The effectiveness of disinfection and flushing procedures to prevent coliform persistence in aircraft water systems

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ABSTRACT

A full-scale reproduction of an aircraft drinking water system was conditioned using municipal tap water with a mixture of free chlorine and chloramines, and subsequently contaminated with coliforms. Disinfection was undertaken using chlorine dioxide, ozone and a mixed oxidant solution followed by flushing until no disinfectant residual remained. Results showed that coliforms were not persistent on the aircraft plumbing surfaces, and coliforms were not detected after disinfection and flushing with any disinfectant. The one exception was the aerator installed in the lavatory faucet, which was coliform positive after disinfection with ozone and mixed oxidants. These data suggest that the faucet aerators could be a source of coliform contamination that may result in coliform positive samples. Further experiments conducted on disinfection of aerators with glycolic acid and quaternary ammonia (both commonly used by the airlines) showed no detectable coliforms on coliform contaminated aerators after 30 minutes of soaking in the disinfectants.

Key words | aircraft, coliform, disinfection, drinking water, flushing, plumbing

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INTRODUCTION

In 2009, the United States Environmental Protection Agency (EPA) promulgated the Aircraft Drinking Water Rule (ADWR) (USEPA 2010). The rule allows air carriers to comply with the Safe Drinking Water Act (SDWA) and national primary drinking water regulations (NPDWRs) while still taking into consideration the unique aspects of aircraft water systems that differentiate them from traditional stationary public water systems. The rule sets a schedule for disinfection and flushing and coliform/*Escherichia coli* sampling in addition to instituting best practices and operator training. If coliforms or *E. coli* are detected in an aircraft water system, the rule sets forth corrective actions and public notification that must take place within certain time periods.

Data on coliform disinfection in the peer-reviewed literature have focused on stationary drinking water distribution system infrastructure and home plumbing materials, with no studies focusing on coliform persistence or disinfection of aircraft water systems. Therefore, data on coliform persistence and decontamination in aircraft water systems are novel and can help commercial air carriers maintain the quality of their water. Coliform bacteria have been shown to persist and possibly regrow on drinking water infrastructure such as iron and polyvinyl chloride (PVC) (Camper *et al.* 1996; LeChevallier *et al.* 1996; Szabo *et al.* 2006; Juhna *et al.* 2007; Culotti & Packman 2014). Other studies have indicated that long-term persistence of coliforms in drinking water, especially when a disinfectant residual or high levels of shear (e.g. from flushing) is present, is unlikely (Fass *et al.* 1996; McMath *et al.* 1999; Abberton *et al.* 2016; Oder *et al.* 2018). However, other research has

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shown that coliforms persisting or colonizing biofilms are protected from disinfectants (Williams & Braun-Howland 2003; Szabo *et al.* 2006). Recent research on the microbial diversity of aircraft water systems found that coliforms are rarely detected, suggesting that coliforms may come from transient, external contamination (Handschuh *et al.* 2017).

This study was designed to determine coliform persistence in an aircraft water system and the effectiveness of commonly used disinfection and flushing procedures. A 'mock' or replica of a Boeing 737 aircraft water system was built for this study. The water system was conditioned using municipal tap water, and subsequently contaminated with coliforms isolated from commercial passenger aircraft. Persistence of the coliform bacteria was monitored on the water system tubing, couplings, and lavatory faucet. Decontamination of coliform bacteria was evaluated using Purogene® (chlorine dioxide) or ozone, which are commonly used by US-based air carriers, or a mixed oxidant solution. Results from the disinfection and flushing studies led to further evaluation of coliform bacteria persistence on the faucet aerators.

METHODS

Isolation of coliform bacteria

Coliform bacteria were isolated from ADWR samples provided by US-based air carriers. The bacteriological medium from total coliform-positive aircraft water samples was shipped at 4 °C to EPA's laboratory where aliquots were diluted and cultured on MacConkey agar. Individual colonies displaying a typical coliform appearance (red-centered colony, often accompanied by pink bile precipitation around the colony, indicative of lactose fermentation), were chosen for further study. Identification of the isolates was performed using the BBL™ Crystal™ bacterial identification system (Becton, Dickinson and Company, Sparks, MD).

Bacterial isolates were chosen for colonization experiments using demonstrated ability to form biofilms. Briefly, 4-inch sections of aircraft water tubing (Hydraflow, Fullerton, CA) were sterilized by ethylene oxide and sealed using autoclaved rubber stoppers. Bacterial isolates were grown overnight in Luria–Bertani (LB) broth and diluted such that all suspensions were of an equal optical density. Ten millilitres of LB broth and 50 µl of cell suspension were added to each of three sterilized tube sections. Blank tube sections were also prepared, with LB broth only. The tube sections were incubated overnight at 35 °C with shaking. The broth culture was removed, the optical density measured (600 nm) ($OD_{culture}$) and any unattached cells were removed by rinsing the tube sections three times with Butterfield's Phosphate Buffer. After air-drying, 10 ml of a crystal violet stain solution (0.1%) were added to each tube with a 15-minute incubation at room temperature, followed by gently rinsing three times with Butterfield's Phosphate Buffer. After air-drying again, 10 ml of ethanol were added to the tubes to collect any stain adsorbed to attached bacterial cells. Measuring the optical density of this ethanol–crystal violet solution (600 nm) (OD_{CVtest}) and normalizing the data based on the optical density of the bacterial cultures in the tube sections allowed the calculation of a biofilm formation factor ($OD_{CVtest} - OD_{CVblank}/OD_{culture}$) (Wakimoto *et al.* 2004).

Mock aircraft water system

Figure 1 shows the pilot-scale aircraft drinking water system, which includes key components of a Boeing 737 water system. These components include a 0.15 m^3 (40-gallon) aircraft water tank (Yokohama Rubber Company, Tokyo, Japan), the water supply lines and couplings (Hydraflow, Fullerton, CA), and a timed, swivel-head lavatory faucet (Adams-Rite, Fullerton, CA). The aircraft water system was operated by introducing tap water through the fill fitting, filling the tank to approximately 0.15 m^3 (40 gallons), and then pressurizing the tank head space to 2.4 atm (35 psi). Water was dispensed through the lavatory faucet at a flow rate of approximately 1.9 L/min (0.5 gallons per minute (gpm)).

Conditioning of the mock system

Once the mock system was assembled, it was disinfected with 100 mg/L Purogene® (Bio-Cide International, Norman, OK) for 2 hours, and then conditioned for 1 month. Water demand was simulated by depressing the lavatory faucet daily from 6:00 am to 8:00 am, 9:00 am to 11:00 am, and 12:00 pm to 2:00 pm and 3:00 pm to 5:00 pm, with no

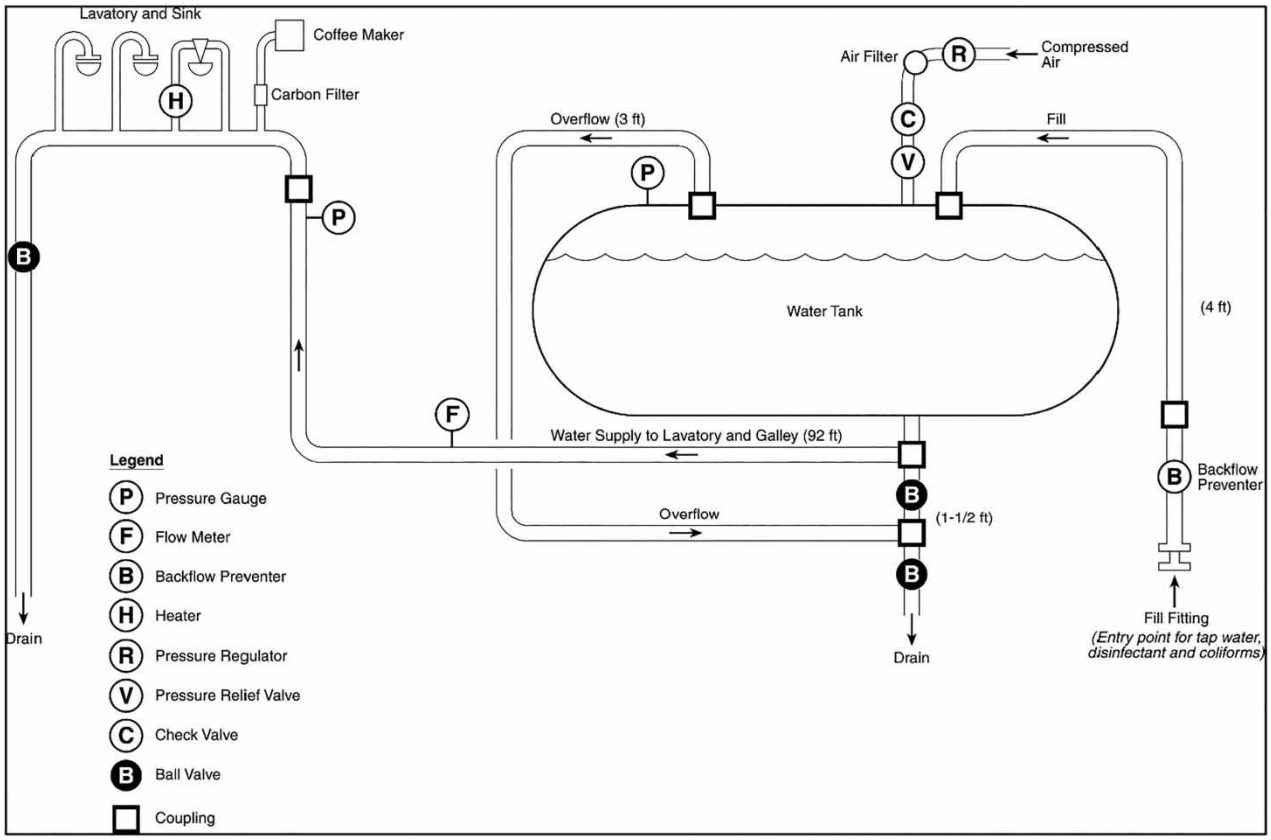

Figure 1 | Schematic of the mock aircraft water system.

demand in between. These intervals were meant to represent periods of flight with down time in between. It was assumed that passengers use the lavatory every 6 to 12 minutes while in 'flight' (five to 10 people using the lavatory per hour), and that water usage lasted 10 seconds with every lavatory visit. At the end of the day (6:00 pm), the faucet and counter tops were disinfected by spraying them with Celeste® Sani-Cide disinfectant (Celeste Corp., Eaton, MD) and wiping them down with paper towels. This schedule was maintained for 4 weeks on Monday through Friday with stagnation on Saturday and Sunday. This conditioning strategy was developed with the input from two United States-based air carriers and Boeing Commercial Airplanes.

Commercial aircraft fill their potable water tanks as needed. Depending on the city the aircraft is in, this could be chlorinated or chloraminated water, which often results in a mixture of both disinfectants in the tank. This was simulated by feeding the mock aircraft system with chlorinated Cincinnati tap water (0.9 to 1.1 mg/L free chlorine) dosed with stock solutions of ammonium sulfate (472 mg/L) and free chlorine (6% or 60,000 mg/L) to create both free chlorine and total chlorine/monochloramine. Target free-chlorine levels in the aircraft water tank were less than 0.1 mg/L and total chlorine and monochloramine levels were less than 0.3 mg/L, which is representative of what has been observed on board long- and short-haul aircraft (Handschuh *et al.* 2015). The volumes of ammonium and free-chlorine stock solutions added to the water in the tank varied based on ambient temperature and water age.

Contamination, disinfection and flushing and sampling

After the 1-month conditioning period, the mock aircraft water system was spiked with coliforms recovered from positive coliform ADWR samples. After spiking, coliform levels in the water tank were 10^5 to 10^6 MPN/100 ml total. Contamination of the aircraft water system was

conducted by depressurizing the tank, draining it and refilling it with 0.04 m^3 (10 gallons) of disinfectant-free granular activated carbon (GAC) filtered water. Coliforms were spiked into the 0.04 m^3 (10 gallons), and the tank was filled to 0.15 m^3 (40 gallons). The tank was re-pressurized to 2.4 atm (35 psi), and the lavatory faucet was depressed for 5 minutes to ensure that the coliforms were spread throughout the aircraft water system. The water system then sat stagnant for 24 hours to ensure that coliform bacteria had adequate contact with all surfaces.

After the 24-hour contact period, 100 ml water samples were collected from the faucet and tank, and swab samples were collected from the stainless steel coupling and tubing surfaces at six points in the water system (Figure 1). Disinfection and flushing occurred by adding Purogene® (chlorine dioxide) at either 100 mg/L in the tank with a contact time of 2 hours, 0.94 L (1 quart) of Purogene® diluted in the 0.15 m^3 (40-gallon) tank with a contact time of 2 hours, or 3.8 L (1 gallon) of Purogene® diluted in the 0.15 m^3 (40-gallon) tank for 5 min. Ozone was applied at a minimum of 1 mg/L for a contact time of 5 minutes. Mixed oxidant was applied at a target concentration of 4 mg/L in the 0.15 m^3 (40-gallon) tank with a contact time of 24 hours.

Purogene® was added to and flushed through the mock aircraft water system in the same manner as the coliform bacteria. Ozone was supplied by a ClearWater Tech, LLC CD2000 ozone generator (San Luis Obispo, CA). Ozonated water was flushed through the water system by depressing the faucet and allowing water to flow until the 5-minute contact time was achieved. Mixed oxidant was produced using an on-site generator from REDO Water Systems GmbH (Groß-Zimmern, Germany), which generates mixed oxidant through a proprietary electrolysis process using table salt and water. Ten grams of sodium chloride can generate 1 L of the mixed oxidant solution at 400–600 ppm free chlorine. After treatment with the disinfectants, the system was flushed with tap water (containing both free chlorine and combined chlorine) until no ozone, chlorine dioxide or mixed oxidant (as determined by free chlorine) was detected. At the conclusion of the test, the conditioning process described in the previous section was repeated in preparation for another contamination experiment.

Aerator disinfection

Aerators obtained from US-based air carriers were individually soaked in a 100 ml solution of coliform bacteria recovered from positive coliform ADWR samples for 1 hr. Coliform levels in each 100 ml volume were approximately 10^3 MPN/100 ml total, with equal proportions of the coliform species used. After soaking nine aerators for 1 hr, three aerators were aseptically transferred to 100 ml sterile phosphate buffer with $MgCl_2$ (Hardy Diagnostics, Santa Maria, CA), three to 100 ml Lysol® (Reckitt Benckiser, Parsippany, NJ), and three to 100 ml Glyco-San® (Celeste® Corp., Eaton, MD) for 30 minutes. The aerators were then aseptically transferred to sterile phosphate buffer with $MgCl_2$, vortexed for 10 seconds, and the aerators aseptically removed. The remaining buffer was analyzed for residual coliform bacteria that came off the aerators after soaking in disinfectant or sterile buffer. Like the coliform soaking solution, aerators were individually placed in sterile buffer or disinfection solution. Identical experiments were performed with brushing of the aerators with steel wool to remove any external deposits before soaking in the coliform solution. Brushing with steel wool occurred until no visible dirt or deposits were observed on the aerators.

Analytical methods

Table S1 in the Supplementary Information (available with the online version of this paper) summarizes the analytical methods used in this study. All methods, quality assurance/quality control checks and calibrations were performed in accordance with the method or manufacturer recommendations.

RESULTS AND DISCUSSION

Isolation of coliform bacteria

A total of 161 bacterial isolates were recovered from 38 total coliform-positive ADWR water samples over 2 years. Most of the isolates belonged to the genera *Enterobacter* and *Klebsiella*, but isolates from *Citrobacter, Serratia, Hafnia, Kluyvera, Pseudomonas, Stenotrophomonas, Shigella, Myroides* and *Escherichia* were also recovered, as well as unidentified isolates.

Since these bacteria came from aircraft water systems, the isolates with the greatest ability to form biofilms were ideal for seeding coliforms in the mock aircraft systems. Therefore, 24 isolates were chosen for biofilm-forming ability. Of these, 12 isolates yielded a biofilm formation factor of >2.0 and were considered to be potential water-plumbing colonizers. Three of these isolates (one *Enterobacter*, one *Klebsiella* and one unidentifiable) were chosen for use in the mock water system contamination experiments.

Aircraft water quality

Over the course of 1 year, free chlorine, monochloramine and total chlorine in the aircraft water tank averaged 0.05 ± 0.08 ($n = 324$), 0.23 ± 0.16 ($n = 275$) and 0.28 ± 0.17 ($n = 325$) mg/L, respectively, and 0.01 ± 0.02 ($n = 307$), 0.04 ± 0.07 ($n = 310$) and 0.11 ± 0.11 ($n = 266$) mg/L, respectively, at the lavatory faucet. Turbidity was 1.3 ± 0.5 and 0.9 ± 0.5, and pH was 8.1 ± 0.2 and 8.0 ± 0.2 at the faucet and in the tank, respectively. Heterotrophic plate count (HPC) was higher at the faucet with levels of $6.3 \times 10^4 \pm 6.5 \times 10^4$ MPN/ml compared with $3.6 \times 10^3 \pm 1.4 \times 10^4$ MPN/ml in the tank. These data are summarized in more detail in the Supplementary Information (available with the online version of this paper).

Coliform persistence in the aircraft water system

Before the effectiveness of disinfection and flushing were examined, coliform bacteria were injected into the mock system with dechlorinated water and their persistence examined. Results are summarized in Figure 2. Coliforms were introduced into the tank and flushed through the mock system at time zero. Thereafter, the contaminated water sat stagnant until day 4. On day 4, the contaminated water in the tank was dumped, the tank was refilled with clean dechlorinated water and the entire mock system flushed for 10 minutes. This process was repeated a second time. After the second flush, water was sampled from the faucet for the next 6 days. Coliform samples were taken in duplicate at two different times (four samples total) on days 4 to 7, and at one duplicate sampling time (two samples) on day 10. The number of coliforms detected was 2 to 3 logs lower than before flushing occurred, with the average number of coliforms dropping to an average of 13 MPN/100 ml at day 4 and 1 MPN/100 ml at day 10. Relative standard deviation of the samples taken from days 4 to 10 ranged from 40% to 60% due to the low numbers of coliforms detected.

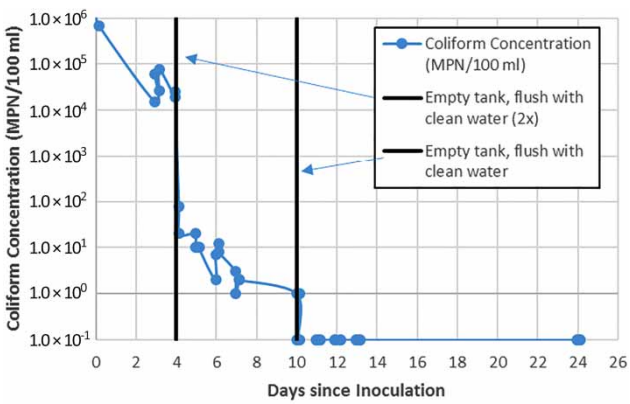

Figure 2 | Coliform persistence in the mock aircraft system water phase in dechlorinated water.

At day 10, the tank was again dumped, filled with coliform-free dechlorinated Cincinnati water and the system flushed for 10 minutes. Coliform bacteria were sampled from the faucet for an additional 2 weeks. During this time, no coliforms were detected at the faucet. At day 24, the six couplings in the water system were opened, and the stainless steel coupling and inner tubing surfaces were swabbed and analyzed for coliforms. No coliforms were detected on any surface. The swab data and the bulk phase data collected after flushing suggest that the coliforms introduced into the mock aircraft water system do not persist on the surfaces and are released into the water phase over the long term. If any coliforms did persist in the water system it was between days 4 and 10. However, these could have been residual coliform bacteria suspended in the water phase that were not removed by the flushing and draining of the tank.

Disinfection and flushing

Table 1 summarizes the results of the disinfection and flushing experiments. As indicated in the left column, coliforms were introduced into the tank with dechlorinated water at approximately 10^5 to 10^6 MPN/100 ml, flushed through the water system and allowed to stagnate for 24 hours. After 24 hours, the tank was emptied, refilled with water containing disinfectant, and this was flushed through the

Table 1 | Summary of coliform persistence after disinfection and flushing

Coliform inoculation[a] (MPN/100 ml) Mean	Disinfectant/Contact time	Disinfectant concentration at the tap (mg/L)	Fitting/Tubing coliform sampling (all six points[b]) MPN	Inside faucet coliform sampling MPN	Aerator coliform sampling MPN
3.7×10^5	Purogene/2 hr	120	ND	ND	ND
9.2×10^5	Purogene/2 hr	140	ND	ND	ND
2.4×10^5	Purogene (1 qt)/2 hr	18	ND	ND	ND
4.6×10^5	Purogene (1 gal)/5 min	34	ND	ND	ND
5.6×10^5	Ozone/5 min	1.25	ND	ND	8.5×10^0
2.6×10^6	Ozone/5 min	1.04	ND	ND	8.8×10^1
4.1×10^5	MIOX/24 hr	2.9	ND	ND	3.1×10^0
2.7×10^5	MIOX/24 hr (6-day coliform contamination)	4.0	ND	ND	2.6×10^3

[a]Mean values are from eight samples from both the tap and tank.
[b]Six fitting and six tubing sections were swabbed during each experiment. No coliforms were detected in any swab samples after disinfection.
ND: None detected (detection limit 1 MPN/100 ml).

system and allowed to sit in contact with the plumbing for a certain amount of time (e.g., 5 min for ozone, 2 hrs for Purogene®, etc.). The disinfectant concentration achieved at the tap is shown in the third column. In one experiment with mixed oxidant, the coliforms were left in contact with the aircraft plumbing system for 6 days instead of 24 hours.

The fourth column summarizes results from all six coupling sections (both stainless steel fittings and tubing) that were opened, swabbed and analyzed for coliform bacteria. After disinfection and flushing in all conditions shown in Table 1, no coliforms were detected on any of the swabbed surfaces in the mock aircraft water system. The same is true for the inside of the Adams-Rite timed swivel-head faucet after the aerator was removed. In addition, during the mixed oxidant tests, extra tubing sections were swabbed at 30 min and 1, 2, 6 and 24 hours after disinfection began. No coliforms were detected on these tubing sections, and none were detected in the water exiting the faucet after mixed oxidant was added. This suggests that coliform inactivation in the mock system takes place much sooner than the 24-hour hold time. The only place that coliforms were detected after disinfection and flushing was in the timed swivel-head faucet aerator after ozone and mixed oxidant treatment. Overall, the data suggest that any residual coliforms in the mock aircraft water system were inactivated by the Purogene®, ozone and mixed oxidant disinfection and flushing methods.

Air carriers using ozone routinely achieve concentrations of 4 to 5 mg/L during disinfection. Ozone levels higher than those achieved in this study may have inactivated coliforms adhered to the aerator. Furthermore, many air carriers have procedures for the disinfection or replacement of the aerators during each disinfection procedure, effectively eliminating this potential source of coliform contamination. Finally, for the mixed oxidant experiment with a 6-day coliform contact, water from the Adams-Rite faucet was sampled for free chlorine and coliforms at six points evenly spaced over two and a half weeks after the decontamination experiment ended. No coliforms were detected at any sample point, and free chlorine was detected at each sample point. Free chlorine decreased from 4 mg/L during the experiment to 1.5 mg/L after two and half weeks.

Aerator disinfection

During routine disinfection and flushing of the aircraft, air carriers can either disinfect or replace the faucet aerators. Table 2 summarizes the results of the aerator disinfection study. The aerators were soaked in a coliform suspension of approximately 10^3 MPN/100 ml (shown at the far left). After soaking for one hour, the aerators were transferred to sterile buffer, Lysol® or Glyco-San®, and then transferred to sterile buffer, vortexed and the coliforms released into the buffer were analyzed. Table 2 shows that after soaking in sterile buffer, some coliforms were released from the

Table 2 | Disinfection of coliforms attached to aircraft faucet aerators

	No brushing	With brushing
Coliforms in aerator soaking solution	1,228 ± 572 MPN/100 ml	641 ± 127 MPN/100 ml
Coliforms removed from aerator after sterile buffer rinse	3.4 ± 1.6 MPN/100 ml	2.0 ± 1.6 MPN/100 ml
Coliforms removed from aerator after Glycosan disinfection	None detected (<1 MPN/100 ml)	None detected (<1 MPN/100 ml)
Coliforms removed from aerator after Lysol disinfection	None detected (<1 MPN/100 ml)	None detected (<1 MPN/100 ml)

Data are shown as average ± standard deviation.

aerators. However, after soaking in undiluted Lysol® or Glyco-San®, no coliforms were recovered from the aerators. This suggests that soaking aerators in Lysol® or Glyco-San® is effective at inactivating any coliforms that are persisting on the aerators. Furthermore, if disinfection and flushing with Purogene® or ozone does not inactivate all coliforms in the faucet aerators, the secondary disinfection step with Lysol® or Glyco-San® will.

CONCLUSIONS

The original conclusions from this study are as follows:

- When introduced at 10^6 MPN/100 ml, coliform bacteria did not persist in the water phase or on the water system surfaces over 24 days. This suggests that coliform bacteria are not persistent on aircraft plumbing infrastructure.
- Disinfection and flushing with Purogene®, ozone or mixed oxidant was effective and resulted in no detection of coliform bacteria on the aircraft water system surfaces or the water phase. The only exception was the faucet aerator when using ozone at 1.04 to 1.25 mg/L for 5 minutes and mixed oxidant at 2.9 to 4.0 mg/L for 24 hours.
- Results suggest aerators contaminated via soaking in a coliform solution can be disinfected with either Lysol® or Glyco-San®. After soaking in neat solutions of Lysol® or Glyco-San® no coliforms were detected on the aerators.

Maintaining safe water on board commercial aircraft is critical to the safety of passengers and compliance with regulations. The conclusions from this study can help commercial air carriers deliver safe water by indicating that coliform bacteria are not persistent on aircraft plumbing surfaces, and that coliforms are not detected in aircraft water systems after routine disinfection and flushing procedures are implemented. Faucet aerators can harbor coliforms, but they can be removed by disinfection with common commercially available cleaning agents.

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