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Sanitization of an Automatic Reverse-Osmosis Watering System: Removal of a Clinically Significant Biofilm

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During environmental monitoring of our institution's rodent watering systems, one vivarium was found to have high bacterial loads in the reverse-osmosis (RO) automatic water system. These findings prompted evaluation of the entire RO water production and distribution system. Investigation revealed insufficient rack and RO system sanitization, leading to heavy biofilm accumulation within the system. Approximately 2 wk after discovery in the water system, one of the bacterial organisms isolated in the water supply, *Sphingomonas paucimobilis*, was isolated from a peritoneal abscess of a severely immunodeficient B6.Cg-*Slc11a1*^{r} Rag1^{tm1Mom}/Cwi mouse housed in the same vivarium, suggesting that rodents drinking from this system were being exposed randomly to fragments of biofilm. Plans were developed to sanitize the entire system. Hypercholorination was used first, followed by treatment with a combination of peracetic acid and hydrogen peroxide. Between system sanitizations, a low-level chlorine infusion was added to the system as a biocide. Heterotrophic plate counts and bacterial isolation were performed on water samples obtained before and after sanitization procedures. We here discuss the process of identifying and correcting this important water-quality issue.

Abbreviations: PRS, pressure-reducing stations; RO, reverse-osmosis.

The reliable provision of high-quality drinking water is essential for both the welfare of laboratory rodents and the scientific integrity of the studies for which they are used.[17] The *Guide for the Care and Use of Laboratory Animals* indicates that "watering devices, such as drinking tubes and automated water delivery systems, should be checked frequently to ensure appropriate maintenance, cleanliness, and operation," yet no specific recommendations are provided on how to perform such maintenance; how frequently devices should be checked; or what criteria institutions should aim to meet.[14] Veterinarians and animal facilities staff members at the University of Washington deemed that they needed to develop a single plan for maintenance of all animal-watering systems across campus. The University of Washington's Department of Comparative Medicine cares for approximately 780,000 animals across approximately 45,000 ft^2 of space. The program encompasses 6 main centralized animal vivaria; the water-delivery system is slightly different in each. This report focuses on a single vivarium located on the medical center campus. The vivarium is 5362 ft^2 and houses mice and rats in 49 racks of individually ventilated caging that are connected to an automated watering system. Baseline testing of this watering system and investigation into past maintenance revealed its upkeep to be insufficient, resulting in substantial bacterial contamination and biofilm development within the production and distribution components of the system. Bacterial counts were high, and shortly after discovery of the sizable biofilm, a clinical case believed to be directly associated with the biofilm's existence was noted. The current report discusses our findings, the clinical case associated with the watering system biofilm, and the steps taken to sanitize and update the automatic rodent-watering system.

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Materials and Methods

Watering system. The vivarium discussed waters the mice and rats that it houses via an automated watering distribution system (Edstrom Industries, Waterford, WI) supplied by an animal-specific reverse-osmosis (RO) production plant (Siemens Industry, Warrendale, PA). The system is a flow-through (that is, single-pass) RO system (Figure 1). The source of water is the City of Seattle municipal supply, which is filtered through an activated carbon filter prior to the RO purification process. Water is transferred into a fiberglass storage tower (150 to 175 gal [568 to 662 L]). The water is pressurized via a bladder tank prior to being pumped into the distribution lines. Distribution lines to and from the pressure-reducing stations (PRS) and into the holding rooms are constructed of stainless steel. From the point of the PRS, the distribution lines are flushed into the drain lines that empty into sinks located in various rooms of the vivarium. The system originally was designed to operate without the use of biostatic or biocidal agents. When required (for example, loss of pressure in an animal-specific RO system, power failure), back-up water supply is provided by a 'house RO' system (Siemens Industry). This system is a RO, service deionization system in which the distribution loop is fully recirculated, with a return loop that passes through a UV disinfection unit (Aquafine, Valencia, CA) and then through 0.2-µm pleated filters before the water returns to storage towers (capacity, 1500 gal each). Since its installation the system has been maintained, under contract, by the vendor (Siemens Industry).

Sample collection and bacterial isolation. Two points in the automatic watering system were chosen as water collection sites: the point where distribution lines drain to the sinks (distribution lines) and at the manual drains on the back of the

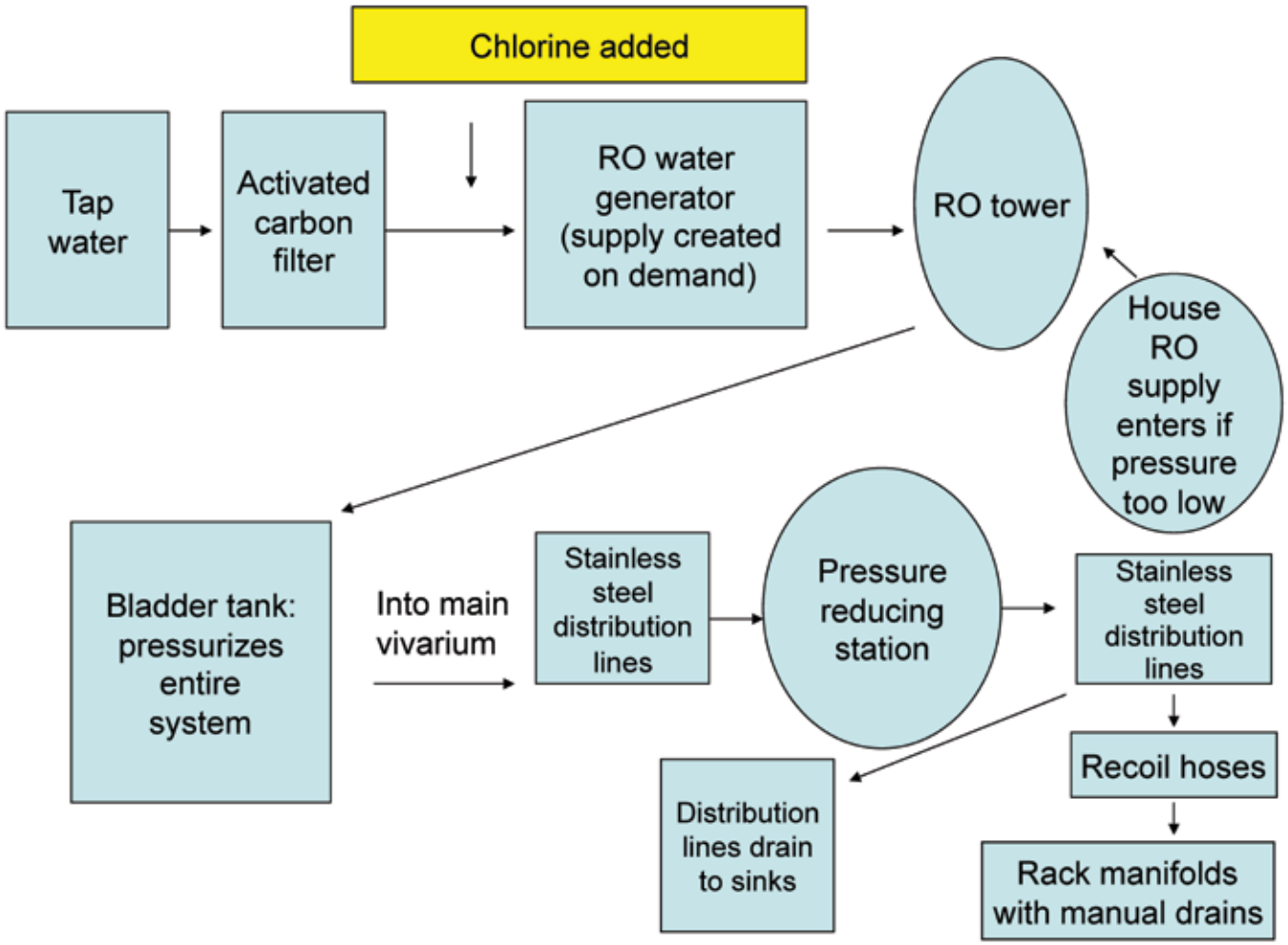

Figure 1. Schematic diagram of the automatic water system. The system is a single-pass RO system fed by municipal city water. Water samples were taken at the end of the distribution system, where the distribution lines drain into the sinks in animal holding rooms, and from manual drains on the back of individual rack manifolds. As part of the system sanitization procedures, a low concentration of chlorine (1–4 mg/L) was added to the system as a preventative biocidal agent. Chlorine is added to the water after it passes through the activated-carbon filter but before the water enters the RO membranes of the generator. At the start of the investigation, the back-up house RO, which is activated when water pressure drops, was cultured similarly to other components on the system; yeast, fungal, and bacterial counts all were less than 1 cfu/mL H_2O.

rack manifolds (racks); this site enables testing of the rack in addition to the watering system to which it is attached. Before sample collection, the external portion of the sample site was cleaned with 70% ethanol alcohol, and 'free-catch' samples were obtained after water was run from each site for 30 to 60 s. From each site, 500 mL water was collected in a sterile container. Sampling containers were capped immediately, chilled during transport from the facility, and stored at 4 °C. Samples were sent on ice to a commercial environmental laboratory (AmTest Laboratories, Kirkland, WA) for heterotrophic plate, fungal, and yeast counts. Bacterial plates were sent to a second laboratory (LabCor, Seattle, WA) for identification. Identification (genus or species or both) was made of novel-appearing bacterial colonies from original culture plates. The testing laboratories followed standard industry protocols for bacterial isolation and culture.[11,12]

Microbial water-quality goals. Acceptable microbial counts for campus automatic RO watering systems were based on previous testing and the evaluation of water samples obtained from campus facilities that provide water to rodents via water bottles. Water-bottle–only facilities provide autoclaved, acidified (pH 2.4 to 2.8) water (that is, sterile water), with heterotrophic plate counts of less than 1 cfu/mL H_2O, yeast counts of less than 1 organism per milliliter H_2O, and fungal counts of less than 1 organism per milliliter H_2O. The goal for our automatic RO systems was to achieve results similar to those of the acidified, autoclaved water from water bottles. Heterotrophic bacterial plate, yeast, or fungal counts greater than 1 but less than 10 cfu/mL were considered to be equivocal, and any heterotrophic bacterial plate, yeast, or fungal counts greater than or equal to 10 cfu/mL were considered to indicate unacceptable water quality.

Sanitization methods. ***Hyperchlorination.*** A portable deionized-water production unit, with a new inline 0.2-µm supply filter, was connected to the animal watering system to rinse and fill the RO tower. After the system's automated flushing cycle, the RO tower was drained. All recoil hoses were disconnected from racks. The RO storage tower was treated with 20 mg/L chlorine bleach (Clorox Company, Oakland, CA.). Chlorine test kits (Hach, Loveland, CO.) were used to verify that the chlorine level of the deionized water in the RO storage tower reached a concentration of at least 20 mg/L. The valve on the RO line at the level of the bottle filler was opened and allowed to drain water into the bottle filler drain for 4 to 6 min. Valves on the 2 PRS located at the farthest ends of the distribution system were opened, and water was drained into portable barrels for

4 to 6 min. Manual flush sequences were initiated twice from the system's control panel, which opened all solenoids in the PRS and allowed treated water to pass through the PRS into each set of room lines. Water from the distribution system was tested to ensure that a concentration of at least 20 mg/L chlorine was reached throughout the system at the end of the second manual flush. All wall-mounted quick-disconnects were activated, allowing treated water to exit. Once a level of 20 mg/L chlorine was verified throughout the system, a 1-h contact time was started.

After this contact time, the RO storage tower was flushed, drained, and rinsed with deionized water that had been filtered through a 0.2-µm filter until a chlorine level of 0 mg/L was reached. The valve on the RO line at the bottle filler was opened, and water was drained into the bottle filler drain for 6 min. Valves on the 2 PRS located at the farthest ends of the distribution system were opened, and water was drained into portable barrels for 4 to 6 min. Water from the RO storage tower was tested to ensure that chlorine treatment levels were reduced to 0 mg/L.

Once 0 mg/L was reached in the RO storage tower, the distribution system was flushed. Flushing continued until a chlorine level of 0 mg/L was reached; this goal was accomplished by initiating 2 manual-flush sequences from the system's control panel. This action opened all solenoids in the multiple PRS to allow deionized rinse water to pass through the PRS into each set of room lines. Chlorine levels at the room line drains were tested and determined to be 0 mg/L. All wall-mounted quick-disconnects were activated, allowing clean water to exit. The animal RO tower was drained of deionized water and refilled with RO water. All wall-mounted quick-disconnects were reconnected. Every rack in each room was flushed manually to ensure the absence of air bubbles and air locks in the rack manifold lines and to ensure that water flowed appropriately within each rack.

Treatment with peracetic acid–hydrogen perioxide. A portable deionized water-production unit, with a new inline 0.2-µm supply filter, was connected to the animal watering system to rinse and fill the RO tower. After the system's automated flushing cycle, the RO tower was drained. All recoil hoses were disconnected from racks. The RO storage tower was treated with peracetic acid–hydrogen perioxide solution (1% Minncare Cold Sterilant, MarCor Purification, Philadelphia, PA). Test strips (MarCor Purification) were used to verify that the deionized water in the RO storage tower reached a concentration of at least 1% Minncare. The valve on the RO line at the level of the bottle filler was opened and allowed to drain water into the bottle filler drain for 4 to 6 min. Valves on the 2 PRS located at the farthest ends of the distribution system were opened, and water was drained into portable barrels for 4 to 6 min. A manual flush sequence was activated twice from the system's control panel, which opened all solenoids in the PRS, allowing treated water to pass through the PRS into each set of room lines. At the end of the second manual flush, water from the distribution system was tested to ensure that a concentration of at least 1% Minncare was reached. All wall-mounted quick-disconnects were activated, allowing treated water to exit. Once 1% Minncare was verified throughout the system, a 1-h contact time was started. After this contact time, the RO storage tower was flushed, drained, and rinsed with deionized water that had passed through a 0.2-µm filter. The valve on the RO line at the bottle filler was opened, and water was drained into the bottle filler drain for 6 min. Valves on the 2 PRS located at the farthest ends of the distribution system were opened, and water was drained into portable barrels for 4 to 6 min. Water from the RO storage tower was tested with test strips to ensure that Minncare treatment levels were reduced to 0 mg/L. Two manual flush sequences from the system's control panel were initiated, opening all solenoids in the multiple PRS to allow deionized rinse water to pass through the PRS into each set of room lines. Minncare levels were tested at the room line drains and determined to be 0 mg/L. All wall-mounted quick-disconnects were activated, allowing clean water to exit. The animal RO tower was drained of deionized water and refilled with RO water. All wall-mounted quick-disconnects were reconnected. Every rack in each room was flushed manually to ensure that no air bubbles or air locks were present in the rack manifold lines and that water flowed appropriately within each rack.

Recoil hose sanitization. On a monthly basis, recoil hoses are disconnected from rack manifolds and transferred to the dirty side of the cagewash unit, where they are placed in baskets and run through the tunnel washer (model T230, North Star Better Built, Vancouver, BC, Canada). The following tunnel washer parameters are used: prerinse, ambient water temperature; wash cycle, 60 °C (140 °F); rinse cycle, 85 °C (185 °F); and final rinse, 88 °C (190 °F). The maximal final rinse temperature achieved is verified weekly by testing with Temp-a-Sure Chemical Indicator Strips (190 °F [88 °C] maximum, Steris, Mentor, OH). In the clean side of the cagewash unit, the recoil hoses are connected to the automated recoil hose flush station (Edstrom Industries). An automated cycle is run: hoses are flushed with RO water for 2 min, soaked in chlorinated (15 mg/L) RO water for 30 min, and flushed with RO water for 1 min. The hoses undergo a compressed-air drying cycle of 30 s. Afterward, cleaned recoil hoses are coupled to themselves and autoclaved in bins via a gravity cycle with pressures between 18 to 22 psi, temperature of 121 °C, and cycle time of 30 min. A dry time of 40 min follows. Autoclave sterilization is verified weekly by testing with Thermalog-S Steam Chemical Integrator Strips (3M, St Paul, MN) and monthly by using biologic indicators (B/T Sure, Barnstead International, Dubuque, IA).

Rack manifold sanitization. Every 6 mo, individually ventilated caging racks are disconnected from the automatic watering system. Water is not drained from the rack manifolds. After a rack has been dissembled and sprayed with a cleaner–degreaser (Crystal Simple Green, Sunshine Makers, Hunting Beach, CA) to remove accumulated dust and dirt, it is loaded into the rack washer (Basil 4600, Steris). Racks undergo a series of wash cycles with the final rinse cycle reaching a maximal temperature of 82 °C (180 °F), which is verified by weekly testing with Temp-a-Sure Strips (190 °F [88 °C] maximum, Steris). Rack manifolds are drained of water before removal from the rack washer. Racks then are connected to the automated chlorine injection station (Edstrom Industries). The injection station flushes the rack manifold with RO water, fills the rack with chlorinated water (20 to 30 mg/L) for a 60-min soak period, and then flushes racks with RO water. After the cycle is complete, the rack manifold is flushed again with chlorinated and RO water. Racks manifolds are drained thoroughly and left empty until reuse.

Case Report

Baseline heterotrophic plate counts, yeast counts, and fungal counts were obtained at the start of maintenance program development (February 2011). The baseline values obtained at all testing sites from the watering system in question failed to meet the previously established microbial water-quality goals. Fungal and yeast counts were less than 1 cfu/mL for all sites tested, but bacterial counts were well above 10 cfu/mL (Table 1), averaging

Table 1. Heterotrophic plate counts at system distribution lines

	Room A	Room B	Room C	Room D	Room E	Room F	Room G	Room H
Initial	175	285	500	490	365	295	350	460
2 d after hyperchlorination	120	29	29	750	100	129	4	17
1 wk after hyperchlorination	1750	900	1200	1100	1650	1850	1850	1750
1 d after PA–HP; 12 wk after hyperchlorination	74	20	7	13	4	34	2	1
2 wk after PA–HP	15,000	4000	5500	6450	3500	6500	7000	5500
3 wk after PA–HP	1700	108	395	3650	108	189	5500	1645
5 wk after PA–HP	5000	19	41	2800	35	71	375	153
6 wk after PA–HP	550	20	18	2600	55	55	145	23
7 wk after PA–HP	205	2	3	16	39	22	10	5
9 wk after PA–HP	11	1	2	15	6	5	7	2
10 wk after PA–HP	2	1	1	5	5	1	1	1
11 wk after PA–HP	1	1	1	2	3	2	1	1
13 wk after PA–HP	2	1	1	16	5	1	1	1
14 wk after PA–HP	1	1	2	4	4	1	2	1
16 wk after PA–HP	7	24	37	29	25	15	285	300
17 wk after PA–HP	9	9	38	4	9	8	45	38
18 wk after PA–HP	3	4	8	6	19	4	40	19
19 wk after PA–HP	32	27	62	34	60	52	110	105
21 wk after PA–HP	305	43	93	60	50	175	13	37
24 wk after PA–HP	73	8	8	28	21	95	9	14
26 wk after PA–HP; 1 d before second PA–HP	525	690	690	600	515	465	405	600
1 d after second PA–HP	1	97	0	13	1	0	0	0
1 wk after second PA–HP	800	795	615	400	0	925	890	950
3 wk after second PA–HP	0	1	1	1	1	1	23	0
5 wk after second PA–HP	0	0	0	0	0	0	0	0
9 wk after second PA–HP	3100	2300	3000	5500	1150	3150	2850	2600
11 wk after second PA–HP	2600	1800	1150	980	1750	2350	940	825
14 wk after second PA–HP	4700	745	850	475	585	750	915	
18 wk after second PA–HP	1	0	1	0	0	0	0	0

Heterotrophic plate counts were obtained from water samples collected from distribution line drains in 8 housing rooms before and after sanitization with chlorine and peracetic acid–hydrogen peroxide (PA–HP). Yeast and fungal counts were less than 1 cfu/mL H_2O for all testing sites at all testing points. Due to a mechanical issue at the point of the testing, no sample was obtained at 14 wk after the second PA–HP for room H. This issue was rectified by the next testing point.

365 cfu/mL over the 8 distribution line sites tested and 240 cfu/mL over the 23 rack sites tested (data not shown). The bacterial species identified included *Actinomycetes* spp., *Brevundimonas vesicularis*, *Corynebacterium gentilium*, *Corynebacterium pseudodiptheriticum*, *Empedobacter brevis*, *Pseudomonas alcaligenes*, and *Sphingomonas paucimobilis*. To our knowledge, none of the identified bacterial species had previously been cultured in association with clinical animal cases in this vivarium. At the same time that baseline samples were obtained, maintenance records for the system were gathered and reviewed. This vivarium was opened in 1996. Review of available documents confirmed that system sanitization was inadequate, with no historical record of sanitization of the RO production system or supply lines. In addition, room distribution lines and racks were only sporadically documented as being sanitized. Rack recoil hoses had been sanitized regularly. Water samples obtained in February 2011 from the two 1500-gal storage tanks associated with the back-up house RO water system were cultured similarly to other components of the main animal-specific RO system. Yeast, fungal, and bacterial counts in these samples were less than 1 cfu/mL. The house RO system is tested routinely for bacterial counts and is sanitized on an as-need basis according to testing results.

As the maintenance of the automatic watering system in this vivarium was being reviewed, a 2-mo-old, male, immunocompromised B6.Cg-*Slc11a1*^{r} Rag^{tm1Mom}/Cwi mouse in the same vivarium became ill despite no experimental manipulation. An abdominal mass was noted on physical examination, and the mouse was euthanized. Gross necropsy revealed a peritoneal abscess. The abscess was cultured, as was the spleen of the mouse. Both tissue samples were positive for *Sphingomonas paucimobilis*, which previously had been cultured only from the watering system of this vivarium. The cultures obtained from the spleen and abscess of the mouse were sent to a different laboratory (Specialty VetPath, Shoreline, WA) than that used for the water samples, yet both laboratories cultured *S. paucimobilis*. This case was the first, and only, suggestion of a clinical consequence secondary to biofilm formation and high bacterial counts in the automatic watering system of this vivarium. In light of the baseline microbial water quality values, information gathered regarding system maintenance, and what we considered to be a clinical consequence of biofilm formation in a rodent housed on the system, plans were developed for full system sanitization of the automatic watering system.

The first attempt at sanitization (23 May 2011) consisted of hyperchlorination of the entire system; sanitation of recoil hoses and rack manifolds began in June 2011. During this process, 20 mg/L chlorine was added to the system at the level of the RO storage tower for a contact time of 60 min. All racks were run through the rack washer, with water remaining in the manifold lines. After removal from the rack washer, rack manifolds were

drained and connected to the automated chlorine injection station and flushed twice with 20 to 30 mg/L chlorine. Afterward, rack manifolds were rinsed and drained until reuse. All recoil hoses were disconnected from the room distribution lines and rack manifolds, washed via the tunnel washer, and connected to the automated recoil hose flush station, where they were flushed with 15 mg/L chlorine. Recoil hoses subsequently were autoclaved and stored until reuse.

Water from the RO system was tested at 2 d and at 1 wk after hyperchlorination; at this time, only the distribution line drain sites were tested. At 2 d after chlorination, bacterial counts at 7 of 8 of the testing sites were lower (average, 147 cfu/mL over all 8 sites) than baseline counts, yet counts from all sites failed to meet our microbial quality goals. At 1 wk after chlorination, bacterial counts from all 8 distribution testing sites averaged 1506 cfu/mL, a value that not only was higher than the day 2 results but also higher than baseline values (Figure 2). Hyperchlorination had started the process of disrupting the biofilm within the watering system, but our group decided that another product might be more suitable for the size and density of the biofilm that we suspected.

The product chosen for the second round of sanitization was a commercially available combination of peracetic acid and hydrogen peroxide, which was used at a concentration of 1%. Before the use of this agent could occur, the RO system had to be modified to remove the original rubber-bladder pressurization tank, replacing it with a new fiberglass tank (Figure 3). After completion of system modifications (August 2011), the peracetic acid–hydrogen peroxide compound was added to the RO tower and flushed throughout the entire system. Follow-up testing again was limited to the distribution lines and occurred at 24 h and 2 wk after sanitization. The 24-h bacterial counts averaged 19 cfu/mL, and the 2-wk counts averaged 6681 cfu/mL, which were higher than those obtained after hyperchlorination. Water testing of the distribution lines was repeated at 3, 5, and 6 wk after peracetic acid–hydrogen peroxide sanitation. For all testing sites, the bacterial counts at 3 wk after treatment were lower than at 2 wk (Figure 2). Values at 5 and 6 wk were usually, but not always, lower than at those at 3 wk.

At 5 wk after sanitization with peracetic acid–hydrogen peroxide, the automated system flushes were increased from 2 to 4 times daily. At 6 wk after peracetic acid–hydrogen peroxide sanitization, the RO system was modified such that after the activated carbon filters, at the level of the RO membranes, a low level of chlorine (2 mg/L) was infused (Figure 1). Water-quality testing strips (Hach Company, Loveland, CO) were used to measure the free and total chlorine levels in the watering system at the level of the water tower and PRS and from rack lines of various rooms within the facility. Testing occurred weekly after the addition of chlorine. No free or total chlorine was detected downstream of the water tower for multiple testing periods. Increasing the amount of chlorine added to the storage tower to 3 mg/L again failed to lead to detection of free or total chlorine downstream. The frequency of flushing the watering system was reduced back to 2 times daily and the amount of chlorine added to the system was increased to 4 mg/L. These actions led to the detection of 0.5 mg/L chlorine in a single rack of one of the animal holding rooms closest to the water tower. This first positive result for free chlorine n came almost 5 mo after the initial introduction of low-level chlorine into the system. After this first positive finding, more testing sites registered chlorine values during each subsequent testing period, followed by chlorine levels uniformly rising at each of these sites. Chlorine infusion levels then were decreased to 2 mg/L at the RO storage tower and the flushing frequency remained at twice daily to achieve free chlorine values of 0.5 mg/L throughout the system.

In February 2012, while chlorine levels were being optimized, a third system-wide sanitization of the system was performed. This sanitation was accomplished by again adding peracetic acid–hydrogen peroxide to the system for a 60-min contact time, as described earlier; initiation of this round of sanitization was delayed until system modifications were completed, including several electrical upgrades, replumbing to accommodate a second pressurization pump as back-up for the original system pump, and reconfiguration of the RO systems' layout in the mechanical space. Prior to starting peracetic acid–hydrogen peroxide sanitation, the distribution system had to be flushed free of chlorine, because the mixture of chlorine and peracetic acid–hydrogen peroxide potentially could result in the formation of toxic chlorine gas. This issue was handled by turning off the chlorine delivery pump to the RO system 24 h prior to starting peracetic acid–hydrogen peroxide treatment while maintaining the automated twice-daily flushes of the system. Postsanitization testing continued as before.

Similarly to the first time that peracetic acid–hydrogen peroxide was used to sanitize the system, the bacterial counts obtained 24 h after sanitization were lower (average 13.8 cfu/mL) than those obtained for several weeks after sanitization: average bacterial counts were 620 cfu/mL at 1 wk afterward and 16 cfu/mL at the 3-wk point. At 5 wk after the addition of peracetic acid–hydrogen peroxide, bacterial counts at all of the distribution line testing sites were less than 1 cfu/mL. It is important to note that the 3-wk time point after peracetic acid–hydrogen peroxide sanitization coincided with the first measurable chlorine values in the system downstream of the RO water tower.

Testing of the water system continued on a biweekly to monthly basis after the third system-wide sanitization. At 9 wk after the second sanitization with peracetic acid–hydrogen peroxide, bacterial counts for all distribution site testing points rose, averaging 2956 cfu/mL; yeast and fungal counts remained less than 1cfu/mL. Over the course of the subsequent 5 wk, bacterial levels dropped slightly but remained increased over results obtained earlier after sanitization (Figure 2). At 18 wk after the second peracetic acid–hydrogen peroxide treatment, bacterial counts returned to values of 1 cfu/mL or less.

Discussion

Water quality is undeniably an important aspect of animal care, yet industry standards on this topic do not exist. Statements on this subject have suggested that water provided to our laboratory animals should be colorless, odorless, tasteless, and free of all contamination.[22] A heterotrophic plate count of less than 500 bacterial colonies per milliliter of water is acceptable in human public drinking water.[9] A 1996 survey questioned 9 research institutions regarding the operation and monitoring of their watering systems. The acceptable bacterial load for these institutions varied greatly, some similar to ours (less than 1cfu/mL) and others less stringent (less than 1000 cfu/mL).[6] Previous articles have addressed the potential sources of biofilm in automated rodent watering systems and bacterial growth within these biofilms, as well as the difficulties of sanitizing the water manifold lines of racks connected to automated water systems.[3,19] The criteria for bacteria, mold, and fungi that we set for our institution's automated RO water facilities were based on the fact that more stringent values were considered normal with sterilized acidified (pH 2.4 to 2.8) water that was being provided in our water-bottle–only rodent facilities. In

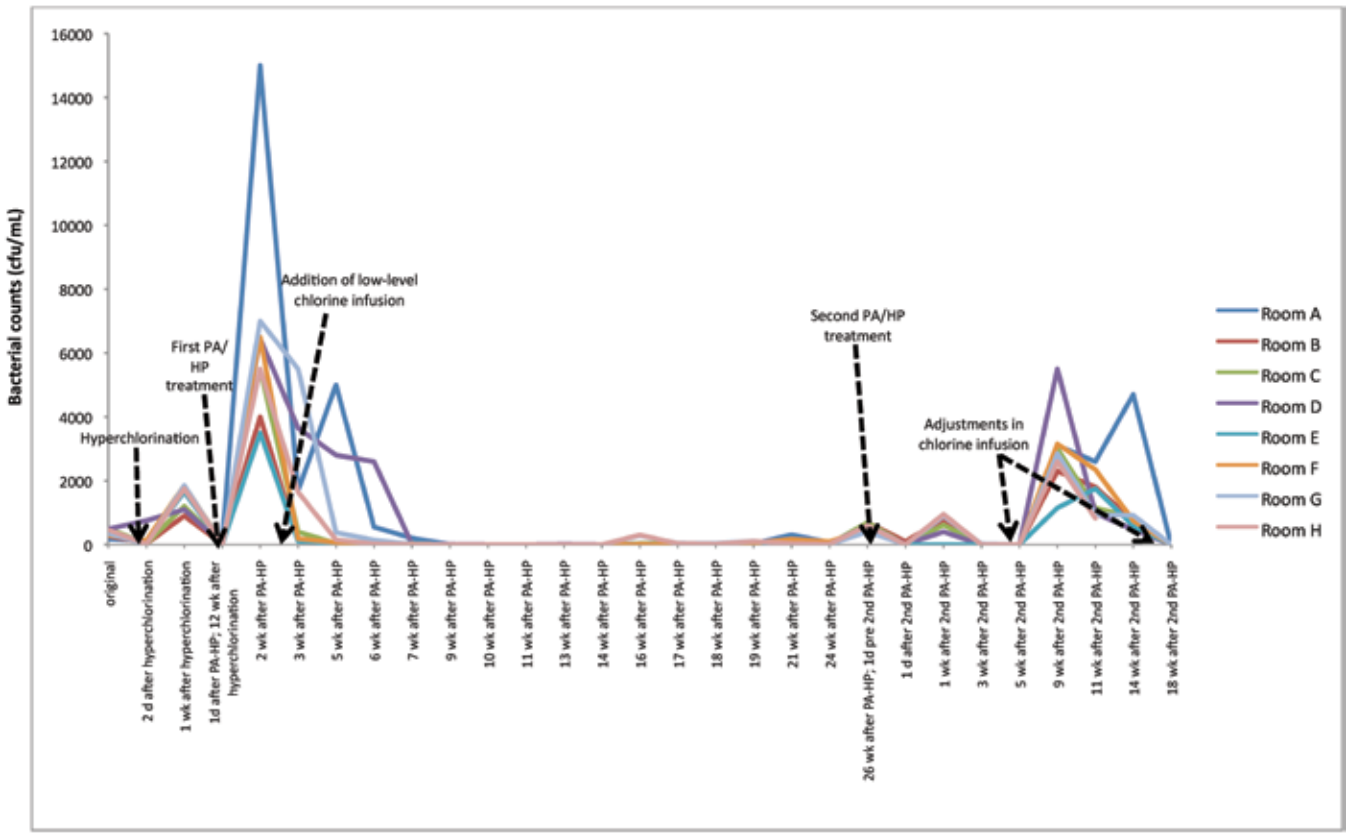

Figure 2. Sanitation timeline and bacterial counts at distribution lines. Heterotrophic plate counts were obtained from water samples collected from distribution line drains in 8 housing rooms before and after sanitization with chlorine and peracetic acid–hydrogen peroxide (PA–HP). Yeast and fungal counts both were less than 1 cfu/mL H_2O for all testing sites at all testing points. Individual sanitization-related events are indicated over the time course.

Figure 3. Distribution system before (left) and after (right) modifications. The rubber-lined bladder tank on the old system (gray) was replaced by a fiberglass tank (blue). This modification required moving the nearby electrical box. A chlorine tank (yellow) was added to the remodeled system so that this product could be used as a biocide.

addition, the criteria set for the automated RO water system in this facility were being met in water samples from other campus automated water facilities.

Biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primary polysaccharide material.[5] Biofilms can form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, and other natural aquatic systems. Biofilms are not continuous-monolayer surface deposits but rather are heterogeneous, containing microcolonies of bacterial cells encased in an extracellular polymeric substances matrix and separated from other microcolonies by interstitial voids (water channels).[16] Biofilm formation within a water distribution system is practically inevitable, because once water comes in contact with a charged surface, organic molecules are deposited and bacteria

subsequently are attracted to the surface.[8,13,20] Especially in areas of low flow or piping 'elbows', bacteria adhere to and secrete extracellular polymeric substances, attracting more bacteria to the area and ultimately resulting in a weblike structure or scaffolding. Bacteria avidly attach to the surface of this scaffolding, grow, multiply, adapt, and expand the biofilm. Periodically, if the biofilm is in contact with turbulent flow, lone or small groups of bacteria will slough from the surface of the biofilm. The 3 main processes for biofilm detachment caused by physical forces are: erosion or shearing (continual removal of small portions of the biofilm), sloughing (rapid and massive removal), and abrasion (detachment due to collision of particles from the bulk fluid with the biofilm).[1] In an automated animal-watering system, periodically sloughing of biofilm components means that animals may be exposed to sporadic showers of biofilm in their drinking supply and the bacteria populations contained within. Therefore, biofilm formation should be minimized as much as possible. Thick biofilms eventually will cross what has been deemed the 'threshold of interference', which is defined as the level at which a biofilm becomes unstable, leading to a greater degree of sloughing.[10] When a bacterial species, cultured initially only from the biofilm in the watering system, subsequently was cultured from a sick mouse presenting with a septic peritoneal abscess, we concluded that this mouse likely consumed a portion of the sloughed biofilm.

The sanitization of the watering system featured in this case study was a multistep process, including modification of recoil hose and rack manifold cleaning and disinfection procedures; sanitation of the entire RO system and delivery lines with 2 different compounds at 3 different time periods; system component replacement, additions, and reconfigurations; electrical component upgrades; and biocide addition (Figure 3). Modification of the process for rack manifold sanitization included maintaining water in all rack manifold lines before placing them inside of the rack washer. Because the water is heated to 82 °C during the sanitation cycle, the water in the lines aided the sanitization of the internal surfaces of the rack manifold lines via thermal disruption of accumulated biofilm.

The initial hyperchlorination sanitization event of the RO system started the process of breaking down the biofilm. Chlorine is an oxidizing agent that is effective in killing planktonic and biofilm bacteria.[2,4,15,23] In addition, chlorine degrades the polysaccharide substances that provide the weblike structure of the biofilm. Chlorine binds to and kills bacteria as it diffuses through a biofilm. However, the efficacy of chlorine decreases with thickness of a biofilm. Chlorine can be bound quickly by sloughed bacteria or those in the outermost biofilm layer, potentially resulting in very low levels of chlorine actually permeating through, and consequently disrupting, the middle and bottom layers of the biofilm.[2,4,15,23] We surmise that our system contained a thick layer of biofilm because we were unable to obtain written records of previous sanitation of the entire RO system and because we did not observe a large disruption and dispersal in the biofilm after hyperchlorination. Because our initial posthyperchlorination bacterial load averages were low, averaging 1506 cfu/mL, we modified future sanitization efforts.

The combination of 4.5% peracetic acid and 22% hydrogen peroxide is an oxiding bactericidal product routinely used for RO system sanitization in medical, pharmaceutical, and industrial settings. The use of this compound, with an appropriate contact time, should result in a 6-log reduction of bacteria at a 1% concentration.[18] We selected this compound for the second and subsequent sanitation events because this product had previously been used successfully by commercial vendors for RO system sanitization.[18] This compound is incompatible with certain materials such as rubber; therefore prior to its use, the original rubber-lined bladder tank of our system was replaced with a new fiberglass tank (Figure 3). Evaluation of other RO production and distribution system components did not yield additional material incompatibilities with the peracetic acid–hydrogen peroxide compound. The high bacterial plate counts (6681 cfu/mL) that we obtained from multiple testing sites after using this product suggested that it was more effective at disrupting the full thickness of the biofilm, resulting in showers of bacteria that ultimately made their way to the distribution line sample sites.

We believe that the delay seeing increased bacterial numbers after the addition of either chlorine or peracetic acid–hydrogen peroxide into the system was due to the fact that our testing points were at the very end of the watering system, requiring more than 48 h for this area to be representative of the rest of the system. Heterotrophic plate counts are used routinely for enumerating bacterial loads in drinking water.[7] These counts reflect the number of planktonic or free-floating bacteria in the collected water sample. Results from these counts will reflect bacteria removed from a biofilm after a recent disruption, such as hyperchlorination or peracetic acid–hydrogen peroxide sanitization. However, they can also underestimate the actual bacterial load of an established biofilm, or a portion of biofilm, especially if it has been exposed recently to a means of disruption. Some authors state that as many as 1000 sessile microorganisms may be present for each planktonic cell detected.[21] Therefore, although the testing methods we used may not directly assess biofilm size, we feel that the increase in bacterial load detected in the samples taken after hyperchlorination and peracetic acid–hydrogen peroxide sanitization reflected the sloughing of biofilm and release of bacteria and that sloughing was a sign that our methods of sanitization resulted in biofilm disruption.

The change in frequency of automated flushing of the room distribution lines prior to chlorine infusion from 2 to 4 times daily was done in attempts to physically flush out portions of the disrupted biofilm present in various sections of the system, especially at the level of the PRS toward the distribution drain lines. The addition of a low level of chlorine (2 mg/L) into the system at the level of the RO membranes and into the RO storage tower after hyperchlorination and peracetic acid–hydrogen peroxide sanitization events was a preventative measure to limit additional biofilm development. Fortunately, this level of chlorination also aided in the destruction of bacteria present in shearing, sloughing, or abraded portions of the biofilm still present in the system and most likely did not actively diffuse into or through the remaining biofilm present. As stated previously, chlorine is quickly bound to organic matter, so it will be most effective in killing bacteria present in agitated portions of biofilm.

In addition, while still in the process of biofilm removal, the low-level chlorine was likely quickly bound at the outermost layer of the remaining portions of biofilm throughout the system. It took almost 5 mo after the addition of 2 mg/L chlorine to the system, plus subsequent increases to 3 and then 4 mg/L with a decrease in frequency of automated flushing from 4 to 2 times daily before we were able to detect free chlorine throughout the RO system. This scenario most likely reflects the fact that large amounts of biofilm and thus bacteria remained in the system and quickly bound the supplemental chlorine. The reduction in frequency of automated flushes most likely increased the chlorine contact time on the biofilm remaining in the system.

Our repeated system-wide sanitization events increased the number of free bacterial organisms in the system via disruption of the biofilm. This situation was supported when, approximately 5 wk after the second peracetic acid–hydrogen peroxide treatment, chlorine infusion was halted because high (greater than 4 mg/L) chlorine levels were detected throughout the system. The closest testing point after cessation of active system chlorination (9 wk after the second peracetic acid–hydrogen peroxide treatment) resulted in much higher bacterial counts than those seen shortly after the second peracetic acid–hydrogen peroxide sanitization treatment. The chlorine infusion was resumed 1 wk after its removal and then was increased from 0.5 mg/L to 1 ppm over 1 mo. At that time, approximately 18 wk after the second peracetic acid–hydrogen peroxide sanitization, bacterial levels returned to low values of 1 cfu/mL or less, when 1.0 mg/L chlorine was being infused into the system regularly.

Sphingomonas paucimobilis, the bacterial organism cultured from a peritoneal abscess in a severely immunocompromised (lacking B and T cells) mouse housed in the vivarium in question, had only previously been cultured from the watering system in this same vivarium. Until this incident, *S. paucimobilis* had not been cultured in association with any known clinical rodent cases in this or any other vivarium on campus. *S. paucimobilis* is a gram-negative bacillus that formerly was known as *Pseudomonas paucimobilis*. This organism thrives in both soil and water; has been isolated from human hospital water systems, distilled water, and respiratory equipment; and has been reported as a cause of infection for both immunocompetent and immunocompromised persons.[24,25] Genotyping determined the same strain of *S. paucimobilis* that was recovered from the water supply in the hematologic unit of a human hospital in Finland was the cause of septicemia in several leukemic patients housed in the unit.[24]

In our situation, the cage of severely immunodeficient B6.Cg-*Slc11a1*^{r} Rag1^{tm1Mom}/Cwi mice may have been exposed to random showers of biofilm containing this organism as it was being removed passively from the automated watering system serving these mice. A second mouse in this cage was found dead around the same time that the mouse with the abdominal mass was noted. However, this dead mouse was severely autolyzed, and neither necropsy nor cultures were performed. Three additional mice from the same cage were in good condition and did not go on to develop any clinical abnormalities. It is interesting to note that for the remaining duration of the cleaning, disinfection, and upgrades of the automatic watering system, no other case of clinical disease was associated with exposure to bacterial species present within the biofilm of our system. This scenario suggests that relatively high bacterial levels combined with a severely immunocompromised status may be required to result in clinical disease associated with ingestion of bacteria from biofilm. This case also highlights the inherent randomness of the showering of biofilm components throughout a rodent automatic watering system. The hyperchlorination and peracetic acid–hydrogen peroxide sanitizations performed on our system were started early in the morning and were completed by early afternoon. This timing may have played a part in the lack of clinical consequences noted, in that perhaps the largest portions of removed biofilm were actively flushed from the system when the rodents were not highly active and therefore not readily drinking water from the system.

Water system care and maintenance is an important aspect of animal care, yet this subject that has received little attention in the laboratory animal community. As more genetically manipulated rodent models are developed and used, especially those that result in severe immunologic deficits, exposure to unanticipated bacterial organisms via portions of biofilm from the water supply needs to be considered as an experimental variable and potential source of infection and should be prevented. Regular water system monitoring is paramount so that potential problems can be identified quickly and rectified. We now test the automatic watering systems in all of our centralized animal vivaria via fungal, yeast, and bacterial cultures regularly throughout the year, and each watering system is sanitized in its entirety (rather than only sanitizing specific system components) at least annually. The consequence of an equivocal testing result is increased testing, with subsequent data evaluation aimed to determine the potential cause for the result. Continued equivocal or unacceptable results are criteria for additional overall system sanitization. Completely removing biofilm from our system is unrealistic. Our goal is to minimize biofilm size and, between regular system sanitizations, to keep the levels of bacteria low by maintaining a low level of free chlorine in the system.

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