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Evaluation of disinfectant efficacy against biofilm and suspended bacteria in a laboratory swimming pool model

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Abstract

Laboratory reactor systems designed to model specific environments enable researchers to explore environmental dynamics in a more controlled manner. This paper describes the design and operation of a reactor system built to model a swimming pool in the laboratory. The model included relevant engineering parameters such as filter loading and turnovers 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 free chlorine or 10 mg/L 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 free chlorine resulted in the greatest log reduction in biofilm density. The research demonstrated the importance of studying a biofilm in addition to the planktonic bacteria to assess the microbial dynamics that exist in a swimming pool model.

Keywords: Biofilm; Swimming pools; Laboratory models; Disinfectants; Efficacy comparison

1. Introduction

Research on bacteria in swimming pools dates back to the 1920s [1]. In the following decades, researchers compared the efficacy of various disinfectants in both field and laboratory studies [2–6]. 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 [7–9], 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 [10] in addition to the bulk water measurements.

Prior to the 1990s, the typical laboratory evaluations of swimming pool disinfectants were conducted in flasks

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that incorporated neither the engineering components of a swimming pool system nor the water chemistry [11,12]. Systems became more relevant when in 1990 Yahya et al. [13] reported the bulk fluid efficacy results from a meso-scale system operated with representative microbial and nutrient loading. Judd and Black [14] utilized a meso-scale swimming pool system that included the addition of a body fluid analog to evaluate disinfectant by-product formation. Borgmann-Strahsen included relevant water chemistry and bacteria in her efficacy studies [15].

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.

2. Materials and methods

2.1. Reactor system design

The reactor vessel was a 10 L plastic vessel with an overflow valve at the 6.6 L water mark, as shown in Fig. 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

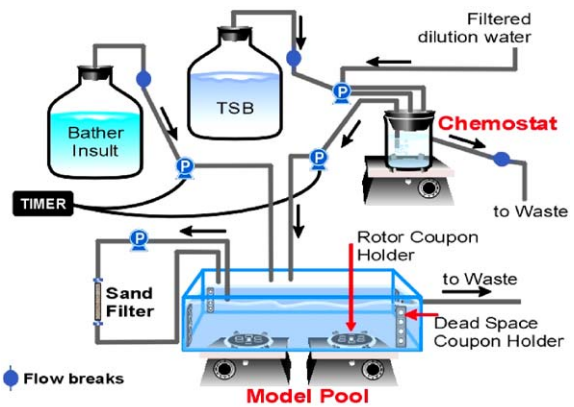

Fig. 1. Illustration of the laboratory swimming pool model.

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 × 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 through a sand filter with a surface area of 1.5 cm^2, a filter loading rate of 12.2 mL/min/cm^2 and a residence time of 6 h (four turnovers 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°C for 4 h to remove organic carbon.

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 L/day [16]. Typically the growth chemostat had a steady state cell concentration of 3.3×10^6 cfu/mL 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 Tryptic Soy Broth (TSB), had an 8.3 h 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 L/day 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 [16].

2.2. Reactor water chemistry

The water chemistry was adjusted daily to fall within the following American National Standards Institute/

National Spa and Pool Institute (ANSI/NSPI) recommended guidelines: pH 7.2–7.6, alkalinity 60–120 mg/L and total hardness 200–400 mg/L [17]. 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.

2.3. Analytical methods

Alkalinity was determined according to standard method 2320 B [18]. *pH measurements* were read on a calibrated pH meter. *Polyhexamethylene biguanide* (*PHMB*) was measured according to a modification of the Dawson and Brown method [19]. *Total and free chlorine* were determined according to standard method 4500-Cl G [18]. *Total hardness* was determined according to standard method 2340 C [18]. *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.

2.4. 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 and greater than or equal to 200 cfu/mL. Ranges were chosen based upon United States regulatory criteria of fewer than 200 cfu/mL of heterotrophic bacteria in swimming pool water [20], with the notable difference in incubation time and temperature. The LOD was 5 cfu/mL.

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 s using a Tekmar–Dohrmann tissue homogenizer [21]. 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 s and sonicated for 30 s, 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 of dried sand.

2.5. Experimental protocol

2.5.1. 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 *ppm free chlorine control*: Free chlorine was maintained between 1 and 3 ppm in the bulk fluid.

Sixty-four hour super-chlorination experiment: Free chlorine concentration was maintained between 1 and 3 ppm. Every Friday, the system was dosed with 10 ppm free chlorine. On Monday, the chlorine concentration was measured then increased with additional chlorine or lowered with sodium thiosulfate. The experiment was repeated twice.

Sixteen hour super-chlorination experiment: Free chlorine concentration was maintained between 1 and 3 ppm. Every Wednesday, the system was dosed with 10 ppm free chlorine. On Thursday, the chlorine concentration was measured and adjusted as described previously. The experiment was repeated twice.

10 *ppm PHMB control*: The PHMB concentration was maintained at 10 ppm.

10 *ppm PHMB and quaternary biocide (quat) experiment*: PHMB concentration was maintained at 10 ppm 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 L 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 ppm PHMB and quaternary biocide and hydrogen peroxide experiment: Experiment lasted for two months to accommodate two doses of hydrogen peroxide. PHMB concentration was maintained at 10 ppm. 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 ppm hydrogen peroxide. Coupons in rotor 1 were sampled on day 30 then replaced with a sterile rotor and coupons. The filter and coupons in rotors 1 and 2 and dead space 2 and 4 were sampled on day 60.

2.6. 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 [22]. 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. [23].

3. Results

3.1. 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).

3.2. Bulk water

Table 1 presents the distribution of heterotrophic bacterial densities for each treatment. Samples containing 200 cfu/mL 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.

The mean TOC concentrations, associated standard error of the mean and ranges are presented in Table 2. For the untreated control, chlorine and 16 h super-chlorination experiments, the TOC values held constant in the ranges listed (Fig. 2). The TOC concentration for the 64 h super-chlorination and the PHMB/quat treatments held constant with the exception of two samples for the 64 h 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 1
Bulk water: distribution of heterotrophic bacterial densities for each treatment

Experiment	<LOD[a] (%)	>LOD and <200 (%)	⩾200 (%) (±95% CI[b])	No. of samples
Untreated control[c]	0	0	100 (74, 100)	10
Chlorine	72	28	0 (0, 15)	18
64 h super-chlorination	56	28	15 (5, 30)	39
16 h 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[d]	3	31	66 (54, 77)	77

[a] LOD = less than the level of detection.
[b] CI = confidence interval.
[c] Two week experiment.
[d] Two month experiment.

Table 2
Bulk water: range and mean TOC concentration ± standard error (SE) for each treatment

Experiment	Range (mg/L)	Mean (mg/L) ± SE	No. of samples
Untreated control[a]	1.34–5.65	3.47 ± 0.46	10
Chlorine	1.30–6.27	3.54 ± 0.29	20
64 h super-chlorination	2.28–23.75	7.58 ± 0.72	29
16 h 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[b]	15.03–130.16	25.49 ± 2.53	56

[a] Two week experiment.
[b] Two month experiment.

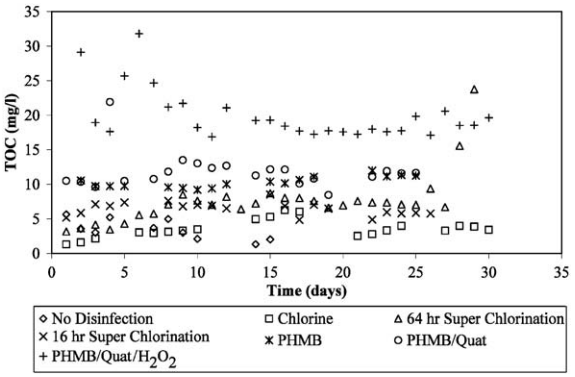

Fig. 2. TOC concentrations measured during the seven experiments.

3.3. Coupons

The log reduction in biofilm density on the coupons and associated standard error for each treatment are presented in Table 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.

3.4. Filter

The log reduction in filter biofilm and associated standard error for each treatment are presented in Table 4. The mean bacterial density for the untreated control was 9.04 $\log_{10}$ cfu/g. 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.

4. 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 [9] observed bacteria surviving water purification by existing in clusters surrounded by a protective coat. Seyfried [7] reported in 1980 that a slime layer enabled *Pseudomonas aeruginosa* to survive in a chlorinated swimming pool. Storey [8] stated that once a *Pseudomonas* biofilm was established on a swimming pool surface, nothing short of physical removal would eliminate it. These findings were particularly important from the standpoint of public safety because biofilm may be a potential reservoir for pathogenic bacteria [10,24].

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 on the filters. This work also demonstrated that a 16 h, or overnight super-chlorination was as effective as a 64 h, or weekend super-chlorination. In an actual swimming pool, applying a 16 h treatment is much more feasible. The addition of quat/H_2O_2 to PHMB improved the nonoxidizing disinfectant's performance against

Table 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
64 h super-chlorination	0.03	6.07	0.37
16 h 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[a]	1.06	5.04	0.46

[a] Two month experiment.

Table 4
Filters: log reduction (LR) and associated standard error (SE) for each treatment

Experiment	Accumulation $\log_{10}$ (cfu/g)	Log reduction $\log_{10}$ (cfu/g)	SE of the LR
Chlorine	2.34	6.70	0.67
64 h super-chlorination	0.10	8.94	0.12
16 h 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[a]	1.23	7.81	0.33

[a] Two month experiment.

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 free chlorine and 10 mg/L 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 requirement. A large log reduction in mean biofilm density on the coupons (Table 3) and in the filter (Table 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.

5. 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:

- 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.
- Shock treatments were effective at minimizing biofilm accumulation in filters and on coupons, although care must be given to disinfect the detached biofilm.
- Biofilm accumulation was found in the filter and on coupon surfaces in reactors consistently maintained with a residual free chlorine level of 1–3 and 10 mg/L PHMB.
- In this system, a 16 h super-chlorination was as effective as a 64 h super-chlorination against biofilm accumulation in the filter and on coupon surfaces.

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