Predictive modeling for hot water inactivation of planktonic and biofilm-associated *Sphingomonas parapaucimobilis* to support hot water sanitization programs

Authors: Laura (Kaatz) Wahlen, Al Parker, Diane Walker, Mark Pasmore & Paul Sturman.

Kaatz Wahlen L, Parker A, Walker D, Pasmore M, Sturman P “Predictive modeling for hot water inactivation of planktonic and biofilm-associatedSphingomonas parapaucimobilis to support hot water sanitization programs,” Biofouling, 2016 Aug;32(7):751-61.

Predictive modeling for hot water inactivation of planktonic and biofilm-associated *Sphingomonas parapaucimobilis* to support hot water sanitization programs

Laura (Kaatz) Wahlen[a], Al Parker[b,c], Diane Walker[b], Mark Pasmore[a] and Paul Sturman[b]

[a]Sterility Assurance, Baxter Healthcare Corporation, Round Lake, IL, USA; [b]Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA; [c]Department of Mathematical Sciences, Montana State University, Bozeman, MT, USA

ABSTRACT

Hot water sanitization is a common means to maintain microbial control in process equipment for industries where microorganisms can degrade product or cause safety issues. This study compared the hot water inactivation kinetics of planktonic and biofilm-associated *Sphingomonas parapaucimobilis* at temperatures relevant to sanitization processes used in the pharmaceutical industry, *viz.* 65, 70, 75, and 80°C. Biofilms exhibited greater resistance to hot water than the planktonic cells. Both linear and nonlinear statistical models were developed to predict the log reduction as a function of temperature and time. Nonlinear Michaelis–Menten modeling provided the best fit for the inactivation data. Using the model, predictions were calculated to determine the times at which specific log reductions are achieved. While ≥80°C is the most commonly cited temperature for hot water sanitization, the predictive modeling suggests that temperatures ≥75°C are also effective at inactivating planktonic and biofilm bacteria in timeframes appropriate for the pharmaceutical industry.

KEYWORDS

Biofilm; planktonic; sanitization; hot water; inactivation

Introduction

Hot water sanitization is commonly employed by the pharmaceutical and food and beverage industries. Pharmaceutical manufacturing facilities utilize hot water to sanitize production piping, tanks, and associated com-ponents, while food processing facilities employ hot water to reduce the number of viable microorganisms on fruit, vegetables, meat, and food-contact surfaces (Castillo et al. 1998; Collentro 2002; Selma et al. 2008). Ample liter-ature exists for studies that assess the efficacy of hot water sanitization in the food industry, such as pasteurization literature(Castillo et al. 1998; Pearce et al. 2001; Fan et al. 2008). However, few studies can be found for its usage in the pharmaceutical industry. The literature acknowledges that hot water is an effective method for sanitization of production components, along with steam, chemicals, and ozone (Collentro 2002). Temperatures > 80°C are frequently referenced as the process temperature for hot water sanitization (Collentro 1995; Junker et al. 1997; Jahnke 2001). Lower temperatures may be acceptable, and are commonly used in the water industry in which microbial limits are higher than in the food and phar-maceutical industries. One study recommends that 60°C is the minimum temperature applied when heat-treating water systems containing potential pathogens in plank-tonic, or free-floating form (Spinks et al. 2006), while a long-term study using hot water inactivation in conjunc-tion with ionization and ozonation found that increas-ing the application temperature from 50°C to 65°C was a more effective method of control than either disinfectant applied at the lower temperature (Blanc et al. 2005). Other process parameters such as exposure time and frequency of sanitization are also not widely discussed in the lit-erature, although a group tasked to investigate thermal disinfection in a number of hot water distribution systems indicated that 65°C should be adequate against *Legionella* but recommended a system flush of at least 10 min or more at that temperature, and, as regrowth was found within a month after treatment, the regular application of control measures was encouraged (Mouchtouri et al. 2007). In contrast, Collentro (1995) suggests sanitization process parameters for a purified water system should be correlated to moist heat sterilization (temperature of

121°C) and that sanitization should be performed at 95°C for > 100 min. The data reported in the literature were generated almost exclusively using planktonic microorganisms and few data exist related to heat inactivation of biofilms (sessile bacteria).

Biofilms are defined as communities of microorganisms adhering to and growing on surfaces and are often embedded in an extracellular polysaccharide matrix (Parkar et al. 2004). These microbial communities can be found wherever there is a combination of moisture, nutrients, and a surface, including in high purity water systems. For example, biofilms can form on the surfaces of stainless steel equipment in pharmaceutical manufacturing environments, which is not only detrimental to product quality but also has the potential to adversely affect patients if sufficient microbial control practices have not been implemented. Although biofilms are recognized as a threat in the manufacturing environment, most microbial disinfection and sanitization studies are performed using suspensions of planktonic microorganisms or suspensions spotted onto surfaces. However, research has indicated that biofilms are typically more resistant to disinfection treatments than their planktonic counterparts (Frank & Koffi 1990; Norwood & Gilmour 2000; Olson et al. 2002), and that there may be as many as 10^3 sessile organisms for each planktonic cell detected in a system (Momba et al. 2000). When evaluating the efficacy of chemical disinfectants against a thermophilic bacilli biofilm grown on stainless steel, Parkar et al. (2004) observed that the reduction in cell numbers decreased as application temperatures decreased below 75°C, stressing the importance of considering biofilm resistance when determining effective sanitization parameters.

Preliminary studies evaluated the relative tolerance of waterborne microorganisms to hot water treatment, including *Pseudomonas aeruginosa*, *Sphingomonas paucimobilis*, *Sphingomonas parapaucimobilis*, and *Ralstonia pickettii*, among others (unpublished data). Among the organisms tested, *S. parapaucimobilis* was the most tolerant to 60°C water in the planktonic state (unpublished data). This organism was isolated from a pharmaceutical manufacturing system that is regularly hot water sanitized at 80°C, evidencing its ability to resist heat. *Sphingomonas* spp. are widely distributed in nature and are commonly found in all types of water systems including sea and river water, waste water, drinking water, and hospital water (Koskinen et al. 2000). Sphingomonads are known to produce a more viscous slime at lower than optimal growth temperatures (30–35°C), which may explain their ability to form biofilms in ambient water or solution transmission systems (Koskinen et al. 2000). Also, it has been reported that biofilm formation on reverse osmosis membranes in water systems is initiated and dominated by *Sphingomonas* spp. (Bereschenko et al. 2010). The ability of *S. parapaucimobilis* to form biofilms, resist heat, and its prevalence in water systems made the species an appropriate test organism for this study.

The purpose of this study was to investigate and compare the hot water inactivation kinetics of planktonic and biofilm-associated *S. parapaucimobilis*. The comparison of these kinetics can then be used to determine the relative challenges presented during the inactivation of free-floating versus sessile contamination, and through use of statistical modeling make inactivation predictions to assist in the development of effective hot water sanitization programs.

Materials and methods

Bacterial culture and growth conditions

Planktonic

S. parapaucimobilis (Baxter Healthcare Corp., Deerfield, IL, USA, manufacturing facility isolate) was grown in tryptic soy broth (TSB; Becton, Dickinson, and Co., Franklin Lakes, NJ, USA) at 30–35°C[1] (Caron Inc., Marietta, OH, USA, model 6030) for 20–24 h with shaking at 120 rpm (IKA Works, Inc., Wilmington, NC, USA, model HS 260). Immediately before hot water treatment was conducted, a planktonic cell test suspension was prepared by diluting the 20–24 h TSB culture 1:10 in sterile water (Sterile Water for Irrigation, USP; Baxter Healthcare Corp.) to achieve a concentration of ~7 or 8 log(CFU ml^{-1}).

Biofilm

S. parapaucimobilis was grown in TSB at 20–25°C for 20–24 h with shaking at 110 rpm (New Brunswick Scientific Co., Inc., Edison, NJ, USA model G24). A CDC reactor (BioSurface Technologies, Inc., Bozeman, MT, USA) was used for biofilm growth (Figure 1). The CDC reactor is a continuous-stirred tank reactor that holds eight rods, each inserted with three coupons of known surface area on which to grow biofilms. The ASTM Standard Method E2562-12, 'Quantification of *Pseudomonas aeruginosa* biofilm grown with high shear and continuous flow using CDC biofilm reactor,' was followed with modifications appropriate to the establishment of a *S. parapaucimobilis* biofilm with respect to continuous flow (CF) nutrient concentration, duration, and flow rate (ASTM International 2012A). Other modifications included inoculum culture incubation duration and temperature, volume of inoculum, and coupon material. A CDC reactor with 316L stainless steel coupons (surface area per coupon = 4.13 cm^2) was autoclaved with 500 ml of 1:100 strength TSB medium. Once cooled, the reactor was inoculated with a 5 ml aliquot of the 20–24 h culture (average log(CFU ml^{-1}) = 8.48). The reactor was operated

Figure 1. CDC biofilm growth reactor (BioSurface Technologies, Inc.).

in batch mode (no flow of medium) for 24 h to facilitate attachment. After this period, 1:100 strength TSB influent was started at a flow rate of 2.5 ml min^{-1} at room temperature (20 ± 0.5°C[1]) for 72 h until a mature biofilm was formed. The total time for biofilm establishment, including batch mode, was 96 h. Biofilm growth optimization studies indicated that these conditions (eg temperature and time) produce mature biofilms, confirmed by microscopic and enumeration methods (unpublished data).

Hot water treatment

Planktonic

Five ml aliquots of the planktonic test suspension were aseptically pipetted into sterile glass screw cap tubes (Pyrex No. 9826, 25 ml; Corning Inc., Corning, NY, USA) taking care not to drip or splash the suspension onto the inner surface of the tube. An appropriate number of tubes were prepared to achieve three replicates for each test group (heat treated and controls at room temperature) outlined in Table 1. Additionally, 5 ml aliquots of sterile water were pipetted into two tubes. These tubes contained thermocouples and were held under the same conditions as the test samples to evaluate the temperature of the heat treated and room temperature samples. Since the same type of tube was used for all experiments, the liquid level (5 ml) in the tube was always at the same height.

The methods for planktonic hot water treatment were performed for each of the four water temperature and sample exposure time combinations for the test and control samples outlined in Table 1. Each of the four experiments outlined was independently performed three times. For each temperature tested, a circulating water bath (Cole Parmer, Vernon Hills, IL, USA) was set to the high end of the target temperature range (ie 66°C for 65°C (range 64 ± 2°C, reference Table 1)). The water level in the bath was maintained at the same level for all experiments so that the liquid samples in the test tubes were always submerged. The temperature of the water bath was monitored with a thermometer. Heat treatment was initiated when the temperature of the water bath reached the set point (ie 66°C for the example above). The tubes containing the *S. parapaucimobilis* in water suspension designated for heat treatment were placed into the water bath along with a tube containing water and a thermocouple and a timer was started. The room temperature controls were maintained at room temperature with a tube containing water and a thermocouple. When the temperature reading on the thermocouple in the tube in the water bath reached the low end of the target temperature range (ie 62°C for 65°C (range 64 ± 2°C)) the timer was stopped. This time period was the time it took for the heat treated tubes to reach the target temperature range. A timer was then started to monitor the heat treatment exposure time after the samples reached the target temperature. When the exposure time reached the time period for each treatment group (Table 1), the tubes were removed from the bath and immediately placed into an ice bath. This was repeated for each test group, including the room temperature controls.

Table 1. Hot water treatment parameters for planktonic suspensions and biofilms on stainless steel.

	Sample exposure times (min)			
	Planktonic		Biofilm	
Water temperature (range)	Heat treated	Controls (room temp)	Heat treated	Controls (room temp)
65°C (64 ± 2°C)	1, 3, 5, 10, 15, 20	0, 20	15, 30, 45, 60, 90	0, 45, 90
70°C (69 ± 2°C)	1, 3, 5, 7, 10, 15	0, 15	10, 20, 30, 40, 60	0, 30, 60
75°C (74 ± 2°C)	1, 3, 5, 7, 9, 10	0, 10	5, 10, 20, 30, 40, 60	0, 60
80°C (79 ± 2°C)	0.5, 1, 1.5, 2	0, 2	1, 2, 5, 10, 15	0, 10, 15

Biofilm

Following the single tube method (ASTM E2871-12), control coupons were placed in 10 ml of buffered water (0.0425 g KH_2PO_4 and 0.405 g $MgCl{\cdot}6H_2O$ l^{-1} of distilled water and filter-sterilized (Eaton et al. 2005)) and held at room temperature (20 ± 0.5°C) (ASTM International 2012B). For direct comparison, biofilm coupons for heat exposure were also placed in 10 ml of buffered water, but were then placed in a Precision water bath that was maintained at the same water level throughout testing (Thermo Scientific, Waltham, MA, USA) set to treatment temperatures. To monitor temperatures of the heat treatment, an additional tube containing 10 ml of buffered water, a stainless steel coupon and a thermocouple was included with each experiment. Once the target temperature was reached (± 2°C, reference Table 1), a rod from the CDC reactor was aseptically removed and gently rinsed in buffered water to remove any planktonic or loosely attached biofilm bacteria. The coupons were transferred from the rod to a sterile sampling platform. Using flame-sterilized forceps, each coupon was then gently submerged directly into a tube of pre-heated buffered water located in the water bath (splashing onto the sides of the tubes could result in some bacteria not being exposed to the full heat treatment). A timer was started to monitor the exposure time for each coupon (Table 1). After the timed exposure, thermocouple readings were documented and the heat treated tubes were directly placed into an ice bath.

Bacterial enumeration

After the specified exposure time (Table 1), the heat treated planktonic and biofilm samples were cooled in an ice bath for ~5 min. The planktonic cell suspensions were serially diluted in sterile water and plated by spreading 100 µl aliquots onto R2A agar plates or pour plating 1 ml with molten R2A agar (Becton, Dickinson, and Co. Franklin Lakes, NJ, USA), in duplicate. The plates were incubated at 30–35°C for a minimum of 48 h before the colonies were counted. Planktonic colony forming units (CFUs) were converted to a log density (LD) ml^{-1} by multiplying the average colony count among duplicate plates by the dilution factor. Prior to enumerating the biofilm-associated cells, the biofilm was removed from each coupon and disaggregated into suspension by a vortex/sonication series of 30 s at 45 kHz. Vortex/sonication was repeated a second time and the procedure was completed with a final vortex at 30 s. This removal and disaggregation procedure follows ASTM standard test method E2871-12 (ASTM International 2012B). A dilution series was performed, and then drop plated using an electronic pipettor to withdraw 100 µl and dispense five 10 µl aliquots of cell suspension onto each of two R2A agar plates. The plates were incubated at 36 ± 2°C for 48 ± 2 h. At the lowest countable dilution, the number of CFUs was enumerated. CFUs were converted to a LD cm^{-2} of surface area using the following equation:

$$\begin{aligned} LD &= LOG_{10}\left(CFU\, cm^{-2}\right) \\ &= LOG_{10}\left[\left(10^{d}\, CFU\, 100\mu l^{-1}\right)(V/SA)\right] \end{aligned} \quad (1)$$

where V, the sonicated sample volume, was 10 ml; d is the dilution of the enumerated sample; and SA, the surface area of each coupon, was found using the surface area formula for a cylinder with height of 4 mm and a radius of 6.35 mm:

$$SA = 2(\pi)\left(0.635^2\right) + 2(\pi)(0.635)(0.4) = 4.12947612\, cm^2$$

Data and statistical analysis

The response of bacteria subjected to the hot water treatment regimen was the log reduction (LR), which is the mean LD of the time 0 untreated control samples (ie maintained at room temperature) minus the mean LD of heat treated samples at each time point with the assumption that LR = 0 at time 0. Whenever zero CFUs were observed on the plates at all dilutions, 0.5 CFU was substituted in at a single plate at the lowest dilution plated (United States Environmental Protection Agency 1998), and then scaled up to a LD that represented the minimum possible LD, or the limit of detection, for the test. Since the 0th dilution was always enumerated using two duplicate plates, the limit of detection for the planktonic samples was $LOG_{10}[0.5/2]=-0.60$; for the biofilm samples, 0.5 was substituted into Equation 1 and the limit of detection was:

$$LOG_{10}\left[\left(0.5\, 100\mu l^{-1}\right)(10/4.13)\right] = LOG_{10}\left[12.11\, CFU\, cm^{-2}\right] = 1.08$$

Four different regression models were investigated to describe the effect of time on the LRs for each temperature separately: linear, quadratic, nonlinear (two parameter) Michaelis–Menten, and a four-parameter logistic model (Pinheiro & Bates 2000). The quadratic model output

Table 2. Linear regression equations of the LRs as a function of time (*T*) and temperature.

	Planktonic			Biofilm		
Temp(°C)	Regression equation	*p*-value	R^2	Regression equation	*p*-value	R^2
65	3.41 + 0.2944 *T*	< 0.0001	90%	2.80 + 0.0193 *T*	0.001	98%
70	6.20 + 0.1775 *T*	0.002	47%	3.86 + 0.0523 *T*	0.064	73%
75	7.12 + 0.1754 *T*	0.004	53%	6.53 + 0.0075 *T*	0.106	52%
80	8.21 – 0.0417 *T*	0.833	64%	5.92 + 0.0883 *T*	0.324	32%

model parameters were challenging to interpret in terms of microbial inactivation, and can only be interpreted over the range of times actually observed. The logistic regression model could not always be fit to the data and, when it could be fit, did not result in substantially better model fits as indicated by R^2 values. Thus, only results for the linear and Michaelis–Menten models are presented here. Due to lack of fit of the linear models, the zero LRs at time zero were only included when fitting the Michaelis–Menten models. In order to compare the LRs across all temperatures and time points, a mixed effects linear model was fit with covariates for time and temperature.

The repeatability of the control LDs across time was assessed *via* ANOVA, and the trend of the control LDs across time was assessed *via* regression. Because the data were generated from multiple experiments (three planktonic experiments and one biofilm experiment per temperature) all of the models described above included a random effect for experiment. All reported p-values were generated by follow-up t-tests. These models were fit using R v.3.0.2 (R Core Team 2013) package *nlme* (Pinheiro et al. 2013).

Results

Hot water inactivation of S. parapaucimobilis *and statistical modeling*

Planktonic

The mean LDs for each hot water temperature are plotted in Figure 2. The average initial LD of the test suspensions was 7.85. There was a high level of repeatability (ie a low level of variation) across experiments of the controls' LDs at room temperature (repeatability SD = 0.28 $\log_{10}$(CFU ml^{-1})) which suggests that if a new experiment is performed, then a randomly chosen 1 ml volume in the new experiment using the same protocol would be about 0.28 $\log_{10}$(CFU ml^{-1}) from the true mean LD of the control planktonic bacteria. Nor was there a statistically significant linear trend of the control LDs across time at room temperature (trend = 0.0017 $\log_{10}$(CFU ml^{-1}) min^{-1}, p-value = 0.568). This indicates that exposure to water alone (without heat) over the time of the experiment did not affect the LD of the suspensions. For the heat treated suspensions, there were statistically significant increases in the LR as temperature and contact time increased (p-value ≤ 0.0001). Complete inactivation (to the limit of detection, LD = −0.60) occurred within 20, 10, 5, and 2 min at 65, 70, 75, and 80°C, respectively.

Linear regression models of the planktonic LRs as a function of time for each temperature separately are presented in Table 2. The curvature in the LRs as a function of time and the low R^2 values suggest that a line is not an appropriate model for the data. Nonlinear two-parameter

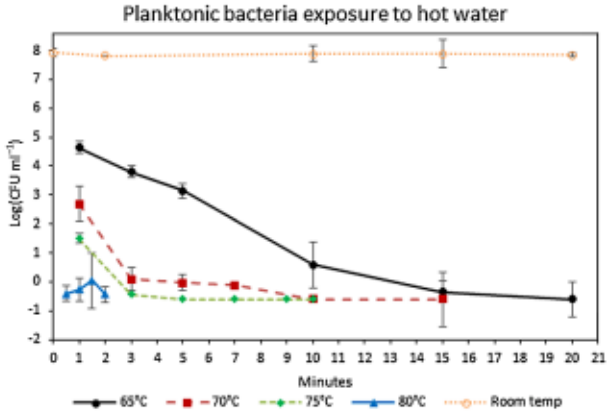

Figure 2. Average LDs of planktonic *S. parapaucimobilis* exposed to hot water treatment at 65, 70, 75, and 80°C, over various contact times.
Notes: The values for LD are the means of nine replicates from three separate experiments. Error bars indicate the SD of the mean LD for a single time point from all experiments at a certain temperature. The limit of detection for this test was a LD of −0.60.

Michaelis–Menten models, $\mathrm{LR} = \left(\frac{\mathrm{LR}_{max}\,\mathrm{Time}}{T_{1/2}+\mathrm{Time}}\right)$, were also fit to the LRs for the planktonic data and are provided in Table 3. Visualizations of the Michaelis–Menten models for each temperature separately are provided in Figure 3, with the exception of 80°C because the model (with positive parameter values) cannot describe such quick inactivation, ie such an extreme increase in LR over a short time.

Biofilm

The inactivation kinetics of different temperature and contact times were assessed in experiments using biofilms grown on stainless steel coupons in the CDC reactor. Figure 4 depicts the LD results of all four temperatures and contact times on stainless steel. The average initial LD of the test coupons was 8.00. Analyses indicate that there was no significant effect on the LDs due to contact time in water at room temperature (p-value ≥ 0.801). There was a statistically significant linear increase in the LR of the biofilm as temperature increased (p-value < 0.0001), and as contact time increased (p-value = 0.006). The 65 and 70°C exposures were unable to achieve inactivation to the limit of detection (LD = 1.08 or ~12 CFU), by the end of the exposure period. The LR at 65°C at 90 min was 4.6, while the LR at 70°C at 60 min was 6.9. Conversely, 75 and 80°C exposures achieved complete inactivation to the limit of detection by 20 and 5 min, respectively, correlating to LRs > 6.9.

The linear regression equations of the LR as a function of time for each temperature separately are presented in Table 2. Similar to the planktonic linear analyses, the curvature in the LRs as a function of time and the low R^2 values suggest that a line is not an appropriate model for the data, with the exception of the 65°C data, which was the only biofilm experiment that exhibited a significant

Table 3. Nonlinear Michaelis–Menten model equations of the LRs as a function of time (T) and temperature.

	Planktonic		Biofilm	
Temp(°C)	Nonlinear regression equation	R^2	Nonlinear regression equation	R^2
65	$LR = 10.13T/(3.96 + T)$	94.5%	$LR = 4.64T / (8.91 + T)$	99.1%
70	$LR = 8.76T / (0.69 + T)$	98.4%	$LR = 7.54T / (9.10 + T)$	94.9%
75	$LR = 9.03T / (0.39 + T)$	99.5%	$LR = 6.91T / (0.39 + T)$	99.8%
80[1]	$LR = 8.16$	64.8%	$LR = 7.35T / (0.39 + T)$	98.1%

[1]For 80°C, the nonlinear Michaelis–Menten model could not be fit for positive values of the parameters for the planktonic data. The value of LR = 8.16 for 80°C is the mean of the non-zero LRs from all planktonic experiments at 80°C.

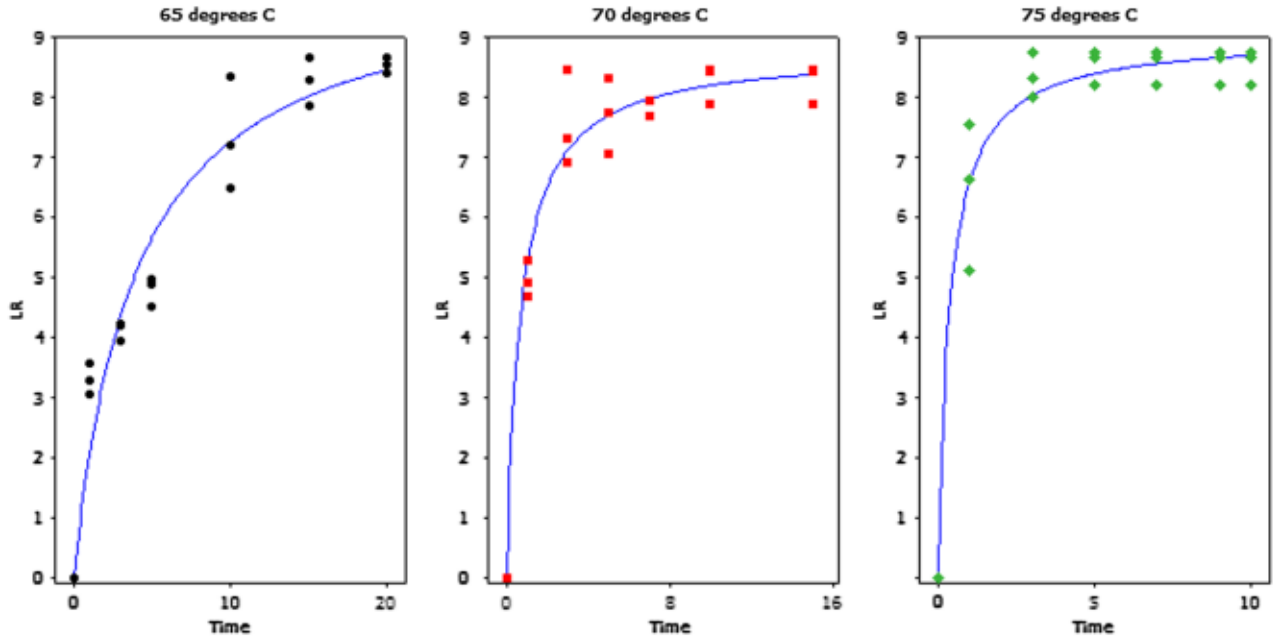

Figure 3. Michaelis–Menten models of the planktonic log reductions (LR) fit separately for each temperature.
Notes: Each point in the preceding graph is a LR for a specific temperature and time combination in a single experiment. The vertical axes in all three panels are scaled from 0 to 9. Solid lines depict the nonlinear regression curves. The plot for 80°C is not shown because the nonlinear Michaelis–Menten model could not be fit for positive values of the parameters.

linear trend (p-value = 0.001). The two-parameter nonlinear Michaelis–Menten model provided a better fit to the LRs over time (Table 3 and Figure 5).

Hot water inactivation predictions

Since the nonlinear Michaelis–Menten model provided the best fit to both the planktonic and biofilm lethality data at each temperature tested, prediction intervals were used to determine the time at which the LR from a single experiment would be between 1 and 6 with 90% confidence. In other words, an upper one-sided prediction interval (PI) with 90% confidence was calculated by first generating a two-sided PI with 80% confidence. Figures 6 and 7 depict 80% PIs for the planktonic and biofilm data, respectively. These intervals predict, with 80% confidence, the range of LRs to be attained from a new experiment conducted with the same protocol as for the present study. From these figures the time points can be found for which the models first predict that the LR from a future experiment will be larger than some specified target, eg a target of 6. Table 4 summarizes the times at which the models predict LR targets between 1 and 6. The times at which these targets are attained with 90% confidence are also presented (Table 5).

Discussion

The efficacy of a range of temperatures in the hot water inactivation of planktonic and biofilm-associated *S. paraupaucimobilis* was investigated. The data show that biofilms are more resistant to hot water treatment than the planktonic cells, as evidenced by the longer time periods predicted for biofilm lethality (Table 5). This finding is supported by studies found in the literature for other disinfection treatments, that is, biofilms are typically more resistant than their planktonic counterparts (Frank & Koffi 1990; Norwood & Gilmour 2000; Olson et al. 2002; Stewart 2015). Although hot water inactivation data for biofilms are not widely reported in the literature, it has

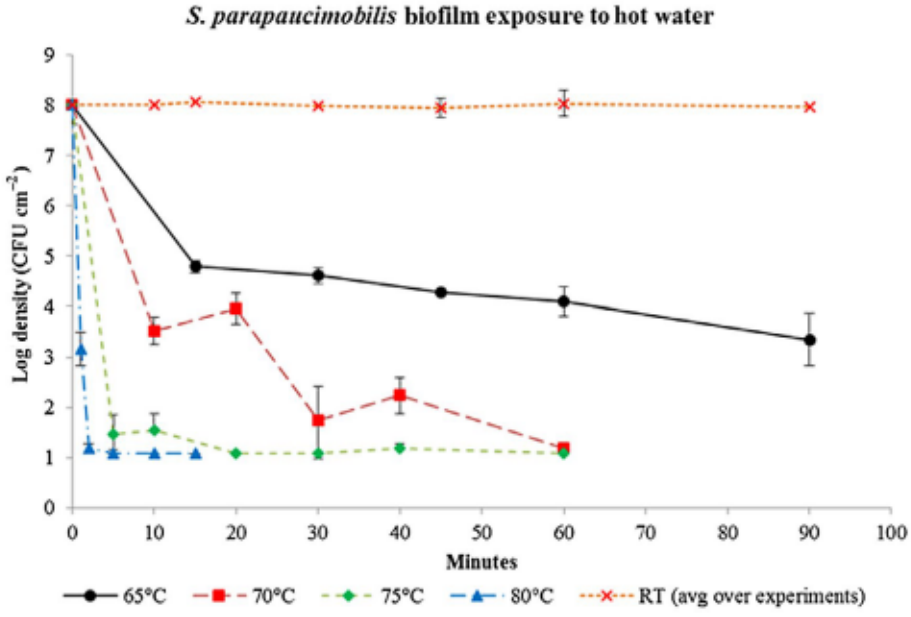

Figure 4. Average LDs of *S. parapaucimobilis* biofilms on stainless steel exposed to hot water treatment at 65, 70, 75, and 80°C over various contact times.
Notes: The values for LD are the means of three coupon replicates (with the following exceptions: two replicates at 5 min for 75°C and at 5 and 15 min for 80°C) from each single experiment at a water temperature, with the exception of the room temperature data which are compiled averages for all experiments. That is, time zero controls were sampled for all four experiments and averaged and the 70°C and 75°C experiments each had 60 min contact time controls and these were averaged. The limit of detection for this test was a LD of 1.08. Error bars indicate the SD of the mean LD for a single experiment.

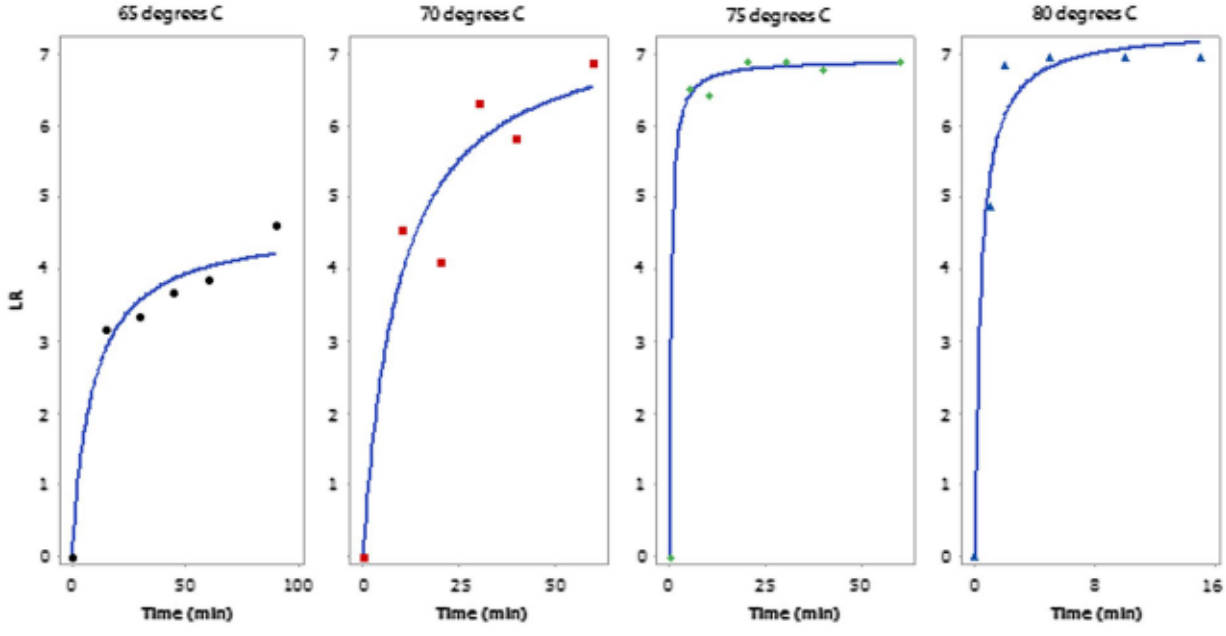

Figure 5. Plots of the Michaelis–Menten models fit to the biofilm LRs for each temperature separately.
Notes: Each point is a LR for a specific temperature and time combination in a single experiment. The solid lines depict the nonlinear regression curves.

been reported that planktonic bacteria are easily killed at temperatures ≥ 65°C, with the time to achieve 1 LR (ie the D-value) as low as 6–19 s for several waterborne organisms (Spinks et al. 2006).

Statistical modeling was utilized to make predictions about the LR of bacteria in a single experiment when exposed to a temperature for a certain contact time. After considering multiple statistical models, it was determined that the

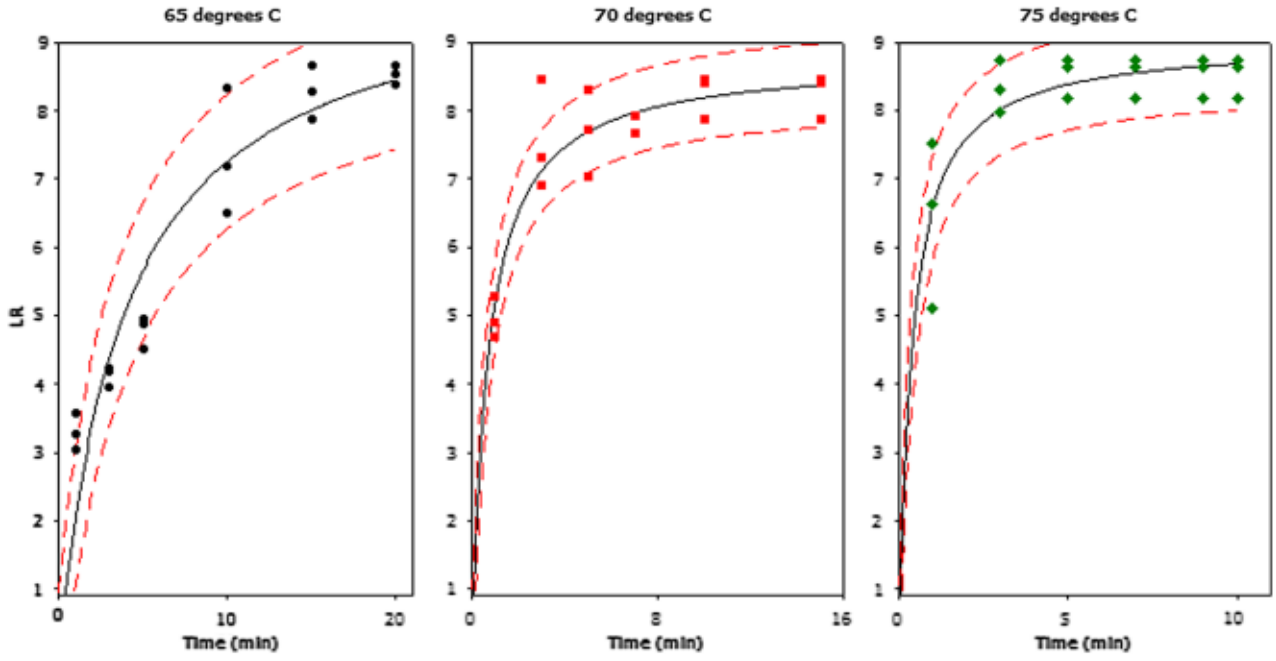

Figure 6. Prediction intervals for planktonic LRs based on Michaelis-Menten kinetic lethality models.
Notes: The solid lines depict the regression model. Prediction intervals are depicted with dashed lines. Prediction intervals for 80°C are not shown because the nonlinear Michaelis-Menten model could not be fit for positive values of the parameters.

nonlinear Michaelis–Menten model provided the best fit to the data. Using the nonlinear model, it was of interest to determine when the model predicts target LRs between 1 and 6. The upper target value of LR = 6 was chosen because ≥ 6 logs represents a higher bioburden level than what is normally found in the pharmaceutical manufacturing environment, making this target LR a worst-case challenge. The confidence limits account for the statistical uncertainty in the predicted times to attain the target log reductions.

The model predicts that, in a single experiment, it will take > 60 min to achieve a greater than 6 LR (with 90% confidence) at 70°C in biofilms, compared to 2 min 8 s for planktonic cells. However, at the higher temperatures of 75 and 80°C, the differences between the time predictions are less substantial and a greater than 6 LR is predicted to be achieved in ≤ 4 min 14 s for both planktonic and biofilm cells at both temperatures. This suggests that 75 and 80°C treatments are very effective at inactivating bacteria in both states. In the planktonic state, 80°C treatment is so effective that the model could not be fit for positive values of the parameters due to the rapid inactivation. It is important to note that it took an average of 1 min 46 s for the planktonic cell suspensions to heat up to the target temperatures. It is expected that some level of inactivation occurred during that heat-up time, especially at the higher temperatures. This is unavoidable when working with planktonic suspensions and is representative of the sanitization process that occurs in industry. However, a time zero sample was not analyzed to determine the LR over heat-up time. For data analysis purposes, a LR = 0 at Time = 0 was assumed. The heat-up time was not included in the exposure time referenced in the graphs and tables depicting the planktonic data and predictions; the times reported are for samples exposed at the target temperature. On the other hand, the biofilm samples were added to water that was already heated up to temperature, removing the unknown level of inactivation during heat up time that was experienced in the planktonic cell treatment. Therefore, a greater than 6 LR of biofilms exposed to target temperatures without the added lethality during heat-up time can be achieved in less than 2 min 34 s on average (4 min 14 s to achieve a LR of 6 with 90% confidence) at 75–80°C. This is encouraging because 80°C is the most commonly cited temperature utilized in hot water sanitization. These results may support legitimacy of sanitizations that are unable to reach or fall below 80°C for all or part of the process, but still remain ≥ 75°C. Based on the modeling predictions, it appears that 65°C is not an effective temperature for sanitization purposes. According to the model, it is not possible to say with 90% confidence that the LR in a single experiment will reach greater than 6 against a biofilm, while a LR of 6 against planktonic cells is attained after 8 min 46 s. While 65°C may not be effective in sanitizations involving a high bioburden consisting of biofilm, inactivation did occur (Figures 2 and 4), suggesting that it may be an effective temperature in maintaining microbial control of planktonic organisms and preventing biofilm formation in hot loops/systems.

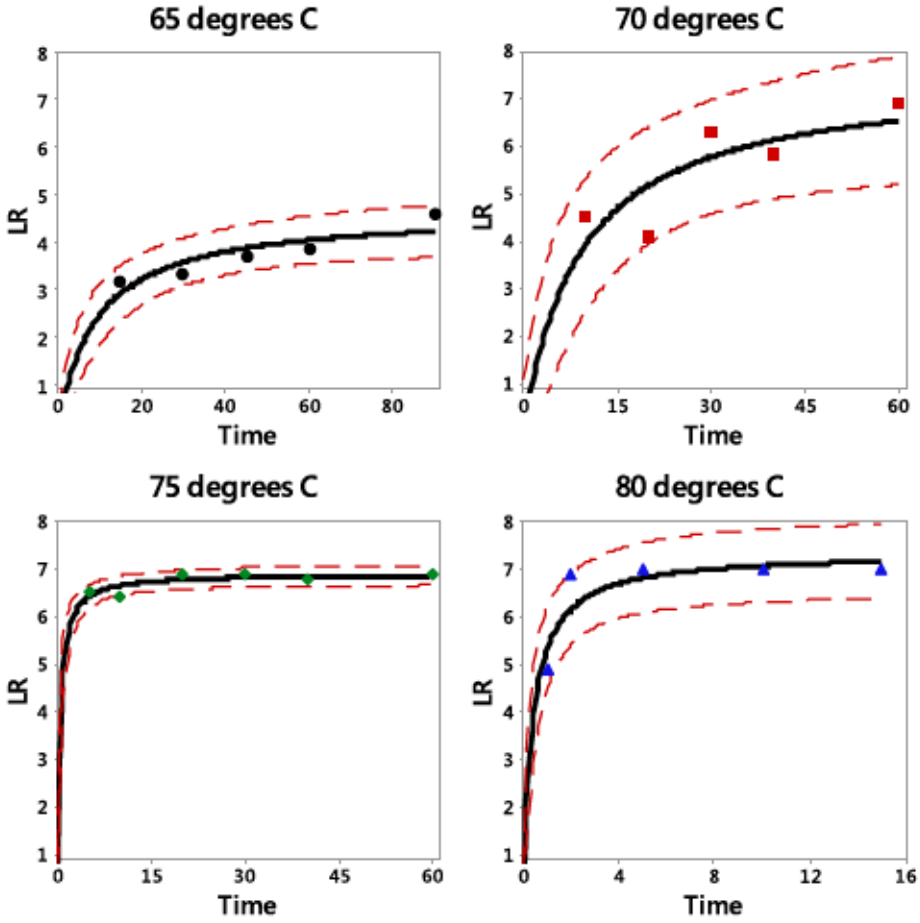

Figure 7. Prediction intervals for biofilm LRs based on Michaelis–Menten kinetic lethality models.
Notes: The solid lines depict the regression model. Prediction intervals are depicted with dashed lines.

The LR values in the range of 1–6 follow the expected relationship between time, temperature, and microbial inactivation; ie that the time to achieve the same LR decreases as the temperature increases (Table 4). For example, the time to achieve a 4 LR in biofilms at 70°C is 10 min 17 s, while the time to achieve the same LR at 75°C is 32 s. To account for the variability present in microbial testing the estimated LR values were also calculated using 90% confidence (Table 5). These calculations indicate a longer contact time, but provide a higher level of confidence than those in Table 4. It is not intuitive that the predicted time to attain a LR greater than 6 with 90% confidence in biofilms is slightly longer at 80°C (4 min 14 s) than at 75°C (3 min 52 s). One possible reason that the nonlinear model predicts a longer time to achieve > 6 LR at 80°C *vs* 75°C (with 90% confidence) is because the biofilm limit of detection (LD = 1.08) is quickly reached at these temperatures. This produces a plateau effect around 6 LR since the highest LR achievable in the study was ~ 6.9. This effect may not be present if the experimental protocol

Table 4. Predicted times to achieve a log reduction in a single test between 1 and 6.

	Planktonic				Biofilm			
	Time (min:s)				Time (min:s)			
Log reduction	65°C	70°C	75°C	80°C[1]	65°C	70°C	75°C	80°C
1	00:27	00:05	00:03	<00:30	2:27	1:23	00:04	00:04
2	00:58	00:12	00:07	<00:30	6:45	3:17	00:10	00:09
3	1:40	00:22	00:11	<00:30	16:18	6:01	00:18	00:16
4	2:35	00:35	00:19	<00:30	55:41	10:17	00:32	00:28
5	3:52	00:55	00:29	<00:30	Never[2]	17:55	1:01	00:50
6	5:45	1:30	00:46	<00:30		35:27	2:34	1:44

[1]For 80°C, the nonlinear Michaelis–Menten model could not be fit for positive values of the parameters.
[2]Never because 4.64 is the largest LR that is predicted at 65°C against biofilms (Table 3).

Table 5. Predicted times to achieve a minimum log reduction between 1 and 6 in a single test with 90% confidence (ie prediction intervals).

	Planktonic				Biofilm			
	Time (min:s)				Time (min:s)			
Log reduction	65°C	70°C	75°C	80°C[1]	65°C	70°C	75°C	80°C
1	00:58	00:10	00:05	<00:30	4:57	4:20	00:09	00:08
2	1:40	00:18	00:10	<00:30	11:42	7:41	00:17	00:16
3	2:37	00:31	00:16	<00:30	27:52	12:26	00:30	00:28
4	3:52	00:48	00:26	<00:30	NC[2]	20:40	00:50	00:47
5	5:46	1:16	00:40	<00:30	Never[3]	44:46	1:32	1:27
6	8:46	2:08	1:05	<00:30		NC[4]	3:52	4:14

[1]For 80°C, the nonlinear Michaelis–Menten model could not be fit for positive values of the parameters.
[2]NC = Not calculable because it is not possible to say that a 4 LR is achieved with 90% confidence within the range of times actually tested (up to 90 min).
[3]Never because 4.64 is the largest LR that is predicted at 65°C against biofilms (Table 3).
[4]Not calculable because it is not possible to say that a 6 LR is achieved with 90% confidence within the range of times actually tested (up to 60 min).

was modified in order to lower the limit of detection. Even so, the difference between the 6 LR predictions (at 90% confidence) at 75 and 80°C is just 22 s, suggesting that at these temperatures the inactivation is quite comparable.

This study suggests that 75 and 80°C are appropriate temperatures for hot water sanitization of planktonic and biofilm bacteria that are potentially present in process equipment (not including spore-forming organisms). These temperatures are effective at achieving greater than 6 LR in both planktonic and biofilm associated *S. parapaucimobilis* in ≤ 4 min 14 s with 90% confidence, according to the Michaelis–Menten model.

The predictions presented in this article are based on one test organism under ideal laboratory conditions. Manufacturing systems are significantly larger and more complex than laboratory systems; therefore, the predictions presented in this article should only be used as a starting point when setting sanitization parameters and a safety factor should be included based on a thorough understanding of the facilities' equipment, systems, and microbial flora. It is also recommended that the user performs further experimentation with facility environmental isolates, including biofilm testing, before implementing a hot water sanitization program. Biofilm testing is important because it represents the worst case contamination in terms of heat resistance, as evidenced by the data presented in this study. Also, the effect of hot water in the removal of biofilms was not investigated in this study. Therefore, if a gross contamination or biofilm is suspected in production lines, the use of additional treatments in combination with hot water sanitization may be necessary to ensure complete remediation.

Note

1. The planktonic and sessile bacteria were grown at different temperatures because optimization studies (unpublished data) indicated that these conditions produced robust microbial challenges for hot water treatment.

Acknowledgements

The authors acknowledge the technical support of Denise Pride (Sterility Assurance, Baxter Healthcare Corp.), microbiologists Lindsey Lorenz and Kelli Buckingham-Meyer, and interns Blaine Fritz and Danielle Goveia of the Standardized Biofilm Methods Laboratory at the Center for Biofilm Engineering (Montana State University).

Disclosure statement

No potential conflict of interest was reported by the authors.

References

ASTM International. 2012A. E2562-12 standard test method for quantification of *Pseudomonas aeruginosa* biofilm grown with high shear and continuous flow using CDC biofilm reactor. Vol. 11.05. West Conshohocken, PA: ASTM International.

ASTM International. 2012B. E2871-12 Standard test method for evaluating disinfectant efficacy against *Pseudomonas aeruginosa* biofilm grown in CDC biofilm reactor using single tube method. Vol. 11.05. West Conshohocken, PA: ASTM International.

Bereschenko LA, Stams AJM, Euverink GJW, van Loosdrecht MCM. 2010. Biofilm formation on reverse osmosis membranes is initiated and dominated by *Sphingomonas* spp. Appl Environ Microbiol. 76:2623–2632. doi:http://dx.doi.org/10.1128/AEM.01998-09.

Blanc DS, Carrara PH, Zanetti G, Francioli P. 2005. Water disinfection with ozone, copper and silver ions, and temperature increase to control *Legionella*: seven years of experience in a university teaching hospital. J Hosp Infect. 60:69–72. doi:http://dx.doi.org/10.1016/j.jhin.2004.10.016.

Castillo A, Lucia LM, Goodson KJ, Savell JW, Acuff GR. 1998. Use of hot water for beef carcass decontamination. J Food Prot. 61:19–25.

Collentro WV. 1995. Microbial control in purified water systems – case histories. Ultrapure Water. 12:30–38.

Collentro AW. 2002. Practical microbial control techniques for pharmaceutical water purification systems. Ultrapure Water. 19:53–58.

Eaton A, Clesceri LS, Rice EW, Greenberg AE, editors. 2005. Standard methods for the examination of water and

wastewater. 21st ed. Washington (DC): American Public Health Association; p. 9–19.

Fan X, Annous BA, Beaulieu JC, Sites JE. 2008. Effect of hot water surface pasteurization of whole fruit on shelf life and quality of fresh-cut cantaloupe. J Food Sci. 73:M91–M98. doi:http://dx.doi.org/10.1111/j.1750-3841.2008.00695.x.

Frank JF, Koffi RA. 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J Food Prot. 53:550–554.

Jahnke M. 2001. One-way distribution system for water for injection: process management, microbiological quality control, and meeting regulatory requirements. PDA J Pharm Sci Technol. 55:3–9.

Junker BH, Stanik M, Adamca J, LaRiviere K, Abbatiello M, Salmon P. 1997. An ambient water loop system for USP purified water. Bioprocess Eng. 17:277–286. doi:http://dx.doi.org/10.1007/s004490050386.

Koskinen R, Ali-Vehmas T, Kampfer P, Laurikkala M, Tsitko I, Kostyal E, Atroshi F, Salkinoja-Salonen M. 2000. Characterization of *Sphingomonas* isolates from Finnish and Swedish drinking water distribution systems. J Appl Microbiol. 89:687–696. doi:http://dx.doi.org/10.1046/j.1365-2672.2000.01167.x.

Momba MNB, Kfir R, Venter SN, CLoete TE. 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. Water SA. 26:59–66.

Mouchtouri V, Velonakis E, Hadjichristodoulou C. 2007. Thermal disinfection of hotels, hospitals, and athletic venues hot water distribution systems contaminated by *Legionella* species. Am J Infect Control. 35:623–627. doi:http://dx.doi.org/10.1016/j.ajic.2007.01.002.

Norwood DE, Gilmour A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. J Appl Microbiol. 88:512–520. doi:http://dx.doi.org/10.1046/j.1365-2672.2000.00990.x.

Olson ME, Ceri H, Morck DW, Buret AG, Read RR. 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res. 66:86–92.

Parkar SG, Flint SH, Brooks JD. 2004. Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. J Appl Microbiol. 96:110–116. doi:http://dx.doi.org/10.1046/j.1365-2672.2003.02136.x.

Pearce LE, Truong HT, Crawford RA, Yates GF, Cavaignac S, de Lisle GW. 2001. Effect of turbulent-flow pasteurization on survival of *Mycobacterium avium* subsp. paratuberculosis added to raw milk. Appl Environ Microbiol. 67:3964–3969. doi:http://dx.doi.org/10.1128/AEM.67.9.3964-3969.2001.

Pinheiro J, Bates D. 2000. Mixed effects models in S and S-PLUS. New York: Springer. doi:http://dx.doi.org/10.1007/978-1-4419-0318-1.

Pinheiro J, Bates D, DebRoy S, Sarkar D, and the R Development Core Team. 2013. *nlme*: Linear and nonlinear mixed effects models. R package version 3.1-111.

R Core Team. 2013. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available from: http://www.R-project.org/

Selma MV, Ibáñez AM, Allende A, Cantwell M, Suslow T. 2008. Effect of gaseous ozone and hot water on microbial and sensory quality of cantaloupe and potential transference of Escherichia coli O157:H7 during cutting. Food Microbiol. 25:162–168. doi:http://dx.doi.org/10.1016/j.fm.2007.06.003.

Spinks AT, Dunstan RH, Harrison T, Coombes P, Kuczera G. 2006. Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. Water Resour. 40:1326–1332. doi:http://dx.doi.org/10.1016/j.watres.2006.01.032.

Stewart PS. 2015. Antimicrobial tolerance in biofilms. Microbiol Spectrum. 3:MB-0010-2014. doi:http://dx.doi.org/10.1128/microbiolspec.MB-0010-2014.

United States Environmental Protection Agency. 1998. Guidance for data quality assessment: practical methods for data analysis. EPA QA/G-9 Ver. QA96. Washington (DC): Office of Research and Development.