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Preliminary Assessment of the Interaction of Introduced Biological Agents with Biofilms in Water Distribution Systems

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Preliminary Assessment of the Interaction of Introduced Biological Agents with Biofilms in Water Distribution Systems

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Abstract

Basic research is needed to better understand the potential risk of dangerous biological agents that are unintentionally or intentionally introduced into a water distribution system. We report on our capabilities to conduct such studies and our preliminary investigations. In 2004, the Biofilms Laboratory was initiated for the purpose of conducting applied research related to biofilms with a focus on application, application testing and system–scale research. Capabilities within the laboratory are the ability to grow biofilms formed from known bacteria or biofilms from drinking water. Biofilms can be grown quickly in drip–flow reactors or under conditions more analogous to drinking–water distribution systems in annular reactors. Biofilms can be assessed through standard microbiological techniques (i.e, aerobic plate counts) or with various visualization techniques including epifluorescent and confocal laser scanning microscopy and confocal fluorescence hyperspectral imaging with multivariate analysis. We have demonstrated the ability to grow reproducible *Pseudomonas fluorescens* biofilms in the annular reactor with plate counts on the order of 10^5 and 10^6 CFU/cm^2. Stationary phase growth is typically reached 5 to 10 days after inoculation. We have also conducted a series of pathogen–introduction experiments, where we have observed that both polystyrene microspheres and *Bacillus cereus* (as a surrogate for *B. anthracis*) stay incorporated in the biofilms for the duration of our experiments, which lasted as long as 36 days. These results indicated that biofilms may act as a safe harbor for bio–pathogens in drinking water systems, making it difficult to decontaminate the systems

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1 INTRODUCTION

Basic research is needed to better understand the potential risk of dangerous biological agents that are introduced (either unintentionally or intentionally by a bio–terrorist) into a water distribution system. Studies are needed to answer questions such as: 1) do biofilms enhance or reduce the initial risk of toxic exposure to a host of biological agents? 2) what effect does a biofilm have on restoration and remediation activities after an attack has been carried out? and, 3) do biofilms add a latency dose that will remain in the pipelines even after shocking with chlorine or other typical cleaning methodologies?

A biofilm is a colony of microorganisms attached to a surface and encased in extracellular polymeric substances (EPS) (Figure 1). 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 (Mattila–Sandholm and Wirtanen, 1992). The pipe interiors within water distribution systems are often covered with biofilms. Quignon et al. (1997) and Storey and Ashbolt (2001) found that viruses are adsorbed by biofilms. However, the extent that biofilms can permanently remove microorganisms is unclear. It is possible that due to boundary layer effects or the protective coatings on biofilms (Momba et al., 2000) some microorganisms could be unaffected. If microorganisms are adsorbed by biofilms then the question of how readily they are remobilized needs to be addressed. Another issue is that bacteria adsorbed by a biofilm are less likely to be killed by a chemical disinfectant, such as chlorine. Therefore, there is also a potential danger of release of microorganisms when removing biofilms or if biofilms degrade. Also, the current clean–up strategies that include shocking systems with chlorine may not be as effective as previously thought if contaminants can be protected within a biofilm. A better understanding of the effects of biofilms on microorganism transport is critical to determining the risk of terrorist attack and the most effective method for sanitizing a water distribution system after such an attack.

There has been much public speculation about terrorists releasing airborne *Bacillus anthracis,* the bacteria that causes anthrax, and the subsequent effects of *B. anthracis* inhalation. However, what if *B. anthracis* is released into a drinking water distribution system? Gastrointestinal anthrax has a high mortality rate (though the few documented cases are due to eating contaminated meat) (Pile et al., 1998; Inglesby et al., 1999). In addition, the symptoms of gastrointestinal anthrax appear 2 to 7 days after ingestion (Pile et al., 1998). This latency period would allow for ingestion of *B. anthracis* by many of the water system users before the problem was detected. Any other bacteria, protozoan, or viral agent are potential threats to water distribution systems if they have a high rate of mortality after ingestion, can persist in the water supply, and are available to terrorists. Teter and Brady (2003) indicate that sabotage of water–supply systems with biological agents is a legitimate threat. Burrows and Renner (1999) list 10 replicating agents, including *B. anthracis*, and 9 biotoxins that are threats or probable threats to water systems. The authors anticipate that the number of threatening agents will increase. There is very little research on how agents behave in water systems, thus increasing the uncertainty of any such risk or vulnerability assessment.

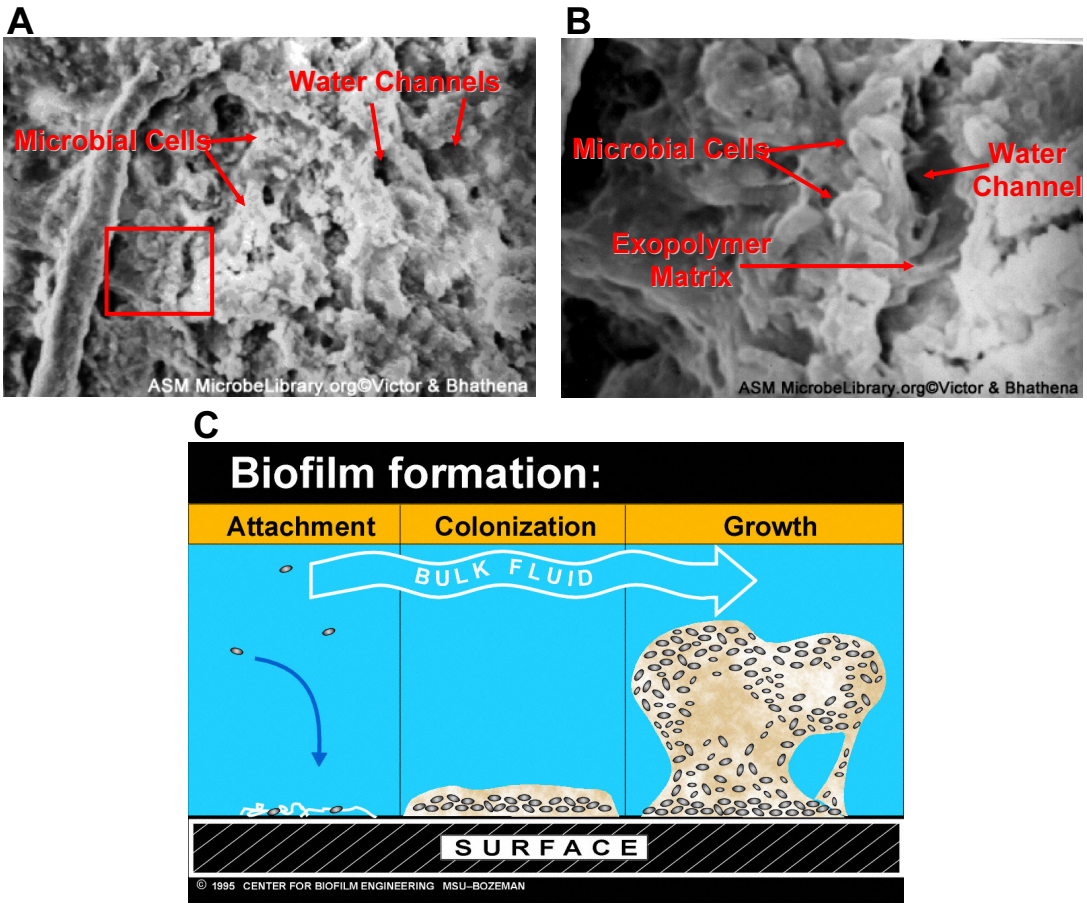

Figure 1: Scanning Electron Micrographs of a heterogeneous, drinking–water biofilm at 543× (A) and 2459× (B) (from American Society of Microbiology), and schematic showing the 3 stages of biofilm formation: attachment, colonization and growth of complex biofilm structures (from the Montana State University, Center for Biofilm Engineering).

The Cryptosporidium outbreak in Milwaukee (estimated over 50 fatalities [Hoxie et al., 1997] and 403,000 cases watery diarrhea [MacKenzie et al., 1994], or 50% of the approximately 800,000 people to which the Milwaukee Water Utility pumps water [Fox and Lytle, 1996], or 25% of the approximately 1.61 million Milwaukee population) demonstrate the possibility of the spread of a contaminant through a water distribution system. Cryptosporidium is a parasite that causes diarrhea and is potentially fatal to children, elderly, and adults with deficient immune systems. If *B. anthracis* is disseminated in a manner similar to the Cryptosporidium in Milwaukee, it is feasible that fatalities could be 25% of the population, or on the order of 10^5 to 10^6 people in the U.S.'s 25 largest cities. However, given the uncertainty in the problem, it is unclear how realistic this extrapolation is from the Cryptosporidium outbreak to a *B. anthracis* attack.

There are many costs associated with the risk of a bioterrorist attack to a water distribution system. First, there is the cost of minimizing vulnerability. Assuming a system is compromised, there are medical, production losses, and clean–up costs. The cost of the Milwaukee

Cryptosporidium outbreak is estimated to be on the order of almost $100 million: $31.7 million in medical costs and $64.6 million in productivity losses (Corso et al., 2003).

Currently, municipalities are required to conduct a vulnerability assessment of the threat of a terrorist attack on their water supply systems as mandated by the Bioterrorism Act. Medium and large municipalities have conducted these assessments and found that water distribution systems are highly vulnerable to terrorism because there are virtually an infinite number of access sites to the system downstream of the treatment sites (consider the number of valves outside homes). It is difficult to protect water distribution systems and tools to evaluate the risk are just now being developed. It is possible that attacks would be ineffective in water distribution systems because of dilution of the contaminant. However, there remains great uncertainty in the efficacy of dilution, at least for some contaminants. It is also possible that eliminating vulnerabilities could be prohibitively expensive. Risk must be quantitatively assessed further, including developing a clear understanding of what effect biofilms have on the transmission of potential terrorist threat agents. There is considerable uncertainty surrounding the potential terrorist agents and their behavior in water systems that research into agent interactions with biofilms is a component of the necessary research portfolio.

This document reports on preliminary research with the goal of acquiring data to quantify the uncertainty associated with the risk of pathogenic biological agents being introduced into water distribution systems and the effects of biofilms on this risk. The document describes our laboratory facilities in which we are conducting the research (Section 2), experiment run to grow reproducible biofilms in a environment similar to drinking water distribution systems (Section 3), experiments examining the integration of biopathogen surrogates with biofilms (Section 4) and our methods for growing biofilms from drinking water (Section 5).

2 BIOFILMS LABORATORY OVERVIEW

The Biofilms Laboratory is used to conduct applied research related to biofilms. The focus of the research conducted in the Biofilms Laboratory is on application, application testing and system–scale research. This research is intended to be complimentary to the more fundamental research being conducted at Sandia National Laboratories. Development of the Biofilms Laboratory was initiated in early 2004. To date, research conducted in the laboratory has focused on 1) biofilms in water distribution systems, as discussed in this report, 2) biofouling of membranes for use in water treatment systems, and 3) preliminary research related to cell–to–cell communications in mixed community biofilms. The current capabilities of the Biofilms Laboratory are discussed below.

2.1 Reactors

Currently the Biofilms Laboratory has two types of reactors with which to grow biofilms: drip–flow reactors (BioSurface Technologies, Corporation, Model DFR 110) and annular reactors (BioSurface Technologies, Corporation, Model 1120 LJ). The drip–flow reactors are useful for growing biofilms quickly and easily under a low shear environment. The annular reactor is used to simulate drinking water distribution system conditions by controlling the residence time and shear force of the water.

The drip–flow reactor consists of four channels into which slides with approximate dimensions of 2.5 × 7.5 cm can be placed (Figure 2). The slides can be glass microscope slides or constructed of a material of choice. Solutions are dripped into one end and discharged from the other end. Solutions generally consist of the media containing the microorganisms and nutrient

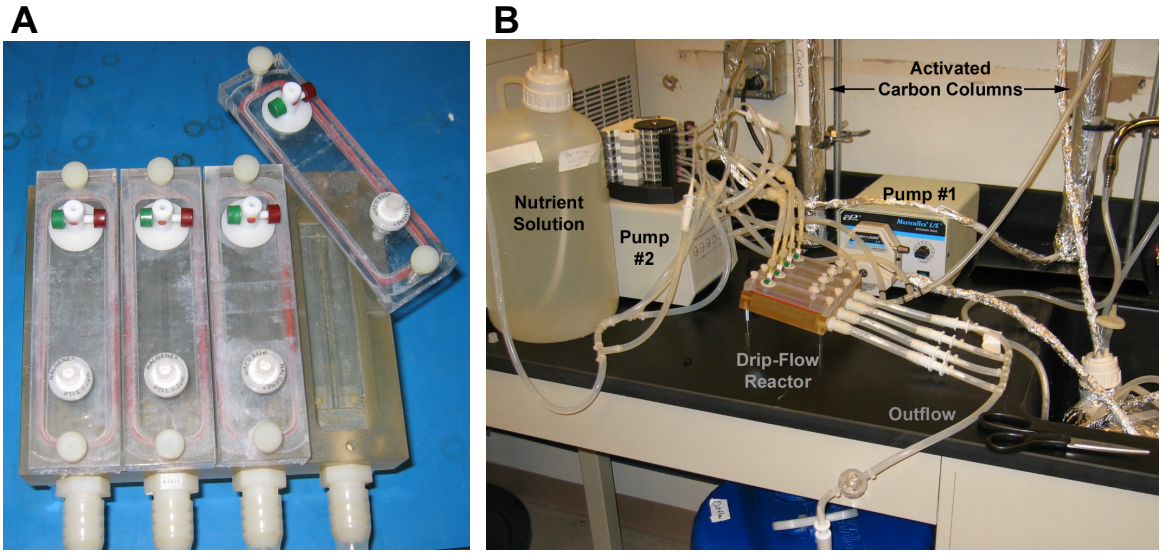

Figure 2: Photographs of drip–flow reactor showing 4 channels where slides can be placed (A) and the reactor set up to grow biofilms from drinking water (B).

solutions. If a microorganism inoculum (cells added to start a culture) is used to make the biofilms, this inoculum is typically injected into the system and allowed to sit for 24 hours prior to starting flow. Flow rates into the reactor are relatively slow (usually less than 1 ml/min). The reactor is sloped by approximately 10 degrees so that water will flow in a low–shear environment from inflow to discharge end. The time it takes to grow biofilms is dependent on the nutrient solution concentration and the inoculant type and concentration. Though, biofilms can typically be grown on the slides in just a few days. The slides are individually removed when it is time to study the biofilms.

The annular reactors are used to conduct experiments simulating drinking–water distribution system conditions (Figure 3, Section 3.1.1). The reactor consists of an inner cylinder into which twenty slides can be places. The slides are 15 × 1.25 cm. Slides can be made of any materials including polycarbonate, PVC, cast iron, and steel. The inner cylinder rotates in order to control the shear force on the biofilms. Another cylinder is used to contain the solutions from which the biofilms will be grown. Finally, an optional third cylinder can be used to contain water of known temperature in order to control the temperature of the system. This water does not interact with the solution in contact with the rotating cylinder. Solutions flow into the different areas from ports on the top of the reactor and discharge through ports at the bottom of the reactor.

The drip–flow reactor has been used to grow biofilms in order to test staining and visualization techniques, grow biofilms from drinking water (Section 5), and grow biofilms in order to develop a method for measuring biofilm thickness by cryosectioning (see Section 2.2). The

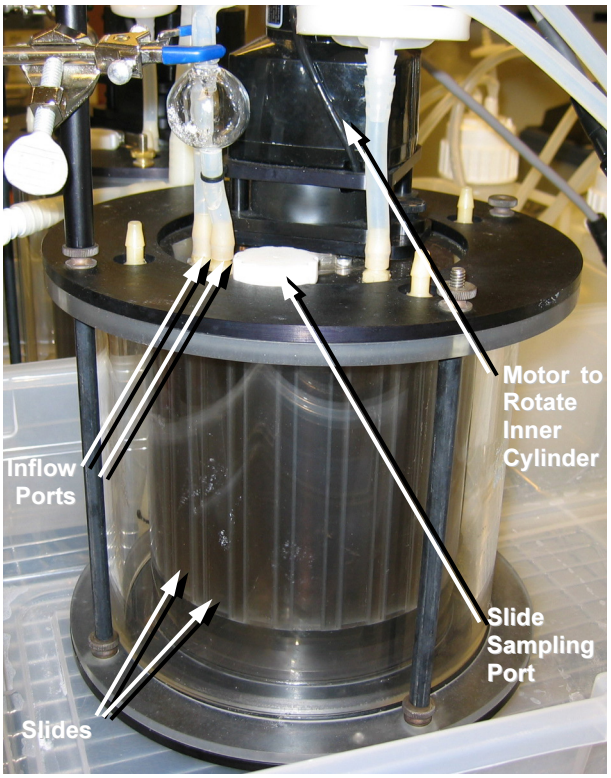

Figure 3: Photograph of annular reactor. See Figures 8 and 9 for a schematic and photograph of annular reactors set up for use in biofilms–growth experiments.

annular reactors have been used to conduct biofilm–growth (Section 3) and pathogen–introduction (Section 4) experiments.

2.2 Biofilm–Growth Measurement Techniques

We predominantly conduct heterotrophic plate counting to measure biofilm growth. In order to do this, samples (slides) are collected from reactors and scraped and sonicated according to the methods described in Appendix A. The solution is then serially diluted (Appendix B) and enumerated on appropriate media. Plates are kept in an incubator at 30 ºC for at least 24 hours and then counted. The unit for this type of plate counting is Colony Forming Units (CFU) per surface area of the slide on which the biofilms are grown (CFU/cm^2). In general, at least 4 replicated plates are made for each sample in order to quantify the amount of variability in our measurement.

Because some of our experiments (see Section 4) require the plating of both *Pseudomonas fluorescens* and *Bacillus cereus*, we conducted a study to determine how well we can selectively plate gram–negative (*Pseudomonas fluorescens*) and gram–positive organisms (*Bacillus cereus*). These media are discussed further in Section 4.1. The most common media used in the laboratory include Trypticase Soy Agar (TSA) and R2A Agar. We also use Polymyxin B (an antibiotic) in TSA to selectively plate for gram–negative organisms. To a smaller extent, we have worked with Eosin Methylene Blue (EMB) and MacConkey agars.

In addition to heterotrophic plate counting, we are initiating a program to cryosection biofilms in order to measure thickness. This method involves freezing a biofilm and making thin–sections of the film. These thin–sections can then be observed through a microscope in order to measure a thickness. Fluorescent staining of the biofilms is often used to enhance visualization.

Finally, visualization techniques (Section 2.3) are used to qualitatively assess biofilm growth.

2.3 Visualization Techniques

The purpose for visualization of biofilms is to: 1) visualize that we have indeed grown biofilms and can see the microorganisms and EPS, 2) determine whether the pathogens are integrated in the biofilms in the pathogen–introduction experiments (Section 4), 3) have another metric available for qualitatively assessing biofilm growth, and 4) measure biofilms thickness (still in progress). Three visualization techniques have been predominantly used: epifluorescence microscopy, confocal laser scanning microscopy (CLSM), and confocal fluorescence hyperspectral imaging with multivariate analysis. All three measurements are available for use by our laboratory.

Epifluorescent microscopy has the advantage of being able to attain images relatively quickly on stained biofilms. Staining allows for differentiation of microorganisms and EPS, gram–positive and gram–negative microorganisms, alive and dead organisms, etc. This is done by selecting stains that emit fluorescence at different wavelengths. For example, we currently use two nucleic acid stains to differentiate gram–positive from gram–negative organisms: SYTO®9 and

hexidium iodide (purchased at Molecular Probes). SYTO®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®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). The laboratory currently uses a Nikon 80i Epifluorescent microscope equipped with several different objectives and filter cubes. Examples of epifluorescent images that we have obtained are presented in Figure 4.

The advantage of CLSM is that it allows for visualization of one plane in a sample without the interference of having to look through the other planes such as seen in Figure 4. Numerous planes can be imaged and recorded sequentially and a 3–dimensional image attained. Like the

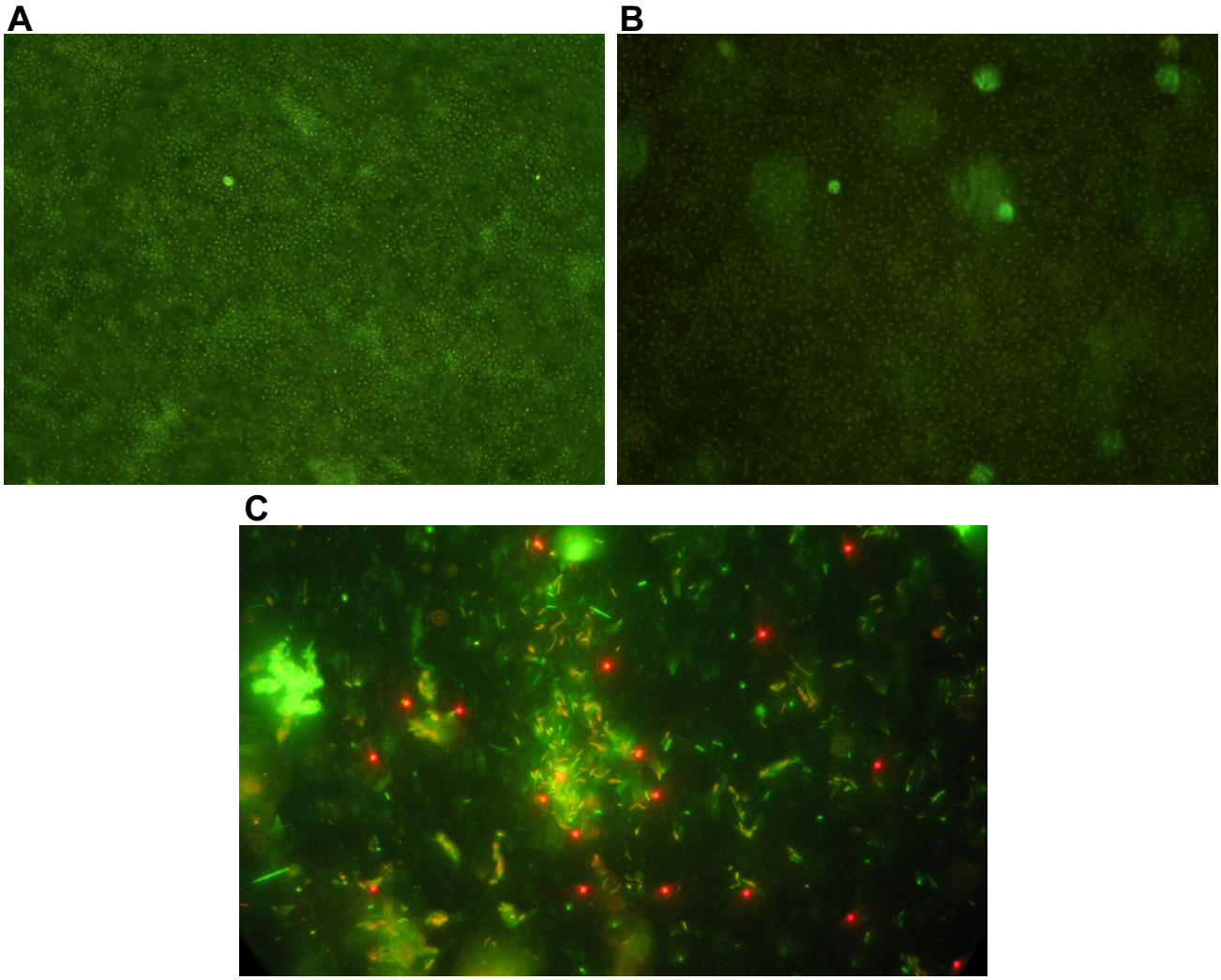

Figure 4: Images taken with epifluorescent microscope. Image of a drinking–water biofilm stained with hexidium iodide and SYTO®9 using a 60× water dip objective (A) and a 100× oil objective (B), and image of a *Pseudomonas fluorescens* (green) biofilm from a pathogen–introduction experiment (Section 4) with 1 μm-diameter polystyrene microspheres (red) and *B. cereus* (orange) imbedded in it (C). Biofilm was stained with hexidium iodide and SYTO®9 and image was taken using a 100× oil objective.

epifluorescent microscope imaging, staining can be used in order to make specific differentiations. An example of a CLSM image that we have obtained is presented in Figure 5.

A custom built 3–dimensional confocal fluorescence hyperspectral imaging microscope (Sinclair et al., in preparation) along with multivariate analysis techniques (Van Benthem et al., 2002; Haaland et al., 2003) is available at Sandia National Laboratories. The advantage of using hyperspectral imaging and Multivariate Curve Resolution (MCR) is that we can simultaneously extract several overlapping fluorescence species in these biofilm 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. At each location a hypercube of data are collected. This hypercube consists of 3 spatial dimensions and a spectral dimension (Figure 6). The spectral dimension consists of 512 wavelengths ranging from 490–800 nm. The spatial dimension can vary depending on the desired image size. 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). This imaging can be performed on both unstained and stained samples. It is possible to use this technique on unstained samples because it can detect weakly emitting auto–fluorescence and because of the method's ability to differentiate overlapping fluorescence spectra. Figure 7 presents a hyperspectral image attained on a *Pseudomonas fluorescens* biofilm using a 60× objective. An example of the use of the hyperspectral imaging to view drinking–water biofilms is discussed in Section 5.

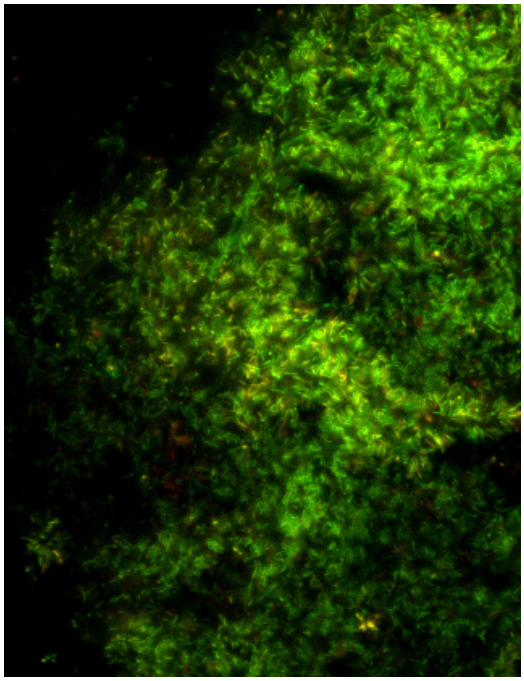

Figure 5: Images taken with Laser Scanning Confocal Microscope. Image of a drinking–water biofilm stained with hexidium iodide and SYTO®9 using a 63× objective.

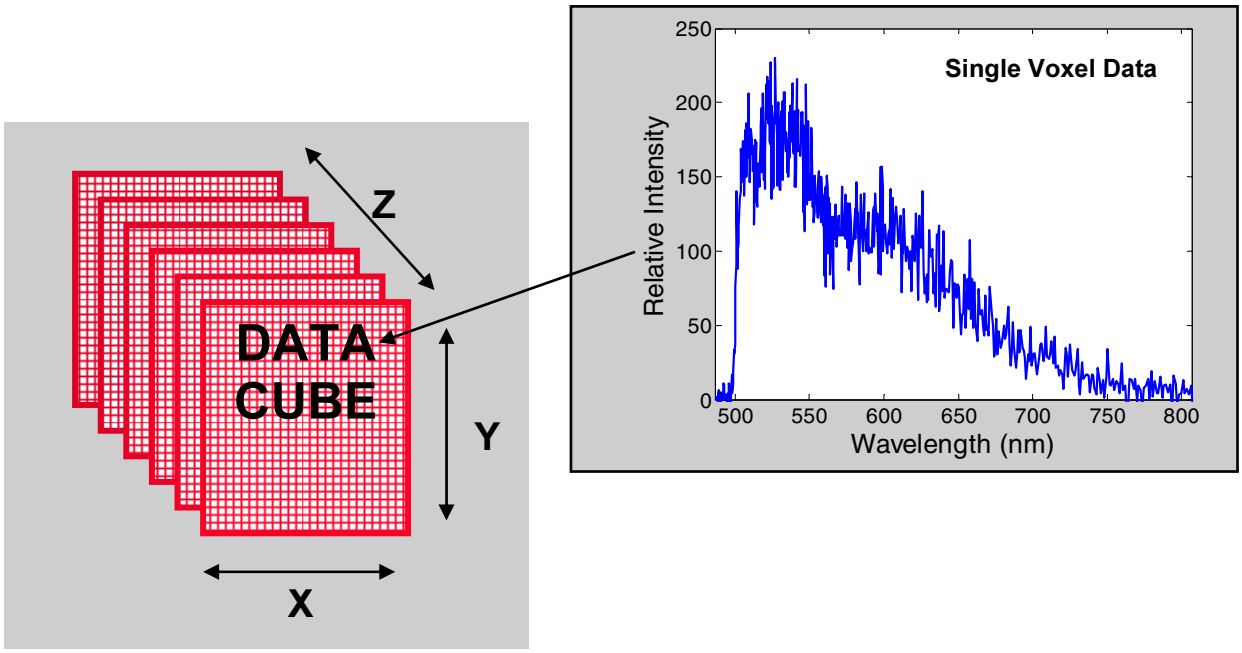

Figure 6: **Representation of data consisting of three spatial dimensions (X, Y, Z) and a spectral dimension. In this example we have 6 slices of data in the Z dimension.**

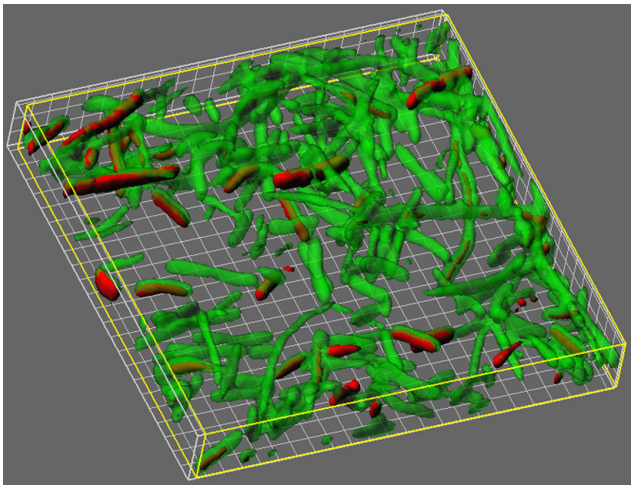

Figure 7: **Three–dimensional rendering of a hyperspectral image of a *Pseudomonas fluorescens* biofilm stained with hexidium iodide and SYTO®9 using a 60× objective.**

3 BIOFILM–GROWTH EXPERIMENTS

The primary goal of the biofilm–growth experiments were to demonstrate that we could grow repeatable biofilms in the annular reactor. The final result is a protocol for growing biofilms prior to running the pathogen–introduction experiments. A series of eight experiments were run (Table 1) in order to:

- Determine a flow rate such that counts of planktonic bacteria were well below the biofilms' CFU counts,
- Determine NaOH concentration such that the pH of the solution was close to neutral,
- Determine how many days the biofilms should be grown to reach a stationary phase, and
- Demonstrate we can grow repeatable biofilms.

Table 1: Summary of Biofilm–Growth Experiments.

Experiment #	Dates	NaOH Solution Concentration (mg/L)	Inner Cylinder Rotation Rate (RPM)
1	09/08/2004 – 09/17/2004	3	100
2	10/11/2004 – 10/22/2004	3	100
4	11/05/2004 – 11/19/2004	3	100
6	12/01/2004 – 12/21/2004	3	100
7	01/07/2005 – 02/24/2005	3 – 5	90
8	03/18/2005 – 05/11/2005	4.5	60
9	05/04/2005 – 08/19/2005	4.5	60
10	05/13/2005 – 06/27/2005	4.5	60

3.1 Methods

3.1.1 Flow System Description

For all of the experiments an annular reactor (Biosurfaces Technologies, Corporation, Model 1120 LJ, see Section 2.1 for more details) was used to grow and sample the biofilms. polycarbonate slides were used. The rotation rate of the inner cylinder varied from experiment to

experiment (Table 1). The final rotation rate was chosen based on what was reported in Camper et al. (2003)

The components of the flow system are diagramed in Figure 8 and photographed in Figure 9. A 20–L carboy filled with sterile nutrient solution (Table 2, Appendix C) and a second 20–L carboy filled with sterile NaOH solution (Appendix D) were pumped into the reactor at equal flow rates. A peristaltic pump [Masterflex L/S model # 7554–80 with two HV–77201–60 pump heads] was used to control the flow rate from the carboys into the reactor. Starting with experiment #2, a system was set up such that one carboy could be aseptically backfilled from a second carboy (or third, if necessary) sitting further down the lab bench in Figure 9B. Backfilling of the carboys was controlled by a second pump [Masterflex L/S model # 7524–50

Table 2: Nutrient Solution Concentrations

Nutrient	Concentration (mg/L)
Glucose	5
Peptone	2.5
Yeast	1.25

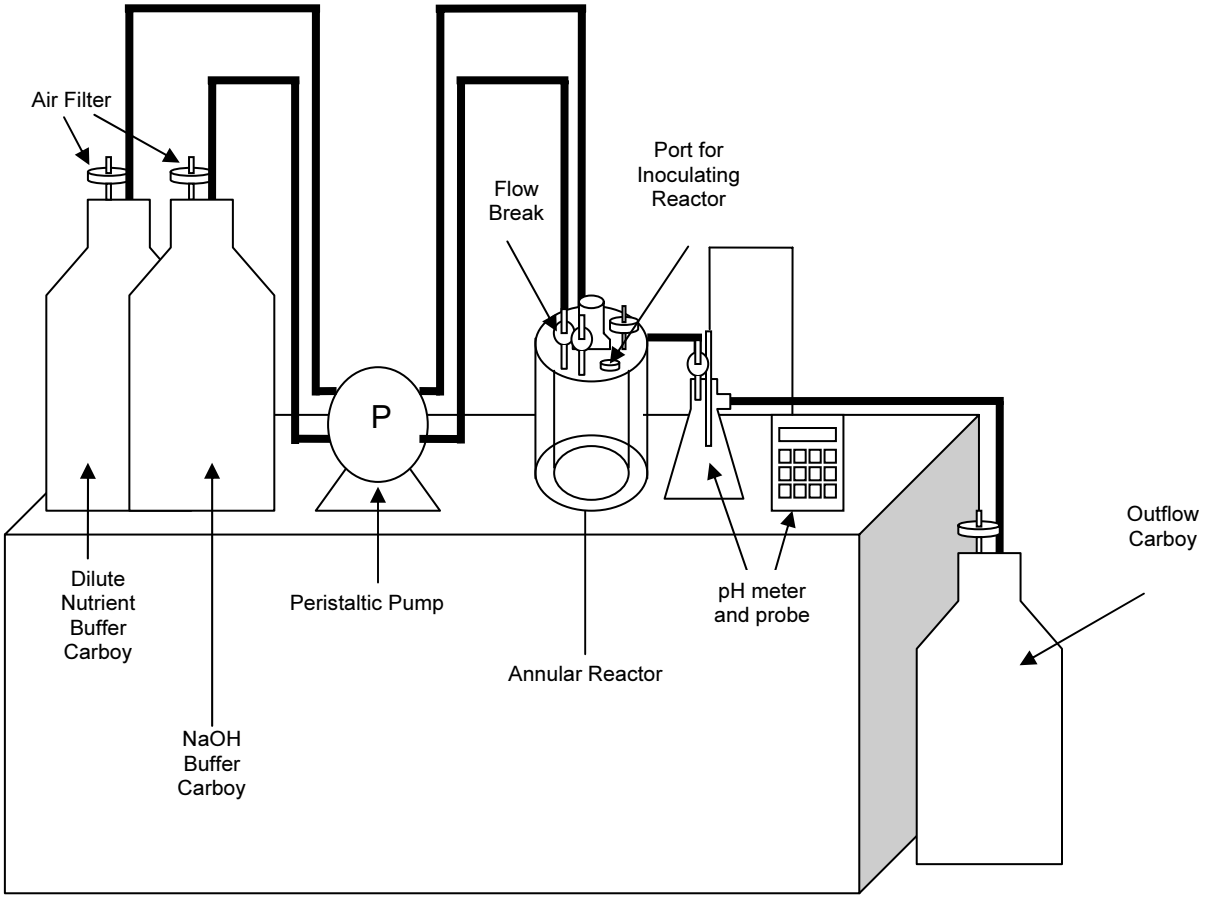

Figure 8: Schematic of annular reactor setup for biofilms–growth experiments.

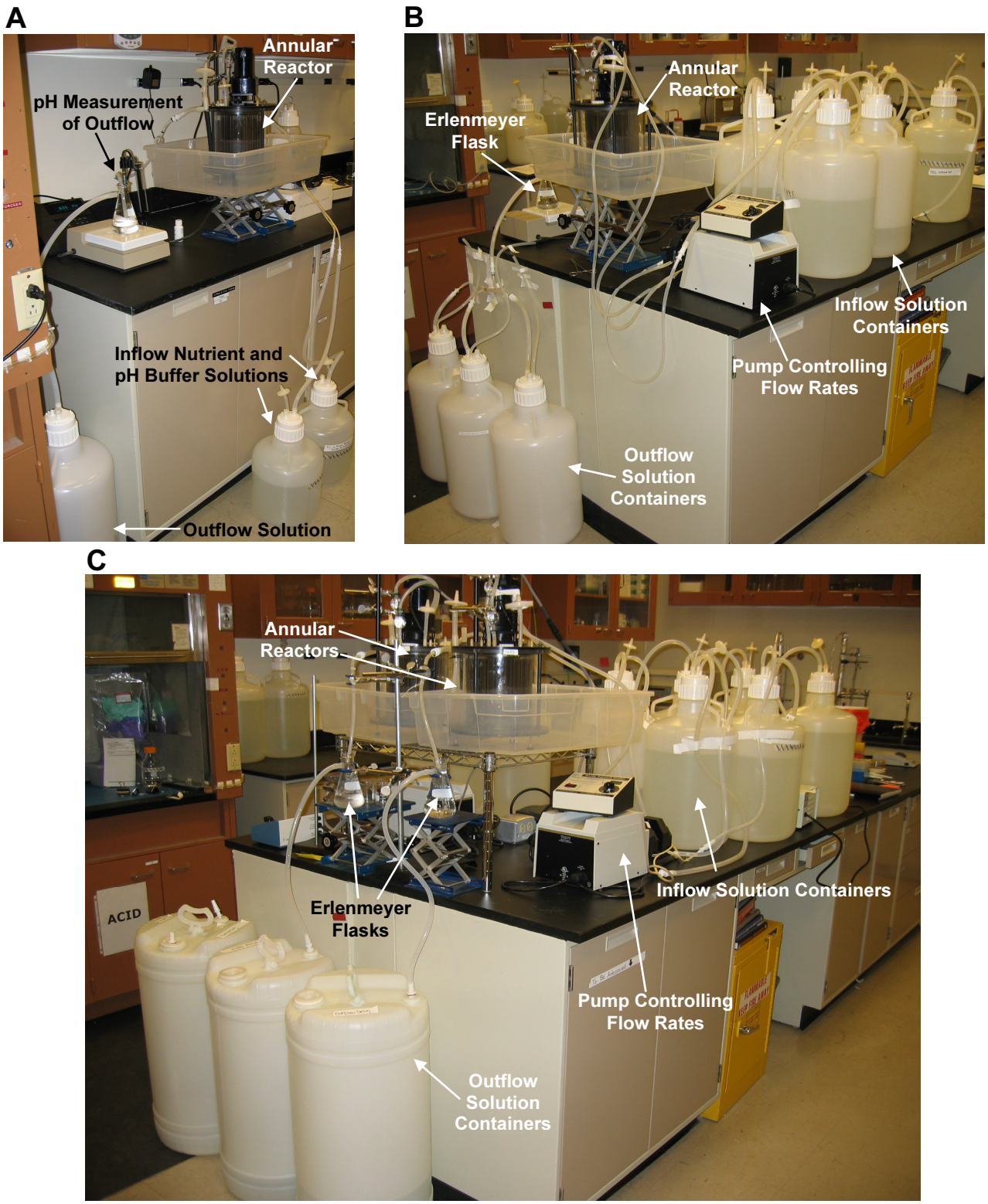

Figure 9: **Photographs showing evolutions of biofilms–growth experiment set-up. Experiment #1 most closely resembles (A) and Experiments #2 – #7 resemble (B). Starting with Experiment #8 two experiments could be run simultaneously and 15–gallon barrels were used to collect the outflow solution, as shown in (C).**

with a dual channel pump head HV–77202–60] that was turned on periodically. This pump did not control the flow rate into the reactor. This setup minimized the likelihood of contamination of the system.

Outflow was collected in a 20 L carboy for experiments #1, #2, #4, and #6 (Figure 9A). In experiment #7, 3 20 L carboys were used to collect waste (Figure 9B). The three carboys were connected to a single outlet line via two T–connectors such that the outflow solution could empty into any of the three carboys. Air vents are extended above the T–connectors to ensure that none of the carboys overflow. The design of the system changed so that the experiment could run for more than one day without having to empty one of the carboys. During experiment #8, the method for collecting the reactor outflow solution was changed. Fifteen gallon barrels (57 L) replaced the 20–L carboys (Figure 9C). Use of these larger containers meant we did not have to use the complicated three–carboy system. These barrels were also used for experiment #10.

Flow rate was one of the variables for the experiments. Appendix E contains tables summarizing the flow rates for each experiment. In experiment #1 the flow was such that the residence time was 1 hour and 51 minutes. Starting in experiment #2 a slower flow rate (with a residence time of 11 hours 6 minutes) was used for the first 3 days (or 10 days for experiment #2) to maximize bacterial adhesion to the coupons. Starting with experiment #7, the flow rate after the initial slower flow rate was increased so the residence time was 1 hour 14 minutes The increased flow rate decreased the amount of planktonic cells in the reactor. Experiments #8, #9, and #10 had more complicated flow rate patterns because we had to inoculate the reactor more than once. However, the general pattern was that residence time of 11 hours 6 minutes was used for three days after inoculation, after which the flow rate was increased such that the residence time was 1 hour and 14 minutes. The flow rates were also changed for a short time if a pathogen was introduced to the reactor (see Section 4).

The NaOH solutions was used to maintain a constant pH of approximately 7. The concentrations of the solution used for all experiment are shown in Table 1. The concentrations used in experiment #7 are shown in Table 3.

Table 3. NaOH Concentrations During Experiment #7.

Date concentration changed	Days Since Inoculation	NaOH concentration (mg/L)
1/10/2005	0	3.0
1/11/2005	4	4.0
1/12/2005	6	4.5
1/17/2005	10	5.0
2/14/2005	38	4.5

3.1.2 Inoculant

Pseudomonas fluorescens was chosen as the biofilm organism because it is a Biosafety Level 1 (BSL 1) organism and has been successfully used in previous biofilm–growth experiments. Also, since *P. fluorescens* is a gram–negative organism, it can be differentiated from the gram positive *Bacillus cereus* (a pathogen surrogate used in the pathogen–introduction experiments, Section 4) with fluorescence gram–indicating stains. Preparation procedures for the *P. fluorescens* are summarized in Appendix F. Table 4 summarizes the dates, times, volumes of inoculation and the initial concentration of the inoculant.

Table 4. Summary Inoculation Information for Biofilm–Growth Experiments

Experiment #	Inoculation Date	Inoculation Time	Volume (ml)	Mean (Standard Deviation) Inoculum Concentration (CFU/ml)
1	09/07/2004	9:00 AM	5	1.79×10^9
2	10/08/2004	12:37 PM	5	5.80×10^8
4	11/05/2004	2:06 PM	5	4.68×10^7 (2.22×10^6)
6	12/01/2004	1:57 PM	5	5.38×10^7 (2.75×10^6)
7	01/07/2005	10:00 AM	5	8.15×10^7 (7.33×10^6)
8	03/18/2005	2:00 PM	5	4.70×10^7 (6.32×10^6)
8	03/29/2005	12:30 PM	5	1.21×10^9 (1.41×10^8)
9	05/05/2005	9:50 AM	5	7.35×10^8
9	05/17/2005	3:42 PM	5	3.33×10^8 (1.71×10^7)
9	05/26/2005	2:35 PM	5	3.33×10^8 (1.71×10^7)
9	06/21/2005	9:15 AM	9	2.98×10^8 (2.63×10^7)
9	07/11/2005	15:54 AM	9	1.92×10^9 (2.70×10^8)
10	05/13/2005	12:50 PM	5	3.3×10^8 (1.71×10^7)
10	05/26/2005	02:33 PM	5	3.3×10^8 (1.71×10^7)
10	06/15/2005	07:30 AM	9	2.90×10^8 (2.58×10^7)

3.1.3 Data Collection

Slides from the annular reactor were collected and sampled for heterotrophic plate counts to quantify biofilm growth. Methods for sampling and scraping slides are summarized in Appendix A. Reactor water was sampled starting with experiment # 6 to determine the amount of planktonic *P. fluorescens* in the system. A 1 ml sample was collected from the inoculation port of the annular reactor using a sterile pipette. All samples were serially diluted and enumerated on TSA (Appendix B). Finally, outflow solution was collected periodically to measure pH. The pH was measured using an Orion 520A+ pH meter. Two–point calibrations were performed daily using the Orion Application Solution with pH standards of 4.01 and 7.00.

3.2 Results and Discussion

Results of biofilm–growth experiments show that *P. fluorescens* biofilms can be grown reproducibly (Figure 10, Appendix H). The stationary phase growth is typically reached 5 to 10 days after inoculation with counts between 10^5 and 10^6 CFU/cm^2. These counts could be maintained for the 48 days that experiment #7 was run. Experiment #9 achieved stationary growth with counts generally above 10^7 CFU/cm^2 15 days after a 5th inoculation. It is at this time that pathogen–introduction experiment #3 was initiated (Section 4). For initial pathogen–introduction experiments EMB agar was used to selectively enumerate *P. fluorescens*. After further evaluation, it was decided that the EMB agar too easily became contaminated. Therefore, these higher counts are thought to be erroneous. The dip in counts for experiment #8 can be explained by the increase in residence time from 1.23 hours to almost 6 hours for pathogen–introduction experiment #1 (Appendix H). After the residence time was returned to 1.23 hours,

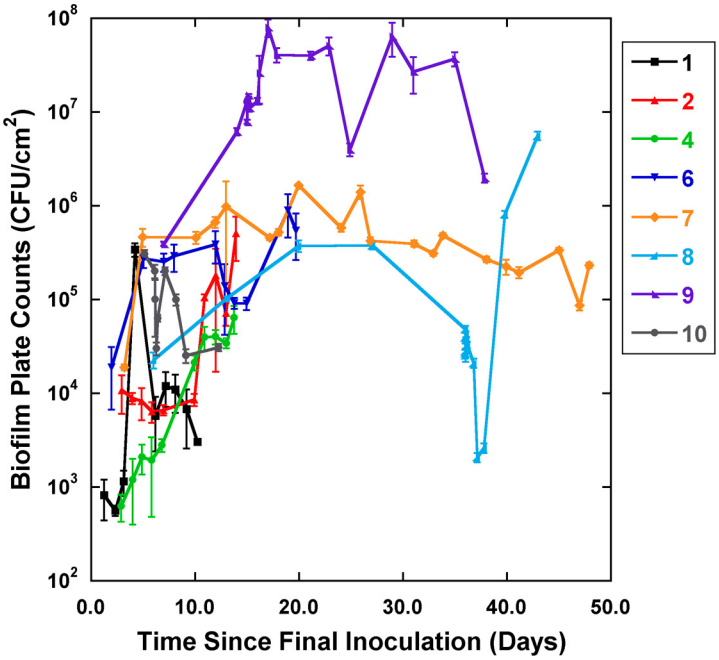

Figure 10: Results of plating biofilms scrapings for biofilms–growth experiments.

the counts increased to even higher levels than measured before. Difficulties in achieving adequate plate counts in experiments #8 – #10, as well as experiment #10 never reaching stationary phase, where attributed to the *P. fluorescens* inoculum. As a result, it has been decided *P. fluorescens* inoculum will be propagated from frozen stock before each experiments in the future (Appendix G).

Lower plate counts from the reactor water in experiments #7, #8, #9 and #10 suggest that increasing flow rates in these experiments successfully lowered the amount of planktonic bacteria in the reactor (Figure 11, Appendix I). The higher count for the last measurement in experiment #9 is most likely due to using the EMB agar.

pH measurements generally ranged from 6 to 8.5 (Figure 12). The earlier experiments had pH values less than 7, thus the NaOH concentration was increased in order to raise the pH. During experiment #7, the NaOH concentration was varied and pH measured in order to determine the best concentration (Figure 13). Bacterial growth in the flask where the pH was measured was possibly influencing the measurements. Therefore, after day 18 in experiment #7, the method of collecting water to measure pH was changed, so that the water was collected into a clean beaker each time. Based on the results of experiment #7, a NaOH concentration of 4.5 mg/L was used in subsequent experiments. However, pH measurements in experiments #9 and #10 being consistently (with a few exceptions) above 7 indicate that the NaOH concentration should be further decreased.

Based on our confidence that we can consistently grow *P. fluorescens* biofilms, a protocol for growing the biofilms was developed for use in the pathogen–introduction experiments (Appendix J).

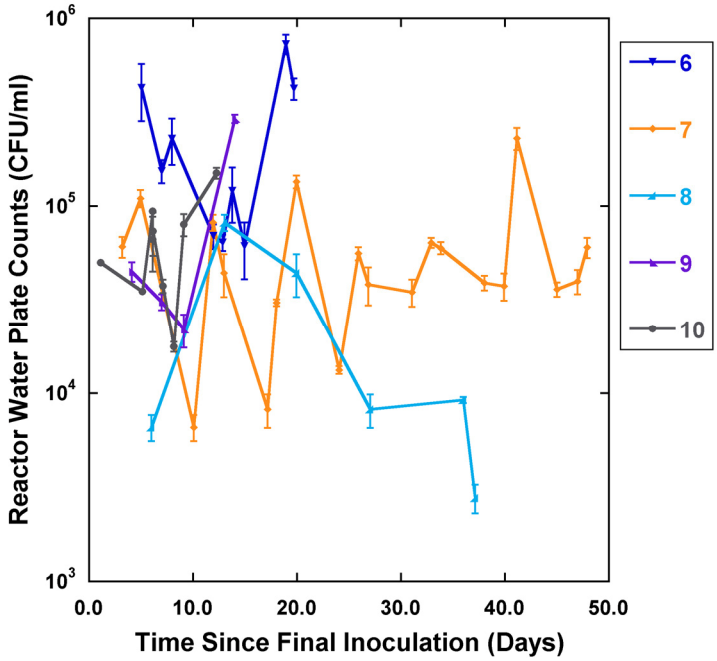

Figure 11: Results of plating of reactor water sampling for biofilm–growth experiments.

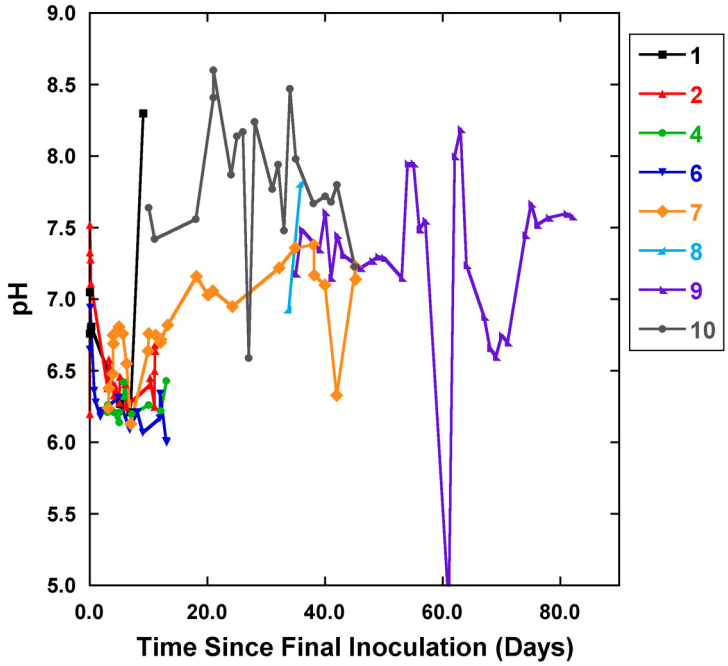

Figure 12: Results of pH measurements of reactor water for biofilms–growth experiments.

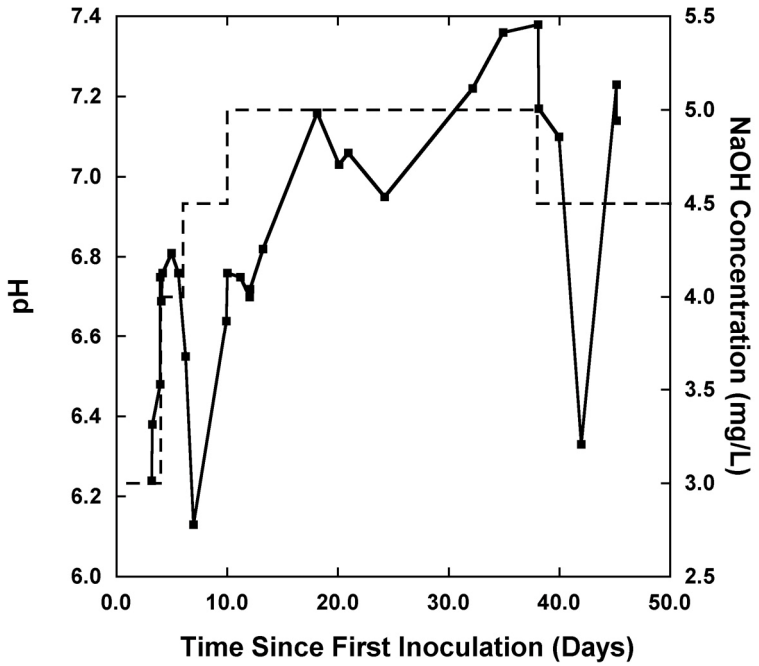

Figure 13: Results of pH measurements of reactor water for biofilm–growth experiment #7.

4 PATHOGEN–INTRODUCTION EXPERIMENTS

The purpose of the pathogen–introduction experiments is to determine whether bio–pathogen surrogates incorporate into biofilms grown in a simulated water distribution system. If the bio–pathogens are observed to incorporate into the biofilms, then the next goal is to determine how long they remain incorporated. For this experiment, separate solutions of polystyrene microspheres and *B. cereus* spores were used as the bio–pathogen surrogates. An annular reactor simulated the water distribution system and the biofilms were grown from *P. fluorescens*. A biofilm was first grown in the annular reactor. Either polystyrene beads or polystyrene beads and *B. cereus* spores were introduced into the reactor and their concentration in the reactor water and biofilms was carefully monitored.

4.1 Methods

Methods for growing the biofilms prior to pathogen introduction are described in Section 3.1. Refer to Table 5 for the connection between the biofilm–growth experiments and the pathogen–introduction experiments. In all cases biofilms were grown under a total reactor water residence time of 1 hour, 14 minutes, with an inner cylindrical rotation of 60 RPM. Residence time in the annular reactor was increased to 5 hours 58 minutes just prior to the pathogen introduction and maintained at that flow rate for 5 days for experiment #1 and 1 day for experiments #2 – #4. In all cases 1 ml samples of the pathogens were injected into the system. TransFluoSpheres® Carboxylate–Modified Fluorescent Microspheres with a diameter of 1 μm were used as the polystyrene microspheres. Preparation of the *B. cereus* spores is described in Appendix K.

To date a total of four experiments have been run (Table 5). The first two experiments used the microspheres as the bio–pathogen surrogate. The second two experiments used both microspheres and *B. cereus* spores. For the first three experiments, 1 slide was sampled for each sampling period, while 2 slides were sampled during experiment #4. Thus, experiments #1 – #3 had 4 points (4 plates per slide) describing the standard deviation of the measurements and experiment #4 had 8 points (2 slides, 4 four plates per slide).

Table 5. Summary of Pathogen–Introduction Experiments

Pathogen–Introduction Experiment #	Biofilm–Growth Experiment #	Number of Polystyrene Microspheres Introduced	Number of Initial *Bacillus cereus* spores Introduced	Date of Injection	Experiment Duration (Days)
1	8	9.91×10^8	N/A	05/04/2005	7.0
2	10	9.72×10^8	N/A	06/21/2005	6.1
3	9	9.72×10^8	2.35×10^7	07/26/2005	36.1
4	N/A	3.47×10^9	4.93×10^7	09/06/2005	13.1

Once the pathogen was introduced into the reactor both reactor water and biofilm samples were collected over the duration of the experiment. Slides from the annular reactor were sampled to measure 1) the amount of post–introduction *P. fluorescens* biofilm growth (experiments #1 and #2 only), 2) microsphere concentration within the biofilms, and 3) the amount of *B. cereus* spores integrated into the biofilms. Slides were collected, scraped and sonicated following the methods in Appendix A. This method leaves us with 10 ml of sample.

Reactor water from the annular reactor was sampled to determine 1) the concentration of microspheres in the reactor water, and 2) the amount of planktonic *B. cereus* spores. A 10 ml sample of reactor water was collected from the inoculation port of the annular reactor using a sterile pipette.

For both the reactor water and the biofilm samples, 3 ml of sample was put in two cuvettes (6 ml total) that were used for analysis of the microsphere concentrations using fluorescence spectrophotometry on a Varian Cary Eclipse Fluorescence Spectrophotometer (system ID Eclipse–EL00073126). A 1ml sample was serially diluted and enumerated on appropriate media (Appendix B). TSA was used in experiments #1 and #2 to quantify *P. fluorescens* biofilm growth. TSA mixed with Polymyxin B (an antibiotic that inhibits gram–negative growth) was used to quantify *B. cereus*. For experiments #3 and #4, Levine Eosin Methylene Blue Agar (EMB), a gram–negative selective agar, was used to quantify *P. fluorescens*. The counts from the EMB agar were not accurate, possibly due to contamination. In the future, we will make fresh batches of EMB agar daily to avoid contamination. Therefore, we do not report *P. fluorescens* growth for experiments #3 and #4 after the *B. cereus* introduction.

4.2 Results

After approximately 1 day, the concentration of the microspheres in the reactor water approached the detection limit (Figure 14). Only for experiment #3 did the microspheres remain in the reactor water for more than 1.8 days. For an unexplained reason, the microspheres remained in the reactor water at or near the detection limit of 10^3 particles/ml for the duration of experiment #3. In contrast to the microspheres, the *B. cereus* remains in the reactor water for the duration of experiment #3 and #4 (Figure 14). With one exception, counts remained above 10^4 CFU/ml for experiment #3 and above 5×10^5 CFU/ml for experiment #4. For experiment #3, the concentrations appeared to be decreasing with time, though a measurement was made on the order of 10^5 CFU/ml on day 36 of the experiment. During experiment #4 *B. cereus* concentrations remained on the order of 10^5 and 10^6 CFU/ml for the duration of the experiment.

With the exception of experiment #2, the microspheres remained integrated in the biofilms with concentrations between $10^3 – 10^5$ particles/cm^2 for the duration of the experiments (Figure 15). Experiment #2 did not have well formed biofilms, explaining the lower concentrations and decrease in concentration over time, compared to the other experiments. Like the microspheres, *B. cereus* remains integrated in the biofilms over the duration of the experiment (Figure 15). The amount *B. cereus* appears to be increasing through day 16 in experiment #3, though this increase in not observed in experiment #4, where plate counts appear to remain relatively constant between 10^6 and 10^7 CFU/cm^2, with one exception.

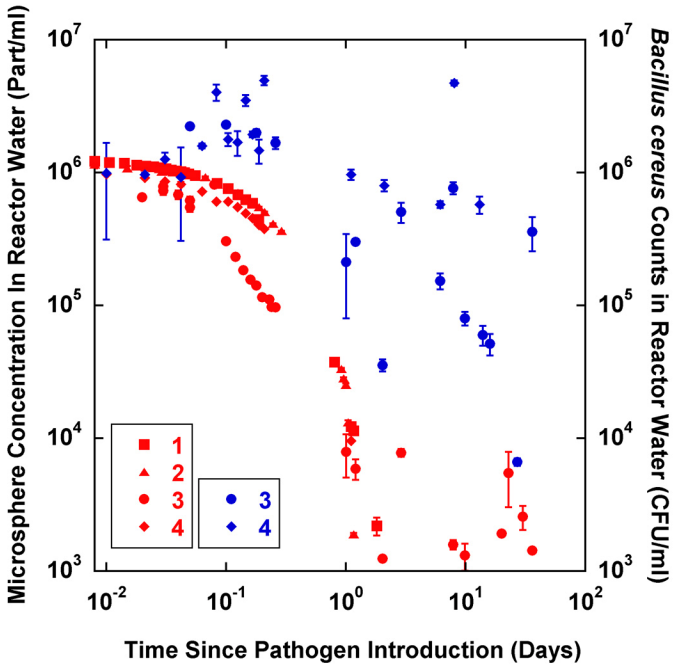

Figure 14: **Concentrations of microspheres and *Bacillus cereus* in reactor water during pathogen–introduction experiments. Numbers in the legend refer to the experiment number and red and blue indicate microspheres and *Bacillus cereus*, respectively.**

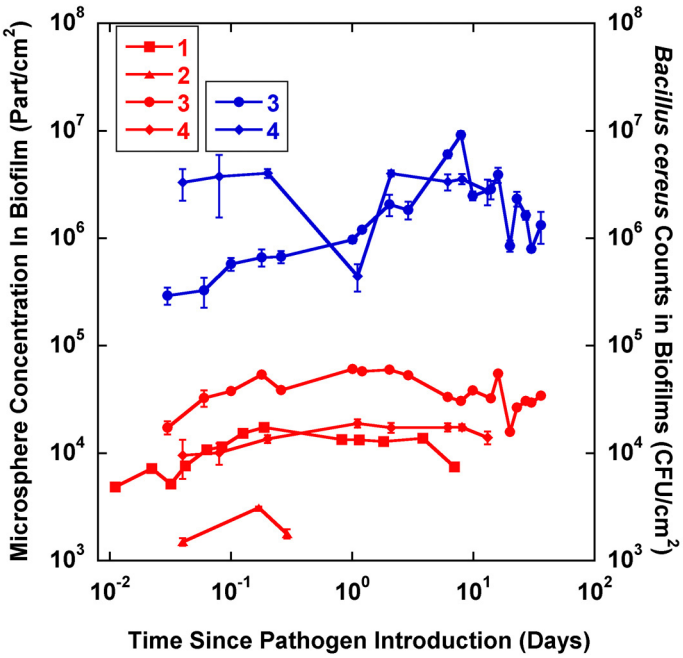

Figure 15: **Concentrations of microspheres and *Bacillus cereus* in biofilms during pathogen–introduction experiments. Numbers in the legend refer to the experiment number and red and blue indicate microspheres and *Bacillus cereus*, respectively.**

The *B. cereus* is clearly replicating. While approximately 10^7 spores were introduced into the system (Table 5), integrating under the reactor water curves (Figure 14), yields counts on the order of $10^9 – 10^{10}$ CFU (accounting for an approximate 1–liter reactor volume). In addition, the *B. cereus* counts in the biofilms are approximately 2 orders of magnitude higher than the microsphere concentrations (Figure 15) despite less spores being introduced to the system than the microspheres.

There is no indication that the microspheres or *B. cereus* are sloughing off into the reactor water, unless the *B. cereus* is sloughing of at approximately the same rate it is being incorporated in the reactor water. Concentrations of both in the biofilms remain relatively constant over the duration of the experiments. Unfortunately, the concentrations of microspheres in the biofilms are low enough that they would be below the detection limit of the Fluorescence Spectrophotometer once diluted in the reactor. The concentration of *B. cereus* in the reactor water is high enough, such that any *B. cereus* removed from the biofilms would not be detected.

5 DRINKING–WATER BIOFILMS

5.1 Introduction

The biofilms laboratory has the capability to grow biofilms with an origin from drinking water. As this research is focused on the impact of biofilms on drinking–water quality and the decontamination of drinking–water distribution systems, this capability is important. In the future, experiments as described in Sections 3 and 4 could be conducted with drinking–water biofilms instead of *P. fluorescens* biofilms. Also, these biofilms are being used to study biofouling of water treatment membranes.

5.2 Methodology

5.2.1 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 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 (see Section 2.1 for a description of the reactor). Biofilms were then grown on 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 results of the biofilm growth were assessed by scrapping and sonicating the biofilms (Appendix A) and enumerating the solutions on appropriate media (Appendix B). TSA and R2A agar were used for total heterotrophic plate counts. Polymyxin B is an antibiotic that inhibits the growth of gram–negative bacteria. TSA was created with 100 IU per ml of Polymyxin B to create the gram–positive selective media. Finally, Eosin Methylene Blue (EMB) agar was used as a gram–negative selective media.

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 KNO_3, 0.15 mg/L K_2HPO_4, and 0.11 mg/L KH_2PO_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.

5.2.2 Imaging Drinking–Water Biofilms

Two samples were prepared by growing the biofilm onto two microscope slides (as described in Section 5.2.1). One sample remained unstained and the other sample was stained using the LIVE *Bac*Light™ Bacterial Gram Stain Kit (Molecular Probes). The kit contained SYTO®9, which labels both live gram–negative and gram–positive bacteria and hexidium iodide, which preferentially labels gram–positive bacteria. The hexidium iodide will displace the SYTO®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 μm 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 hyperspectral microscope and exciting the biofilm samples with a 488 nm laser. Several locations (5–6) were explored on each sample to ensure a representative interrogation of these biofilm samples. 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 μm. This confocal hyperspectral microscope has a lateral resolution of 0.24 μm, an axial resolution of 0.6 μm 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 unstained biofilm samples, it was necessary to increase the laser power by a factor of 1000 and the exposure time by a factor of 4 over what was necessary for the stained sample.

5.3 Results and Discussion

With the high concentration of 1:100 TSB, we were able to grow thick biofilms within days (Figure 16). Heterotrophic plate counts were on the order of 10^8 CFU/cm2. 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/cm2) 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 TSA or R2A agar were used. When the nutrient solution more similar to that of drinking water was used the heterotrophic plate counts

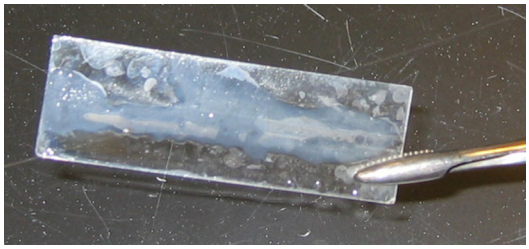

Figure 16: Photograph of biofilms grown from drinking water on a 75 × 25 mm slide.

were between 10^4 and 10^5 CFU/cm^2 for both TSA and R2A agar.

The hyperspectral imaging provided excellent images of the biofilms. Four pure components were detected in the unstained biofilm sample (Figure 17a). 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.

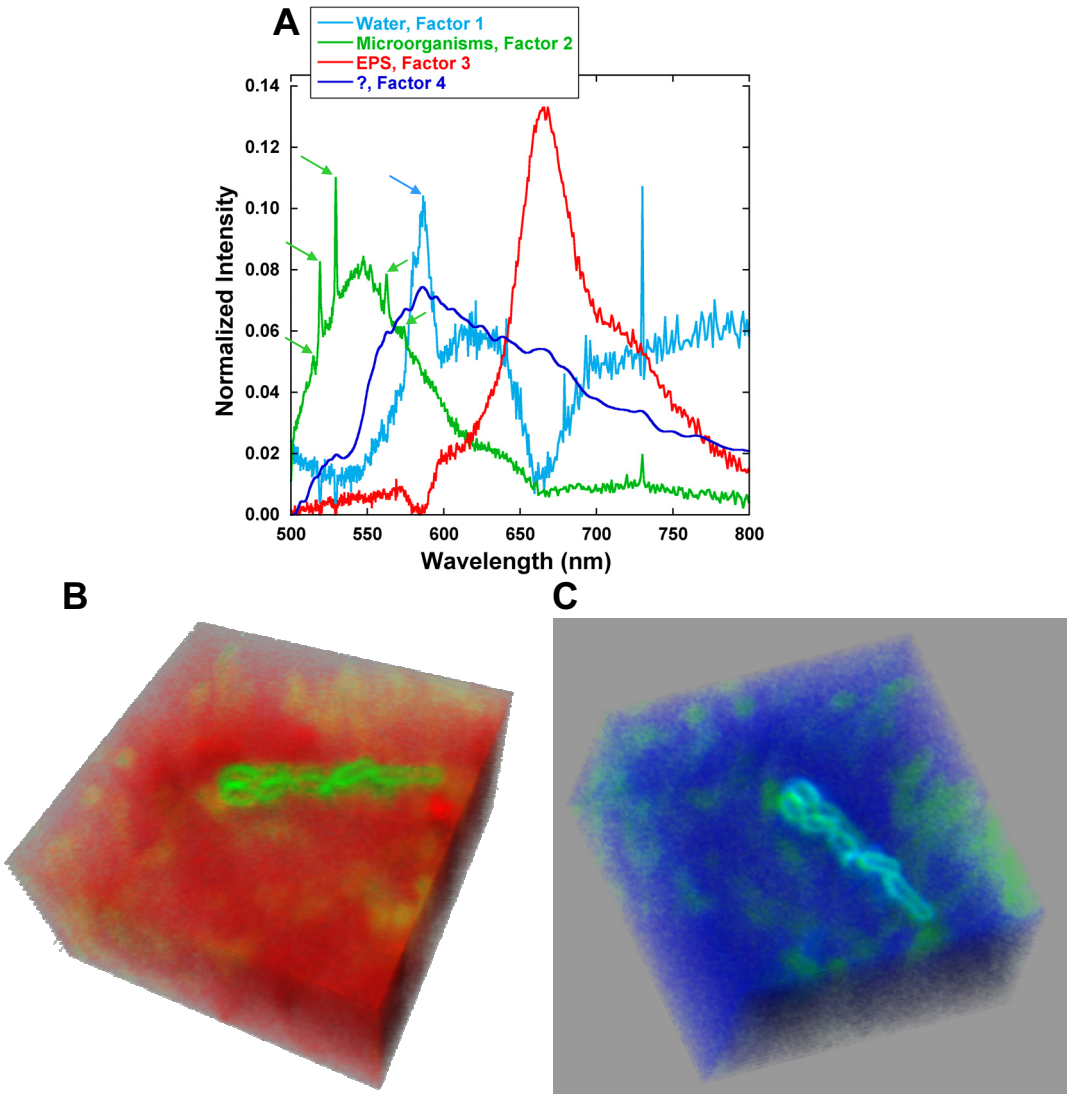

Figure 17: Four factors seen in the unstained drinking-water biofilms (A) and images taken of the biofilm showing Factors 2 (green) and 3 (red) (B) and Factors 2 (green) and 4 (blue) (C). Factor 1 is most likely from the water in the system. Factor 2 is thought to be from the microorganisms and Factors 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.

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 17C). It is unclear what causes Factor 4. While Figure 17a 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 17C). The colors represented in the images in Figure 17 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 17C is a result of the spatial co–location of the Factor 2 (green) and Factor 4 (blue).

Three factors were detected in the stained drinking–water biofilm (Figure 18A): Factors 1 and 2 are the different stains, SYTO®9 and hexidium iodide, respectively. The SYTO®9 appears to have stained most of the microorganisms, indicating that they are gram–negative. This result is consistent with the results of gram–selective enumeration. 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 18B).

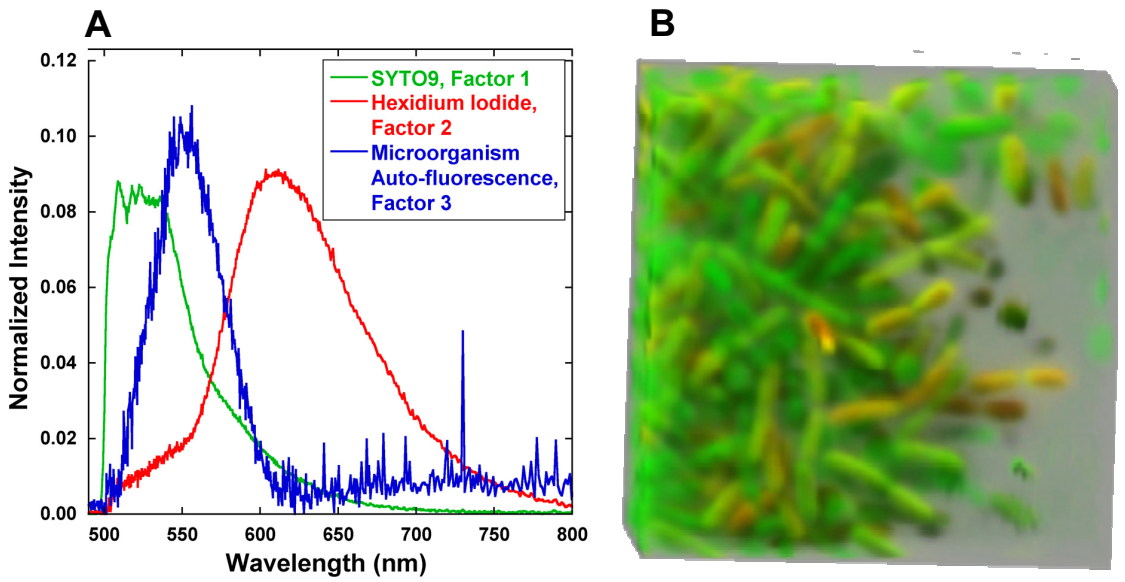

Figure 18: Three factors seen in the stained drinking–water biofilms (A) and image taken of the biofilm showing only Factors 1 and 2 (B). Microorganisms appear to be primarily gram negative.

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APPENDIX A: METHODS FOR PREPARING BIOFILMS FOR PLATING

Appendix A
Methods for Preparing Biofilms for Plating

Materials Needed:

- One sterile 100 ml beakers (or other sterile container), filled with 9 ml of sterile deionized water and covered with foil for each slide sampled.
- 95% ethanol
- Retrieving tool and stainless steel hemostat
- Scraping tool
- Ethanol burner
- Dilution tubes (test tubes) containing 9 ml sterile diluent
- 1 ml pipette
- 10 ml pipette
- Sterile pipette tips (1 ml and 10 ml)
- Two sterile, empty test tubes

Biofilm Scraping Procedure:

1. Disinfect lab bench with a 10:1 bleach solution.
2. Organize all materials on the lab bench.
3. Flame–sterilize the retrieving tool using 95% ethanol and a small, open flame.
4. Aseptically retrieve the coupon from the reactor and carefully place it into the sterile beaker with the biofilm facing upward. The coupon should lean against the inside of the container.
5. Flame–sterilize the hemostat.
6. Holding the top of the coupon with the sterile hemostat, firmly scrape the slide from top to bottom with a sterile scraper. Periodically stir or gently tap the scraper into the resulting diluent now contained in the beaker. Scrape the slide 3–4 times.
7. Carefully rinse the slide by pipetting the diluent down the slide at least 3 times using a sterile pipette tip.
8. Gently swirl the contents in the container to capture any cells attached to the container walls. Carefully transfer this fluid into an empty, sterile test tube.
9. Rinse the beaker with 1 ml sterile DI water and transfer it to the test tube containing the biofilm sample. This is the 10^{o} (or undiluted) sample.
10. Sonicate the sample using the following technique.

Sonication Procedure

1. Tightly cap the biofilm sample test tube.
2. Place the sample into the modified rack in the sonicator.
3. Fill the sonicator with tap water to the line indicated.
4. Sonicate the sample for at least 5 minutes.
5. Remove and vortex the sample.

(Modified from Biofilm Methods Workshop Handouts, Center for Biofilm Engineering, February 26–27, 2004)

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APPENDIX B: DILUTION AND POUR PLATING METHODS

Appendix B
Dilution and Pour Plating Methods

Items needed

- Test tube racks
- Sterile test tubes with 9 ml of DI water in them.
- 1 ml pipette
- 1 ml pipette tips
- Trypticase™ Soy Agar (TSA)

Trypticase™ Soy Agar (TSA) Preparation

1) Weigh out 40 g of Trypticase™ Soy Agar powder.

2) Mix powder with 1 L of Nanopure water in a 2 L beaker on a hot plate with a magnetic spinning function. Turn on the heater.

3) Boil the solution for approximately one minute to completely dissolve the powder.

4) Pour the solution into sealable autoclavable bottles, label, and autoclave at 121 ˚C for 15 minutes.

5) Allow solution to cool slightly in the autoclave prior to opening the door. Seal bottle immediately once the autoclave is opened and place the bottles in a 50 ˚C water bath until use.

Dilution Procedure

1) Vortex the 10 ml sample that has already been sonicated.

2) Withdraw 1 ml of liquid from the sample solution and inject it into a sterile test tube with 9 ml of DI water in it.

3) Vortex the test tube that now has 10 ml of liquid in it, this is considered the first dilution.

4) Repeat the procedure of taking 1 ml of liquid from the 10 ml test tube and placing it into a 9 ml DI test tube. Keep track of which tube is what dilution number.

5) Stop the dilution process based on the concentration of inoculum and the counts from previous samples. For example, a 24–hour growth culture of *P. fluorescens* usually is at a concentration of 10^6 CFU/ml. Therefore, plating dilutions 4–6 would be adequate.

Plating and Counting Procedure

1) Label sterilized Petri dishes.

2) Aseptically take 1 ml samples from the dilution tubes and place in the center of the Petri dish. Place cover back on.

3) Pour enough media to cover the bottom of the Petri dish and gently swirl to mix sample with media.

4) Allow media to solidify. Invert dishes to prevent condensation from altering dilution of sample. Incubate at 30°C for 24 hrs.

5) For the biofilm–growth experiments samples are plated in quadruplicate.

6) Count the number of visible colonies after approximately 24 hrs of incubation.

7) Calculate the CFU/cm^2:

$$\frac{CFU}{cm^2} = (\text{average CFU})(10^{\text{dilution counted}})\left(\frac{\text{volume } H_2O \text{ scraped into}}{\text{surface area of slide}}\right)$$

where the surface area of the reactor slide = 18.85 cm^2

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APPENDIX C: PROCEDURE FOR MAKING NUTRIENT SOLUTION

Appendix C
Procedure for Making Nutrient Solution

1) Make concentrate stock solution
   - Pour approximately 200 ml of DI water into a 500 ml volumetric flask
   - Weigh out 5 g of dextrose on a weigh boat.
   - Pour glucose into the flask rinsing the weigh boat with DI water.
   - Weigh out 2.5 g of peptone on a weigh boat.
   - Pour peptone into the flask rinsing the weigh boat with DI water.
   - Weigh out 1.25 g of yeast extract on a weigh boat.
   - Pour yeast extract into the flask rinsing the weigh boat with DI water.
   - Fill the volumetric flask to the fill line.
   - Pour solution into an autoclavable bottle and sterilize solution at 121 °C for 15 minutes.
   - Store solution in the refrigerator at 4 °C.
2) Sterilize DI water for use in experiment
   - Assume an inflow solution of 19.5 L of DI water.
   - Add 19.5 L of DI water to an autoclavable carboy.
   - Autoclave carboy at 121 °C for 2 hours.
3) Dilute concentrate stock solution for use in experiment
   - Pour approximately 10 ml of sterilized nutrient stock solution into a beaker.
   - Draw up 9 ml of nutrient stock solution from the beaker and place into a 500 ml volumetric flask.
   - Fill the volumetric flask up to the fill line.
   - Pour the solution into an autoclavable bottle and sterilized the solution at 121 °C for 15 minutes.
4) Make final dilute solution for use in experiment
   - Aseptically pour the sterilized 500 ml dilute nutrient solution into the sterilized 19.5 L DI water carboy.

(Modified procedure from Oliveira et al., 1994, and Pereira et al., 2002)

APPENDIX D: PROCEDURE FOR MAKING NaOH SOLUTION

Appendix D
Procedure For Making NaOH Solution

1) Sterilize DI water for use in experiment.
   - Assume an inflow solution of 19.5 L of DI water.
   - Add 19.5 L of DI water to an autoclavable carboy.
   - Autoclave carboy at 121 °C for 2 hours.

2) Sterilize 500 ml of NaOH solution to pour into 20 L carboy
   - Add approximately 200 ml of DI water to a 500 ml volumetric flask.
   - Using the below table draw up the desired volume of purchased 500,000 mg/L NaOH solution and place into the 500 ml volumetric flask.

NaOH Concentration	Volume NaOH to add
3.0 mg/L	120 µL
3.5 mg/L	140 µL
4.0 mg/L	160 µL
4.5 mg/L	180 µL
5.0 mg/L	200 µL

   - Fill the volumetric flask up to the fill line.
   - Pour the solution into an autoclavable bottle and sterilized the solution at 121 °C for 15 minutes.

3) Making NaOH buffer solution for use in experiment.
   - Aseptically pour the sterilized 500 ml NaOH solution into the sterilized 19.5 L DI water carboy.

APPENDIX E: FLOW RATES FOR BIOFILM–GROWTH EXPERIMENTS

Appendix E
Flow Rates for Biofilm–Growth Experiments

Flow Rates and Residence Times, Experiment #1

Time Since 1st Inoculation (days)	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0	4.2	8.4	1 hr 51 min

Flow Rates and Residence Times, Experiment #2

Time Since 1st Inoculation (days)	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0	0.7	1.4	11 hrs 6 min
10	4.2	8.4	1 hr 51 min

Flow Rates and Residence Times, Experiment #4, 6

Time Since 1st Inoculation (days)	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0	0.7	1.4	11 hrs 6 min
3	4.2	8.4	1 hr 51 min

Flow Rates and Residence Times, Experiment #7

Time Since 1st Inoculation (days)	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0	0.7	1.4	11 hrs 6 min
3	6.3	12.6	1 hr 14 min

Flow Rates and Residence Times, Experiment #8

Time Since 1st Inoculation (days)	Time Since 2nd Inoculation (days)	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0	——	0.7	1.4	11 hrs 6 min
4	——	6.3	12.6	1 hr 14 min
12	1	0.7	1.4	11 hrs 6 min
14	3	6.3	12.6	1 hr 14 min
47	36	1.3	2.6	5 hrs 58 min
52	41	6.3	12.6	1 hr 14 min

Table 2: Flow Rates and Residence Times, Experiment #9

Time Since 1st Inoculation (days)	Time Since 2nd Inoculation (days)	Time Since 3rd Inoculation (days)	Time Since 4th Inoculation (days)	Time Since 5th Inoculation (days)	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0	0	0	0	0	0.7	1.4	11 hrs 6 min
11	0	0	0	0	6.3	12.6	1 hr 14 min
12	0	0	0	0	0.7	1.4	11 hr 6 min
47	35	26	0	0	0	0	0
47	35	26	0	0	0.7	1.4	11 hrs 6 min
53	41	32	6	0	6.3	12.6	1 hr 14 min
63	51	42	16	0	0.7	1.4	11 hrs 6 min
67	55	46	20	0	0	0	0
67	55	46	20	0	0.7	1.4	11 hrs 6 min
71	59	50	24	4	6.3	12.6	1 hr 14 min
82	70	61	35	15	1.3	2.6	5 hr 58 min
83	71	62	36	16	6.3	12.6	1 hr 14 min

Table 2: Flow Rates and Residence Times, Experiment #10

Time Since 1st Inoculation (days)	Time Since 2nd Inoculation (days)	Time Since 3rd Inoculation (days)	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0	——	—	0.7	1.4	11 hrs 6 min
21	8	—	6.3	12.6	1 hr 14 min
33	20	0	0	0	0
33	20	0	6.3	12.6	1 hr 14 min
39	26	6	1.3	2.6	5 hours 58 min
40	27	7	6.3	12.6	1 hr 14 min

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APPENDIX F: PREPARATION OF *PSEUDOMONAS FLUROESCENS* INOCULUM

Appendix F
Preparation of *Pseudomonas fluorescens* Inoculum (Original Method)

Materials Needed:

- Tryptic Soy Broth (TSB)
- 16 × 110 test tubes
- rack
- centrifuge
- *Pseudomonas fluorescens* stock
- 1 ml pipette tips and pipette
- 10 ml serological pipettes and pipette
- sterile deionized water

Procedure:

8) Prepare TSB according to package directions.

9) Aliquot 9 ml TSB into 2 test tubes. Transfer 1 ml *Pseudomonas fluorescens* stock to each of two tubes of fresh TSB.

10) Incubate overnight at 30ºC.

11) Vortex tubes and centrifuge at max speed, 10,000 RPM, for 15 minutes.

12) Remove supernatant using 10 ml serological pipette.

13) Reconstitute pellet using 9 ml sterile deionized water.

14) Vortex and centrifuge at max speed, 10000 RPM for 15 minutes.

15) Remove supernatant. Reconstitute pellet in 10 ml deionized water.

16) Combine the contents of two tubes in sterile 50 ml centrifuge tube.

17) Store in refrigerator.

This method usually yields a solution of 10^7 CFU/ml.

APPENDIX G: PROPAGATION OF *PSEUDOMONAS FLUROESCENS* CULTURE FROM CRYO TUBES

Appendix G
Propagation Of *Pseudomonas fluorescens* culture from CRYO tubes

Materials Needed:

- Tryptic Soy Broth (TSB)
- Phosphate Buffer Solution (PBS)
- 16 × 110 test tubes
- rack
- centrifuge
- *Pseudomonas fluorescens* stock in frozen CRYO tube
- 1 ml pipette tips and pipette
- 10 ml serological pipettes and pipette

Procedure:

1) Prepare TSB according to package directions.
2) To 9 ml sterile TSB add 1 CRYO Bead from *P. fluorescens* frozen stock. Vortex.
3) Incubate 24 hours at 30ºC.
4) Vortex tube. Transfer 1 ml of *P. fluorescens* overnight growth to 9 ml sterile TSB. Vortex. Incubate 24 hours at 30ºC
5) Vortex tube and centrifuge at max speed, 10,000 RPM, for 10 minutes.
6) Remove TSB supernatant using 10 ml serological pipette.
7) Reconstitute pellet with 9 ml PBS.
8) Vortex and centrifuge at max speed, 10,000 RPM, for 10 minutes.
9) Remove the PBS supernatant and reconstitute the pellet in 6 ml PBS.
10) Obtain concentration of *P. fluorescens* suspension by enumerating on TSA. (Appendix E)
11) To inoculate experiment, add 9 ml TSB to the *P. fluorescens* 5 ml solution just before addition to the annular reactor. The new mixture will be 14 ml.
12) Add the solution to the reactor, without flow, at 60 RMP for 4 hours.
13) Turn pump on at the recommended flow rate to begin biofilm growth.

This method should yield a solution exceeding 10^7 CFU/ml.

APPENDIX H: BIOFILM–GROWTH RESULTS

Appendix H
Biofilm–Growth Results

Table H1: Biofilm Heterotrophic Plate Counts For Experiment #1

Time Since Inoculation (Days)	# Slides Sampled	# Plates per Slide	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
0.89	2	1	2	0.00E+00	0.00E+00
1.25	2	1	2	8.20E+02	3.79E+02
2.31	2	1	2	5.65E+02	7.13E+01
3.13	2	1	2	1.15E+03	3.49E+02
4.19	2	1	2	3.42E+05	5.63E+04
6.19	2	1	2	5.78E+03	3.38E+03
7.15	2	1	2	1.20E+04	4.84E+03
8.09	2	1	2	1.10E+04	4.80E+03
9.17	2	1	2	6.79E+03	4.20E+03
10.23	2	1	2	3.05E+03	1.88E+02

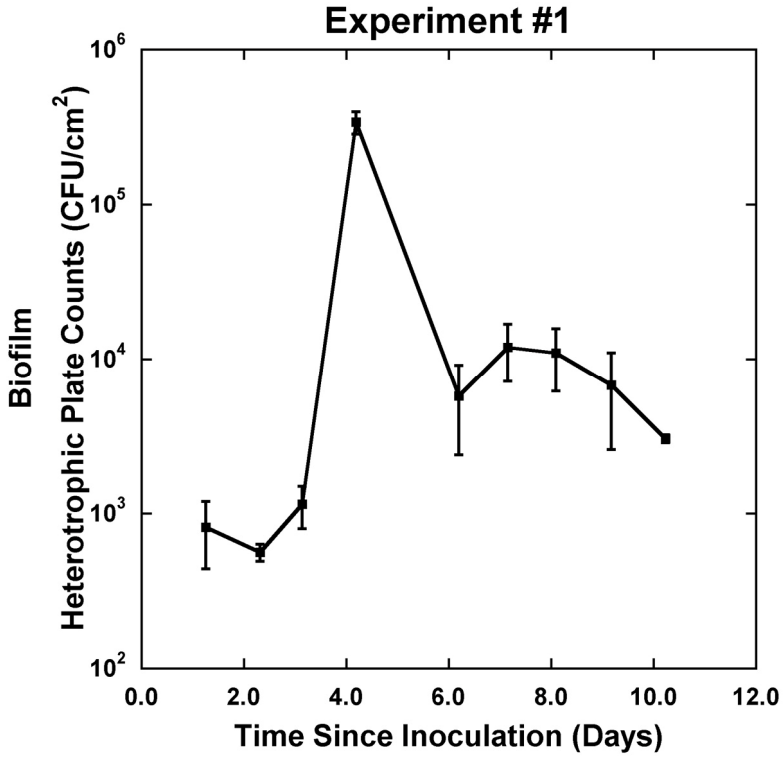


Table H2: Biofilm Heterotrophic Plate Counts For Experiment #2

Time Since Inoculation (Days)	# Slides Sampled	# Plates per Slide	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
2.94	2	4	8	1.08E+04	4.77E+03
3.96	2	4	8	8.89E+03	1.23E+03
4.86	2	4	8	8.29E+03	3.11E+03
5.84	2	4	8	6.45E+03	1.58E+03
6.95	2	4	8	6.66E+03	8.31E+02
9.92	2	4	8	8.61E+03	1.28E+03
10.90	2	4	8	1.06E+05	7.36E+03
11.98	2	4	8	1.83E+05	1.66E+05
12.96	2	4	8	7.25E+04	2.00E+04
13.93	2	4	8	5.11E+05	2.53E+05

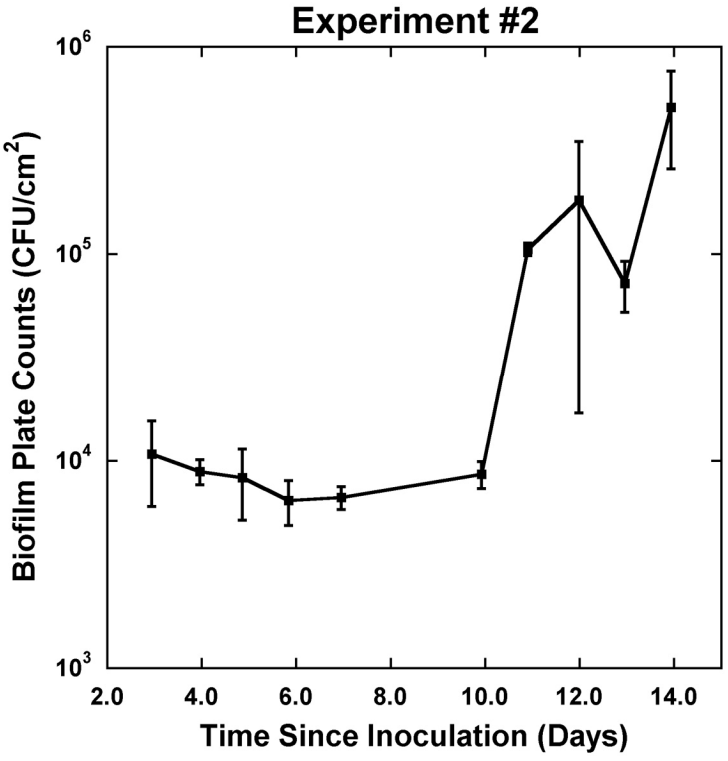


Table H3: Biofilm Heterotrophic Plate Counts For Experiment #4

Time Since Inoculation (Days)	# Slides Sampled	# Plates per Slide	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
2.88	2	4	8	6.29E+02	2.00E+02
3.99	2	4	8	1.20E+03	8.02E+02
4.87	2	4	8	2.10E+03	7.36E+02
5.81	2	4	8	1.94E+03	1.46E+03
6.81	2	4	8	2.81E+03	4.40E+02
9.95	2	4	8	2.14E+04	3.91E+03
10.90	2	4	8	3.97E+04	1.15E+04
11.95	2	4	8	4.04E+04	7.09E+03
12.99	2	4	8	3.44E+04	4.16E+03
13.75	2	4	8	6.45E+04	2.14E+04

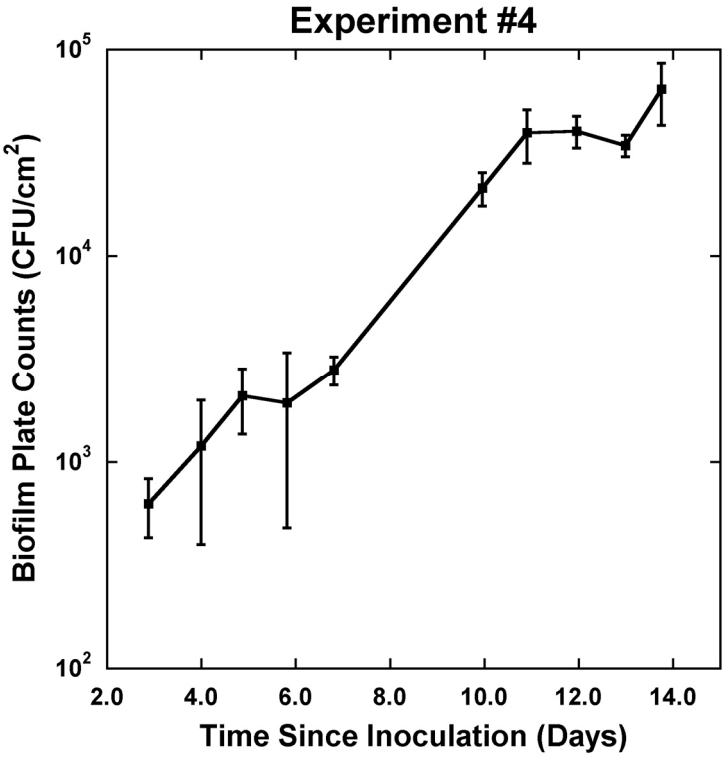


Table H4: Biofilm Heterotrophic Plate Counts For Experiment #6

Time Since Inoculation (Days)	# Slides Sampled	# Plates per Slide	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
1.95	2	4	8	1.90E+04	1.23E+04
5.03	2	4	8	2.75E+05	5.78E+04
6.99	2	4	8	2.53E+05	5.61E+04
7.96	2	4	8	2.92E+05	9.38E+04
11.96	2	4	8	3.90E+05	1.47E+05
12.83	2	4	8	1.40E+05	9.80E+04
13.79	2	4	4	9.20E+04	1.24E+04
14.94	2	4	8	9.12E+04	1.42E+04
18.92	2	4	8	8.96E+05	4.37E+05
19.71	2	4	8	5.46E+05	2.82E+05

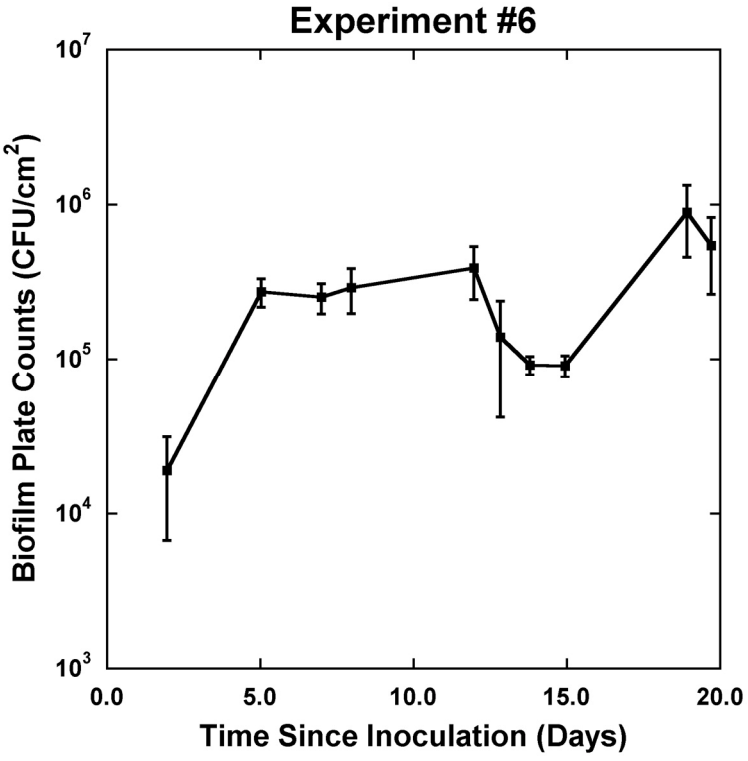


Table H5: Biofilm Heterotrophic Plate Counts For Experiment #7

Time Since Inoculation (Days)	# Slides Sampled	# Plates per Slide	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
3.19	1	4	4	1.90E+04	1.40E+03
4.95	1	4	4	4.66E+05	1.02E+05
10.05	1	4	4	4.60E+05	6.81E+04
11.92	1	4	4	6.70E+05	9.05E+04
12.97	1	4	4	9.83E+05	8.36E+05
17.15	1	4	4	4.60E+05	2.95E+04
18.02	1	4	4	5.24E+05	4.36E+04
19.95	1	4	4	1.66E+06	5.08E+04
24.05	1	4	4	5.88E+05	5.82E+04
25.91	1	4	4	1.41E+06	2.31E+05
26.85	1	4	4	4.24E+05	3.75E+04
31.05	1	4	4	3.95E+05	3.08E+04
32.91	1	4	4	3.12E+05	2.00E+04
33.85	1	4	4	4.83E+05	2.91E+04
38.04	1	4	4	2.69E+05	1.91E+04
39.91	1	4	4	2.25E+05	4.17E+04
41.14	1	4	4	1.95E+05	2.65E+04
45.00	1	4	4	3.38E+05	1.86E+04
46.98	1	4	4	8.69E+04	1.01E+04
47.91	1	4	4	2.33E+05	1.56E+04

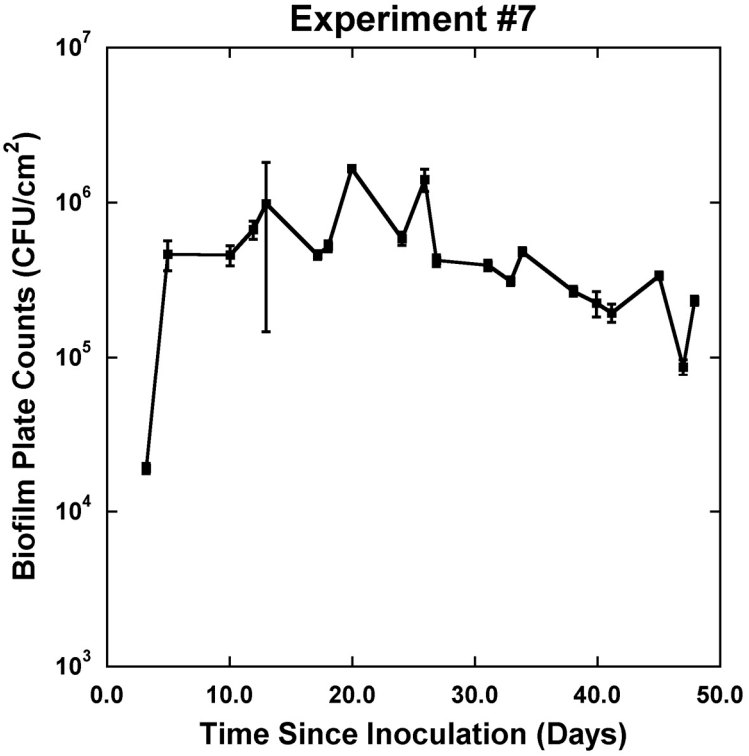


Table H6: Biofilm Heterotrophic Plate Counts For Experiment #8

Time Since 1st Inoculation (Days)	Time Since 2nd Inoculation (Days)	# Slides Sampled	# Plates per Slide	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
3.03	—	1	4	4	0.00E+00	0.00E+00
9.96	—	1	4	4	0.00E+00	0.00E+00
16.95	6.01	1	4	4	2.28E+04	4.46E+03
23.97	13.03	1	4	4	1.03E+05	4.46E+03
30.90	19.96	1	4	4	3.74E+05	5.24E+04
37.98	27.04	1	4	4	3.79E+05	3.51E+04
46.92	35.98	1	4	4	4.84E+04	4.09E+03
46.93	35.99	1	4	4	3.91E+04	5.08E+03
46.94	36.00	1	4	4	2.68E+04	3.14E+03
46.95	36.01	1	4	4	2.82E+04	1.75E+03
46.96	36.02	1	4	4	2.53E+04	3.71E+03
46.98	36.04	1	4	4	4.08E+04	4.94E+03
47.00	36.07	1	4	4	3.20E+04	7.61E+03
47.04	36.11	1	4	4	3.78E+04	2.05E+03
47.11	36.17	1	4	4	3.29E+04	2.53E+03
47.74	36.80	1	4	4	2.11E+04	2.43E+03
48.07	37.13	1	4	4	2.08E+03	2.39E+02
48.74	37.80	1	4	4	2.61E+03	3.10E+02
50.74	39.80	1	4	4	8.13E+05	7.08E+04
53.89	42.95	1	4	4	5.60E+06	6.07E+05

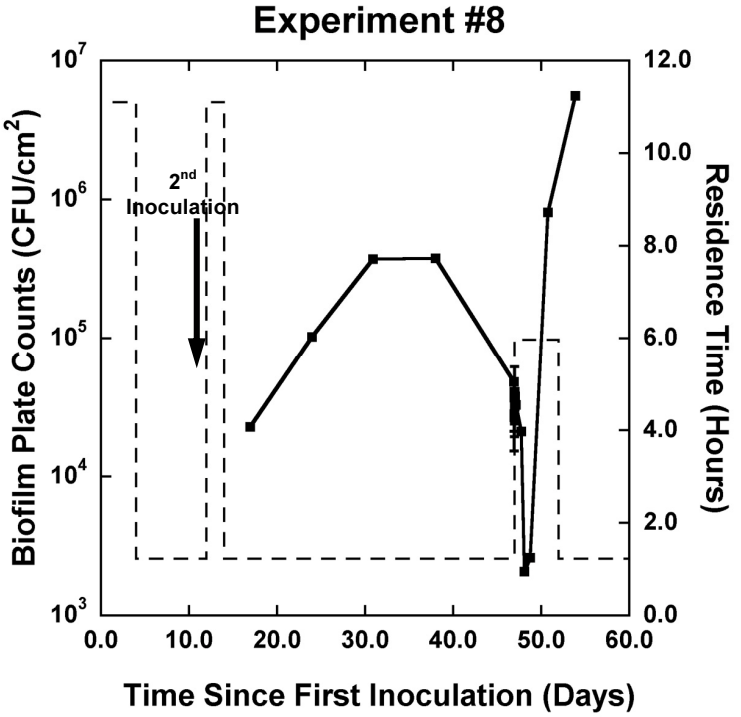


Table H7: Biofilm Heterotrophic Plate Counts For Experiment #9

Time Since 1st Inoculation (Days)	Time Since 2nd Inoculation (Days)	Time Since 3nd Inoculation (Days)	Time Since 4th Inoculation (Days)	Time Since 5th Inoculation (Days)	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
10.71	0	0	0	0	4	0.00E+00	0.00E+00
46.15	33.9	24.95	0	0	4	8.28E+02	3.99E+01
53.23	40.98	32.03	6.25	0	4	1.84E+04	1.81E+03
55.14	42.9	33.94	8.17	0	4	9.73E+04	7.67E+03
62.09	48.84	39.89	14.11	0	4	3.29E+04	4.96E+03
75.01	61.77	52.82	27.04	6.97	4	3.93E+05	1.98E+04
81.05	68.81	59.86	34.08	14.01	4	6.21E+06	5.61E+05
82.03	69.78	60.83	35.05	14.98	4	1.29E+07	2.31E+05
82.05	69.8	60.85	35.07	15	4	7.86E+06	3.91E+05
82.07	69.83	60.87	35.09	15.03	4	1.29E+07	6.44E+05
82.11	69.87	60.91	35.13	15.07	4	1.35E+07	3.08E+05
82.19	69.95	61	35.22	15.15	4	1.51E+07	5.00E+05
82.28	70.03	61.08	35.3	15.23	4	1.11E+07	9.74E+05
83.03	70.78	61.83	36.05	15.98	4	1.31E+07	1.11E+06
83.23	70.98	62.03	36.25	16.18	4	2.61E+07	1.38E+07
84.05	71.8	62.85	37.07	17	4	8.01E+07	1.71E+07
84.91	72.66	63.71	37.93	17.86	4	4.08E+07	7.16E+06

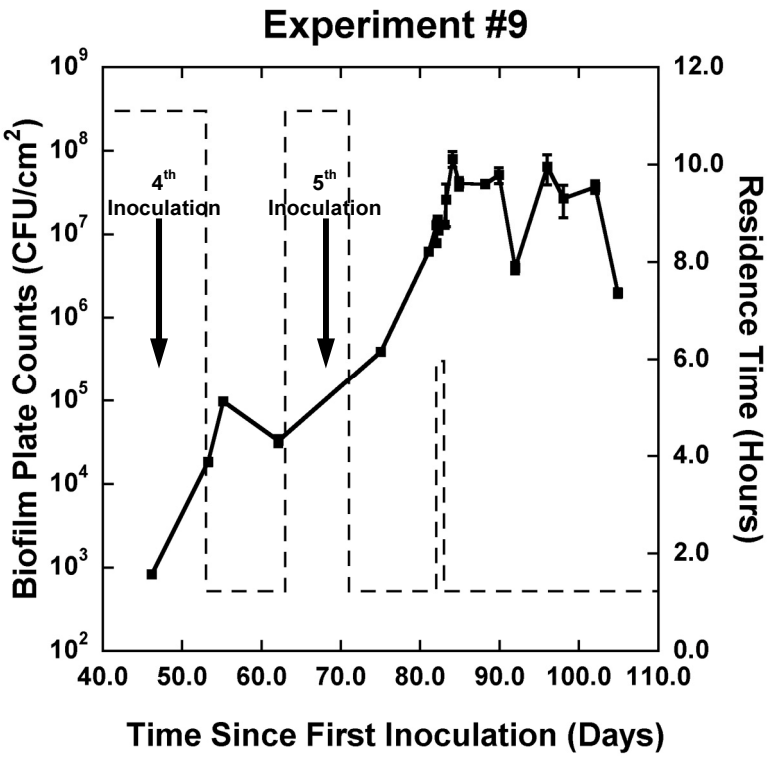
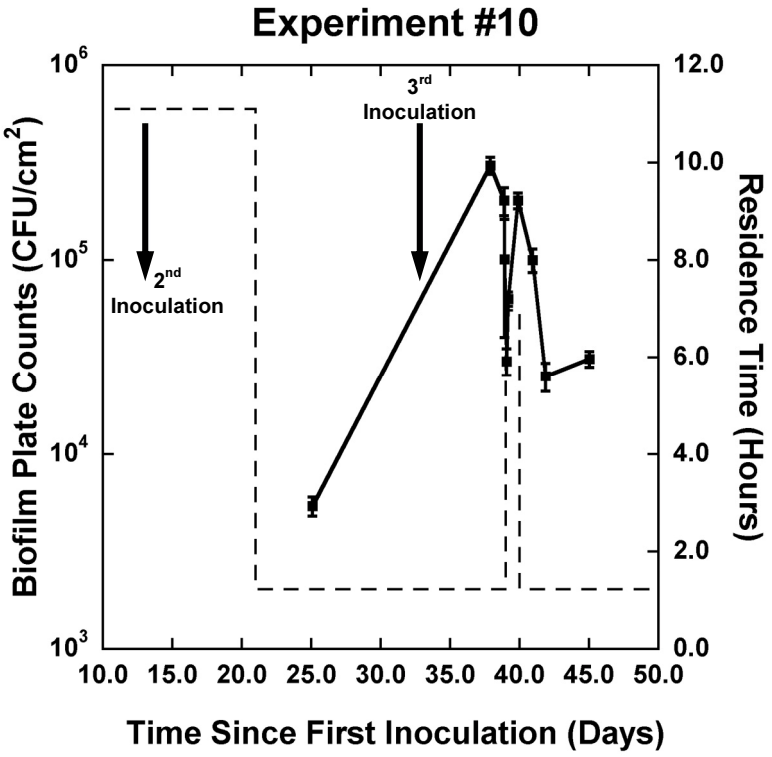

Experiment #9
Biofilm Plate Counts (CFU/cm2)
Residence Time (Hours)
Time Since First Inoculation (Days)
4th Inoculation
5th Inoculation
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
12.0
10.0
8.0
6.0
4.0
2.0
0.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0
110.0

Table H8: Biofilm Heterotrophic Plate Counts For Experiment #10

Time Since 1st Inoculation (Days)	Time Since 2nd Inoculation (Days)	Time Since 3rd Inoculation (Days)	# Slides Sampled	# Plates per Slide	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
25.09	12.02	0.0	1	4	4	5.41E+03	6.05E+02
37.91	24.83	5.13	1	4	4	3.06E+05	3.13E+04
38.89	25.82	6.11	1	4	4	2.02E+05	3.27E+04
38.93	25.86	6.15	1	4	4	1.01E+05	6.09E+04
39.06	25.99	6.28	1	4	4	3.02E+04	4.76E+03
39.18	26.11	6.40	1	4	4	6.33E+04	5.28E+03
39.89	26.82	7.11	1	4	4	2.02E+05	1.89E+04
40.94	27.86	8.16	1	4	4	9.99E+04	1.38E+04
41.88	28.81	9.10	1	4	4	2.53E+04	4.21E+03
45.04	31.97	12.26	1	4	4	3.10E+04	2.89E+03



APPENDIX I: REACTOR WATER SAMPLING RESULTS

Appendix I
Reactor Water Sampling Results

Table I1: Reactor Water Sampling Results for Experiment #6

Time Since Inoculation (Days)	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
5.03	4	4.28E+05	1.45E+05
6.99	4	1.54E+05	2.16E+04
7.96	3	2.29E+05	6.29E+04
11.96	4	6.95E+04	1.05E+04
12.83	4	6.40E+04	6.38E+03
13.79	4	1.21E+05	3.95E+04
14.94	4	6.13E+04	2.06E+04
18.92	4	7.30E+05	8.79E+04
19.71	4	4.23E+05	5.56E+04

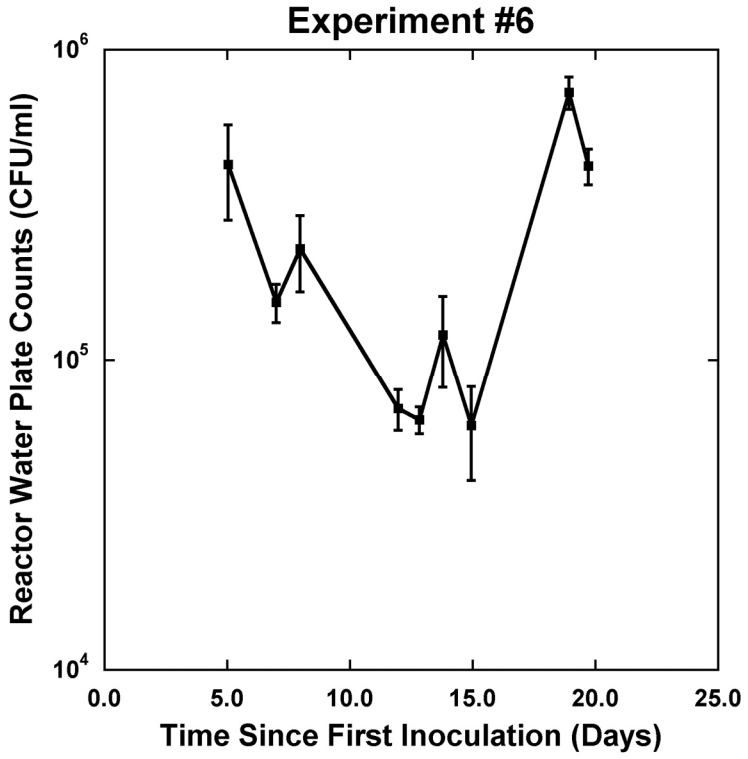


Table I2: Reactor Water Sampling Results for Experiment #7

Time Since Inoculation (Days)	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
3.19	4	6.08E+04	7.80E+03
4.95	4	1.10E+05	1.16E+04
10.05	4	6.60E+03	1.04E+03
11.92	4	8.18E+04	8.26E+03
12.97	4	4.40E+04	1.13E+04
17.15	4	8.20E+03	1.66E+03
18.02	4	3.05E+04	1.29E+03
19.95	4	1.35E+05	1.06E+04
24.05	4	1.33E+04	6.06E+02
25.91	4	5.60E+04	4.55E+03
26.85	4	3.83E+04	8.85E+03
31.05	4	3.48E+04	5.81E+03
32.91	4	6.38E+04	3.86E+03
33.85	4	5.98E+04	4.50E+03
38.04	4	3.90E+04	3.56E+03
39.91	4	3.75E+04	6.24E+03
41.14	4	2.30E+05	3.11E+04
45.00	4	3.60E+04	3.16E+03
46.98	4	3.98E+04	5.91E+03
47.91	4	6.03E+04	7.37E+03

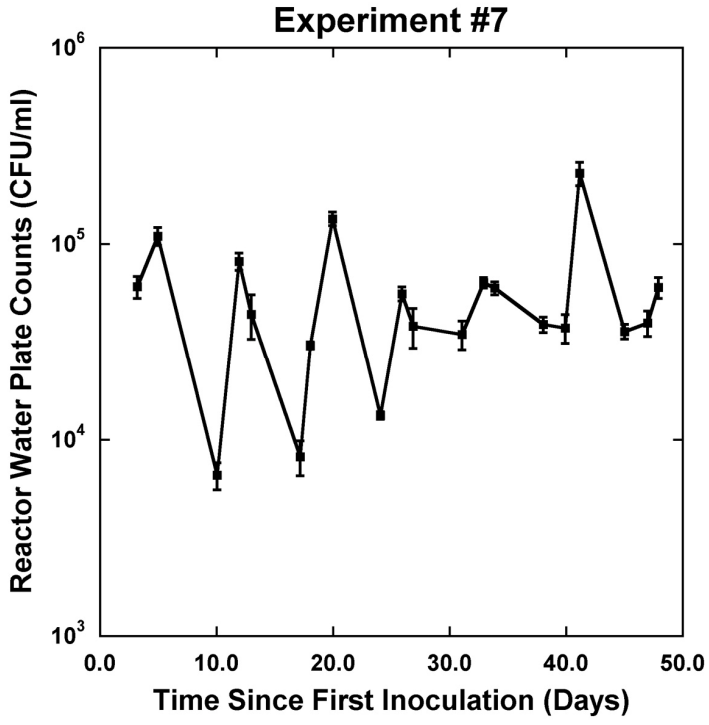


Table I3: Reactor Water Sampling Results for Experiment #8

Time Since 1st Inoculation (Days)	Time Since 2nd Inoculation (Days)	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
3.03	—	4	0.00E+00	0.00E+00
9.96	—	4	0.00E+00	0.00E+00
16.95	6.01	4	6.60E+03	1.04E+03
23.97	13.03	4	8.18E+04	8.26E+03
30.90	19.96	4	4.40E+04	1.13E+04
37.98	27.04	4	8.20E+03	1.66E+03
46.92	35.98	4	9.20E+03	3.56E+02
48.07	37.13	4	2.78E+03	4.79E+02

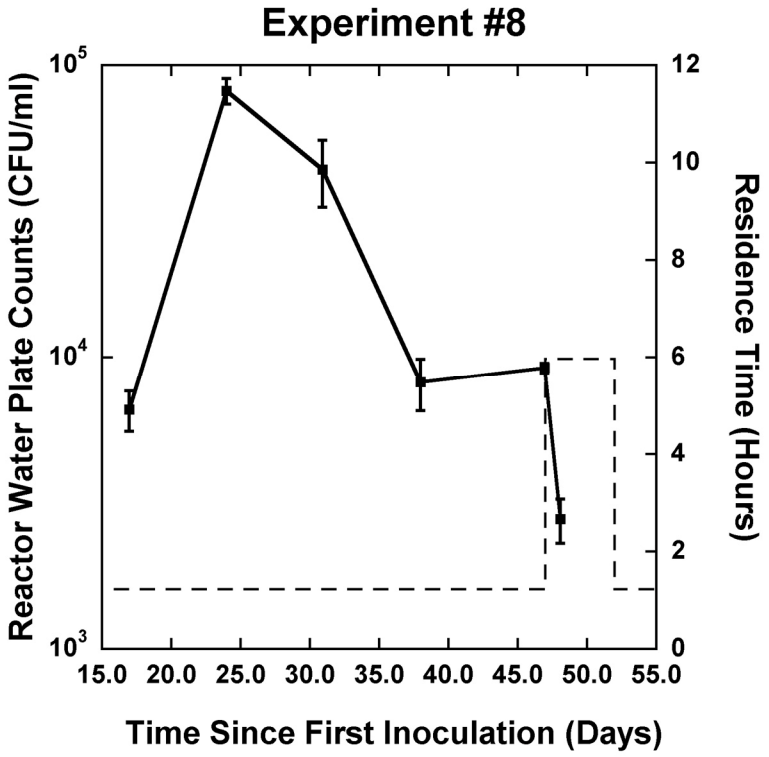


Table I4: Reactor Water Sampling Results for Experiment #9

Time Since 1st Inoculation (Days)	Time Since 2nd Inoculation (Days)	Time Since 3rd Inoculation (Days)	Time Since 4th Inoculation (Days)	Time Since 5th Inoculation (Days)	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
11.21	——	——	——	——	4	0.00	0.00
13.20	0.95	——	——	——	1	TNC (2)	N/A
18.14	5.89	——	——	——	1	NG (1–3)	N/A
20.00	7.75	——	——	——	1	NG(1–3)	N/A
26.06	13.82	4.86	——	——	1	3.10E+02	N/A
28.17	15.93	6.98	——	——	1	3.90E+02	N/A
32.00	19.76	10.81	——	——	1	1.02E+03	N/A
32.94	20.70	11.74	——	——	1	8.90E+02	N/A
33.94	21.70	12.75	——	——	1	8.90E+02	N/A
39.06	26.81	17.86	——	——	1	1.97E+03	N/A
42.03	29.78	20.83	——	——	1	3.20E+03	N/A
46.03	33.79	24.83	——	——	1	6.60E+03	N/A
48.13	35.89	26.93	1.15	——	4	6.40E+05	5.48E+04
53.23	40.98	32.03	6.25	——	4	1.06E+04	1.25E+03
62.09	48.84	39.89	14.11	——	4	3.43E+02	4.99E+01
70.93	57.69	48.74	22.96	2.89	4	TNC (3)	N/A
72.16	58.92	49.97	24.19	4.12	4	4.48E+04	5.38E+03
75.01	62.77	52.82	27.04	6.97	4	3.08E+04	3.03E+03
77.14	64.90	54.95	29.17	9.10	4	2.19E+04	4.36E+03
82.05	69.81	59.85	33.08	14.01	4	2.92E+05	1.52E+04

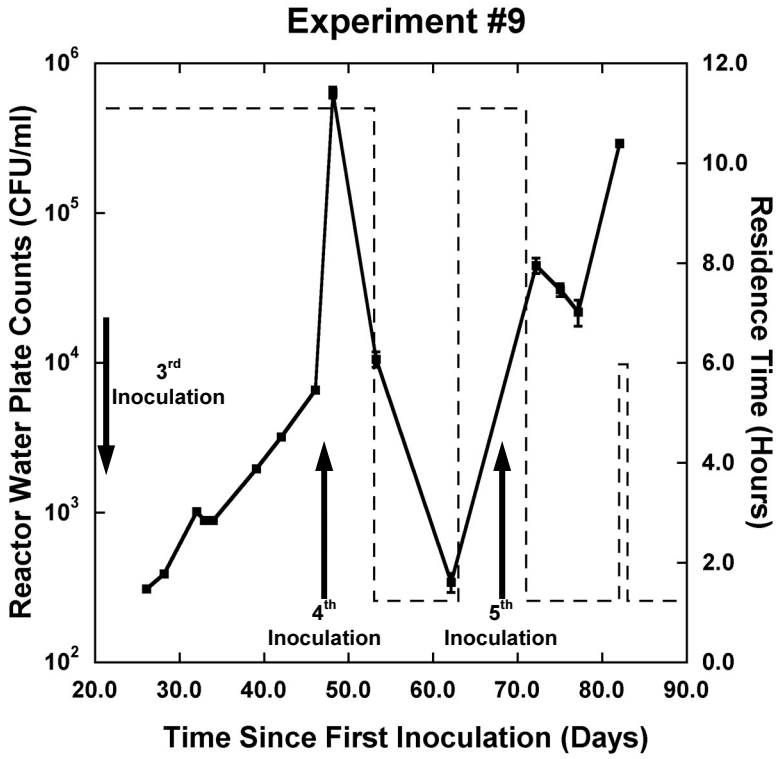
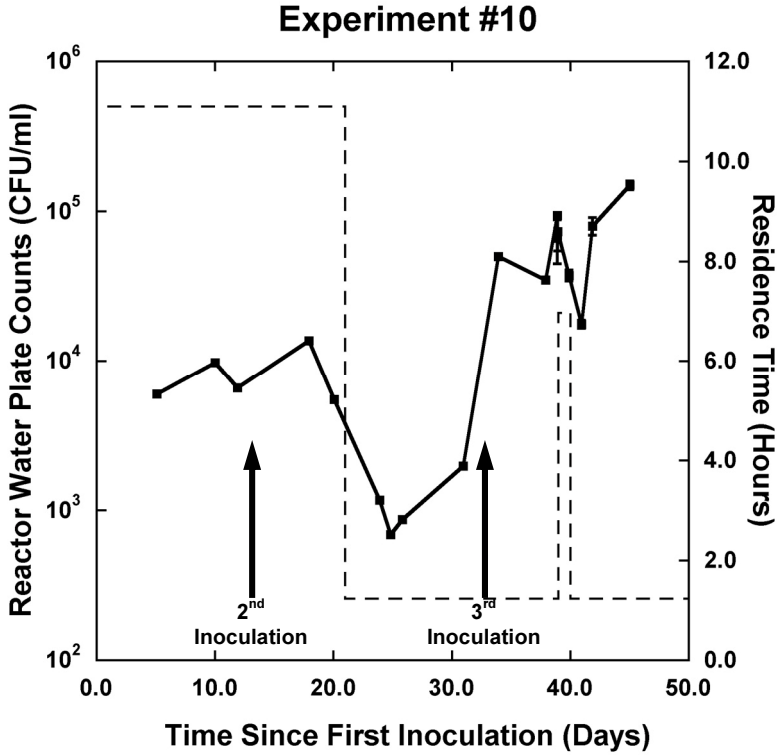

Experiment #9
Reactor Water Plate Counts (CFU/ml)
Residence Time (Hours)
Time Since First Inoculation (Days)
3rd Inoculation
4th Inoculation
5th Inoculation
10^6
10^5
10^4
10^3
10^2
12.0
10.0
8.0
6.0
4.0
2.0
0.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0

Table I5: Reactor Water Sampling Results for Experiment #10

Time Since 1st Inoculation (Days)	Time Since 2nd Inoculation (Days)	Time Since 2nd Inoculation (Days)	n	Mean (CFU/cm^2)	Standard Deviation (CFU/cm^2)
5.07	0.00	0.00	1	6.00E+03	N/A
10.01	0.00	0.00	1	9.80E+03	N/A
11.87	0.00	0.00	1	6.60E+03	N/A
17.93	4.86	0.00	1	1.37E+04	N/A
20.05	6.98	0.00	1	5.50E+03	N/A
23.88	10.81	0.00	1	1.17E+03	N/A
24.82	11.74	0.00	1	6.90E+02	N/A
25.82	12.75	0.00	1	8.70E+02	N/A
30.93	17.86	0.00	1	1.98E+03	N/A
33.90	20.83	1.12	1	5.00E+04	N/A
37.91	24.83	5.13	1	3.50E+04	N/A
38.89	25.81	6.11	1	9.40E+04	N/A
38.89	25.82	6.11	4	6.63E+04	2.15E+04
38.93	25.86	6.15	4	7.35E+04	1.91E+04
39.89	26.82	7.11	4	3.75E+04	3.11E+03
40.94	27.86	8.16	4	1.77E+04	1.07E+03
41.88	28.81	9.10	4	8.00E+04	1.08E+04
45.03	31.96	12.25	4	1.50E+05	1.00E+04



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APPENDIX J: PROTOCOL OF GROWING BIOFILMS FOR PATHOGEN–INTRODUCTION EXPERIMENTS

Appendix J
Protocol for Growing Biofilms for Pathogen–Introduction Experiments

1. Goals

- Produce repeatable *P. fluorescens* biofilms in an annular reactor until growth becomes stationary (Heterotrophic Plate Counts are expected to be approximately 10^4 CFU/cm^2, or higher).
- Use successfully grown biofilms for subsequent pathogen–introduction experiments using *B. cereus* and 1–μm diameter polystyrene microspheres as the pathogen.

2. Biofilm Growth and Sampling

2.1 Biofilm Organism

Biofilm Organism: *P. fluorescens* is a Biosafety Level 1 (BSL 1) organism and has been successfully used in previous biofilm–growth experiments. Also, since *P. fluorescens* is a gram negative organism, it can be differentiated from the gram positive *B. cereus* (a pathogen surrogate) with a gram stain and gram selective agar.

2.2 System Components

Inflow containers:

Prepare 20 L each of nutrient solution and NaOH buffer solution inflow containers. Concentrations of nutrient solution ingredients are listed in Table J1. The NaOH buffer inflow solutions should be maintained at 4.5 mg/L to comply with the recommendations following biofilm–growth experiment #7.

Table J1: Nutrient Solution Concentrations, Biofilm–Growth Experiment Protocol

Nutrient	Concentration (mg/L)
Glucose	5
Peptone	2.5
Yeast	1.25

Outflow containers:

Collect reactor water outflow solutions (waste) in 15 gallon barrels so that the system may run for more than one day before being emptied. Decontaminate outflow solutions in these containers by adding ¼ chlorine tablets to each filled container. After approximately 24 hours, the chlorinated solutions may be carefully drained into the sink. Record all waste water discharge in the appropriate log. To determine the efficacy of this decontamination procedure, the outflow containers may be randomly sampled and plated during the experiment using a 1 ml sample of the outflow solution on a spread plate with Tryptic Soy Agar (TSA). If growth is not

observed on the plates after one–day incubation, the outflow solution may be appropriately discarded.

Pumps:

Use a Masterflex L/S model #7524–50 with a dual channel pump head HV–77202–60 (pictured in Figure 1) for aseptic transfers of sterile solutions between refill inflow containers; do not use this pump for flow rate control. Use the Masterflex L/S model #7554–80 with two HV–77201–60 pump heads (pictured in Figure 1) to fill the primary inflow containers.

Reactor:

Use an annular reactor from Biosurface Technologies with an inner cylinder rotation rate of 60 RPM and polycarbonate slides.

2.3 Methods

System assembly:

1. Clean and sterilize all components (including tubing) of the annular reactor for 40 minutes. Assemble the sterile apparatus aseptically. Attach the nutrient tubing to the system tubing, squirting ethanol over the tubing to decrease contamination from the air and operator's hands. Connect the water line tubing to the water source the same way. Unclamp all tubing. Remove the clamp beneath the air vent on the effluent line. It is important to reattach all tubing aseptically.
2. Pump both nutrient and NaOH inflow solutions (with the Masterflex L/S model #7554–80) through the reactor until the unit is full (using an arbitrary flow rate).
3. Run the system for approximately 2 hours until the pH has stabilized and no leaks occur.

Reactor maintenance:

A series of supplemental inflow containers filled with sterile nutrient and NaOH solutions will maintain the reactor for the duration of the experiment. Use a peristaltic pump to draw each solution into the reactor from the primary inflow solution containers. These containers should be refilled daily with sterile solutions pumped from adjacent inflow containers. A third set of containers, controlled by a second pump, should be prepared in advance to fill the first set of refill containers.

Inoculation:

1. Prepare the *P. fluorescens* inoculant to create an initial concentration of more than 1×10^7 CFU/ml. The resulting solution may be used for subsequent inoculations using the method described in Appendix A for up to 2 months before it expires and a new *Pseudomonas* culture must be generated.

2. Determine and record the actual concentration of *P. fluorescens* injected into the reactor using Trypticase™ Soy Agar (TSA).

3. Add the solution to the reactor and incubate, without flow, at 60 RMP for 4 hours.

4. Turn the pump on at the recommended flow rate to begin biofilm growth.

Flow rates and Residence times:

The flow rates for each inflow solution container, total flow rates, and the reactor water residence times associated with each flow rate that should be used in the biofilm–growth experiment are presented in Table 2. The times shown in Table 2 may be altered, based on results from reactor water monitoring. During inoculation, hold the total flow rate at 1.4 ml/min to maximize inoculant cell adhesion to the coupons. Increase the total flow rate to 12.6 ml/min when reactor water samples (collected after the first day of the experiment) yield CFU counts above 10^3. This flow rate should minimize the amount of planktonic bacteria in the reactor water so that only biofilm cells are collected. Maintain the 12.6 ml/min total flow rate until the start of the pathogen–introduction experiment, at which point the total flow rate should be decreased to 2.6 ml/min to increase contact time between the colloids and biofilm. Return the total flow rate to 12.6 ml/min 24 hours after the pathogen introduction.

Table J2: Flow Rates and Residence Times, Biofilm–Growth Experiment Protocol

Approximate Time Since Inoculation	Flow Rate per Inflow Container (ml/min)	Total Flow Rate (ml/min)	Residence Time
0–4 hours	0.0	0.0	0.0
4 hours–~7 days	0.7	1.4	11 hrs 6 min
~7 days–Path. Intro.	6.3	12.6	1 hr 14 min
Path. Intro.	As prescribed by specific experiment		
24 hours after Path. Intro.	6.3	12.6	1 hr 14 min

2.4 System Sampling

Reactor water sampling

Reactor water should be sampled periodically (beginning approximately 24 hours after the initial inoculation) to determine the amount of planktonic *P. fluorescens* in the system. Sampling should be at least two times a week. Collect a 1 ml sample from the inoculation port of the annular reactor using a sterile pipette. Perform serial dilutions of the 1 ml sample and quantify by pour plating. Record the time / date sampled and quantification results in the appropriate laboratory notebook. Sample the reactor water twice per week until the start of the pathogen–introduction experiment.

Biofilm sampling

A biofilm slide should be sampled the day before the pathogen–introduction experiment is scheduled to commence to ensure that CFU counts are 10^4 CFU/cm^2, or higher. Sampling of Biofilm slides should be sampled, scraped, and plated. Record the time / date sampled, quantification results, and additional observations in the correct laboratory notebook.

pH measurements

Record daily pH measurements of the outflow solution from the annular reactor using an Orion 520A+pH meter. Perform 2–point calibrations before each measurement using the Orion Application Solution with pH standards of 4.01 and 7.00. Record all measurements and calibration results (slope) in the correct laboratory notebook.

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APPENDIX K: PREPARATION METHODS FOR *BACILLUS CEREUS* SPORES

Appendix K
Preparation Methods for *Bacillus cereus* Spores

Materials Needed:

- **2X SG agar (sporulation agar)**
  per liter:

Difco nutrient broth	16.0 g
KCl	2.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Agar	17.0 g

  Adjust the pH to 7.0, autoclave, and then add the following sterile component solutions to cooled medium:

1 M $Ca(NO_3)_2$	1.0 ml
0.1 M $MnCl_2 \cdot 4H_2O$	1.0 ml
1 mM $FeSO_4$	1.0 ml
50% (w/v) glucose	2.0 ml

1) Grow up *B. cereus* in 50 ml Tryptic Soy or Nutrient broth overnight, 30°C shaking.
2) Plate 0.5 ml overnight culture on several sporulation agar plates, incubate at 30°C for five days.
3) After incubation, most of the *B. cereus* should have sporulated or died. Colonies will turn brown, and can be checked for sporulation microscopically.
4) Using flame sterilized policeman and sterile water, scrape the colonies from the plates into sterile centrifuge tubes. Use 50 ml tubes, 35 ml cold, distilled water, and put one plate in each tube. Centrifuge at 10,000g, 10 min. Wash three or four times. Suspend pellet in 25 ml 50% ethanol and store at 4°C overnight.
5) The following day, wash spores in cold water 2–3 times as before. Combine two tubes, but keep volume the same. Wash a couple more times, but at 20,000 for 20 minutes. During the last wash, if there is a noticeable top layer that is loose and slides down the tube—it can be discarded. Again store in 50% ethanol overnight.
6) Wash spores as before to remove ethanol (2–3 times). At this point, check the solutions with phase–contrast microscope, spores will appear as phase–bright bodies, or refractile bodies with light microscope. If there is still a lot of cell debris and cell in suspension, either continue the daily water wash for several more days or try the following;
7) Suspend pellet(s) in 2 ml of 20% meglumine diatrizoate (Sigma), then layer the 2 ml of suspension over 10–20 ml of 50% meglumine diatrizoate in a centrifuge tube. Centrifuge for 30 minutes at ~14,500g. The upper portion of the pellet will be cells and debris, the lowest portion free spores. Wash pellet 3–4 times to remove chemical.
8) Spores clump and will need to be sonicated before use.

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