MONTANA STATE UNIVERSITY

Center for Biofilm Engineering

proceedings. . .

Technical Advisory Conference

February 8–9, 2007

Bozeman, Montana

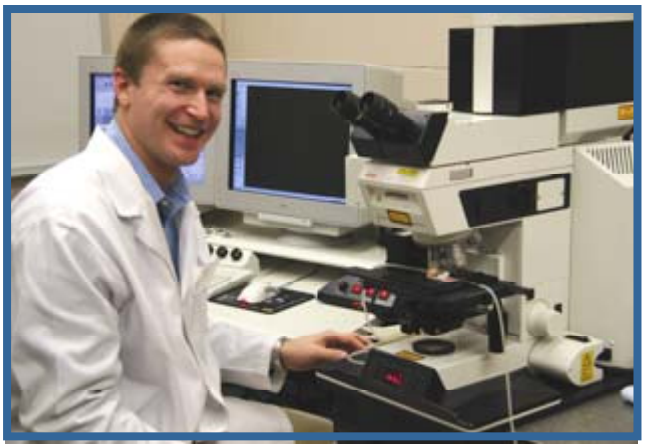
GENERAL INFORMATION

CBE LEADERSHIP

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

A BRIEF HISTORY OF THE CBE

The CBE was established in 1990 with a grant from the National Science Foundation's Engineering Research Centers program. The NSF-ERC program was created to increase U.S. industrial competitiveness and to re-invent science and engineering education in U.S. universities. Under the leadership of its founding director, Bill Characklis, the new engineering center also drew support from the state of Montana, Montana State University and industrial partners gathered during its pre-1990 work as the Institute for Biological and Chemical Process Analysis. Since the beginning, CBE researchers have been recognized nationally and internationally for leading-edge biofilm research, and for taking an interdisciplinary approach to the study of microbial growth on surfaces. In the spring of 2001, the CBE's 11-year period of NSF-ERC program support drew to a close. The CBE's continued success is built on the foundation of many years of productive university-industry-government collaboration in pursuit of its vision to be a world leader in fundamental research, science and engineering education, and industrially relevant technology.



CBE students help conduct a day of workshops offered in conjunction with each Technical Advisory Conference.

Stewart Clark received the CBE's 2007 W.G. Characklis Award during the February Technical Advisory Conference.

MISSION AND GOALS OF THE CBE

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

The CBE has identified goals in three areas of activity.

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

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

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

Industrial Associates Program Benefits

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

CBE Technical Advisory Conferences

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

Education and Training Workshops

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

Research/Testing Projects

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

Product/IP Development Consulting

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

Regulatory Interactions

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

For More Information

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

Presentation and Poster Abstracts
Technical Advisory Conference: February 8–9, 2007

Session 1: Medical/Oral Biofilms

Session 2: Biofilm Ecology

Session 3: Biofilm Methods

Session 4: Regulatory Session

Session 5: Biofilm Control

Posters

SPEAKER ABSTRACTS

SESSION 1: Medical / Oral Biofilms

W07-S03
Keynote Presentation: The role of autoinducer-2 in the development of oral multi-species biofilms

Alex Rickard, *Assistant Professor, Dept. of Biological Sciences, Binghamton University–State University of New York, Binghamton, NY*

Bacteria are not solitary units of life. They are social organisms that congregate in communities that we, as microbiologists, call biofilms. These communities can be composed of hundreds of species of bacteria and develop in nearly every environment common to nature. From the hulls of ships to the human oral cavity, bacteria within multi-species biofilm communities possess a combined metabolic potential to perform tasks that the individual component species could not achieve alone. For example, biofilms can cause corrosion of surfaces and have a 1000-fold decreased susceptibility to antimicrobials. Whilst much research has focused on the properties of laboratory grade single-species biofilms, very little is understood about the underlying mechanism that promotes the development of natural multi-species biofilm. Furthermore, the processes that contribute to the cohabitation of natural biofilms by numerous species have yet to be fully described. It is the aim of this talk to describe two forms of ubiquitous oral bacterial inter-species communication that are potentially connected. These are coaggregation (the recognition and adhesion of different oral species to one another) and the production and detection of the chemical cell-cell signal, autoinducer-2 (AI-2, an umbrella term for a family of inter-convertible signal molecules that mediate inter-species cell-cell communication). Coaggregation is a mechanism that brings together oral species into close proximity. This process is not random; instead, any one particular species of oral bacteria coaggregates with a specific group of partner species. The union allows for different species to be in close proximity. We hypothesize that coaggregation brings specific species together and AI-2 is used by the coaggregating species to detect the presence of the other. As a consequence of coaggregation, AI-2 concentration around the cells increases, and this may act as a trigger for gene expression. Depending upon the species, AI-2 concentration may induce changes in gene expression that can promote mutualism or competition interactions. With reference to recent findings by ourselves and others, a role for AI-2 in the development and maintenance of oral multi-species biofilm communities will be discussed.

W07-S04
In-vitro models of oral biofilm

Elinor deLancey Pulcini, *Research Manager, Medical Biofilm Laboratory, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

No single model is an exact replicate of the conditions that the system is designed to represent. The oral cavity contains over 500 species of bacteria living in an ecosystem with variations in pH, oxygen availability, surface characteristics, saliva flow and composition. Despite the complexity of the natural system they emulate, a number of in-vitro oral models developed and used in the CBE Medical Biofilms Laboratory have provided useful data regarding treatment regimes and have also fit the criteria regarding experimental variation. These include 96-well format models, rotating disc reactors, flow cells and drip flow reactors. Each of these model types has been adapted and used to address specific system and testing requirements. In-vitro biofilm models based on the 96-well format—including the Minimal Biofilm Eradication Concentration model (MBEC) system—are able to generate data in a relatively short period of time. This format allows for numerous replicate samples utilizing small volumes, so these are excellent choices for the preliminary screening of compounds in which the compounds are expensive or difficult to make. A disadvantage with these models is that they contain a small volume that may be affected more by variations in such parameters as temperature, oxygen or shaking. Rotating disc reactors have proven to be useful models for growing biofilms under shear fluid flow conditions. The coupons can be made from a range of materials ranging from glass to polystyrene to hydroxyapatite and provide replicate samples. Flow-through systems use flow cells in which media is pumped through the system. Flow rates (fluid shear forces) can be more easily and precisely controlled in this system. Glass flow cells have proved to be valuable instruments for real-time visualization of oral biofilms and treatment effects using the CSLM. The drip flow reactor (DFR) is designed to model a low shear environment and has been used for both single species and mixed species oral biofilm research using both glass and hydroxyapatite (HA)-coated glass slides. Some biofilms grown in this reactor have been shown to have great resistance to treatments.

W07-S05
Role of biofilms in chronic wounds

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

The incidence and prevalence of chronic wounds—diabetic foot ulcers, pressure ulcers and venous leg ulcers—have been increasing at alarming rates. Multiple factors have been implicated in the failure of these wounds to heal, many of which are the result of underlying medical conditions, such as diabetes mellitus or vascular disease. Nonetheless, patients with chronic wounds also incur wounds that heal within a normal time frame (i.e. acute wounds), indicating that other factors may be involved. The similarities between chronic wounds and biofilm diseases have led to speculation that biofilms may be involved. We examined specimens from fifty chronic wounds along with specimens from twenty acute wounds and found that those from chronic wounds were statistically significantly more likely to harbor biofilms (Fishers exact test, P=0.01). We also analyzed bacterial DNA extracted from chronic wounds and found diverse microbial communities that included strictly anaerobic bacteria. However, the role of bacteria in wound healing is poorly understood. Infection of wounds with specific pathogens such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Clostridium perfringes* has been long recognized as a barrier to healing, and the control of "bioburden" is a well-recognized aspect of wound care. Recent studies have also indicated that the production of proteinases and exotoxins by bacteria, such as *Pseudomonas aeruginosa*, may have negative impacts on chronic wounds. Some studies, on the other hand, have indicated that the presence of bacteria may play a beneficial role in wound healing. It has been proposed that sub-infective populations of bacteria promote the inflammatory response, thereby accelerating healing. Nonetheless, prolonged inflammation is also well-recognized as an important factor in delayed wound healing. Certainly research indicates that bacteria are present in all wounds, except perhaps those incurred by fetuses. Thus, normal wound healing seems to involve a balance between microbial activity and immune response. In the case of chronic wounds, impaired immune function due to age or systemic disease may shift the balance in favor of the microbes. A considerable amount of research will be necessary to elucidate the role of microorganisms and biofilms in wound healing. In the meantime, approaching chronic wounds as a biofilm disease may lead to advances in treatment of this growing problem.

W07-S06
Molecular biology of chronic wound biofilms

Pat Secor, *PhD Candidate, Cell Biology, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Chronic wounds are a frequently encountered problem in elderly and bedfast patients and are produced by trauma or pathologic insult. Chronic wounds include a loss of skin or underlying tissue which does not heal with conventional treatment. The main focus of this research was the identification of the different bacterial species that form biofilms in chronic wounds, using several different molecular techniques. Research has shown that not all bacterial species can be cultured in the laboratory; therefore the vast majority of bacteria in chronic wounds may not be detected by simple culture tests. For example, strict anaerobes have not been successfully cultured from debridement samples in the clinical laboratory. Fresh debridement samples were provided from different areas of the wound bed to analyze the spatial heterogeneity of wound biofilms. DNA was extracted from the debridement samples, and primers that targeted the conserved regions of the bacterial 16S ribosomal subunit gene were used to amplify segments of the 16S genes using the polymerase chain reaction (PCR). The amplified 16S DNA population was then analyzed using denaturing gradient gel electrophoresis (DGGE). This method provided the means to estimate the diversity of the microbial populations inhabiting these chronic infections. Some of the 16S gene fragments were also cloned and sequenced to determine which species of bacteria were present. The DNA from strictly anaerobic bacteria were found to be present in the debridement samples. The specificity of different primer sets was also considered: some primer sets were more specific towards the 16S gene than others. Primer sets that were less stringent pulled out genes that came from various organisms including host, canine, and mouse genes. Primer sets that were more specific towards their targeted region in the 16S gene pulled out 16S genes with 80% efficacy. These results indicate that the use of molecular-based methods may prove to be a helpful diagnostic tool to assess wounds for the presence of bacterial species not usually detected using traditional culture-based methods.

SPEAKER ABSTRACTS

W07-S07

Biomaterials and bacteria: Strategies for medical devices

Buddy Ratner, *Director, University of Washington Engineered Biomaterials (UWEB), Seattle, WA*

Millions of implanted biomaterials are used in humans each year. Though medical devices made of synthetic biomaterials are largely successful, bacterial infection remains a chronic problem costing the healthcare system millions of dollars and killing thousands. The central foci of this lecture are medical devices, bacteria and what we can do to address this problem. Bacteria attach to most any surface, proliferate after adhesion, modulate their phenotype and exude a self-protective biofilm. Bacteria employ multiple mechanisms for attachment, implying that surfaces useful for inhibiting bacterial colonization may also require a set of "tricks" to foil the multipronged bacterial sticking. Combining methods may create surfaces that anticipate bacterial adhesion/proliferation and address the problem before strong adhesion and growth can be established. Non-fouling surfaces function by inhibiting adsorption (often proteinaceous) to the substratum. Non-fouling surfaces have been synthesized by RF-plasma grafting of thin films resembling poly(ethylene glycol)(PEG). These surfaces almost completely inhibit initial bacterial attachment. Other types of non-fouling surfaces are also being explored. To deal with low levels of bacterial adhesion on even a non-fouling surface, a Ciprofloxacin™-releasing material has been developed that elutes the antibiotic at low levels over long times. This is accomplished by an RF-plasma deposition to coat a hydrophobic barrier on a Cipro (or other antibiotic)-loaded core. At the surface, the level of Cipro induces 100% bacterial kill. Micrometers from the surface, the Cipro concentration is diluted and has little effect on non-adherent bacteria. By using a sustained-release approach, the antibiotic dosage is minimized and so is the possibility of promoting resistant strains. Another variant of this rate-limiting release barrier approach, when activated by ultrasound, releases Cipro upon demand. This strategy, explored in collaboration with Montana State University, shows promise for the removal of mature biofilms.

SESSION 2: Biofilm Ecology

W07-S09

Heterogeneity and distribution of biofilm on reverse osmosis and nanofiltration membranes

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

Membrane filtration enables the production of safe potable water by removing microorganisms, inorganic and organic compounds. Biofilm formation on reverse osmosis and nanofiltration (RO/NF) membranes raises the applied filtration pressure and causes a flux decline. To observe the biofilm formation potential on membranes, one RO membrane (BW30) and one NF membrane (FilmTec Corp., Dow Chemical Co.) were examined in a two-phase study. The study was carried out in three Rotating Disk Reactor (RDR) systems where membrane samples were affixed to reactor coupons. Each reactor was operated for 31 days continuously during Phase-I and for 28 days during Phase-II. To enhance the growth of biofilm, feed water consisted of biologically treated water amended with nutrients. The average CFU/ml in the BAC treated water was 1.4~2.0 x 10^4 cfu/ml. The temperature was ambient (25 ^oC) and a rotational speed of 50 rpm was used in all reactors. For analysis, 5.0 mm membrane slices were cryo-sectioned and observed with an epifluorescence microscope with a 20X object. LIVE/DEAD BacLight™ Bacterial Viability Kit (Molecular Probes) was used for live/dead staining of cells on the surface of the membrane. The stained membranes were observed under the epifluorescence microscope with a 100X objective. Contact angle measurements were performed during Phase-II; the advanced and receding angles were measured at 10 different points on the surface of the membranes. After drying and coating by gold (Au) and palladium (Pd), SEM images were taken. During Phase-I, AFM analysis and, during Phase-II, XPS analyses were done on the surface of membranes before use and after removing the biofilm. Biofilm thickness in Phase-I was obtained at 31 days and the overall accumulation rate was lower than in Phase-II. In Phase-II, the biofilm accumulation rate was measured from cryo-sectioning analysis, and was greater initially than at the end of operation (21 days). This suggests that biofilm thickness continues to decline with time. AFM images illustrated that the microporous and mesoporous spaces of the

membranes were blocked and/or covered by biofilms. Usually the formation of biofilm along the micro-channels raised the applied filtration pressure as well as flux decline. There was no significant change of hydrophobicity of the NF surface after 28 days, which indicates that physical cleaning alone was sufficient to maintain the same surface properties. In the case of the RO membrane, the hydrophobicity increased from 15^{o} to 32^{o}. This increase of contact angle indicated the loss of membrane permeability due to the attached biofouling macromolecules and that physical cleaning alone was not adequate. SEM images indicated that the coverage of biofoulants on membrane surfaces increased with time and that bacterial cells can be directly attached to the surface or integrated into the EPS matrix which forms a mat of biomass. Within a few days after membrane exposure to nutrients and bacteria, the carbon content on the surface was reduced to 30–35%, which was confirmed by XPS analysis. After that, insignificant change was observed, indicating that adhesion and EPS interaction did not further influence the amount of carbon on the surface of the membranes. The amount of silica increased from 0–10.2%. Reproducibility of Phase-II research is good, based on the results of live/dead images of cells and also average thickness or accumulation rate by cryostat analysis. Currently, membrane researchers consider biofilm formation to be equivalent to biofouling and treat it as a "black box." A better understanding of the progress and growth of biofilms would decrease operational costs and membrane replacements and increase process performance. Although the analyses described here are somewhat simplistic, they provide one approach toward better understanding membrane biofilm formation and biofouling.

W07-S10
Escherichia coli 0157:H7 requires colonizing partner for biofilm formation and development

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

E. coli 0157:H7 is an important pathogen both in food and water systems. We examined the ability of an *E. coli* 0157 strain (tagged with DsRed) isolated from a drinking water distribution system to colonize a glass capillary flow cell and further develop microcolonies when grown alone and in combination with *Pseudomonas aeruginosa* (tagged with GFP). *E. coli* was unable to attach to the glass surface after 48 hours of continuous cell inoculation. However, when inoculated simultaneously with *P. aeruginosa* cells (co-inoculation), confocal microscopy revealed that *E. coli* immediately (<15min) adhered to the surface of the glass, with an initial attached population of 1.4×10^{3} cells/cm^{2}. When *E. coli* cells were introduced into a flow cell pre-colonized with a steady-state *P. aeruginosa* biofilm (pre-colonization), approximately an order of magnitude more cells were captured than in the co-inoculated case, with an initial attached population of 2.1×10^{4} cells/cm^{2}. The predominant location of captured cells was between the mature *P. aeruginosa* biofilm and the glass surface, not at the biofilm-nutrient interface as previously hypothesized. Both species were monitored non-destructively by time-lapse confocal microscopy, direct microscopy of filtered effluent, and effluent plate counts. Despite more *E. coli* cells initially present in the pre-colonized system, *E. coli* generally was only able to develop into microcolonies in the co-inoculated system. Image analysis software was used to calculate volumes of each species over time, and revealed a repeatable pattern of formation whereby *E. coli* predominantly occupied the outer 200 microns of the flow path, while *P. aeruginosa* occupied the centerline (500 microns). While *E. coli* comprised only about 1% of the total biomass in the reactor, it occupied, in some cases, more than 50% of the total biomass at the edges of the flow path. This research presents a novel engineering approach to quantifying and modeling growth at multiple scales of observation from the reactor-level to the single-cell level.

W07-S11
Retention of a model pathogen in a porous media biofilm

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

The introduction of bacterial pathogens into drinking water systems from inadvertent and deliberate addition can lead to potential public health consequences. There is little information available on the entrainment of these organisms in engineered biofilm systems. In this study, a porous media biofilm reactor was used to investigate the ability of an established mixed-species drinking water biofilm to trap *E. coli* 0157:H7 as a model pathogen. This opportunistic human pathogen has the ability to survive in drinking water distribution systems by

integrating into biofilms. Quantitative PCR targeting the Shiga-like toxin 1 (stx1) gene, plate counts, and direct microscopic counts was used with a cyan-labeled *E. coli* O157:H7 strain to determine the fraction of organisms retained in a natural biofilm grown under different nutrient conditions in a porous media reactor. Threshold cycle (CT) values from quantitative PCR were correlated with plate counts and direct counts. The results will be compared with those where inert fluorescent latex beads have been added to reactors as a surrogate for bacteria. Control and test experiments were performed to differentiate between inherent trapping by the reactor and biofilm-induced trapping. Control reactors were sterilized and fed with filter-sterilized, dechlorinated drinking water, while test reactors were fed with an inoculum of drinking water distribution system microorganisms supplemented with organic carbon at 0.5 mg/l. Biofilm was established in the test reactors for either two or three weeks prior to inoculation. An inoculum of approximately 109 *E. coli* 0157:H7 cells was added as a 1 ml slug dose to porous media biofilm reactors being fed at 25 ml/min. Five effluent pore volumes were collected in half-pore volume increments before draining the reactor and performing destructive sampling to enumerate the retained fraction. Plating was done immediately upon collecting samples. Direct counts and DNA extractions were done within twenty-four hours with the samples stored at 4˚C. As in the experiments conducted with fluorescent latex beads, most of the inoculum passed through the reactor for control experiments. Test experiments revealed a correlation between the duration of biofilm formation and pathogen trapping efficiency. The percent recovery based on plate counts—which, for control experiments, was near 100%—varied appreciably for experiments with pre-colonized biofilm. Additionally, Ct values from quantitative PCR showed inhibition for test experiments when undefined environmental biofilm was present.

SESSION 3: Biofilm Methods

W07-S13
Using flow cytometry to distinguish between live and dead cells

Anne Camper, *Professor, Civil Engineering, Center for Biofilm Engineering; Associate Dean of Research, COE, Montana State University, Bozeman, MT*

An issue of critical concern in microbiology is the ability to detect viable cells by methods other than culturing. It is well known that traditional culture methods for detecting indicator and pathogenic bacteria in food and water may underestimate numbers due to sublethal environmental injury, inability of target bacteria to take up nutrient components in the medium, and other physiological factors which reduce culturability. Alternatives are needed—approaches that reduce selectivity, decrease bias from sample storage and incubation, and reduce assay time. Flow cytometry (FCM) is a sensitive analytical technique that can rapidly monitor physiological changes of microorganisms, especially when related to membrane integrity. Optimized flow cytometry was used for detecting the viability of several bacterial species through the selection of appropriate dyes and operating conditions. It was assumed that cells having intact membranes are alive and those with damaged membranes are dead or theoretically dead. To improve the ability to detect cells with intact membranes, a nucleic acid double-staining assay based on analytical flow cytometry was developed. The method required assays to select the best dye for several bacterial species as well as optimized instrument settings to minimize noise and signals from detritus. By comparing cell numbers from FCM with those that can be cultured, it is possible to identify the number of individual cells in different physiological conditions. Using heat treatment, cells were stressed and damaged, and the methods established using these mid-log phase cultures were used to determine the fraction of live, dead (membrane compromised) and culturable cells. Two complete sets of data to enumerate the viable but non-culturable (VBNC) and viable-culturable (VC) stages of gram-negative bacteria were obtained. The best dyes were identified based on greatest sensitivity. The main objectives of this research were to establish the quickest, most accurate and easiest ways to estimate the proportions of VBNC, VC, and dead cells as indicated by membrane integrity. This research was carried out on four gram-negative bacteria of concern in the water and food industry (*E. coli* O157:H7, *P. aeruginosa*, *P. syringae*, and *S. typhimurium*). Staining

was done with five separate dyes; SYTO 9, STYO 13, SYTO 17 and SYTO 40 (which stain all cells) and propidium iodide (PI) (which stains only dead cells or cells with damaged membranes). The premise was that different organisms would be optimally stained by some but not all dyes because the nucleic acid binding capacity of these dyes differs. By comparing results from pure cultures cultured at mid-log phase, 1–64% of cells were non-culturable, 40–98% were culturable, and 0.7–4.5% had damaged cell-membranes and were therefore theoretically dead. The results illustrate the usefulness of flow cytometry for the rapid detection of bacteria in various states of membrane damage. The specific methods most likely can be applied directly to other organisms, or modest modifications may be required for optimization.

W07-S14
Use of propidium monoazide for live-dead distinction in microbial ecology

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

During the last decade cultivation-independent molecular techniques have provided new insights into the composition of bacterial communities. This is critical because uncultured organisms comprise the vast majority of the microbial world. Genotypic profiling of PCR-amplified DNA extracted from environmental samples has become a commonly used molecular methodology in microbial ecology. Techniques like denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP) analysis are used to study microbial communities from various matrixes and community dynamics as a result of changing environmental parameters. One of the prerequisites to obtain meaningful data, however, is to limit analysis to the live portion of microbial communities. Only if the profiles reflect the current community status can changes in community patterns and responses of the community composition to changing conditions be interpreted. The obstacle in this respect is that DNA in the environment can be very stable and can persist for extended time periods even when cells have lost their viability. Propidium monoazide (PMA) has recently been shown to be useful in combination with quantitative PCR for bacterial live-dead distinction. This chemical is a membrane-impermeant dye that is efficiently excluded from cells with intact membranes. However, it readily penetrates cells with compromised membranes, which can be considered dead. Once inside the cells, PMA intercalates into the DNA and can be covalently cross-linked to it upon light exposure. This modification of DNA from dead cells strongly inhibits PCR amplification. Consequently, the analysis of bacterial communities can theoretically be limited to cells with intact cell membranes. This study was performed to evaluate the benefit of treating community samples with PMA using end-point PCR in combination with DGGE. Four experiments were performed to study the usefulness of PMA treatment of mixed bacterial communities comprising both intact and compromised cells in combination with end-point PCR: (1) profiling of defined mixtures of live and isopropanol-killed cells from pure cultures of random environmental isolates; (2) profiling of a waste water treatment plant influent sample spiked with defined ratios of live and dead cells; (3) profiling of selected environmental communities and (4) profiling of a water sediment sample exposed to increasing heat stress. Regions of 16S rRNA genes were PCR-amplified from extracted genomic DNA, and PCR-products were analyzed using denaturing gradient gel electrophoresis (DGGE). Results from the first two experiments show that PMA treatment can be of value with end point PCR by suppressing amplification of DNA from dead cells. The last two experiments suggest that PMA treatment can affect banding patterns in DGGE community profiles and their intensities, although the intrinsic limitations of end point PCR have to be taken into consideration.

W07-S15
Magnetic resonance microscopy analysis of biofilm polymer dynamics and bioreactor transport

Sarah Codd, *Assistant Professor, Mechanical and Industrial Engineering, Montana State University, Bozeman, MT*

Magnetic resonance microscopy (MRM) allows characterization of the spatial heterogeneity of materials on the 10 μm imaging scale of molecular-level properties such as magnetic relaxation and translational molecular diffusion which provide nanometer-scale molecular dynamic information. Several groups have applied MRM techniques to study transport in biofilm systems. Dynamic MRM has been used to characterize the bulk advective transport in a biofilm capillary reactor. The biofilm generates non-axial flows that are up to 20% of the maximum axial velocity, indicating mixing by a complex flow field. The characteristics of the

transport indicate a scaling of the observed oscillatory flow with the capillary diameter and hydrodynamic similitude; ways of statistically evaluating these observations are being explored. Understanding the basic aspects of the fluid dynamics in biofilm-impacted systems will allow for advances such as controlled transport of antimicrobial agents over a range of system sizes by applying scaling relations to design systems with known hydrodynamics after biofilm growth. Of primary interest in this presentation is the treatment from a materials science perspective of the EPS as a polymeric gel. The biofilm matrix influences macroscale transport through the material properties of the EPS gel, impacting the boundary condition for momentum, mass and energy conservation at the wall. Magnetic resonance microscopy (MRM) techniques are capable of spectrally and spatially resolving diffusion coefficients, and their application to polymeric materials is well established. We are applying these methods to the biofilm matrix and observing the impact of antibiotic activity on the diffusion of the various spectral peaks. The detailed composition of the EPS is unknown but contains polysaccharides, proteins and even DNA. The polymer translational diffusion indicates multiple populations of polysaccharides and other biopolymers with diffusion coefficients ranging from the order of 10^{-9} to 10^{-13} m^2/s, indicating a broad size range and composition of biopolymers. The amount of biopolymeric substance in highly restricted environments due to polymer size or gel crosslink structure can be determined from the spectrally resolved diffusion data. By observing changes in the diffusion, we are able to monitor variations in the polymer material properties during aging and various antimicrobial treatments to determine whether the antimicrobial is acting by changing the cell or components of the EPS structure. Spatial resolution of NMR spectra has been used to infer metabolism in biofilms as a function of depth. Extension of our spectrally resolved diffusion experiments to the spatial dimension measures changes that occur in the biofilm material structure as a function of depth due to environmental conditions and antimicrobial assaults.

SESSION 4: Regulatory Session

W07-S17
Regulatory methods: The registration and efficacy evaluation of biofilm disinfectants

Marc Rindal, *Microbiologist, Office of Pesticide Programs, EPA, Fort Meade, MD*

The U.S. Environmental Protection Agency (EPA) recognizes biofilm as a pest. Therefore, products which claim to prevent, destroy, repel, or mitigate biofilm require registration under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). As a component of registration, product efficacy must be demonstrated to support a specific claim. The EPA's Office of Pesticide Programs has responsibility for regulating antimicrobial products (pesticides) and is evaluating options for the registration of biofilm disinfectants. As part of this initiative, the OPP Microbiology Laboratory is developing capacity to conduct and evaluate available protocols for growing and treating biofilm. The laboratory is currently conducting ASTM Standard Method E2196, Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with Shear and Continuous Flow Using a Rotating Disk Reactor. Alternative scraping methods/materials for biofilm removal and an enumeration technique using 3M Petrifilm™ will be discussed. Practices related to quality control and standardization will be emphasized.

W07-S18
Antimicrobial-coated medical devices: Regulatory perspective

Chiu Lin, *Division Director, Anesthesiology, General Hospital Infection Control and Dental Devices, Center for Devices and Radiological Health, FDA*

Biofilm in medical devices is a common occurrence in many implants, indwelling catheters and some reusable complex medical devices. Biofilm in these devices plays an important role in device-induced infection. The control of the biofilm formation in medical devices is a subject of interest to many device manufacturers, researchers, and public health workers. Current strategies designed to control biofilm formation include: antimicrobial coated or impregnated devices; surface modification of device material; chemical (disinfectant) and heat treatment; and filtration of water supply for medical procedures. It is uncertain whether these strategies

are clinically effective. Currently, there is no standardized and scientifically sound validation test method and criteria that can be used to assess the effectiveness of these strategies in controlling biofilm formation, especially in clinical situations. The FDA encourages an open discussion, such as this conference, and offers an opportunity for collaboration to arrive at clinically meaningful evidence to support clearance or approvals of products with biofilm claims.

W07-S19
Strategic plan for creating standardized biofilm methods

Darla Goeres, *Senior Research Engineer, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

This presentation will provide an overview of the work being done in the standardized biofilm methods research area. In October 2006, a strategy for the approval of biofilm methods was proposed to ASTM Subcommittee E35.15. A review of this strategy and the status of the biofilm methods currently under review by E35.15 will be presented. In addition, the presentation will include a report on the progress of the method for testing the efficacy of dental unit water line disinfectants and growing a repeatable *Staphylococcus aureus* biofilm in the drip flow reactor. Finally, an update on the steering committee for regulatory test methods for biofilm disinfectants will be given.

SESSION 5: Biofilm Control

W07-S21
Activities of ceragenins in eradicating biofilms and preventing biofilm formation

Paul Bennett Savage, *Professor, Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT*

Bacterial biofilms are the causative agents of many chronic infections, including medical-device related infections. Conventional antibiotics, while effective against planktonic bacteria, are often much less effective in eradicating biofilms. We have developed a new class of antimicrobial agents called ceragenins, which are derived from a cholic-acid backbone. The ceragenins are bactericidal against a broad spectrum of Gram-negative and Gram-positive strains, including *Acinetobacter baumannii*, *Clostridium difficile*, tobramycin-resistant *Pseudomonas*, MRSA, VRE, and VRSA. They also display activity against fungi and lipid-enveloped viruses. While these membrane-active compounds mimic the activity of LL-37 and other antimicrobial peptides, they are aminosterols—not peptides—and are far simpler and less expensive to produce in quantity. Lacking peptide bonds, they are also not subject to protease degradation and are stable even at high temperatures. Ceragenins may be used as a part of a medical device coating or incorporated into a polymeric material itself to provide long lasting protection against bacterial colonization and biofilm formation. Ceragenins selectively target bacterial membranes and are capable of eradicating existing biofilms at concentrations close to their MIC values. Because the ceragenins are surface active and do not need to enter into the bacteria to effect cell death, these antimicrobial agents retain activity when covalently attached to polymers. Attachment of ceragenins to a polymer decreases their high water solubility and provides a mechanism of sustained release from a medical device. Cerashield™, a medical-grade polyurethane foam coated with a polyurethane-ceragenin conjugate, provides sustained release of the highly bactericidal polymer for over 21 days in an aqueous environment and completely eradicates inocula of 10^6 CFU of MRSA in solution in under 6 hours throughout repeated daily testing. Use of this coated foam may prevent medical-device related infections by eliminating bacteria at skin-device interfaces over extended periods. In-vitro testing has shown Cerashield™ to be more effective and of longer duration than a chlorhexidine-based comparison product. The FDA's Office of Combination Products has designated Cerashield™ as a combination product assigned to CDRH as the lead agency to be reviewed as a PMA.

SPEAKER ABSTRACTS

W07-S22
Analysis of antibiotic tolerance mechanisms in staphylococcal biofilms

Suriani Abdul Rani, *NovaCal, Inc.; recent MS graduate, Chemical and Biological Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Spatial patterns of DNA synthetic activity were imaged and quantified in *Staphylococcus epidermidis* biofilms using immunofluorescent detection of pulse-labeled DNA. This method revealed highly stratified patterns of DNA synthetic activity, revealing regions in which bacteria were active and regions in which bacteria were present but exhibited no activity. In a colony biofilm system, the dimension of the zone of anabolism at the air interface ranged from 16 to 38 microns, and corresponded with the depth of oxygen penetration measured with a microelectrode. A second zone of activity was observed along the nutrient interface of the biofilm. Much of the biofilm was anabolically inactive. To test whether the inactive cells were protected from killing by antibiotics, colony biofilms were treated with rifampin, ampicillin, or ciprofloxacin and then pulse-labeled to reveal the pattern of DNA synthetic activity. The resulting patterns support the interpretation that the antibiotics were able to penetrate the biofilm, but were not able to kill bacteria well, even in the replicating regions of the biofilm. Antibiotics reduced DNA synthetic activity in the aerobic and anaerobic zones of the biofilm to similar extents.

W07-S23
Visualization of antimicrobial action in biofilms

Willy Davison, *PhD candidate, Chemical and Biological Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

The action of antimicrobial agents against bacterial biofilms of *Staphylococcus epidermidis* was visualized by a time-lapse microscopy technique that allowed spatial and temporal patterns to be discerned non-invasively. Biofilm was grown in a continuous-flow glass capillary reactor for 24 h, and then pre-stained with Calcein-AM. This fluorogenic esterase substrate loads cells with an unbound green fluorescent dye that remains trapped inside the cell as long as the cell membrane is intact. If membrane integrity is compromised—for example by an antimicrobial agent—the dye leaks out and the cell becomes dark. Using confocal scanning laser microscopy, the action of glutaraldehyde, chlorine, a quaternary ammonium biocide, and the antimicrobial peptide nisin were observed under flow conditions. Each antimicrobial exhibited a distinct spatio-temporal pattern of action in biofilm clusters. During chlorine treatment, fluorescence loss occurred in a small (~10 micron) layer at the periphery of the biofilm and progressed toward the center while the clusters were simultaneously eroded. This pattern could be attributed to limited penetration of chlorine due to a reaction-diffusion interaction. Treatment with the quaternary ammonium compound resulted in biphasic loss of fluorescence in biofilm clusters. A fraction of the cell population mostly located in the interior of the clusters remained bright for a longer time. This pattern suggests two populations within the biofilm: one that is rapidly permeabilized by the agent and a second that is much less susceptible. During glutaraldehyde treatment, biofilm clusters maintained most of the initial fluorescence. This retention of fluorescence suggests that this biocide does not cause cellular envelope permeabilization. Treatment with the antimicrobial peptide nisin resulted in rapid, uniform fluorescence loss within the biofilm clusters. The rapid fluorescence loss during this treatment implies that all the cells within the biofilm lost their membrane integrity. Image analysis was applied to extract estimates of the penetration time and to quantify relative rates of action of the antimicrobial agents.

W07-S24

A 3D computer model analysis of three hypothetical biofilm detachment mechanisms

Jason Chambless, *recent PhD graduate, Chemical and Biological Engineering, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Three hypothetical mechanisms of detachment were incorporated into a three-dimensional computer model of biofilm growth and development. The model integrated processes of substrate utilization, substrate diffusion, growth, cell advection, and detachment in a cellular automata framework. The purpose of this investigation was to characterize each of the mechanisms with respect to four criteria: the resulting biofilm structure, the existence of a steady state, the propensity for sloughing events, and the dynamics during starvation. The three detachment mechanisms analyzed represented various physical and biological influences hypothesized to affect biofilm detachment. The first invoked the concept of fluid shear removing biomass that protrudes far above the surface and is therefore subjected to relatively large drag forces. The second pathway linked detachment to changes in the local availability of a nutrient. The third pathway simulated an erosive process in which individual cells are lost from the surface of a biofilm cell cluster. The detachment mechanisms demonstrated diverse behaviors with respect to the four analysis criteria. The height dependent mechanism produced flat, steady state biofilms that lacked sloughing events. Detachment based on substrate limitation produced significant sloughing events. The resulting biofilm structures included distinct, hollow clusters separated by channels. The erosion mechanism produced neither a non-zero steady state nor sloughing events. A mechanism combining all three detachment mechanisms produced mushroom-like structures. The dynamics of biofilm decay during starvation were distinct for each detachment mechanism. These results show that detachment is a critical determinant of biofilm structure and of the dynamics of biofilm accumulation and loss.

W07-P375
Microbially enhanced geologic sequestration of supercritical CO_2

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Geologic sequestration of CO_2 is one strategy to reduce the emission of greenhouse gases generated through the combustion of fossil fuels. Geologic sequestration of CO_2 involves the injection of CO_2 into underground formations such as oil-bearing formations, deep un-minable coal seams, and deep saline aquifers. There are several conditions which must be met for successful sequestration in these formations including: 1) temperature and pressure conditions must be such that CO_2 will be supercritical, 2) the aquifer must have a suitable aquitard trap, and 3) the receiving aquifer should have appropriate porosity and permeability. During operation of geologic CO_2 sequestration sites, supercritical CO_2 (sc CO_2) would be injected into the receiving formation, resulting in elevated pressure in the region surrounding the point of injection. As a result, an upward hydrodynamic pressure gradient may develop across the trapping aquitard. Upward "leakage" of CO_2 could occur due to the primary permeability of the aquitard, through fractures or near injection wells.

The research to be presented is part of the Zero Emission Research and Technology (ZERT) program, a collaborative research effort focused on understanding the basic science of underground carbon dioxide storage. ZERT is a partnership involving DOE laboratories (Los Alamos National Laboratory, Lawrence Berkeley National Laboratory, National Energy Technology Laboratory, Lawrence Livermore National Laboratory, and Pacific Northwest National Laboratory) as well as Montana State University and West Virginia University. The research to be presented is based on the hypothesis that engineered microbial biofilms can be used to significantly decrease the permeability of high permeability areas in deep aquifers, such as near well casings or in fractures and increase the mineralization (i.e. carbonate formation) after sc CO_2 injection. This part of ZERT focuses on microbially based strategies and technologies for controlling leakage of supercritical CO_2 (sc CO_2) during geologic sequestration. We used a high pressure (1300 psi), moderate temperature (≥ 32 °C) system to grow biofilms in 2.54 cm diameter, 5 cm long, 40-millidarcy Berea sandstone cores. Biofilm growth resulted in a two order-of-magnitude reduction in permeability and permeability did not increase significantly in response to starvation and scCO_2 challenges. The inoculum of *Shewanella fridgidimarina* was replaced by other species that were likely introduced with the sandstone core such as *Bacillus mojavensis* and a *Citrobacter* sp., both known biofilm- and EPS-forming subsurface organisms. Light, epifluorescence and electron microscopy of the rock core revealed substantial biofilm accumulation in rock pore channels. Viable population assays of organisms in the effluent indicate survival of the bacteria after sc CO_2 challenges. These observations are encouraging for the prospective use of engineered biofilm barriers for controlling leakage of geologically sequestered CO_2. Future research will continue to examine the concept of using engineered microbial biofilm barriers as a method for 1) reducing permeability of confining aquitard layers in the vicinity of sc CO_2 injection, 2) providing a zone of reduced permeability to mitigate further migration of CO_2 leaks which have penetrated the aquitard and reached overlying strata, and 3) stimulating irreversible precipitation of mineral phases for long-term permeability reduction.

W07-P390
The influence of cellulose utilization on metal and radionuclide mobility and reduction

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Cellulose is one of the most abundant forms of biomass. Cellulose utilization by microorganisms has become an important aspect of many bioprocesses, including biotransformation into alternative fuels, waste management, and involvement in the pulp and paper industry. In addition, cellulose utilization may play an important role in contaminant mobility and reduction at many Department of Energy low level waste (LLW) sites. LLW sites contain cellulose waste which may be present in the form of paper towels, Kimwipes, and cardboard. This waste is often contaminated with heavy metals and radionuclides such as chromium and uranium. Previous research has shown that many fermentative bacteria are capable of degrading these cellulolytic materials and producing C-6 sugars, such as glucose, as well as short chain organic acids,

alcohols, and hydrogen. Furthermore, some bacteria, such as *Cellulomonas spp*. ES6, are capable of reducing chromium and uranium to less soluble forms. Unfortunately, the organic acids produced through cellulose degradation can potentially increase the mobility of these metals and radionuclides through chelation. To date, these interactions have received little attention and may hold important information that could influence remediation processes at LLW sites. Using ICP-MS analysis and a diphenylcarbazide assay, abiotic studies have been conducted to determine sorption isotherms for a chromium-cellulose system. This data provided a baseline that is needed for future studies determining chromium speciation and mobility in systems containing *Cellulomonas spp*. ES6. In addition, *Cellulomonas spp*. ES6 biofilms have been grown on cellulose-coated slides in a drip flow reactor using simulated groundwater medium. This demonstrates the ability of *Cellulomonas spp*. ES6 to grow in biofilms on a cellulose-coated surface. Future work will examine cellulose utilization by these biofilms with cellulose being the sole carbon source and in the presence of chromium and uranium. Chromium toxicity tests have also been conducted with *Cellulomonas spp*. ES6 to determine its growth response to hexavalent chromium with concentrations ranging from 50 to 500mg/L Cr(VI). Future plans for this research include comparing batch experiments with biofilm and column studies and their influence on chromium and uranium mobility and reduction. Different cellulose sources as well as cellulose utilization products will also be studied.

W07-P391
Inhibition of biofilm formation using lactoferrin and xylitol

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The influence of lactoferrin and xylitol were evaluated using an in-vitro laboratory model system to simulate wound biofilms. Lactoferrin, found in milk and exocrine secretions, has therapeutic properties that potentially include antibacterial, antibiofilm, antiviral, antifungal, anti-inflammatory, antioxidant and immunomodulatory activities. Xylitol has been shown to impact both dental plaque and in-vitro model biofilms. This research evaluated these compounds for the prevention of biofilm formation by chronic wound isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in a flow cell system. This system enabled imaging of the biofilms and integrated sample coupons enabled quantitative analysis by plate count. Log Reduction (LR) in biofilm cell density for each treatment was calculated relative to untreated controls. LR values for xylitol (10% w/v) reduced biofilm formation by 1.45 for *S. aureus* and 1.90 for *P. aeruginosa*. LR values for lactoferrin (0.02%) prevented biofilm formation by 0.75 for *S. aureus* and 1.8 for *P. aeruginos*. Overall, these results demonstrate that lactoferrin and xylitol inhibit biofilm formation by bacteria that are common in chronic wound infections and may have potential for wound therapy. On-going research will further evaluate the effects of lactoferrin and xylitol as well as combinations of these agents on biofilms.

W07-P392
Development of immobilized chitosan-coated beads for biogrowth control

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The main purpose of this research was to observe how chitosan may prevent the growth of biofilm in a porous media reactor, as well as its ability to reduce suspended bacterial counts and improve filtrate quality. Chitosan (poly b-(1 → 4) N-acetyl-D-glucosamine) is a deacetylated product of chitin obtained from crab or krill. Reactor C-1 contained 3 mm glass beads without coating; Reactor C-2 had glass beads coated only by Rhoplex; and Reactor C-3 contained beads coated with Rhoplex emulsion and 1% chitosan. The volume of each reactor was 1072 ml, and the hydraulic retention time (HRT) was 5 hrs. The chitosan-coated bead reactor (C-3) reduced 99~99.9% (2~3 log) of the influent cells for nearly four weeks, but the C-2 and C-1 reactors were ineffective after one week. These results show that the beads can capture cells at a significant rate for a very specific time period, after which their efficacy declines. From the stereoscope images, the thickest biofilm was formed on the chitosan-coated beads. The effluent total organic carbon of C-3 reactor was the lowest prior to the increase in bacterial growth; there was no change or a slight increase in TOC across the other two reactors. During the first few days, it appears that there was release of material from the chitosan coating which led to a spike in turbidity, and then decreased to the lowest value of the three reactors. Interestingly, this increase in turbidity did not correlate with a detection of chitosan or chitosan fragments by HPLC. The

average ORP values of the influent and effluents of the C-2 & C-1 reactors were 215±20 mV, but the initial ORP of the effluent of the C-3 reactor was very high (285mV). This value decreased sharply with time and at the end of operation the ORP was 245 mV. The probable reason for this decrease is a decline in the number of chitosan reactive sites that influence the potential for oxidation and reduction. To assay for the release of chitosan or its breakdown products, the influent and effluents were assayed by HPLC. The molecular weight of constituents in the influent varied from 7,003,594 D to 10,711D, but that of the effluents of all three reactors were below the detection limit. The oligosaccharides from the BAC, C1 and C2 reactors varied from 15 D to 100 D (lower than the MW of a chitosan monomer) and those from the C3 reactor were below detection initially and then rose slightly to 12 D which is still smaller than a chitosan monomer. These results suggest that the bead column system is good at capturing higher molecular weight dissolved molecules.

Biofilm on the beads was extracted in three different layers (bottom, middle and top layers) of each reactor. The heterotrophic plate counts (HPC) of the C-1 and C-3 reactors were the highest. The extracted DNA was also quantified and the C-3 reactor values were the highest in each layer compared to layers in the C-2 and C-1 reactors. To obtain an initial understanding of the community structure in the three reactors in the three layers, the extracted biofilm DNA was analyzed via denaturing gradient gel electrophoresis gels (DGGE). The community structure varied between reactors, but did not vary within a single reactor. Cloning of samples gave a 56-clone library of each sample. We are now using DNA sequencing to obtain information about the respective species. Parallel to the community analysis, batch experiments using indigenous organisms in BAC-treated water and different Gram-negative bacteria (*E. coli* O157:H7, *P. aeruginosa*, *S. typhimurium*, and *P. syringae*), Gram-positive bacteria (*S. epidermidis* and *S. mutans*) and yeasts (*C. albicans* and *S. cerevisiae*) were done to obtain the disinfection kinetics for three different doses (51.5 ppm, 103.0 ppm and 154.5 ppm) of chitosan. The selection of these doses was based on the amount of chitosan added on the beads in C-3. The initial concentration of cells was 10^4 cfu/ml. The disinfection kinetics followed the first order decay equation and fit the Delayed Chick-Watson model. In the absence of bacteria, the chitosan was stable but declined in contact with cells. The disinfection rate constant, 'k' and 'CT' for 90% inactivation of cells were determined. The most important parameter is the ratio between cell and available chitosan. The chitosan-coated porous bead column can be used not only for polishing purposes, but also can be added with other filtration and adsorption devices to improve influent quality to drinking water quality standards.

W07-P393
To build a microbial factory: Investment cost and operating cost analysis of metabolic networks

***R. Carlson**, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

Evolutionary success requires strategic allocation of scarce resources. Under conditions of nutrient sufficiency, metabolic networks are well known to be regulated for thermodynamic efficiency; however, efficient biochemical pathways are anabolically expensive to construct. A cost-benefit analysis of an in silico *Escherichia coli* network revealed the relationship between pathway proteome synthesis requirements, thermodynamic efficiency, substrate affinity, and DNA coding sequence length. Pathway proteome synthesis requirements appear to have shaped metabolic network structure and regulation. Under conditions of nutrient scarcity and other general stresses, *E. coli* expresses pathways with relatively inexpensive proteome synthesis requirements instead of more efficient but also anabolically more expensive pathways. This evolutionary strategy provides a cellular function-based explanation for common network motifs like isozymes and parallel pathways and possibly explains 'overflow' metabolisms observed during nutrient scarcity. The study provides fundamental clues into evolutionary pressures and competitive metabolic designs.

W07-P394
Investigations of dormant cells in *Pseudomonas aeruginosa* biofilms

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It was hypothesized that *Pseudomonas aeruginosa* biofilms are protected from killing by antimicrobials due to the presence of dormant cells within mature biofilms. A *P. aeruginosa* strain containing a stable, inducible green fluorescent protein (GFP) was used to visualize and characterize the metabolically dormant and active cell populations within the biofilm. In up-shift experiments, cells were labeled by introduction of the inducing agent to mature biofilms. Only about 30% of the cells within the biofilm turned bright, thus labeling the active cells with GFP. Dormant cells were labeled by growing biofilms to maturity in the continuous presence of inducer, then switching to media lacking inducer. This down-shift treatment produced a biofilm in which only the most dormant cells within the biofilm were bright after an extended period on media lacking the inducer. By disaggregating the biofilms and sorting each population by flow cytometry (FCM), it was possible to separate and collect populations based on GFP expression in which cells expressing GFP were characterized as bright events (GFP+) while the non-GFP expressing cells were characterized as dim (GFP-). When plating the GFP+ population of dormant cells for viability, 17% of bright events produced colony forming units (cfu). Similarly, when considering active cells, 21% of bright events were found to yield viable cells. This novel approach of labeling cells in intact biofilms by their metabolic state was used in two ways. We explored the susceptibility of both active and dormant cells to antibiotic treatment and we devised a method of physically separating the metabolically active, top layer of the colony biofilm from the dormant lower layers. In up-shifted colony biofilms, top layers that showed > 90% GFP+ events and bottom-most layers that showed >80% GFP- events were retained for RNA extraction in preparation for determination of genome-wide differences in gene expression between the two populations. For the resistance studies, after FCM sorting into GFP+ and GFP- populations, each population of cells was plated for viability, and their susceptibility to either ciprofloxacin or tobramycin was determined. In the down-shift experiments, active cells, compared to the dormant cells (GFP+), showed a 7-fold and 35-fold greater decrease in log cfu, respectively, after treatment with ciprofloxacin and tobramycin. In the up-shift experiments where the active cells were GFP+, the dormant cells experienced an actual increase (negative log reduction) in log cfu after treatment with the antibiotics, while the active cells again showed reductions in log cfu after exposure to the antibiotics. These data suggest that the antibiotic tolerance expressed by *P. aeruginosa* biofilms is affected by the presence of metabolically dormant cells within the biofilm.

W07-P395
Molecular analysis of the microbial communities in constructed wetland plant rhizospheres

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Molecular techniques were used to determine the microbial community structure and activity in the rhizosphere of constructed wetlands and to identify microbial community differences by using a variety of 16S group-specific primer combinations. Denaturing gradient gel electrophoresis (DGGE) fingerprints initially obtained from rhizosphere samples using universal bacterial primers revealed a poor resolution of differences largely because real differences were likely masked by the great complexity of the profiles. In order to visualize and compare between plant species and various regions of a root surface, a variety of group-specific primers were selected. The specific groups considered were *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and Bacillus. Universal 16S primers were also used for each sample as a standard for comparison. Molecular analysis of rhizosphere communities was accomplished by PCR amplification of 16S rDNA with primers targeting variable regions of the rDNA gene and using a reverse primer containing a GC-rich "clamp" to facilitate gel separation of distinct genotypes. DGGE was performed using a 40% to 70% denaturation gradient and an 8% to 12% gradient in the polyacrylamide. Breaking down this complex community into smaller more specific groups made further analysis and visualization by DGGE more informative. By creating community profiles with a more simplistic scope, a more complete microbial view of this highly diverse habitat was obtainable.

POSTER ABSTRACTS

W07-P396
Development of a rapid molecular technique for detection of HAA degraders in drinking water distribution systems

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The chlorination of drinking water has largely contributed to the drastic reduction of water-borne disease. However, this chlorination process is known to produce a variety of disinfection by-products (DBPs), including haloacetic acids, a group of probable carcinogens. In a sub-set of drinking water treatment plants, the concentration of haloacetic acids (HAAs) decreases along the course of the water treatment and distribution system. It is therefore of obvious importance to determine if microbial degradation plays a role in remediation of this recalcitrant molecule. Drinking water distribution systems were evaluated for the presence of HAA degrading bacteria contained within the bulk fluid and pipe wall biofilms. Several isolates were found with the potential to utilize monochloro-acetic acid (MCAA) and dichloro-acetic acid (DCAA) as the sole carbon and energy source. In bacteria that have the ability to utilize and dechlorinate HAAs, the dehalogenase enzyme is critical to the degradative pathway. Two subfamilies of dehalogenases—DehI and DehII—have been molecularly identified in known HAA degrading strains. The deh genes provided a target for non-cultivation based molecular detection of HAA degraders. Published degenerate primers were used to detect dehalogenase genes in bacterial isolates enriched on HAAs. To date, all MCAA and DCAA degraders, isolated from water distribution systems, contain dehII and not dehI genes. Sequences of dehII genes from the HAA enriched strains were aligned, and specific primers were designed in conserved regions. These non-degenerate primers were then successfully used in real-time PCR detection of deh genes. This technique will be further validated, providing a rapid indication of the HAA degrading capacity of a water treatment plant.

W07-P397
Development of a rapid biofilm analysis kit

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The Center for Biofilm Engineering at Montana State University and a Montana-based company, BioSurface Technologies Inc. (BST), have collaborated to develop a rapid biofilm analysis kit. The kit will utilize the tetrazolium salt CTC (5-cyano-2, 3-ditolyl tetrazolium chloride). CTC is a dye that is used to stain bacteria in biofilm. When the bacteria are active, they take up CTC and reduce it to a fluorescent, insoluble crystal. In certain field applications, for instance a distribution system, biofilm needs to be monitored and treated with anti-biofilm products. To monitor bacteria in the field, viable plate counts (VPCs) are commonly used. However, VPCs require at least 24 hours to produce results, as well as the user's basic knowledge of microbiology and aseptic techniques. VPCs are known to underestimate the actual number of active bacteria in industrial and environmental systems. There is currently a need in the market for a rapid biofilm analysis kit. A rapid biofilm analysis kit should be inexpensive, simple, rapid, repeatable, accurate, and field applicable. The technologies currently on the market for rapid test kits include ATP (adenosine triphosphate) analysis, nucleic acid based assays, and antibody-based assays. Each of these methods has advantages and disadvantages, but none meet all of the desired characteristics for a rapid analysis kit. CTC was chosen because the results are relatively rapid, it has been successfully used to measure bacterial activity in biofilm, it can be used on a wide range of organisms, and the method does not require expensive equipment. The method requires staining biofilm with a known concentration of CTC for a specified time, eluting the CTC with ethanol, and reading the absorbance of the solution with a spectrophotometer. CTC does have a few limitations, one of which is that its use in the laboratory is not standardized. The phase one goal for this project is to standardize and optimize the concentration and incubation time of CTC for measuring biofilm bacteria grown in the CDC reactor. Data collected includes CTC absorbance readings, viable plate counts, and total cell counts using DAPI and CTC.

W07-P398
Biofilms on ice: Why cold temperature research is HOT!

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The biofilm mode of growth is increasingly being recognized as the preferred mode in many environments. In icy systems, organisms may be active for only a short period of time, depending upon the availability of liquid water. Their survival is tied to the ability to scavenge nutrients and energy; hence keeping these in close proximity affords an advantage to organisms growing in biofilms. This poster presents three examples of cold temperature biofilm research here at MSU: the use of Earthly analogs in the search for life on other planets, cold temperature enzymes, and hydrocarbon degradation.

W07-P399
Characterization of *Escherichia coli* biofilm detachment in mixed species biofilms grown in capillary flow cells

***P. Volden**, B. Klayman, and A. Camper, Center for Biofilm Engineering at Montana State University, Bozeman, MT*

A quantitative description of biofilm detachment is important in obtaining a full understanding of biofilm dispersal, as well as in developing treatment and preventative methods for biofilm related fouling and illness. Using *Pseudomonas aeruginosa*, a well known biofilm-forming species implicated in bio-fouling and illness, and *Escherichia coli* O157: H7 isolated from a water distribution system, we observed variations in biofilm detachment under two inoculation conditions. *E. coli* was either inoculated at the upstream end of a flow cell reactor containing *P. aeruginosa* biofilm pre-colonized for a 72 hour period (pre-colonization), or simultaneously inoculated with *P. aeruginosa* (co-inoculation). Epifluorescence microscopy, image analysis, and viable cell counts were used to quantify biofilm detachment and cluster size from effluent samples collected at the downstream end of the reactor. Microscopy was also used to qualitatively describe detached cells and clusters. As observed through direct cell counts and analysis of membrane filtered effluent areas, *E. coli* biofilm under co-inoculation conditions showed roughly an order of magnitude detachment increase 24 hours after initial inoculation, reaching a pseudo-steady detachment state. *E. coli* inoculated under pre-colonization conditions reached, within only 3 hours, the pseudo-steady state detachment levels observed in co-inoculation and eventually surpassed this level. This suggests an influence by *P. aeruginosa* pre-colonized biofilm on detachment of *E. coli*. Cluster size analysis revealed a higher average cluster size in the pre-inoculated *E. coli*; however the maximum detachment size did not seem to be affected by the inoculation conditions. Additionally, epifluorescence images provided qualitative evidence for the association of *E. coli* cells within *P. aeruginosa* clusters during detachment events. This observation was supported further by data-plots showing detachment of *E. coli* about one order of magnitude less, but parallel to that of *P. aeruginosa*—a pattern that supports an associated detachment mechanism for the mixed species. Our work shows that *E. coli* O157:H7 detachment and cluster size is influenced by both a competing biofilm forming species and the development of the competing species as a biofilm prior to inoculation.

W07-P400
Toxic effects of select metals and organic acids to *Acidithiobacillus caldus*

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Acidithiobacillus caldus is a chemolithotrophic autotrophic acidophile that oxidizes reduced sulfur compounds and grows mixotrophically with glucose or yeast extract coupled with sulfur or tetrathionate. *At. caldus* is ubiquitous to bioleaching environments, where it leaches metals by oxidizing metal sulfides. To date, few studies have elucidated the interactions between *At. caldus* and its environment. Organic acids are often released in bioleaching environments by heterotrophic acidophiles. It is believed that organic acids may cause de-coupling in low pH environments where *At. caldus* thrives. In addition, metals are known to interfere in enzyme and DNA function. The inhibitory effects of malate, acetate, pyruvate, fumarate, succinate, oxaloacetate, and α-ketoglutarate were examined. In addition, effects of the metals lead, zinc, and copper were determined. Cultures were grown in triplicate at 45 °C, pH 2.5, and shaken at 150 rpm. Sodium tetrathionate was added as an electron donor (1 g/L) in an aerobic, defined medium. Organic acids or metals were

added in varying concentrations. Inhibition was characterized by changes in specific growth rates and cell yields, determined via direct cell counts. Concentrations of organic acids were measured using capillary electrophoresis. ICP-MS was used to quantify metal concentrations during growth. Acid, metal, and cell-free controls were used as appropriate. Oxaloacetate had the lowest IC50 at 28 ±1.5 µm. Malate had the highest IC50 at 84 ±7.2 µm C50's of 39 ± 2.5 µm 180 ± 3.7 µm and 2370 ± 130 µm were observed for lead, zinc, and copper respectively. In the case of both organic and metal inhibition, yields were reduced by 2- to 3-fold at the IC50 concentration. Conclusion: All organic acids and metals tested exhibited an inhibitory effect. Oxaloacetate, the only acid tested with a pKa below the medium pH, exhibited the highest level of inhibition of the organics tested. Of the metals tested, it appeared that *At. caldus* is relatively tolerant to high copper concentrations.

W07-P401
Initial changes in bacterial community structure of a denitrifying fluidized bed reactor treating uranium-contaminated groundwater

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Uranium is a major groundwater contaminant at U.S. Department of Energy (DOE) sites. Former waste ponds in the Y-12 National Security Complex located in Oak Ridge (TN) pose several challenges for uranium bioremediation. These sites are marked by acidic conditions, high concentrations of nitrate, chlorinated solvents, and heavy metals. These contaminants need to be removed from the groundwater before uranium reduction by indigenous bacteria can occur. A fluidized bed reactor (FBR) was set up to remove nitrate from pre-treated groundwater through the denitrification process by indigenous microorganisms. The bacterial community of the FBR was studied by constructing clonal libraries of the SSU rRNA gene at several timepoints. The timepoints (12d, 34d, 48d, 69d, and 118d) were selected according to the nitrate levels of the influent in the FBR. The inoculum of the FBR was also analyzed. At earlier timepoints, the FBR was predominated by *Azoarcus* species, however, species richness increased throughout the operation time with the appearance of *Dechloromonas*, *Pseudomonas*, and *Hydrogenophaga* species. The bacterial community in the inoculum was also diverse, with the detection of *Acidovorax*, *Diaphrobacter*, *Dechlorosoma*, *Alkaligenes*, and *Sporumusa* species and Perchlorate-reducing bacterium; however, no *Azoarcus* species was detected. Some clones are related to uncultivated microorganisms and some have less than 95% identity with previously observed microorganisms. To achieve bioremediation potential, it is important to understand the changes in microbial diversity in the FBR. The analysis of the bacterial community in the FBR may provide insight to the conditions required to maintain a stable bacterial community that promote the denitrification process to eventually achieve in situ uranium bioremediation.

W07-P402
Identification of molecular and cellular responses of *Desulfovibrio vulgaris* biofilms under culture conditions relevant to field conditions for bioreduction of heavy metals

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Desulfovibrio vulgaris ATCC29579 is a sulfate reducing bacterium that is commonly used as a model for direct and indirect heavy metal reduction, and can also be a causative agent of metal corrosion. Objective: Characterize *D. vulgaris* biofilms and identify key proteins necessary for biofilm formation and maintenance. Results: During growth with lactate and sulfate, internal carbohydrate levels increased throughout exponential phase, and peaked as the cells transitioned to stationary phase. The carbohydrate-to-protein ratio (C:P) peaked at 0.05 µg/µg as the cells transitioned to stationary phase, and then declined to 0.02 µg/µg during extended stationary phase. In contrast, a strain of *D. vulgaris* that does not contain the megaplasmid (rmp), maintained higher internal carbohydrate levels and the C:P ratio peaked 2-fold higher compared to wild-type. The C:P ratio in extended stationary phase was 4-fold higher compared to the wild-type. Under the tested growth conditions, we observed biofilm formation in wild-type cells, but the rmp strain

formed less biofilm (2-fold decrease). In addition, carbohydrate levels in the culture supernatant were approximately 2-fold increased for wild-type cells compared to rmp cells. We hypothesized that carbohydrate was reallocated to the external cell proper for biofilm formation. However, biofilm contained little carbohydrate (0.6 to 1.0 µg/ml) and had a similar C:P ratio compared to wild-type early stationary phase cells. Staining with calcafluor white also indicated the presence of little external carbohydrate in *D. vulgaris* biofilms. The formation of biofilm was hindered by the presence of protinease K, trypsin, and chymotrypsin; however, the growth of planktonic cells was not. In addition, when *D. vulgaris* biofilm was treated with a protease, biofilm was degraded. In comparison, the biofilm of *Shewanella oneidensis* contained more carbohydrate, and the *S. oneidensis* biofilm was not significantly affected by protease treatment. Electron micrographs indicated the presence of filaments between the biofilm cells, and filaments were susceptible to protease degradation. Biofilm filtrates contained soluble protein, and SDS-PAGE analysis suggested different polypeptide profiles between filtrates, planktonic, and biofilm samples. The results indicated that *D. vulgaris* changes carbohydrate distributions in response to growth phase, the megaplasmid contains genes important for carbohydrate distribution and biofilm formation, and *D. vulgaris* biofilms contain extracellular filaments that may be important for the initial stages of biofilm formation.

W07-P403
Deletion of a predicted sensory box gene in *Shewanella oneidensis* MR-1 causes pleiotropic phenotypes

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Shewanella oneidensis MR-1 can utilize a variety of electron donors and acceptors for energy, including oxygen and heavy metals. Understanding the physiological responses of MR-1 to environmental stresses is important in assessing the potential impact of such perturbations on metal-reducing activity. Here the possible physiological roles of a conserved hypothetical protein—SO3389—were characterized. The ORF contains PAS, PAC, EAL and GGDEF domains, and these domains have been implicated in multiple phenotypes. The exact physiological role(s) of proteins that contain these domains, however, have not been fully established. In addition, the possible role of a protein with this domain composition and architecture has not been previously described. Initial studies revealed that the mutant was impaired in motility and biofilm formation when grown aerobically compared to wild-type. The mutant lagged for 35–40 h when transferred from aerobic to anoxic medium, but growth rate was similar to wild-type once growth was initiated. Interestingly, when the mutant was transferred from anoxic growth, the subsequent lag was diminished. However, motility and biofilm formation were still impaired. This suggested that the mutant was affected in transitions from aerobic to anoxic conditions, motility, and biofilm. However, the phenotypes could be decoupled in subsequent transfers. In order to determine the cytochrome-c content of wild-type and mutant cells, spheroplast- and periplasmic-fractions were obtained. When the fractions from aerobic or anoxic grown cells were compared, some minor differences were observed. However, when cells were harvested during the transition from aerobic to anoxic conditions, major differences were observed in the spheroplast fraction, most noticeably for polypeptides of 57, 33, and 20 kDa. These results indicated that rSO3389 was also affected in c-type cytochrome content. This is the first report of a multi-domain PAS protein involved in biofilm formation. The data suggested that O_2 may be a major signal that is sensed by SO3389, but further work is needed to elucidate the respective signal(s) and the mechanism(s) of signal transduction.

W07-P406
Measuring growth rates at the single cell level in mature biofilms

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The goal of this research project is to develop a method to quantify individual cell and cell cluster movement within developing and mature *Pseudomonas aeruginosa* PAO1 biofilms. This work builds largely on top of previous research done at the Center for Biofilm Engineering (CBE) which quantified replication, emigration, and movement events in young (3-10 µm thick) PAO1 biofilms (Rice et al., 2003). Building on recent improvements in fluorescent labeling technology, time-lapse images of maturing dual-labeled PAO1 biofilm are

captured using Scanning Confocal Laser Microscopy (SCLM) so that individual cells and cell clusters can be visualized within larger clusters. Tracking volume and shape factor measurements for individual cell pockets over time will lead to information regarding cell displacement and colony formation within the biofilm as a whole. Differential growth rates can be observed and measured at various locations within a biofilm, allowing for visualization of slower-growing regions within a biofilm, which is of interest to those studying antibiotic application and detachment phenomena. This information can also be used to improve biofilm cellular automata (CA) models by more accurately describing cell movement and displacement following cell division. Current CA models assume a random displacement vector (Hunt et al., 2003), which can be updated to fit observations seen in this system.

W07-P407
Extended lifetime of unstable GFP in biofilms

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Cells within a biofilm have different access to oxygen, nutrients and metabolic wastes. Due to these microenvironments, anabolic activity is heterogeneously distributed in biofilms. In the course of applying methods to determine spatial patterns of activity in *Escherichia coli* K12 colony biofilm, it was discovered that a green fluorescent protein (GFP) considered to be relatively unstable was, in fact, persisting for periods exceeding 24 hours. Understanding spatial variation of cell activity within biofilm will facilitate design of new control strategies. Labeling regions of active DNA synthesis revealed two zones of replication. One was at the air interface (21 microns wide on average) and a thinner zone (7 µm) was located along the membrane interface. Much of the interior of the biofilm exhibited no DNA synthetic activity. Regions of active protein synthetic activity were identified using an inducible unstable GFP. A 4-hour induction with IPTG (an inducer) revealed a pattern of activity similar to the one observed for DNA synthesis: proteins were synthesized at the air interface (43 µm wide) and in a thinner zone adjacent to the membrane (14 µm). An alternative technique for probing anabolic patterns is to use an unstable GFP fusion to a growth-rate dependent promoter. The fis promoter is highly expressed during the early exponential phase. Due to the fusion with unstable GFP[LVA], cells which are growing fast should appear bright; slow growing or non-growing cells should be dim or dark. During the 48 hours of growth, colony biofilms have to be transferred to fresh media plates to renew access to nutrients. Unexpected patterns of growth activity were observed, as they depended on the number of these transfers. No fluorescence was observed in absence of transfer, one thin zone of activity was observed in the middle of the biofilm after one transfer at the 24th hour, two zones appeared after a 24- and 44-hour transfer, three zones after 12-, 24- and 36-hour transfers. Stripes observed on the fis strain patterns are the result of transfers to fresh media. GFP[LVA] fluorescence persists in biofilms though it is an unstable variant of GFP that decays within a few hours in planktonic cultures. Unstable GFP variants
have been developed as a way of monitoring recent gene expression, thanks to their relatively fast turn-over. However, in biofilms, unstable GFP reporter fusions may not reflect only the recent gene expression, but also expression which has occurred during the whole experiment.

W07-P408
Nitrification rate in PVC and copper distribution systems

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Nitrification in drinking water distribution systems is overwhelmingly prevalent in the U.S.A., with occurrence as high as two thirds of all significant distribution systems, according to a 1996 study published in American Water Works Association. This statistic lends itself well to further analysis, as nitrification is known to be a potentially dangerous problem. Once the issue of nitrification in the water distribution system has been established, a specific question regarding the type of system arises. The lion's share of the household plumbing in the U.S.A. is done through plastic or copper pipe, and copper has been indicated to be an inhibitor of nitrification. It is a reasonable assumption, therefore, that a copper distribution system would have a slower rate of nitrification than a comparable PVC distribution system. The protocol in this experiment follows. Two nitrification systems were made, one for PVC and one for copper. Each consisted of four replicate reactors, all with biofilm cultivated from the residential water supply in Bozeman. In order to

create a reasonable facsimile of the distribution system, the ratio of surface area to volume in a typical copper or PVC pipe was determined and translated to the reactor. The rate of flow in a typical system was also determined and was applied as above. The reactors were kept in darkness at all times, except when sampling. Influent pumps were activated every eight hours for five minutes to supply the reactors with, among other things, a known amount of ammonia. The ammonia level was measured every half hour until the biofilm had oxidized it completely. This protocol was run for six days in each of the eight reactors, for a total of 24 runs for both PVC and copper systems. The resulting correlation was unambiguous. The biofilms on the copper-based reactors were slower in reducing the level of ammonia to zero in every trial. In fact, the reactors were generally an hour—approximately 50%—slower. This result seems to indicate that a copper-based distribution system would have slower rates of nitrification than a comparable PVC system. Among other considerations, it could be a factor in deciding which material to use when building new systems or refurbishing obsolete systems.

W07-P409

Bacteriophage interaction with *Staphylococcus aureus* biofilm

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Bacteriophages are viruses that target bacteria; they use the host cell's machinery to self-replicate and either lyse the host cell, or incorporate themselves into the host-cell chromosome and become a prophage. Bacteriophages are known to attack planktonic cells with relative ease. However, the effectiveness of phage K against biofilm-forming *S. aureus* has been largely undocumented. The purpose of this research is to investigate the relationship of phage K and clinical isolates of *S. aureus* in both planktonic and biofilm settings. The use of bacteriophage has the potential to make a large impact on the medical biofilm industry. Chronic wound research, in particular, may benefit from bacteriophage research. Currently, many chronic wounds are being treated with a mixture of various antibiotics. However, with the increase in antibiotic resistance that is occurring, the chronic wound becomes harder to heal. Bacteriophage therapy may prove to be a beneficial choice in chronic wound care because, since only bacterial cells are targeted, the chance of side-effects for the patient is relatively low.