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Quality Assessment of Treated Reverse Osmosis Drinking Water in a Specific Pathogen Free Rodent Barrier

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ABSTRACT

This study investigated the quality of treated reverse osmosis drinking water in a specific pathogen free rodent facility. The water bottles placed in the animal rooms were evaluated on days 10, 14, and 21 for room-level assessment. For cage-level assessment, the water was stored for 14 days inside the room and then evaluated on days 2 and 5 (group A – 5 mice) and days 2, 5, 7, 9, and 14 (group B – 2 mice) after provision to the mice in individually ventilated cages. At the room level, a significant decrease in free chlorine was observed from days 10 to 21 ($p<0.0001$). The concentrations of heterotrophic bacteria and *Pseudomonas aeruginosa* were consistently low (<1 CFU/mL and <1 CFU/100 mL, respectively). Cage-level assessment revealed a significant decrease in free chlorine at days 7 ($p = 0.0291$) and 14 ($p = 0.0231$) for Group B. Adenosine triphosphate was detected on days 2 and 5 (Group A) and days 5, 7, 9, and 14 (Group B). Heterotrophic bacteria were found in Group A, day 5 (460 CFU/mL), and Group B, days 9 (2 CFU/mL) and 14 (2000 CFU/mL). The level of *Pseudomonas aeruginosa* remained low in both groups. There was no significant change in the pH at the room or cage level. The findings suggest that water bottles filled with treated reverse osmosis water can be stored unused in rooms for 14 days before being distributed to cages with at most two animals for 7 days. For higher stocking densities, it is recommended to change water bottles every two days.

Keywords: Drinking water, Heterotrophic bacteria, *Pseudomonas aeruginosa*, Reverse osmosis, Water quality

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INTRODUCTION

The water that animals consume can have a direct impact on their health. Hence, it is vital to verify that it is safe and contaminant-free. Providing drinkable, uncontaminated drinking water to laboratory animals is an important part of successful animal husbandry. It is performed not only to ensure the animal's overall well-being but also to reduce experimental variables (Allen et al., 2017). Water quality is guaranteed by routine or periodic monitoring of water parameters such as pH, chlorine, and microbiological or chemical contamination (National Research Council, 2011).

Specific pathogen free (SPF) rodent facilities host several strains of animals, some of which are immunologically compromised. These animals have a weakened immune system and are susceptible to opportunistic infections. Thus, municipal or city water that is suitable for human consumption may not be sufficient to supply laboratory animals. In most SPF facilities, the water fed to animals undergoes a purification process known as reverse osmosis (RO) to remove particles, chemicals, and biological pollutants from the water. After this, the RO water undergoes post-treatment, such as chlorination and acidification, to maintain potable drinking water that is free from microorganisms (Allen et al., 2017). However, studies have shown that chlorine levels in drinking water kept in bottles fall over time to levels insufficient to maintain the self-sterilizing property of the water (Bywater & Kellett, 1977). This makes water susceptible to the growth of potential pathogens such as heterotrophic plate count bacteria (HPC) and *Pseudomonas aeruginosa (Bywater & Kellett, 1977; Edstrom Industries, 2015; Peveler et al., 2015).* HPC is often employed as a microbiological quality indicator in drinking water (Bartram et al., 2003), and it represents a diverse spectrum of bacterial species, such as *Escherichia, Klebsiella, Enterobacter, Citrobacter, Serratia, and Helicobacter*. *P. aeruginosa*, on the other hand, is another major microbe that has the potential to contaminate water supplies and is normally prohibited from most SPF facilities. This bacterium is an opportunistic pathogen that lives in water and can cause septicemia in immunocompromised animals, which can be fatal in severe cases (Fox et al., 2015).

To date, most SPF facilities have transitioned to using automatic watering systems to deliver water to animals housed in individually ventilated cages (IVCs). However, the use of an automated system alone has yet to become universal, and the traditional way of using water bottles or a combination of water bottles and an automated watering system is still preferred or required by some researchers due to experimental or animal welfare reasons. The use of an automated watering system requires some learning curve for the animals to learn the use of the valve. Likewise, pre-weanling mice may not have sufficient strength to trigger a water valve, which leads to dehydration (Gordon & Wyatt, 2011; National Research Council, 2011). Furthermore, researchers are concerned about flooding, whose occurrence is greater in automated watering systems than in water bottles (Gonzalez et al., 2011).

In most facilities, the water bottles provided for the animals were not always freshly prepared. The Guide for the Care and Use of Laboratory Animals recommends regular checking and maintaining watering devices for cleanliness and functionality. It suggests replacing water bottles rather than refilling them to avoid microbiological cross-contamination (National Research Council, 2011). Since water bottles were stored in the animal holding room before being provided to the animals, regular changes are necessary to ensure sterility. Currently, there is no general standard on the changing frequency of water bottles containing treated RO water, both as stock stored in the room and in the IVC with animals. During room-level storage, water bottles are usually stored until they are completely used. Meanwhile, at the cage level, water bottles were replaced whenever they ran low or became visibly cloudy, whichever came first.

In this study, we propose a comprehensive investigation into the quality of treated RO drinking water in an SPF rodent facility, focusing on both room-level and cage-level dynamics. The study hypothesizes that extending the duration of storage will not significantly impact the microbial contamination of unused drinking water when stored properly, even with degraded chlorine levels. Additionally, the hypothesis suggests that water contamination at the cage level may occur before visible indicators such as water cloudiness appear. Finally, the hypothesis anticipates a correlation between the number of animals in the cage and the microbial contamination of the water, suggesting that higher population densities will result in an increased microbial load in the water. Through these hypotheses, this study provides insights into maintaining high-quality drinking water standards within SPF rodent facilities.

MATERIALS AND METHODS

Preparation and Storage of Drinking Water

The primary water supply was city water, regulated by the Environmental Public Health (EPH) and adhering to the WHO Guidelines for drinking water quality. This city water was then purified using the facility's RO filtering system (Edstrom Industries, Waterford, WI). The pre-treated city water was forced through the RO membrane, separating the purified permeate from the concentrate. The resulting

Figure 1. Water bottles arranged in a tray for room storage. Prior to placement, the bottles and nozzles undergo autoclaving for sterilization and are subsequently filled with treated reverse osmosis (RO) water. To maintain cleanliness and minimize contamination risks, a green cloth is used to cover the bottles when stored inside the animal holding room.

RO water was subsequently treated with 5% sodium hypochlorite (Prime Products Pte Ltd., Singapore) to achieve chlorine concentrations ranging from 2 to 3 mg/L. The water pH was maintained between 6.5 and 7.0 using 8% sulfuric acid (Prime Products Pte Ltd., Singapore).

Autoclaved 250 ml polyetherimide (PEI) water bottles (GM500 SEALSAFE Plus, Tecniplast, Buguggiate, Italy) were filled with the treated RO water at the water bottle refilling station inside the facility. The bottles were capped with autoclaved metal nozzles, placed into dedicated crates covered with autoclaved, woven fabric, and stored in an animal holding room (Figure 1).

Water Quality Testing

This study included both in-house and external laboratory tests. The Singapore Test Services were used for the external testing of water samples. Room-level water assessment utilized an external testing laboratory, while cage-level water assessment utilized both in-house testing and external laboratory testing.

Room-level Water Quality Testing

Water bottles were aged at days (d) 10, 14, and 21 within the animal holding rooms to mimic the storage of stock drinking water bottles in the room. For each timepoint, 200 mL of water was collected per water bottle (n = 4) and transferred into a sterile cell culture flask (Nunc™, Nunclon™ Delta Surface, Apogent, Denmark). This is the minimum volume of water sample required by the external testing laboratory to perform the analysis. The samples were collected in a class II biological safety cabinet (Gelman, Singapore) to limit the risk of contamination. All water samples were sealed with paraffin film, labeled, and packed into Ziplock bags before being sent to the external laboratory for testing of water pH, free chlorine, HPC, and *P. aeruginosa*.

The pH and conductivity were tested using the standard electrometric method of the American Public Health Association (APHA). Moreover, chlorine testing was performed using the Hach methods. For pathogen testing, HPC was tested using APHA methods, while the *P. aeruginosa* test was carried out using the International Organization for Standardization (ISO) standards of detection.

Cage-Level Water Quality Testing

In the cage-level water quality assessment, 69 male and female mice (FVB/NJ and BALB/c strains, aged 5-6 weeks) were used. The animals were obtained from the in-house breeding colony of the Biological Resource Centre, an AAALAC International-accredited and Bizsafe Level 3-certified animal facility in Singapore. The animals were kept in IVC (GM500 SEALSAFE Plus, Tecniplast, Buguggiate, Italy) with autoclaved corncob bedding (BioCOB, Biosys, Singapore). The animals had unlimited access to irradiated diets (Altromin 1324, Altromin, Lage, Germany), and treated RO water was provided via water bottles. The cages were maintained in a humidity (30-70%) and temperature (22-24°C) controlled environment with a 12:12 hour light:dark cycle and 10-15 air exchanges per hour. Health surveillance was performed quarterly in the colony using soiled bedding sentinels. The animals were free of the following pathogens, according to the institution's exclusion list: Sendai virus, Pneumonia virus of mice, Mouse hepatitis virus, Minute virus of mice, Mouse parvovirus, Theiler's murine encephalomyelitis virus, Reovirus 3, Rotavirus, Mouse adenovirus types 1 and 2, Polyoma virus, Lymphocytic choriomeningitis virus, Mouse cytomegalovirus, Hantaan virus, Ectromelia virus, Mouse Norovirus, *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Citrobacter rodentium*, *Helicobacter hepaticus*, *H. bilis*, *H. rodentium*, *Clostridium piliforme*, *Salmonella* spp., *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Pasteurella pneumotropica*, *P. multocida*, *Pseudomonas aeruginosa*, *Streptococci beta-Hemolytica* Groups A, B and C, *Klebsiella pneumoniae*, *K. oxytoca*, *Mycoplasma pulmonis*, *Encephalitozoon cuniculi*, ectoparasites (i.e., fleas, furmites, and lice), endoparasites (i.e., pinworms, tapeworms, roundworms, and other helminths), *Tritrichomonas m*uris, and *Entamoeba muris*. All procedures involving animals were approved by the institution's Institutional Animal Care and Use Committee. The procedures were also

conducted in accordance with the local guidelines (i.e., NACLAR guidelines) and the Guide for the Care and Use of Laboratory Animals, 8th edition.

The 69 mice were randomly distributed into 2 groups: Group A (Grp A), with 5 mice housed per IVC, and Group B (Grp B), with 2 mice housed per IVC. The groupings represent the maximum and minimum number of animals that can be socially housed in IVC. Water samples were tested at d2 and 5 for Grp A and at d2, 5, 7, 9, and 14 for Grp B. In Grp A, 3 cages were allocated for d2, and 4 cages were allocated for d5 (a total of 7 cages). Meanwhile, in Grp B, 3 cages each were allocated for d2, 5, 7, 9, and 5 cages for d14 (a total of 17 cages). The number of cages for each timepoint in both groups was determined based on the estimated volume of water left at each timepoint to perform both in-house and external water testing, which require at least 12 mL and 200 mL of water samples, respectively, to run the water analysis. The estimation of the volume of water left at the end of each timepoint was based on individual adult mice's daily water consumption of 6.7 mL/day (Fox et al., 2015). In accordance with the facility's standard operating procedure (SOP), which mandates cage changing every two weeks, no cage changes were performed during the study.

All water bottles were stored for 14 days inside the animal holding room before being provided to the animals. Fourteen days of aging were chosen based on the results obtained from the room-level testing of water samples. For each timepoint, the following in-house water tests were performed: pH analysis using commercially available universal pH indicator test strips (LabRat Supplies, USA), free chlorine analysis using a MD100 photometer system (Lovibond® Water Testing, Tintometer® Group, London, UK), and water ATP analysis using Hygiena Aquasnap™ swabs (Hygiena, California, USA) with a Hygiena SystemSURE Plus luminometer (Hygiena, California, USA).

To measure the water pH, 1 mL of water was collected with a 3 mL sterile syringe (BD 3 mL Syringe Luer-Lok™, Becton, Dickinson and Company, Laagstraat, Temse, Belgium) and dripped onto the pH indicator strips. The pH value was determined by comparing the color changes to the strip reader. For free chlorine testing, approximately 10 mL of water was collected using a sterile 10 mL syringe (BD 10 mL Syringe Luer-Lok™, Becton, Dickinson and Company, Laagstraat, Temse, Belgium) and placed into a 10 mL vial of the photometer system unit. The unit was then calibrated using a vial containing the water sample. The same sample was tested by dissolving one MD 100 chlorine duo tablet (DPD No. 1, Lovibond®, London, United Kingdom) before being reinserted into the calibrated unit. Calibration of the photometer-system unit was performed for every sample. For water ATP testing, another 1 mL of water was removed using a sterile syringe and separated into two 0.5 mL aliquots in 1.5 mL Eppendorf tubes. The first aliquot was used to determine the total ATP, and the second aliquot was used to determine the free ATP using a Hygiena luminometer. Aquasnap™ water testing devices utilize both Aquasnap™ total and Aquasnap™ free devices to screen ATP levels in water. Aquasnap™ total measures both ATP contained within living cells and particulate matter (microbial) as well as ATP dissolved in water (nonmicrobial or dead microbial ATP). Aquasnap™ free measures only the dissolved ATP outside of living cells. Microbiological contamination was determined by subtracting the free ATP concentration from the total ATP concentration (Hygiena Technical Sheet, 2014). All tests were performed inside a class II biological safety cabinet.

For the external laboratory analysis, the following tests were carried out: pH, free chlorine, HPC, and *P. aeruginosa*. Water samples from the different bottles were collected and pooled in a sterile cell culture flask at the end of each timepoint to meet the 200 mL minimum volume of water required by the testing laboratory to perform the analysis. All water samples were sealed with paraffin film, labeled, and packed into Ziplock bags. The pooling of samples was performed inside a class II biological safety cabinet.

Statistical analysis

All the statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). The Shapiro–Wilk normality test was used to determine the data distribution. When comparing two variables, a paired t test was used for normally distributed data, and the Wilcoxon test was used for non-normally distributed data. For comparisons between groups, a one-way ANOVA was used for normally distributed data, and a Tukey post hoc test was used for further analysis. For non-normally distributed data, the Kruskal–Wallis test with Dunn's post hoc test was used. Statistical significance was set to $p<0.05$.

RESULTS

Room-Level Water Quality Testing

The free chlorine concentration decreased as the storage time increased. Compared to those at baseline, significant differences were observed at all timepoints (d10, $p<0.0001$; d14, $p<0.0001$, and d21 $p<0.0001$). The baseline used was the latest result of the facility's routine testing of the treated RO water from the refilling station. Comparisons between timepoints revealed a significant decrease in values between d10 and d21 ($p = 0.0002$) and between d14 and d21 ($p = 0.0254$). There was no significant difference between d10 and d14 (Figure 2). Across all timepoints,

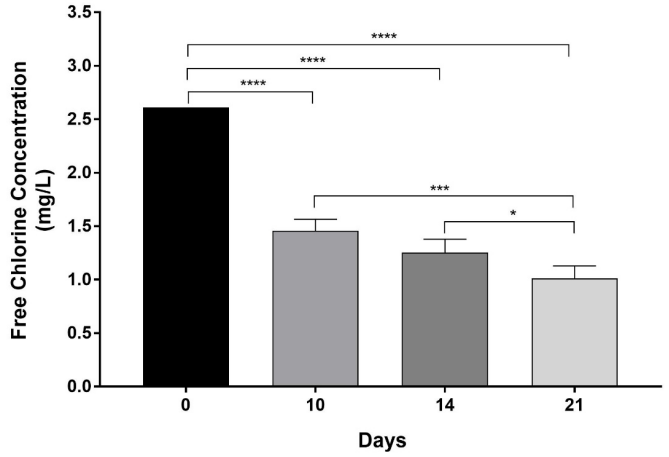

Figure 2. Variation in free chlorine concentration in stored drinking water over time. Water samples were collected and analyzed by an external testing laboratory on different days following storage within the animal holding room. The free chlorine concentration decreased as the storage time increased ($p<0.05$, one-way ANOVA and Tukey's multiple comparison test).

the HPC concentration was <1 CFU/mL, and the *P. aeruginosa* concentration was <1 CFU/100 mL. These values are the same as those at baseline. The mean pH values obtained ranged from 6.7 to 6.8. No significant changes were observed when the pH values were compared to the baseline values or between timepoints.

Cage-Level Water Quality Testing

The concentration of free chlorine in the drinking water supplied to the animals is depicted in Figure 3. In both experimental groups, all timepoints showed a decrease in free chlorine levels when compared to their respective baselines. However, statistical analysis revealed that the differences were not significant, except for Grp B on d7 (gBd7, $p = 0.0291$) and d14 (gBd14, $p = 0.0231$). The baseline values (referred to as d0) were obtained from 14-day-old water samples stored in the animal holding room prior to provision to the animals. Pooled water samples collected at each timepoint and analyzed by an external testing laboratory indicated a free chlorine concentration of <0.02 mg/L at d5 (Grp A) and from d5 onwards (Grp B) (Table 1). It is worth noting that this value represents the minimum detection limit of the external testing laboratory for free chlorine concentration.

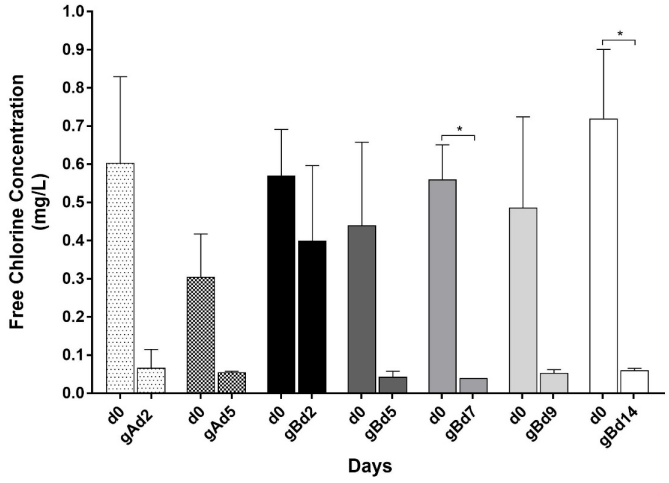

Figure 3. Free chlorine concentration in drinking water provided to animals. Drinking water, stored for 14 days prior to provision to the animals, was sampled at days 2 (gAd2) and 5 (gAd5) for Group A (cages with 5 animals each), and at days 2 (gBd2), 5 (gBd5), 7 (gBd7), 9 (gBd9), and 14 (gBd14) for Group B (cages with 2 animals each). Analysis was conducted in-house using the MD100 photometer system. In both groups, all timepoints showed a decrease in free chlorine levels when compared to respective baselines. Significant differences were observed in Grp B, d7 and 14 ($p<0.05$, paired t-test for normally distributed data, and Wilcoxon test for non-normally distributed data).

For the in-house water ATP test, baseline values were also taken from 14-day-old water samples stored in the animal holding room prior to provision to the animals. Notably, the mean values obtained from five out of seven timepoints exhibited a lower total ATP content compared to free ATP content, indicating minimal microbial contamination. This discrepancy was attributed to the presence of the extractant in the Aquasnap™ total assay. A low level of microbial contamination (1.3 ± 2.3 RLU) was recorded at the baseline of Grp B, d7 samples. Microbial contaminants were detected at d2 (4.7 ± 3.1 RLU) and d5 (46.5 ± 72.5 RLU) in Grp A. Conversely, in Grp B, microbial contaminants emerged at d5 (5.3 ± 1.2 RLU), persisting until d14 (9.2 ± 6.8 RLU) but were absent at d2 (Table 2). When the water samples for each timepoint were pooled and analyzed by an external testing laboratory, the presence of HPC was detected at d5 in Grp A (460 CFU/mL) and at d9 (2 CFU/mL) and d14 (2000 CFU/mL) in Grp B. These timepoints yielded the highest ATP readings during in-house testing. Remarkably, the detection level of *P. aeruginosa* remained consistently below 1 CFU/100 mL across all samples (Table 1).

There were no significant changes in the pH of the water in both groups, across all timepoints. All water samples examined for in-house testing yielded a pH reading of 6. For the pooled sample analysis by an external testing laboratory, a slight increase in pH was observed from d2 (pH 6.3) to d5 (pH 6.7) in Grp A. Meanwhile, an inconsistent pattern was observed in Grp B (Table 1).

DISCUSSION

The provision of clean water to research animals is imperative for ensuring their welfare and health. Regular water changes are recommended to prevent contamination. Guidelines typically recommend a free chlorine level of 2 to 3 mg/L to maintain microbial-free water (Edstrom Industries, 2015). However, a previous study indicated bacterial growth in treated water when free chlorine concen-

Table 1. External laboratory analysis results of pooled water samples collected and analyzed at various timepoints.

Group	**Timepoint** (days)	**pH**	**Free Chlorine** (mg/L)	**HPC** (CFU/mL)	***P. aeruginosa*** (CFU/100mL)
A	2	6.3	0.03	<1	<1
	5	6.7	<0.02	460	<1
B	2	6.4	0.32	<1	<1
	5	6.9	<0.02	<1	<1
	7	6.6	<0.02	<1	<1
	9	6.3	<0.02	2	<1
	14	6.4	<0.02	2000	<1

Table 2. ATP test results (Mean±SD) for in-house analysis of water samples at various timepoints.

Group	**Timepoint** (days)	**Total ATP** (RLU)	**Free ATP** (RLU)	**Microbial Contamination** (RLU)
A	0	0.0 ± 0.0	0.3 ± 0.6	-0.3 ± 0.6
	2	9.3 ± 6.8	4.7 ± 3.8	4.7 ± 3.1
	0	1.3 ± 1.5	2.3 ± 3.3	-1.0 ± 2.7
	5	95.0 ± 118.6	48.5 ± 47.0	46.5 ± 72.5
B	0	1.7 **± 2.9**	2.7 ± 4.6	-1.0 ± 6.6
	2	1.0 ± 1.0	1.3 ± 1.2	-0.3 ± 2.1
	0	0.0 ± 0.0	0.7 ± 1.2	-0.7 ± 1.2
	5	11.0 ± 2.0	5.7 ± 1.2	5.3 ± 1.2
	0	1.7 **± 2.9**	0.3 ± 0.6	1.3 ± 2.3
	7	11.3 ± 5.1	7.0 ± 1.0	4.3 ± 6.1
	0	0.0 ± 0.0	1.3 ± 2.3	-1.3 ± 2.3
	9	9.3 ± 11.8	4.0 ± 4.4	5.3 ± 7.5
	0	0.2 ± 0.4	0.2 ± 0.4	0.0 ± 0.7
	14	31.6 ± 19.0	22.4 ± 13.9	9.2 ± 6.8

trations decreased to less than 2 mg/L (Bywater & Kellett, 1977). Notably, our study demonstrated that even with a lower free chlorine concentration (<1 mg/L), treated RO water remains free of microbes when stored unused. This preservation of water quality can be attributed to the use of sterile, autoclaved water bottles and proper storage within the animal holding room, mitigating external contamination sources.

The degradation of free chlorine was observed both in the unused water stored in the room and in the water provided to the animals. The decay of free chlorine in drinking water due to storage can be affected by several factors, one of which is the storage temperature. A study showed that free chlorine tends to degrade faster in unrefrigerated water (Sheikhi et al., 2014). For this study, water bottles were stored inside the animal holding room at a controlled environmental temperature of approximately 22°C. Moreover, treated RO water contains low levels of dissolved minerals, causing chlorine to react with the water itself, resulting in its rapid degradation.

Animals play an important role in contaminating drinking water with low chlorine concentrations. The longer the drinking water was provided to the mice, the greater the HPC. Additionally, drinking water provided to a larger number of animals was more likely to be contaminated earlier by this group of bacteria. Aside from the normal oral flora of animals as a possible source of water contamination, the microenvironment inside the IVC can also play a part. Bedding, for instance, can be contaminated with animal urine, feces, and even skin dander, which can potentially contaminate water via water bottle sippers (Haist et al., 2004). HPC represents a diverse range of bacterial types, and a published study revealed that even with existing measures, such as chlorination of water, regrowth of HPC is possible (Chowdhury, 2012). In a study conducted in individually housed mice utilizing freshly prepared, monochloramine-treated filtered tap water, it remained potable for up to two weeks. No coliforms were detected, but there was a low percentage of gram-positive bacilli, *Staphylococcus*, *Micrococcus*, *Streptococcus*,

and *Pantoea* species (Haist et al., 2004). One limitation of the present study is that no differentiation was performed on the isolated HPC to identify the specific bacteria present in the water samples. This is something that should be considered in future studies to understand whether the microbial contaminants were normal commensals of the animals or from external sources. This could help in designing a proper workflow and deciding whether more stringent measures are needed to strengthen biosecurity. The *P. aeruginosa levels, however, remained consistently low across all timepoints and* yielded the same values as the initial readings even with decreasing chlorine concentration. It is known that this microorganism is resistant to lower concentrations of chlorine (Edstrom Industries, 2015). Hence, the current study proved that contamination of water with *P. aeruginosa* is highly unlikely to occur in a clean, SPF environment or in animals that are negative for *P. aeruginosa*.

Despite previous suggestions that pH could indicate water contamination (Saalidong et al., 2022), this study revealed that stable pH values are unaffected by microbial contaminants. This finding concurred with a previous study using unused RO and autoclaved water, which documented that the pH of rodent drinking water is stable even with prolonged storage (Peveler et al., 2015). The pH stability during storage can be attributed to the RO filtration system, which produces water that contains very little dissolved solids such as minerals and other elements that can potentially affect the pH (Allen et al., 2017). Low levels of dissolved solids in treated RO water aid in pH stabilization even in the presence of microbial contaminants. Furthermore, the degradation of free chlorine has little effect on the pH of water. This study revealed that the pH remained within the recommended range (i.e., pH 5 to 7), which does not require a higher concentration of chlorine to ensure sterility (Edstrom Industries, 2015). The visual evaluation of pH, nevertheless, is not reliable for measuring water pH using commercially available strips due to its limitations. Most pH indicator strips provide limited reading capacity. The use of pH meters that can provide readings in decimals should be considered.

The cage changing frequency of the facility adheres to the recommended intervals established by published references. Furthermore, the use of corncob bedding has been demonstrated to be highly effective in controlling ammonia levels within IVCs (Ferrecchia et al., 2014; Fox et al., 2015; Tataryn et al., 2021). Consequently, ammonia accumulation in the cages over the duration of the study is not anticipated to significantly affect chlorine degradation and pH levels of the drinking water. This is corroborated by the findings of the current study, which indicated no significant alterations in pH levels up to day 14, with chlorine degradation commencing as early as day 2.

Based on the findings, stock water bottles can be stored for 14 days in an animal holding room without the risk of microbial contamination. Even though room-level testing established microbial-free water at 21 days of storage, the length of time that the water bottles remained in the cage should be considered. Water bottles stocked for 14 days inside the animal holding rooms can be provided to IVCs with at most two animals for 7 days. Moreover, changing water bottles every two days is recommended for cages with a relatively high stocking density.

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