

# **SANDIA REPORT**

SAND2006-7988

Unlimited Release

Printed February 2006

## **Exploratory Research into Pathogen Surface Interactions**

Todd W. Lane, Julie Kaiser, Susan J. Altman, Lucas McGrath, Caroline Souza, Mike Sinclair, Howland Jones, and Roberto Rebeil

Prepared by  
Sandia National Laboratories  
Albuquerque, New Mexico 87185 and Livermore, California 94550

Sandia is a multiprogram laboratory operated by Sandia Corporation,  
a Lockheed Martin Company, for the United States Department of Energy's  
National Nuclear Security Administration under Contract DE-AC04-94AL85000.



**Sandia National Laboratories**

Issued by Sandia National Laboratories, operated for the United States Department of Energy by Sandia Corporation.

**NOTICE:** This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government, nor any agency thereof, nor any of their employees, nor any of their contractors, subcontractors, or their employees, make any warranty, express or implied, or assume any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represent that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government, any agency thereof, or any of their contractors or subcontractors. The views and opinions expressed herein do not necessarily state or reflect those of the United States Government, any agency thereof, or any of their contractors. Printed in the United States of America. This report has been reproduced directly from the best available copy.

Available to DOE and DOE contractors from  
U.S. Department of Energy  
Office of Scientific and Technical Information  
P.O. Box 62  
Oak Ridge, TN 37831  
Telephone: (865) 576-8401  
Facsimile: (865) 576-5728  
E-Mail: [reports@adonis.osti.gov](mailto:reports@adonis.osti.gov)  
Online ordering: <http://www.osti.gov/bridge>



SAND2006-7988  
Unlimited Release  
Printed February 2006

# Exploratory Research into Pathogen Surface Interactions

Todd W. Lane, and Julie Kaiser  
Biosystems Research Department  
Sandia National Laboratories  
PO Box 969  
Livermore, CA 94550-0969

Susan J. Altman, and Lucas McGrath  
Geohydrology Department

Caroline Souza  
Chemical and Biological Systems

Mike Sinclair  
Microsystems Materials

Howland Jones  
Biomolecular Analysis and Imaging

Roberto Rebeil  
Biomolecular Interfaces and Systems  
Sandia National Laboratories  
PO Box 5800  
Albuquerque, NM 87185

## Abstract

In this short-duration project the research team was able to achieve growth of both drinking water biofilms and monospecific biofilms of *Legionella pneumophila*. Preliminary comparative proteomic analyses were carried out on planktonic and biofilm-associated *Legionella*. After delay for completion of permitting and review by the director of the National Institutes for Allergy and Infectious Disease, the Utah 112 strain of *Francisella novicida* was obtained and preliminary culture and comparative proteomic analyses were carried out. Comprehensive literature searches and data mining were carried out on all research topics.

This page intentionally left blank.

## Contents

Abstract.....	3
Executive Summary.....	6
Introduction.....	8
Drinking Water Biofilms.....	10
Growing Drinking Water Biofilms.....	10
Imaging Drinking Water Biofilms.....	11
Results and Discussion.....	13
<i>Legionella pneumophila</i> Biofilms.....	15
Introduction.....	15
Experiment.....	16
<i>Legionella pneumophila</i> culturing and biofilm generation.....	16
2D gel electrophoresis.....	16
Results and Discussion.....	16
Generating planktonic and biofilm associated <i>L. pneumophila</i> cells.....	16
2D gel protein analysis.....	17
Culture of <i>Francisella novicida</i> Utah 112.....	19
<i>F. novicida</i> Utah 112 Culture Media and Reagents.....	19
<i>F. novicida</i> Utah 112 Growth Analysis.....	21
Initial Proteomic Analysis of <i>Francisella novicida</i> Utah 112.....	23
Conclusions.....	25
References.....	26
Distribution List.....	27

## List of Figures

Figure 1. Photograph of set-up to grow drinking water biofilms.....	11
Figure 2. Data representation.....	12
Figure 3. Photograph of a drinking water biofilm.....	13
Figure 4. Image analysis of unstained drinking-water biofilms.....	14
Figure 5. Image analysis of stained drinking-water biofilms.....	15
Figure 6. Light and ultraviolet (UV) micrographs of <i>L. pneumophila</i> biofilms.....	17
Figure 7. Analysis of <i>Legionella pneumophila</i> proteins via 2D-gel electrophoresis.....	18
Figure 8. Growth characteristics of <i>Francisella novicida</i> .....	22
Figure 9. SDS PAGE of <i>F. novicida</i> .....	25

## List of Tables

Table 1. Media formulations.....	19
Table 2. Determination of culture titers in colony forming units.....	23
Table 3. <i>F. novicida</i> doubling rates.....	23
Table 4. Protein concentration determination.....	24

This page intentionally left blank.

## Executive Summary

We successfully grew biofilms from the drinking water in Building 823. To do this we first dechlorinated the water by running it through two columns of granulated activated carbon. The drinking water was then mixed with an equal volume of 1:100 Trypticase Soy Broth (TSB) solution and dripped into a drip-flow reactor. With this higher concentration of nutrient solution we were able to grow very thick biofilms. We used hyperspectral imaging with multivariate analysis techniques (Multivariate Curve Resolution) to image the drinking water biofilms. The hyperspectral imaging was able to produce excellent images of the bacteria in the drinking water biofilms. There is also evidence from the hyperspectral imaging that some substance (extracellular matrix?) is integrated with the outer parts of some of the micro-organisms.

*Legionella pneumophila* was cultured in buffered Yeast extract liquid media supplemented with activated charcoal, L-cysteine, ferric pyrophosphate. Cells grown in tubes without agitation formed a robust and highly cohesive biofilm with few planktonic cells present in the media. To stimulate planktonic cell formation, culture tubes were placed in shaker incubators (37 °C, 250 RPM) to simulate turbulent flow. Total proteins were extracted from cells and analyzed by two dimensional electrophoresis.

*L. pneumophila* grown without agitation formed aggregates which were confirmed to be biofilms. As expected, bacterial cells were suspended and held together by an amorphous matrix, presumptively an extracellular polysaccharide. Proteins were extracted from these macrocolonies for further study. *L. pneumophila* grown under turbulent flow yielded a turbid culture that did not contain macrocolonies. In addition, very little Congo Red dye bound to the cells indicating that the cells were indeed in a planktonic state. Further optimization will be required, yet clearly *L. pneumophila* planktonic and biofilm cells display different protein contents even though they are growing in identical nutrient media.

A comprehensive literature search was completed for both the biofilms and the *Francisella tularensis* efforts of this project. Permits were obtained from the department of agriculture and the Centers for Disease control for the transfer of *Francisella tularensis* subspecies *novicida* strain Utah 112 to Sandia National Labs. The Principal investigator registered with the NIH sponsored Biodefense and Emerging Infections Research Resources Repository. After meeting all of these regulatory requirements the strain was transferred to Sandia National Labs and cell culture experiments were initiated. *F. novicida* was grown under a variety of culture conditions and harvested at different growth phases. Proteomic analysis of cell from each sample was carried out by gel electrophoresis.

This page intentionally left blank.

## Introduction

The primary goal of this research was to compare molecular pathways in two microorganisms in the hope of determining which molecular pathways are shared and which ones are specific to a particular model system. The two model pathogens chosen for this study are *Francisella tularensis* and *Legionella pneumophila*. The secondary goal of this research was to grow biofilms with an origin from drinking water. The intent of this second goal is to take the first steps needed so that we can study cell surface interactions that are involved in the association of the *F. tularensis* and *L. pneumophila* (herein referred to as the model pathogens) in multicellular communities – the drinking-water biofilms.

These two model pathogens were selected because of their importance to homeland security and water quality. *Francisella tularensis* (the causative agent of the disease tularemia) presents a major terrorism threat given its extreme infectiousness (10 bacteria by the respiratory route can cause disease in humans). Teter and Brady (2003) identified it as one of the top 20 pathogens that pose a terrorist threat to our public water systems. This ranking was due to *F. tularensis*' relatively long latency period (3 – 5 days), which allows it to disseminate through the system, its ease of dissemination because of its low infectious dose, the ease of obtaining the agent by potential terrorist groups. *Legionella* (the causative agent of Legionnaire's disease) infects an estimated 8,000-18,000 people every year in the United States, and has lethality rate of 5-30% (CDC estimate). Populations at an increase risk of infection are the elderly and immunosuppressed patients. Contaminated municipal or institutional water supplies are the most frequent sources of infection. *Legionella* replicates in water pipes and reservoirs by establishing a parasitic relationship in species of amoebas frequently found in most potable water sources. In addition, *Legionella* persist on the surfaces of water heaters and pipes for long periods of time by forming bacterial biofilms. *Legionella* biofilms are particularly problematic since, in addition to being a constant source of infection, they protect the bacteria from many bacteriocidal water treatments. Due to its pathogenesis and lifestyle, *Legionella* is a bacterial pathogen of significant interest to the Environmental Protection Agency (EPA). Unfortunately, factors important in biofilm formation remain understudied. The link of the model pathogens to drinking-water biofilms has been chosen because of the importance of biofilms to energy and infrastructure surety and therefore the interest of the Department of Energy (DOE).

By examining two very different organisms that share a similar lifestyle we can determine which molecular pathways are shared and are likely to be generalizable to bacteria in general and which ones are specific to a particular model system and are likely to be unique features of that organism. Both model pathogens persist in the environment and are found associated with biofilms. Both organisms survive phagocytosis or engulfment by free-living amoebas such as *Acanthamoeba castellanii*. Genomic sequence information is available for *F. tularensis* and *L. pneumophila*. In the case of *Francisella* it has been demonstrated that its ability to survive engulfment by macrophages is related to its ability to survive in amoeba. The ability to survive in biofilms and amoebas also has implications for water quality assurance. Bacteria that reside in these environments are often more resistant to chemical disinfectants and antibiotics.

## Drinking Water Biofilms

One goal of this research was to grow biofilms with an origin from drinking water. The intent of this goal was to take the first steps needed to study cell surface interactions that are involved in the association of the *F. tularensis* and *L. pneumophila* in multicellular communities - the drinking-water biofilms. The capability to grow multicellular communities is also of interest in other related fields. For example, there is an interest on the impact of biofilms on drinking-water quality and the decontamination of drinking water distribution systems. Also, these biofilms could be used to study biofouling of water treatment membranes.

A biofilm is a colony of microorganisms attached to a surface and encased in extracellular polymeric substances (EPS). They are pervasive in most environments due to their ability to trap nutrients for their own growth and protect themselves from antibiotics and other anti-microbial agents. Cell-to-cell signaling is thought to be an important component of biofilm development. Thus, the study of cell-to-cell signaling should help to better understand biofilm development, pathogen integration with biofilms and potential methods to monitor and limit biofilm growth.

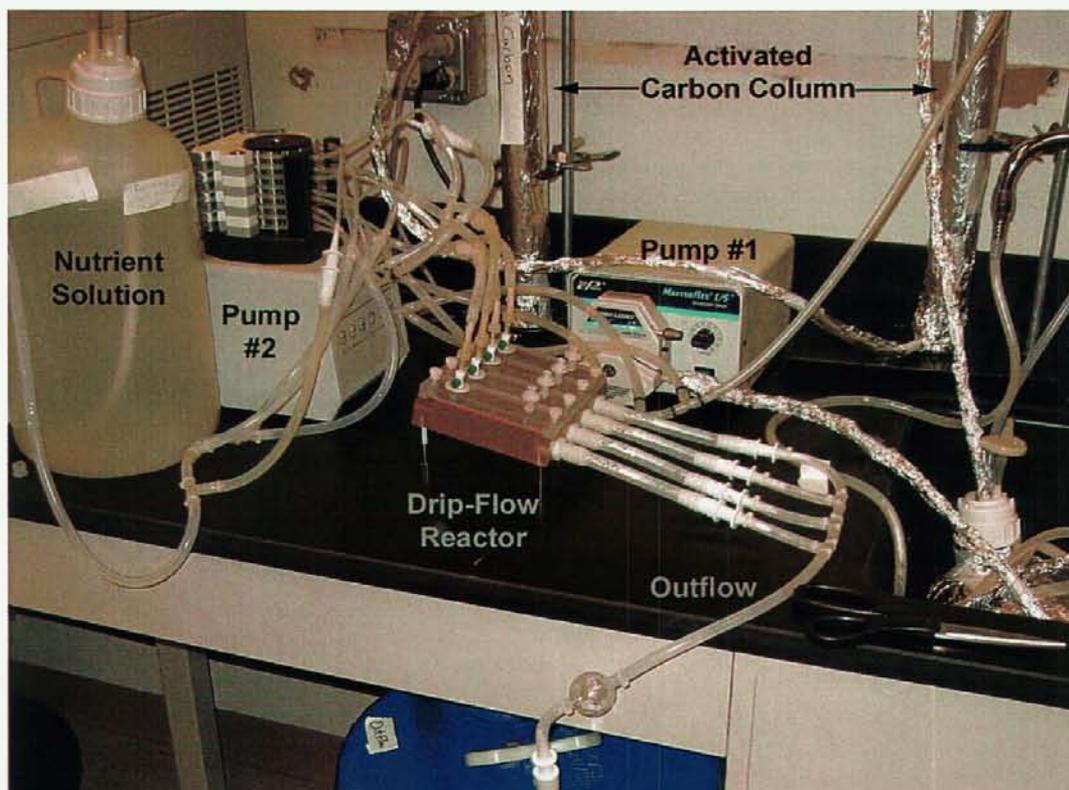
## Growing Drinking Water Biofilms

Drinking water biofilms were grown from the tap water in Sandia National Laboratories, Albuquerque, New Mexico, Building 823. The first step was to generate biologically activated carbon by filling a glass column with activated carbon (Calgon Filtrasorb 820) and running tap water through the column for approximately three months. The column was considered biologically activated when we could get growth when plating the water on Trypticase Soy Agar (TSA). After this step was completed, we added a second column to the system so that the water would flow through a column of activated carbon prior to flowing through the column of biologically activated carbon. The flow direction was from the bottom of the columns to the top of the columns. The outflow solution was then mixed with an equal volume of a nutrient solution dripped into a drip-flow reactor. Biofilms were then grown on the glass slides within the reactor. Flow rates through the carbon columns were approximately 48 ml/min, leading to a residence time of approximately 10 minutes per column.

The drip flow reactor system is shown in Figure 1. A pump was used to control the rate at which the drinking water and nutrient solutions were dripped into the reactor. The drip flow reactor consists of four channels where glass slides were placed on the bottom. The channels have a slope of approximately 10 degrees, so that the biofilms can be grown under a low shear environment.

The results of the biofilm growth were assessed by scraping the biofilms and plating the solutions using different types of agar. TSA and R2A were used for total heterotrophic plate counts. Polymyxin B is an antibiotic that inhibits the growth of gram-negative bacteria. TSA agar was created with 100 IU per mL of Polymyxin B to create the gram-positive selective agar. Finally Eosin Methylene Blue (EMB) agar was used as a gram-negative selective agar.

Two different nutrient solutions were used. In the first iteration, the nutrient solution was 1:100 Trypticase Soy Broth (TSB). The equal volume mixture of the drinking water and TSB was dripped into the reactor at 0.83 ml/min. As a second iteration, a nutrient solution more like what would be available in drinking water distribution systems was used. This consisted of carbon-source solution containing 4.9 mg/L of L-Glutamic acid and a nitrogen and phosphorous source solutions containing 1.7 mg/L of  $\text{KNO}_3$ , 0.15 mg/L  $\text{K}_2\text{HPO}_4$ , and 0.11 mg/L  $\text{KH}_2\text{PO}_4$ . The solutions are mixed at equal flow rates of 0.21 ml/min and combined with drinking water at a flow rate of 0.42 ml/min giving a total flow rate to each channel of 0.84 ml/min.



**Figure 1. Photograph of set-up to grow drinking water biofilms.**

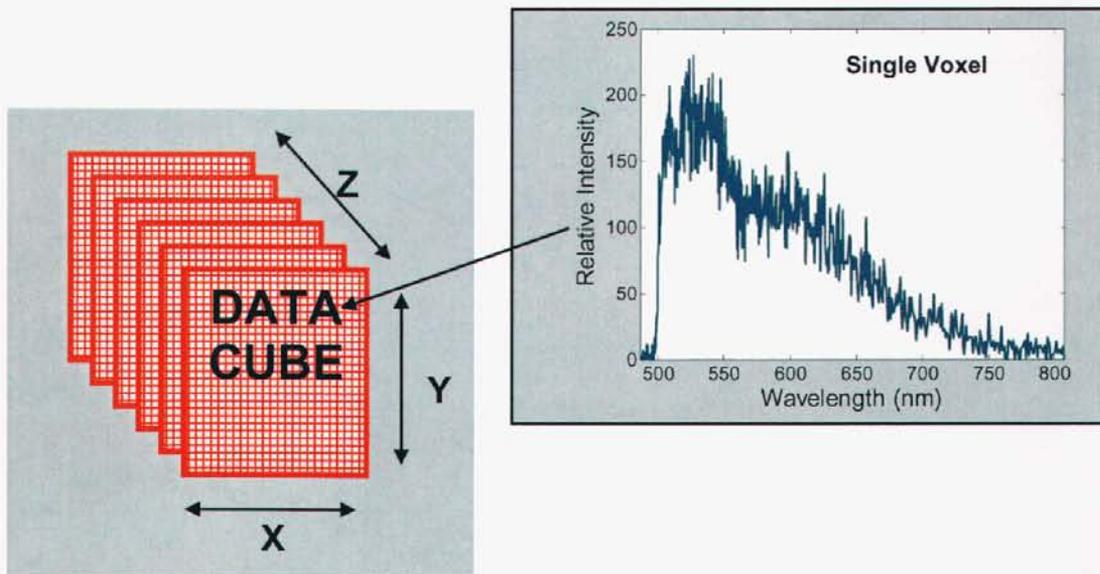
Pump #1 controls the flow rate through the activated carbon columns. Pump #2 controls the flow rate of the drinking water and nutrient solution through the drip-flow reactor.

## Imaging Drinking Water Biofilms

A custom built 3D confocal fluorescence hyperspectral imaging microscope (Sinclair et al., in preparation) along with multivariate analysis techniques (Van Benthem et al., 2002; Haaland et al., 2003) were used to image the drinking water biofilms. The advantage of using hyperspectral imaging and Multivariate Curve Resolution (MCR) is that we can simultaneously extract several overlapping fluorescence species in these bio-film samples. Furthermore, this can be accomplished without “*a priori*” knowledge of either the number of fluorophores present or the emission spectra of the fluorescent species, i.e., we can “discover” the pure emission spectra for all emitting species and obtain their relative concentrations based on the spectral emission data alone. Once we have the concentrations of all the emitting species, we can reconstruct the 3D images of the biofilm samples using a 3D image analysis software package (Imaris MeasurementPro by Bitplane AG Scientific Solutions).

Two samples were prepared by growing the biofilm onto two microscope slides (as described in section 2.1). One sample remained unstained and the other sample was stained with two nucleic acid stains: SYTO<sup>®</sup>9 and hexidium iodide (purchased at Molecular Probes). SYTO<sup>®</sup>9 labels both live gram-negative and gram-positive bacteria. In contrast, hexidium iodide preferentially labels gram-positive bacteria. The hexidium iodide will displace the SYTO<sup>®</sup>9 stain, thus gram-negative bacteria should fluoresce at a wavelength of 500 nm (green) and the gram-positive bacteria should fluoresce at 625 nm (red). Both samples were prepared for imaging by placing 170  $\mu\text{m}$  thick glass cover slips onto these samples and then using an ultra-fast drying fingernail polish along the edges of the coverslips to provide an air tight seal.

The image data collection consisted of using a 60× oil immersion objective on the confocal microscope and exciting the bio-film samples with a 488 nm laser. Several locations (5-6) were explored on each sample to ensure a representative interrogation of these biofilm samples. At each location we collected a hypercube of data. This hypercube consists of 3 spatial dimensions and a spectral dimension (Figure 2). The spectral dimension consists of 512 wavelengths ranging from 490-800 nm. The spatial dimension can vary depending on the desired image size. For both the unstained and stained samples we collected an image size of 105 × 104 pixels × 21 slices. This equates to 229,320 total spectra for the entire image with an image size of 12.5 × 12.5 × 6 mm. This hyperspectral confocal microscope has a lateral resolution of 0.25 mm, an axial resolution of 0.75 mm and a spectral resolution of 3 nm. The amount of laser light exposure to the sample can also be varied with this microscope. When there are strongly emitting species present, such as the gram stain, then the exposure time and laser power can be decreased to prevent the CCD from saturating. However, when only weakly emitting auto-fluorescence species are present, it may be necessary to increase the light exposure and laser power. For the exposure of unstained biofilm samples, it was necessary to increase the laser power by a factor of 1000 and the time by a factor of 4 over what was necessary for the stained sample.



**Figure 2. Data representation.**

Representation of data consisting of three spatial dimensions (X, Y, Z) and a spectral dimension. In this example we have 6 slices of data in the Z dimension.

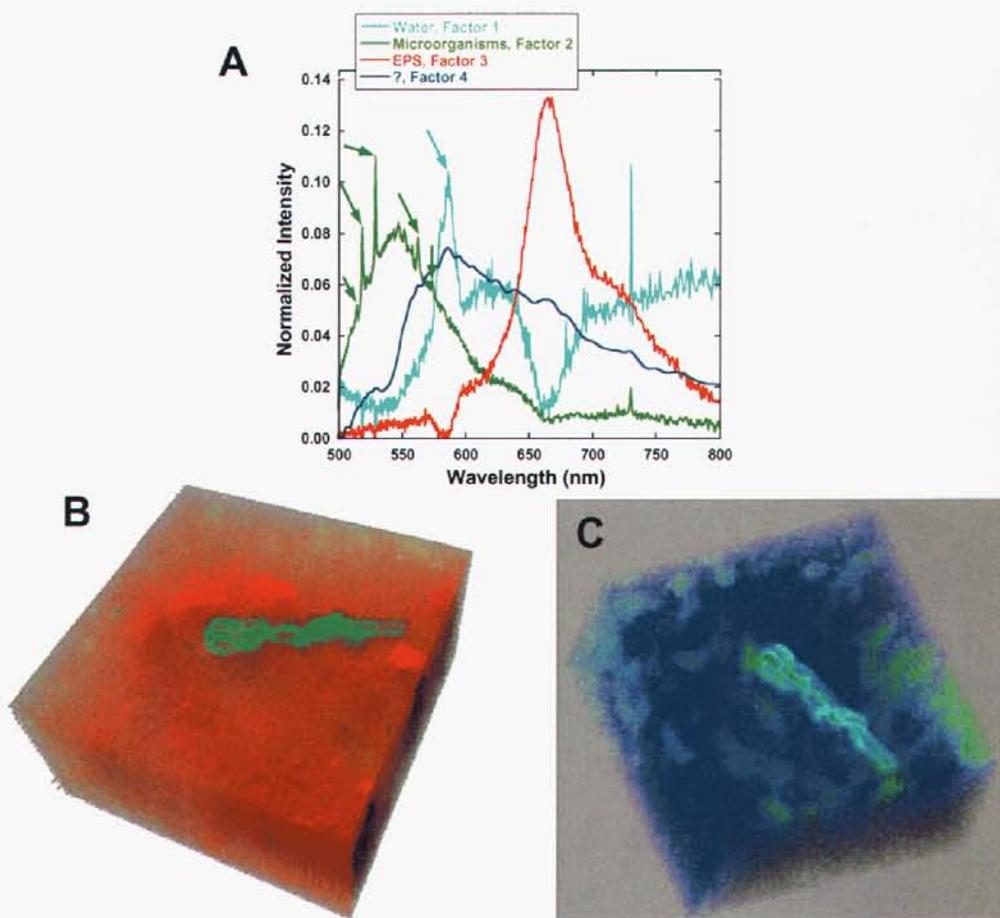
## Results and Discussion

With the high concentration of 1:100 TSB, we were able to grow very thick biofilms within days (Figure 3). Heterotrophic plate counts were on the order of  $10^8$  CFU/cm<sup>2</sup>. Based on the use of gram-specific agars, the majority of the organisms appear to be gram negative. However, some gram-positive organisms (on the order of  $10^6$  CFU/cm<sup>2</sup>) were present. Heterotrophic plate counts did not change significantly over the time periods the biofilms were grown (12 to 28 days). Heterotrophic plate counts were similar whether Trypticase Soy or R2A agars were used. When the nutrient solution more similar to that of drinking water was used, the heterotrophic plate counts were between  $10^4$  and  $10^5$  CFU/cm<sup>2</sup> for both Trypticase Soy or R2A agars.



**Figure 3. Photograph of a drinking water biofilm.**  
Cells were grown on a 75 x 25 mm glass slide.

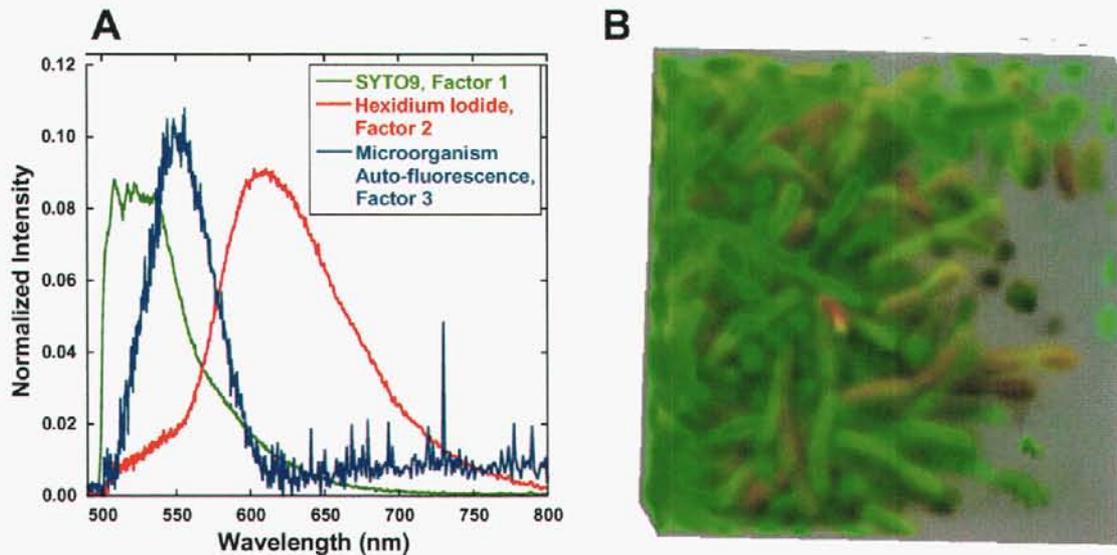
The hyperspectral imaging provided excellent images of the biofilms. Four pure components were detected in the unstained biofilm sample (Figure 4a). Factor 1 is a very noisy component that is due to Raman spectrum of water (see arrows). This component mixes with the other biofilm components because it is hard to separate this ubiquitous water feature. Factor 2 represents the auto-fluorescence and Raman spectral features (arrows) of the microorganisms. The Raman spectral features are the result of having to increase the laser power and exposure time on the unstained sample. We believe that these Raman spectral features are due to the carotenoids present in the microorganisms (Andreeva et al., 2005). Factor 3 most likely represents the auto-fluorescence of the EPS, due to the overwhelming presence of this component everywhere. Finally, there is a fourth component that was detected in only one of the locations imaged on the biofilm, but is ubiquitous in this area (see blue in Figure 4C). It is unclear what causes Factor 4. While Figure 4a shows some overlap between Factors 1 and 4, analysis shows they are two separate components. There are some suggestions that Factor 4 and the string of microorganisms are associated with each other. This evidence comes from the fact that the concentrations for Factor 4 increase when in closer proximity to that of the microorganisms (cyan in Figure 4C). The colors represented in the images in Figure 4 are as follows: Factor 2 is green (microorganisms), Factor 3 is red (possibly auto-fluorescence of the EPS) and Factor 4 is blue (unknown, but possibly a second auto-fluorescence component of EPS). Cyan as seen in Figure 4C is a result of the spatial co-location of the Factor 2 (green) and Factor 4 (blue).



**Figure 4. Image analysis of unstained drinking-water biofilms.**

Four factors seen in the unstained drinking-water biofilms (A) and images taken of the biofilm showing Factors 2 (green) and 3 (red) (B) and Factors 2 (green) and 4 (blue) (C). Factor 1 is most likely from the water in the system. Factor 2 is thought to be from the microorganisms and Factors 3 and 4 appear to be EPS due to their ubiquity in the different areas imaged. Note that Factor 4 was detected in one location imaged, but not in the others. Also note that there appears to be overlap between some of the microorganisms and Factor 4, as seen by the cyan (green + blue = cyan) in C. Arrows indicate Raman spectral features.

Three factors were detected in the stained drinking-water biofilm (Figure 5A): Factors 1 and 2 are the different stains, SYTO<sup>®</sup>9 and hexidium iodide, respectively. The SYTO<sup>®</sup>9 appears to have stained most of the microorganisms, indicating that they are gram-negative. This result is consistent with our gram-specific agar plating results. Due to the decrease in the laser power and exposure time, we do not see the Raman peaks in the auto-fluorescence factor (Factor 3). The hyperspectral imaging provided a very clear image of the microorganisms in the biofilm. (Figure 5B).



**Figure 5. Image analysis of stained drinking-water biofilms.**

Three factors seen in the stained drinking-water biofilms (A) and image taken of the biofilm showing only Factors 1 and 2 (B). Microorganisms appear to be primarily gram negative.

## ***Legionella pneumophila* Biofilms**

### **Introduction**

The study of disease causing organisms, specifically bacterial pathogens, in their native microenvironments is essential for the development of disease prevention strategies and novel treatment methods. Biofilms are complex micro-colonies attached to surfaces via an extra cellular matrix usually made of polysaccharide. Biofilm formation requires bacteria to first bind to surfaces in a non-specific manner, surface proteins such as pili play a major role in this first step. As the adhered bacteria divide, they secrete a polysaccharide extra cellular matrix that provides tighter adhesion between the bacteria and the surface being colonized (5, 7).

*L. pneumophila*, the cause of a lethal pneumonia named legionnaire's disease, is a bacterial pathogen of increasing interest in public health and water quality. The most common victims of Legionnaire's disease are elderly residents of nursing homes and other long term care facilities. HIV infected, organ transplant and cancer treatment patients also suffer significant illness and mortality. *L. pneumophila* can be found free living or associated with biofilms formed by multiple organisms that are also found growing in drinking water (6). In addition, *L. pneumophila* is also found dividing inside drinking water amoebas (1, 3). During the course of normal water use, *L. pneumophila* is aerosolized, enters the lungs of susceptible hosts and causes disease. A number of studies have focused on *L. pneumophila* genetic factors involved in virulence (2, 4). Unfortunately, very little is known about how *L. pneumophila* interacts with drinking water biofilms and what factors are important in this stage of its life cycle. One of the aims of proposed study was to characterize the protein profiles of *L. pneumophila* during planktonic and biofilm growth to understand how the organisms interacts with different surfaces.

## Experiment

### ***Legionella pneumophila* culturing and biofilm generation.**

*L. pneumophila* was cultured from frozen stocks in buffered (ACES/KOH pH = 6.9) Yeast extract liquid media supplemented with activated charcoal, L-cysteine, ferric pyrophosphate. The previously mentioned liquid media was also supplemented with Congo Red dye to confirm biofilm formation. Cells grown in tubes without agitation formed a robust and highly cohesive biofilm with few planktonic cells present in the media. To stimulate planktonic cell formation, culture tubes were placed in shaker incubators (37 °C, 250 RPM) to simulate turbulent flow.

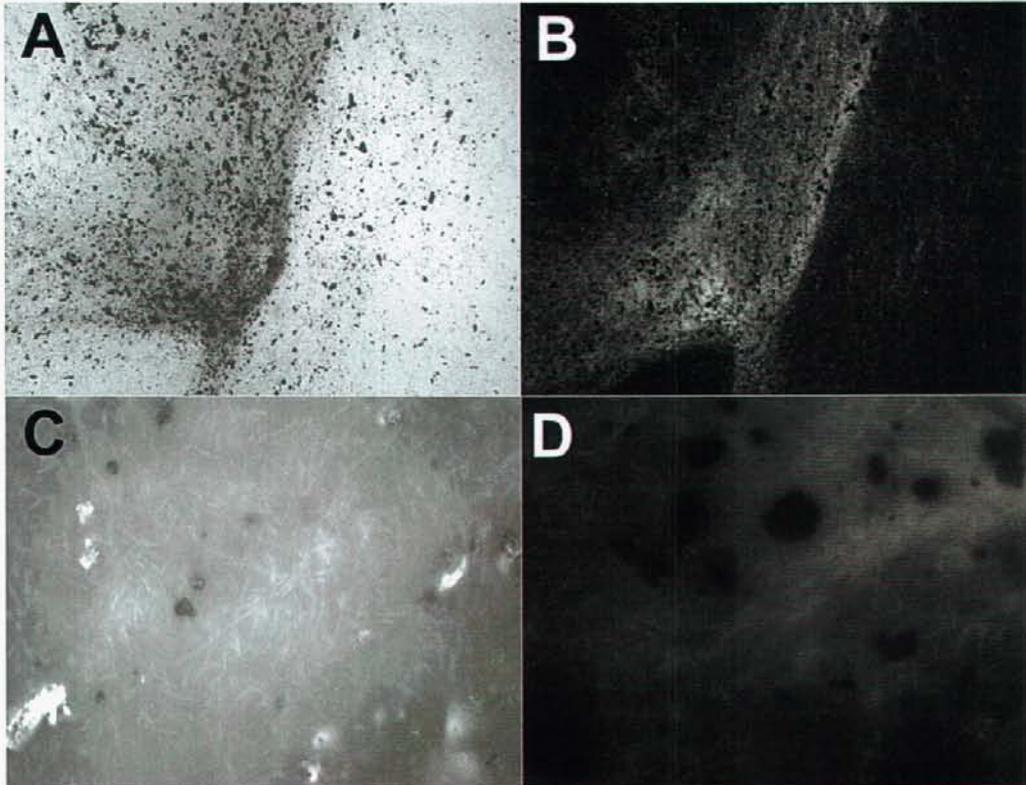
### **2D gel electrophoresis.**

Total proteins were extracted from cells using rehydration/sample buffer containing 9M Urea, 4% CHAPS, 50 mM DTT, and 0.2% biolyte (BioRad). Insoluble debris was cleared using high speed centrifugation, followed by ultracentrifugation ( $r_{fc} = 150,000$  g) for one hour. The solubilized protein was then stored at -20 °C until the day of the analysis. Proteins were then diluted to the desired concentration using rehydration/sample buffer. Proteins were introduced to 3-10 IPG strips (BioRad) by passive rehydration for 12 hours followed by isoelectric focusing using a PROTEAN IEF cell (see results and discussions for protocol). Immediately after isoelectric focusing, cells were separated by size using 12% polyacrylamide gels in a PROTEAN II xi multi-cell following manufacturer's recommendations. Planktonic and biofilm protein samples were always separated in parallel samples on the same run to ensure comparable results. PAGE gels were stained using the Sigma ProteoSilver Plus silver stain kit and images of the stained gels were obtained for analysis.

## Results and Discussion

### **Generating planktonic and biofilm associated *L. pneumophila* cells.**

Liquid media was generated in the laboratory that is based on the solid plate media utilized for growing *L. pneumophila* cells. *L. pneumophila* is slow growing compared to other Gram negative organisms, with visible growth only appearing 3 days after inoculation and incubation. It generally took > 7 days to obtain sufficient growth for protein analysis. Rather than growing and causing generalized turbidity of the media like other bacterial species, *L. pneumophila* grew in large cohesive macrocolonies that were difficult to disrupt even after tube agitation. To confirm that the macrocolony observed was a biofilm, Congo Red dye was added to the media (Congo Red has been shown to stain bacterial colonies organisms generating biofilms). In our experiments, the *L. pneumophila* macrocolonies bound most of the Congo Red molecules present in the media (Fig. 1 A-D). To confirm our finding fragments of the Congo Red stained macrocolony were placed on a slide and examined via light and fluorescent microscopy. As expected, bacterial cells were suspended and held together by an amorphous matrix, presumptively an extracellular polysaccharide, which fluoresced due to the presence of the Congo Red dye (Fig. 1 B and D). Proteins were extracted from these macrocolonies for further study. Since not enough free swimming (Planktonic) bacterial cells were present in *L. pneumophila* cultures, a new growth method had to be used. Tubes that had just been inoculated with *L. pneumophila* frozen stocks were placed in an incubator/shaker (250 rpm) to simulate growth under turbulent flow. This growth method yielded a turbid culture that did not contain *L. pneumophila* macrocolonies. In addition, very little Congo Red dye bound to the cells indicating that the cells were indeed in a planktonic state. The cells generated were pelleted and washed with PBS twice for protein extraction.

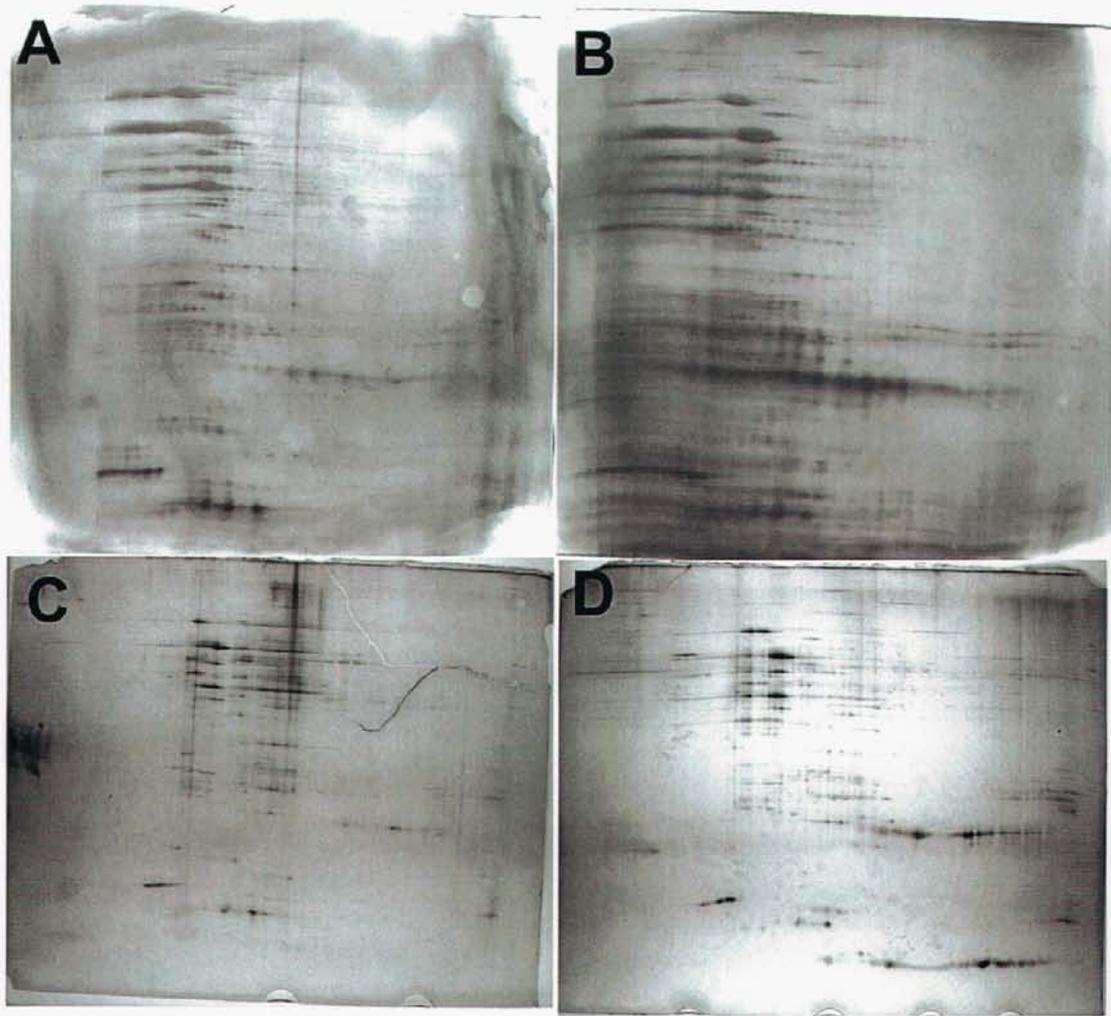


**Figure 6. Light and ultraviolet (UV) micrographs of *L. pneumophila* biofilms.**

Cells grown in liquid media: A) light at 10x objective, B) UV at 10x objective, C) Light at 100x oil immersion objective, and D) UV at 100x oil immersion objective. Biofilms were stained with Congo Red dye (Fluorescent component) which binds to the extrapolsaccharide matrix of biofilms.

### **2D gel protein analysis.**

The protocol for protein separation had to be optimized to obtain satisfactory separation and reduction of contaminating background. The first set of gels that were generated displayed large amounts of contaminating background and poor focusing of abundant protein species (Fig. 2 A and B). The effect was likely due to 1) overloading (0.6 mg total protein), 2) insoluble components, 3) and too rapid a voltage slope or insufficient focusing time. In the first attempt we had only clarified the solubilized protein with high-speed centrifugation. An additional step, ultracentrifugation at an  $r_{fc} = 150,000$  for 1 hour, was added to the protocol to ensure removal of insoluble material. The amount of protein loaded was also reduced to 0.06 mg total protein, and a slow voltage ramping method was used. The optimized protein separation protocol yielded much better results (Fig. 2 C and D). Different protein species were now resolved, and differences between *L. pneumophila* cells grown under different conditions began to be detected. Further optimization will be required, yet clearly *L. pneumophila* planktonic and biofilm cells display different protein contents even though they are growing in identical nutrient media.



**Figure 7. Analysis of *Legionella pneumophila* proteins via 2D-gel electrophoresis.**

Proteins analyzed were extracted from cells growing in Biofilms (A = 0.6 mg total protein and C = 0.06 mg total protein) and from planktonic cells (B = 0.6 mg total protein and D = 0.06 mg total protein). Proteins were separated by isoelectric focusing on 3-10 IPG strips followed by separation by size via 12% PAGE, and silver stained. The acidic end of the strips was located on the left edge of the gel image.

## Culture of *Francisella novicida* Utah 112

The initiation of experiments with *Francisella novicida* were unexpectedly delayed because of the arbitrary decision made by the American Type Culture Collection to suspend distribution of the organism. This delay was further compounded by the additional NIH approval process required to obtain the organism from the only alternative source, Biodefence and Emerging Infections Research Resources Repository. This delay in addition to the late funding of this project severely limited the time available for the performance of milestones that were dependent on culturing of the organism. Nonetheless we were able to make significant progress that will play an important role in newly funded LDRDs and in future external grant applications.

*F. novicida* was cultured in three different liquid media, Modified Mueller-Hinton, Trypticase Soy Broth and Chamberlain's Defined Broth. In addition cultures were maintained on Trypticase Soy Agar and Cystine Heart Agar.

### *F. novicida* Utah 112 Culture Media and Reagents

Most of the formulations of growth media for culturing *F. novicida* Utah 112 are scattered through the literature. Some are in papers that are more than forty years old. In order to facilitate ongoing research in this organism we have included the collected media formulations below.

**Table 1. Media formulations.**

**"TSBC" Tryptic Soy Broth with 0.1% Cysteine**

Lauriano et al, Proc Natl Acad Sci U S A. 2004 Mar 23;101(12):4246-9

3 %	Trypticase Soy Broth Powder, BBL # 211768	30 grams per 1 L
0.1%	Cysteine	1 gram per 1 L

Combine 30 grams TSB powder and 1 gram of cysteine with 975 mL di-water  
Autoclave for 30 minutes.

**"TSBC Agar" Tryptic Soy Broth Agar with 0.1% Cysteine**

Lauriano et al, Proc Natl Acad Sci U S A. 2004 Mar 23;101(12):4246-9

4 %	Trypticase Soy Agar Powder, BBL # 211043	40 grams per 1 L
0.1%	Cysteine	1 gram per 1 L

Combine 40 grams TSB Agar powder and 1 gram of cysteine with 975 mL di-water  
Autoclave for 30 minutes.

Cool to 50-60 C.

Pour into petri dishes.

**"MMH BROTH" MODIFIED MUELLER HINTON BROTH**

Maier et al, App Env Micro, Dec 2004, p 7511-7519

2.1 %	Mueller Hinton Broth Powder, Difco #275730	21 grams per 1 L
0.1 %	Glucose	5 mL of 20%
0.025 %	Ferric Pyrophosphate	10 mL of 25 mg/mL or 2.5% or 100X
2 %	BBL IsoVitaleX, Difco #211876	20 mL, resuspended as instructed

Combine 21 grams MH broth powder with 965 mL di-water.

Autoclave for 30 minutes.

Cool to 50-60 C.

Add glucose, ferric pyrophosphate, and isovitalex aseptically; filter if needed.  
Check that pH is 7.0 +/- 0.1 at 25C.

**"MMH AGAR" MODIFIED MUELLER HINTON AGAR**

Maier et al, App Env Micro, Dec 2004, p 7511-7519

3.8 %	Mueller Hinton Agar Powder, Difco #225250	38 grams per 1 L
0.1 %	Glucose	5 mL of 20%
0.025 %	Ferric Pyrophosphate	10 mL of 25 mg/mL or 2.5% or 100X
2 %	BBL IsoVitaleX, Difco #211876	20 mL, resuspended as instructed
2.5 %	Fetal Bovine Serum, Invitrogen #10082147	25 mL

Combine 38 grams MH agar powder with 940 mL di-water.

Autoclave for 30 minutes.

Cool to 50-60 C.

Add glucose, ferric pyrophosphate, isovitalex, and FBS aseptically.

Pour into petri dishes.

Check that pH is 7.0 +/- 0.1 at 25C.

**"CDM BROTH" CHAMBERLAIN'S CHEMICALLY DEFINED MEDIUM BROTH**

Chamberlain, App Micro, March 1965

Cherwonodgrodzky et al, Vaccine 1994 vol 12 no 9

Final concentration:	Component	Add per 1000 mL:
10 mg/mL	NaCl	100 mL of 100 mg/mL
2 mg/mL	L-proline (hydroxy-L-proline free) CAS 147-85-3	40 mL of 50 mg/mL
2 mg/mL	DL-threonine (allo free) CAS 80-68-2	40 mL of 50 mg/mL
4 mg/mL	glucose	20 mL of 200 mg/mL
0.4 mg/mL	DL-isoleucine CAS 443-79-8	16 mL of 25 mg/mL
0.4 mg/mL	DL-methionine CAS 59-51-8	16 mL of 25 mg/mL
0.4 mg/mL	L-tyrosine CAS 60-18-4	16 mL of 25 mg/mL
1 mg/mL	K <sub>2</sub> HPO <sub>4</sub>	10 mL of 100 mg/mL
1 mg/mL	KH <sub>2</sub> PO <sub>4</sub>	10 mL of 100 mg/mL
0.4 mg/mL	L-aspartic acid CAS 56-84-8	8 mL of 50 mg/mL
0.2 mg/mL	L-histidine (free base) CAS 71-00-1	8 mL of 25 mg/mL
0.4 mg/mL	L-leucine (methionine free) CAS 61-90-5	8 mL of 50 mg/mL
0.4 mg/mL	DL-serine CAS 302-84-1	8 mL of 50 mg/mL
0.4 mg/mL	DL-valine CAS 516-06-3	8 mL of 50 mg/mL
0.4 mg/mL	L-arginine (free base) CAS 74-79-3	4 mL of 100 mg/mL
0.2 mg/mL	L-cysteine-HCl CAS 52-89-1	4 mL of 50 mg/mL
0.4 mg/mL	L-lysine (mono HCl) CAS 657-27-2	4 mL of 100 mg/mL
0.04 mg/mL	spermine phosphate CAS 3891-79-0	2 mL of 20 mg/mL
0.004 mg/mL	thiamine HCl CAS 67-03-8	2 mL of 2 mg/mL
0.135 mg/mL	MgSO <sub>4</sub> -7H <sub>2</sub> O	1.35 mL of 100 mg/mL
0.002 mg/mL	DL-calcium pantothenate CAS 63409-48-3	0.5 mL of 4 mg/mL

All components are prepared as individual solutions.

Combine ingredients with appropriate amount of di-water.

Adjust pH to 6.2-6.4 with 3N HCl.

Filter sterilize.

## "CDM AGAR" CHAMBERLAIN'S CHEMICALLY DEFINED MEDIUM AGAR

Chamberlain, App Micro, March 1965

Cherwonodgrodzky et al, Vaccine 1994 vol 12 no 9

Final concentration:	Component	Add per 1000 mL:
1%	bacto-Agar	10 grams
10 mg/mL	NaCl	100 mL of 100 mg/mL
2 mg/mL	L-proline (hydroxy-L-proline free) CAS 147-85-3	40 mL of 50 mg/mL
2 mg/mL	DL-threonine (allo free) CAS 80-68-2	40 mL of 50 mg/mL
4 mg/mL	glucose	20 mL of 200 mg/mL
0.4 mg/mL	DL-isoleucine CAS 443-79-8	16 mL of 25 mg/mL
0.4 mg/mL	DL-methionine CAS 59-51-8	16 mL of 25 mg/mL
0.4 mg/mL	L-tyrosine CAS 60-18-4	16 mL of 25 mg/mL
1 mg/mL	K <sub>2</sub> HPO <sub>4</sub>	10 mL of 100 mg/mL
1 mg/mL	KH <sub>2</sub> PO <sub>4</sub>	10 mL of 100 mg/mL
0.4 mg/mL	L-aspartic acid CAS 56-84-8	8 mL of 50 mg/mL
0.2 mg/mL	L-histidine (free base) CAS 71-00-1	8 mL of 25 mg/mL
0.4 mg/mL	L-leucine (methionine free) CAS 61-90-5	8 mL of 50 mg/mL
0.4 mg/mL	DL-serine CAS 302-84-1	8 mL of 50 mg/mL
0.4 mg/mL	DL-valine CAS 516-06-3	8 mL of 50 mg/mL
0.4 mg/mL	L-arginine (free base) CAS 74-79-3	4 mL of 100 mg/mL
0.2 mg/mL	L-cysteine-HCl CAS 52-89-1	4 mL of 50 mg/mL
0.4 mg/mL	L-lysine (mono HCl) CAS 657-27-2	4 mL of 100 mg/mL
0.04 mg/mL	spermine phosphate CAS 3891-79-0	2 mL of 20 mg/mL
0.004 mg/mL	thiamine HCl CAS 67-03-8	2 mL of 2 mg/mL
0.135 mg/mL	MgSO <sub>4</sub> ·7H <sub>2</sub> O	1.35 mL of 100 mg/mL
0.002 mg/mL	DL-calcium pantothenate CAS 63409-48-3	0.5 mL of 4 mg/mL
0.002 mg/mL	FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 mL of 4 mg/mL

All components are prepared as individual solutions.

Combine 10 g bacto-Agar with 670 mL di-water. Autoclave 30 minutes.

Cool to 50-60 C.

Combine other components and filter sterilize; add to cooled agar.

Adjust pH to 6.2-6.4 with 3N HCl.

Pour into petri dishes.

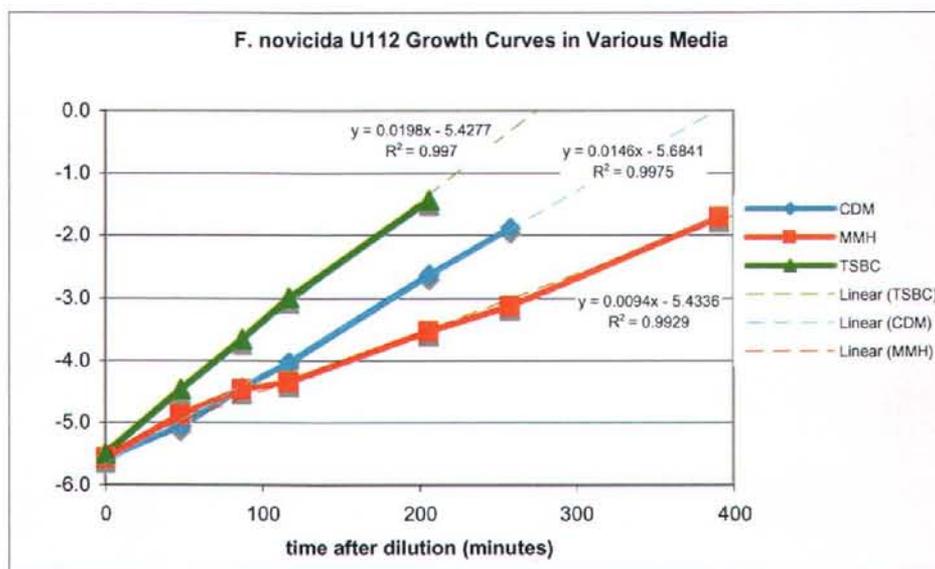
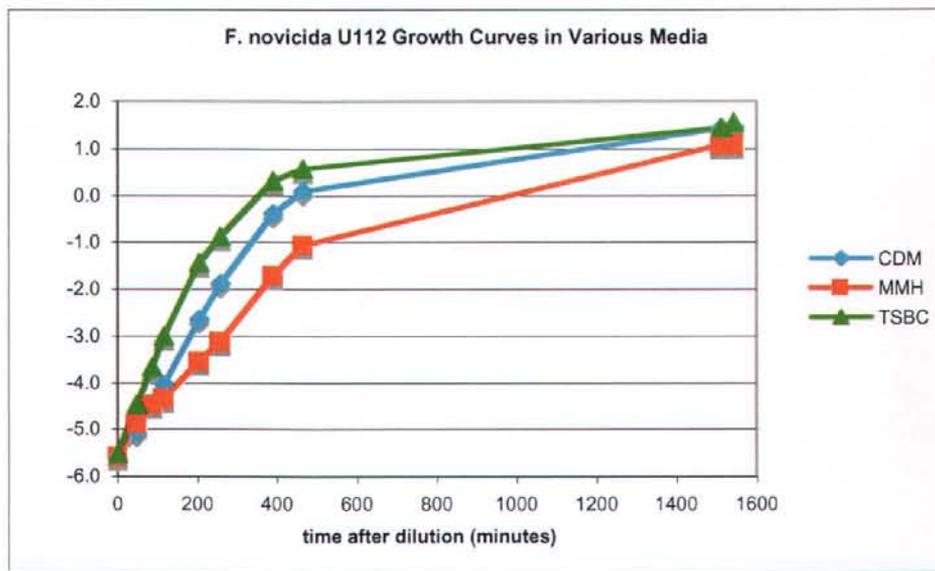
## ***F. novicida* Utah 112 Growth Analysis**

*F. novicida* Utah 112 cells streaked onto CHA agar plates appear bluish-white in color after 24 hours at 37°C and turn white after 3-4 days at 37°C. If 1-2 day old plates are stored at 4°C, the cells will retain the bluish-white color for about 2 weeks, and eventually they will become white. Viability is significantly reduced after 2 weeks and is lost after 4 weeks at 4°C.

Cells plated on TSB agar supplemented with cysteine appear clear to slightly opaque, and develop smaller colonies than on CHA plates. Long-term viability on TSB agar plates is unknown.

Glycerol and DMSO stocks were prepared with cells grown in TSBC to stationary phase; glycerol and DMSO concentrations used were 10, 15, 20, 25, and 30%. Many viable cells were recovered from the 10% glycerol stock onto TSBC and CHA plates; viability of the remaining stocks is not known.

Growth of cells in various liquid culture media was analyzed by measurement of OD at 600 nm over time (Figure 7), and the doubling times of the cells in each media were determined (Figure 8). Results are summarized below:



**Figure 8. Growth characteristics of *Francisella novicida*.**

The doubling times of *F. novicida* Utah 112 in different culture media were determined from two independent experiments (graphs for second experiment not shown). Serial dilutions of exponentially growing and stationary phase *F. novicida* Utah 112 cultures were plated on TSBC plates to determine the culture titers in CFU/mL. Plates were incubated for 24 hours at 37°C, and colonies were counted.

**Table 2. Determination of culture titers in colony-forming units.**

Media	OD 600 nm	10 <sup>-4</sup> dilution	10 <sup>-5</sup> dilution	10 <sup>-6</sup> dilution	10 <sup>-7</sup> dilution	10 <sup>-8</sup> dilution		
<b>NUMBER OF COLONIES AFTER 24 HOURS:</b>								
CDM	0.161	n/a	too many	92	11	1		
MMH	0.086	n/a	272	23	2	0		
TSBC	0.369	n/a	too many	493	41	7		
CDM	2.70	too many	too many	1000	n/a	n/a		
MMH	1.96	too many	too many	1000	n/a	n/a		
TSBC	2.83	too many	too many	1000	n/a	n/a		
<b>CALCULATED CFU/ML FROM COLONY COUNTS:</b>							<b>AVERAGE:</b>	<b>CFU/OD:</b>
CDM	0.161	n/a	n/a	9.20E+08	1.10E+09	1.00E+09	1.01E+09	6.25E+09
MMH	0.086	n/a	2.72E+08	2.30E+08	2.00E+08	n/a	2.34E+08	2.72E+09
TSBC	0.369	n/a	n/a	4.93E+09	4.10E+09	7.00E+09	5.34E+09	1.45E+10
CDM	2.70	n/a	n/a	1.00E+10	n/a	n/a	1.00E+10	3.70E+09
MMH	1.96	n/a	n/a	1.00E+10	n/a	n/a	1.00E+10	5.10E+09
TSBC	2.83	n/a	n/a	1.00E+10	n/a	n/a	1.00E+10	3.53E+09

**Table 3. *F. novicida* doubling rates.**

Date	09-08-2005	10-18-2005
CDM	68.5 min	82.0 min
MMH	106.4 min	117.6 min
TSBC	50.5 min	70.4 min

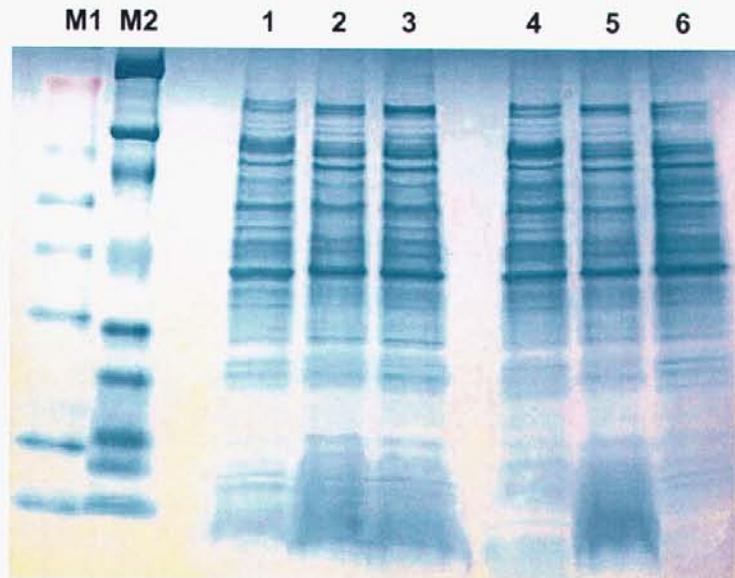
## Initial Proteomic Analysis of *Francisella novicida* Utah 112

Samples of exponentially growing and stationary *F. novicida* Utah 112 cultures were lysed, and concentration of total protein was determined using the BCA protein assay (Pierce). 500 uL of cultures in various media at various times were lysed in 150 uL of 1X BugBuster (Novagen). The following dilutions of lysates were tested in the assay: 1:1, 1:5, 1:10, 1:20, and 1:30. BSA was used as a standard, and the assay was carried out as in the instruction manual. The relationship between optical density of the culture and the protein content was determined. Multiplying culture OD by 437 will provide an approximate protein concentration in the culture in ug/mL.

**Table 4. Protein concentration determination.**

Media	Timepoint	OD600	ug/mL in lysate	ug/mL in culture	ug/mL in culture per OD	mean ug/mL in culture per OD
CDM	1T3	0.18	296.3	88.9	496.5	<b>437</b>
CDM	1T5	0.67	757.9	227.4	339.8	
CDM	1T6	1.35	1538.7	461.6	341.9	
CDM	2T6	2.28	2101.2	630.3	276.5	
CDM	4T4	0.16	220.2	66.1	410.3	
CDM	4T6	0.77	744.5	223.3	290.1	
MMH	4T4	0.09	144.6	43.4	504.6	
MMH	4T6	0.30	479.3	143.8	473.0	
TSBC	1T3	0.49	1106.9	332.1	676.3	
TSBC	1T5	1.00	1957.3	587.2	589.6	
TSBC	1T6	2.03	2920.1	876.0	431.5	
TSBC	2T6	2.81	3262.4	978.7	348.3	
TSBC	4T4	0.37	728.6	218.6	592.4	
TSBC	4T6	1.25	1456.6	437.0	349.6	

Protein from *F. novicida* Utah 112 lysates was separated by SDS-PAGE and stained with SimplyBlue SafeStain (Invitrogen). Each well contains approximately 15 ug of total protein estimated by multiplying the culture OD by 437. The volume of 1X BugBuster added to the cell pellets was such that the lysate would contain about 10 ug/uL protein. A representative gel is shown in Figure 9. Clear differences in the protein profile can be seen between the cells grown in the different culture media.



**Figure 9. SDS PAGE of *F. novicida*.**

Analysis of total protein from *F. novicida* Utah 112 lysates by SDS-PAGE on a 4-16% Tris-glycine gel. M1: SeeBlue Plus2 marker (Invitrogen); M2: Broad Range protein marker (Bio-Rad); 1-3: lysate from exponentially growing 6.5 hour old cultures in CDM, MMH, and TSBC, respectively; 4-6: lysate from stationary cultures (24 hours old) in CDM, MMH, and TSBC, respectively.

Although we were limited (by time constraints) to one dimensional SDS PAGE we were able to see clear differences in the protein profile of cells grown in different medium. This is an important result to follow up on in our attempts to develop robust proteomic signatures for this agent. Future work will include analysis by two dimensional PAGE and followed by mass spectroscopy. This will enable to better characterize the growth condition and growth stage dependent differences that are likely present.

## Conclusions

This short-term research project has produced a number of results that can be applied to new and ongoing programs. We have successfully cultured multispecies drinking water biofilms and monospecific biofilms of *Legionella pneumophila*. We have demonstrated the efficacy of the hyperspectral imaging system for analyzing these systems. We have demonstrated our ability to culture the highly fastidious *Francisella novicida* and detected growth state dependent differences in the protein profile of the organism.

## References

- Andreeva, A., Velitchkova, M. "Resonance Raman spectroscopy of carotenoids in photosystem I particles," *Biophysical Chemistry* 114 (2005)129-135.
- Belyi, Y. 1999. Intracellular parasitism and molecular determinants of *Legionella* virulence. *Int Microbiol* 2:145-54.
- Byrne, B., and M. S. Swanson. 1998. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infect Immun* 66:3029-34.
- Cirillo, J. D., S. L. Cirillo, L. Yan, L. E. Bermudez, S. Falkow, and L. S. Tompkins. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infect Immun* 67:4427-34.
- Forsbach-Birk, V., T. McNealy, C. Shi, D. Lynch, and R. Marre. 2004. Reduced expression of the global regulator protein CsrA in *Legionella pneumophila* affects virulence-associated regulators and growth in *Acanthamoeba castellanii*. *Int J Med Microbiol* 294:15-25.
- Haaland, D. M., J. A. Timlin, M. B. Sinclair, M. H. Van Benthem, M. J. Martinez, A. D. Aragon and M. Werner-Washburne: Multivariate Curve Resolution for Hyperspectral Image Analysis: Applications to Microarray Technology. In *Spectral Imaging: Instrumentation, Applications, and Analysis* (R. M. Levenson, G. H. Bearman and A. Mahadevan-Jansen, eds.), Vol. 4959, paper 06, International Society for Optical Engineering, San Jose, CA, 2003.
- Hall-Stoodley, L., J. W. Costerton, and P. Stoodley. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2:95-108.
- Sinclair, M. B., Haaland, D. M., Timlin, J. A., and Jones, H. D. T., *Hyperspectral Confocal Microscope*. (To be submitted to *Applied Optics*)
- Sinclair, M. B., Haaland, D. M., Timlin, J. A., and Jones, H. D. T., *Hyperspectral Confocal Microscope*. (To be submitted to *Applied Optics*)
- Van Benthem, M. H., Keenan, M. R. and Haaland, D. M. (2002) Application of equality constraints on variables during alternating least squares procedures. *J. Chemometrics*, 16, 613-622.
- van der Kooij, D., H. R. Veenendaal, and W. J. Scheffer. 2005. Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. *Water Res* 39:2789-98.
- Webb, J. S., M. Givskov, and S. Kjelleberg. 2003. Bacterial biofilms: prokaryotic adventures in multicellularity. *Curr Opin Microbiol* 6:578-85.

## Distribution List

1	MS 9004	Terry Michalske, 8300
1	MS 0701	Peter Davies, 8100
1	MS 1413	Grant Hefflefinger, 8330
1	MS 9291	Glenn Kubiak 8320
1	MS 9292	Malin Young, 8321
1	MS 0735	Ray E. Finley
1	MS 1413	Paul Dressendorfer
3	MS 9292	Todd Lane, 8321
1	MS 0735	Susan Altman, 6115
1	MS 1413	Roberto Rebeil, 8331
1	MS9292	Julie Kaiser, 8321
2	MS 9018	Central Technical Files, 8945-1
2	MS 0899	Technical Library, 9616
1	MS 0323	D. Chavez