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Major Article

Toilet plume aerosol generation rate and environmental contamination following bowl water inoculation with *Clostridium difficile* spores

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A B S T R A C T

Introduction: *Clostridium difficile* is the leading cause of health care–associated gastric illness. Environmental contamination with *C difficile* spores is a risk factor for contact transmission, and toilet flushing causes such contamination. This work explores toilet contamination persistence and environmental contamination produced over a series of flushes after contamination.
Methods: A flushometer toilet was seeded with *C difficile* spores in a sealed chamber. The toilet was flushed 24 times, with postflush bowl water samples and settle plates periodically collected for culturing and counting. Air samples were collected after each of 12 flushes using rotating plate impactors.
Results: Spores were present in bowl water even after 24 flushes. Large droplet spore deposition accumulated over the 24-flush period. Droplet nuclei spore bioaerosol was produced over at least 12 flushes.
Conclusions: Toilets contaminated with *C difficile* spores are a persistent source of environmental contamination over an extended number of flushes.

Clostridium difficile is an endospore-forming anaerobic bacteria that is responsible for thousands of *C difficile* infections (CDIs) throughout the United States[1]. The morbidity and mortality rates are increasing,[1,2] and this organism has become the leading cause of gastric illness in the form of infectious diarrhea, pseudomembranous colitis, and toxic megacolon in the health care setting.[3] CDI appears as a complication of antibiotic use, disproportionately affecting the elderly and women.[3] The relapse rate is high, with each successive infection reported as more severe than the previous infection. Those with secondary CDI as a result of antibiotic therapy had the worst outcomes.[3] Therefore, it has become extremely important to identify and address routes of contamination in order to prevent colonization and infection of patients.

Previous research has examined both environmental contamination and aerosol recovery of *C difficile* endospores (spores). Kim et al[4] demonstrated that spores could remain on surfaces at least 5 months, where spores were commonly found, and showed what appeared to be a positive association between hand cultures and environmental spore load. Other research indicated that rates of contracting CDI were greater in rooms that previously housed patients with active CDI infection, and when sporicidal cleaners were used according to manufacturer's directions, they were effective.[5] Unfortunately, microbiological models indicated terminal cleaning, the cleaning that occurs after patient discharge, was inadequate in most cases.[6] This meant that contaminated surfaces were implicated as a transmission method, with the probability of multiple strains of infectious pathogens building up over time.[7] Strains were also recovered in rooms with asymptomatic patients, and the researchers suggested aerial dissemination should be investigated further.[7] Air sampling with a portable cyclone outside of a hospital bathroom resulted in collection of positive air samples, although they were unable to successfully repeat the collection the next year.[8] A study, which utilized an Air Trace slit-to-agar impactor, (Particle Measuring Systems, Boulder, CO) resulted in positive air samples recovered from the bedside of confirmed CDI patients, as well as a control patient room, during activities such as room cleaning, curtain moving, and food delivery.[9] An in situ test of toilets in a hospital ward examined the potential for aerosolization of *C difficile* from contaminated toilets and resulted in the collection of positive air and environmental samples, with the greatest measurable

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contamination occurring immediately postflush.[10] This is in line with Gerba et al,[11] who noted that their organisms of interest, *Escherichia coli* and MS2 bacteriophage, persisted in the toilet bowl after multiple flushes. Their reported 2-3 log concentration reduction after the first flush and eventual plateauing was also noted by Barker and Bloomfield[12] in 2000, Barker and Jones[13] in 2005, and Johnson et al[14] in 2017. The literature review of Johnson et al in 2013 and a later study in 2017 indicated that the toilet bowl may serve as an ongoing contamination source.[14,15]

As an aerosol generation device, a toilet may continue to generate an aerosol and the resulting droplet nuclei could contaminate the environment when settling on surrounding surfaces and fomites. The purpose of this study was to further characterize bacterial endospore persistence in toilet bowl water after a series of consecutive flushes, aerosol generation from the toilet over the series of flushes, and large droplet contamination of surrounding surfaces.

METHODS

Culture preparation and concentration determination

All experiments took place in a pre-existing 5 m^3 volume water closet (WC) experimental apparatus (Fig 1) equipped with a Flushometer-style toilet (Model 3461, American Standard, Piscataway, NJ; Model 111-1.2 valve, Sloan, Franklin Park, IL). The chamber was fitted with an airtight door, filtered water supply, high-efficiency particulate air (HEPA)-filtered air supply and exhaust systems, and an outfall to allow capture of flushed water.[16] A nonpathogenic strain of *C difficile* spores (ATCC 700057; Microbiologics, St Cloud, MN) was used to prepare a spore stock suspension. The starting lyophilized pellet was cultured in 500 mL of brain heart infusion broth medium (Bacto Laboratories PYI Ltd, New South Wales, Australia) and anaerobically incubated at 37°C for 10 days, after which the culture was heat shocked at 80°C for 20 minutes to kill all remaining vegetative bacteria. The culture was then centrifuged at 5,000 g for 15 minutes, the supernatant removed via pipette, and the pellet resuspended in sterile deionized water. Centrifugation, supernatant removal, and resuspension were performed a total of 4 times. The suspension was then separated into sterile conical tubes in 50-mL aliquots.

Fig 1. Water closet chamber showing the placement of slit-to-agar bioaerosol samplers, East (E.) and West (W.), and the marked floor locations where surface collection took place.

All culture plates were made using *C difficile* chromogenic agar (CHROMagar, Paris, France) and were batch prepared according to manufacturer directions. In previous studies, *C difficile* was cultured on cycloserine-cefoxitin-fructose-egg yolk agar or on 1 of the many altered versions of this base agar formula.[17] The formulas were then adjusted over time and through experimentation by either increasing or decreasing the amounts of cycloserine and cefoxitin, changing antibiotics, adding bile salts, or adding lysozyme.[17,18,19,20,21,22] Perry et al[17] tested a new media, a chromogenic agar with a proprietary blend of antibiotics, growth factors, and chromogenic mix, against 5 distinctive selective agars. The chromogenic agar was highly selective, sensitive, and differential. CHROMagar for *C difficile* allowed for confirmatory identification of positive colonies after 24 hours through the use of short-wave ultraviolet (UV) illumination. To determine the source suspension spore concentration, serial dilutions of the stock suspension were prepared and plated on 100 x 15-mm plates and incubated anaerobically at 37°C for 24 hours. The resulting colonies were counted under 365-nm UV illumination, and the indicated concentrations were averaged to provide the concentration estimate.

Preparation and use of the WC

Prior to all experimental runs, the toilet bowl was cleaned with a standard toilet brush and a chlorine bleach solution. The solution was prepared as 100 mL of 8.25% chlorine bleach added to the bowl water, which averaged 5 L. The solution was allowed to stand in the bowl for at least 1 hour. After cleaning, the toilet was flushed until the free chlorine residual measured <0.1 mg/L. The water used for this study was public utility water, which was filtered to remove suspended solids. Prior to all experimental runs, water samples were obtained from the toilet bowl and from the pressure tanks, where the water was held to confirm the water was not previously contaminated with *C difficile*. The HEPA-filtered air supply and exhaust systems were adjustable to provide a net positive or negative pressure in the WC relative to surrounding areas. The system purged the WC under positive pressure of 7.5 Pa ± 1.2 Pa when removing background aerosol prior to a flush, and under negative pressure of 7.5 Pa ± 1.2 Pa when exhausting *C difficile* aerosol after a flush. The toilet seat was in the down position, as might be expected during actual use. The lid was in the up position, simulating the no-lid toilets found in hospitals.

Experiment A: Environmental surface contamination from droplets and toilet bowl water persistence

After the blank samples were obtained, a 50-mL aliquot of suspension was added to the toilet (seeding), then sterile deposition surfaces (inverted 100-mm diameter culture plates) were placed in 7 premarked positions on the floor surrounding the toilet (Fig 1). Contact plates were used for 2 reasons: (1) prior studies utilized contact plates in the collection of *C difficile* spores in the environment,[2,4,5,7,8,23] and (2) RODAC plates required less CHROMagar media than agar-filled settle plates. The WC door was sealed and the first flush was initiated. After allowing 1 minute for large droplets to settle onto the plates, the HEPA air supply was turned on and allowed to purge the WC under negative pressure for 30 minutes prior to opening the door and collecting the settle plates and a bowl water sample. A 50-mL postflush water sample was obtained and the spores

collected on the deposition surfaces were transferred to CHROMagar RODAC plates for incubation and counting. Transfer from surface to RODAC plate was accomplished by pressing for at least 30 seconds to ensure transfer of the spores from the collection surface to the contact plate. New sterile deposition surfaces were then placed, and the door resealed for the next trial. This process was repeated for flushes 2-4, then the procedure was altered to switch from every flush to every fifth flush, so that postflush water and environmental samples were obtained after flushes 1-4, 9, 14, 19, and 24. The overall series of trials was replicated 3 times.

RODAC plates were anaerobically cultured at 37°C for 24 hours and counted under UV illumination. The postseeding water sample was serially diluted and surface plated for incubation and counting. All other water samples were filtered without dilution through 37-mm membrane filters, and the filters were placed directly on a CHROMagar plate for incubation and counting.

Experiment B: Aerosol generation

The toilet was seeded, and a postseed sample was obtained. Sterile deposition surfaces and 2 Air Trace slit-to-agar bioaerosol impactor samplers were placed as shown in Figure 1. The air samplers were placed 1 m above the floor and 0.5 m from the toilet rim to avoid capturing large-droplet aerosol. The 150 x 15-mm Air Trace plates were placed in the samplers, and their initial position was marked through the inlet slit with a sterile glass coverslip. The samplers operated at a flow rate of approximately 28 L/min and were programmed to start after the door was sealed and the HEPA purge was complete. The sampler was allowed to operate for 5 minutes before the first flush. After the first flush, there was a flush every 15 minutes for a total of 12 flushes. After completion of the trial, the HEPA was run for 30 minutes under negative pressure prior to unsealing the WC. After removing the WC door, the Air Trace plates were immediately lidded and removed to prevent contamination. RODAC plates were used to transfer collected spores from the deposition surfaces, and a 50-mL bowl water sample was obtained. The overall series of trials was replicated 5 times. RODAC and Air Trace plates were incubated as previously described. The Air Trace plates were counted using a template that indicated the plate area spanned by the sampler during each postflush sampling period.

Experiment C: Aerosol generation with purge

This experiment was a follow-up to experiment B, in which the chamber was not purged with particle-free air prior to each flush. It could not be stated with confidence that any spores collected in later flushes were not simply droplet nuclei left over from earlier flushes. In this experiment, the toilet was flushed a total of 6 times after initial contamination, with a 15-minute air sampling period, followed by 30 minutes of chamber purge after each flush. The effective purge rate was 18 air changes per hour. The Air Trace sampler remained in operation throughout the entire experiment. The lack of deposited spores on the plate segments corresponding to the purging periods verified spore-free air in the WC prior to the next flush. The bowl water samples were collected immediately after seeding and after the last flush.

RESULTS

The stock spore suspension concentration was estimated to be 5.75×10^5 colony-forming units (CFU)/mL (95% confidence interval, 3.97×10^5, 7.53×10^5).

Water samples

All pretrial and water supply water samples were negative for *C difficile*. *C difficile* bowl water concentrations for the 3 trials in experiment A are shown in Table 1. Spores were captured in all postflush water samples in all trials, indicating persistent contamination through at least 24 flushes. Figure 2 shows that the fractional reductions in bowl water were consistent with the results of Darlow and Bale,[24] Newsom,[25] Gerba et al,[11] Yahya et al,[26] Barker and Jones,[13] and Johnson et al,[14] in which bowl water concentrations of various microorganisms and inert microspheres were reduced by approximately 3 logs with the first flush and a total of approximately 4-5 logs after 3 flushes. Initial bowl water spore concentrations were similar in experiment C, averaging 1.97×10^4 CFU/mL.

Experiment A and B environmental samples

For experiment A, in which the settle plates were removed after flushes 1-4, 9, 14, 19, and 24, *C difficile* colonies were identified on RODAC plates through flush 19, though not on every plate. Counts were usually 1 or 2 CFU, with a maximum count of 4 CFU. Over the series of 3 trials, all plate positions had at least 1 positive sample.

For experiment B, in which 1 set of plates remained in place through a series of 12 flushes, each plate position had at least 1 positive sample in the 5 trials. The counts were generally low, with only 3 of the 35 plates having more than 3 CFU. These results and those of experiment A suggest that the settle plate sampling process was governed by Poisson probabilities.

Areal deposition density of large-droplet spore bioaerosol settling on floor surfaces around the toilet was calculated as $\textit{Areal density} = \frac{\Sigma N_i}{A_s}$ in which cumulative CFU counted through a particular flush is divided by the total area of the RODAC plates counted (25 cm^2 per plate). The cumulative buildup in average areal deposition density is shown in Figure 3. The cumulative areal density for all plates combined was 533 CFU/m^2 after 24 flushes. Approximately 75% of this level was attained after only 4 flushes, and 90% of this level was attained after only 9 flushes. The cumulative average areal deposition density over 12 flushes in the 5 trials of experiment B was 446 CFU/m^2, in general agreement with the pattern seen in Figure 3.

Experiments B and C air sampling results

One of the Air Trace samplers was discontinued after failing during trials 2 and 3 of experiment B. Average CFU counts for the first 3 flushes were approximately 8, 3, and 2.5, respectively, equivalent to the average airborne droplet nuclei spore aerosol concentrations of

Table 1
Initial and postflush bowl water concentrations (CFU/mL)

Run	0	1	2	3	4	9	14	19	24
A1	1.60E+03	5.24E+00	1.24E+00	5.20E-01	2.80E-01	1.60E-01	2.00E-02	2.00E-02	2.00E-02
A2	1.47E+04	8.40E-01	1.32E+00	2.80E-01	8.00E-02	2.00E-01	4.00E-02	8.00E-02	4.00E-02
A3	9.40E+03	5.20E-01	3.20E-01	1.60E-01	1.20E-01	2.00E-02	2.00E-02	2.00E-02	2.00E-02

CFU, colony-forming units.

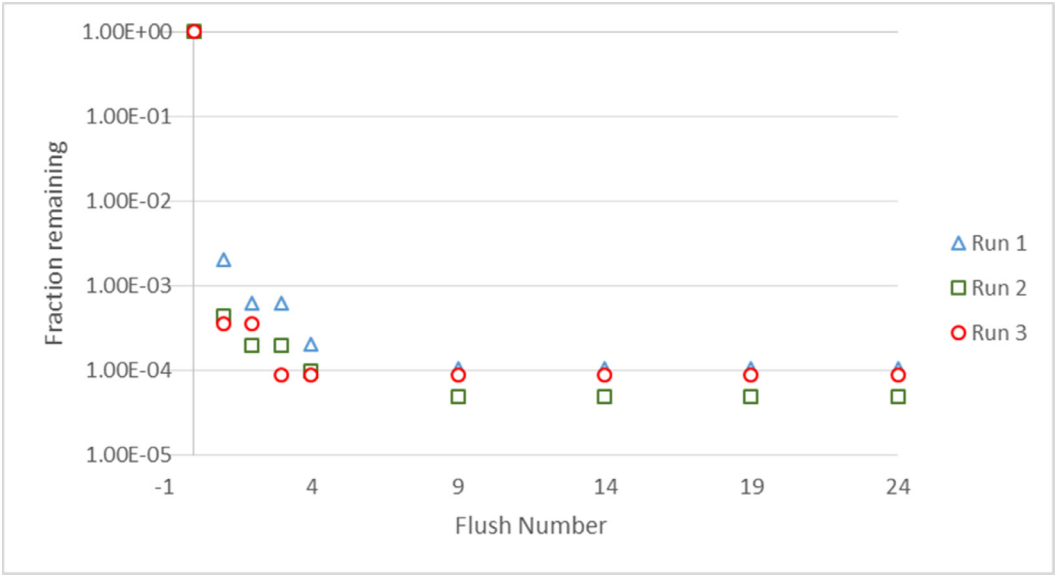

Fig 2. Fractional reductions in bowl water concentration over the series of 24 flushes. *Run 1*, blue triangles; *Run 2*, green squares; *Run 3*, red circles.

approximately 10.9, 3.8, and 3.4 CFU/m^3. For the 5 m^3 WC chamber volume, this indicates droplet nuclei bioaerosol generation rates of approximately 54, 19, and 17 CFU per flush over the first 3 flushes. The overall pattern of bioaerosol concentrations produced over the 12-flush series is shown in Figure 4. Owing to the small number of CFU counted after each flush, the data are presented as Poisson means and 95% confidence intervals.[27] It is notable that the droplet nuclei bioaerosol generation reflected in Figure 4 did not decline in proportion to the decrease in bowl water concentration reflected in Figure 2. This was consistent with observations by others that indicated a 3-log reduction in bowl water concentration resulted in only 50%-60% reduction in droplet nuclei bioaerosol production per flush.[11,13,24,26]

Air sampling results for the 15-minute postflush measures in experiment C are shown in Figure 5. No colonies were observed on plate segments corresponding to the purge periods between flushes. Although the initial bowl water concentrations in experiments B and C were similar at approximately 10^4 CFU/mL, the estimated mean spore aerosol concentration after the first flush was somewhat lower in experiment C than in experiment B. The 2 experiments were not significantly different and exhibited similar patterns of decline in airborne concentration over the flush series, with each showing a 95% decline in airborne concentration over the first 4 flushes. The experiment C results appear to indicate that the experiment B measures were not influenced by residual droplet nuclei spore aerosol.

DISCUSSION

To our knowledge, this study is the first to report the bowl water contamination persistence and toilet plume bioaerosol generation potential of commercial toilets contaminated with *C difficile* spores. Our findings are consistent with observations by others for much shorter series of sequential flushes, using various microorganisms and inert surrogate microspheres, and extend them to show persistence and aerosol generation for many more

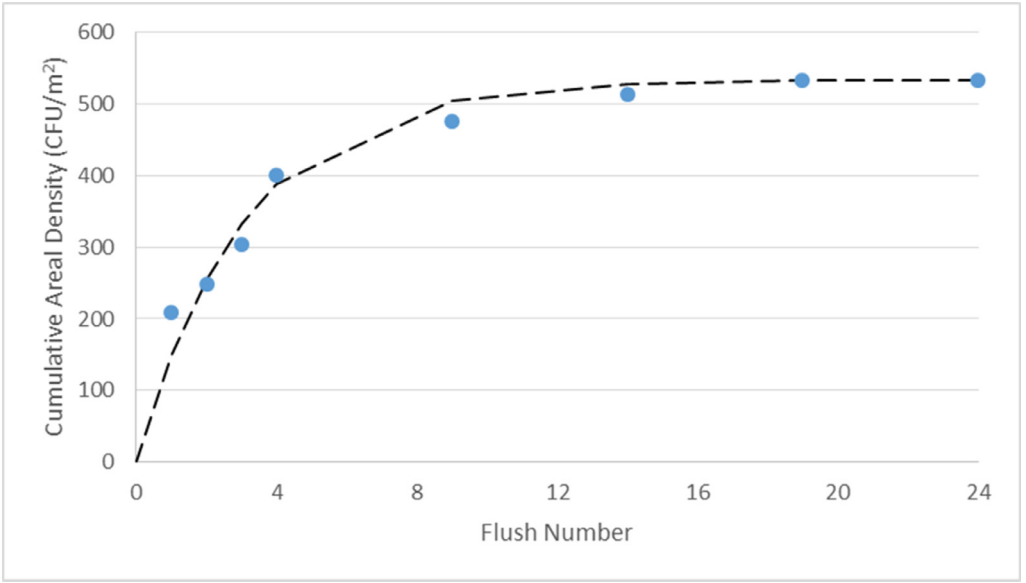

Fig 3. Cumulative areal density of *Clostridium difficile* large droplet deposition in experiment A. *CFU*, colony-forming units.

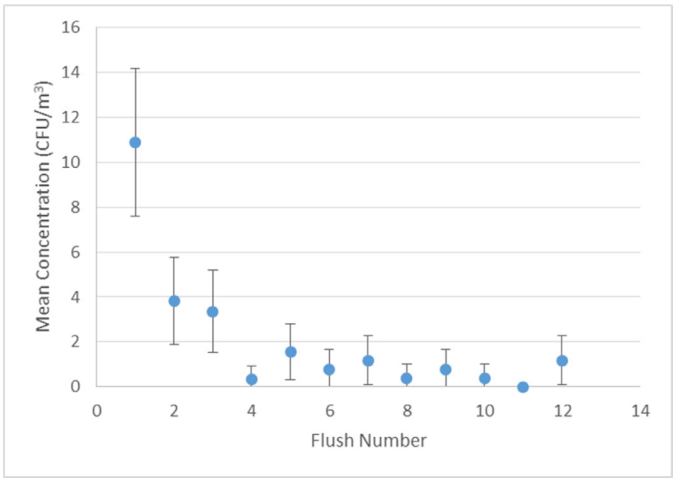

Fig 4. Poisson mean and 95% confidence interval of air concentration over 12 flushes. Experiment B with no purge between flushes. *CFU*, colony-forming units.

flushes than might have been expected. HEPA purges removed aerosolized spores from the WC, and negative pressure purges and neutral pressure sampling did not result in spores re-entering the WC from outside the chamber.

This study is also the first, to our knowledge, to use CHROMagar for air sample collection and analysis. The agar was effective for slit-to-agar direct impaction of spores, culturing of MCE filters on the agar surface, and surface plating of water samples. Previous studies, which in general had difficulty detecting airborne *C difficile*, used other agars. A direct comparison of CHROMagar and other agars via side-by-side air sampling would provide useful information regarding which is most effective.

Utilizing contact plates instead of settle plates may have resulted in underestimation of bacterial colonies due to clumping of endospores; however, previous studies have suggested that gram-positive bacteria, and likely endospores, are more sensitive to recovery by contact plate and that 30 seconds of contact time between surface and contact plate is sufficient in laboratory-controlled experiments.[28]

These results have significant implications for public access environments where persons shedding *C difficile* spores, or other gastrointestinal pathogens such as norovirus, in stool may contaminate a toilet that is subsequently used by many other persons. These users may be exposed to either accumulated surface contamination or inhaled bioaerosol, potentially leading to disease transmission. Previous research on *C difficile* infectious dose (ID) by Lawley et al[29] using murine models indicated that a very low spore count, <1 spore/cm^2, could induce disease in a susceptible, antibiotic-treated population, whereas infectious dose$_{50}$ was approximately 5 spores/cm^2 and all exposed mice contracted disease at 100 spores/cm^2. This indicates that there is a possible hazard from both the immediate deposition from the initial flush and from the buildup of spores on surfaces after many flushes. In health care environments, more frequent and aggressive toilet bowl decontamination may be desirable to limit ongoing room surface contamination, and there is a potential for migration of *C difficile* droplet nuclei bioaerosols, with air currents produced by care provider movement or heating, ventilation, and air conditioning system operation, to contaminate surfaces outside of the contact isolation room.

CONCLUSIONS

These results demonstrate that toilets contaminated with *C difficile* spores will produce numerous large droplet and droplet nuclei bioaerosols that can contaminate surfaces close to and distant from the source. Further, the microbial contamination will persist in the bowl water for many flushes after initial contamination and produce bioaerosol with each flush. *C difficile* surface contamination will accumulate as large droplet and droplet nuclei spore bioaerosol continue to be produced for many flushes, and droplet nuclei *C difficile* spore aerosol may be expected to travel with air currents throughout the facility and contaminate surfaces outside the CDI patient room.

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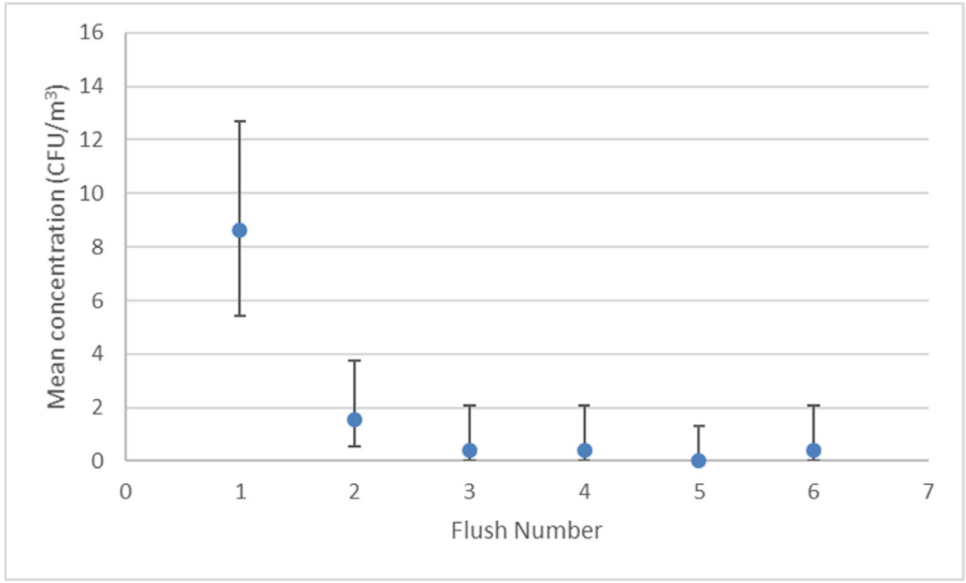

Fig 5. Poisson mean and 95% confidence interval of air concentration over 6 flushes. Experiment C with purge between flushes. *CFU*, colony-forming units.

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