DESIGN OF MODEL REACTOR SYSTEMS FOR EVALUATING DISINFECTANTS AGAINST BIOFILM BACTERIA

by

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ABSTRACT

Laboratory reactors are devices which grow biofilms under controlled and/or field relevant conditions for the purpose of developing biofilm control strategies. In disinfectant efficacy testing, as well as in research, choosing the best reactor to use is important because different laboratory reactors impart unique characteristics in the biofilm that influence everything from biofilm architecture and protein regulation to the response of biofilm bacteria to disinfection. In this dissertation, three laboratory reactors and associated standard operating protocols were developed to provide a reliable, standardized assessment of disinfectant efficacy against biofilm bacteria that grow: 1) in a moderate to high fluid shear environment, 2) in hot tubs, and 3) in swimming pools. The reactors and protocols were then evaluated for the statistical characteristics of repeatability, ruggedness, and responsiveness, required criteria for the standardization of a method.

In the CDC biofilm reactor (CBR), biofilm was engineered to possess the characteristics of a field biofilm grown under moderate to high fluid shear. The laboratory hot tub and swimming pool reactors were each engineered to generate a field-relevant biofilm by modeling the physiochemical and biological characteristics present in such recreational water. Testing demonstrated that the CBR and laboratory hot tub system were repeatable and rugged. The hot tub and swimming pool systems were responsive to disinfectant type and concentration. This research demonstrated the feasibility of incorporating relevant engineering design into practical laboratory methods used to evaluate disinfectant efficacy.

CHAPTER 1

INTRODUCTION

The focus of this dissertation is the engineering design of three biofilm reactor systems and the development of associated standard operating procedures. Laboratory reactors are devices which grow biofilms under controlled and/or field relevant conditions for the purpose of evaluating biofilm control strategies. The reactors presented in this dissertation were designed to provide a reliable, standardized assessment of disinfectant efficacy against biofilm bacteria grown in a moderate to high fluid shear stress (~0.02 - 0.06 N m^{-2}) environment, in a laboratory hot tub reactor and in a laboratory swimming pool reactor.

Background

The discovery that bacteria predominantly exist as biofilm in natural ecosystems (Costerton *et al.*, 1978; Costerton, 2004; Donlan & Costerton, 2002) lead to the realization that better designed reactor systems were needed for disinfectant efficacy testing against biofilm bacteria. Biofilm has a unique architecture depending upon the conditions under which it forms. For example, a *Pseudomonas fluorescens* biofilm formed under high shear is more dense and tightly adhered to the surface as opposed to a *P. fluorescens* biofilm formed under low shear, which is fluffy (Pereira *et al.*, 2002). Bacteria possess a genetic plasticity that enables them to express a different phenotype depending upon whether or not

they are part of a biofilm (Loo *et al.*, 2000; Sauer *et al.*, 2002). A biofilm most likely includes cells in all states of growth, from dormant cells to actively dividing cells (Sternberg *et al.*, 1999). Finally, in a natural ecosystem, biofilm consists of a variety of microorganisms, including pathogenic and/or opportunistic bacteria (Murga *et al.*, 2001; Armon *et al.*, 1997).

Biofilm bacteria present an increased public health risk for three reasons. First, it is accepted in the scientific community that biofilm is more difficult to kill than bacteria suspended in a flask or a suspended bacterial culture dried on a surface. Second, detached biofilm clumps retain this increased resistance (Fux *et al.*, 2004). Lastly, detached biofilm clumps may contain enough bacteria to be an infective dose (Wilson *et al.*, 2004). The development of new control strategies specific to biofilm, therefore, require new laboratory reactors and methods to grow, treat, sample and analyze biofilm bacteria.

Fundamental research, especially in microbiology, utilizes laboratory reactors as a necessary piece of equipment for generating biofilm bacteria. Although fundamental research may result in the creative new design of a biofilm reactor to meet a specific need, rarely is reactor design the focus of the research. Conversely, for applied research, biofilm is grown in laboratory reactors engineered to model the most important physiochemical qualities of the environment of interest. Often times these reactor systems are complex and expensive to operate. In addition, biofilm is ubiquitous in nature, industrial systems, and medical infections, suggesting that numerous laboratory reactor designs are necessary to obtain biofilms relevant to the diverse environments.

The literature contains several examples of different laboratory reactors and methods used in both fundamental and applied biofilm research. Some of the more referenced reactor

designs are: the rotating disk reactor (Zelver *et al.*, 1999), annular reactor (Characklis, 1990; Stoodley & Warwood, 2003), flow cell (Stoodley & Warwood, 2003), modified Robbins device (Kharazmi *et al.*, 1999), drip flow reactor (Stewart, 2001), porous media column (Reinsel, 1996), biofilm grown in tubing (Sauer *et al.*, 2002), and the constant depth film fermentor (Wilson, 1999). In each of these systems, media continuously flows through the reactor. An advantage of the continuous flow systems is the ability to achieve a steady state biofilm. Other examples of biofilm reactors include batch systems used in traditional microbiology research modified to grow biofilm; e.g., the colony biofilm (Anderl *et al.*, 2000), microtiter plate (Pitts *et al.*, 2003), Calgary Device (Ceri *et al.*, 1999), biofilm grown on a glass slide placed on top of a colony biofilm (Charaf *et al.*, 1999), and biofilm grown on a coupon suspended in a batch culture (Yu *et al.*, 1993). In batch reactors, there is no continuous supply of media, although some of the methods that use these reactors include a step to refresh the nutrients. Finally, a different approach from growing laboratory biofilm in reactors is to construct a synthetic biofilm by trapping suspended bacteria in an artificial matrix (Gilbert *et al.*, 1998).

For years, biofilm reactors were successfully used in research, but the standardization of laboratory biofilm reactors and methods for acceptance by a standard setting organization began only recently. Although other methods have been suggested as potential candidates for standardization (Ceri *et al.*, 1999; Charaf *et al.*, 1999; Gilbert *et al.*, 1998; Luppens *et al.*, 2002; Pitts *et al.*, 2003), to date, a method titled "Standard Test Method for the Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with Shear and Continuous Flow using a Rotating Disk Reactor" is the only biofilm reactor and method approved by a

standard setting organization (ASTM, 2006).

Standard methods development is the creation of laboratory protocols for the purpose of comparison, both within a single laboratory and among various laboratories. Researchers choose to use a standard method for various reasons. For instance, a standard method is useful for teaching proper laboratory protocol or monitoring equipment performance. The impetus for the development of many microbial standard methods, though, is disinfectant efficacy testing for product registration with a regulatory agency such as the US Environmental Protection Agency (US EPA) or the US Food and Drug Administration (US FDA). To protect the public's health, regulatory agencies require efficacy data when a product is registered. For this purpose, standardized methods that are repeatable, reproducible, rugged and responsive (Table 1.1) are absolutely required. A standard method should also be reasonable, meaning it should utilize equipment that is "typical" for a laboratory and it should not require an excessive amount of time, supplies or highly specialized training. Relevancy is an important requirement not included in the above list. Detailed discussions exist in the microbiology literature on the relevancy of certain factors, such as which species and/or strain of bacteria is the best surrogate to use (Bloomfield, 1995; Springthorpe & Sattar, 2005). There is a need, however, for standard methods that properly model the field conditions where a disinfectant will be applied when testing the efficacy of disinfectants against biofilm bacteria (Luppens *et al.*, 2002).

Table 1.1 Definitions for the statistical characteristics that a standard method must possess.

Statistical characteristic	Definition
Repeatable	Independent repeats of the same experiment in the same laboratory produce nearly the same result, as indicated by a small standard deviation (SD). An analysis of variance (ANOVA) is done to calculate the SD and to determine how the variance partitions into the with-in experiment and between experiment components.
Rugged	Slight changes to the standard operating procedure (SOP) do not result in a significantly different response from what is predicted. Ruggedness is quantified by the regression coefficients in a least squares multiple regression analysis. Equivalence testing or a t-test may also be used.
Responsive	An SOP is able to distinguish between treatments of high and low efficacy. A responsive SOP is also called sensitive. A trend test of the slope of a least squares regression line where concentration is the prediction variable is used to evaluate responsiveness. An ANOVA F-test may also be used to look for significance between two log reduction values associated with two different treatments.
Reproducible	Repeats of the same experiment run independently by different researchers in different laboratories produce nearly the same result, as indicated by a small SD calculated from an ANOVA. A collaborative study is required to determine a method's true reproducibility.

Similar to standard methods developed to test the efficacy of disinfectants against a culture of suspended bacteria, some of the biofilm reactors and methods being suggested for standardization are not specific to a single simulated system (Ceri *et al.*, 1999; Charaf *et al.*, 1999; Gilbert *et al.*, 1998; Pitts *et al.*, 2003). These tests are well-defined, easy to run, and yield a large number of replicates. They are potentially useful as screening methods for identifying presumably active formulations. The risk is that the simplified design may not correctly predict a disinfectant's performance in the field. The challenge when designing a

laboratory system for estimating real world efficacy is to find the proper balance between field relevancy and practicality while achieving the statistical specifications required of a standard method.

For various reasons, laboratory reactors and methods that become approved standards lag behind the reactors and methods being used in research. Typically, standard methods are based upon research methods that have a record of documented success in more than one laboratory. By the time a research method has demonstrated potential, been standardized and evaluated, the method is no longer novel, and most likely the scientific community has turned its attention to new techniques. Once a method is approved and accepted as a standard and the federal agencies have approved product claims based upon the method, it takes years of effort, a considerable amount of money, and the cooperation many groups to update the existing method or get a new method approved and fully accepted.

Researchers at academic institutions and fundamental research facilities have access to highly specialized and expensive equipment that requires specially trained operators. It is not practical to standardize methods that require such a high level of sophistication until the technology becomes more affordable. Many biofilm research methods can uncover intriguing scientific insights even though the results are qualitative. However, regulatory authorities and standards approval organizations prefer a quantitative measure of efficacy.

A unique opportunity currently exists in the field of standard methods development. The public, industry and government agencies are aware of biofilm and concerned that uncontrolled biofilm may threaten public health. This concern has created a demand for standard methods to grow, treat, sample and analyze a repeatable laboratory biofilm for the

purpose of disinfectant efficacy testing. There are two strategies for creating such methods. The first strategy engineers a biofilm to have specific characteristics that match a field biofilm, matching for example, the architecture, thickness, and strength of attachment. The second strategy uses a reactor that incorporates the most important physiochemical and biological characteristics in the environment of interest.

Both strategies require the development of a standard operating procedure (SOP) to clearly define how the method is executed in a laboratory. A SOP must meet the statistical characteristics defined in Table 1.1 before it can be approved as a standard method. The statistical tools listed in the Table 1.1, such as analysis of variance and least squares multiple regression analysis, are used to optimize the procedure. For example, parameter settings are grossly altered until an acceptable mean is achieved for the response variable of interest, such as the biofilm viable cell log density. Then, the parameter settings are finely adjusted until the repeatability standard deviation is at a minimum, which indicates that the parameters are set at the optimum levels. If an ANOVA then shows that the variance among technicians is small to negligible, then there is a good indication that the SOP is satisfactory. Ruggedness testing is used to indicate the reproducibility of a procedure by uncovering any possible interactions among the various parameters and quantitatively describing the extent slight variations made to the SOP affect the response variable mean. If at any time during the development of the SOP the statistical requirements are not met, then the procedure and/or reactor is modified and retested. The development of a SOP that meets the criteria described in Table 1.1 therefore requires numerous repeated experiments and a lot of resources.

This dissertation describes three engineered reactor systems and associated SOPs

designed for the purpose of standardization and disinfectant efficacy testing. The first engineered system was designed to grow a laboratory biofilm with specific characteristics and may be used in efficacy testing of any disinfectant that would be used against a similar type of biofilm. The second and third engineered systems were designed specifically to test the efficacy of disinfectants used to control biofilm growth in hot tubs and swimming pools, respectively.

Research Goal and Objectives

The goal of this research was to design, build and test laboratory reactors that incorporate the relevant engineering specifications of field systems to recreate a growth environment for predicting the efficacy of chemical disinfectants against biofilm bacteria. Specific objectives were as follows:

1. Develop a laboratory reactor and method that grows biofilm under high fluid shear so that it meets the statistical criteria of repeatability, reproducibility, ruggedness and responsiveness required for standardization.
2. Design and build scaled down laboratory models of recreational water systems, specifically a hot tub model and a swimming pool model.
3. Develop a standard operating procedure (SOP) for the hot tub and swimming pool models to assess the performance of recreational water disinfectants against biofilm and suspended bacteria.

The previous three objectives are addressed in the three main chapters of this dissertation. Chapter 2, first published in the journal *Microbiology* in2005, describes the

CDC biofilm reactor (CBR), associated standard operating procedure and the testing that was done to define the repeatability and ruggedness of this system. In the CBR, biofilm is engineered to possess the characteristics of a field biofilm grown under moderate to high fluid shear, estimated to equal approximately 0.02 - 0.06 N m^{-2}. Chapter 3 and Chapter 4 describe the design, development and testing of a laboratory hot tub model and laboratory swimming pool model, respectively. Chapter 4 was first published in the journal *Water Research* in 2004. Both of these systems were engineered to generate a field-relevant biofilm by modeling the physiochemical and biological characteristics present in recreational water. The laboratory hot tub model was also tested for the statistical characteristics of repeatability, ruggedness and responsiveness. Finally, Chapter 5 summarizes the main conclusions from this work and how all three engineered systems contribute to the field of standardized biofilm methods development.

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CHAPTER 2

STATISTICAL ASSESSMENT OF A LABORATORY METHOD FOR GROWING BIOFILMS

Reproduced with permission from Goeres, D.M., Loetterle, L.L., Hamilton, M.A., Murga, R., Kirby, D.W. & Donlan, R.M. (2005) Statistical Assessment of a Laboratory Method for Growing Biofilms. *Microbiology* 151:757-762.

Introduction

Many *in vitro* systems have been developed for growing and testing microbial biofilms. These systems include simple batch/static systems (O'Toole & Kolter, 1998), batch systems with introduced shear (Ceri *et al*., 1999), flow cells (Mittelman *et al*., 1992), perfused biofilm fermenters (Allison *et al*., 1999), and systems that can be operated under continuous flow conditions such as the Rotating Disc Reactor (Zelver *et al*., 1999), Modified Robbins Device (Nickel *et al.*, 1985; Kharazmi *et al.*, 1999), and the Annular Reactor (Camper *et al*., 1996). These systems may operate under batch or continuous flow configurations and generally provide a surface that can be removed and examined once it is colonized to assess biofilm formation. Donlan *et al.* (2002) developed a reactor (CDC Biofilm Reactor, CBR) that incorporated 24 removable biofilm growth surfaces allowing biofilm formation under moderate to high shear in batch or continuous flow conditions. Studies that utilized this reactor showed that it could be used for detecting biofilm formation, characterizing biofilm structure (Donlan *et al.*, 2004) and assessing the effect of antimicrobial agents on the biofilm (Donlan *et al.,* 2002). However, a thorough statistical

evaluation that incorporated an assessment of reproducibility, repeatability, and ruggedness of this system had not been conducted.

In this study, preliminary experiments were performed to generate biofilms of several different clinically relevant microorganisms, including *Pseudomonoas aeruginosa, Klebsiella pneumoniae,* and *Streptococcus pneumoniae*. Then a standard operating procedure (SOP) was created for growing a *P. aeruginosa* biofilm. This procedure was incorporated into an intra-laboratory evaluation to determine the repeatability and ruggedness of the method. We present a description of the CDC biofilm reactor system, preliminary results on biofilm grown in that system, a SOP for *P. aeruginosa* and the results of a study designed specifically to evaluate the repeatability and ruggedness of the SOP.

Materials and Methods

CDC Biofilm Reactor (CBR) Description

The CBR (Biosurface Technologies, Bozeman, MT)[1] consisted of a one-liter glass vessel with an effluent spout positioned to provide approximately 350 ml operational fluid capacity (Figure 2.1). An ultra-high molecular weight polyethylene top supported eight independent and removable polypropylene rods, a medium inlet port, and a gas exchange port. Each rod held three removable coupons (biofilm growth surfaces) for a total of 24 sampling opportunities. Each coupon was a disk (1.27 cm diameter, 0.3 cm thick). The glass vessel was placed atop a digitally controlled stir plate to provide constant rotation of the baffled stir bar at a designated rpm. Rotation of the baffle provided constant mixing and

[1]Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

consistent shear to the coupon surface. The intensity of shear experienced by the coupons was a function of the speed at which the baffle rotated and the distance from the outer edge of the baffle to the coupon face. The estimated range for the fluid shear stress in the CBR was 0.02 - 0.06 N m^{-2} for the rpm settings used in this study (Appendix A). Sterile medium was pumped into the glass vessel using a peristaltic pump (Cole Parmer, Vernon Hills, IL). The CBR was operated as a continuous flow stirred tank reactor, i.e., nutrients continuously flowed into and out of the reactor at a chosen, fixed rate (Characklis & Marshall, 1990). For experiments in which an increased reactor temperature was required, the glass vessel was placed into a water bath. Temperature was maintained using a digitally controlled temperature controller (Digi-sense, model no. 89000-00, Cole Parmer, Vernon Hills, IL) and heating element (Heet-O-Matic, model 324, Cole Parmer).

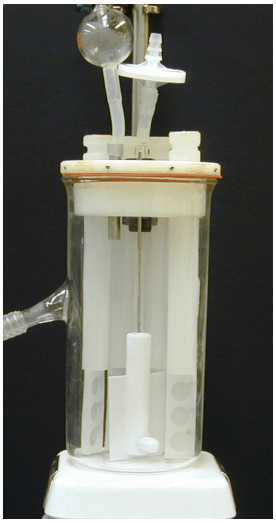

Figure 2.1 The CDC biofilm reactor (CBR). Rods have been removed to expose the baffled stirrer.

Preliminary Experiments

P. aeruginosa (ATCC 7700) and *K. pneumoniae* (CDC culture no. DMDS Lab

92-08-28a) were grown separately on R2A agar plates (Difco Laboratories, Detroit, MI) passaged twice from frozen stocks, incubated at 30°C for 24 h, then suspended in phosphate-buffered saline (PBS) to a concentration equivalent to a 0.5 McFarland standard. Stainless steel 316L coupons were cleaned in 70% ethanol, rinsed in filter-sterilized reverse osmosis (RO) water, placed in the CBR, and then the assembled reactor was sterilized by autoclaving. The sterile reactor was filled with filter-sterilized medium containing 0.05 g yeast extract, proteose peptone #3, casamino acids, dextrose, 0.03 g sodium pyruvate and dibasic potassium phosphate, and 0.005 g magnesium sulfate (all from Difco Laboratories, Detroit, MI) per liter of RO water. After inoculation, the reactor was placed in a water bath to maintain a temperature of 30°C. The water bath was placed onto the surface of a mixing plate set to provide constant mixing at 100 rpm. The system was operated under batch conditions for 72 h then under continuous flow conditions by pumping a 1/10 dilution of the medium defined above at a flow rate of 1 ml min^{-1} (providing a residence time of 6.6 h) for 24 h prior to sampling all 24 coupons. The batch conditions provided additional time for the attachment of organisms prior to initiation of flow. The biofilm growth protocol was based upon Murga *et al*. (2001).

S. pneumoniae (clinical isolate from the Boston's Children's Hospital, Boston, MA, provided by Paul Edmonds, Georgia Institute of Technology, Atlanta, GA) was transferred from a frozen stock onto Trypticase soy agar containing 5% sheep's blood (Blood Agar) (BD Microbiology Systems, Cockeysville, MD) and incubated at 35°C in a 5% CO_2 incubator overnight. A single colony was picked and inoculated into a 10-ml tube of Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI), and incubated for 12 h at 35°C in a 5% CO_2 incubator. For the *S. pneumoniae* experiments, the CBR contained 1.27 cm

diameter Teflon coupons. The reactor was sterilized in an autoclave, then filled with 400 ml of filter-sterilized full strength BHI broth. Nine ml of the 12 h culture was added to the reactor, providing 2.13 x 10^7 cfu ml^{-1} in the reactor as determined by plating on blood agar incubated at 35°C in a CO_2 incubator. A filter sterilized mixture of 85% nitrogen, 10% carbon dioxide, and 5% oxygen was continually supplied to the CBR to provide an atmosphere of supplemental CO_2. The entire reactor was placed into a heated water bath to maintain a temperature of approximately 35°C for the duration of the experiment. The system was operated in batch for 12 h, then in continuous flow by pumping a 1/10 dilution of BHI broth at a flow rate of 0.5 ml min^{-1}(providing a residence time of 13.3 h) for 24 h prior to sampling coupons.

In each preliminary experiment, all 24 coupons in the reactor were sampled and analyzed. Each rod was placed into a biological safety cabinet and three coupons from one rod were aseptically removed. Each coupon was rinsed twice in Butterfield Buffer to remove planktonic cells and placed into a tube containing 10 ml of PBS. Biofilm bacteria were recovered by subjecting coupons to three alternating 30-s cycles of sonication at a frequency of 42 kHz (model 2510 sonicating water bath, Branson Co., Danbury, NY), followed by vortexing (Vortex Genie 2, Scientific Products Co., Bohemia, NY). The removed biofilm was disaggregated by homogenizing the suspension with a tissue homogenizer (Polyscience Tissue Homogenizer Model K-120, Polysciences Co., Niles, IL) at 16,000 rpm for 60 s. The disaggregated biofilm was then processed to quantify the number of viable cells. For *P. aeruginosa* and *K. pneumoniae*, this entailed spread plating serial dilutions of the suspension onto R2A agar (Difco Laboratories, Detroit, MI), incubating the plates at 35°C for 48 h and enumerating the colonies. For *S. pneumoniae*, the diluted

suspension was spread plated onto blood agar and colonies enumerated after incubating for 24 h at 35°C in a CO_2 incubator.

Following the preliminary experiments, the experimental protocol was modified to provide conditions optimal for the *P. aeruginosa* strain used in the ruggedness tests. The sample and analysis steps were also evaluated and modified (see the standard operating procedure below).

Standard Operating Procedure (SOP)

Polycarbonate coupons were sonicated for 30 seconds in a detergent solution; specifically, a 1-2% solution of Micro-90® (International Products Corporation). Then each was rinsed and sonicated for another 30 seconds in reagent grade water. Alternate rinsing and sonication was repeated until no soap was left on the coupon surface. The coupons were then soaked for 2 hours in 2M HCl, rinsed, and allowed to air dry prior to use. One coupon was positioned into each hole of the reactor rods so that the face of the coupon was flush with the rod surface that faced the baffle. A set screw was tightened to hold the coupon in place. The reactor system was then assembled and 500 ml of a 300 mg tryptic soy broth (TSB) l^{-1} (Difco Laboratories, Detroit, MI) was added. The assembled system, minus the pump and stir plate, was autoclaved.

After the system cooled to 23°C, the glass vessel was set on a digital stir plate and inoculated with 1 ml of a 10^8 cfu ml^{-1} suspension of *P. aeruginosa* (ATCC 700888). The *P. aeruginosa* suspension was prepared by inoculating 100 ml of a 300 mg TSB l^{-1} broth with a single colony collected from a bacterial isolation plate. The suspension was incubated for 18-24 h at 37°C in a shaker. Immediately following the addition of *P. aeruginosa* to the

reactor, the stir plate was set to rotate at 125 rpm. The biofilm was allowed to establish for 24 hours in batch phase while the baffle rotated. A 24 hour continuous flow phase followed immediately. While the baffle continued to rotate, a 100 mg TSB l^{-1} nutrient broth was pumped into the reactor at a rate equal to 11.7 ml min^{-1}, resulting a 30 minute residence time.

The stir plate and pump were turned off after 24 hours of continuous flow and the biofilm was harvested from a predetermined number of randomly selected coupons. For each selected coupon, the rod holding that coupon was removed through the top of the reactor. The set screw that held the coupon in place was loosened and the coupon was removed using a flame sterilized hemostat. Care was used not to disturb the surface of the coupon that faced the baffle because that surface held the biofilm sample that was analyzed. Following the procedures described in Zelver *et al.* (1999), the biofilm was then scraped from the surface, homogenized to create a uniform cell suspension, serially diluted, plated on R2A agar, incubated for 18 - 24 hours at 37+/- 2˚C and enumerated. In brief, to remove the biofilm from the coupon, the coupon was held with a sterile clamp. Using a sterile applicator stick, the surface of the coupon was thoroughly scraped for about 1 minute. During that time, the stick was occasionally stirred in 9 ml of sterile buffered water to remove material attached to the stick. After sufficient scraping, the coupon surface was rinsed with 1 ml of sterile buffered water. The final volume in the sample test tube was 10 ml. Then a sterile homogenizer probe was inserted into the homogenizer and the sample tube was homogenized at ~20,500 rpm for 60 seconds. The disaggregated biofilm was then processed to quantify the number of viable cells. This entailed drop plating serial dilutions of the suspension onto R2A agar (Difco Laboratories, Detroit, MI), incubating the plates at 37°C for 24 h, and enumerating the colonies (Herigstad *et al.,* 2001).

The key measurement was the $\log_{10}$ density, where density was expressed in units of cfu cm^{-2}, for each sampled coupon, calculated as in equation 2.1.

$$\log \text{density} = \log_{10}(\text{mean cfu per plate}) + \log_{10}(\text{volume scraped into}) + \log_{10}(\text{dilution}) - \log_{10}(\text{volume plated}) - \log_{10}(\text{area of coupon face}) \quad \text{(Eq. 2.1)}$$

The "area of coupon face" was 1.267 cm^2. For this and all subsequent statistical calculations performed on the $\log_{10}$ scale, five or more significant figures past the decimal point were carried, rounding occurred only at the conclusion of the calculations.

Ruggedness and Repeatability Evaluation Study

A series of experiments were conducted to estimate the repeatability standard deviation, denoted by s_r, and the regression coefficients that measure the ruggedness of the CBR SOP. In these experiments, the SOP was not followed exactly, instead some of the operational factors were purposely varied slightly; e.g., some experiments were conducted at 20˚C rather than at the SOP temperature of 23˚C. For the ruggedness tests, we purposely altered the settings of four operational factors – temperature, rpm of the rotating baffle, time in batch, and nutrient concentration during continuous flow. Three settings were selected for each factor (Table 2.1).

Table 2.1 The three settings for each of the four operating conditions that were studied in the ruggedness test.

	Settings[†]		
Operating conditions	Low	Medium	High
Temperature (˚C)	20	23	26
Baffled stir bar rotation speed (rpm)[‡]	125	180	225
Time in batch (h)	4	18	24
Nutrient[*] concentration during continuous flow (mg l^{-1})	50	100	200

[†] Gray shading indicates standard operating procedure values
[‡] rpm (revolutions per minute)
[*] Tryptic soy broth

A complete factorial experimental design for testing all combinations of the settings would entail $3^4 = 81$ experiments plus some replicates for purposes of calculating s_r. Instead of running a complete factorial design, a response surface design was used. Experimentation was conducted in two phases, with replicates in each phase for purposes of calculating s_r. The first phase used a fractional factorial design (half fraction of a 2^4 factorial) plus replicate runs at the SOP (AOAC, 1998 – Appendix C). The data from the first phase were evaluated to see if the log density was unaffected by any of the factors; if so, those factors could be dropped from the next phase. All of the factors had an effect, therefore none were dropped. The second phase consisted of one-at-a-time experiments, where three of the factors were held at their SOP settings and the fourth factor was at a non-SOP setting. Several experiments in this phase were conducted in which only SOP settings were used. For final analysis, data from both phases were combined and the results were based on a total of 21 separate experiments with five or six randomly sampled coupons in each experiment, resulting in a total of 124 sampled coupons.

Ruggedness was quantified by the regression coefficients in a least squares multiple regression analysis where the response variable was log density and the predictor variables were temperature, rpm, $\log_{10}$-transformed time in batch, and $\log_{10}$-transformed nutrient concentration during continuous flow. To do the analysis, the four predictor variables were entered as covariates into the General Linear Model component of the analysis of variance (ANOVA) module in the computer software package Minitab® (Release 13; Minitab, Inc., State College, PA, USA).

The ANOVA also provided a variance component analysis to assess the variance within experiments and the variance between experiments. The square root of the sum of

those two variances was s_r, which was interpreted as the typical difference, sign neglected, between the log density for a single (randomly chosen) experiment and the average log density across many independent, identical (same operational factor settings) experiments.

Results

Preliminary Experiments

Preliminary testing demonstrated that the CBR, when operated with shear under a combination of batch and continuous flow conditions, was capable of generating biofilms of three different organisms on replicate surfaces. Based on a sample of 24 coupons, the mean $\log_{10}$ density ($\pm$ standard deviation) was 6.77 ($\pm$0.30) $\log_{10}$ cfu cm^{-2} for *K. pneumoniae*, 5.33 ($\pm$0.22) $\log_{10}$ cfu cm^{-2} for *S. pneumoniae*, and 5.63 ($\pm$0.17) $\log_{10}$ cfu cm^{-2} for *P. aeruginosa.*

Coupon Position

Each rod in the CBR held three coupons in vertical alignment. Data from the preliminary experiments with *K. pneumonia, S. pneumoniae*, and *P. aeruginosa* were submitted to an analysis of variance. Neither the 8 rods nor the 3 coupon positions significantly affected the average log density (p-value > 0.50 for each of the three organisms). For the SOP ruggedness test experiments, the mean differences in log densities were 0.18 (bottom minus middle), 0.10 (bottom minus top), and -0.08 (middle minus top). The mean log densities for the three positions were not significantly different (p-value = 0.22). These results indicate that the 24 coupons in the reactor were equally representative of the log density.

Repeatability

For the SOP ruggedness test experiments, replicate experiments were conducted to allow assessment of repeatability of the SOP. The mean $\log_{10}$ *P. aeruginosa* density (cfu per cm^2) was 7.0590, independent of coupon position within the reactor. The estimated within-experiment variance was 0.1884 (estimated with 103 degrees of freedom) and the estimated between-experiment variance was 0.1656 (estimated with 8 degrees of freedom). Therefore, $s_r = (0.1884+0.1656)^{1/2} = 0.59$ of which 53% was attributable to within-experiment variation and 47% to between-experiment variation. This s_r pertained to a protocol that sampled only one coupon per experiment. The repeatability standard deviation for a protocol that requires sampling n coupons per experiment is

$$s_r = [(0.1884 / n) + 0.1656]^{1/2}$$

According to this equation, the s_r for the biofilm mean log density based on three coupons (n = 3) would equal 0.48 of which 27% is attributable to within-experiment variation and 73% to between-experiment variation. For the most intense sampling protocol possible where all n = 24 coupons in the reactor are sampled, $s_r = 0.42$, which is 95% attributable to between experiment- variation.

Because there is an important between-experiment variance, it would be reasonable to choose an operating procedure that requires m independent experiments, n coupons per experiment. In this case, the log density estimate would be the mean log density across all n·m coupons where the repeatability standard deviation is

$$s_r = [(0.1884 / (n \cdot m)) + 0.1656 / m\ ]^{1/2}$$

Ruggedness

Equation (2.2) is the least squares regression model for log density. It shows the estimated log density associated with any pattern of operating conditions, as long as those operating conditions are "near" the SOP specifications. Figure 2.2 shows the relationship between the predicted and observed log density for each coupon, where the predictions are based on equation (2.2). The correlation coefficient between the predicted and observed values (the multiple correlation coefficient) is 0.89, indicating that the regression model is a good fit to the log densities.

$$\log_{10}(\text{cfu cm}^{-2}) = 7.0590 + 0.0191(\text{Temperature} - 23) - 0.00867(\text{rpm} - 125) + 1.2014 \log_{10}(\text{Time in Batch} / 24) + 2.1258 \log_{10}(\text{Nutrient Concentration} / 100) \quad \text{(Eq. 2.2)}$$

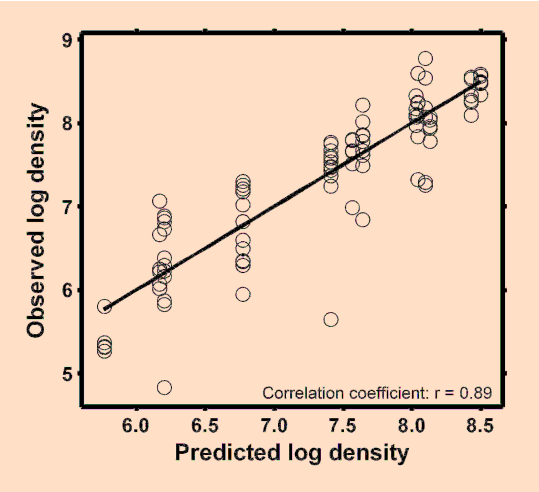

Figure 2.2 Relationship between the estimated (Eq. 2.2) and observed biofilm log densities. The line of equality is shown.

Equation (2.2) provides a quantitative ruggedness assessment because the sizes of the regression coefficients indicate the extent to which the associated condition affects the average log density. If the experiment was conducted at a temperature 2°C higher than specified in the SOP, the average log density would be increased by only 0.0191(2) = 0.04.

If the experiment was conducted with the baffle rotating at 10 rpm faster than specified in the SOP, the average log density would be decreased by only 0.00867(10) = 0.09. If the experiment was conducted with the time in the batch mode increased by 10% to 26.4 h the average log density would be increased by only $1.2014(\log_{10}(1.1)) = 0.05$. Finally, if the experiment was conducted with the nutrient concentration during the continuous flow mode decreased by 10% to 90 mg TSB ml^{-1}, the average log density would be decreased by only 0.10 because $2.1258(\log_{10}(0.9)) = -0.10$.

Discussion

Statistical Evaluation

Guidelines for evaluating a microbiological laboratory method usually include a list of desirable characteristics such as repeatability, reproducibility, and ruggedness (Feldsine *et al*., 2002; ISO, 1993). A method is considered repeatable if independent repeats of the same experiment in the same laboratory produce nearly the same results. The conventional measure of repeatability is the standard deviation or some multiple of the standard deviation (ASTM, 2002a; Feldsine *et al*., 2002).

A method is reproducible if the same result occurs when the same experiment is run independently by different researchers in different laboratories. A collaborative study involving several laboratories is required to assess reproducibility. The results of the collaborative study are summarized conventionally by a standard deviation, called the reproducibility standard deviation, which can be no smaller, and is usually significantly larger, than the repeatability standard deviation (AOAC, 1995; ASTM, 2002a; Feldsine *et al.,* 2002).

A rugged method is one for which the outcome is insensitive to minor perturbations of critical factors or conditions. There is no conventional quantitative measure for ruggedness, though several have been suggested (Thompson *et al*., 2002; AOAC, 1998 – Appendix C; ASTM, 2002b). One reason for conducting a ruggedness test is to provide a single laboratory approximation to a collaborative study. If the operational factors are changed by the amounts that one expects them to vary among laboratories, the standard deviation of the results should be similar to the reproducibility standard deviation. That expectation may be too optimistic. Although there could be considerable savings of resources if in fact a single laboratory ruggedness test could be substituted for a collaborative study, it is not possible at present to design such a test due to lack of knowledge about all relevant factors (Thompson, 2000). A ruggedness test can highlight the critical components of a laboratory method so that practitioners know which steps or conditions require special attention or which parameters should be optimized before a collaborative study is performed. Our informal examination of the literature indicates that ruggedness evaluations of new microbiological methods are seldom conducted. Moreover, we are unaware of other studies describing a quantitative assessment of the influence of individual operational factors on microbiological methods in general and biofilm methods in particular. The work presented here demonstrates that quantitative ruggedness evaluations of microbiological methods can be feasible and informative. The statistical design and analysis strategy presented here could also be used to evaluate other microbiological methods.

This study used a response surface experimental design (ASTM, 2002b; NIST/SEMATECH, 2003), starting with a fractional factorial design (Youden & Steiner,

1975), to identify the smallest number of experiments necessary for a multiple regression analysis. This two-step approach was efficient and provided the desired measure of the influence of each factor.

The choice of which operational factors to study and the range of settings for each factor were subjective steps in the ruggedness evaluation. It was infeasible to study all operational factors; therefore, we relied on experience gained during the development of the reactor and SOP to choose the important factors for evaluation. The relevant settings for each factor were those that fell within the range expected when competent researchers faithfully followed the SOP. We observed responses at three or more levels of each factor. This made it possible to inspect the data for a potential nonlinear association between log density and the factor. There was no evidence of nonlinearity within the range of settings used for any of the four factors. The most reliable regression coefficient estimates occur when one chooses lower and upper settings of the operational factor as far apart as possible within the range of linearity (Thompson *et al.*, 2002).

The interpretation of the influence of operational factors is complicated if there is an interaction, i.e., if the regression coefficient for one factor is dependent on one of the other factors. We did not expect interactive effects to occur within the ranges of factor settings investigated. However, it was infeasible to run the large number of experiments required to check whether all potential interactions were in fact negligible. Note that model of equation (2.2), which does not contain interaction terms, fits the data (Figure 2.2).

The CDC System

Laboratory-grown biofilms are engineered to emulate a specific real-world environment. By altering parameters, such as flow dynamics and reactor configuration, it is possible to grow biofilms differing in structure (e.g., thickness) and function (e.g., nutrient consumption). A case in point is the effect of shear conditions on biofilm structure. Stoodley *et al.* (1999) showed that under high shear, biofilms consisted of dense elongated cell clusters while under low shear biofilms were comprised of less dense round cell clusters. Neither is the uniformly correct laboratory biofilm; each emulates a different growth environment. Because the choice of reactor affects the laboratory biofilm, it is important for the investigator to choose the appropriate reactor and growth conditions. The biofilm reactor (CBR) described in this paper provided a useful tool for growing repeatable biofilms under constant shear using a variety of organisms. By standardizing the design of the reactor, establishing an SOP, and performing multiple experiments, it was possible to conduct a statistical evaluation.

The ruggedness results showed that the baffle rotational speed could be an influential factor if it is not carefully controlled. For example, if the protocol was followed except that the stir plate was set so that the baffle rotated at a speed of 180 rpm instead of 125 rpm as specified by the protocol, the mean log density for a coupon would be estimated to be 6.58, a decrease of 0.47. We recommend that a digital stir plate be used with the CBR to provide that control.

The CBR can be operated under a wide range of controllable conditions. With modifications to the SOP, we believe it can be used to grow a standard biofilm for purposes of addressing diverse research questions.

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CHAPTER 3

A LABORATORY HOT TUB MODEL FOR DISINFECTANT EFFICACY EVALUATION

Introduction

Hot tubs, also known as spas or spa pools, are approximately 1400 liter pools of hot water used for recreational and therapeutic soaking. Hot tub standards recommend draining, cleaning, and refilling public hot tubs at a water replacement interval (days) equal to *(1/3)(spa volume, U.S. gallons)/(max no.of daily uses)* or when the total dissolved solids (TDS) in the water exceeds the source water TDS by 1,500 ppm (ANSI/NSPI-2, 1999). In the interim, the water quality is maintained by the addition of chemicals and recycling the water through a skimmer and filter. Recommended ranges for oxidizing disinfectants, stabilizers, pH, alkalinity, calcium hardness, and TDS are found in the American National Standards for Public Spas (ANSI/NSPI-2, 1999), American National Standards for Portable Spas (ANSI/NSPI-6, 1999) and American National Standards for Permanently Installed Residential Spas (ANSI/NSPI-3, 1999). Guidelines for the skimmer and filter design are found in Circulation System Components and Related Materials for Swimming Pools, Spas/Hot tubs (ANSI/NSF 50, 2001). Compliance rules for bacterial contamination in hot tubs are set by local health departments.

Maintaining a balanced water chemistry in a hot tub is challenging for three reasons. Bathers introduce an organic load on the water chemistry, thereby neutralizing the

disinfectant within the water (Judd & Black, 2000; Kim *et al.*, 2002). The water in a hot tub may reach a maximum temperature of 40°C (ANSI/NSPI-2, 1999), and hot tubs are outfitted with hydrojets and air blowers to enhance the bathing experience. These devices also create aerosols that bathers breathe while soaking.

Hot tub users may be exposed to waterborne pathogens and are at risk of illness, including respiratory infections (Kahana *et al.*, 1997; Mangione *et al.*, 2001; Rickman *et al.*, 2002), Pontiac Fever (Fallon & Rowbotham, 1990; Fields *et al.*, 2001; Spitalny *et al.*, 1984a), gastrointestinal disease (Holmes *et al.*, 1989), urinary tract infections (Salmen *et al.*, 1983), or ear infections (Havelaar *et al.*, 1983), eye infections (Insler & Gore, 1986; Samples *et al.*, 1984) and skin infections (Gustafson *et al.*, 1983; McCausland & Cox, 1975; Spitalny *et al.*, 1984b). Folliculitis that occurs as a result of hot tub use, which accounts for the majority of hot tub illness related reports, are most often linked to *Pseudomonas aeruginosa*. More recent reports indicate the importance of nontuberculous *Mycobacteria* (Mangione *et al.*, 2001) infections in hot tubs. The presence of *Legionella* in hot tubs has been observed and associated with Pontiac Fever or Legionnaires's Disease (Thomas *et al.*, 1993).

The Centers for Disease Control and Prevention (CDC) tracks recreational water disease outbreaks and reports their findings in the "Surveillance for Waterbome-Disease Outbreaks, United States" (http://www.cdc.gov/mmwr/). Even though diseases associated with hot tub use are well-documented in the literature, many incidences acquired in private hot tubs are probably never reported, especially in the case of folliculitis, which is self-limiting and often does not require medical attention.

The risk of infection and disease can be reduced by the proper application of effective

hot tub disinfectants. To provide assurance that a disinfectant is effective, federal regulatory authorities in the US require that hot tub disinfectants pass both the laboratory suspension test AOAC Method for Water Disinfectants for Swimming Pools against each of *Escherichia coli* and *Streptococcus faecalis* (AOAC, 1990) and a field test (EPA, 1979). The AOAC method was developed to test the "germicidal activity" of swimming pool disinfectants (Ortenzio & Clark, 1964). When hot tubs became a part of the recreational water market, the swimming pool standards were applied even though hot tubs are different from swimming pools in many important ways. Hot tubs operate at higher temperatures, are more aerated due to the hydrojets and air blowers, have a greater surface area to volume ratio, serve more bathers per water volume under typical use, and more frequently recycle the water through filters. Based upon these differences, there has been a call for a new set of standards designed specifically for hot tubs (Crandall & MacKenzie, 1984; Kush & Hoadley, 1980). It is important to note that the currently accepted methods do not monitor or measure the extent of biofilm growth.

Biofilm bacteria may well be a significant source of microbial contamination in hot tubs (Price & Ahearn, 1988). Biofilms often harbor pathogens (Murga *et al.*, 2001; Primm *et al.*, 2004). Bacteria in biofilms are more tolerant to treatment than planktonic bacteria (Costerton *et al.*, 1999; Highsmith & Favero, 1985; Lewis, 2001). Bacteria in detached biofilm particles can be more pathogenic than individual planktonic bacteria; e.g., in causing a characteristic pulmonary injury in guinea pigs, 10 to 100 times more suspended cells of *Legionella pneumophila* were required than if the cells were contained in artificial microcolonies (Wright, 2000). For these reasons, there is a pressing need for a laboratory

hot tub disinfectant test against biofilm bacteria as well as planktonic bacteria (Favero, 1984; Hamilton, 2002).

This chapter presents a laboratory hot tub (LHT) system and an associated standard operating procedure (SOP) that together are suitable for assessing the efficacy of hot tub disinfectants against biofilm and planktonic bacteria. A series of experiments was conducted to evaluate the practicality, repeatability, sensitivity, and ruggedness of the LHT and SOP.

Materials and Methods

Laboratory Hot Tub (LHT)

The LHT was designed to represent the engineering, water chemistry and microbial components of a 1420 liter (375 gallon) hot tub built to accommodate four people. In a typical hot tub, the water passes through a skimmer which removes the larger particles, a filter which removes smaller particles, and a heat exchanger to maintain the temperature within a specified range. Then the water recycles back into the tub. The hot tub water must make this cycle every 30 minutes. ANSI/NSF recommends that surface-type filters for use in public hot tubs should receive a maximum loading of 1.5 ml min^{-1} cm^{-2} (ANSI/NSF 50, 2001).

The tub component of the LHT consisted of a one liter beaker, called a reactor, that possessed no spouts or pour lips. A one inch stir bar was placed in the bottom of the reactor (Figure 3.1a) for gentle mixing. Two reactors were placed on a submersible stir plate with digital rpm control in a constant temperature water bath maintained at 38°C (Figure 3.1d). One reactor was randomly selected to be the untreated control and the other reactor received

a treatment. The efficacy of the treatment was based on a comparison of the microbial accumulation (planktonic and biofilm) in the control reactor with the ostensibly lower accumulation in the treated reactor. By running the control and treated reactors simultaneously the comparison was not influenced by inherent, unknown, or uncontrolled factors that change from day to day and potentially affect microbial accumulation.

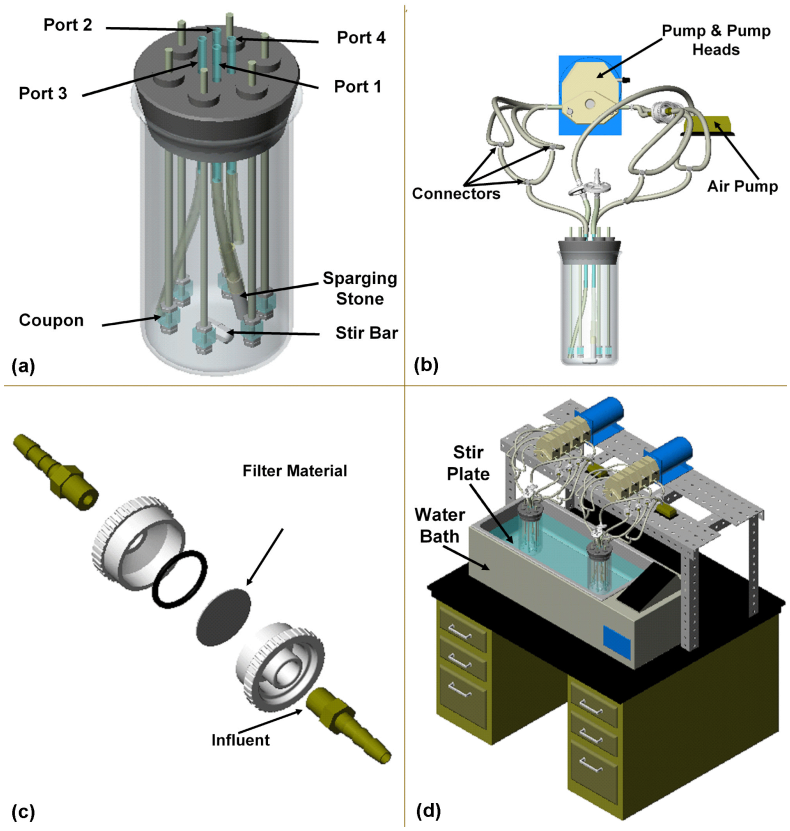

Figure 3.1 Schematics of the various components that comprise the laboratory hot tub system. Panel (a) illustrates the ports, coupons, sparging stone and stir bar. Panel (b) demonstrates how the laboratory reactor is plumbed. Panel (c) is a blown-up schematic of one filter. Panel (d) is an illustration of the complete system as it rests in a water bath on a stir plate.

Each reactor was capped with a #15 rubber stopper that contained six holes sized to fit #3 rubber stoppers and four barbed ports (Figure 3.1a). Each #3 stopper contained a

Teflon™ rod fitted with a 1cm^3 polycarbonate coupon for biofilm sampling. Although polycarbonate is not a typical construction material for hot tubs, previous research has shown that coupon construction material was not a critical design criteria for the laboratory evaluation of recreational water disinfectants (Goeres *et al.*, 2004).

Ports 1 and 2 were used for air exchange. A piece of tubing with an air sparging stone was connected to the bottom side of port 1 and an 0.2 µm air filter followed by an air pump was connected to the top side of port 1. The air pump was the same style that is used in household fish tanks and was included to simulate the hydrojets. A 0.2 µm air filter was connected to port 2 to provide an escape route for the sparged air.

Ports 3 and 4 were the effluent and influent for the filter recycling loop. A piece of tubing that extended to the bottom of the reactor was connected to the bottom of port 3. Three Y connectors were attached to the top of port 3 in such a way that four individual lines resulted (Figure 3.1b). A minimum amount of tubing was used for this configuration. Each of the four lines were then fed through a pump head. All four pump heads were driven from one pump. An on-line filter (Pall Gelman Laboratory; 25 mm in-line filter holder, Delrin) was attached to the end of the tubing that came out of the pump head. Each on-line filter contained a piece of commercial hot tub filter material (Unicel C-6430) cut into a circle with a 2.5 cm diameter where the effective filter diameter was 2.17 cm (Figure 3.1c). The total filter surface area was 14.8 cm^2. Only new commercial hot tub filter material that contained no chemical or biological residue was used. Following the filters, the Y connectors were reversed and hooked to the top of port 4 and a piece of tubing located just below the liquid/air interface was connected to the bottom of port 4. To achieve a filter loading rate

of 1.02 ml min^{-1} cm^{-2} and 30 minute residence time required an overall flow rate of 15.1 ml min^{-1} through the four filters and a reactor volume of 450 ml. Collectively, both reactors, tubing, filters, water bath and pumps were labeled "the LHT system."

The LHT Standard Operating Procedure (SOP)

Before the start of an experiment the reactors and associated tubing were cleaned. The system was assembled, minus the filters, a 1% bleach solution was added and the pumps were turned on for 30 minutes. After the bleach soak, 10 ml of a 78,750 mg l^{-1} sodium thiosulfate stock solution was added and the pumps turned on for another 30 minutes. The liquid was drained and all pieces thoroughly rinsed with tap water. A scrub brush and soap were used to remove any residual debris on the reactor pieces and to wash the filter holders. This process was followed by a thorough rinsing. If any reactor piece was visibly fouled, it was sonicated for 10 - 15 minutes for extra cleaning. Each piece was then allowed to air dry.

Twenty-four hours before the reactors were inoculated, they were filled with 450 ml of nano-pure water and sterilized by autoclaving. The filters were sterilized separately and the entire system assembled once the pieces cooled. The assembled system was placed in the 38°C water bath on top of the stir plate and the tubing was fed through the pump heads and the air pump was connected to the end of the filter attached to port 1. The stir plate was adjusted to rotate at 130 rpm.

The water chemistry in the laboratory hot tub was adjusted to fall within the ANSI/NSPI recommended guidelines for a public hot tub (ANSI/NSPI-2, 1999) which achieved a near neutral saturation index (SI). The calcium hardness and alkalinity and were

manipulated to equal 200 mg $CaCO_3$ l^{-1} and 120 mg $CaCO_3$ l^{-1}, respectively, in the bulk fluid with the addition of 1.35 ml of an autoclaved 31.74 g $MgCl_2$ l^{-1} and 73.99 g $CaCl_2$ l^{-1} solution and a 1.40 ml addition of a filter sterilized 56.03 g $NaHCO_3$ l^{-1} solution (AOAC, 1995). Using aseptic technique, the pH was adjusted to equal 7.2. At this point, one reactor was randomly designated as a treatment reactor and the other as the control reactor.

Chlorine, added as sodium hypochlorite, was used as the model disinfectant during SOP development, repeatability testing and ruggedness testing. For the sensitivity analysis both chlorine and bromine, added as a powder that contained active ingredients of sodium dichloro-s-triazinetrione and sodium bromide, were utilized.

Twenty-four hours prior to inoculating the reactors, disinfectant was added to the treated reactor to meet the demand the system itself placed on the disinfectant. For chlorine, the demand was generally met with 5 - 10 mg free available chlorine (FAC) l^{-1}. The demand was successfully met when a measurable disinfectant residual was found. At the end of 24 hours, the disinfectant concentration in the treated reactor was measured, and if necessary, the concentration was adjusted to the target value.

At time zero, each reactor was inoculated with a mixture that contained 0.1 ml of a 10^8 cfu ml^{-1} suspended culture of *Pseudomonas aeruginosa*, originally isolated from a hot tub, and 1.0 ml of a sterile solution that contained 9.0 g tryptic soy broth (TSB) l^{-1} and 3.6 g urea l^{-1}. This concentration of undefined carbon and urea is consistent with the ANSI/NSPI 50 test method (Annex H of ANSI/NSF 50, 2001).

Immediately, the air pump was started and both reactors sparged for 20 minutes. At the end of 20 minutes, the air pump was turned off and the disinfectant concentration was

measured in the treated reactor. At 24 hours post inoculation, a second disinfectant concentration was measured in the treated reactor; also, a bulk water, two coupons and four filter samples were collected from both the treated and control reactors and analyzed for viable bacteria. Bacterial samples were collected after 24 hours to allow time for injured cells to recover and biofilm to form. The hardness, alkalinity and pH were then measured.

Chemical Analysis

Alkalinity was measured with a Hach test kit (Cat. No. 24443-01) based upon the sulfuric acid titration method (APHA, 1995). pH measurements were read on a calibrated pH meter. Free available chlorine (FAC) was measured according to the DPD method (APHA, 1995) using a standard curve prepared with Hach free chlorine packets (Cat. No. 14077-99) and potassium permanganate. Bromine was measured according to the DPD method (APHA, 1995) using a standard curve prepared with Hach total chlorine packets (Cat. No. 14076-99). Calcium hardness was measured with a Hach test kit (Cat. No. 1457-01) based upon the EDTA titration method (APHA, 1995).

Microbial Methods

Bulk water samples were serially diluted in sterile buffered water. The first dilution tube for both the treated and control samples contained sodium thiosulfate to neutralize the chlorine or bromine. The samples were plated on R2A agar, incubated for 18 - 24 hours at 37+/- 2°C and enumerated. Coupon biofilm samples were collected by gently dipping a randomly selected coupon in sterile dilution water to remove any unattached cells, scraping the side of the coupon that faced the inside of the reactor into sterile buffered water that

contained sodium thiosulfate, sonicating for 2 minutes at 50/60 Hertz, serially diluting and plating on R2A agar. The plates were then incubated for 18 - 24 hours at 37+/- 2°C and enumerated. Filter biofilm samples were harvested by placing the entire filter into 10 ml sterile buffered water containing sodium thiosulfate. The sample was sonicated for 30 seconds and vortexed for 30 seconds for two cycles. Finally, the sample was serially diluted, plated on R2A, incubated for 18 - 24 hours at 37+/- 2°C and enumerated.

Experimental Design and Analysis

The purpose of the experimental design and analysis was to determine the statistical properties of repeatability, sensitivity and ruggedness for the LHT and associated SOP. For the microbial samples, log density, log reduction and the associated repeatability standard deviation were calculated according to the method described by Zelver *et al.* (2001).

A quantitative method is said to be "repeatable" if the quantitative response exhibits a small standard deviation when the method is repeated on multiple occasions. The mean log density and repeatability standard deviation were calculated for the control data. The outcome indicates the typical growth found in the reactors when no treatment was present. The repeatability of the log reduction values was assessed at each of the three chlorine concentrations. Each log reduction data point was the result of a paired treated and control experiment. In the repeatability study, there were three technicians who worked in two different laboratories, each located at the Center for Biofilm Engineering (CBE). Technician 1 completed six experiments, technician 2 completed 14 experiments and technician 3 completed 16 experiments for a total of 36 experiments. Repeatability was evaluated by

submitting the log density values to an analysis of variance (ANOVA) where laboratory, technician, experiment and samples were nested random effects factors.

A method is considered "sensitive" if it is able to distinguish between treatments of high and low efficacy. The sensitivity of the hot tub model was tested against three concentrations of free chlorine (1, 3 and 5 mg l^{-1}) and two concentrations of bromine (1 and 3 mg l^{-1}). A trend test of the slope of a least squares regression line was used to evaluate the sensitivity of the log reduction values to increasing chlorine concentration, where disinfectant concentration was the regression predictor and laboratory, technician and experiment were nested random effects factors. An ANOVA F-test was used to look for significant differences in the log reduction values associated with a chlorine versus bromine treatment. For this ANOVA, disinfectant was a fixed effects factor, and laboratory and experiment were nested random effects factors.

A method is considered "rugged" if slight changes to the SOP do not result in a significantly different quantitative response from what is predicted. Small departures from the SOP may well occur when the method is implemented in different laboratories, and ruggedness testing is a tool for predicting inter-laboratory variability. The SOP was altered to create the parameter settings listed in Table 3.1. The "Low" setting was chosen to discourage microbial growth and the "High" setting was chosen to encourage microbial growth. Each setting was tested 5 or 6 times. The disinfectant concentration was 3 mg FAC l^{-1} for each experiment. An ANOVA was used to calculate the standard error of each mean log density or log reduction value at the low and high settings. For this ANOVA, the parameter setting was a fixed effects factor; experiment and sample were sequentially nested

random effects factors. The difference between means, high setting minus low setting, was evaluated by conducting a two-sample t-test and finding the associated confidence limits.

Table 3.1 Ruggedness test parameter settings.

Settings	Mixing (rpm)	Water chemistry	Inoculum (log_{10} cfu ml^{-1})	Organic contribution (mg l^{-1})
Standard operating procedure (SOP)	130 1 in. stir bar	SI -0.06	7	20 TSB 8 urea
Low	200 1 in. stir bar	SI -0.70	6	10 TSB 4 urea
High	200 ½ in. stir bar	SI +0.70	8	40 TSB 16 urea

In the SOP, the treated reactor is conditioned for 24 hours with approximately 5 - 10 mg l^{-1} disinfectant to meet the demand the system placed upon the disinfectant. After 24 hours, the residual was measured and the disinfectant concentration adjusted to the target value. This 24 hour conditioning method was compared to an alternative method of conditioning the treated reactor just prior to inoculation. The reactor was considered conditioned when a stable disinfectant concentration was measured. Chlorine, at a final concentration of 3 mg FAC l^{-1}, was used for both approaches. Each conditioning method was tested in three or four independent experiments. The difference in mean log reductions was evaluated by conducting a two-sample t-test and finding the associated 90% confidence interval.

All statistical analyses were done using Minitab, Release 13 (Minitab, Inc. State College, PA).

Results

The statistics associated with the repeatability of the control data are presented in Table 3.2. The mean log density reported is the bacterial growth that occurred in the control reactor where no disinfectant was present. The repeatability standard deviation ranged from 0.31 for the suspended bacteria in the bulk water to 0.56 for biofilm bacteria harvested from the coupons. For the control data, the analysis of variance isolated the variability attributable to the sources: laboratory & technician-to-laboratory & technician, experiment-to-experiment and sample-to-sample. No consistent trend was found for the percent contribution each made to the repeatability standard deviation.

Table 3.2 Statistical summary of the log densities in the control reactors when operated according to the standard operating procedure.

	Mean	Repeatability standard deviation	Percentage contribution the total variance		
			Laboratory & technician	Experiments	Samples
Bulk water $\log_{10}$cfu ml^{-1}	7.2	0.31	40%	60%	NC[†]
Coupons $\log_{10}$cfu cm^{-2}	5.3	0.56	45%	34%	21%
Filters $\log_{10}$cfu cm^{-2}	6.6	0.50	28%	56%	16%

[†]Not Calculable because only one bulk water sample was collected for each experiment.

Figure 3.2 displays the variability of control log density values observed during an eight month period. There was no time trend for the bulk water ($p = 0.9$) and filters ($p = 0.2$). The coupon data, Figure 3.2b, had a statistically significant trend ($p < 0.001$), but it amounted to an increase of only 1 cfu cm^{-2} day^{-1} which is not of practical importance.

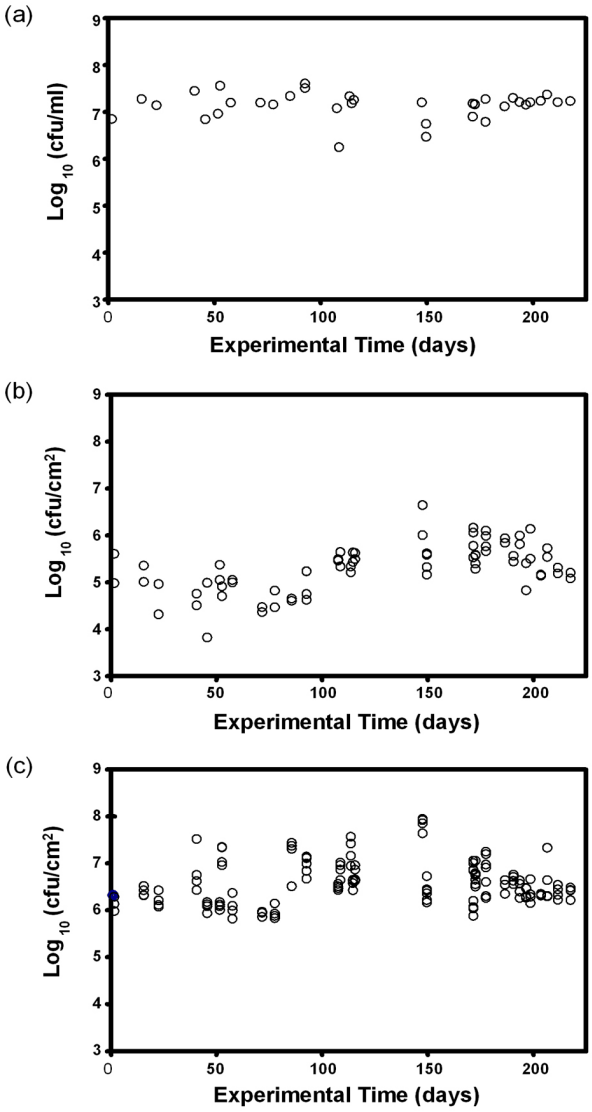

Figure 3.2 Time series plots of control bacterial counts illustrating the time trend associated with the method. Data is pooled for all technicians and both laboratories. Panel (a) contains the bulk water data, panel (b) the coupon data and panel (c) the filter data. Points aligned vertically are either samples collected from different experiments completed on the same day and/or multiple samples collected from the same experiment.

The mean, repeatability standard deviation and variance component results for the log reduction repeatability experiments are presented in Table 3.3. The experiments were conducted using 1, 3 and 5 mg l^{-1} free chlorine. The log reduction repeatability standard

deviation was always greatest at the lowest treatment concentration and lowest for the highest treatment concentration. For the log reduction data, the variance was attributed to variability from laboratory & technician-to-laboratory & technician or experiment-to-experiment. Once again, no consistent trend was found for the percent contribution either made to the log reduction repeatability standard deviation.

Table 3.3 Statistical summary of the log reduction values associated with each chlorine treatment when reactors operated according to the standard operating procedure. The numbers of independent experiments were n= 5, 7, and 4 for chlorine concentrations of 1, 3, and 5 mg FAC l^{-1}, respectively.

	Chlorine concentration (mg l^{-1})	Mean	Repeatability standard deviation	Percentage contribution to the total variance	
				Laboratory & technician	Experiment
Bulk water log_{10}cfu ml^{-1}	1	5.1	2.56	89%	11%
	3	6.5	0.67	10%	90%
	5	7.3	0.35	51%	49%
Coupons log_{10}cfu cm^{-2}	1	3.9	2.21	47%	53%
	3	4.8	0.55	44%	55%
	5	5.0	0.48	0	100%
Filters log_{10}cfu cm^{-2}	1	4.2	2.93	86%	14%
	3	5.5	1.24	61%	38%
	5	6.5	0.21	38%	62%

For the sensitivity analysis, regression trend tests showed that the log reduction increased with chlorine concentration for the bulk water ($p = 0.016$), coupons ($p = 0.09$) and filter ($p = 0.005$). These results indicate that the method can detect the effect of increased chlorine concentration.

Table 3.4 compares the mean log reductions resulting from a chlorine treatment and

a bromine treatment. Both treatments were tested at 1and 3 mg l^{-1}. The calculated p-values pertain to the null hypothesis that there was no difference between the chlorine and bromine treatments. In all cases tested, the chlorine resulted in the greater log reduction, indicating greater efficacy, although not all cases achieved statistical significance. For both the chlorine and bromine, the log reduction was always the greatest for the 3 mg l^{-1} treatment concentration.

Table 3.4 Comparison of log reduction values calculated for chlorine and bromine, each tested at two concentrations. The p-values pertain to the true difference in mean log reductions, chlorine minus bromine.

	Disinfectant concentration (mg l^{-1})	Mean for chlorine	Mean for bromine	2-tailed p- Value
Bulk water log_{10}cfu ml^{-1}	1	5.5	3.7	0.004
	3	6.4	5.0	0.08
Coupons log_{10}cfu cm^{-2}	1	4.4	2.6	0.06
	3	4.5	3.5	0.1
Filters log_{10}cfu cm^{-2}	1	4.8	2.3	0.007
	3	5.3	4.3	0.08

The ruggedness test results for the control data are presented in Table 3.5 and the log reduction results are presented in Table 3.6. The large p-values indicate that there was no statistically significant difference between the high and low settings for either control log densities or treated reactor log reductions. One can be 95% confident that the difference in true mean control log densities, high setting minus low setting, is less than 0.65 log cfu ml^{-1} for bulk water samples, less than 0.88 log cfu cm^{-2} for coupon samples, and less than 0.97 log cfu cm^{-2} for filter samples (Table 3.5). The difference in mean log reductions, high

setting minus low setting, could conceivably be positive or negative. The 95% confidence intervals in Table 3.6 show that the data do not discredit a difference between log reductions as large as -2.9 for coupons.

Table 3.5 Statistical summary of the bacterial log densities in the control reactors for each ruggedness test setting. The standard error of the mean (SEM) applies to both means in the row. The p-value and upper confidence limit pertain to the true log density mean difference, high setting minus low setting.

Sample	Mean for growth setting		SEM	2-tailed p- value	95% upper, one-sided confidence limit
	Low	High			
Bulk water $\log_{10}$cfu ml^{-1}	7.1	7.4	0.12	0.1	0.65
Coupons $\log_{10}$cfu cm^{-2}	5.5	5.9	0.19	0.2	0.88
Filters $\log_{10}$cfu cm^{-2}	6.7	7.0	0.24	0.4	0.97

Table 3.6 Statistical summary of log reductions due to a chlorine treatment of 3 mg l^{-1} as observed for each ruggedness test setting. The standard error of the mean (SEM) applies to both means in the row. The p-value and confidence interval pertain to the true mean log reduction difference, high setting minus low setting.

Sample	Mean for growth setting		SEM	2-tailed p- value	90% confidence interval
	Low	High			
Bulk water $\log_{10}$cfu ml^{-1}	7.1	6.3	0.48	0.3	-2.1, 0.4
Coupons $\log_{10}$cfu cm^{-2}	5.3	4.2	0.68	0.3	-2.9, 0.7
Filters $\log_{10}$cfu cm^{-2}	6.4	6.4	0.43	0.96	-1.1, 1.1

The results for the reactor conditioning techniques are presented in Table 3.7. The large p-values indicate that the data do not discredit the null hypothesis that there is no

difference between the true mean log reductions for the two conditioning techniques. The associated confidence intervals for the true differences show that the observed data do not discredit a true difference as large as 2.0 for filter samples.

Table 3.7 Statistical summary of log reductions for the two chlorine conditioning methods when the reactor is treated with 3 mg FAC l^{-1}. The standard error of the mean (SEM) applies to each of the three means in the row. The p-values and confidence intervals pertain to the true mean difference between log reductions for the two conditioning methods, "Reactor conditioned for 24 hours" minus "No conditioning".

Sample	Mean for each conditioning method		SEM	2-tailed p- Value	90% confidence interval
	No conditioning (chlorine added just prior to time = 0)	Reactor conditioned for 24 hours to achieve a measurable residual			
Bulk water log_{10}cfu ml^{-1}	6.6	6.6	0.35	0.9	-1.0, 0.9
Coupons log_{10}cfu cm^{-2}	5.1	4.9	0.39	0.7	-1.3, 0.8
Filters log_{10}cfu cm^{-2}	6.0	6.8	0.44	0.3	-0.4, 2.0

Discussion

A statistical evaluation of the laboratory hot tub model and SOP demonstrated that the method was repeatable (for effective treatments), sensitive, and rugged. These statistical characteristics are the hallmark of a well-designed standard laboratory method (Hamilton, 2002). The control log densities exhibited an acceptable repeatability standard deviation (SD) indicating that this system creates relatively stable planktonic and sessile bacterial populations. No consistent trend was found when the SD was partitioned into the different sources of variability. This suggests that no single factor is the predominate source of

variability and therefore there is no obvious focal point for improving the system. The statistically significant time trend associated with the coupon control data may be attributed to technician experience. The coupons required the most difficult manipulations, especially for harvesting and disaggregating the biofilm. As technicians become more proficient at those skills, it is plausible that the number of recovered biofilm bacteria would correspondingly increase. But, because the rate of increase is so small and not of practical importance, there is no need to alter either the method or technician training.

The repeatability standard deviations for log reduction values were consistent with other disinfection tests (Tilt & Hamilton, 1999), except at the marginal treatment of 1 mg FAC l^{-1} where the repeatability SDs were 2.6, 2.2, and 2.9 for bulk water, coupon, and filter samples, respectively. That the repeatability SD is higher for the marginal treatment than for the effective treatment is consistent with repeatability results for other disinfection tests (Tilt & Hamilton, 1999). An effective treatment consistently kills all bacteria so that the variability in the log reduction is mostly due to the control data. An ineffective treatment is essentially the same as the control so the SD is similar to that for control data. The ineffective and very effective treatments represent points on opposite ends of a concentration response curve, where the slope of the curve is not changing dramatically. In contrast, marginal treatments correspond to points on the steepest part of the curve where small changes in the system produce big changes in efficacy. For this reason, antimicrobial tests conventionally exhibit less repeatability for marginal treatments than for strong or ineffective treatments.

The results imply that more replicate tests are required for a marginal treatment to

average out the inherent variability and arrive at a precise estimate of efficacy. For example, the results of this investigation indicate that 16 tests of a marginal treatment such as 1 mg FAC l^{-1} are required to produce as precise a log reduction on coupons as would a single test of an effective treatment such as 3 to 5 mg FAC l^{-1}. In spite of the low repeatability for marginal treatments, the regression trend test demonstrated that the laboratory hot tub method was able to distinguish between treatments of high and low efficacy.

Chlorine was chosen as the model disinfectant because of its historical presence in the recreational water industry in both the AOAC Method for Water Disinfectants for Swimming Pools (AOAC, 1990) and DIS/TSS-12 (EPA, 1979). Bromine was chosen as the comparison disinfectant because of its accepted use as a disinfectant in recreational water. For chlorine, the analysis showed a more significant trend for the bulk water and filter results than for the coupon results. The method was also able to show that chlorine had a greater efficacy than an equivalent concentration of bromine. This result is consistent with what other researchers have found (Koski *et al.*, 1966; Shaw, 1984).

Ruggedness testing of conditioning strategies, which specified how the chlorine demand was met, yielded interesting results. Although the log reduction was not greatly affected by whether the demand was met 24 hours previous to inoculating the system or just prior to inoculation, in practice, the first approach was much easier to implement. The ability of the disinfectant to meet the demand both ways may also depend on the type of disinfectant used, so it is an issue that should be examined for each new disinfectant.

The implementation of the LHT model has so far focused on prevention of bacterial contamination in a clean system, although the model is flexible in its use. For instance, the

LHT model has been used to test the effectiveness of treatments administered to kill bacteria and/or kill and remove biofilm from a fouled system. The SOP has been modified to test the efficacy of chlorine against *Escherichia coli* and *Mycobacterium fortuitum* (data not reported), and with a few modifications, a researcher could use the apparatus to measure the disinfection by-products that are generated when organics and chlorine mix, with and without aeration.

It is important to include biofilm contamination as a response variable in both the laboratory and field hot tub tests. Biofilms can and do form in hot tubs, and once established are extremely difficult to remove. To ensure quality and safe water, future chemistries must address the control, kill, and removal of biofilms from hot tubs. The LHT model was designed to mimic actual hot tub design and use conditions and it should provide a indication of how well a treatment is going to work in the field. It is recommended that the laboratory hot tub model be used as a second tier in a three tiered process, in which the first tier is the AOAC test and the final tier is the currently required field test, adapted to include biofilm measurements.

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CHAPTER 4

EVALUATION OF DISINFECTANT EFFICACY AGAINST BIOFILM AND SUSPENDED BACTERIA IN A LABORATORY SWIMMING POOL MODEL

Introduction

Research on bacteria in swimming pools dates back to the 1920's (Mallmann, 1928). In the following decades, researchers compared the efficacy of various disinfectants in both field and laboratory studies (Black *et al.*, 1970; Brown *et al.*, 1964; Gardiner, 1973; Mood, 1950; Powick, 1989). The efficacy studies focused upon maintaining the quality of bulk water and conclusions were based upon the difference in the number of suspended bacteria that survived one treatment as compared to another. Even though researchers noted that "slime protected" bacteria were more resistant to specific treatments (Seyfried & Fraser, 1980; Storey, 1989; Victorin 1974), no researchers published results from a laboratory efficacy study specifically directed towards the evaluation of swimming pool disinfectants against bacteria existing as biofilm. In addition to the efficacy research, some researchers focused upon evaluating the general hygiene of swimming pools. In some later studies, researches began collecting swab samples from the swimming pool edges (Leoni *et al.*, 1999)

in addition to the bulk water measurements.

Prior to the 1990's, the typical laboratory evaluations of swimming pool disinfectants were conducted in flasks that incorporated neither the engineering components of a swimming pool system nor the water chemistry (Fitzgerald & DerVartanian, 1967;Fitzgerald & DerVartanian, 1969). Systems became more relevant when in 1990 Yahya *et al.* reported the bulk fluid efficacy results from a meso-scale system operated with representative microbial and nutrient loading (Yahya *et al.*, 1990). Judd and Black utilized a meso-scale swimming pool system that included the addition of a body fluid analog to evaluate disinfectant by-product formation (Judd & Black, 2000). Borgmann-Strahsen included relevant water chemistry and bacteria in her efficacy studies (Borgmann-Strahsen, 2003).

This paper describes results obtained from applying six chemical treatments in a laboratory reactor system designed to model a swimming pool. The reactor model incorporated engineering parameters such as turn-overs per day, filter loading and relevant construction materials for the evaluation of biofilm growth. The water chemistry in the reactor was maintained within recommended guidelines for the operation of swimming pools. To mimic bathing events, the bulk water was simultaneously inoculated with a mixed bacterial consortium and bather insult, formulated to reproduce the major components of perspiration and urine. Once the system was designed, built and tested, the efficacy of oxidizing and nonoxidizing treatments were evaluated against the accumulation of biofilm and suspended microorganisms.

Materials and Methods

Reactor System Design

The reactor vessel was a 10 liter plastic vessel with an overflow valve at the 6.6 liter water mark (Figure 4.1). The system operated open to the atmosphere. The reactor housed two rotating disks, labeled rotor 1 and rotor 2 from left to right, each containing four coupons made from cement, vinyl, polyvinyl chloride (PVC) or polycarbonate. The diameters of the coupons were: PVC = 1.24 cm, polycarbonate = 1.27 cm, vinyl = 1.21 cm, and cement = 1.25 cm. The reactor sat on two stir plates which slowly spun the disks to create slight water turbulence and mixing. Rubber sheeting (2.8 cm x 10.2 cm) that held the four coupons was velcroed in the four corners. Starting in the upper right corner, the rubber sheeting was labeled dead space 1 - 4 in a counter-clockwise direction. Coupons were placed so that the coupon surface was flush with the surface of the rubber sheeting. The reactor was designed to have a recycle flow rate of 18.3 ml min^{-1} through a sand filter with a surface area of 1.5 cm^2, a filter loading rate of 12.2 ml min^{-1} cm^{-2} and a residence time of 6 hours (four turn overs per day). The filter was constructed from a glass tube filled with #50 - #70 mesh silica sand, typical of swimming pool filters. The sand was held in place by rubber o-rings attached to a stainless steel screen. Liquid entered and exited the filter through glass tubing inserted into rubber stoppers. Prior to each experiment, new sand was baked at 400^{o}C for four hours to remove organic carbon.

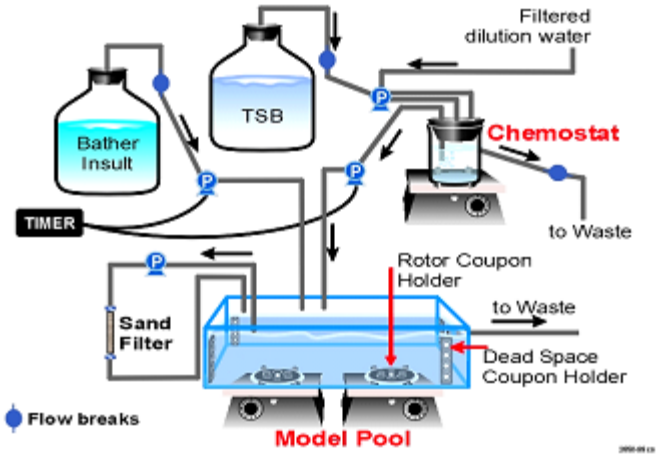

Figure 4.1 Illustration of the laboratory swimming pool model.

The reactor was inoculated from a growth chemostat three times per day, five days a week via a pump connected to a timer. The target inoculum of 5×10^7 viable cells per day was equivalent to 20 bathers 26,500 liters^{-1} day^{-1} (Sandel, 1990). Typically the growth chemostat had a steady state cell concentration of 3.3×10^6 cfu ml^{-1} and a total of 15 ml was added each day. The actual volume of inoculum added to the reactor was adjusted according to the concentration of cells in the chemostat. The chemostat was inoculated from a frozen batch culture which was prepared from a mixed consortium of bacteria obtained originally from an industrial test swimming pool. The consortium was not typed during these experiments. The chemostat was fed a final concentration of 15 mg l^{-1} Tryptic Soy Broth (TSB), had an 8.3 hour residence time and contained an air sparging stone.

Bather insult was added every time the reactor was inoculated. As with the microbial inoculum, an equivalent of 20 bathers 26,500 liters^{-1} day^{-1} or a total volume of 15 ml was added to the reactor per day. One liter of insult included: 62.6 mg urea, 9.7 mg albumin, 4.3 mg creatinine, 3.3 mg lactic acid, 1.5 mg uric acid, 1.2 mg glucuronic acid, 22.1 mg sodium

chloride, 35.3 mg sodium sulfate, 7.0 mg ammonium chloride, 6.7 mg sodium bicarbonate, 11.4 mg potassium phosphate and 10.1 mg potassium sulfate, providing the major components of perspiration and urine (Sandel, 1990).

Reactor Water Chemistry

The water chemistry was adjusted daily to fall within the following American National Standards Institute/National Spa & Pool Institute (ANSI/NSPI) recommended guidelines: pH 7.2 - 7.6, alkalinity 60 - 120 mg l^{-1} and total hardness 200 - 400 mg l^{-1} (ANSI/NSPI, 1991). Water hardness was achieved from the addition of calcium chloride. The initial alkalinity concentration was achieved from the addition of sodium carbonate. Sodium bisulfate lowered the pH, sodium bicarbonate was used to raise the alkalinity.

Analytical Methods

Alkalinity was determined according to standard method 2320 B (APHA, 1995). pH Measurements were read on a calibrated pH meter. Polyhexamethylene biguanide (PHMB) was measured according to a modification of the Dawson and Brown method (Dawson *et al.*, 1983). Total and Free Chlorine were determined according to standard method 4500-Cl G (APHA, 1995). Total Hardness was determined according to standard method 2340 C (APHA, 1995). Hydrogen peroxide concentrations were determined via the thiosulfate titration method. Total Organic Carbon (TOC) was determined as nonpurgeable organic carbon (NPOC) using an infrared carbon analyzer calibrated against a standard stock solution of sucrose.

Microbial Methods

Bulk Fluid Samples-Heterotrophic Bacteria. Samples containing chlorine or potassium monopersulfate were neutralized with sodium thiosulfate. Samples containing PHMB were neutralized with Association of Analytical Communities (AOAC) Azolectin-Tween. Chemostat bulk fluid samples were not neutralized. Samples were serially diluted, spread plated on duplicate R2A medium plates and incubated for 7 days at room temperature (21° - 23°C). Results are reported in ranges set to equal less than the level of detection (LOD), greater than the LOD and less than 200 cfu ml^{-1} and greater than or equal to 200 cfu ml^{-1}. Ranges were chosen based upon United States regulatory criteria of fewer than 200 cfu ml^{-1} of heterotrophic bacteria in swimming pool water (US EPA, 1979), with the notable difference in incubation time and temperature. The LOD was 5 cfu ml^{-1}.

Coupon Biofilm Samples-Heterotrophic Bacteria. Biofilm on a coupon surface (areas defined previously) was scraped into sterile buffered dilution water containing the appropriate neutralizer then homogenized at 20,500 rpm for 30 seconds using a Tekmar-Dohrmann tissue homogenizer (Zelver *et al.*, 1999). Samples were serially diluted, spread plated in duplicate and incubated at room temperature (21° - 23°C) for seven days. Results are reported in as $\log_{10}$cfu cm^{-2}.

Filter Sampling-Heterotrophic Bacteria. The filter was divided into three sections: top, middle, and bottom. Each section was then further broken down into three 5-cm pieces for a total of nine samples. Excess fluid was drained just prior to sampling. Samples were collected using a sterile spatula. Each sample was placed into a dilution tube that contained sterile buffered water. The spatula was rinsed with the appropriate neutralizer into the

dilution tube. The samples were vortexed for 30 seconds and sonicated for 30 seconds, twice, in a bath containing ice and water. Each dilution was serially diluted, spread plated in duplicate and incubated at room temperature (21° - 23°C) for seven days. The sand from each tube was dried in a 60°C oven then weighed on an analytical balance. Results are reported as $\log_{10}$cfu g^{-1} of dried sand.

Experimental Protocol

Operating Procedure. Each experiment was conducted at room temperature (21° - 23°C). Disinfectant and pH levels were tested three times per day, while the remaining water chemistry parameters and the bulk fluid heterotrophic bacterial density were tested daily. The alkalinity, hardness and pH was adjusted to measure within the ranges specified above when necessary. The heterotrophic bacterial density in the chemostat was tracked daily. Each experiment lasted for one month, unless otherwise described. Coupons in rotor 1 and in dead space 1 and 3 were sampled on day 14 and the remaining coupons and filter were sampled on day 30, unless otherwise described. In total, one control and six treatments were tested, described as follows:

Untreated Control. System failure due to excessive plugging of the tubing resulted in the experiment lasting two weeks. Coupons and filter were sampled on day 14.

1 - 3 mg l^{-1} Free Chlorine Control. Free chlorine was maintained between 1 - 3 mg l^{-1} in the bulk fluid.

64 Hour Super-chlorination Experiment. Free chlorine concentration was maintained between 1 - 3 mg l^{-1}. Every Friday, the system was dosed with 10 mg l^{-1} free chlorine. On

Monday, the chlorine concentration was measured then increased with additional chlorine or lowered with sodium thiosulfate. The experiment was repeated twice.

16 Hour Super-chlorination Experiment. Free chlorine concentration was maintained between 1 - 3 mg l^{-1}. Every Wednesday, the system was dosed with 10 mg l^{-1} free chlorine. On Thursday, the chlorine concentration was measured and adjusted as described previously. The experiment was repeated twice.

10 mg l^{-1} PHMB Control. The polyhexamethylene biguanide (PHMB) concentration was maintained at 10 mg l^{-1}.

10 mg l^{-1} PHMB & Quaternary Biocide (quat) Experiment. PHMB concentration was maintained at 10 mg l^{-1} in the bulk fluid. Once a week, 0.15 µl of a 49.8% n-Alkyl dimethyl benzyl ammonium chloride solution was added to the 6.6 liter reactor. The filter was sampled on day 14. A new, sterile filter was put on-line for the remaining 14 days. At the end of 30 days, the remaining coupons and second filter were sampled. The experiment was repeated twice, but the second experiment only included the day 14 coupon sampling.

10 mg l^{-1} PHMB & Quaternary Biocide & Hydrogen Peroxide Experiment. Experiment lasted for two months to accommodate two doses of hydrogen peroxide. PHMB concentration was maintained at 10 mg l^{-1}. Once a week, 0.15 µl of a 49.8% n-Alkyl dimethyl benzyl ammonium chloride solution was added and once a month the system was shocked with 27 mg l^{-1} hydrogen peroxide. Coupons in rotor 1 were sampled on day 30 then replaced with a sterile rotor and coupons. The filter and coupons in rotor 1 and 2 and dead space 2 and 4 were sampled on day 60.

Statistical Methods

For evaluating the system, the log density values were submitted to an analysis of variance where the coupon surface material was a fixed effects factor and the coupon location was a random effects factor (Neter *et al.*, 1996). The reported p-values are for the analysis of variance F-tests. For the percentage of bulk water samples that exceed regulatory limits, the confidence intervals were calculated by the exact (Clopper-Pearson) procedure in Minitab (Release 13 for Windows; http://www.minitab.com). For the coupons and filters, the log reduction was calculated by subtracting the treated mean log density from the control mean log density. We report the mean and standard error of the log reduction values across independent repeats of the experiment. The standard error of the log reduction was calculated as described in Zelver *et al.* (Zelver *et al.*, 2001).

Results

System Evaluation

The effects of coupon construction material and placement were evaluated in the model laboratory swimming pool for the treatment experiments. The results from the super-chlorination experiments were right at the LOD, so this data was not considered in the analysis. The mean log densities for the four different coupon surface materials ranged over 0.2 logs (p-value= 0.6). The mean log densities across coupon placement positions ranged over 1.0 logs, (p-value < 0.001).

Bulk Water

Table 4.1 presents the distribution of heterotrophic bacterial densities for each treatment. Samples containing 200 cfu ml^{-1} exceeded the accepted minimum level for heterotrophic bacteria in recreational water. The 95% confidence intervals for samples that exceeded the standard are listed in parenthesis. The number listed in the No. of Samples column is the combined total samples for all replicates. As expected, the untreated control resulted in 100% of the samples out of compliance. The chlorine treatment resulted in the smallest percentage of incidences that exceeded the minimum acceptable level.

Table 4.1 Bulk water: distribution of heterotrophic bacterial densities for each experiment.

Experiment	<LOD[†] (%)	>LOD and <200 (%)	≥200 (%) (± 95% CI [‡])	No. of Samples
Untreated control[*]	0	0	100 (74, 100)	10
Chlorine	72	28	0 (0, 15)	18
64h Super-chlorination	56	28	15 (5, 30)	39
16h Super-chlorination	59	28	14 (4, 31)	29
PHMB	7	27	67 (38, 88)	15
PHMB/n-alkyl dimethyl benzyl ammonium chloride (quat)	17	52	31 (15, 50)	29
PHMB/quat/hydrogen peroxide[**]	3	31	66 (54, 77)	77

[†]LOD = less than the level of detection
[‡]CI = confidence interval
[*]Two week experiment
[**]Two month experiment

The mean total organic carbon (TOC) concentrations, associated standard error of the mean and ranges are presented in Table 4.2. For the untreated control, chlorine and 16 hour super-chlorination experiments, the TOC values held constant in the ranges listed (Figure 4.2). The TOC concentration for the 64 hour super-chlorination and the PHMB/quat

treatments held constant with the exception of two samples for the 64 hour super-chlorination and one sample for the PHMB/quat treatment. The TOC concentration measured during the PHMB/quat/H_2O_2 experiment was generally higher than the other treatments.

Table 4.2 Bulk water: range and mean TOC concentration ± standard error (SE) for each treatment.

Experiment	Range (mg l^{-1})	Mean (mg l^{-1}) ± SE	No. of Samples
Untreated control†	1.34 - 5.65	3.47 ± 0.46	10
Chlorine	1.30 - 6.27	3.54 ± 0.29	20
64h Super-chlorination	2.28 - 23.75	7.58 ± 0.72	29
16h Super-chlorination	3.46 - 9.08	6.47 ± 0.21	20
PHMB	9.20 - 11.98	10.32 ± 0.20	17
PHMB/n-alkyl dimethyl benzyl ammonium chloride (quat)	8.45 - 34.90	11.83 ± 0.57	21
PHMB/quat/hydrogen peroxide ‡	15.03 - 130.16	25.49 ± 2.53	56

†Two week experiment
‡Two month experiment

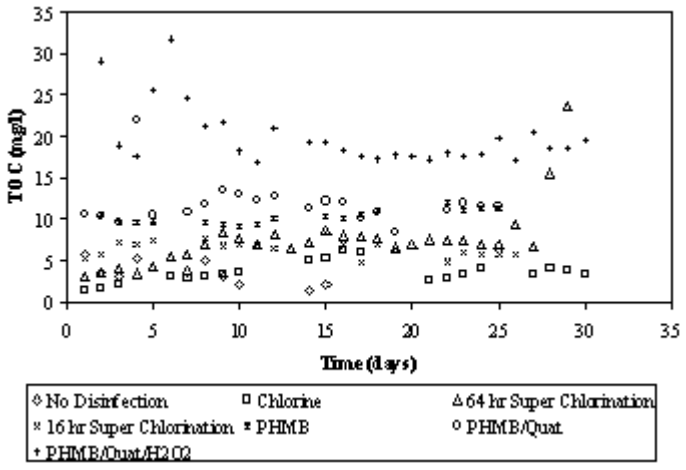

Figure 4.2 TOC concentrations measured during the seven experiments.

Coupons

The log reduction in biofilm density on the coupons and associated standard error for each treatment are presented in Table 4.3. The mean bacterial density for the untreated control was 6.10 $\log_{10}$cfu cm^{-2}. All treatments tested resulted in at least a four log reduction in biofilm density, although the super-chlorination treatments achieved the greatest log reduction, approximately equal to 6 logs.

Table 4.3 Coupons: log reduction (LR) and associated standard error (SE) for each treatment.

Experiment	Accumulation $\log_{10}$(cfu cm^{-2})	Log reduction $\log_{10}$(cfu cm^{-2})	SE of the LR
Chlorine	0.57	5.53	0.46
64h Super-chlorination	0.03	6.07	0.37
16h Super-chlorination	0.03	6.06	0.37
PHMB	1.39	4.70	0.43
PHMB/n-alkyl dimethyl benzyl ammonium chloride (quat)	1.29	4.81	0.57
PHMB/quat/hydrogen peroxide[†]	1.06	5.04	0.46

[†]Two month experiment

Filter

The log reduction in filter biofilm and associated standard error for each treatment are presented in Table 4.4. The mean bacterial density for the untreated control was 9.04 $\log_{10}$ cfu g^{-1}. The two super-chlorination treatments resulted in the greatest log reduction, approximately 8.9. The chlorine treatment resulted in a 6.7 log reduction. The log reductions associated with the nonoxidizing treatments increased with each additional treatment chemical.

Table 4.4 Filters: log reduction (LR) and associated standard error (SE) for each treatment.

Experiment	Accumulation $\log_{10}$(cfu g^{-1})	Log reduction $\log_{10}$(cfu g^{-1})	SE of the LR
Chlorine	2.34	6.70	0.67
64h Super-chlorination	0.10	8.94	0.12
16h Super-chlorination	0.12	8.92	0.14
PHMB	4.36	4.68	0.26
PHMB/n-alkyl dimethyl benzyl ammonium chloride (quat)	1.92	7.12	0.52
PHMB/quat/hydrogen peroxide[†]	1.23	7.81	0.33

[†]Two month experiment

Discussion

These experiments demonstrated the ability of biofilm to form in a laboratory simulated swimming pool even when the water chemistry and disinfectant were maintained within the recommended ranges for operation. Early swimming pool research supports this finding. In 1974, Victorin observed bacteria surviving water purification by existing in clusters surrounded by a protective coat (Victorin, 1974). Seyfried and Fraser reported in 1980 that a slime layer enabled *Pseudomonas aeruginosa* to survive in a chlorinated swimming pool (Seyfried & Fraser, 1980). Storey stated that once a *Pseudomonas* biofilm was established on a swimming pool surface, nothing short of physical removal would eliminate it (Storey, 1989). These findings were particularly important from the standpoint of public safety because biofilm may be a potential reservoir for pathogenic bacteria (Leoni *et al.*, 1999; Leoni *et al.*, 2001).

In the laboratory model, coupon placement resulted in a 1 log variation in mean

biofilm density. In an actual swimming pool, biofilm accumulation may vary at the air/water interface and at a swimming pool's overflow drain where a surface is intermittently covered by a thin film of water. The practical significance of the laboratory result is that multiple samples are required to understand biofilm accumulation in a field swimming pool. This study showed no statistical difference in mean biofilm density for the polycarbonate, PVC, vinyl and cement coupons. Therefore, surface material was not an important factor in this system. On a practical level, however, some surfaces are easier to maintain in a field swimming pool.

Of the treatments tested, super-chlorination was the most effective against biofilm accumulation on coupons and in the filter. Super-chlorination resulted in a mean biofilm accumulation less than or right at the level of detection on the coupons and 0.1 $\log_{10}$ cfu g^{-1} on the filters. This work also demonstrated that a 16 hour, or overnight super-chlorination was as effective as a 64 hour, or weekend super-chlorination. In an actual swimming pool, applying a 16 hour treatment is much more feasible. The addition of quat/H_2O_2 to PHMB improved the nonoxidizing disinfectant's performance against biofilm accumulation in the filter and on the coupon surfaces. The ability of shock treatments to increase the log reduction of biofilm was important to understand because the biofilm did accumulate when the laboratory model was maintained with 1- 3 mg l^{-1} free chlorine and 10 mg l^{-1} PHMB. Shock treatments may provide one means of controlling biofilms in swimming pools.

A critical percentage of samples contained bulk water bacteria out of compliance for the PHMB/quat/H_2O_2 treatment. The percentage of super-chlorination bulk water counts out of compliance was borderline. Only the chlorine treatment was not higher than the 200 cfu

ml^{-1} requirement. A large log reduction in mean biofilm density on the coupons (Table 4.3) and in the filter (Table 4.4) combined with a variable response in TOC data suggested that biofilm detachment evolved, as a result of the shock treatments, contributing to the high bulk water microbial counts. More research is required to understand the correlation between shock treatment and biofilm detachment. Swimming pool operators applying one of these treatments may want to consider the importance of disinfecting detached biofilm before the swimming pool is used.

Conclusions

In this research, a laboratory reactor was created that modeled important design parameters, water chemistry guidelines and microbial loading equivalent to a full scale swimming pool. Experiments conducted using this model led to the following conclusions:

1. To understand the microbial dynamics that existed in a laboratory scale swimming pool, it was important to assess biofilm activity in the filter and on surfaces in addition to bulk water samples.
2. Shock treatments were effective at minimizing biofilm accumulation in filters and on coupons, although care must be given to disinfect the detached biofilm.
3. Biofilm accumulation was found in the filter and on coupon surfaces in reactors consistently maintained with a residual free chlorine level of 1 - 3 mg l^{-1} and 10 mg l^{-1} PHMB.
4. In this system, a 16 hour super-chlorination was as effective as a 64 hour super-chlorination against biofilm accumulation in the filter and on coupon surfaces.

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CHAPTER 5

CONCLUSIONS

This dissertation describes three laboratory reactors and associated standard operating procedures designed to provide a reliable assessment of disinfectant efficacy against biofilm bacteria grown in a moderate to high fluid shear environment, in a laboratory hot tub reactor, and in a laboratory swimming pool reactor.

Chapter 2 describes the CDC biofilm reactor (CBR), a laboratory reactor system engineered to grow biofilm under moderate to high fluid shear stress. The reactor incorporates 24 removable biofilm growth surfaces (coupons) for purposes of sampling and analyzing the biofilm. Following preliminary experiments to verify the utility of the CBR system for growing biofilms of several clinically relevant organisms, a standard operating procedure (SOP) for growing a *Pseudomonas aeruginosa* biofilm was created. A rigorous, intra-laboratory, statistical evaluation was then conducted to determine the repeatability and ruggedness of the reactor and SOP. For the statistical evaluations, the outcome of interest was the density (cfu per cm^2) of viable *Pseudomonas aeruginosa*. The repeatability of the log density was based upon the results of replicate experiments. The mean *P. aeruginosa* $\log_{10}$ density was 7.1, independent of the coupon position within the reactor. The repeatability standard deviation of the log density based on one coupon per experiment was 0.59. Analysis of variance showed that the variability of the log density was 53% attributable to within-experiment sources and 47% attributable to between-experiments sources.

The ruggedness evaluation applied response surface design and regression analysis techniques, similar to those often used for performing sensitivity analyses in other fields of science and engineering. This approach provided a quantitative description of ruggedness; specifically, the amount the log density was altered by small adjustments to four key operational factors – time allowed for initial surface colonization, temperature, nutrient concentration, and fluid shear stress on the biofilm. The small size of the regression coefficient associated with each operational factor showed that the method was rugged; that is, insensitive to minor perturbations of the four factors. These results demonstrate that the CBR system is a reliable experimental tool for growing a standard biofilm under moderate to high fluid shear.

Chapter 3 describes a laboratory hot tub model and associated SOP designed to reproduce the key biological, chemical and engineering parameters associated with recreational hot tubs. Disinfectant efficacy, as measured quantitatively by log reduction values, was determined against both biofilm and planktonic bacteria. When the laboratory model hot tub was run according to the SOP, with no antimicrobial treatment, a consistent level of bacterial contamination occurred. The mean log densities ($\pm$ repeatability standard deviation) were 7.2 $\log_{10}$ cfu ml^{-1} ($\pm$ 0.31) for the bulk water, 5.3 $\log_{10}$ cfu cm^{-2} ($\pm$ 0.56) for the coupons and 6.6 $\log_{10}$ cfu cm^{-2} ($\pm$ 0.50) for the filters. Concentration versus response trend tests showed that the log reduction increased with chlorine concentration for samples of planktonic bacteria in the bulk water ($p = 0.016$), biofilm bacteria on the coupons ($p = 0.09$) and biofilm bacteria on the filter ($p = 0.005$), indicating that the method was responsive to chlorine concentration. The method was also able to differentiate between chlorine and

bromine treatments. In every case, chlorine produced a greater log reduction than did the same concentration of bromine. The model and SOP were ruggedness tested with respect to slight changes in fluid shear, water chemistry, inoculum density, organic loading and alternative strategies for meeting chlorine demand. The results showed that the system was rugged relative to these factors. The laboratory hot tub model is suitable for use as the second tier in a three tiered process for determining the efficacy of disinfectants for use in hot tubs, in which the first tier is a simple screening test and the final tier is a field test.

Chapter 4 describes the design and operation of a laboratory reactor system engineered to model a swimming pool. The model included relevant engineering parameters such as filter loading and turn-overs per day. The water chemistry in the system's bulk water was balanced according to standard recommendations and the system was challenged with a bacterial load and synthetic bather insult, formulated to represent urine and perspiration. The laboratory model was then used to evaluate the efficacy of six chemical treatments against biofilm and planktonic bacteria. Results showed that the biofilm was able to accumulate on coupons and in the filter systems of reactors treated with either 1 - 3 mg l^{-1} free chlorine or 10 mg l^{-1} polyhexamethylene biguanide (PHMB). All the treatments tested resulted in at least a 4 log reduction in biofilm density when compared to the control, but shock treatments were the most effective at controlling biofilm accumulation. A once weekly shock dose of 10 mg l^{-1} free chlorine resulted in the greatest log reduction in biofilm density. This research demonstrated the importance of incorporating relevant engineering design into the test methods used to evaluate the efficacy of swimming pool disinfectants against biofilm and planktonic bacteria.

Table 5.1 summarizes how the reactors and associated standard operating procedures developed for this dissertation met the required statistical characteristics defined in Table 1.1 (Chapter 1) plus the desired qualitative characteristics of relevance and reasonable.

Table 5.1 A summary of how well the CBR, hot tub model, and swimming pool model met the statistical characteristics necessary for standardization. xxx indicates the criteria were completely met, xx indicates the criteria were met, and x indicates the criteria were partially met.

	CBR	Hot tub model	Swimming pool model
Repeatable	xxx	xx	x
Rugged	xxx	xxx	not determined
Responsive	not determined	xx	xxx
Reproducible	not determined	not determined	not determined
Relevant	xx	xxx	xxx
Reasonable	xxx	xx	x

Of the three methods developed, the CBR best satisfies the statistical characteristics of repeatability and ruggedness required of a standard method. The CBR is the least relevant of the three methods, although less relevant, more defined methods are usually more repeatable. The CBR protocol is also reasonable. An experiment lasts only one week, and besides the reactor, digital stir plate and pump, the SOP uses supplies that are commonly found in a microbiology laboratory. In addition, subsequent testing of the CBR has shown that this reactor and SOP are responsive (data not reported). These results suggest that the CBR would preform well in a collaborative study.

The laboratory hot tub method falls between the CBR and swimming pool method as to how well it meets the required statistical criteria. The hot tub method did exhibit good

ruggedness and repeatability for the control data and disinfectants with a high level of efficacy in a field relevant system. Although, this method is more challenging than many standard methods and the results of the repeatability and responsiveness testing indicate that additional experimentation is required to gain the same level of confidence in the true log reduction value for disinfectants with marginal to low efficacy. The laboratory hot tub method is still more efficient and cost effective than a field study, which makes it a useful tool. Considering all the data, the laboratory hot tub method would perform acceptably in a collaborative study.

The laboratory swimming pool method is the most relevant but also the most complex and least repeatable and reasonable of three methods. The swimming pool method did exhibit good responsiveness. The method correctly predicted the efficacy of swimming pool disinfectants that have performed similarly in actual swimming pools, and the laboratory study would still cost less than an actual field test.

In disinfectant efficacy testing, as well as in research, reactor choice is critical. Laboratory reactors impart unique characteristics in the biofilm that influence everything from biofilm architecture and protein regulation to the response of biofilm bacteria to treatment. Currently, there is a need for standardized laboratory biofilm reactors and associated standard operating procedures engineered to grow repeatable, rugged, responsive, and relevant biofilms for use in disinfectant efficacy testing. Biofilm's ubiquitous nature suggests that several laboratory reactors and associated methods are required to fully meet this need. The three engineered laboratory systems that were created and evaluated for this dissertation provide reliable, standardized tools for crafting disinfectant strategies to control

biofilm bacteria in some important field conditions.

APPENDICES

APPENDIX A

CDC BIOFILM REACTOR WALL SHEAR CALCULATIONS

CDC - Biofilm Reactor Wall Shear Calculation

Goal: Calculate the wall fluid shear that the coupons experience in the CDC Biofilm Reactor

Assumptions:
1. Temperature = 20 C
2. Bulk fluid has the properties of water
3. Reactor is modeled by two concentric cylinders

Constants:

1. density (rho)=	998.23 kg/m^3
2. viscosity (mu)=	0.001005 kg/(m*s)
3. radius of outer cylinder (R_o)=	3 cm
4. radius of inner cylinder (R_i)=	2.5 cm
5. ratio of inner to outer cylinder (alpha)=	0.833333333

Calculate Reynold's number
(Characklis, W.G & Marshall, K.C. (1990) *Biofilms* pp. 288. New York, NY: John Wiley & Sons, Inc.)
Re= omega*alpha*R_o^2*rho/mu
where omega = rotational speed of inner baffle (in rpm)

Transition Re for laminar to turbulent flow
(Bird, R.B., Stewart, W.E. & Lightfoot, E.N. (2002) *Transport Phenomena* 2nd Edition pp. 92. New York, NY: John Wiley & Sons, Inc.)
Re_trans=41.3/(1-alpha)^1.5
Re_trans= 607

Calculate surface friction (f)
Assume a smooth surface
For turbulent flow f= 0.0791/Re^0.25 Blasius Formula
For laminar flow f= 16/Re Hagen-Poiseuille Equation

Calculate shear stress at wall (tau in N/m^2)
For turbulent flow tau= f*rho*vel^2/2
Where vel^2= omega^2*R_i*R_o

omega (rpm)	Re	flow regime	f	velocity (m/s)	tau (N/m^2)
125	1552	turbulent	0.0126	0.0571	0.0205
180	2235	turbulent	0.0115	0.0822	0.0388
225	2794	turbulent	0.0109	0.1027	0.0573