SBM Team, in collaboration with BioSurface Technologies, completed a project to develop the CDC reactor into a system suitable for mass production. Mr. Ricardo Murga and Dr. Rodney Donlan of the Centers for Disease Control and Prevention (CDC) are responsible for the original design. Ms. Marion Osterud and Dr. Marty Hamilton presented the results of the ruggedness testing at the February 2003 TAC meeting. This talk will discuss technology transfer strategies for the reactor and associated operating procedure.

S03-S14

Development of a national standard for testing dental unit water line antimicrobials

Paul Sturman, *Research Engineer, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Over the past 30 years it has become increasingly evident that dental unit water lines have the potential to become heavily colonized with biofilm. Dental unit water lines are characterized by intermittent flow, long periods of stagnation, and very small diameters (1.5mm). Most dental units receive tap water influent, and residual chlorine from municipal treatment is often exhausted within the lines. Biofilm growth during stagnation periods often leads to the detachment of large pieces of biofilm during flow, potentially causing patient infection. The American Dental Association (ADA) and the Organization for Sepsis and Aseptic Procedures (OSAP) recognize the potential for patient infection from untreated dental units, as do many dentists and manufactures of dental units. The ADA has adopted a maximum standard of 200 cfu/ml in dental unit process water. Options for treating dental unit water include the addition of antimicrobials, water filtration, and the use of externally supplied water; however, no standard methods exist for efficacy testing of these products. Both EPA and FDA recognize the need for standard testing methods, and have endorsed the ADA and OSAP in their development. In concert with an ADA/OSAP sponsored task force, the CBE has assisted this methods-development effort and is now in the process of building and testing a prototype reactor system to test antimicrobial compounds and other dental unit water line treatment products. Following initial testing, the reactor system will undergo round-robin evaluation in three laboratories (CBE included) and finally be proposed as a standard testing method.

SESSION 3: Fungal and Mycobacterial Biofilms

S03-S15
Candida albicans as a model biofilm system

Peter Suci, *Assistant Research Professor, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The primary focus of most biofilm studies has been on bacterial, or more precisely, prokaryotic, systems. This emphasis is changing slightly, in large part due to recent interest in biofilm formation by the opportunistic pathogen *Candida albicans*, a eukaryotic fungus. Fungi form a relatively tight phylogenetic group, but exhibit great diversity in both cell physiology and multicellular behavior. A paradigm shift has occurred with the realization that many prokaryotic biofilm communities are organized at the multicellular level. While elaboration of clearly defined functionally specialized morphological structures is the exception for prokaryotic systems, it is commonplace for fungal systems. In this talk, a brief introduction will be given to fungal systems, including an allusion to the origin and types of general fungicides. *C. albicans* will be discussed in a little more detail, including a discussion of the medical relevance of *C. albicans* biofilm formation, and its ability to switch between yeast and filamentous forms. Issues of *C. albicans* biofilm resistance to antimicrobial agents will be discussed with reference to results obtained by the presenter. Results from other researchers suggesting that bacterial-fungal interactions can be exploited to inactivate fungi will be presented. Finally, some summary points will be offered as a stimulus for discussion.

S03-S16
Mycobacterial biofilms: A sticky wicket**

Luanne Hall-Stoodley, *Adjunct Professor, Microbiology, MSU Veterinary Molecular Biology, Montana State University–Bozeman, 59717*

The ability of Mycobacteriaceae to form biofilms is of industrial relevance for several reasons. While the majority of the over 80 species of mycobacteria commonly found in soil and water are environmental saprophytes, some, like other biofilm bacteria, are capable of causing disease. Many diseases associated with mycobacteria may be linked to their ability to survive in potable water systems, and particularly, to survive high temperatures. Mycobacteria are also extremely recalcitrant to biocides and may survive when other more prevalent species have been killed off in a water system. For these reasons, mycobacteria are increasingly a problem of industrial and pharmaceutical importance. Mycobacteria are particularly important in the biofouling of reverse osmosis membranes, oil/water mixtures and synthetic water based products. Notably, mycobacteria are increasingly implicated in emerging infectious diseases from hot tub lung to pulmonary disease. Finally, mycobacteria are capable of biotransformation and may be prime candidates for bioremediation because of their hardiness. However, much remains to be learned about their ability to attach to surfaces and the environmental factors that facilitate their growth in biofilms.

**Something that is sticky, spec. (a) an adhesive material; (b) a sticky wicket. Also fig., esp. in phr. to bat (or be) on a sticky wicket: to contend with great difficulties (colloq.)., from OED

SESSION 4: Environmental Biofilms

S03-S17
Session introduction

Al Cunningham, *Professor, Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

S03-S18
A conceptual model of biofilm processes in porous media

Al Cunningham, *Professor, Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

A series of porous media reactor studies was performed to characterize the development and structure of thick biofilms in porous media. Dye tracer studies revealed that flow channels (advection channels) develop within the biofilm-porous media matrix. Channel configuration and location was observed to change significantly during daily observation. Studies with bioluminescent *Vibrio fischeri* showed that regions of active cell growth (and

hence biotransformation of substrate and oxygen) tend to occur along active or recently active flow channels. Mesoscale packed column studies have further revealed that biofilm accumulation decreases free pore space thereby decreasing media porosity and permeability while increasing hydrodynamic dispersion. These observations provide a conceptual model for biofilm accumulation in porous media and the subsequent effects on media hydrodynamics, mass transport and microbial activity to better understand and control environmental biofilms in porous media. This insight will promote biofilm-based technologies applied to subsurface injection systems (i.e. MEOR), subsurface contaminant bioremediation, and biofilm filtration systems.

S03-S19
Influence of electron shuttling compounds and iron mineral surfaces on redox-reactions in the environment

Robin Gerlach, *Postdoctoral Researcher, Civil Engineering & Environmental Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

It has become evident that surface and electron shuttling compound-mediated reactions significantly influence the fate of redox-reactive compounds in the environment. Humic substances (HS) and iron minerals are some of the most abundant naturally occurring redox-reactive compounds in the environment. Iron is one of the most abundant elements in the Earth's crust, and it has been shown over the last decade that iron-reducing bacteria can significantly influence the fate of redox-reactive compounds in the environment. HS are naturally formed from the decomposition of organic matter in aquatic and subsurface environments. HS are complex, high molecular weight compounds containing aromatic and heterocyclic systems with a large number of functional groups such as carboxyl-, hydroxyl-, ketone-, and quinone-groups. Due to their chemistry, HS can influence the fate of organic and inorganic compounds in the environment in several ways. In anaerobic environments, there are at least three processes of importance. First, the complexation of metals or sorption of organics by colloidal HS can increase their mobility in the environment since colloidal HS might be transported faster than dissolved compounds. Second, sorbed or colloidal HS can function as terminal electron acceptors for microbial growth, therefore competing for electrons otherwise available for microbial reduction of these compounds. Thirdly, the quinone moieties of HS can function as electron shuttles, thus increasing the reduction rates of redox-reactive compounds by increasing electron transfer rates.

This presentation will demonstrate the importance of surfaces and electron shuttling compounds in microbial, electron shuttle-, and surface-mediated reactions. These findings are not only important in determining the fate of environmental contaminants but can also enhance our understanding of antibiotic or biocide action and thus have an impact on the development of biofilm control processes since some of the electron shuttling compounds have similar structures and characteristics as some antibiotics.

S03-S20
Uranium immobilization by sulfate-reducing biofilms

Haluk Beyenal*, Assistant Research Professor, Chemical Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Hexavalent uranium [U(VI)] was immobilized using biofilms composed of the sulfate reducing bacterium (SRB), *Desulfovibrio desulfuricans* G20. Biofilms were grown in flat-plate continuous-flow reactors using lactate as the electron donor and sulfate as the electron acceptor. U(VI) was continuously fed into the reactor for 32 weeks at a concentration of 126 μM. During this time, the soluble U(VI) was removed (between 88 and 96% of feed) from solution and immobilized in the biofilms. The dynamics of U immobilization in the sulfate reducing biofilms were quantified by estimating: 1) microbial activity in the SRB biofilm, defined as the hydrogen sulfide (H_2S) production rate, and estimated from the H_2S concentration profiles measured using microelectrodes across the biofilms; 2) concentration of dissolved U in the solution; and (3) the mass of U precipitated in the biofilm. Results suggest that U was immobilized in the biofilms as a result of two parallel processes: (1) enzymatically; and (2) chemically, by reacting with microbially generated H_2S. Analytical tests showed that sulfide species and U(VI) react to produce a black precipitate. Synchrotron-based U L3-edge x-ray absorption near edge structure (XANES) spectroscopy analysis of U precipitated abiotically by sodium sulfide indicated that U(VI) had been reduced to U(IV). Selected area electron diffraction patterns and crystallographic analysis of transmission electron microscope lattice fringe images confirmed the structure of precipitated U as being that of uraninite.

SPEAKER ABSTRACTS

S03-S21
Control of acid production from mine tailings through the addition of dissolved organic carbon

Paul Sturman, *Research Engineer, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Mine tailings are the fine-grained waste material left after valuable minerals have been extracted from crushed rock ore. At many hard-rock mines, tailings contain high concentrations of sulfide-bearing materials, typically pyrite (FeS_2). When exposed to atmospheric oxygen, pyrite is oxidized to ferrous iron, sulfate and acidity. In tailings impoundments, aerobic chemolithotrophic iron-oxidizing bacteria (IOB) utilize ferrous iron as an electron donor, generating ferric iron. Ferric iron then further oxidizes pyrite to accelerate acidification. A potential solution to accelerated acid mine drainage is to prevent the infiltration of oxygen by stimulating heterotrophic bacterial populations through the addition of organic carbon.

Laboratory tailings columns were used to assess the effectiveness of molasses, whey and methanol in reducing acid and dissolved metals in effluent water. Treatments were tested sequentially in two test columns. A control column received only unamended water. Microbial populations within the columns were assessed on a monthly basis. Whey was more effective than molasses in raising effluent pH and lowering dissolved metals within the columns; however, between-column variation was dramatic. While Test Column 1 (TC1) performed well throughout the experiment, Test Column 2 (TC2), which received identical treatments, generated significantly more metals and acidity. Heterotrophic bacterial populations were slightly stimulated in both test columns, while IOB were approximately unchanged. SRB populations in TC1 were 2–3 orders of magnitude higher than in TC2, suggesting that SRB are primarily responsible for column performance, rather than heterotrophs. Subsequent analysis of tailings solids indicated the presence of iron-oxidizing bacteria that are also capable of heterotrophic growth under high organic carbon conditions. Populations of these newly identified bacteria, generally gram positive species of the genus *Sulfobacillus*, may be enhanced by heterotrophic growth through organic carbon additions. When organic carbon again becomes limiting, these bacteria revert to iron- and sulfur-oxidation metabolism, contributing to acidification. These results underscore the importance of SRB as the mechanism of acid mine drainage reduction, rather than aerobic heterotrophs in the near-surface zone of the tailings.

Methanol was also tested as a carbon source in an effort to selectively enhance SRB growth in the columns. Atmospheric gas content measurements from the near surface in the columns show that oxygen levels at 12” depth in the columns increased markedly following methanol addition, while carbon dioxide levels remained low. Conversely, whey addition results in a large increase in CO_2, suggesting the activity of heterotrophs, many of which are likely also capable of iron oxidation.

This work indicates that organic carbon addition can increase pH and decrease dissolved metals in tailings effluent; however close attention to microbial populations is necessary to insure optimal performance.

S03-S22
Use of molecular techniques to study changes in an oilfield consortium exposed to nitrate and nitrite

Birthe Venø Kjellerup, *Visiting Scientist, Danish Technological Institute*

Identification and enumeration of bacteria and study of bacterial physiology in oil fields has previously been based mainly on cultivation-based methods. However, cultivation of bacteria from industrial environments (that often are extreme regarding temperature, substrates, pressure etc.) is very difficult, and may provide little information and lead to wrong conclusions, resulting in ineffective control strategies. Several studies have shown that <10% of industrial environment bacteria can be detected by use of cultivation-based methods, not reflecting the real diversity occurring in the system.

In the last decade a number of new molecular biological techniques and isotope techniques have been developed for identification and determination of bacterial physiology in complex environmental and industrial systems. Application of these techniques has shown a heretofore unknown diversity regarding identity and physiology among microorganisms. In addition these *in situ* techniques are faster to perform when compared to the traditional cultivation-based techniques that often require two or more weeks (especially in anaerobic systems) before results are obtained. Molecular biological methods can therefore

improve knowledge about bacteria, and thereby provide control strategies reducing the risk of incorrect conclusions and actions. Furthermore, faster action can be taken in order to control the problems occurring instead of waiting two or more weeks before the results are ready.

In this study *in situ* techniques for detection of bacterial identity of oil field biofilms were applied. The purpose of the experiments was to evaluate the efficiency of treatment with nitrate, nitrite, and molybdate and nitrite, respectively, in order to control sulfide production. Identification of bacteria was performed by molecular biological methods, allowing detection directly in the systems without previous cultivation. For direct detection and enumeration of the bacteria by microscopy, fluorescence *in situ* hybridization (FISH) by rRNA targeted fluorescently labeled oligonucleotide probes was applied. For evaluation of possible microbial population changes due to treatment with nitrate and nitrite, PCR and DGGE were used. Furthermore, sequencing of clones followed by a BLAST analysis was performed to evaluate the identity and diversity of the microbial population in the biofilm. In particular, sulfate reducing bacteria (SRB) and nitrate reducing bacteria (NRB) were studied.

The results showed that nitrite and molybdate reduced sulfide production, while nitrate (at the concentration applied here) had no effect. No long-term effect on sulfide production was observed for any of the treatments, showing that the chemicals must be consistently present in order to reduce SRB activity. The number of SRB was not influenced by the treatments. However, a significant increase of SRB was observed in all reactors 4 weeks after termination of the treatments. Evaluation of general corrosion based on weight loss of mild steel coupons and scanning electron microscopy (SEM) showed that none of the treatments increased corrosion rates significantly.

SESSION 5: Emerging Research

S03-S23
Confocal microscopy: Changing our perceptions of biofilms

Betsey Pitts, *Research Associate & Microscopist, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

In the past year, the CBE Microscope Facility has undergone dramatic changes. Through a grant funded by the Murdock Charitable Trust (written by L. Hall-Stoodley) the CBE recently acquired a new Leica SP2-AOBS confocal and a Leica/Spectra Physics MaiTai 2-photon confocal system. These two pieces of equipment have already altered our perceptions of biofilms by giving us new imaging tools to use and by expanding our existing confocal imaging capabilities. The acquisition of this new technology has pushed Center Microscope users, as a group, to re-examine what makes a good biofilm image, to ask what about our images is real and what is artifact, and to raise our standards regarding useful, quality biofilm images.

S03-S24
Biofilms and bioterrorism: CBE's developing research in defense

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

The ability of biofilms to retain pathogens and toxins is of interest in the detection and examination of natural contamination events as well as of deliberate contamination events, when these organisms and chemicals may be added as an act of bioterrorism. Regardless of the nature of the event, we know that these organisms/compounds can be retained in biofilms, and that these agents may persist there for periods of time exceeding that for their transport and disinfection/inactivation in the water. Consequently, the biofilm may be a reservoir for further public health threat, or it may be viewed as a tool to help determine if contamination occurred. Research is currently underway to determine the transport of potential contaminants to biofilms and their retention in surfaces in water systems. Work also includes determining the best ways to detect the retained

organisms. Efforts include concentration of biofilm and the associated pathogens, improved enumeration/detection methods, and a modeling component to provide insight into the behavior of these agents in water systems.

SESSION 6: Industry Forum

S03-25

Diane Cummins, *Worldwide Director, Oral Care, Colgate-Palmolive*

Abstract not available.

S03-26
Smith & Nephew Implants

David Vogel, *Microbiology Manager, Smith & Nephew*

Abstract not available.

SESSION 7: Medical Biofilms

S03-27
Session introduction

Mark Shirtliff, *Assistant Research Professor, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

S03-28
Compromised host defense on *Pseudomonas aeruginosa* biofilms: Characterization of neutrophil and biofilm interactions

Al Jesaitis, *Professor, Microbiology, Montana State University–Bozeman, 59717*

Pseudomonas aeruginosa is an opportunistic pathogen that forms biofilms on tissues and other surfaces. We characterized the interaction of purified human neutrophils with *P. aeruginosa* growing in biofilms, with regard to morphology, oxygen consumption, phagocytosis, and degranulation. Scanning electron and confocal laser microscopy indicated that the neutrophils retained a round, unpolarized, unstimulated morphology when exposed to *P. aeruginosa* PAO1 biofilms. However, transmission electron microscopy demonstrated that neutrophils, although rounded on their dorsal side, were phagocytically active with moderate membrane rearrangement on their bacterial proximal surfaces. The settled neutrophils lacked pseudopodia, were impaired in motility, and were enveloped by a cloud of planktonic bacteria released from the biofilms. The oxygen consumption of the biofilm/neutrophil system increased sixfold and eightfold over that of the biofilm alone or unstimulated neutrophils in suspension, respectively. H_2O_2 accumulation was transient, reaching a maximal measured value of 1 ìM. Following contact, stimulated degranulation was 20–40% (myeloperoxidase, â-glucuronidase) and 40–80% (lactoferrin) of maximal when compared to fMet-Leu-Phe plus cytochalasin B stimulation. In summary, after neutrophils settle on *P. aeruginosa* biofilms, they become phagocytically engorged, partially degranulated, immobilized and rounded. The settling also causes an increase in oxygen consumption of the system, apparently resulting from a combination of a bacterial escape response and the neutrophil respiratory burst, but with little increase in the soluble concentration of H_2O_2. Thus, host defense becomes compromised as biofilm bacteria escape while neutrophils remain immobilized with a diminished oxidative potential.

S03-S29
Oxygen and nitrate modulate antibiotic susceptibility of *Pseudomonas aeruginosa* in biofilms

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The *in vitro* susceptibility of *Pseudomonas aeruginosa* biofilms to a number of antibiotics (including ciprofloxacin, tobramycin, carbenicillin, chloramphenicol and tetracycline) and its dependence upon anaerobic growth conditions (oxygen limited and oxygen limited with nitrates) was evaluated. Anaerobic growth conditions were tested, since mature biofilms have been previously shown to contain anoxic regions using oxygen microelectrodes. The depth of oxygen penetration into the biofilm was approximately 50 microns. Polycarbonate membranes seeded with bacteria and overlaid on tryptic soy agar were used for colony biofilm growth at 37°C. Colony biofilms grown for either 4 h (young) or 48 h (mature) were tested for their antimicrobial susceptibility by transferring the colony to a fresh plate supplemented with the desired antibiotic for 12 h. Under aerobic conditions, young biofilms demonstrated rapid growth, while mature biofilms exhibited stationary phase growth kinetics. While antibiotic efficacy was

greatly reduced in mature colony biofilms, young biofilms exhibited a much higher sensitivity to antibiotics like their planktonic counterparts. The impact of oxygen-limited growth was tested by challenging young colony biofilms to antibiotics in anaerobic bags. All of the antibiotics were less effective when tested under anaerobic conditions. In order to test the effect of sustained anoxic growth prior to antibiotic treatment like that seen in mature biofilms, oxygen starvation was applied to young biofilms for 24 hours before exposure to antibiotics in an anaerobic environment. A slight reduction in the susceptibility of the ordinarily vulnerable 4 h colony biofilms was noted, and the trend approached the levels seen in 48 h biofilm susceptibility profiles. In contrast, when nitrates were added to the medium in the anaerobic system, the bacterial biofilm growth was enhanced while the antibiotic susceptibility was further reduced in all the cases. These results obtained with this *in vitro* system suggest that the absence of oxygen and presence of nitrate in the airway mucus of the cystic fibrosis lung may contribute to increased antibiotic tolerance of *P. aeruginosa* in the host.

S03-S30
Proteomics and transcription profiling of Staphylococcal biofilms: A comparative approach

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Staphylococcus aureus is able to cause a number of persistent infections and bacterial fouling of surfaces through a biofilm mode of growth. This research sought to identify biofilm-specific gene products and key genetic regulators of *S. aureus* biofilm formation through two-dimensional gel electrophoresis. *S. aureus* biofilms were grown at 37^{o}C in a once-through reactor system in silicon tubing. Biofilm samples were harvested at various times post-inoculation (8 hr, and 2, 7 and 14 days) to represent early-to-late developmental stage biofilms. For *in vitro* planktonic cultures, aliquots were obtained at logarithmic, stationary, and post-stationary growth phases. Protein samples were prepared, quantified, and resolved by two-dimensional electrophoresis. Protein spot identification was accomplished by mass spectrometry of trypsin-digested fragments. Globally, approximately 20% of the proteins derived from mature biofilms demonstrated statistically significant differences when compared to proteins from stationary phase planktonic bacteria. A number of metabolic enzymes had upregulated production in older biofilms (7 day and 14 day) including those coding for components of the Krebs cycle. In addition, transcription regulators such as the sae regulatory locus (SaeR and SaeS) and a membrane-bound potassium transporting ATPase showed high level production. Specifically, the membrane-bound potassium transporter was immunogenic in rabbits and may represent a potential vaccine candidate or target for directing labeled monoclonal antibodies for novel biofilm diagnostic technologies. These data were compared to those obtained for microarray experiments in which biofilm and planktonic samples (grown and harvested at the times described above) had RNA extracted, quantified, equalized, and reverse transcribed. CY3/CY5 aminoallyl labeled probes were applied to DNA microarrays constructed to include all of the open reading frames of four *S. aureus* species, including COL, N315, Mu50, and MW2. These microarray experiments validated proteomic data by demonstrating that gene expression profiles of *S. aureus* growing in biofilm and stationary planktonic cultures were similar, and several transcription factors and metabolic enzymes were uniquely expressed in the biofilm growth mode.

SESSION 8: Biofilm Detachment/Removal

S03-S31
Session introduction

Paul Stoodley, *Assistant Research Professor, Microbiology and Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

S03-S32-33
From colonization to infection: *Staphyloccocus aureus* biofilms in an *in vitro* catheter infection model

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Background: The expanding use of invasive procedures in modern medicine has resulted in an increase in *S. aureus* infections which are feared for their high mortality and rising rates of antibiotic resistance. Catheter-related bloodstream infections are preceded by the bacterial colonization of the device.

Under continuous flow conditions, bacteria form a biofilm and eventually cause metastatic infections by detached particles. Despite their increasing clinical importance, the pathophysiological steps leading to catheter infections are not well defined.

Methods: We studied growth and detachment dynamics of a *Staphylococcus aureus* biofilm in a glass flow cell using time-lapse microscopy. Detached particles were analyzed for their size, number of bacteria and their resistance to oxacillin. The MIC and MBC of whole biofilm effluent and large clumps were measured before and after the ultrasonic disruption of multicellular particles. The results were compared with planktonic cultures in logarithmic and stationary growth phase.

Results: Growing biofilm showed an important degree of structural variability over time. We documented different modes of dispersal, including continuous growth, detachment and reattachment of single cells and clumps, as well as the rolling motility of microcolonies. Detached particles ranged from single cells to particles with more then 1,000 cells. Emboli with 11 to 100 cells were the main source of detached biomass.

The MBC of large biofilm clumps exceeded 20mg/ml, but decreased to 23µg/ml after the dissolution of the clumps. Stationary phase planktonic cultures in spent medium showed resistance similar to large clumps, but regained antibiotic susceptibility after the addition of fresh medium.

Discussion: In *S. aureus* biofilms, dispersal is a continuous process involving different mechanisms. Detachment can be characterized and seems to show species-specific patterns. The detachment of multicellular emboli with biofilm-typical antibiotic tolerance explains the high virulence of SA biofilm infections. The striking differences in antibiotic susceptibility between stationary and logarithmic phase planktonic cultures illustrate the direct correlation between nutritional supply, metabolic activity and antibiotic susceptibility. The regained antibiotic susceptibility of dissolved clumps stresses the paramount importance of diffusion limitation for the resistance of large biofilm emboli. Additional, biofilm-specific resistance mechanisms to killing seem to be of minor importance.

Our observations suggest that the characterization of species-specific detachment may predict the virulence of biofilm emboli. The established *in vitro* model allows studying the impact of varying shear stress or antibiotic therapies on resistance and detachment. Further studies could help to explain the clinical differences between left- and right-heart endocarditis or to evaluate the safety and efficacy of therapeutic antibiotic catheter-locks.

S03-S34
Proteomics of detachment in *Staphylococcus aureus* biofilms

***Krista Cooperstein**, Undergraduate Researcher, Microbiology, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Methicillin Resistant *Staphylococcus aureus* (MRSA) is a common cause of disease in humans. MRSA can form biofilms and detach as infectious emboli (detached particles) from the mass of biofilm in dynamic flow. Our goal was to determine if MRSA biofilm differs from the detached populations of MRSA by proteomic evaluation. A reactor system for simulated dynamic flow produced both detached populations and biofilm for collection after seven days of growth. Analysis of the proteomic structure was performed using 2-D gel electrophoresis (for isolation of proteins demonstrating altered expression upon comparison) and matrix assisted laser desorption/ ionization-time of flight mass spectrometry (MALDI-TOF MS) for identification of proteins.

S03-S35
Predicting the rate of large particle detachment events

***Gordon Cord Hamilton**, Statistician, CBE; Marty Hamilton, Professor Emeritus of Statistics, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The risk of infection from a medical indwelling device such as a catheter may well be dependent on the size of the particles detaching from the biofilm. Although the body's defense systems can inactivate individual bacterial cells, large clumps of detached biofilm may persist and cause an infection. Similarly, biofouling in industrial systems may be detrimental only if large clumps of biofilm detach. This investigation uses detachment data from *Pseudomonas aeruginosa* biofilms to estimate the rate at which large particle detachment events occur.

A laboratory biofilm was grown in a flow cell. At a specified time, effluent from the reactor was captured and carefully filtered using a technique that caused

thick clumps of biofilm to flatten into a single cell-thick pancake. The area covered by each flattened detached particle was calculated using image analysis software. Image analysis also provided a calibration showing the average area per bacterial cell. That information was used to convert the measured area of a detachment event into the number of cells contained in that event. For the biofilms grown in this study, single cell detachment events predominated, then two cell detachment events, and so on, with steadily declining frequency for increasing event sizes. Large detachment events did occur, but very sporadically. We converted these observations into the rate at which large particles detach by fitting a three-parameter Pareto distribution to the data. The Anderson-Darling minimum discrepancy technique was used to find the best fit. This presentation will briefly review the laboratory and statistical methods and describe the results.

S03-S36

Detachment and dispersal mechanisms in biofilms

***Paul Stoodley**, Assistant Research Professor, Microbiology and Civil Engineering, Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

Confocal and time-lapse microscopy have revealed the complex nature of biofilm structural and temporal development. These observations suggest that biofilm formation may follow a biologically determined sequential development. Observations based largely on *Pseudomonas aeruginosa* biofilms depict this sequence as: 1) Attachment: motile cells in the planktonic phenotype swim to an uncolonized surface, 2a) Growth: the attached cells switch to a biofilm phenotype, divide and produce an extracellular slime matrix. 2b) Maturation: the biofilm forms complex structures such as “mushrooms”, “mounds” and “streamers” which, given a particular shear stress and nutritional environment, optimize surface survival. 3) Detachment: single cells within the clusters revert to the planktonic phenotype, swim out of the biofilm, presumably to colonize new surfaces. In addition to swimming motility, *P. aeruginosa* can also migrate across surfaces by type IV pilus-mediated twitching motility and through rippling. However, *in vitro* observations of the non-motile species, *Staphylococcus aureus*, reveal similar complexity in biofilm development. In *S. aureus* biofilm, detachment is primarily of clumps containing hundreds to thousands of cells. The clumps are resistant to oxacilin in a similar manner seen in attached biofilms. *S. aureus* biofilm microcolonies can also disperse by shear mediated rolling or sliding across surfaces. Based on these observations we present a hypothesis that environmental selection pressure has allowed diverse species to develop different biofilm dispersal strategies. *P. aeruginosa* cells disperse primarily as single motile cells. Although the cells are in the relatively sensitive planktonic phenotype, they can actively move away from hostile environments through chemotactic motility. This is analogous to animal locomotion. The non-motile *S. aureus* take advantage of the fluid flow to disperse, but are passive in terms of determining their destination. However, dispersal in biofilm clumps affords a higher level of homeostasis which will protect the dispersing colony as it passes through, or attaches in, hostile environments. This dispersal strategy is analogous to the airborne dispersal of dandelion seeds. The seeds are somewhat protected by an outer coat, and although locomotion is not actively controlled, the dandelion takes advantage of the wind to disperse, while sheer numbers increase the probability of survival of some of the progeny. A third strategy is the formation of protected spores by non-motile species such as *Bacilli*. This case allows the passive dispersal of single cells in a highly protected state. Given the metabolic and genetic diversity in prokaryotes, it is not surprising that environmental selection pressure may have allowed the development of different survival strategies. However, similarities of life in fluid environments (shear stress and mass transport mechanisms) have resulted in convergent biofilm behavior. Commonality of viscoelasticity in diverse biofilms may reflect one convergent property. Although the physiology of many prokaryotes has been characterized in great detail a better, understanding of community behavior in biofilms is required to develop more effective control strategies in industry and medicine.

S03-P302

Identification of siderophores produced by the halo-alkaliphile *Halomonas campisalis*

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Halomonas campisalis strain 4A has been identified as capable of producing siderophores under halo-alkaliphilic growth conditions. Siderophore production was confirmed through the use of the chrome azural S (CAS) agar plate method which showed a red orange halo around the bacterial colonies indicative of siderophore production. These siderophores are produced under conditions of both high salinity and pH, with a salt concentrations ranging from 0.4–1.8 M NaCl and a pH ranging from 8–11. The siderophores produced have been determined to be of the hydroxamate class via the Csáky method. The culture supernatant of *Halomonas campisalis* responded negatively to the Arnow assay which indicates the siderophore produced does not contain any catechol moieties in its chemical structure. It was found that maximum siderophore production was equivalent to approximately 400 mM desferrioxamine and occurred during mid stationary phase. Similar results were found at pH 8, 10 and 11. Because of the scarcity of iron under the alkaline conditions in which *Halomonas campisalis* strain 4A thrives, we hypothesize that the siderophores secreted by *Halomonas campisalis* strain 4A and other alkaliphilic bacteria will have a stronger affinity for binding and solubilizing ferrous iron than siderophores produced by mesophilic bacteria. It is further hypothesized that siderophores from alkaliphilic bacteria will also have a corresponding higher affinity to heavy metal and radionuclide contaminants, thus increasing the mobility of these metals in subsurface environments influencing contaminant fate and transport in subsurface environments.

S03-P305

Bacterial biofilms in sinusitis

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Sinusitis is a widespread bacterial infection that can develop into a chronic condition which cannot be cleared with antibiotics. In the event of a chronic infection, the most common bacterial species involved in sinusitis, *Streptococcus pneumoniae*, may form a biofilm that is resistant to treatment on sinus tissue. This project focuses on identifying these bacteria in a mouse model of sinusitis to determine if chronic cases do, in fact, contain biofilms. The technique used for locating and identifying the *strep* on the tissue is a procedure called Fluorescence *in situ* Hybridization (FISH). This procedure uses complimentary strands of DNA with fluorescent tags to bind to the bacterial rRNA and cause them to fluoresce. Mouse sinuses are dissected, sectioned and mounted on slides for this procedure, allowing the samples to be viewed on an epifluorescent microscope subsequent to FISH probing. Images of the tissue samples will be taken under fluorescent light and analyzed to determine amount of bacteria and the degree of biofilm formation. If successful, this study will assess the significance of biofilms in sinusitis.

S03-P307

Detachment and antimicrobial resistance of single cells and cell clusters from *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms

***Suzanne Wilson,** Center for Biofilm Engineering at Montana State University–Bozeman, 59717*

The detachment of cell clusters from biofilms enables them to disseminate, flow downstream, and reattach, establishing another biofilm event. In this study detached biomass from biofilms grown under a constant flow rate were filtered from the effluent and microscopically examined to determine the size distribution (number of cells per particulate) and detachment frequency from biofilms composed of gram positive and gram negative bacteria. Biofilms were grown from three individual strains of *Pseudomonas aeruginosa*, and also from *Staphylococcus aureus* to compare with a gram positive non-motile species. Biofilms were grown in

glass flow cells for seven days under laminar flow. Effluent samples were taken on various days during the course of the experiment; data from day five were selected for statistical analysis. The detachment distribution from each of the *Pseudomonas* strains was similar (PAO1 averaged 3.5 cells/cluster, JP1, 3.1 and FRD, 2.9 respectively). Most of the detached particulates from the *Pseudomonas* biofilms occurred predominantly as single cells (c.a. 75%), ranging from 55% for PAO1 to 90% for FRD1. Detaching particulates from the *S. aureus* biofilm were larger on average (11.2 cells/cluster) and more evenly distributed among cluster size.

Detached particles were also evaluated for increased antibiotic resistance over planktonic cells. Sonicated particulates were exposed to a range of oxacillin between 0.05 µg/ml and 20 mg/ml, and the MBC values were compared to that found using either stationary or exponential planktonic cells as inoculum. There was no significant difference ($P < .01$) between the antibiotic resistance of the clump and the stationary planktonic inoculum, while the exponentially growing cells expressed normal sensitivity. This would indicate that the low metabolic rate of the cells within the detached particles would account for the high MBCs.

S03-P309

Biogeochemistry of uranium under reducing and re-oxidizing conditions: An integrated laboratory and field study

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EPA's National Priority List (NPL) lists 54 sites highly contaminated with U (VI) in the USA. The treatment of uranium-contaminated waters is typically based on reducing the soluble U(VI) to sparsely soluble U(IV), which can be done chemically and biologically. Previous work in our laboratories shows that *Desulfovibrio desulfuricans* G20 can reduce U(VI) to U(IV) while associated with hematite, goethite, ferrihydrite, and quartz surfaces; and that the composition of the secondary mineral phase precipitates was significantly different than previous work where no Fe-mineral phase was initially present. In addition, using ion specific microelectrodes, we have shown that the aqueous phase chemistry near a hematite surface, and in accumulations of hematite-associated *D. desulfuricans*, was significantly different than near a quartz surface. Our results indicate that the composition of the mineral substratum also significantly affects rates of metal precipitation and immobilization, and that the underlying mineral phase affects the rate and extent of U(IV) reoxidation and subsequent mobilization. Finally, our use of open flow reactors that are more representative of *in situ* conditions indicates that, in the field, groundwater hydrodynamics and a continual influx of substrate and contaminants can yield significantly different results than obtained with closed serum bottles.

The progress of uranium removal from contaminated waters needs to be monitored using sensitive analytical methods. The most popular technique used to determine concentrations of hexavalent uranium, Kinetic Phosphorescence Analysis (KPA), is expensive and difficult to use. Electrochemical techniques are less costly compared to KPA, but their use is limited to relatively clean waters contaminated with uranium. It is important to adapt these electrochemical methods to waters contaminated with organics, and we have explored the possibility of using adsorptive stripping voltammetry (ASV) for that purpose.

We have adapted known procedures for analysis of U(VI) in groundwaters and wastewaters containing organics using differential pulse voltammetry (DPP). Wet acid digestion with 5% HNO_3 (w/w) was used to reduce interference of organic matter. Prior to analysis, uranium was chelated using cupferron ($C_6H_6N_2O_2$) and acetate buffer 0.01 M at pH 4.5. The procedure permits detection of trace levels of U(VI), both in wastewater under sulfate reducing conditions and in groundwater, and has a detection limit of 0.09 ppb. We use the following conditions to perform the analysis: -0.1 V deposition potential, 3 min concentration time, 80 μ M/l concentration of cupferron. Since the characteristics of wastewater under sulfate reducing conditions vary, we use standard addition method with blank subtraction to determine uranium concentration in the samples. The error of the method is about 10% at the ppb detection level.

S03-P310
Ultrasound, hydrogel and biofilms

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Medical devices are routinely employed in healthcare settings since they provide clinicians with a useful means of administering nutrients, drawing blood samples and drug delivery. In spite of these advantages, local and systemic infections are frequently associated with their use. In fact, implanted devices often provide a highly suitable surface for bacterial adhesion and colonization resulting in the formation of complex, differentiated and highly structured communities known as biofilms. Once a biofilm infection is established, conventional treatments frequently fail, since bacteria growing in biofilms are much more resistant to antibiotics than their planktonic counterparts. As a result, a variety of implantable drug-delivery systems have been developed. However, drug release tends to decay over time, and these systems are prone to uncontrollable leaching. To overcome this problem, the University of Washington Engineered Biomaterials (UWEB) group developed a novel drug-delivery polymer matrix consisting of a poly 2-hydroxyethyl methacrylate hydrogel coated with ordered methylene chains forming an ultrasound-responsive coating. This system is able to keep the drug inside the polymer in the absence of ultrasound, but will show a significant drug release when low intensity ultrasound is applied. The drug embedded within the polymer is ciprofloxacin, an antibiotic well known for its action against gram negative bacteria. In collaboration with UWEB, we have designed a flow cell system incorporating the hydrogel coatings which allows simultaneous real time digital or confocal time-lapse microscopy and the application of ultrasound.

Pseudomonas aeruginosa biofilms were grown on hydrogel surfaces in flow cells with a bulk fluid flow of 1 ml/min. The development of cell clusters could clearly be resolved on the hydrogels using transmitted light, and single GFP expressing cells could be observed using epi-fluorescence microscopy. The CDC Reactor was used to grow biofilm on polycarbonate coupons. Some of the coupons were treated with ciprofloxacin and ultrasound in order to study the bioacoustic effect. The bioacoustic effect is defined as the synergistic effect of ultrasound and antibiotics to kill bacteria. When a power density of 10 mW/cm^2 was applied to the coupons—with and without ciprofloxacin—there was no notable enhanced killing of bacteria due to the bioacoustic effect. However, when a power density of 100 mW/cm^2 used, there was some killing due to the ultrasound only (0.2 log reduction). There was minimal killing due to the bioacoustic effect. Further testing will include the measurement of biofilm cell viability after exposure to ultrasound at different frequencies and power densities. The results of our studies may ultimately facilitate future development of medical devices sensitive to external impulses (ultrasound) capable of treating or preventing biofilm growth via "on demand" drug release.

S03-P311
Microbial fuel cells

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Today, more than ever, we are dependent upon fuel and electricity, so it is important to expand our power production capabilities by investigating all feasible options. One particular way to produce power for remote locations is via microbial fuel cells. Minute amounts of power allow us to run microsensors that can detect concentrations of many different chemicals, such as heavy metals, and transmit that data to a receiver. This type of power may be beneficial in trying to obtain data from hard-to-reach places, such as deep in the ocean, or in places where constant monitoring is important, such as acid mine drainage sites. Microbial fuel cells are adequate in these settings because the microbial fuel cells use the natural environment to produce electricity. Another reason we want to use a fuel cell in such a place is because a microbial cell never needs to be recharged, allowing us to collect readings from the microsensor continuously. Our studies in the lab have shown that a microbial fuel cell creates enough electricity to power a microsensor and transmit data 100 feet away to a receiver that will collect the data, such as temperature.

S03-P313
Reproducibility of *Pseudomonas aeruginosa* biofilms

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The goal of this project is to determine the reproducibility of *Pseudomonas aeruginosa (ATCC#700829)* biofilms with respect to areal and textural parameters characterizing biofilm structure. Each experiment lasts approximately four weeks and is conducted under controlled conditions. The reactors are treated identically; for example each reactor receives the same amount of feed, and both reactors are fed from the same source. The biofilms are grown in parallel reactors positioned on the stage of an inverted microscope. Images are acquired at the same time each day using a COHU Digital camera with Flash Point frame grabber. The images are then analyzed with respect to areal and textural parameters using the ISA 3 software developed by the Structure and Function Group at the Center for Biofilm Engineering. Textural parameters are calculated directly from the grayscale image, while areal parameters are calculated from a binary representation of the grayscale image. The textural parameters are energy, textural entropy, and homogeneity. The areal parameters are porosity, fractal dimension, diffusion distances, run lengths and perimeter. The analyzed data is then run through a computational statistical program using an ANOVA test. This test isolates the sources of variance introduced into the experiment and allows us to determine the extent of reproducibility between the biofilms. After running the ANOVA, a threshold for reproducibility is chosen, and the reproducibility of *Pseudomonas aeruginosa* biofilms, with respect to the calculated parameters is evaluated.

S03-P314
Modeling the interaction of biological agents in drinking water systems using AQUASIM

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Biofilms in drinking water systems have been shown to play an important role in the survival and growth of pathogenic bacteria. Water distribution systems have the ability to disseminate infectious agents or toxins widely among the population. The developed world is faced with many terrorist challenges, but one of the most troublesome involves the addition of chemical or biological agents to water distribution systems. Drinking water distribution systems could be potential targets for terrorist operations to quickly infect a large population with a chemical or biological agent. Little is known about how chemical or biological agents interact and persist in natural biofilms, or how quickly these agents could travel through water distribution systems.

The goal of this project is to detect, predict, and remove pathogenic agents from water distribution systems. Laboratory experimentation coupled with mathematical model formulae will allow for accurate determination of how these agents will propagate in a biologically active aqueous environment.

The simulation software AQUASIM was chosen to model biofilm growth due to its specialized ability to fit parameters in aqueous environments and biofilm systems. An application model was created and logistically altered to determine the interaction of hypothetical agents with a simulated biofilm. Results of our current work suggest that the AQUASIM model will be a useful tool for quantifying the transport and fate of pathogens in water distribution systems.

S03-P315
Role of oxygen limitation in *Pseudomonas aeruginosa* biofilm antibiotic tolerance

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Pseudomonas aeruginosa biofilms have been implicated in many health problems including colonization of foreign bodies such as implants and catheters, as well as causing chronic bronchitis in some cystic fibrosis patients. Treatment of these infections by usual means has often proven ineffective.

Oxygen limitation and how it affects antibiotic susceptibility in *Pseudomonas aeruginosa* biofilms was explored. *P. aeruginosa* biofilms were grown in continuous flow reactors for 72 hours. It was found that, in comparison to planktonic cells, these biofilms were protected from killing by both ciprofloxacin and tobramycin. Green fluorescent protein expression was also shown to occur only near the bulk fluid/air interface. The region of protein expression was

concurrent with the aerobic region of the biofilm as measured by microelectrodes. When the gas phase surrounding the biofilm was changed from air to pure nitrogen or to pure oxygen during treatment, susceptibility changed in some cases. Bacteria were re-suspended and treated under aerobic and anaerobic conditions. It was found that susceptibility was partially restored in both cases.