

# The Efficacy of Ozonated Water in Biofilm Control in USP Purified Water Circulation and Storage

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This article presents a case study for the use of ozone to reduce the amount of biofilm contaminant in a pilot UPW production and delivery system designed to represent typical large 316L stainless steel systems in biopharmaceutical companies.

**S**torage and distribution systems for water and water-based fluids are both critical and ubiquitous in every industry, geography, and culture. Large scale industrial water handling systems require water storage and distribution for a range of applications from simple thermal control (cooling and heating systems) to Purified Water (PW) production and delivery.

For more than 40 years, manufacturers of pharmaceutical products have been concerned about potential microbial contamination of their water systems. The action and alert limits commonly cited in literature are based on sampling of the water from a use point, inoculation and incubation of a nutrient plate, and counting the resulting bacteria. These point-of-use samples are simply the planktonic concentration of the bacteria in the water and may not represent other contamination sources, i.e., biofilms.

This article provides a case study for the use of ozone to reduce the amount of biofilm contaminant in a pilot PW production and delivery system designed to represent typical large 316L stainless steel systems in biopharmaceutical companies.

While there are a number of potential sources of contamination in storage and delivery systems for purified and sterile water, one of the most common problems facing PW production and delivery is the prevention and removal of biofilms.

First described by Henrici<sup>1</sup> and Zobel<sup>2</sup> more than 60 years ago, these tenacious thin films form on almost any natural or synthetic surface and wherever surface-associated microbes are present. Once established, biofilm-producing microbes Excrete Polymeric exopolysacharrides (EPS) film that cannot be effectively removed using conventional antimicrobial Reagents.<sup>3,4</sup> The EPS provides a matrix where nutrients are retained and microbes can thrive, thereby continuously contaminating the PW storage and distribution system. Biofilms occur in a wide variety of systems that can range from the biological (e.g., plant life, gastrointestinal tracts, etc.) to the highly technological (e.g., medical and dental implants).<sup>5-12</sup>

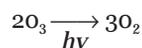
The removal of biofilms from the wetted surfaces of PW systems is thus a prerequisite for the maintenance of high purity water quality in many industries. Elimination of this source of microbial contamination is critical, but can be exceedingly difficult.

Studies have shown that the chemical composition of biofilm matrices varies depending on both the source of the originating microbial contaminant and the environment within which the biofilm grows.<sup>3,13,14</sup> This compositional variability makes targeted destruction of biofilms difficult, encouraging the use of non-specific treatments that can address its heterogeneity. Conventional antimicrobials may not penetrate the protective EPS film, and microbes that in planktonic form can be controlled through the use of a particular biocide may instead become extremely resistant to decontamination when resident within a biofilm matrix. Biofilm removal treatments

also must be biocidal, as removal of the biofilm's EPS matrix liberates the underlying colonizing microbes. Unless destroyed, these underlying microbes will migrate and reestablish at new sites, maintaining the contamination of the water system.

These prerequisites for biofilm control have resulted in a preference in most industrial settings to employ strong oxidant chemistries for biofilm mitigation and removal. Typically, chemicals such as chlorine, organochlorides and peroxychlorides have been preferred. Recently, drawbacks with these conventional approaches, such as water contamination concerns, tightening environmental regulations, and chemical costs, have led different industries to explore the use of ozonated water for the removal of biofilms and destruction of microbial contamination.<sup>3</sup>

Ozone (O<sub>3</sub>), an unstable allotrope of oxygen, reacts rapidly with most hydrocarbons to effectively destroy biofilms, microbes, and organic residue material within these films.<sup>15</sup> As the strongest commercially available oxidant, it has a disinfecting strength 3000 times that of chlorine. At appropriate concentrations, ozone injected in water destroys all microorganisms, viruses, oocysts, and pyrogens, and reduces Total Organic Carbon (TOC) by chemical oxidation. Ozonated water leaves no chemical residues, unlike other chemical purification procedures, and in ambient water ozone reverts back to oxygen within approximately 20 minutes. Any excess or residual ozone also can be easily and immediately destroyed through exposure to Ultraviolet (UV) irradiation according to:



Many treatments can effectively reduce microbial contamination in a water distribution system; however, for PW, it is critical that all microbial contamination be removed. Feinstein, in an article published online in ALN Magazine,<sup>16</sup> provides effective definitions for sanitization, disinfection and sterilization:

**“Sanitization will offer a contamination reduction or bio-burden reduction of 99.9% or 3 log (10<sup>3</sup>). This means that we can expect that out of one million microorganisms, a sanitizer will destroy approximately 990,000 of the organisms leaving behind many viable microorganisms to reproduce. Sanitization is accomplished by utilizing chemicals and gels to achieve this level of cleanliness**

**Disinfection will offer a bio-burden reduction of 99.99% and up to 99.999% or up to 5 log (10<sup>5</sup>). This means that we can expect that out of one million microorganisms, a disinfectant will destroy up to 999,990 of the organisms leaving behind very few, but still some, viable organisms. Disinfection is accomplished by utilizing many different chemicals or ultraviolet light.**

**Sterilization is the statistical destruction of all microorganisms and their spores. This is defined as 6 log (10<sup>6</sup>) or a 99.9999% reduction. Statistically, this definition is accepted as zero viable organisms surviving. Sterilization is accomplished via several methods including ionized hydrogen peroxide or other hydrogen peroxide based solutions, high heat, ultraviolet light, ozone, radiation, and chemicals (chlorine, formaldehyde, glutaraldehydes, etc.).”**

For PW production, especially for pharmaceutical applications, the latter category should be achieved within production, storage and distribution systems to ensure that planktonic biofilm microbes are not sampled, potentially providing increased readings for tested parameters. Strong continuing mitigation of biofilm may ensure compliance of the water system.

The advent of ASTM standard E2500<sup>17</sup> removed a number of impediments to the implementation of ozone-based purification in pharmaceutical manufacturing, encouraging improvements in Process Analytical Technology (PAT) through well-documented, robust and flexible manufacturing capabilities. Since then, the confluence of continuously rising energy costs, process simplicity and political pressure for lower pharmaceutical prices has helped define newer technologies, such as ozone, to replace heat shock (hot water sanitization, steam, etc.) and chemical disinfection using chlorine, chlorides, peroxides, etc. Simple injection and mixing of gaseous ozone into the water is sufficient to produce concentrations suitable for microbe-free PW. Ozone is both safe and economical to use since it can be reliably generated on-site as needed, avoiding the handling and costs associated with strong oxidant transportation and storage. It is generated at ambient temperature and is soluble in ambient temperature water, increasing ease of operation. The infrastructure requirements for thermochemical sterilization and subsequent decontamination are significant and the use of ozonated water can greatly reduce capital, operations, and maintenance costs of water treatment.<sup>18</sup>

This study describes tests in which a pilot scale USP purified water storage and distribution system was challenged using a minimum of 10<sup>6</sup> logs of *E. coli* (ATCC #8739) that were either inoculated into the recirculating purified water system in planktonic form (Challenge Test A) or established as biofilms on stainless steel coupons placed in the distribution system (Challenge Test B). The efficacy of ozonated water treatment for *E. coli* biofilm removal and system sterilization was tested by ozone treatment of these contaminations at three different ozone concentrations at three time periods. Resulting counts of test Colony-Forming Units (CFUs) determined the amount of log reduction of the microbial contamination.



Figure 1. The full experimental test skid and water system used in this study.

## Equipment and Procedures

Figures 1 and 2 are photos of the purified water storage and distribution system employed in this study. Figure 3 provides a schematic diagram detailing the components of this experimental test bed. The purified water storage and distribution skid, consisting of a 30 ft, 316L stainless steel water loop, was designed and manufactured for this study. Configured within the loop were an automated integrated water ozonation system, a 200 liter closed storage tank equipped with an ozone destruct unit, a recirculation pump, and a sample coupon rack for the sterile stainless steel coupons. Treatment products used to create Deionized (DI) process water, a conductivity meter, and other non-ozonated related components were supplied for this study. For all references, see manufacturers' identification at the end of this article.

The ozonated water within the loop was monitored for ozone concentration using an external dissolved ozone concentration monitor with a range of 0 to 10 ppm. A separate conductivity meter measured the water's conductivity. The integrated automated water ozonation system provided up to 30 gpm (113.6 liters per minute, lpm) of ozonated USP PW by an ozone generator fed by an oxygen concentrator. The automated water ozonation system comes equipped with standard components of an ozone generator, Pressure Swing Absorption (PSA) oxygen concentrator, dissolved ozone monitor (0 to 10 ppm range), and process water flow meter, with integrated degas capability and safety monitoring. The unit's optional UV destruct attachment was included for purposes of this evaluation.

Two test procedures were employed in the study. In the first (Challenge Test A), the recirculating ozonated water was inoculated to achieve at least  $10^6$  CFU/mL of *E. coli* in the system. After inoculation the system was run with an ozone concentration of 2 ppm in the process water, and the bacteria contamination level was monitored. In the second procedure

(Challenge Test B), six duplicate stainless steel coupons were aseptically inoculated with at least  $10^6$  CFU/coupon of *E. coli*. After the formation of a surface biofilm of at minimum  $10^6$  CFU/coupon, the coupons were placed into the coupon holder in the recirculating ozonated water loop. Coupon decontamination was evaluated at three separate ozone concentrations of 0.5, 2, and 5 ppm respectively. Coupons were collected for testing for *E. coli* after 2, 5, and 10 minutes exposure to each of the various concentrations of ozonated water. Each experiment was performed with new coupons inoculated according to the same procedure. An additional coupon experiment with no ozone (0 ppm) was run to establish a comparative baseline.

## Methodology

### Pilot USP PW Water Storage and Distribution System Testing

#### Initial Test System Sanitization

Prior to initiation of the test series, the USP PW water system was twice drained and refilled with fresh DI water to purge any contaminants, and the ozone monitors were recalibrated to a zero setpoint. The system was then sanitized by ozonated



Figure 2. Close-up of the storage tank and coupon sampling system.

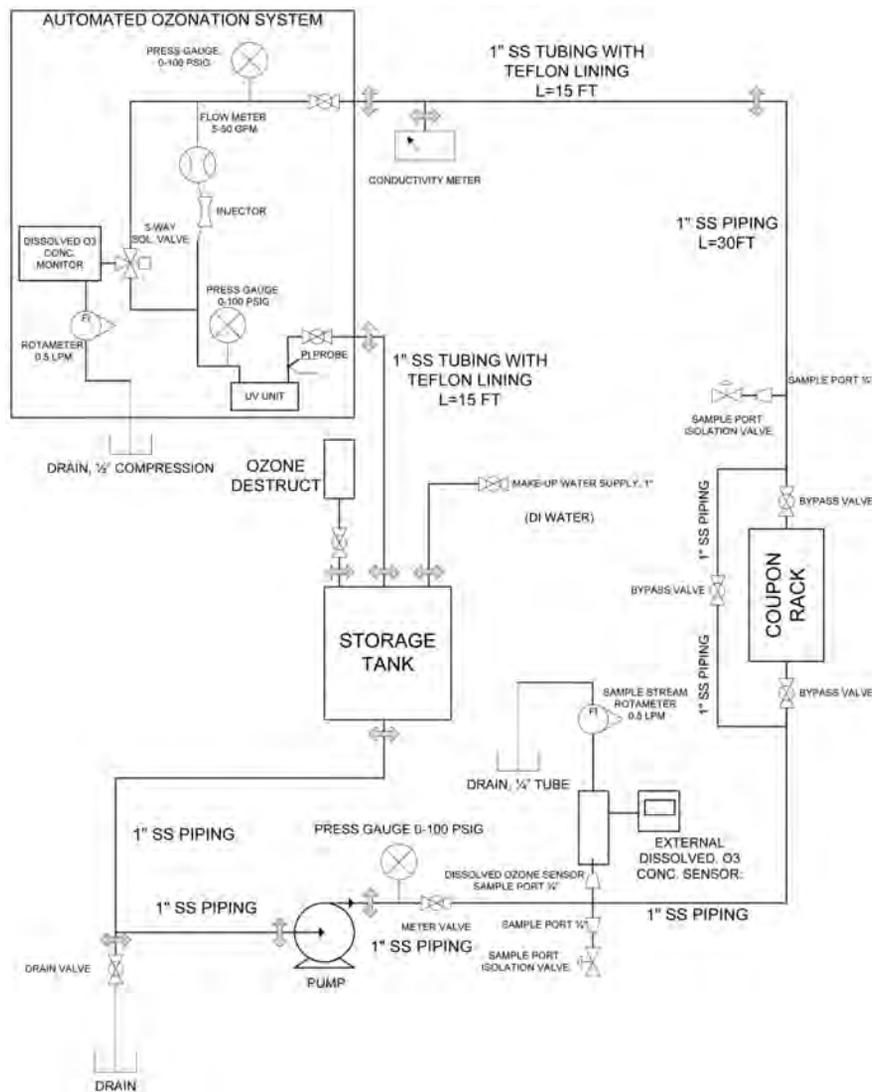


Figure 3. Schematic of the purified water storage and circulation test bed around the automated ozonation system.

ing the recirculating water for one hour using the automated ozonation system at a setpoint of 5 ppm. After the hour-long sanitization, the ozone generator was turned off and the UV was activated to destroy any residual ozone. The system then ran for an additional 30 minutes with the UV destruct operational to lower the ozone concentration to the lower measurement limit of the ozone monitor ( $< 40$  ppb). At this point, the water was sampled and its conductivity measured to ensure the water met USP PW criteria as described in USP  $<1231>$ , of Heterotrophic Plate Count (HPC)  $< 100$  CFU/mL, TOC  $\leq 500$  ppb, and conductivity  $< 1.3$   $\mu$ S/cm. Figure 4 shows the ozone profile for this initial sanitization as measured at the ozonation system.

After an initial rapid increase of the dissolved ozone concentration in the recirculating PW, the temporary concentration drops briefly as the automated ozonation system adjusts

the ozone generator power to achieve the optimal long-term setpoint. After the time period is completed, the final measurable ozone is quickly destroyed by turning off the generator and activating the UV destruct.

### Challenge Test A: Planktonic *E. coli* Testing

After the production of USP PW within the water storage and distribution system had been confirmed, the efficacy of ozonated water for the decontamination of planktonic *E. coli* was tested (Challenge Test A).

In this initial test, baseline water samples were first obtained and measured. The UV destruct was then turned off and an inoculum preparation volume appropriate to achieve  $10^6$  CFU/mL concentration of *E. coli* in the USP water recirculation loop was aseptically transferred to the system using the internal sampling port with a sterile funnel. Following the transfer, water was allowed to circulate for approximately 5 minutes at 12 gpm to ensure uniform distribution of the inoculum throughout the system. The challenge populations of *E. coli* within the system were determined by aseptically collecting 120 mL of system water from the drain port after the coupon rack and analyzing the sample. Samples were refrigerated immediately following collection. The system water control samples were tested by preparing dilutions in PB (Phosphate Buffer) water

and plate dilutions of  $10^{-1}$  through  $10^{-6}$  to Tryptic Soy Agar (TSA) in duplicate. The plates were incubated and *E. coli* counts determined as described above. The system challenge

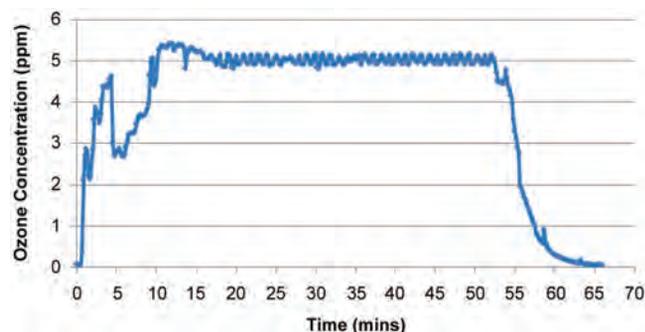


Figure 4. Ozone concentration profile at generation system return - initial system sanitization at 5 ppm.

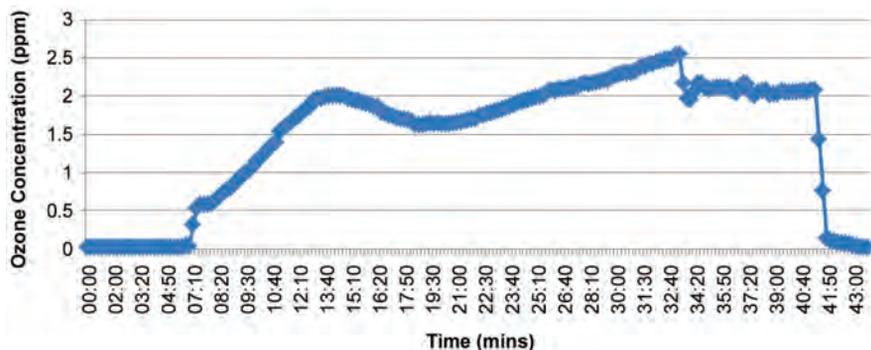


Figure 5. Ozone concentration profile during the planktonic system challenge test (Challenge Test A) – 2 ppm ozone challenge.

analysis had to exhibit at least a  $1.0 \times 10^6$  CFU/mL population of the challenge organism for acceptance.

The inoculums for these tests were prepared as follows: 4L of Trypticase Soy Broth (TSB) was inoculated with *E. coli* (ATCC# 8739) and incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 48 hours. The inoculum population in the TSB was confirmed by preparing dilutions in sterile Phosphate Buffer (PB) water and plating to Tryptic Soy Agar (TSA) plates. These plates were incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 18 to 24 hours and the colonies counted to confirm the concentrations in the inoculums. Once the control population of *E. coli* was established within the water storage and distribution system, ozonation tests were performed. Following collection of a control sample, the automated ozonation water system was turned on and set to achieve a concentration of ppm ozone. Figure 5 shows the ozone concentration profile over the duration of the test. 120 mL samples of the system water were aseptically collected from the sample port located after the coupon rack at 2, 5, 10, and 30 minutes after the initiation of ozonation. Samples were refrigerated until they could be analyzed. Ozone concentrations within the system were determined for each sample collection.

After all samples had been collected, the ozone generator was turned off and the UV destruct was initiated. The system was run for 30 minutes or until the measured ozone concentration was below 40 ppb, the lowest possible measurement threshold for the ozone monitor. This residual ozone should not influence testing results, as lower measurements cannot be detected with accuracy. The system was then allowed to continuously recirculate process water.

After an initial rapid increase of the dissolved ozone concentration in the recirculating PW, the temporary concentration drop is caused when the automated ozonation system adjusts the ozone generator power to achieve its long-term setpoint. Any measurable ozone is quickly destroyed after turning off the generator and activating the UV destruct.

Each collected sample was analyzed as follows: dilutions of  $10^{-1}$  through  $10^{-6}$  were aseptically plated to TSA in duplicate. 1.0 mL, 10 mL, and 100 mL samples were aseptically

filtered and rinsed using USP Fluid D, and the filters transferred to individual TSA plates. Plates were labeled with the sample time and dilution. All plates were incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 24 to 48 hours after which the colonies were counted and the CFU/mL for the system was determined for each sample time point. Using the CFU/mL at a given sample time and the initial challenge population, the log reduction in the system was determined for each time point.

After Challenge Test A, the water system was drained, refilled with DI water, and sanitized with ozone using the automated ozonation system. The system water was then verified as meeting USP Purified Water criteria per <1231> prior to commencing the next series of testing.

#### Challenge Test B: Adherent *E. coli* Biofilm Testing on Coupons

In Challenge Test B, four sequences of testing were conducted by varying the ozone setpoint concentration at 0 ppm, 0.5ppm, 2.0 ppm, and 5ppm. The effect of ozone on a biofilm of *E. coli*-inoculated on 316L stainless steel (SS) coupons was then measured at three different exposure time periods of 2 minutes, 5 minutes, and 10 minutes per concentration, totaling six coupons per concentration. The negative test without ozone (0 ppm) was run before the first ozonation sequence test to establish a comparative baseline for the experiment.

To ensure the destruction of any residual ozone in the time period between the concentration tests, the water system was allowed to run continuously with both the pump and the UV destruct on, ensuring both recirculating water flow and ozone destruction via the UV system. The ozone limit was confirmed to be < 40 ppb, the lowest possible measurement threshold for the ozone monitor. The production of USP Purified water also was confirmed before commencing each challenge test.

Inoculums for coupon testing were prepared as follows: a biofilm of *E. coli* was grown on a TSA plate and incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 48 hours. The plate was then harvested using a sterile hockey stick and PB water to prepare the inoculums stock. The inoculums stock population was verified by preparing dilutions in PB water and plating to TSA. The plates were incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 18 to 24 hours after which the colonies were counted and the stock population confirmed.

Sterile stainless steel coupons were aseptically inoculated with the *E. coli* inoculums described in the preceding paragraph to achieve at least  $1 \times 10^6$  CFU/coupon upon recovery. The inoculums were spread on each coupon using a sterile glass hockey stick and allowed to dry for 15 to 30 minutes.

Inoculated coupons were aseptically transferred into separate covered sterile sample containers and stored covered until use.

Two positive control samples were prepared as above and retained for the determination of the challenge CFU/coupon. The challenge CFU/coupon was determined by first placing each control coupon into a sterile covered container with 100 mL of sterile PB water. The container with the coupon and PB water was then sonicated at 40 Hz for 10 minutes. Dilutions of  $10^{-1}$  through  $10^{-5}$  were prepared for each control coupon and plated to TSA plates which were incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 24 to 48 hours. Following incubation, the colonies on each control coupon were counted and the average CFU/coupon was determined for the challenge.

The positive control coupon acceptance criterion was the demonstration of at least  $1.0 \times 10^6$  CFU/coupon of the challenge organism.

### *Baseline Test (Inoculated Coupons; Ozone Concentration at 0 ppm)*

At the beginning of the Baseline Tests, two *E. coli*-inoculated coupons prepared as described above were placed into the coupon rack (Figure 2) using a wire mesh holder designed to keep the coupons vertical during the test. The coupon holder was then sealed and the system circulation initiated without ozone present in the system. After 2 minutes, the coupons were aseptically removed from the coupon rack and placed into 100 mL of PB water in a separate covered container labeled with the sample time point. This procedure was repeated with two new inoculated coupons with the only variation being that the coupons spent 5 minutes in the coupon rack exposed to the system water. This procedure was repeated a third time with an additional two new coupons and an exposure time of 10 minutes in the water system. All coupons were refrigerated immediately after collection. Each coupon was extracted by first sonicating the container, coupon and PB water for 10 minutes at 40 Hz, then preparing and plating dilutions of  $10^{-1}$  through  $10^{-5}$  in duplicate onto TSA. The plates were then incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 24 to 48 hours after which the colonies were counted and the average CFU/coupon determined. This analysis was repeated for each sample coupon. Using the average CFU/coupon and the initial challenge population as determined above, the average log reduction of the coupons was determined for each time point.

### *Ozone Tests (Inoculated Coupons; Ozone Concentrations at 0.5, 2.0, 5.0 ppm)*

At the start of the first test sequence, an ozone concentration of 0.5 ppm was established in the circulation system. Once the system ozone concentration had stabilized at 0.5 ppm, the following procedure was used for test sequence #1:

1. Two inoculated coupons, prepared as described above, were aseptically placed into the coupon rack using a wire mesh holder designed to keep the coupons vertical during the test.
2. The coupon rack was sealed and the coupons exposed to the recirculating ozonated water for a period of 2 minutes.
3. At the end of this time, the coupons were aseptically removed from the coupon rack and placed into 100 mL of sterile PB water in a separate covered container labeled with the time point. The ozone concentration in the system at each sample time point was recorded.

Steps 1 through 3 were repeated in test sequences #2 and #3 which each employed fresh inoculated coupons and one difference in the procedure: for test sequence #2, the exposure time was 5 minutes; for test sequence #3, the exposure time was 10 minutes. All samples were refrigerated until they were extracted. The samples were extracted by first sonicating the coupon/PB water containers for 10 minutes at 40 Hz. Dilutions of  $10^{-1}$  through  $10^{-5}$  in duplicate were prepared and filtered for each coupon and transferred to TSA plates. The plates were then incubated at  $32.5 \pm 2.5^\circ\text{C}$  for 24 to 48 hours, after which the colonies were counted and average CFU/coupon was determined. Using this value and the initial challenge population, the log reduction for the sample time point was calculated.

All three of the above test sequences and analyses were repeated using ozone concentrations of 2 and 5 ppm. Between each test sequence, the water system was drained, refilled, and ozone sanitized using the automated water ozonation system. The system water was verified as meeting USP Purified Water criteria per <1231> prior to commencing each test sequence.

### *Negative Coupon Controls (Non-inoculated coupons; not used in ozone system)*

Negative coupon controls were prepared by placing a sterile SS coupon that had not been inoculated with *E. coli* in a sterile sample container with 100 mL of PB water. The coupon was then sonicated in the PB water container for 10 minutes at 40 Hz and then the entire 100 mL was aseptically filtered, rinsed with Fluid D and the filter transferred to a TSA plate. The plate was incubated at  $32.5 \pm 2.5^\circ\text{C}$  with the test samples.

### *Log Tabulation*

The microbiological test protocol was designed so that a series of dilutions would be plated to ensure countable plates. The lowest dilution plated from the coupon was 1:10. Therefore, if there was no growth on the plate, it would be reported as < 10 with a log value of 1. The log recovered would be subtracted from the challenge Log 6.4. Therefore, the sensitivity of the dilutions only allowed for total log reduction reporting of  $\geq 5.4$ .

Recirculation Time	Ozone Concentration	Population Recovered
5 min	0 ppm	$2.6 \times 10^6$ CFU/ml

Table A. Baseline for Challenge Test A: Population of *E. coli* in the USP PW storage and distribution system, following inoculation and prior to ozone treatment.

Exposure Time	Ozone Concentration		Log Reduction
	Ozone Generation System Exit	Test Skid	
2 min	0.825 ppm	0.17 ppm	0.0
5 min	1.651 ppm	0.73 ppm	0.0
10 min	1.790 ppm	1.56 ppm	1.0
30 min	2.112 ppm	0.31 ppm	6.0

Table B. Challenge Test A: Log reduction of planktonic *E. coli* at  $2.6 \times 10^6$  CFU/mL in the USP PW storage and distribution system, using a 2 ppm ozone concentration setpoint.

## Results and Discussion

*E. coli* was chosen to challenge the ozonated water sterilization protocol since it is a well understood microorganism that is known to colonize surfaces and which has been shown to produce biofilms on those surfaces.<sup>18,19</sup> As well, *E. coli* within a biofilm matrix have been shown to be resistant to disinfection using conventional chemical approaches, such as hypochlorous acid or monochloramine.<sup>20</sup>

As such, *E. coli* constitutes an excellent challenge species in determining the efficacy of ozonated water for the removal of biofilms.

At the beginning of each day of testing, positive control coupons were analyzed and the presence of *E. coli* contamination on the coupons' surface at a level of  $2.6 \times 10^6$  CFU/coupon was experimentally verified. This verification was taken as confirmation that the coupons used in subsequent tests met the acceptance criterion.

The results of the planktonic *E. coli* challenge tests are shown in Tables A and B. The data in Table A clearly establish that the inoculum preparation and system loading procedures described above produced contaminant concentrations of planktonic *E. coli* in the pilot scale storage and distribution system that met the test acceptance criterion. The concentration of *E. coli* in the water system was determined to be  $2.6 \times 10^6$  CFU/ml.

After the *E. coli* concentration baseline was established, the ozone generation

Exposure Time	Ozone Concentration	Average Log Recovered	Log Reduction
2 min	0 ppm	4.4	2.0
5 min	0 ppm	4.3	2.1
10 min	0 ppm	4.2	2.2

Table C. Challenge Test B: Log reduction of *E. coli* on inoculated SS coupons at initial value  $2.6 \times 10^6$  CFU/coupon. Ozone concentration in the circulating process water: 0 ppm (Baseline).

began to achieve the 2 ppm setpoint. The following time measurements were taken from when the generator was first turned on, and includes the ramp up and stabilization of the ozone concentration in the complete water system.

The negative control samples evaluated in this test sequence all proved satisfactory. The results of the planktonic challenge are presented in Table B and these data clearly show that a 30 minute ozone sterilization treatment of the inoculated pilot scale USP PW system using 2 ppm ozone concentration reduced the contaminant *E. coli* concentration by the desired 6.0 log reduction. The results of the tests evaluating the efficacy of ozonated water for the removal of *E. coli* and biofilms from stainless steel coupons are presented in Tables C through F.

Table C shows the results of the baseline tests with no ozone (0 ppm) present, where biofilm-inoculated SS coupons were placed in the coupon rack of the USP PW storage and distribution system and PW containing no ozone was circulated over the coupons at a flow rate of 12 gpm.

After exposure to PW at 0 ppm ozone concentration, the average log recovered value for all *E. coli* coupon contamination was 4.3. The average log reduction in surface contamination on these coupons for all test durations was thus 2.1. These results showed that, following an initial, rapid 2.0 log reduction in *E. coli* concentration during the first 2 minutes, the rate of *E. coli* loss from the coupon surface plateaued. It

Exposure Time	Ozone Concentration at Coupon Placement (ppm)		Ozone Concentration at Coupon Removal (ppm)		Total Log Reduction
	OGSI	PC	OGSI	PC	
2 min	0.472	0.54	0.486	0.55	4.9
5 min	0.480	0.58	0.470	0.58	≥ 5.4
10 min	0.491	0.58	0.498	0.59	≥ 5.4

OGSI – Ozone Generation System Input  
 PC – Post-Coupon measurement point  
 Note: Adjusted Log Reduction = Log Recovered (no ozone time point) – Log Recovered (ozone time point)

Table D. Challenge Test B: Log reduction of *E. coli* on inoculated SS coupons at initial value  $2.6 \times 10^6$  CFU/coupon. Ozone concentration in the circulating process water: 0.5 ppm.

Exposure Time	Ozone Concentration at Coupon Placement (ppm)		Ozone Concentration at Coupon Removal (ppm)		Total Log Reduction
	OGSI	PC	OGSI	PC	
2 min	1.941	1.77	1.939	1.80	5.4
5 min	1.930	1.75	1.895	1.83	≥ 5.4
10 min	1.958	1.86	1.993	1.85	≥ 5.4

OGSI – Ozone Generation System Input  
PC – Post-Coupon measurement point

Table E. Challenge Test B: Log reduction of *E. coli* on inoculated SS coupons at initial value  $2.6 \times 10^6$  CFU/coupon. Ozone concentration in the circulating process water: 2.0 ppm.

Exposure Time	Ozone Concentration at Coupon Placement (ppm)		Ozone Concentration at Coupon Removal (ppm)		Total Log Reduction
	OGSI	PC	OGSI	PC	
2 min	5.215	4.92	4.909	4.75	≥ 5.4
5 min	4.636	4.81	4.912	5.01	≥ 5.4
10 min	4.912	4.92	4.782	4.92	≥ 5.4

OGSI – Ozone Generation System Input  
PC – Post-Coupon measurement point

Table F. Challenge Test B: Log reduction of *E. coli* on inoculated SS coupons at initial value  $2.6 \times 10^6$  CFU/coupon. Ozone concentration in the circulating process water: 5.0 ppm.

can be assumed that the sloughing of *E. coli* biofilm from the coupon surface was primarily due to water flow and pressure.

Following the baseline tests, tests for the efficacy of ozone exposure in removal of the *E. coli* and biofilms on the coupon surface were performed.

Table D shows the results from the first series of ozonated water tests in which the biofilm contaminated coupons were exposed to an ozone concentration of 0.5 ppm in the recirculating USP PW and subsequently analyzed for *E. coli* contamination. The results show that 2 minutes exposure to 0.5 ppm ozone in ultrapure water was insufficient to achieve the maximum decontamination, with the total log reduction after 2 minutes measured at a value of 4.9. After 5 minutes, the log reduction of *E. coli* reached a steady state value of ≥ 5.4 with no further reduction observed in the samples that were exposed to the ozonated water for 10 minutes. The baseline and 0.5 ppm ozone concentration tests were performed on the same day.

The results for sterilization tests performed at ozone concentrations of 2 ppm and 5 ppm were performed on the second day of testing and the results are shown in Tables E and F. The coupon challenge for these data indicated that while some of the challenge organism remained on the coupon surface after exposure for 2 minutes to 2 ppm ozonated water, after 5 minutes, no contamination was detectable. For coupons exposed to 5 ppm ozonated water, no contamina-

tion was detectable on any of the coupons at all the time points for exposure to the ozonated water. Note, the “≥ 5.4 log”= figure pertains to ozone removal alone, as shear-related biofilm removal has been subtracted out.

### Conclusion

Ozone is increasingly used as both a sanitant and a sterilizing agent in pharmaceutical facilities. As a non-specific agent, the efficacy of ozone is related to the contact duration (time), method of action against the specific contaminant, ozone concentration, and water parameters such as temperature and conductivity.

In this study, a pilot USP PW production and delivery system using ozone sanitization was designed to represent typical large 316L stainless steel systems run at ambient temperatures in biopharmaceutical companies. Challenge Test A provided an overview of the time required to sanitize a contaminated system using ozone. Under these test conditions, in 30 minutes, ozone had achieved a 6-log sanitization (or sterilization). It is likely that using other methods to achieve an

equivalent sanitation would likely require a longer time, with greater energy expended, plus significant additional minimum grade of USP PW water for system refill and flush to cleanse residuals from the system.

Challenge Test B demonstrated how ozonated water treatment can provide an effective means for biofilm removal and sterilization in UPW PW storage and circulation systems. The results indicate that ≥ 5 minutes exposure to ozonated water at concentrations of 0.5 ppm, 2.0 ppm, or 5.0 ppm ozone is sufficient to produce surface sterilization. The stainless steel coupons contaminated with  $2.6 \times 10^6$  CFU/coupon of *E. coli* biofilm were sterilized under these conditions, with no challenge organism detectable following treatment with the ozonated water.

Through these experiments, ozone has proved to be highly and quickly effective against biofilm, at multiple concentrations and time points. It effectively sanitizes and sterilizes both contaminated water (planktonic biofilm) and contaminated surface biofilm. Ozone is shown to be effective in a matter of minutes, and in higher concentrations (e.g., “shock”) it can impact biofilm even more quickly.

While multiple studies have demonstrated the overall effectiveness of ozone, the tests described above provide a quantifiable real-world simulation for a pharmaceutical facility. Additional studies can be conducted to simulate a larger PW system, and/or test the use of alternative materials

of construction (e.g., PVDF or other non-metallics) for the piping system. The use of a well-designed ozone system able to provide a steady, measurable, and adjustable ozone output concentration allows this technology to prove its effectiveness and ultimately its value in mitigating biofilms and other potential water system contaminants.

## References

- Henrici, A.T., "Studies of Freshwater Bacteria," *Journal of Bacteriology*, 25 (1932) 277.
- Zobell, C.E., and C.E. Allen, "The Significance of Marine Bacteria in the Fouling of Submerged Surfaces," *Journal of Bacteriology*, 29 (1935) 230.
- Tachikawa, M., K. Yamanaka, and K. Nakamuro, "Studies on the Disinfection and Removal of Biofilms by Ozone Water Using an Artificial Microbial Biofilm System," *Ozone: Science and Engineering*, 31, (2009) 3.
- Allison, D.G., "The Biofilm Matrix," *Biofouling*, 19 (2003) 139.
- Vlamakis, H., "The World of Biofilms," *Virulence*, 2, (2011) 431.
- Simoes, M., L.C. Simoes, and M.J. Vieira, "A Review of Current and Emergent Biofilm Control Strategies," *LWT – Food Science and Technology*, 43 (2010) 573.
- Bohus, V., Z. Keki, K. Marialigeti, K. Baranyi, G. Patek, J. Schunk and E. M. Toth, "Bacterial Communities in an Ultrapure Water Containing Storage Tank of a Power Plant," *Acta Microbiologica et Immunologica Hungarica*, 58 (2011) 371.
- Momba, M.N.B., R. Kfir, S.N. Venter, and T.E. Cloete, "An Overview of Biofilm Formation in Distribution Systems and its Impact on the Deterioration of Water Quality," *Water SA*, 26, (2000), 59.
- Mittelman, M.W., "Biofilm Development in Purified Water Systems," in *Microbial Biofilms*, H. M. Lappin-Scott and J. William Costerton, Eds., Cambridge University Press, Cambridge, UK, (1995) pp 133-147.
- Santosh, R.P., J. Veeriah, K. Baskanan, and L. Shu, "Fouling in Reverse Osmosis (RO) Membrane in Water Recovery from Secondary Effluent: a Review," *Reviews in Environmental Science and BioTechnology*, 11 (2012) 125.
- Lindsay, D. and A. von Holy, "Bacterial Biofilms within the Clinical Setting: What Healthcare Professionals Should Know," *Journal of Hospital Infection*, 64 (2006) 313.
- Bixler, G.D. and B. Bhushan, "Biofouling: Lessons from Nature," *Philosophical Transactions of the Royal Society A*, 370 (2012) 2381.
- Meyer, B., "Approaches to Prevention, Removal and Killing of Biofilms," *International Biodeterioration and Biodegradation*, 51 (2003) 249.
- Bridier, A., R. Briandet, V. Thomas, and F. Dubois-Brissonnet, "Resistance of Bacterial Biofilms to Disinfectants: a Review," *Biofouling*, 27 (2011) 1017.
- Greene, A.K., Z.P. Guzel-Seydim, and A.C. Seydim, "Chemical and Physical Properties of Ozone" in *Ozone in Food Processing*, Colim O'Donnell, B.K. Tiwari, P.J. Cullen and R. G. Rice - Editors, John Wiley and Sons, Oxford, UK (2012) pp 19-31.
- Feinstein, S., "How Clean is Clean," *ALN Magazine*, posted 4 November 2010, <http://www.alnmag.com/article/how-clean-clean?page=0,0>
- ASTM Standard E2500, 2012, "Standard Guide for Specification, Design and Verification of Pharmaceutical and Biopharmaceutical Manufacturing Systems and Equipment," ASTM International, West Conshohocken, PA, DOI: 10.1520/E2500-07R12, [www.astm.org](http://www.astm.org).
- "Economic Efficacy Comparison Between Ozone and Hot water Sanitization" presented by Nissan Cohen, ISPE Critical Utilities Conference, February 2013
- Reisner, A., J.A.J. Haagensen, M. A. Schembri, E.L. Zechner, and S. Molin, "Development and Maturation of *Escherichia coli* K-12 biofilms," *Molecular Microbiology*, 48 (2003) 933.
- Beloin, C., A. Roux, and J-M. Ghigo, "*Escherichia coli* Biofilms," *Current Topics in Microbiology and Immunology*, 322 (2008) 249.
- Williams, M.W. and E.B. Braun-Howland, "Growth of *Escherichia coli* in Model Distribution System Biofilms Exposed to Hypochlorous Acid or Monochloramine," *Applied and Environmental Microbiology*, 69 (2003) 5463.

## Additional References

- *ISPE Baseline® Pharmaceutical Engineering Guide, Volume 4 – Water and Steam Systems*, International Society for Pharmaceutical Engineering (ISPE), Second Edition, December 2011, [www.ispe.org](http://www.ispe.org).
- *ISPE Good Practice Guide: Ozone Sanitization of Pharmaceutical Water Systems*, International Society for Pharmaceutical Engineering (ISPE), First Edition, July 2012, [www.ispe.org](http://www.ispe.org).
- Manufacturers' references: Automated Water Ozonation System: LIQUOZON® Ultra by MKS Instruments; PSA: Topaz Plus Dissolved ozone monitors: BMT 964 AQ LC; recirculation pump: ITT Goulds G&L series (model 1HM1D5C5). Purified water storage and distribution skid designed and manufactured for MKS Instruments by GMP Piping, Inc. Deionized (DI) process water treatment products, a conductivity meter, and other non-ozone related components supplied by Northeast Water Services Inc. Test performed at Microtest Labs and supported by Millennium Facilities Resources, Inc.

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