

THE UNIVERSITY OF OKLAHOMA HEALTH SCIENCES CENTER
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DEVELOPMENT, EVALUATION, AND DEMONSTRATION OF AIR SAMPLING
METHODS TO ASSESS AIRBORNE TRANSPORT OF *CLOSTRIDIUM DIFFICILE*
SPORES IN THE HEALTHCARE ENVIRONMENT

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ABSTRACT

Clostridium difficile (*C. difficile*) is a significant healthcare acquired infection worldwide with over 100,000 cases annually in the United States alone. Multiple studies have documented the aerosol generation and airborne dissemination of *C. difficile* endospores. These endospores are resistant to environmental stresses and have the potential for airborne transport from a source to other areas in a healthcare setting, thus enabling environmental contamination. This study evaluated air sampling methods in the collection of *C. difficile* aerosol and aimed to: (1) Evaluate low-volume air sampling devices and mechanisms of collection for laboratory-generated aerosols of *C. difficile* spores; (2) Evaluate the retention of culturable *C. difficile* spores in impinger air samplers; (3) Evaluate high-volume air sampling devices in the collection of nebulizer-generated *C. difficile* spore aerosols in a laboratory environment; (4) Demonstrate effectiveness of high-volume air sampling equipment in the collection of *C. difficile* spore aerosol produced from toilet flushing. In aim 1, mixed cellulose ester (MCE) filters demonstrated significantly higher relative sampling efficiencies compared to slit-to-agar impactor (AirTrace™) and liquid impinger air samplers (AGI-30 and BioSampler™). Both impinger types frequently failed to detect the presence of *C. difficile* aerosol. In aim 2, impinger operation produced significant culturability losses and failed to retain *C. difficile* endospores. In aim 3, high volume air sampling using a military Dry Filter Unit 1000 (DFU-1000) resulted in a significantly higher relative sampling efficiency compared to the commercial Biocapture-650, XMX/2L-MIL, and Microbio MB2. In aim 4, high volume air sampling provided a census air sample in a small enclosure similar to a

patient's bathroom. Toilet flushing from a contaminated toilet generated a mean of 81 colony-forming units during the first three flushes after contamination. Furthermore, high volume air sampling using the DFU-1000 significantly increased the frequency of airborne endospore detection at low airborne concentrations relative to low volume air sampling. This study demonstrated that sampling stress significantly affects the enumeration and detection of airborne *C. difficile* spores. Furthermore, this study demonstrated an economical air sampling method, MCE filtration, to improve air sampling and risk assessment in healthcare facilities.

CHAPTER I

INTRODUCTION and LITERATURE REVIEW

Overview and Objectives

Healthcare-acquired *Clostridium difficile* infection (HA-CDI) is a significant burden to healthcare facilities in the provision of effective care and is the leading cause of healthcare-acquired infection in the United States (Lessa, et al., 2015). Spores of *Clostridium difficile* (*C. difficile*) are infectious, resistant to environmental degradation, and known to survive on environmental surfaces for long periods of time after being shed from an infected patient (Gerding, et al., 2008). While the transmission of CDI from contact with contaminated surfaces and other fomites is well understood, the potential for CDI transmission through aerosol pathways is not well-evaluated. This research comparatively evaluated low-volume and high-volume air sampling systems, under multiple laboratory conditions, for the collection, detection, and enumeration of airborne *C. difficile* spores.

Occurrence of Healthcare-Acquired *Clostridium difficile* and Incidence Trends

In 2013 there were an estimated 453,000 total cases of CDI in the United States. Of these, an estimated 290,300 cases were healthcare-acquired with 107,600 onsets occurring in a hospital, 104,400 onsets occurring in a nursing home, and 81,300 onsets occurring after receipt of outpatient care (Lessa, et al., 2015). Additionally, CDI is the leading cause of gastroenteritis-associated death, causing 14,000 deaths in 2007 alone, responsible for an estimated \$4.8 billion in excess healthcare costs for acute care

facilities, and has become the most common cause of healthcare-acquired infection in the United States (Lessa, et al., 2015).

Incidence rates of HA-CDI are highest among patients 65 years of age and older with an estimated incidence rate of 481.5 cases per 100,000 persons. This compares to 83.1 cases per 100,000 persons for the 45-64 age cohort (Lessa, et al., 2015). While 92 percent of CDI-related deaths occur in the elderly (65 and older) population (Keller & Surawicz, 2014), HA-CDI occurs across multiple age groups and populations including those typically considered low risk. CDI was once considered very uncommon in children, but has recently been classified as an emerging infection with a four-fold increase in HA-CDI cases observed in Ontario, Canada from 2007 to 2012 and a similar increase in community-acquired pediatric cases of CDI (Le Saux, et al., 2014).

Additionally, in a study among active duty United States Armed Forces members, the incidence of HA-CDI has increased significantly from less than 0.5 HA-CDI cases per 1,000 hospitalizations in 1999 to over 2.0 cases per 1,000 hospitalizations in 2010. The active duty military study for HA-CDI incidence rates demonstrates an increasing incidence trend among a relatively healthy and younger population, relative to the overall US population, and who have regular access to healthcare (Gutiérrez, et al., 2013). In a separate study by Gutiérrez (2015), active duty military personnel with a history of CDI experienced increased risk ratios for other chronic gastrointestinal disorders, compared to active duty military personnel with no history of CDI. These gastrointestinal disorders include incident irritable bowel syndrome (RR: 6.1), gastroesophageal reflux disease (RR: 3.3), dyspepsia (RR: 3.3) and constipation (RR: 2.2) (Gutiérrez, et al., 2015). The combination of increasing incidence rates of HA-CDI

and increased chronic gastrointestinal disorders following CDI infection demonstrates the broad burden of CDI across multiple population segments. This study also suggests that CDI may significantly increase military healthcare costs and lost workdays thus affecting the deployment availability of active duty military personnel.

Asymptomatic carriers of *C. difficile* may significantly contribute to the spread of HA-CDI in hospitals. While the transmission of *C. difficile* from symptomatic and confirmed patients to uninfected patients is well documented, the prevalence of asymptomatic carriers in healthcare environments has only recently been studied. A 2014 study by Alasmari et al. of 259 asymptomatic patients in St. Louis, MO admitted to a hospital without diarrhea illness found that 15 percent were colonized with toxigenic *C. difficile* and an additional 4 percent were colonized with non-toxigenic *C. difficile*. This high prevalence of asymptomatic *C. difficile* among the patient population presents a potential reservoir that is not readily identifiable to hospital staff and remains uncontrolled until the patient is discharged from the hospital or develops active CDI (Alasmari, et al., 2014). The number of spores shed by asymptomatic patients can also be high and similar to that of symptomatic patients. A 2014 study by Galdys et al. identified *C. difficile* in the stool of 7 healthy, asymptomatic, individuals with stool densities ranging from less than 10 colony forming units per gram (CFU/g) to 1.6×10^6 CFU/g. The mean stool density was 3.0×10^4 CFU/g among healthy participants compared to 4.0×10^6 CFU/g in symptomatic CDI patients (Galdys, et al., 2014). Additionally, current clinical practice guidelines do not recommend screening of asymptomatic patients for *C. difficile* and do not recommend contact precautions for asymptomatic carriers of *C. difficile* (McDonald, et al., 2018). The increasing disease

burden of HA-CDI, and large number of both symptomatic and asymptomatic reservoirs in health care, demonstrates the value of understanding and assessing all routes of HA-CDI transmission in a healthcare environment including the potential for aerosolization of *C. difficile* from healthcare processes.

Microbial and Infection Characteristics of *Clostridium difficile*

C. difficile is a Gram-stain positive, obligate anaerobic bacterium that forms dormant endospores, allowing for long-term environmental survival (Paredes-Sabja, et al., 2014). Once spores are ingested by a susceptible host and germinate in the colon, the host is colonized and infected. The successful colonization of the colon by *C. difficile* has been linked to the use of proton-pump inhibiting (PPI) medication, whereby stomach acid is reduced thus allowing greater survival of ingested *C. difficile* and entry into the colon for colonization. In a case-control study by Dial et al., the association of CDI and the use of PPI medication had an odds ratio of 2.7 (95%: CI 1.4 – 5.2) compared to patients who did not receive a PPI medication (Dial, et al., 2004). Development of CDI is also associated with the use of antibiotics, whereby the intestinal flora of the body is disrupted, thus allowing the *C. difficile* to proliferate (Dial, et al., 2004). Following colonization and proliferation, illness may occur from *C. difficile* due to the release of toxins, notably enterotoxin TcdA and cytotoxin TcdB, by the vegetative cells of the toxin-producing strains of *C. difficile* (Paredes-Sabja, et al., 2014). An infected host sheds spores through feces and an estimated 10^4 to 10^7 spores are present in every gram of feces from an infected patient (Mulligan, et al., 1979). This shedding of spores would allow for a significant degree of contamination, even if only a

small fraction of shed spores were released into the environment. *C. difficile* spores have been found to survive on environmental surfaces at least 5 months after release (Kim, et al., 1981). This persistence in the environment could allow spores of *C. difficile* to survive aerosolization, transport by air currents, and deposition onto other surfaces and fomites, allowing the spores to infect or colonize other patients and healthcare staff.

The size of *C. difficile* spores is very important for both bioaerosol generation and the sampling of any generated aerosols. Snelling et al. determined the size of single *C. difficile* spores to have a mean length between 1.0 to 1.5 micrometers (μm) with a range of 0.79 – 1.99 μm and a mean diameter between 0.5 – 0.7 μm . Applying Stokes' Law to standard density particles in this size range, Snelling estimated 1 meter settling times of between 2.1 and 13.9 hours in still air (Snelling, et al., 2010). Air movement would be expected in most healthcare environments from heating, ventilation, and air conditioner operation and patient/staff movement in the room; therefore, stirred settling would be expected and could significantly lengthen the spore settling times estimated by Snelling. Additionally, the concentration of monodisperse aerosol particles in a stirred settling environment decays exponentially with time but is never completely removed (Hinds, 1999). The degree that stirred settling could affect an aerosol of *C. difficile* would be dependent on the velocity of air currents and degree of personnel activity or ventilation system operation responsible for the stirring effect. Extended settling times and reduced aerosol elimination could allow a significant amount of time for an aerosolized spore or spore-containing particle to travel on an air current for deposition to other surfaces or patients.

Environmental Contamination and Transport of *Clostridium difficile*

Environmental surface contamination by *C. difficile* spores is well documented in the literature and numerous studies since the early 1980's. *C. difficile* spores that could be cultured from samples have been collected on surfaces in treatment rooms with symptomatic patients, asymptomatic patients, and patients with no evidence of *C. difficile* colonization, although the amount of *C. difficile* was highest in infected patient rooms (Surawicz, et al., 2013). Lawley et al. (2010) estimated the infectious dose from surfaces contaminated with *C. difficile* spores by exposing mice to cage surfaces contaminated with spores. The dose necessary to infect 50% of exposed individuals has been estimated at less than 10 spores per cm². Infections were observed at surface contamination levels of less than 1 spore per cm² (Lawley, et al., 2010). While *C. difficile* spores have been recovered from numerous surfaces and materials in the healthcare environment, only one study to date has demonstrated the environmental stability of vegetative *C. difficile*. A study by Jump et al. demonstrated that vegetative *C. difficile* (whole cells) can survive on moist surfaces such as toilets, sinks, food trays, moist bedding, and dressings for up to 6 hours in an aerobic environment (Jump, et al., 2007). While vegetative *C. difficile* may be a minor contributor to environmental surface contamination, its short-term survival outside of a moist environment makes it an unlikely candidate for widespread persistent surface contamination or aerosolization. In a healthcare environment where temperature and humidity are typically well-controlled, sustained survival of vegetative *C. difficile* would not be expected. Therefore, risk assessment for aerosolized *C. difficile* in healthcare should focus exclusively on aerosolized spores.

Control Methodologies for *Clostridium difficile* in Healthcare

Patient isolation precautions are used to prevent the transmission of infections in healthcare facilities. Centers for Disease Control and Prevention (CDC) guidelines contain two separate tiers of precautions including standard precautions used for all patients regardless of diagnosis and transmission-based precautions used for patients infected with “epidemiologically important pathogens” (Garner, 1996). Transmission-based precautions are further divided into three levels including airborne precautions, droplet precautions, and contact precautions. At this time contact isolation is the transmission-based precaution used for a patient diagnosed with CDI. Contact precautions are recommended from the time a patient is diagnosed with CDI until 48 hours after the cessation of diarrhea (Surawicz, et al., 2013). Contact isolation includes use of a private patient room, use of disposable gloves from patient room entry to departure, use of gowns from patient room entry to departure, and limiting patient transport from the isolation room to essential purposes only (Garner, 1996). Clinical practice guidelines for CDI also recommend the provision of a dedicated toilet to CDI patients (McDonald, et al., 2018). While the use of a private room would limit the transport of generated aerosols to other susceptible patients by increasing the distance from infected patients to susceptible uninfected patients, there is currently no recommendation for additional ventilation or the use of negative pressure rooms for the isolation of CDI patients as is specified under airborne precautions. Airborne precautions are used for microorganisms that are capable of remaining infectious in droplet nuclei and capable of remaining suspended in the air or distributed by air currents and include *Mycobacterium tuberculosis*, Varicella zoster, and measles virus

(Garner, 1996). Airborne precautions require the use of a private room with 6 to 12 air changes per hour of ventilation, outdoor discharge of air or high efficiency particulate filtration of recirculated air, and the use of negative air pressure differentials between the patient room and surrounding areas (Garner, 1996). The effectiveness of negative pressure patient isolation rooms at different negative pressure conditions was evaluated in a 2011 study by Adams et al. This study utilized aerosols of 2 μm diameter polystyrene latex (PSL) spheres and evaluated particle escape under 3 different pressure conditions. The study also evaluated the impact that provider movement into and out of the isolation room has on particle escape into an anteroom transition area (Adams, et al., 2011). A 2 μm PSL sphere is approximately equivalent in size to a *C. difficile* spore (Snelling, et al., 2010). The Adams study showed that the current negative pressure design standard differential of 2.5 Pa is likely inadequate to prevent the escape of airborne pathogens from an isolation room, especially during provider movement into and out of the room. Increasing the pressure differential to 20 Pa significantly decreased particle escape, even with provider movement, compared to the 2.5 Pa standard (Adams, et al., 2011). While airborne isolation precautions are currently not recommended for CDI patients, this study demonstrates that high negative pressure differential isolation could be an effective control for aerosolized *C. difficile* spores if *C. difficile* spores are found outside of the immediate patient vicinity (i.e. patient room and/or bathroom).

Aerosol Generation of *Clostridium difficile* in Healthcare Environments

Numerous healthcare-related processes have been considered as possible sources for the generation of aerosols containing *C. difficile* spores. These include toilet flushing, bedpan washing, bed making, provider movement, housekeeping, and aerosolization or re-aerosolization of spores deposited on surfaces.

Aerosol generation from toilet flushing

Toilet flushing has been identified as a significant generation source of *C. difficile* spore aerosol. This was initially demonstrated in the laboratory by seeding toilets with polystyrene latex (PSL) spheres and measuring the aerosols generated by flushing. A 2013 study by Johnson et al. found that a toilet seeded with 1.9 μm spheres at a mean bowl water concentration of 1.6×10^6 spheres per mL produced an aerosol concentration of 20.1 particles per cubic meter of air when flushed in a 5 m^3 closed water closet. Johnson et al. also found similar generation when 1.0 μm PSL spheres were used (Johnson, et al., 2013). A 1.9 μm particle is similar in size to a *C. difficile* endospore and the 10^6 /mL concentrations used could be representative of a toilet contaminated with feces from an actively shedding CDI patient. Johnson et al. identified a bimodal aerosol size distribution during their trials and suggested that bio-aerosols can be produced by toilet flushing through bubble scavenging and bursting at the bowl water surface and through direct ejection of droplets (Johnson, et al., 2013). Best et al. (2012) conducted a similar laboratory study using a toilet seeded with fecal suspensions containing 10^7 *C. difficile* spores per mL of fecal suspension. After a single flush of a

seeded toilet, *C. difficile* was detected at heights of 10 cm and 25 cm above the seat and spores were found to persist in the air for up to 90 minutes (Best, et al., 2012).

Studies by Aithinne et al. (2019) showed that *C. difficile* spore aerosol can be generated over a series of 12 toilet flushes and that spores can be deposited to nearby surfaces for as many as 24 flushes after an initial contamination event. Surface contamination of more than 500 CFU per square meter on nearby floor surfaces were observed after 24 flushes (Aithinne, et al., 2019). Spores were also found to persist in the air for periods up to 3 hours with serial flushing every 15 minutes of a toilet seeded with approximately 10^4 spores per mL of toilet bowl water (Aithinne, et al., 2019). The Aithinne et al. study also suggested that significant decreases in *C. difficile* bowl water concentrations do not produce a similar decrease in aerosol generation, allowing a toilet with bowl water concentrations as low as 0.02 CFU/mL continuing to sporadically produce spore aerosol. Therefore, toilet flushing is capable of generating a persistent aerosol of *C. difficile* and has the potential to continue aerosol generation after use by a *C. difficile* patient or asymptomatic carrier for several subsequent flushes.

Aerosol generation from bed-making

Bed-making and changing of a patient's bedding have also been identified as potential aerosol generating processes for *C. difficile* spores. During 1 hour air samples collected near CDI patients, Best et al. (2010) found positive air samples for 2 patients when cleaning and bed-making were taking place (Best, et al., 2010). Kim et al. (1981) recovered *C. difficile* on 10 percent of blankets in use by a CDI patient; however, *C. difficile* was not found on clean blankets after laundering (Kim, et al., 1981). Therefore,

removal or disturbance of a used blanket may contribute to the spread of *C. difficile* contamination or aerosol generation. Greene et al. (1962) performed a quantitative microbial contamination study on hospital air and found that the emptying of laundry chutes increased bacterial contamination of the surrounding air. The species of collected bacteria were not determined, however airborne bacterial levels in the chute closet increased by up to an order of magnitude and airborne contamination in a corridor adjacent to the chute closet increased by over 100 percent when compared to levels collected prior to emptying the chute (Greene, et al., 1962). In studies on the aerosolization of methicillin-resistant *Staphylococcus aureus* (MRSA) from patient bed-making, Shiomori et al. (2002) found that mean airborne levels of MRSA increased from 4.7 CFU/m³ to 116.0 CFU/m³. These air samples for MRSA were collected with an Andersen 6-stage microbial impactor placed 1 meter from the patient bedside (Shiomori, et al., 2002). Surface contamination with *C. difficile* spores has also been observed in clinical laundry facilities with positive isolates obtained from surfaces in the receiving and sorting areas of soiled laundry from medical facilities (Michael, et al., 2016). Bedding changes and laundry processing have not specifically been shown to generate *C. difficile* spore aerosol. However, the documented contamination of *C. difficile* spores in soiled patient laundry combined with documented production of other bacterial aerosols by bed-making and laundry processing suggests that these processes are capable of *C. difficile* spore aerosol production.

Bedpan washing and emptying

Bedpan washing and emptying has been identified in previous studies as a potential epidemiological link to the transmission of HA-CDI. In a 1983 study conducted over a two-month period, a cluster of *C. difficile* cases in an intensive care unit was identified near a bedpan washing machine that tested positive for contamination with *C. difficile* (McFarland & Stamm, 1986). No air sampling studies for airborne *C. difficile* have been performed for the emptying and washing of a bedpan; however, bedpan washers are frequently attached directly to a toilet or located in close proximity to a toilet as shown in Figure 1. This presents a risk of aerosol and droplet generation through the use of the sprayer, from pouring wastes into the toilet, and from the aerosol plume generated by the toilet flush as described by Aithinne et al. (2019) and Best et al. (2012).



Figure 1: Bedpan Washing Toilet Station at University of Oklahoma Medical Center

Re-aerosolization of deposited spores

Surface deposition and subsequent re-aerosolization of spores may be a potential cause of airborne *C. difficile* spores in a healthcare environment, especially when spores are deposited on or attached to another environmental fomite, such as a dust particle. The aerosolization of dust particles in indoor environments due to foot traffic or other human activity such as floor sweeping or vacuuming has been documented in the literature. Brauer et al. (1999) conducted measurements in the personal area of a subject in a small dormitory room and demonstrated higher concentrations of fine particulate matter when the subject was moving in the room than when they were sedentary (Brauer, et al., 1999). In residential settings, Ferro et al. (1999) monitored the particulate concentrations in an indoor residential environment and found that coarse particle ($> 2.5 \mu\text{m}$) concentrations increased by 20 times when operating a vacuum cleaner as compared to background levels (Ferro, et al., 1999). While the generation of aerosols from the disturbance of settled particles would be impacted by many factors including type of vacuum cleaner or sweeper used, existing environmental cleanliness (amount of dust present), and type of flooring present, re-aerosolization of fomite-attached *C. difficile* spores should be considered as an aerosol generation source. Hospital rooms are frequently swept and/or vacuumed as part of normal housekeeping activity.

The re-aerosolization of free spores not adhered to a larger particle or fomite has also been studied. Paton et al. (2015) demonstrated the re-aerosolization of *Bacillus atrophaeus* spores deposited by airbrush on polyvinyl chloride flooring and carpeted flooring surfaces. Surface spore contamination of 10^3 to 10^4 spores per square

centimeter was deposited by airbrush onto a flooring surface. Re-aerosolization from light and heavy activity (foot traffic) was assessed at heights ranging from 0 to 1.5 meters above the flooring surface. Spores deposited on carpeted flooring surfaces re-aerosolized at between 114 and 274 times higher on carpet than on PVC flooring depending on whether high or low walking activity was used. Despite the lower re-aerosolization of spores from PVC flooring surfaces, an estimated 1 hour exposure resulted in the breathing of 120 CFU per hour from contaminated PVC flooring. This is in comparison with 14,000 CFU per hour from carpet exposures calculated by the authors and was based on the re-aerosolization of deposited spores assuming a 20 liters per minute (L/min) breathing rate and heavy walking activity (Paton, et al., 2015). Contamination of floors in CDI patient rooms has been demonstrated in previous studies. In a study of 218 CDI patient rooms, 114 (53%) of patient rooms were found to have *C. difficile* contamination on the floor (Deshpande, et al., 2017). While most healthcare facilities use smooth, uncarpeted flooring, this study demonstrates that initial aerosolization or re-aerosolization of deposited *C. difficile* spores by personnel walking activity may occur in healthcare facilities.

Specific Military Applications of *C. difficile* Air Sampling

Long-distance spread of hypervirulent *C. difficile* infection by international patient transfer has been documented in multiple incidences (Rogers, et al., 2011). Spread of norovirus, another enteric pathogen, aboard long-haul aircraft has also been documented, with a single individual contaminating an aircraft and infecting 6 flight attendants over a period of several subsequent flights (Thornley, et al., 2011). For

aeromedical evacuations with multiple patients, as is common during military patient transport, use of recommended *C. difficile* contact precautions is not feasible. For example, placement in separate rooms, use of dedicated toilets, and cohorting of patients with similar diagnoses is not feasible aboard an aircraft, even for large transport aircraft. The US Army Medical Research Institute of Infectious Disease deploys teams equipped with biological containment for aeromedical evacuation, but these teams are only deployed to transport patients infected with biological warfare agents and viral hemorrhagic fevers such as Ebola and Marburg virus (Teichman, et al., 2007). Additionally, current military medical practice for treatment of most combat wounds including those to the chest, abdomen, or extremities recommends the administration of antibiotic prophylaxis to prevent wound infection. Recommended antibiotics include cefazolin and clindamycin (Lloyd, et al., 2018), both of which have been shown to increase susceptibility to *C. difficile* infection (Dial, et al., 2004). While no published data exists on increased *C. difficile* colonization or infection trends in combat injured personnel from the recent conflicts in Iraq and Afghanistan, significant colonization with other healthcare acquired multi drug resistant organisms (MDRO) has been reported at receiving military hospitals in the United States. These reported MDRO colonizations have included enterobacteria and acinetobacter species (Hospenthal, et al., 2011). Therefore, deployable air sampling capability for *C. difficile* may be needed now or in future military operations.

Low-Volume Air Sampling Methods for Bioaerosols

There are four general methods of bioaerosol sampling including impingement, impaction, filtration, and centrifugation. Air sampling methods must be tailored to the organism and setting of interest to maximize efficiency and organism viability (Haas, et al., 2014, pp. 138-140). Additionally, air sampling volumes should be as large as possible without significantly reducing efficiency and organism viability.

Bioaerosol sampling by liquid impingement

Numerous liquid impinger devices have been employed in the collection of bioaerosols. Two of the most common are the All Glass Impinger 30 (AGI-30) (Ace Glass Inc. Vineland, NJ) and the BioSampler™ (SKC Inc. Eighty-Four, PA). These impingers collect airborne particles directly into a liquid and sample at a design flow rate of 12.5 L/min. The AGI-30 accelerates particles in an airstream jet resulting in impaction with the surface of the liquid in the impinger, trapping the particles into a liquid medium. The jet is directed to the liquid surface, producing a high level of turbulence and bubbling. The BioSampler™ uses 3 angled nozzles to direct the airflow and particles to impinge tangentially onto the sides of the collection vessel (Kesavan, et al., 2010). This causes the liquid to swirl in the reservoir, and produces much less turbulence and bubbling than the AGI-30 at the same air flow rate. Impingers have the added advantage of breaking apart aggregated collected cells (Grinshpun, et al., 2016, pp. 3.2.2-2). The BioSampler™ and AGI-30 are shown in Chapter III, Figures 10 and 11, respectively.

The AGI-30 and BioSampler™ impingers have been evaluated in the laboratory for their sampling efficiency for particles in the 1-3 µm aerodynamic size range, similar to that of a single spore of *C. difficile*. The BioSampler™ has a higher collection efficiency than the AGI-30. A study by Kesavan et al. compared the collection efficiencies of the BioSampler™ with the AGI-30. The BioSampler™ was shown to have a 96 percent sampling efficiency for 1 µm aerodynamic diameter polystyrene latex spheres and a 98 percent sampling efficiency for 3 µm polystyrene latex spheres. The AGI-30 was shown to have a collection efficiency of between 6 percent and 27 percent for 1 µm polystyrene latex particles and similar efficiencies for 2 µm sized polystyrene particles (Kesavan, et al., 2010). This differed from earlier work by Lin et al. on AGI-30 impingers with 1 µm sized PSL spheres which obtained collection efficiencies of 88 percent with the AGI-30 (Lin, et al., 1997). A higher degree of re-aerosolization of collected particles in the AGI-30 compared to the BioSampler™ was listed as a possible reason for the AGI-30 significantly underperforming the BioSampler™ (Kesavan, et al., 2010).

In addition to potential losses of captured microbes by re-aerosolization, microbes may be rendered non-culturable by impinger sampling-related stresses, with subsequent underestimation of airborne concentrations. Kesavan et al. compared the retention of culturable microorganisms by seeding the BioSampler™ and AGI-30 with suspensions of bacterial spores (*Bacillus atrophaeus*) and vegetative bacteria (*Pantoea agglomerans*) and operating the impinger for 1 hour. Retention of culturable spores, determined as the ratio of culturable spores in the suspension after sampler operation to that immediately after seeding, was found to be not significantly different ($p > 0.40$) to

the retention of polystyrene latex spheres of a similar size, determined as the ratio of count concentrations. Retention was 79 percent for the BioSampler™ and 67 percent for the AGI-30. Retention of culturable vegetative bacteria was found to be substantially lower, compared to polystyrene spheres and bacterial spores, with a retention of 54 percent for the BioSampler™ and 48 percent for the AGI-30. This result might be expected, as vegetative bacteria are more fragile than spores.

While there are no published studies that utilize the BioSampler™ or AGI-30 in the collection of *C. difficile*, the BioSampler™ has been used both indoors and outdoors in the collection of *Clostridium perfringens* (*C. perfringens*) spores from commercial poultry operations (Brooks, et al., 2010). BioSamplers™ were also used to sample for *C. perfringens* during the land application of biosolids from treated wastewater. *C. perfringens* was sporadically recovered in a small proportion of air samples collected during application of biosolids known to contain *C. perfringens* (Brooks, et al., 2005). BioSamplers™ have also been used in aerosol studies with *Bacillus thuringiensis* used as a surrogate for *C. difficile* by Troup (2017). Troup used the BioSampler™ to evaluate air treatment methods for spore-forming bacteria in an aerosol test chamber (Troup, 2017).

The combination of direct air-to-liquid impingement of collected particles, high collection efficiency in the particle size range of interest, and demonstrated capability in the collection of a related *Clostridium* species indicated that the BioSampler™ and AGI-30 should be evaluated for use in the collection of *C. difficile*, both in the laboratory, and potentially, in healthcare environments. Due to its high collection efficiency in the *C. difficile* particle size range of interest and demonstrated capability to collect a related

Clostridium species, the BioSampler™ could also be utilized as a reference air sampler in laboratory studies involving the evaluation of high- and low-volume air samplers for the collection of *C. difficile*.

Bioaerosol sampling by filtration

Membrane filters are utilized in the collection of bioaerosols and can be examined using either microscopy or culture analysis. Gelatin filters, which are placed directly on agar for culture analysis, are less commonly utilized. One common limitation of membrane filter bioaerosol collection is viability loss due to desiccation from airflow through the filter during collection (Hinds, 1999, p. 398), resulting in an underestimation of culturable bioaerosol counts. However, for the collection of airborne spores the effect of desiccation may be significantly less than in the collection of vegetative bacteria due to their greater resistance to desiccation. Burton et al. (2005) used 3 µm pore size mixed cellulose ester (MCE) filters and 1 µm pore size polytetrafluoroethylene (PTFE) filters to sample for *Bacillus globigii* spores. Sampling times of up to 4 hours were used with both filter types, with vortex and shaker extraction of the spores from the filter followed by surface plating on agar. Physical collection efficiencies were determined by upstream and downstream count concentration measurements with an optical particle counter and were greater than 94 percent. Relative culturability was determined by comparing CFU counts from plated extraction liquid to the number of spores counted on the filter through microscopy prior to extraction, and was greater than 72 percent (Burton, et al., 2005).

Bioaerosols can also be directly cultured by placing the filter in direct contact with agar, i.e. the filter is placed face-up on the surface of an agar plate. This method was used to culture collected environmental bioaerosols using a 0.45 µm pore size MCE filter by Xu et al. and was found to effectively enumerate bacteria collected onto the filter (Xu, et al., 2013). Both MCE and gelatin filters can be placed on wet agars that wick through the filter matrix. Gelatin filters, which are specifically designed for this type of culturing, actually dissolve into the agar but have been shown to dry out and break over prolonged air sampling times (Burton, et al., 2005). Despite published effectiveness with other airborne bacteria, air sampling using filters has not been performed for *C. difficile* or other spore-forming anaerobic bacteria.

Bioaerosol sampling by impaction

Impaction-based devices are the most common bioaerosol air sampling methods and are divided into two classes including slit impactors and multi-jet impactors. Both impactor types direct an aerosol through a nozzle against a flat impaction surface. Particles above the cutoff size have more inertia than the airstream and smaller sized particles and impact on the plate while the airstream and smaller particles continue to the exhaust flow (Hinds, 1999, pp. 121-122). Slit impactors direct air through a slit with typical flowrates ranging from 28 - 50 L/min. An agar-filled plate rotates below the slit providing a collection surface. These samplers are capable of 50 percent cutoff collection efficiencies for particles as low as 0.5 µm diameter in size. Multi-jet impactors use 100 - 500 jets to impact a bioaerosol directly onto an agar-filled plate. These impactors have cutoff sizes as low as 0.6 µm. Shear forces from the high velocity jets

have been shown to reduce sample viability and the results from multi-jet sampling require correction since multiple bioaerosol particles may impact under a single jet and be counted as a single colony instead of multiple colonies (Hinds, 1999, pp. 396-398). This correction is known as positive hole correction and is described in detail in the methodology section. The key advantage of slit impactors over multi-jet impactors and impingers is that time resolution can be obtained on a single sample allowing for an estimate of the time that a colony was collected.

Cyclone air sampling for bioaerosols

Cyclone air samplers direct an aerosol through a tangential inlet into a cylinder and use centrifugal forces to separate particles from the swirling air. During this rotating airstream motion, larger particles sized above a cut size are deposited on the walls and collected (Hinds, 1999, pp. 252-253). For bioaerosols, wet-walled cyclones have been developed that coat the cyclone cylinder walls with a thin film of fluid that is continuously supplied and collected by the system. These wet-walled cyclones typically have 50 percent cut points of approximately 1 μm (McFarland, et al., 2010). The wet film allows the sample to be collected into a liquid medium and would also serve to prevent desiccation of viable organisms. Cyclone bioaerosol air sampling systems have been used in both low-volume and high-volume flow rate applications.

Low-Volume Sampling for *Clostridium difficile*

In Quantitative Microbial Risk Assessment (2014), Haas et al. describe the use and limitations of bioaerosol sampling. There are currently no standard methods for

bioaerosol sampling and air sampling is typically performed to demonstrate the presence of an organism of interest rather than to provide quantification. Additionally, due to limitations in available sampling methods and to the small sample volume typically collected over a limited time period, the absence of an organism in a sample does not guarantee its absence in the environment sampled (Haas, et al., 2014, pp. 138-140). Sampling efficiency, loss of sample viability, and the high degree of variation typically found in the aerosol particle sizes and environmental factors greatly impact the quantitative information provided by air sampling.

Attempts to isolate *C. difficile* from the air in healthcare environments have been made since the early 1980's. The first documented air sampling for *C. difficile* in a healthcare environment was conducted in a surgical intensive care unit and a pediatric care ward located at the University of Michigan, Ann Arbor (Kim, et al., 1981). At the time of air sampling in the ICU, three patients had tested positive for *C. difficile* in stool and two had developed infection, while during the sampling conducted in the pediatric ward, 15 patients had positive stool samples and 5 developed CDI during the study period. This study collected 9 air samples in the ICU and 7 air samples in the pediatric ward using a slit-to-agar (STA) impaction air sampler. A volume of 30 cubic feet of air was collected per sample (approximately 850 liters of air per sample). All 16 collected samples failed to isolate *C. difficile*. An additional study was conducted by Malamou-Ladas et al. (1983) in a London, UK hospital. This study collected 13 samples of 320 L volume in multiple wards of a hospital with active CDI patients present including oncology wards and neonatal wards. This study collected the samples using a

centrifugal air sampler. All 13 air samples collected failed to isolate *C. difficile* using culture analysis (Malamou-Ladas, et al., 1983).

Since 2008, limited studies involving air sampling in close proximity to CDI patients have been conducted in the United Kingdom. A 2008 study conducted by Roberts et al. (2008) in an elderly care ward isolated *C. difficile* from the air using a Burkhard C90M cyclone sampler, (Burkhard Ltd, United Kingdom) sampling at a 16.7 L/min air flow rate for a 15 min sampling period to sample for *C. difficile*. This cyclone sampler deposited the collected airborne particles directly into 1 mL of Ringer's solution (Roberts, et al., 2008). The Roberts et al. study collected 23 positive air samples over a 2 day period with measured air concentrations as high as 426 CFU/m³. The air sampler was placed just outside of a toilet room in the ward, potentially contributing to the high number of positive results and the number of colonies present. Genomic analysis was performed on *C. difficile* organisms harvested from the resulting colonies using polymerase chain reaction (PCR) ribotyping and found that 22 of the 23 samples collected were a genetic match to each other. However, this air sampling was repeated 1 year later in the same elderly care ward and failed to collect any detectable *C. difficile* in the air (Roberts, et al., 2008).

Best et al. (2010) collected air samples near CDI patients for 1 and 10 hour sampling periods using an AirTrace™ STA impactor sampling at 28.3 L/min air flow rate, and obtained a higher proportion of positive air samples from 10 hour samples (70 percent) versus 1 hour samples (12 percent) (Best, et al., 2010). Best et al. concluded that either very dilute aerosols are produced from *C. difficile* patients, aerosols are produced very sporadically, or that limitations in air sampling methodologies and

equipment may have limited and underestimated the collection of airborne *C. difficile*. Specifically, the authors noted that the placement of the air sampler on the floor in a noise-dampening enclosure and sampling through an inlet tube may have reduced the number of airborne spores collected. The Best et al. study further noted that the earlier study by Roberts et al. collected significantly greater amounts of *C. difficile* spores in air (Best, et al., 2010). Best et al. (2010) noted that the airborne *C. difficile* counts obtained by Roberts et al. using a sampler that deposited recovered particles directly into solution were orders of magnitude greater than the *C. difficile* counts obtained using impaction onto solid agar (Best, et al., 2010). This may have been due to the disaggregation of spore clusters in the liquid after collection, whereas spore collection by agar impaction alone would not allow disaggregation of spore clusters. Thus, the number of CFU for liquid media surface-plated on agar would be higher than the CFU observed on an impactor plate for the same number of captured spores (assuming no other influences). However, loss of culturable spores collected by impaction could also have been due to desiccation stresses. Air sampling for *C. difficile* in both laboratory settings and healthcare facilities have successfully utilized slit impactors. Best et al. conducted air sampling in patient care wards with CDI patients used a modified AirTrace™ slit-to-agar (STA) impactor (Particle Measuring Systems, Boulder, CO) that sampled 28.3 L/min. To allow the sampling to take place as close to the patient as possible, the slit-to-agar system was modified by the addition of a Tygon™ tube two meters in length (Best, et al., 2010). This modification may have lowered the capture efficiency of the air sampling instrument, as loss of particles in the tubing may have occurred. Slit impactors operating at 28.3 L/min were also used in the previously described laboratory based

studies conducted by Best et al. (2012) and Aithinne et al. (2019). Based on this combination of demonstrated effectiveness in the field and in the laboratory and current availability, slit impactors may be considered the current best practice or reference method for air sampling for *C. difficile* spore aerosol.

Since the 1981 study conducted at the University of Michigan, no further *C. difficile* air sampling studies in medical facilities have been published in the United States despite advances in bioaerosol air sampling equipment and laboratory analysis methods for *C. difficile*. Air sampling studies conducted outside the United States may not be representative of conditions in U.S. domestic hospitals due to differences in healthcare facility design requirements and differences in patient accommodation (i.e. number of patients per room). For example, the ward sampled in the Roberts et al. study had 6 patient beds separated by curtains (Roberts, et al., 2008), while CDI patients in the United States are typically provided with private rooms (McDonald, et al., 2018).

High-Volume Air Sampling Methods for Bioaerosols

Guidelines, overview, and equipment description

In the Guidelines for Environmental Infection Control in Healthcare Facilities, the CDC recommends that high-volume air sampling be used for environmental air sampling where airborne concentrations of microorganisms are anticipated to be low (Sehulster & Chinn, 2003). However, a definition of high-volume air sampling versus low-volume air sampling devices has not been established and some of the devices defined as low-volume air sampling in one source may be considered high-volume

elsewhere. High-volume air samplers, which might be defined as those sampling at hundreds rather than tens of liters per minute, are primarily used in emergency response and allow for the expedient sampling of a large volume of air following an aerial dispersion of a biological agent (Cooper, et al., 2014). These samplers often have flow rates that are an order of magnitude higher than the low-volume sampling methods. For example, the XMX/2L-MIL and the DFU-1000 have flow rates that exceed 500 L/min. These were developed for, and used extensively in, emergency response for bioterrorism and for the military sampling of biological warfare agents. Currently, the United States Air Force utilizes three high-volume air sampling systems for the air sampling of bioaerosols: the Biocapture-650, the XMX/2L-MIL, and the DFU-1000. These are described below and shown in Chapter III, Figure 16.

The XMX/2L-MIL uses a virtual impactor combined with a liquid impingement system or a dry filter to collect an aerosol sample from a large volume of air into a liquid medium or dry filter for analysis. The first stage of this air sampling system, the virtual impactor, concentrates collected particles above an established cut size into a minor, particle-rich, air flow stream using an acceleration nozzle as shown in Figure 2.

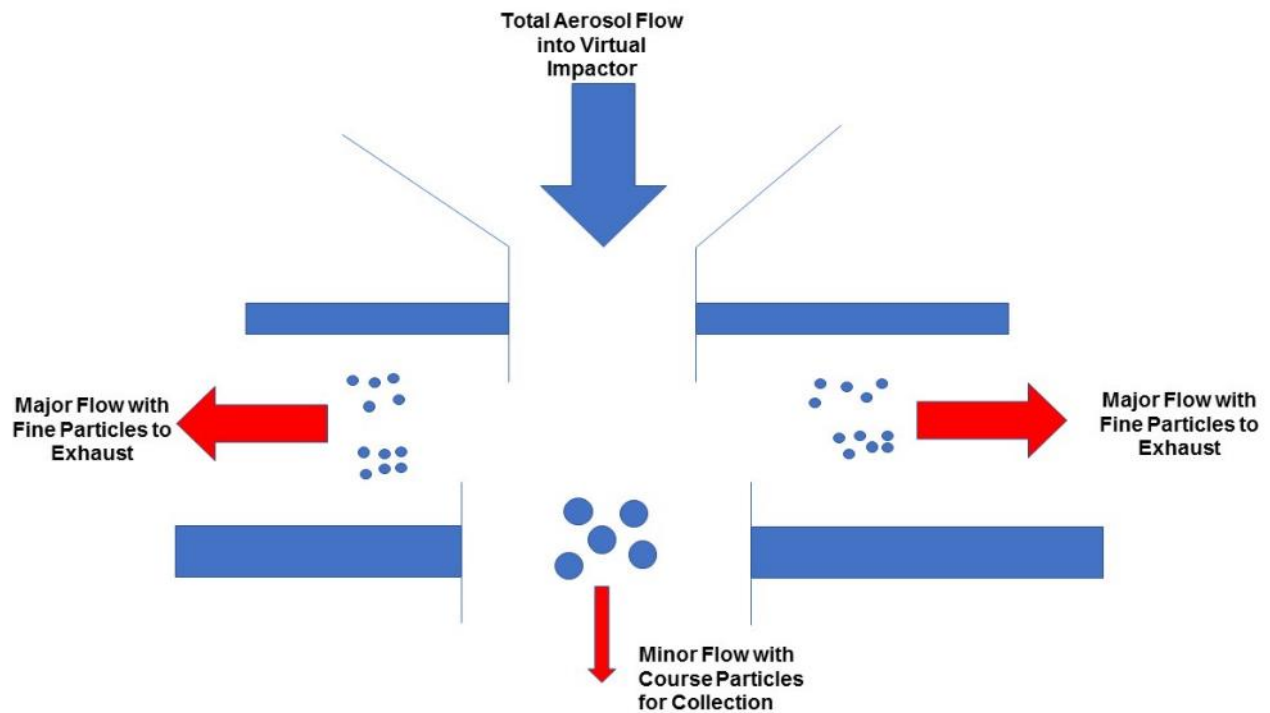


Figure 2: Virtual Impactor Schematic, Adapted from Sioutas & Koutrakis, 1994

After separation, the particle-lean major flow is exhausted while particles from the minor flow stream are collected in the second stage of the air sampling system by liquid impingement or by dry filter (Black, 2011). The XMX-2CV, a civilian version of the XMX/2L-MIL, was utilized by Blatney et al. (2011) in an air sampling study on airborne *Legionella* bacteria from a pulp waste treatment plant and was capable of recovering viable *Legionella* bacteria from air samples collected above an aeration pond. Furthermore, the authors performed a separate study on the XMX-CV to determine if shear forces from the impinger reduce bacterial viability in the collected sample. To do this, the sampling liquid was spiked with vegetative *Legionella* cells and the XMX-CV allowed to sample for 5 minutes, with bacteria counts taken before and after sampling. This comparison of pre- and post-sample analyses found that the sample collection did

not significantly reduce the viability of the cells in the liquid medium (Blatney, et al., 2011).

The Biocapture-650 samples at approximately 200 L/min and uses rotating impeller arms to drive aerosol particles onto an impaction surface, sampling for a period of up to 1 hour with manufacturer-defined sampling time options of 5 minutes, 15 minutes, 30 minutes, and 60 minutes. After the sampling period, the plate is automatically washed into surfactant-containing phosphate-buffered saline (PBS) by the system and the PBS rinse solution is extracted into a collection vial for analysis (Dybwad, et al., 2014). The 1 hour time limit is intended to prevent battery failure since the Biocapture-650 cannot be operated using any other power source. The Biocapture-650 has been used for air sampling in one published veterinary health risk assessment study involving airborne foot-and-mouth virus transmission from swine to cattle (Colenutt, et al., 2016). During this study, air sampling was conducted with the Biocapture-650 at various positions in an animal enclosure. Samples were quantitatively analyzed for infective foot and mouth virus and the Biocapture-650 collected airborne virus at positions within 2 meters of the infected pigs and at positions approximately 3 meters and 5 meters away from the infected pigs. While this study did not specifically intend to evaluate the Biocapture-650 as a sampling device, the results demonstrate that the Biocapture-650 can be utilized in the detection of infectious bioaerosol in the environment (Colenutt, et al., 2016). The RCS High Flow centrifugal bioaerosol collector (Millipore Sigma, Burlington, MA), which samples at 100 L/min, is similar to the Biocapture-650, but uses pre-filled agar strips to collect deposited bioaerosol. The strips are then removed for incubation and counting.

The DFU-1000 uses two 47 millimeter diameter polyester felt filters with a nominal pore size of 1 μm , in parallel, and samples at approximately 850 L/min (i.e. approximately 425 L/min through each filter) (West & Kimber, 2015). The sample is collected directly onto the filters and can be extracted into a liquid medium after collection. The liquid may then be plated on agar for culturing, subjected to genomic analyses, or examined by microscopy; the felt filters are not suitable for placement directly on agar. Since the sample is not collected directly into a liquid medium, evaporation is not a concern and does not limit sampling times. In the adenovirus study by Russell et al. (2006), the DFU-1000 was operated for 12 hours per sample, sampling over 500,000 liters of air per sample collected (Russell, et al., 2006). *C. difficile* spores are expected to have a high resistance to desiccation and the longer sample collection times allowed by the DFU-1000 may offset sample losses due to filter extraction. Therefore, the DFU-1000 should be considered of potential value as a sampling method for the detection of *C. difficile* in a healthcare environment.

High-volume air sampling systems currently used by the United States Air Force have been evaluated in a laboratory environment using test aerosols of endospore-forming bacteria and virus aerosols. Studies were performed by Cooper et al. (2014) for the purpose of evaluating the effectiveness of the XMX/2L-MIL and DFU-1000, relative to the BioSampler™, in collecting viral aerosols. This study used the XMX/2L-MIL, DFU-1000, and the BioSampler™ in side-by-side sampling of a viral aerosol of male-specific coliphage 2 (MS-2) for 5 minutes in an aerosol test chamber. There was no significant difference in the amount of virus collected per liter of air sampled between the XMX/2L-MIL and the BioSampler™. Both the XMX/2L-MIL and BioSampler™

collected significantly more recoverable MS-2 than the DFU-1000 on a per liter of air sampled basis due to sample desiccation and loss during filter extraction (Cooper, et al., 2014). A study conducted by Black and Cooper (2012) used three separate XMX/2L-MILs operated simultaneously to sample for *Bacillus globigii* (BG) from a generated aerosol in an aerosol test chamber. This study found no significant difference between instruments and also determined that in 5 minutes of sampling, the XMX/2L-MIL was effectively able to collect a concentrated sample of BG with 30 CFU BG per milliliter of sampler liquid and higher even when as few as 4 agent-containing particles of BG per liter of air were present in the chamber. An additional study by Enderby conducted a comparative evaluation of the XMX/2L-MIL and the Biocapture-650 to sample for *Bacillus thuringiensis*, a similar anthrax simulant to BG, and MS-2 virus and found that the Biocapture-650 performed as well in the capture of *Bacillus thuringiensis* aerosols as the XMX/2L-MIL on a per volume of air sampled basis (Enderby, 2012). These studies demonstrate that high-volume air sampling equipment, specifically the Biocapture-650 and XMX/2L-MIL, are capable of concentrating a dilute air sample of aerosolized spores into a sample for laboratory analysis using live culture. The XMX/2L-MIL and Biocapture-650 performed similarly in the capture of dilute aerosols of *Bacillus atrophaeus* in laboratory studies (Enderby, 2012). However, the higher sampling rate of the XMX/2L-MIL may provide a significantly lower limit of detection for a given sample duration by concentrating a larger volume of highly dilute aerosol into a single sample.

Laboratory evaluation of high volume air sampling equipment often utilizes a low volume reference or standard air sampler for comparison and determination of

performance of the high-volume sampler on a basis relative to the low-volume sampler. The BioSampler™ has been used as a reference sampler for the evaluation of high-volume air sampling systems in two published studies. Cooper et al. utilized a BioSampler™ as a reference sampler to evaluate the XMX/2L-MIL and DFU-1000 in the collection of aerosols of MS-2 virus (Cooper, et al., 2014). The high-volume MS-2 studies were limited to an indoor aerosol test chamber environment only. The BioSampler™ was also used by An et al. as a reference sampler in the evaluation of the RCS High Flow sampler in the collection of aerosolized spores of *Bacillus subtilis*, a commonly used simulant for *Bacillus anthracis*. For these studies the BioSampler™ was used to comparatively evaluate sampler performance both indoors and outdoors (An, et al., 2004).

Use of high-volume air sampling in health risk assessment

High-volume air sampling equipment has been used on a limited basis for microbial risk assessment studies since the 1960's. A study by Artenstein and Miller (1966) utilized a wet-walled cyclone to sample air for adenovirus-4 in a US Army hospital. The large-volume sampler sampled at 357 cubic feet per minute (approximately 10,000 L/min) and found that high-volume air sampling was capable of recovering adenovirus effectively when present at low concentrations such as the 1 viral unit per 277 cubic feet of air found in this study. The Artenstein and Miller study also showed that low-volume sampling methods such as the AGI-30 would not be effective at determining the presence or absence of microorganisms present at very low concentrations due to the low volumes collected (Artenstein & Miller, 1966). The study

was limited in that samples were analyzed by tissue culture only, as genomic analysis methods were not available at that time. Additional air sampling for adenovirus using modern high-volume air samplers including a DFU-1000 and electrostatic precipitators was performed by Russell et al. (2006) in a Marine Corps recruit barracks that also incorporated genomic sample analysis by PCR. High-volume air sampling allowed for 50 and 42 percent of positive air samples to be collected by the DFU-1000 and ESP respectively. This study is unique in the total volume of sample collected, where the DFU-1000 was allowed to operate for 12 hours per sample. The sample flow rate of the DFU-1000 demonstrates the value of utilizing high-volume air sampling where aerial presence/absence of microorganisms in an area of interest is the desired outcome (Russell, et al., 2006). Both adenovirus air sampling studies were limited in that no laboratory evaluation of the air sampling equipment was performed prior to field use. An approximate limit of detection could not be ascertained, thus decreasing the value in using a negative air sample result to conclude that infectious adenovirus was not present. Furthermore, the Russell et al. studies did not perform tissue culture analysis of the air samples collected; therefore, it is not possible to determine if the virus collected by the DFU and ESP in the positive samples by PCR analysis actually survived air sample collection.

High-volume air sampling for *Clostridium difficile*

The highest volumetric flow rate air sampler used in a published study for aerial dissemination of *C. difficile* was a 100 L/min air sampler used by Keessen et al. (2011) to study aerial dissemination of *C. difficile* on pig farms. This study used an MB1

Microbio Air Sampler (Cantium Scientific, London, United Kingdom), which collects an air sample using a multi-hole single-stage impactor sampling directly onto a nutrient agar. Keessen et al. were able to recover large amounts of *C. difficile* while sampling near pigs, in holding pens, and in building ventilation shafts. Furthermore, they were able to recover detectable levels of airborne *C. difficile* up to 20 meters downwind of the pig barns. Upwind samples were all negative for *C. difficile* (Keessen, et al., 2011). This study demonstrates that a high-volume air sampler can be used to capture a dilute aerosol of *C. difficile* and that when aerosolized, *C. difficile* spores can travel on an air current for distances up to at least 20 meters in agricultural environments. To date, this study by Keessen et al. is the only published study that employed high-volume air sampling in the collection of *C. difficile* spore aerosol. Hathway et al. (2013) used an updated version of the Microbio MB1, a Microbio MB2, to air sample for *Staphylococcus aureus* (*S. aureus*) in open bay hospital wards and collected *S. aureus* in multiple air samples (Hathway, et al., 2013). The Microbio MB2 is a single stage multi-jet impactor that is similar in operation to the Microbio MB1 and operates at 100 L/min. However, the MB2 allows for longer sample collection times and sample volumes than the MB1. The MB2 can sample for up to 100 minutes and collect bioaerosol from up to 10,000 total liters of air per sample (Cantium Scientific, 2019).

The studies by Best et al. (2010) and Roberts et al. (2008) using low-volume samplers demonstrate that *C. difficile* aerosols may be detected in patient care areas, but occur at very low concentrations and/or sporadically. This evidence of low-concentration aerosol generation in healthcare is further supported by laboratory studies on toilet flushing conducted by Best et al. (2012) and Aithinne et al. (2019)

demonstrating that *C. difficile* aerosols produced by toilet flushing have concentrations of less than 1 CFU per cubic meter when aerosolized into a small bathroom or water closet sized space. Air samples collected by Aithinne did not consistently detect *C. difficile* after the third serial flush following initial contamination, potentially due to the low airborne concentrations produced and volume of air sampled (Aithinne, et al., 2019). The studies by Artenstein et al. (1966) and Russell et al. (2006) on adenovirus, and Blatney et al. (2011) on *Legionella*, as well as Black (2012) and Enderby (2012) on *Bacillus* species demonstrate that high-volume air sampling systems can be effectively used to detect the presence of an airborne microorganism at very low concentrations. Black (2012) and Enderby (2012) demonstrated that the XMX/2L-MIL is capable of collecting spores of *Bacillus thuringiensis* and *Bacillus globigii* that are similar in size to those of *C. difficile*. Enderby furthered demonstrated that the Biocapture-650 is capable of collecting spores of *Bacillus thuringiensis* as effectively on a volume of air sampled basis as the XMX/2L-MIL (Enderby, 2012). It seems likely, then, that high-volume air sampling methods should be effective in detecting, and perhaps quantifying, low-concentration *C. difficile* bioaerosol in patient environments.

Culture Analysis of *Clostridium difficile*

Quantitative analysis of environmental bioaerosol samples is typically performed using culture analysis, where a collected sample, or sample aliquot, is grown in a nutrient-rich medium under incubation for a specified time period. Since *C. difficile* is a fecal bacterium, it is typically found in the presence of numerous other bacteria including other species of *Clostridium*. For this reason, a selective medium is typically

used to favor the growth of *C. difficile* present in a sample at the exclusion of other obligate and facultative anaerobic bacteria. A variety of media have been used for the culturing and quantitative analysis of *C. difficile*. Best et al. (2010) used Brazier's cycloserine-cefoxitin-egg yolk agar supplemented with lysozyme for the preparation of slit-to-agar plates and for the analysis of surface and fecal samples (Best, et al., 2010). Lysozyme has been shown to increase recovery of *C. difficile* in environmental samples by inducing spore germination. Wilcox et al. found that incorporating lysozyme into agar more than doubled *C. difficile* recovery from surfaces when compared to agar that did not include lysozyme (Wilcox, et al., 2000). They used an agar medium similar to that used in Best et al. but substituted blood in place of egg yolk as the agar base. Cycloserine and cefoxitin are antibiotics added to the agar medium to prevent the growth of other collected bacteria and selectively allow *C. difficile* to grow.

Chromogenic selective agar medium provides a method of culture analysis to quickly discern *C. difficile* bacteria from other bacteria in both environmental and fecal samples. Chromogenic agar medium causes colonies of *C. difficile* to fluoresce when read using a 365 nanometers (nm) wavelength ultraviolet light after up to 48 hours of anaerobic incubation (Michael, et al., 2016). Chromogenic agar (Chromagar, Paris, France) was used for the analysis of environmental surface samples collected from a clinical laundry facility in 2016 by Michael et al. (2016). Surface swabs were collected and extracted into a liquid medium, and the spores contained in the liquid were analyzed using chromogenic agar medium. Chromogenic agar medium was also used for all quantitative analyses in the toilet-generated aerosol work performed by Aithinne et al. (2019). Aithinne prepared plates of chromogenic agar for use in an AirTrace™

slit-to-agar impactor and found that collected *C. difficile* was clearly distinguishable after anaerobic incubation at 37°C for 24 hours as shown in Figure 3.

Sensitivity of a culture analysis method is determined by analyzing known positive samples (i.e. PCR positive fecal samples) and calculating a ratio or percentage of positives with the culture method out of total samples analyzed. Chromogenic agar has been found to have a sensitivity for fecal analysis ranging from 74.1 percent when read at 24 hours to 87 percent when read at 48 hours, for anaerobic incubation at 37°C. This sensitivity compares to a sensitivity of 70.4 percent for taurocholate cycloserine cefoxitin agar, a non-chromogenic selective agar medium for *C. difficile*, when read at 48 hours under anaerobic incubation at 37°C (Eckert, et al., 2013).

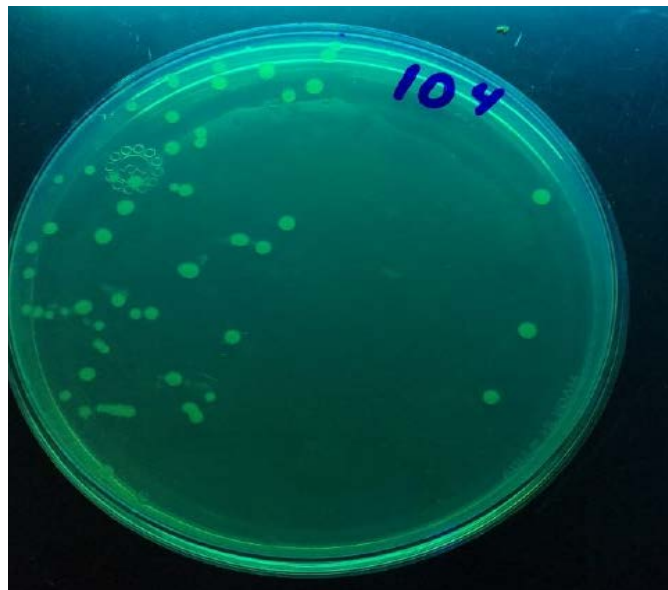


Figure 3: *C. difficile* Colonies on Chromogenic Agar, as Viewed Under 365 nm UV Illumination [Graphic Credit: Aithinne, 2017]

Bacterial analysis of water samples is frequently performed using culture analysis of membrane-filtered water, whereby a volume of water is passed through a filter that is then placed onto nutrient agar medium for incubation, spore germination, colony growth,

and enumeration. This method has been used in the enumeration of *C. difficile* in water samples in a study by Al Saif and Brazier (1996) in New South Wales, Australia. This study passed a 100 mL water sample through a 0.45 µm pore size MCE filter and placed the filter on a selective agar medium for anaerobic incubation at 37°C for up to 5 days. This method was used to identify *C. difficile* in water samples collected from both treated and untreated sources including tap water, swimming pools, lake/river water, and drainage water (Al Saif & Brazier, 1996). Enrichment culture analysis was also performed by Al Saif and Brazier (1996) for the analysis of water samples with very low concentrations of *C. difficile*. These samples were processed using the membrane filtration method described above, but then transferred into a cooked meat nutrient broth containing 8 mg/L of cefoxitin and 250 mg/L of cycloserine to selectively isolate *C. difficile*. The broth was incubated at 37°C for 48 hours and then plated on selective agar and anaerobically incubated for 48 hours at 37°C (Al Saif & Brazier, 1996). While this method would not allow direct quantification of low-concentration liquid samples, it was effectively used to provide presence/absence analysis of low-concentration samples and could potentially be utilized to provide presence/absence analysis of *C. difficile* in liquid media prepared from water, surface, or air samples. Membrane filtration water analysis for *C. difficile* was also used in a study by Aithinne (2017) to determine the concentration of *C. difficile* present in toilet bowl water used for toilet flush aerosol generation experiments. Aithinne (2017) effectively quantified *C. difficile* in water using 0.45 µm pore size MCE filters that were then transferred to chromogenic selective agar medium for enumeration analysis. Samples with high concentrations of *C. difficile* that would have been too numerous to count were serially diluted with sterile

deionized water prior to filtration and analysis (Aithinne, 2017). This study demonstrates that chromogenic selective agar medium could be used to analyze *C. difficile* membrane filter samples prepared from environmental samples into a liquid medium.

Literature Review Summary and Gaps

HA-CDI is a significant disease burden and the generation and transport of spores via aerosol has the potential to expose uninfected hospital personnel, patients, and visitors. Currently, contact isolation with a few additional precautions, such as a dedicated toilet, is the recommended level of isolation for a CDI patient. Additional precautions may be justified for CDI patients if aerosolized *C. difficile* is identified outside of the patient room or shown to be capable of distant transport on air currents. These additional controls may include droplet precautions or airborne isolation. Limited air sampling studies have demonstrated the aerial dissemination of *C. difficile* in close proximity to patients with active infection. These studies have not addressed the extent of aerial dissemination of *C. difficile* or if patients and other persons outside of a patient's room would be at risk from spores transported by aerial dissemination. To enable these air sampling studies, effective air sampling methods must be developed and evaluated. Additionally, while air sampling methodologies have collected aerosols of *C. difficile* in laboratory-based toilet flush studies, high-volume and low-volume air sampling methodologies have not been evaluated or compared in the collection of *C. difficile* aerosols in a controlled laboratory environment. Therefore, additional air sampling studies are necessary in addition to the evaluation of existing air sampling

methods to determine those best suited for these studies. Unevaluated air sampling methodologies, such as slit-to-agar impaction, liquid impingement, and cyclone sampling may underestimate actual *C. difficile* concentrations or fail to detect low concentration *C. difficile* aerosols entirely. A laboratory evaluation of low- and high-volume air sampling methodologies would determine the effectiveness of existing air sampling methodologies in the collection of *C. difficile* aerosols. High-volume air sampling equipment is used in bioterrorism emergency applications where low airborne concentrations are expected. However, this equipment has not been validated in healthcare-based field trials or in the laboratory using *C. difficile*. A study to develop and evaluate air-sampling methods for *C. difficile* could have significant public health implications including the support of microbial risk assessments and a determination of the extent of *C. difficile* transport by aerosol in healthcare environments.

CHAPTER II

PURPOSE AND SCOPE

Problem Statement

The purpose of this work was to assess the comparative utility of low-volume and high-volume air sampling methods in the detection and quantification of *C. difficile* spore bioaerosol. Air sampling equipment and collection methodologies have not been rigorously evaluated for the collection and enumeration of airborne *C. difficile* spores. As a result, current capabilities to collect air samples for *C. difficile* in healthcare environments are limited to low-volume methodologies that may fail to detect or accurately measure airborne *C. difficile* spores. Laboratory evaluation of air sampling equipment and methodologies, particularly high-volume methodologies, will better determine if existing bioaerosol equipment is capable of detecting and quantifying *C. difficile* spore aerosol. This evaluation will better assist healthcare facilities in meeting the CDC's high-volume air sampling recommendations for environmental infection control in healthcare facilities.

Purpose and Specific Aims

This study assesses the effectiveness of commercial low-flowrate and alternative military and commercial high-flowrate air sampling methodologies for collection, detection, and enumeration of airborne *C. difficile* spores. The specific aims of this study are:

1. Evaluate low-volume air sampling devices and mechanisms of collection for laboratory-generated aerosols of *C. difficile* spores. The relative *C. difficile* sampling

efficiencies of four commonly used bioaerosol collection systems, the AGI-30 impinger, BioSampler™ impinger, MCE filter and AirTrace™ slit-to-agar impactor, were assessed in side-by-side laboratory trials with the STA impactor serving as the reference device. The research hypothesis was that there would be no significant difference in the relative collection efficiencies of the air sampling equipment.

2. Evaluate the retention of culturable *C. difficile* spores in impinger air samplers. The BioSampler™ impinger and AGI-30 impinger were evaluated for culturability loss of *C. difficile* spores in sampling media during impinger operation. Re-aerosolization of *C. difficile* spores from liquid impinger media was also evaluated. The research hypothesis was that culturability losses would be similar for the two impingers, but that re-aerosolization would be greater from the AGI-30 than from the BioSampler™. Oxidative stresses on *C. difficile* spores were evaluated by measuring culturable spore losses in fritted glass bubblers operated using air and medical grade nitrogen. The research hypothesis was that there would be higher culturability loss with air bubbling than with nitrogen bubbling.

3. Evaluate high-volume air sampling devices in the collection of nebulizer-generated *C. difficile* spore aerosols in a controlled laboratory environment. Four high-volume air sampling systems including the XMX/2L-MIL, DFU-1000, Biocapture-650, and MB2 were evaluated in the collection of nebulizer-produced aerosols of *C. difficile* spore aerosol. Low-volume STA impactation and filtration air sampling served as reference methods. The research hypothesis was that there would be no difference in the relative efficiency for the evaluated high-volume air sampling systems, and that the high-volume methods would be more sensitive in detecting low-concentration *C. difficile* bioaerosol.

4. Demonstrate use and effectiveness of high-volume air sampling equipment in the laboratory collection of *C. difficile* spore aerosol produced from toilet flushing. Following the evaluation using a relatively high concentration nebulizer-generated aerosol, the most effective and suitable high-volume and low-volume air sampling systems were further demonstrated in the collection of relatively low-concentration *C. difficile* spore aerosols produced from a flush toilet. This experiment utilized a large aerosol chamber to assess the performance of a high-volume sampler in a low-concentration *C. difficile* aerosol environment, such as might be encountered in a CDI patient room or bathroom following toilet flushing while a patient is actively shedding spores. The research hypothesis was that the frequency of aerosol detection would be greater for the high-volume air sampling system compared to the low-volume air sampling system.

CHAPTER III

MATERIALS AND METHODS

Aim 1: Low-Volume Air Sampling Method Evaluation

Aerosol test chamber

Research supporting aims 1 and 2 was performed in a 76 liter aerosol test chamber (ATC) made of polypropylene plastic, shown in Figure 4. *C. difficile* spore aerosol was supplied to the chamber from a 3-jet Collison MRE type nebulizer, with simultaneous sampling from the chamber by three bioaerosol sampling devices. Negative pressure was maintained in the ATC by ensuring that airflows drawn from the ATC by the samplers exceeded airflows into the ATC by the nebulizer. The ATC was placed within a class II biological safety cabinet (BSC) to ensure that generated aerosols were not allowed to escape to the environment, and to ensure that HEPA filtered makeup air flowed into the negative pressure ATC throughout each trial. The placement of the ATC within the class II BSC is shown in Figure 4.



Figure 4: Aerosol Test Chamber in Biological Safety Cabinet

Air mixing in the chamber was conducted using a 3.5 in (8.9 cm) square variable speed cooling fan placed inside the ATC and operated in the “low” setting throughout aerosol trials. Four equally spaced 0.25 inch (6.4 mm) diameter makeup air intake holes supplied HEPA-filtered air from the BSC into the negatively pressured ATC throughout aerosol generation and air sampler operation. A brief summary of the ATC and its setup and operation is included in Appendix A as a peer-reviewed article entitled “A Comparison of Air Sampling Methods for *Clostridium difficile* Endospore Aerosol”. This article was published in *Aerobiologia* in 2019.

Preparation of spore suspension

Concentrated spore suspensions were prepared as described in Aithinne et al. (2019). Non-toxigenic *C. difficile* derived from American Type Culture Collection (ATCC) 700057 (Microbiologics, St. Cloud, MN) was placed in a 500 mL suspension of

brain-heart broth and incubated anaerobically at 37°C for 10 days. This extended incubation time ensured that the brain-heart media was depleted of nutrients, thus enabling sporulation. Following the 10 day incubation period, the brain-heart suspension was heat shocked at 80°C for 20 minutes, mixed, separated into 50 mL aliquots, and placed into conical centrifuge tubes. The suspension was then separated by centrifugation for 15 min at 5,000 x g. Centrifugation ensured that the *C. difficile* spores separated from the medium and formed a pellet at the bottom of the conical tube. The medium was then decanted from the spores, replaced with sterilized water, and separated again for 15 minutes at 5,000 x g. The resuspension and separation process was repeated four times ensuring that the spore suspension was cleaned of nutrient broth, allowing for long-term refrigerated storage. Aithinne et al. (2019) produced spore suspensions of approximately 5.75×10^5 CFU/mL using this method. Following preparation of the spore suspension, a mean suspension concentration was determined by serial dilution and inoculation onto four 90 mm diameter spread plates filled with 17.5 mL of *C. difficile* specific chromogenic agar (Chromagar™). The plates were then incubated for 24 hours at 37°C, and the illuminated colonies were counted under 365 nm UV illumination.

Aerosol generation

Aerosol generation was performed using a 3-Jet Collison nebulizer (CH Technologies, Westwood, NJ). A diluted suspension of non-toxigenic *C. difficile* was prepared by adding 150 µL of source suspension to 100 mL of sterile deionized water and thoroughly mixed. 50 mL of this diluted suspension was transferred to the Collison

nebulizer by a sterile 25 mL pipette with a target concentration of 500-1,000 CFU/mL. HEPA-filtered compressed air was supplied to the Collison nebulizer at 10 L/min flow rate via an air compressor equipped with an in-line HEPA filter. The Collison nebulizer and supply air apparatus is shown in Figure 5. Aerosol generation commenced at the start of the trial and started simultaneously with the air sampling and stopped at the end of the trial and at the same time that air sampling was stopped. The total air sampling flow out of the ATC was greater than the nebulizer air flow into the ATC (see below), ensuring that negative pressure was maintained in the ATC throughout the trials.



Figure 5: Collison Nebulizer Apparatus

Collison Nebulizer Spore Culturability Trials

This study was the first to utilize a Collison nebulizer, or any nebulizer, to produce *Clostridium difficile* spore aerosol in a laboratory. To evaluate the retention of culturable spores by the nebulizer, two 250 µL aliquots of spore suspension were analyzed for each of the 9 MCE filter trials described below. Aliquots were collected immediately prior to nebulizer operation and immediately following 10 minutes of nebulizer operation. These aliquots were cultured on a 90 mm Chromagar™ plate and incubated anaerobically for 24 hours at 37°C. Concentrations prior to and following nebulizer operation were expressed as CFU/mL and compared using a paired analysis.

MCE filter trials

Laboratory-generated aerosols of *C. difficile* spores were simultaneously sampled from the ATC using two 0.45 µm pore size, 47 mm diameter MCE filters in conductive cassettes (MilliporeSigma, Burlington, MA) and an AirTrace™ rotating-plate slit-to-agar (STA) impactor (Particle Measuring Systems, Boulder, CO). The STA impactor served as the reference device, and was selected due to its successful use in three published studies using *C. difficile* aerosols. These included air sampling in patient care areas (Best, et al., 2010), laboratory based toilet flush studies using non-toxigenic *C. difficile* (Aithinne, et al., 2019), and laboratory toilet flush studies using toxigenic *C. difficile* (Best, et al., 2012).

Filter cartridge assembly

MCE filter cartridges were assembled using 47 mm filter cassettes (Catalog No. 225-8497, SKC, Eighty-Four Pennsylvania), and 0.45 μm pore size 47 mm MCE filters (Catalogue No. HAWG04700, MilliporeSigma, Burlington, MA) on 47 mm cellulose support pads (Catalogue No. 225-2903, SKC, Eighty-Four, PA). Filter cartridges were sealed using vinyl plastic electrical tape. An assembled filter cartridge is shown in Figure 6.



Figure 6: Assembled Filter Cartridge

Slit-to-Agar Impactor Air Sampler

A single AirTrace™ slit-to-agar air sampler was used for the filter trials and impinger trials. The slit-to-agar impactor has a design flow rate of 28.3 L/min or 1 ft^3/min and operates by forcing the air sample through a rectangular slit of 44 mm in length by 0.152 mm in width. A 150 mm Chromagar filled plate was used as the collection surface. This slit-to-agar impactor was also used by Best et al. 2010 and

Aithinne et al. (2019) in the collection of *C. difficile* spore aerosol and is shown in Figure 7 below.



Figure 7: AirTrace™ Slit-to-Agar Impactor

Placement of filters and slit-to-agar inlet in ATC

Three sampling positions were used inside the ATC as shown in Figures 8 and 9. Two of the positions had an MCE filter cassette installed (positions 1 and 2 in Figure 8). The cassettes were connected via tubing to air pumps located outside the chamber. The STA was too large to fit in the chamber, so samples were collected from the chamber via a 33.5 in (85 cm) long, 0.5 in (1.27 cm) internal diameter Tygon™ tube as shown in Figures 8 and 9. This sampling apparatus is similar to the AirTrace™ slit-to-agar impactor used by Best et al. (2010) and Best et al. (2012) where a 2 meter length of Tygon™ tubing with an unspecified diameter was connected to the slit-to-agar impactor. This allowed the impactor to collect a sample in close proximity to a patient or at a specified height above a toilet. For this work, the 33.5 inch (85 cm) tubing allowed

the slit-to-agar impactor to collect a sample at any of the three sampling points inside the ATC as shown in Figures 8 and 9.

Nine total trials were conducted with the impactor inlet tube placed, in random order, at each of the three locations for three trials each. MCE filters were placed at the other two sampling locations in the ATC. This study design provided 9 sets of side-by-side aerosol measurements with 1 impactor (reference) measurement and 2 MCE filter measurements in each set. A total of 9 impactor aerosol measurements and 18 MCE filter aerosol measurements were collected.



Figure 8: ATC Setup for Filter/STA Comparison with Numbered Sampling Positions



Figure 9: Top View of ATC Setup for Filter/STA Comparison

Air sampler flow rate measurement

Air flow through the MCE filters was produced by two Vac-U-Go vacuum pumps (Model 228-9605, SKC, Eighty-Four, PA). The sampling flow rate was calibrated to be approximately equal to that of the slit-to-agar (STA) impactor for each filter. Airflow for the STA impactor was set by the manufacturer and could not be adjusted, but was measured to be approximately 25.5 L/min based on an average of 10 measurements collected over a 10 min calibration period. Flow rate calibrations were performed at the beginning and end of each day using a Bios Dry-Cal Defender 530 flow calibrator (Mesa Labs, Butler, NJ).

Sample collection and analysis

Each comparison trial for the STA impactor and MCE filter lasted for 10 minutes. Durations were limited to 10 minutes for all low-volume air sampling trials due to the potential for excessive impinger liquid evaporation at longer durations, as described below. Air samplers and the Collison nebulizer were started simultaneously to ensure that negative pressure was maintained in the aerosol test chamber throughout the trial. The ventilation/HEPA filtered air supply in the BSC was operated for 10 minutes before each trial and throughout each trial. This minimized contamination risk by allowing the BSC ventilation and its HEPA filtration system to remove any spores that might escape from the ATC. For the STA and filter comparison, sampler airflow out of the chamber was approximately 76.5 L/min with nebulizer airflow into the chamber of approximately 10 L/min, ensuring that negative pressure was maintained throughout the trial. Following each trial, a 10 minute HEPA purge of the ATC was conducted to remove remaining aerosolized *C. difficile* spores in the chamber. Exhausted air was purged through a HEPA filter (Whatman HEPA Cap 150, GE Healthcare Biosciences, Pittsburgh, PA) to remove any remaining *C. difficile* spores. This allowed HEPA-filtered air to enter the chamber from the BSC hood through the 0.25 inch (6.4 mm) holes. Filter cartridges and the STA agar plate were then removed and replaced for the following trial. The STA was internally cleaned by wiping the rotation plate, air inlet and plate housing with ethanol between all trials. The Tygon™ inlet hose to the STA impactor was disconnected and replaced with a clean hose prior to subsequent trials. The nebulizer spore suspension was also replaced with fresh suspension. Approximately three trials were conducted per day. Samples from the Collison nebulizer were collected and

analyzed to determine the suspension concentration (in CFU/mL) before operation and after 10 minutes of operation.

At the end of each day, the Collison nebulizer and all Tygon™ tubing were removed from the ATC and BSC hood. Tygon™ tubing, the hose barb attachment to the STA, and the metal parts of the nebulizer were decontaminated by a 10 minute submersion in a 5 % bleach (8.25 percent sodium hypochlorite) and water solution followed by a 10 min submersion in deionized water. Tygon™ R-3603 tubing has been shown to have excellent chemical resistance to immersion in sodium hypochlorite and has been evaluated for immersion periods as long as 28 days in aqueous solutions of sodium hypochlorite concentrations exceeding that of pure commercial bleach (Saint-Gobain Performance Plastics, n.d.). Therefore, decontamination of Tygon™ tubing by a 10 percent bleach water solution does not degrade the Tygon™ tubing over time. Hoses, filter cartridges, and metal components were visually inspected after each decontamination and prior to reuse in subsequent trials. The interior of the ATC was decontaminated at the end of each day with Clorox Fuzion™ (Clorox Corporation, Oakland, CA), a self-neutralizing sodium hypochlorite cleaner used for surface decontamination of *C. difficile* in healthcare settings with a two minute contact time. The use of self-neutralizing cleaner ensured that residual sporicidal agents in the ATC did not somehow impact subsequent trials. At the end of each trial, MCE filters were removed from the cassettes and placed, collection side up, on a 60 mm petri dish filled with 8.5 mL of *C. difficile* specific chromogenic agar (Chromagar™, Paris, France). For each day of trials, a method blank filter was prepared and analyzed on Chromagar™ as well. Blank filters were used to ensure that residual *C. difficile* contamination was not

present in the BSC during sample preparation and to verify the effectiveness of filter cartridge decontamination with sodium hypochlorite bleach. One blank filter was prepared and analyzed for each day of sampling.

Following sample preparation, STA impactor plates and agar plates containing the MCE filters were placed in a 7.5 L sealed box along with three Anaeropack™ anaerobic gas generators and an Anaeropack™ anaerobic indicator tablet (Mitsubishi Gas Chemical, New York, NY). The plates were then incubated anaerobically at 37°C for 24 hours. Following the 24 hour incubation period, the plates were viewed under 365 nm UV illumination, and fluorescing (*C. difficile*) colonies were counted. Indicated airborne spore concentrations were calculated from the spore counts and sampled air volume, and expressed in units of CFU/L. The sampling efficiency of each MCE filter relative to the STA impactor was then calculated as the MCE filter's indicated air concentration divided by the STA impactor's indicated air concentration.

Impinger air sampler comparison trials

Laboratory-generated *C. difficile* aerosols in the ATC were sampled using two types of impinger air samplers, the AGI-30 and the BioSampler™ swirling aerosol collector, and the STA sampler. Impinger measurements were compared to measurements using the STA impactor reference method. A series of nine trials was conducted in the ATC in the same manner as for the MCE filter trials described above, except that the impinger positions and the STA sampling positions rotated using a Latin Square experimental design replicated three times (i.e. each sampler was in each position three times).

Impinger sampling preparation

Phosphate buffered saline (PBS) solution (Sigma Aldridge, St. Louis, MO) was prepared per manufacturer's instructions by dissolving a pre-made tablet into 200 mL of sterile deionized water. Deionized water was sterilized by autoclaving at 121°C for a minimum of 15 minutes and allowed to cool to room temperature prior to use. 20 mL of sterile PBS was placed into each impinger using a sterile 25 mL serological pipette for each trial. The impinger was then assembled and the glass fittings sealed with high vacuum silicone grease (Dow Corning, Midland, MI). A minimum of 15 inches of mercury (in. Hg) of vacuum pressure was supplied to the impingers by one of two SKC Vac-U-Go air pumps. This minimum pressure was sufficient to ensure adequate flow to maintain a critical orifice in the impinger. This pressure provided an air flow rate to the impingers of between 11 and 13 L/min of air using a critical orifice to maintain consistent flow rate. An assembled BioSampler™ and AGI-30 is shown in Figures 10 and 11.



Figure 10: BioSampler™ Impinger



Figure 11: AGI-30 Impinger

A 47 mm MCE filter cartridge was placed in line with the impinger flow exhaust, via a 7 in (17.8 cm) Tygon™ tube, to capture uncollected or re-aerosolized spores

escaping from the impinger. This assembled impinger and filter apparatus is shown in Figure 12.

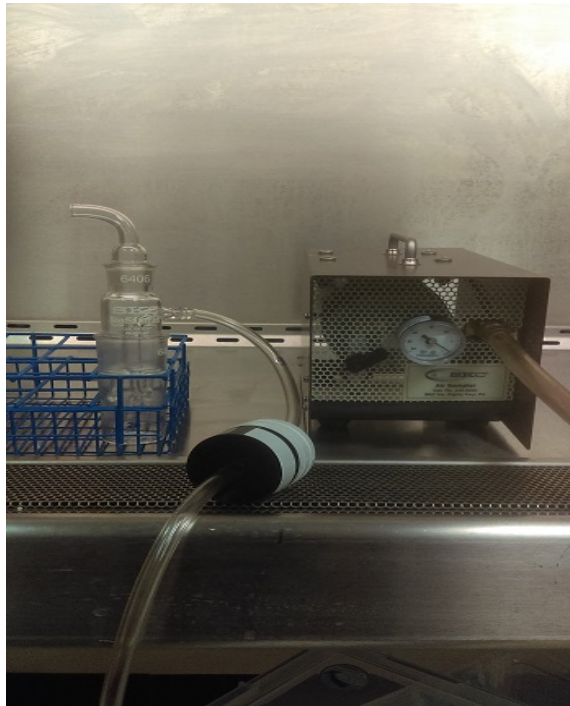


Figure 12: BioSampler™ with Exhaust In-line Filter Cartridges

Air flow to the Collison nebulizer was started at the same time that the AGI-30, BioSampler™, and STA impactor started sampling. The circulating fan was started prior to the Collison airflow and air sampler operation. The impingers and STA impactor were allowed to sample the air in the ATC for 10 minutes as shown in Figure 13. During these trials, sampler airflow out of the chamber was approximately 50 L/min with nebulizer airflow into the chamber was 10 L/min, ensuring negative pressure was maintained in the ATC throughout the trials.



Figure 13: Top View of ATC Set-up for Impinger/STA Comparison. Red Impinger Fluid Added for Illustrative Purposes

After 10 minutes, air flow to the Collison nebulizer was stopped along with the impingers and STA impactor. The ATC was purged, as previously described, with HEPA filtered air for 10 minutes and then opened. Impingers and filter cartridges were removed from the chamber and externally cleaned with an ethanol wipe to remove surface contamination. Liquid from the impingers was then removed and transferred to a 50 mL conical tube. The STA impactor plate was removed and the instrument internally cleaned with an ethanol wipe to remove any contamination present. Following each trial, the ATC, STA impactor (external components), and metal surfaces in the BSC were cleaned with Clorox Fuzion disinfectant previously described. Impinger glass

components were decontaminated after each trial by autoclaving at 121°C for a minimum of 15 minutes. This ensured that residual contamination was not allowed to impact the results of subsequent trials.

Sample preparation and culture analysis

Due to the low concentrations of *C. difficile* spores expected in the impingers, spread plating of an aliquot of the impinger liquid (100 µl or 250 µl) was not a feasible approach for analysis. Therefore, the whole sample of the impinger liquid, supplemented with 20 mL of sterile PBS, was filtered onto a 47 mm, 0.45 µm pore size MCE filter using the membrane filter apparatus shown in Figure 14 below. The additional sterile PBS increased the total volume to facilitate even distribution of spores on the filter surface. A sterile gridded filter and sample cup was placed grid side up on the apparatus. The liquid sample was then added and allowed to pass through the MCE filter using vacuum pressure.

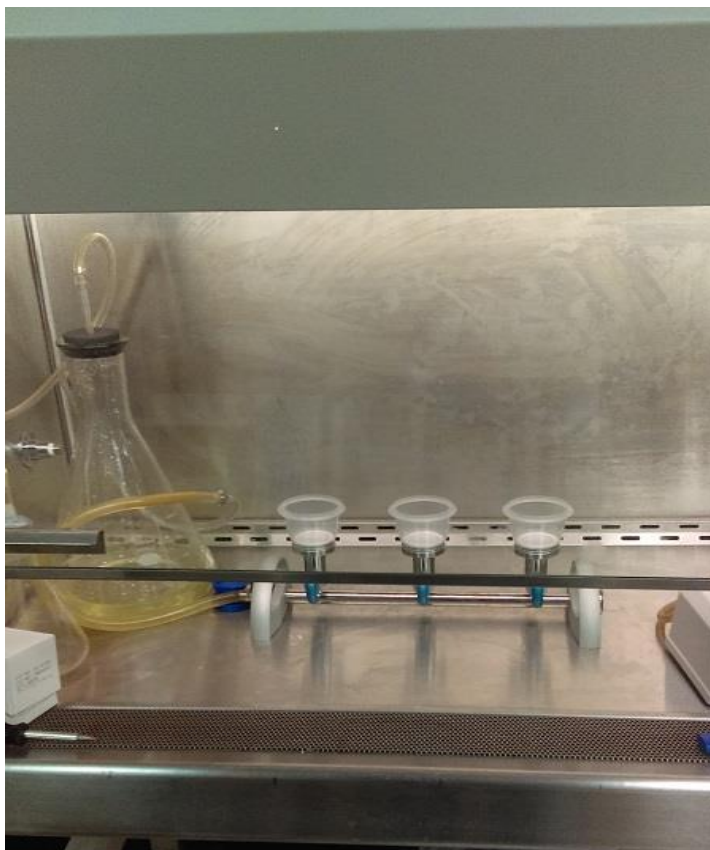


Figure 14: Liquid Membrane Filter Apparatus in BSC Hood

After the liquid sample passed through the filter under vacuum pressure, the filter was removed from the apparatus and transferred grid side up to a 60 mm plate filled with Chromagar™ and placed in an Anaeropack™ box. The MCE exhaust filters were removed from the 47 mm cassette, placed on Chromagar™ plates grid side up, and placed in an Anaeropack™ box. After being packed with plates, Anaeropack™ boxes were sealed with the appropriate number of sachets (1 sachet for a 2.5 L box or 3 sachets for a 7.0 L box) along with an anaerobic indicator and incubated for 24 hours at 37°C. Following the 24 hour incubation period, colonies on the filters were counted visually under ultraviolet light. Colonies counted on the membrane filters used for the

impinger liquid were used to determine the number of colony forming units collected by the impinger. Colonies counted on the MCE filters from the impinger exhaust line were used to determine the number of spores re-aerosolized or not collected during impinger operation.

Aim 2: Impinger Retention of Culturable *C. difficile*

Preparation of seeded impingers

To assess the potential for culturability loss during AGI-30 and BioSampler™ impinger operation as well as for re-aerosolization of collected spores from the impingers, 200 mL of *C. difficile* spore suspension in PBS was prepared each day with a mean target concentration of 500 to 1000 CFU/mL for each day of trials. To facilitate the deagglomeration of aggregated spores, a 0.01% volume/volume concentration of Tween-80 was added to this suspension by adding 20 µl of Tween-80. The suspension was mixed and four 0.1 mL aliquots were collected and cultured on 90 mm Chromagar™ filled plates to provide estimates of the initial *C. difficile* suspension concentration and total seeded *C. difficile* spores prior to impinger operation.

For each trial, the BioSampler™ and AGI-30 were seeded with 20 mL of *C. difficile* spore suspension in PBS solution. After the addition of the *C. difficile* suspension, a 7 in (17.8 cm) Tygon™ exhaust tube was connected to the impinger and connected to a 47 mm filter cartridge. This allowed for the collection and quantification of re-aerosolized spores from the spore suspension during impinger operation. The outlet (exhaust) of the filter was connected to a vacuum pump and operated with a minimum of 15 in Hg vacuum pressure to achieve critical flow in the impinger and a flow

rate of 11 - 13 L/min. Flow rates on the impingers were calibrated at the beginning and end of each day using a Bios Dry-Cal Defender 530 as described in Aim 1. Additionally, each impinger was weighed before and after operation to determine the volume of spore suspension lost during the 10 minutes of operation.

Impinger operation

The two impingers were placed in the 76 liter ATC for each trial run. Prior to the start of each trial, a 20 minute air purge using HEPA-filtered air from the Class II BSC was conducted and ensured that a minimum of 10 air changes of HEPA-filtered air was allowed to purge the chamber. This HEPA air purge removed any residual *C. difficile* spore aerosol in the chamber prior to the start of the subsequent trial. Following the air purge, the impingers were started and allowed to sample HEPA-filtered air supplied to the ATC from the class II BSC for 10 minutes. Ten trials were conducted for each impinger type.

Sample collection, and analysis

Following the 10 minute sampling period, air flow to the impingers was stopped and the impingers removed from the ATC and class II BSC. The collected impinger was immediately weighed and remaining PBS spore suspension was removed and transferred to a sterile 50 mL conical tube. The exhaust line MCE filters were removed from the 47 mm cassettes and placed sample side up on 60 mm Chromagar™ filled plates for culturing. Four 100 µl aliquots of the spore suspension collected prior to impinger operation and after impinger operation were cultured on 90 mm Chromagar™

filled plates. Chromagar™ filled plates containing the MCE filters and 100 µl spore suspension aliquots were incubated anaerobically at 37°C for 24 hours as previously described. Following the 24 hour incubation period, colonies were counted visually using ultraviolet light. Final concentrations (in CFU/mL) and total culturable spores remaining (calculated from the post-sampling suspension concentration and liquid volume) were determined for both the BioSampler™ and AGI-30. Colonies counted from the cultured filters were used to determine the number of culturable spores re-aerosolized from the seeded spore suspension during the 10 minutes of impinger operation.

Fritted glass bubbler trials with nitrogen and air

To assess the effects of oxygen toxicity versus mechanical agitation on culturability, a fritted glass bubbler was seeded with spore suspension and operated using either air or pure nitrogen. Prior to the start of trials, a *C. difficile* spore suspension was prepared with a target concentration of 500 – 1000 CFU/mL in PBS. Additionally, a 0.01% volume/volume concentration of Tween-80 was added to this spore suspension. To determine the starting concentration, four replicate 0.1 mL aliquots of this suspension were collected and cultured using a 90 mm plate filled with Chromagar™ incubated anaerobically for 24 hours at 37°C. Two fritted gas bubblers, as shown in Figure 15, were seeded with 10 mL of *C. difficile* spore suspension in PBS and placed into the 76 liter ATC. Ten replicate trials were completed using fritted bubblers supplied with HEPA filtered air flow supplied to the ATC from the class II BSC drawn by an air sampling pump. An additional 10 replicate trials were completed using a fritted bubbler

supplied with medical nitrogen. Air and nitrogen flow were supplied to the fritted bubbler at a rate of 1.5 L/min. The fritted bubbler was operated for 42 minutes, thus supplying 63 total liters of gas (air or nitrogen) to the bubbler. This total volume of air or nitrogen is approximately equal to the amount of air supplied to the impingers during 10 minutes of sampling, adjusted by half to account for the difference in spore suspension in the fritted bubblers compared to the impingers (20 mL for the AGI-30 and BioSampler™ but only 10 mL for the fritted bubbler). The fritted bubbler was weighed, using an electronic balance, before and after operation to allow for the volume of spore suspension remaining to be calculated. Additionally, to collect spores aerosolized by fritted bubbler operation, a 47 mm cartridge containing a 47 mm diameter 0.45 µm pore size MCE was connected in-line from the exhaust port via a 7 inch (17.8 cm) Tygon™ tube (0.25 in, 0.64 cm ID).



Figure 15: Fritted Glass Bubbler Components and Assembled Device

Ten trials were conducted using air and another 10 trials using medical grade nitrogen as the supply gas for the bubbler (20 trials total). After each trial, the remaining PBS spore suspension was collected and transferred into a 50 mL conical tube. Four replicate 0.1 mL aliquots of this remaining spore suspension were cultured on 90 mm Chromagar™-filled plates and incubated anaerobically at 37°C for 24 hours. Following the 24 hour incubation period, colonies were counted visually using ultraviolet light and a concentration (in CFU/mL) determined. This concentration and the remaining spore volume were then used to determine the total count of spores in the bubbler after operation. A culturable loss fraction was then calculated by comparing the total number of spores in the bubbler after operation to the number of spores placed into the bubbler prior to operation.

Aim 3: High-Volume Air Sampling Evaluation and Demonstration with Toilet Flush Aerosol

Setup of Large Aerosol Chamber (LAC) and aerosol generation

This experiment was conducted in a sealed, 5 m³ volume, HEPA-equipped, Large Aerosol Chamber (LAC) into which *C. difficile* spores were aerosolized using a 3-jet Collison nebulizer. Each trial sampled the air in the LAC using one rotating plate STA impactor sampler, a 47 mm MCE filter operated by an SKC Vac-U-Go pump, and a high-volume sampler. Trials were conducted with a Dycor XMX/2L-MIL modified for dry filter operation, a Biocapture-650, a Cantium Scientific MB2 air sampler, and a Dry Filter Unit 1000 (DFU-1000) as shown in Figure 16. Trials with each high-volume sampler were conducted 15 times for a total of 60 trials, with the experiment blocked based on

high-volume air sampler type. Air flow rates were measured for the DFU-1000 and for the XMX using a hotwire anemometer (Model 9515, TSI, Inc., Shoreview, MN). Due to the device's design, direct flow measurements of the Biocapture-650 were not possible with available equipment. The MB2 was calibrated by the manufacturer prior to use.



Figure 16: High Volume Air Samplers Biocapture-650, DFU-1000, XMX/2L-MIL, and MB2 Shown Clockwise From Top Left

The three air samplers were placed on coated steel tables and their air intake heights were adjusted to be equal and approximately 39.5 inches (100 cm) above the

floor of the chamber as shown in Figure 17. This intake height was at $\frac{1}{2}$ the height, or the midpoint, of the chamber. The air samplers sampled the air in the LAC for a total of 15 minutes per trial. Aerosol generation was conducted using the 3-jet Collison nebulizer placed approximately 20 inches (50 cm) from the rear wall of the chamber, centered between the two side walls, and at a height of approximately 39.5 inches (100 cm). One air circulating fan was placed center in the chamber, approximately 8 inches (20 cm) from the rear wall and 10 inches (25 cm) behind the nebulizer to ensure sufficient air circulation inside the LAC and approximately symmetric air flow from the nebulizer to the air sampling equipment. The nebulizer outlet was directed towards the fan airflow to ensure greater dispersal of the generated aerosol. The LAC with DFU-1000, STA, and MCE filter installed is shown in the left panel of Figure 17. A schematic of the various placements, as viewed from above, is shown in Figure 18. Similar to the blocked experiments conducted in aim 1, air sampler positions were rotated so that five trials were conducted in each location in the LAC.



Figure 17: LAC with Air Samplers

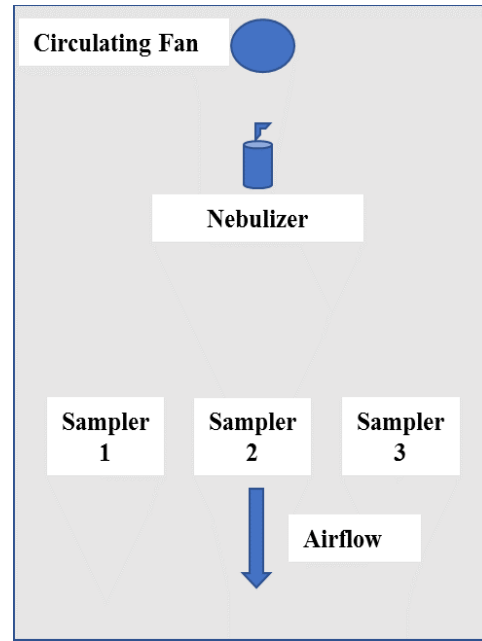


Figure 18: Layout of LAC

One 150 mm Chromagar™-filled plate was placed into the STA impactor. A sterile coverslip inserted through the inlet slit was used to indent the agar to mark the starting location of the rotating STA impactor plate. The nebulizer was then seeded with 50 mL of *C. difficile* suspension in sterile water, with a mean target seeding of 500 - 1000 CFU/mL. The impactor's programmed 15 minute countdown was then started and the LAC's door sealed, and the LAC purged under positive pressure with HEPA-filtered air for 15 minutes to remove any background particles or microbial contamination in the LAC air. After this 15 minute initial purge, airflow of 10 L/min was supplied to the nebulizer to allow aerosol generation and the circulating fan was started. After 5 minutes of aerosol generation, sampling was started using the STA impactor, the MCE filter, and the high-volume air sampler. Aerosol generation continued for the entire 15 minute sampling period. This ensured that aerosol concentrations were sufficient for

the entire sampling period and not excessively removed or stripped by the air sampling systems. The STA impactor, MB2, and Biocapture-650 were timed to automatically stop after 15 minutes of sampling. The DFU-1000, XMX/2L-MIL, and MCE filter air pump were manually stopped after 15 minutes of sampling to coincide with the stop time of the STA impactor. After 15 minutes of sampling, aerosol generation was stopped and the LAC was purged with HEPA filter air under negative pressure for 15 minutes. Following the 15 minute HEPA purge, the ATC was opened and samples collected from the impactor, MCE filter, and high-volume air sampler. The metal tables, exterior equipment surfaces, and stands were cleaned with Clorox Fuzion™ after each trial.

Reference low-volume air samplers

The STA impactor and a 47 mm MCE filter were used as reference air samplers for the high-volume air sampler evaluation. These reference air samplers were placed in two of the three sampling positions during the trials. A relative efficiency for each high-volume air sampler was calculated as the ratio of the concentration of airborne spores indicated by the high-volume air sampler to the concentration indicated by the reference sampler (MCE filter) expressed as a percentage. Additionally, the MCE filter and STA impactor were compared to each other, similar to the Aim 1 trials. This additional comparison was performed to compare the STA impactor, without the use of the inlet sampling tube, to the MCE filter for comparison with Aim 1 measures.

Sample collection and culture analysis

The 150 mm diameter plates were collected from the STA impactor and placed in a sealed 7.0 liter Anaeropack™ box. MCE filters collected from the filter cassettes were transferred sampling-side-up to a 60 mm diameter plate filled with Chromagar™ media and placed in an Anaeropack™ box. The 37 mm diameter filters collected from the XMX/2L-MIL were removed from the filter cartridge and placed sample side up on a 47 mm or 60 mm diameter plates with Chromagar™ media and placed in an Anaeropack™ box. The 90 mm diameter plates collected from the MB2 were placed into an Anaeropack™ box. The two polyester felt filters from each DFU-1000 trial were transferred to separate 50 mL conical tubes, to which 25 mL of PBS was added. The conical tube and filter were then vortexed for 2 minutes to transfer the spores on the filter to the PBS liquid. The samples were then transferred to a 47 mm membrane filter and placed grid side up on 60 mm Chromagar™ plate as previously described. The plate was then placed into a sealed Anaeropack™ box. Samples from the Biocapture-650 are deposited into a collection vial by the system. The sample from this vial was transferred to a 50 mL conical tube and sterile PBS was added until the volume reached 25 mL. The sample was then vortex mixed for a minimum of 15 seconds and transferred to a 47 mm membrane filter using the liquid membrane filtration apparatus. The filter was placed grid side up on Chromagar™ media in a 60 mm plate that was then placed into a sealed Anaeropack™ box. The Anaeropack™ boxes were incubated for 24 hours at 37°C. After this incubation period, colonies were visually counted under ultraviolet illumination. A concentration per liter of air sampled (CFU/L) and total number of spores collected (in CFU) were then determined for each sample.

Positive hole correction for MB2 results

The MB2 used for these trials was a 400-jet one-stage impactor that deposits collected spores onto an agar surface directly below each jet. After culturing, it is impossible to determine whether a colony resulted from a single collected spore or from multiple collected spores deposited by the same jet (Hinds, 1999, pp. 399-400). Without correction, multiple particle collection by a single jet would underestimate the true aerosol concentration present. Positive hole correction is employed to correct measurements made by multi-jet impactors and employs the following equation (Macher, 1989).

$$N_c = N_f \left(\frac{1.075}{1.052 - f} \right)^{0.483} \text{ for } f < 0.95$$

where N_c = total number of viable organisms collected, N_f = number of filled sites with colonies, $f = N_f/N_j$, where N_j = number of jets (400 as configured).

Aim 4: Toilet Flush Aerosol Collection Demonstration

This experiment utilized the HEPA-equipped LAC to assess the performance of a high-volume sampler in a low-concentration *C. difficile* aerosol environment, such as might be encountered in a CDI patient room or bathroom following toilet flushing. This demonstration utilized the high-volume air sampler determined to be most efficient in previous trials. A Flushometer™ toilet was installed in the LAC and plumbed to a filtered water supply system as described by Johnson et al. (2013) and Aithinne et al. (2019). The DFU-1000 and two MCE filters operated by SKC Vac-U-Go air sampling pumps were placed in the chamber, with one to the left of the toilet, one to the right of

the toilet, and one in front of the toilet. These positions were rotated across trials to ensure that the DFU-1000 sampled at all three positions for 4 trials, with 12 total trials conducted. Air samplers were placed at an inlet height of 39.5 inches (100 cm) from the floor of the chamber as before, which was approximately 27 inches (69 cm) above the bowl water surface. At this height fewer large droplets are expected than at lower heights above the bowl water surface, allowing for a greater proportion of droplet nuclei and airborne spores to be sampled. The LAC with the toilet and air samplers installed is shown in Figure 19.

Prior to each trial, the toilet bowl was disinfected by pouring 100 mL of sodium hypochlorite bleach (8.75% concentration) into the bowl, thoroughly brushing the toilet bowl walls, and allowing it to sit for a minimum of 30 minutes. The surfaces of the toilet were externally cleaned by wiping with Clorox Fuzion. The toilet was then flushed multiple times to clear the chlorine out of the bowl before testing the bowl water for residual chlorine using a Chlorine Pocket Colorimeter II (Hach, Loveland, CO). Flushing was continued until the free-available chlorine was reduced to a level of less than 0.2 parts per million, similar to that in a municipal water system. The bowl water was then seeded with 50 mL of *C. difficile* suspension and stirred. The target concentration of the seeded bowl water was approximately 10^4 - 10^5 CFU/mL. A 50 mL bowl water sample was then taken. An additional 50 mL water sample was collected from the pressurized water supply tank to verify the absence of *C. difficile* contamination in the supplied flush water. The LAC was then sealed and purged for 15 minutes using HEPA-filtered air. The toilet was then flushed one time, with air sampling starting immediately after the toilet flush. Air sampling continued for 15 minutes after the flush. The toilet was then

flushed again, followed by another 15 minute air sampling period. This flushing and air sampling was continued for 3 flushes or 45 total minutes of air sampling time. Following the third flush and sampling period, the air samplers were stopped and a 15 minute purge with HEPA-filtered air was conducted. The LAC was then unsealed and opened. Samples from the two low-volume systems and from the high-volume system were collected and replaced with clean sampling media. A bowl water sample was collected, and the LAC resealed and purged with HEPA filtered air for 15 minutes. An additional series of three flushes, without reseeding the toilet, followed by 15 minutes of sampling time was then conducted followed by a 15 minute purge with HEPA-filtered air. Samples were again collected from the high-volume and reference air sampling systems, concluding the trial. This collection of two series of samples per trial allowed for a demonstration of high-volume air sampling for *C. difficile* spores during two conditions. The first condition in flush 1-3 is representative of *C. difficile* spore concentrations immediately after a toilet flush. The second condition, flush 4-6 is representative of those expected over an extended period after initial toilet contamination. Following each trial, the toilet, tables, test stands, and equipment surfaces were cleaned with Clorox Fuzion™ to remove any residual spore contamination.



Figure 19: LAC with Toilet and Air Samplers Installed

Sample collection and culture analysis

Air samples from the MCE filters and DFU-1000 were collected and analyzed as described in Aim 3. The 50 mL water samples collected from the bowl water and from the pneumatic tank were analyzed by membrane filtration using the membrane filter apparatus as described previously. The 50 mL water samples were also collected directly from the toilet bowl water with a 25 mL sterile pipette that was also used to stir the bowl water to ensure that the sample was well mixed. Bowl water samples were collected prior to flush 1, following flush 3, and following flush 6. After the water samples were filtered through a 47 mm diameter, 0.45 μm pore size gridded MCE filter, they were placed grid side up onto Chromagar and incubated anaerobically for 24 hours as described previously.

CHAPTER IV

RESULTS AND DISCUSSION

Aim 1. Evaluation of Low Volume Air Sampling Methods

Survival of C. difficile spores in the Collison nebulizer

Nine paired sets of samples were collected from the Collison nebulizer. These samples were collected immediately after seeding the Collison with spore suspension and after 10 minutes aerosol generation. *C. difficile* concentrations before nebulizer operation ranged from 680 CFU/mL to 1060 CFU/mL. Measured *C. difficile* concentrations after 10 minutes of nebulizer operation ranged from 910 CFU/mL to 1190 CFU/mL. Results from a paired t-test showed a significant ($p = 0.0476$) mean increase of 153.3 CFU/mL (95% CI: 2.07 CFU/mL, 304.6 CFU/mL) after 10 minutes of nebulizer operation. This result suggested that *C. difficile* spore agglomerates initially present in the suspension were disaggregated and that the nebulizer operation did not have a substantial negative effect on spore culturability. Based on these results, the Collison nebulizer was an acceptable aerosol generation method for the setup, air supply flow rates, and operating durations used in this study. Raw data used to calculate the culturability of *C. difficile* spores in the Collison nebulizer are included in Appendix B.

Appearance of cultured C. difficile colonies

Colonies collected and cultured on the 150 mm Chromagar™ plates from the STA impactor were approximately 2-4 mm in size. Colonies collected and cultured on

the 47 mm filters were smaller, approximately 1 mm in size after 24 hours of incubation, but exhibited similar fluorescence properties under ultraviolet light as those cultured directly on the agar surface. Example photos of colonies cultured on the MCE filter and STA impactor plates as shown in Figure 20 and Figure 21.

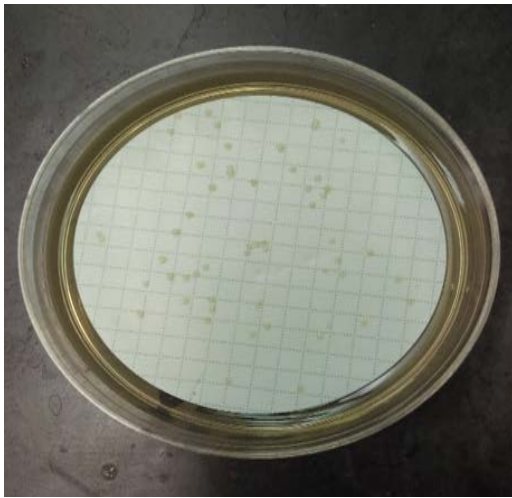


Figure 20: *C. difficile* on MCE filter

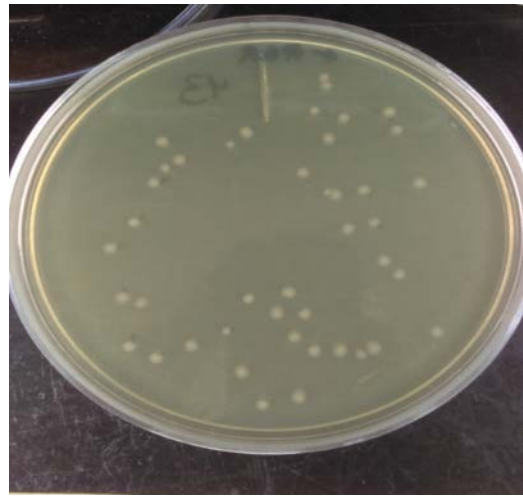


Figure 21: *C. difficile* on STA plate

Comparison of MCE filters to the slit-to-agar impactor

Nine trials consisting of 2 MCE filters and 1 STA impactor sampling side-by-side provided 27 total samples. Mean measured flow rate for the MCE filters was 25.5 L/min (Range: 25.4 – 25.6 L/min), while mean measured flow rate for the STA impactor was 25.5 L/min (Range: 25.4 – 25.5 L/min).

Potential location bias due to incomplete aerosol mixing in the polypropylene aerosol test chamber was assessed through a paired t-test of the two filter measurements in each of the nine trials. Pair differences in relative filter efficiencies based on position in the chamber (left versus right positioned filter) were found to be normally distributed based on the Shapiro-Wilk test ($p = 0.9026$). The mean relative

efficiency of the filter in the left position was 130.40% (95% CI: 112.95%, 147.90%).

The mean relative efficiency of the filter in the right position was 138.89% (95% CI: 118.53%, 159.26%). The paired t-test comparing differences in the relative efficiencies determined that there was no significant difference in the relative efficiency based on filter position ($p = 0.4545$). This result of no significant difference indicates acceptable mixing of generated aerosol in the test chamber and an absence of location bias due to the air sampler position. Mean filter relative sampling efficiencies and 95% confidence intervals by sampling position are shown in Figure 22.

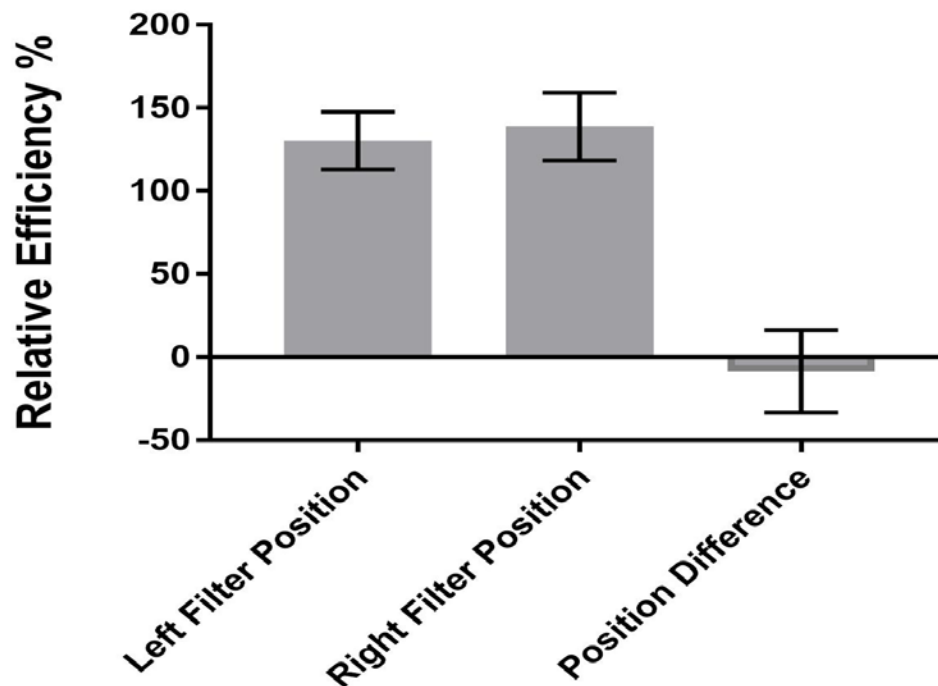


Figure 22: Mean Relative Efficiencies of MCE Filter Air Samples by Position and Pair Difference in Relative Efficiency (Left -Right). Error Bars Represent the 95% Confidence Interval.

The relative efficiencies for the MCE filters were normally distributed based on the results of the Shapiro-Wilk test ($p = 0.9322$). The overall mean relative efficiency of the MCE filter was 134.7% (95% CI: 122.6%, 146.8%) compared to the STA impactor using a Student's t-test. Based on the results of this Student's t-test, the MCE filter is significantly more efficient in the collection of *C. difficile* spore aerosol than the reference STA impactor ($p < 0.0001$). A brief summary of these results is located in a peer-reviewed journal article in Appendix A.

Comparison of the BioSampler™, AGI-30, and STA impactor

Nine air sampling trials were conducted using a BioSampler™, an AGI-30, and an STA impactor. Out of 18 impinger samples, 6 produced no detectable colonies of *C. difficile* (3 samples from the BioSampler™ and 3 samples from the AGI-30). As these were censored data points and produce relative efficiencies of zero, they were recalculated with values equal to one-half of the limit of detection for the instrument. The limit of detection for each impinger was assumed to be 1 CFU, therefore collected values of 0.5 CFU were reassigned for these zero values and a relative efficiency recalculated based on this assigned value. Pair differences between the BioSampler™ and AGI-30 were normally distributed based on the Shapiro-Wilk test ($p = 0.8641$). The BioSampler™ and AGI-30 efficiencies were compared to each other using a paired t-test and not found to significantly differ from each other ($p = 0.4958$). Using these assigned relative efficiency values for the BioSampler™ and AGI-30, the median pooled relative efficiency was 3.16% (Range of 0.93% to 13.22%) relative to the STA impactor. Pooled relative efficiencies for the BioSampler™ and AGI-30 were not normally

distributed based on the Shapiro-Wilk test ($p = 0.0102$). These relative efficiencies were compared to a value of 100%, or equal to the STA impactor, using a Wilcoxon Signed Rank Test and found to collect significantly less *C. difficile* spore aerosol than the STA impactor ($p < 0.0001$). Colonies counted on the impinger in-line exhaust filters ranged from 3 to 14 CFU, which represented less than 10% of the spores entering the impinger as estimated from the slit-to-agar CFU counts.

A comparison plot of the relative efficiencies of the MCE filter, BioSampler™, and AGI-30 relative to the STA impactor is shown in Figure 23. Raw data used to calculate relative efficiencies for each air sampler are included in Appendix B.

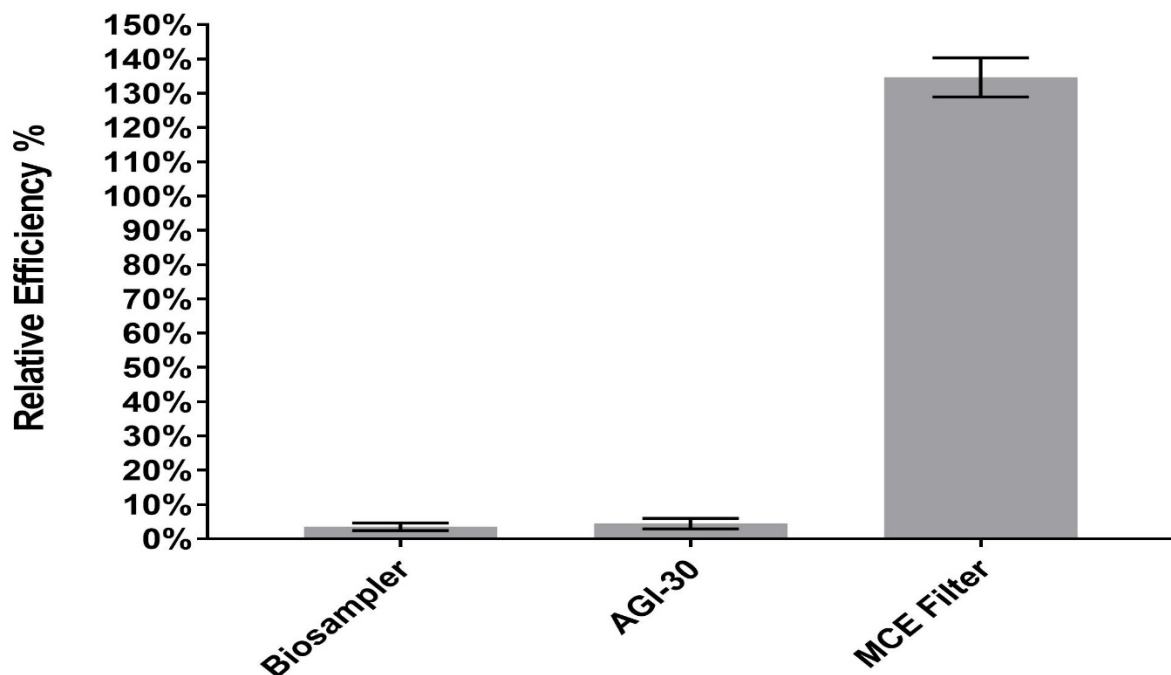


Figure 23: Mean Relative Efficiencies of Low-Volume Air Samplers Relative to Slit-to-Agar Impactor. Error Bars Represent the 95% Confidence Interval

Discussion

This study was the first to produce a nebulizer-generated aerosol of *C. difficile* spores in a laboratory and to demonstrate that *C. difficile* spores are capable of surviving aerosolization in a laboratory environment. Previous studies have either collected *C. difficile* spores directly from the environment such as Best (2010) in patient care or Keessen (2011) in agricultural environments. Spore aerosols have also been produced by toilets in a laboratory setting by Best et al. (2012) and Aithinne et al. (2019). The observed increases in concentration of the *C. difficile* spore suspension in the Collison nebulizer after 10 min was potentially due to deagglomeration of spores by the nebulizer or due to the production and dissemination of sub-micrometer microbe-free water droplets by the nebulizer. Previous studies have shown that sub-micrometer droplets comprise over 90 percent of the total droplets produced by a Collison nebulizer (Crosswy, 1985). Due to the size of a *C. difficile* spore, a sub-micrometer droplet is incapable of containing a spore but would decrease the water in the suspension over the 10 minute period and thus increase the concentration of spores.

The results from the comparison trials demonstrates substantial and unexpected differences in the collection of viable *C. difficile* spores by the two different impingers, MCE filters, and the STA impactor under the evaluated conditions. The results of this study suggest that impingers were particularly ineffective in the collection of culturable *C. difficile* spore aerosol. This suggests that *C. difficile* spores drawn into the impingers were either not captured in the liquid, were captured but became re-aerosolized by fluid turbulence, or were captured but rendered non-culturable by the collection sampling conditions. The extreme underperformance of both the AGI-30 and BioSampler™

compared to the STA impactor is a significant departure from impinger performance in the collection of aerobic spore-forming bacteria demonstrated by previous studies. Grinshpun et al (1997) demonstrated 80 to 90 percent sampling efficiencies for the AGI-30 in the collection of *Bacillus cereus* spores. The penetrative and re-aerosolized *C. difficile* spore fractions of 10% or less, in this study, are consistent with the penetration and re-aerosolization losses observed by Grinshpun et al. (1997). The low penetrative and re-aerosolized impinger spore fractions found in this study for *C. difficile* suggests that uncaptured spores lost due to penetration and re-aerosolization were a small contributor to the underperformance of impinger air sampling. This further suggests that the *C. difficile* spore aerosol in this study was captured but rendered unculturable by the operation of the BioSampler and AGI-30.

The high relative efficiency of *C. difficile* collection by the MCE filter was an unexpected result as well, especially the observed higher collection compared to the reference STA impactor. MCE filters with a 0.45 μm pore size have a high collection efficiency (>99%) for particles in the 1-2 μm size range (Hinds, 1999, p. 203); however, they have also been shown to reduce viability of collected microorganisms due to desiccation losses (Hinds, 1999, p. 398). Therefore, the high relative collection efficiencies observed in this study were an unexpected result and suggests that *C. difficile* spores may not be highly susceptible to desiccation.

The STA impactor used as a reference sampler was modified by the addition of an 85 cm long air inlet tube as described in the methodology. A high relative efficiency of MCE filters compared to the STA impactor could result from under-sampling by the impactor due to spore losses in the tube. The tube was connected to the ATC by

bending the tube approximately 90 degrees with a bend radius of 14 cm. Deposition in sampling lines can produce particle losses as an aerosol travels from the sampling source to the measuring instrument, especially in sampling lines with a bend. Aerosol particle losses in a section of bent tubing can be calculated as described in Kulkarni et al. (2011). The tube bend losses for a particle size of 1.4 μm under these experimental conditions were estimated to be approximately 1.3% (Kulkarni, et al., 2011, p. 94) and would not significantly affect the relative efficiency values.

Limitations

The impinger comparison study was limited by the use of a single collection media (PBS) and a single sampling time (10 min). For air sampling with impingers, extended sampling durations of greater than 30 minutes are not recommended due to the evaporation and re-aerosolization of the liquid media (Lin, et al., 1999). Extended operating times also significantly reduce the retention of captured particles thus decreasing overall sampling efficiency (Kesavan, et al., 2010). The use of non-evaporating mineral oil has been shown to effectively allow bacterial aerosol sampling times of up to 4 hours with the BioSampler™, however mineral oil has not been evaluated for compatibility with chromogenic agar. The scope of this study was also limited to air sampling methods commonly used for bioaerosol assessment and availability of equipment. This study compared the *C. difficile* spore bioaerosol sampling efficiency of the two most commonly used liquid impinger bioaerosol samplers and one type of filter medium (MCE) to that of the STA impactor successfully used for *C. difficile* aerosol sampling in previous studies. Other filter types were considered but

not evaluated based on known limitations. For example, gelatin filter media are susceptible to breakage during extended sampling times that would likely be needed in a healthcare environment, and the compatibility of gelatin with chromogenic agar is unknown. Other filter materials such as PTFE cannot be cultured directly onto agar and would have required extraction from the filter, with associated extraction losses.

This study was also limited by the use of a non-toxigenic strain of *C. difficile* in place of toxigenic *C. difficile* strains, which are typically responsible for HA-CDI. The spores were also prepared and aerosolized in essentially pure suspensions, whereas spores aerosolized in healthcare operations might be incorporated in a matrix of other materials. The surrogate selection was driven by laboratory safety due to the difficulty and risks involved in the use of toxigenic *C. difficile*, particularly for aerosol studies where complete containment cannot be assured. Naturally occurring toxigenic *C. difficile* aerosolized from other media, such as a fecal suspension and sampled and cultured by other methods may not exhibit similar susceptibility to sampling stresses. Environmental survivability of non-toxigenic *C. difficile* has not been compared to toxigenic *C. difficile* and its environmental survivability may also differ. Finally, while this study was the first to perform laboratory air sampling trials of any species of *Clostridia*, the results are limited to *Clostridium difficile* only. Other *Clostridia* species, including those of importance to environmental air sampling such as *C. perfringens* and *C. botulinum*, may exhibit different culturability and aerosol characteristics in air sampling trials.

Aim 2. Evaluation of *C. difficile* Impinger Culturability Loss

Culturability loss in seeded impingers

The BioSampler™ was seeded with 20 mL of *C. difficile* spore suspension with a mean concentration of 751.8 CFU/mL for 10 trials. The AGI-30 was similarly seeded with a mean concentration of 823.5 CFU/mL for 10 trials. Impinger suspension volumes were remeasured after each 10 minute trial and samples collected to determine the post-operation *C. difficile* culturable spore counts. Results are presented in Appendix C and summarized in Figure 24. After 10 minutes of operation for each trial, the mean decrease in culturable spores was 88.73% (95% CI: 86.74% - 90.72%) for the BioSampler™ and 89.90% (95% CI: 87.49% - 92.31%) for the AGI-30. The mean remaining culturabilities for the BioSampler™ and AGI were 11.27% (95%CI: 9.28% - 13.26%) and 10.10% (95%CI: 7.69% - 12.51%). The culturability losses were normally distributed based on the results of the Shapiro-Wilk test for the AGI-30 ($p = 0.2411$) and the BioSampler™ ($p = 0.9885$). The distributions were found to have similar variances based on the results of the folded F-test ($p = 0.5800$). A two-sample t-test was performed to compare culturable spore losses between the AGI-30 and the BioSampler™ and losses were not found to be significantly different ($p = 0.4078$). These calculated culturable spore loss values accounted for any decreases in suspension volume due to evaporation and aerosolization during operation.

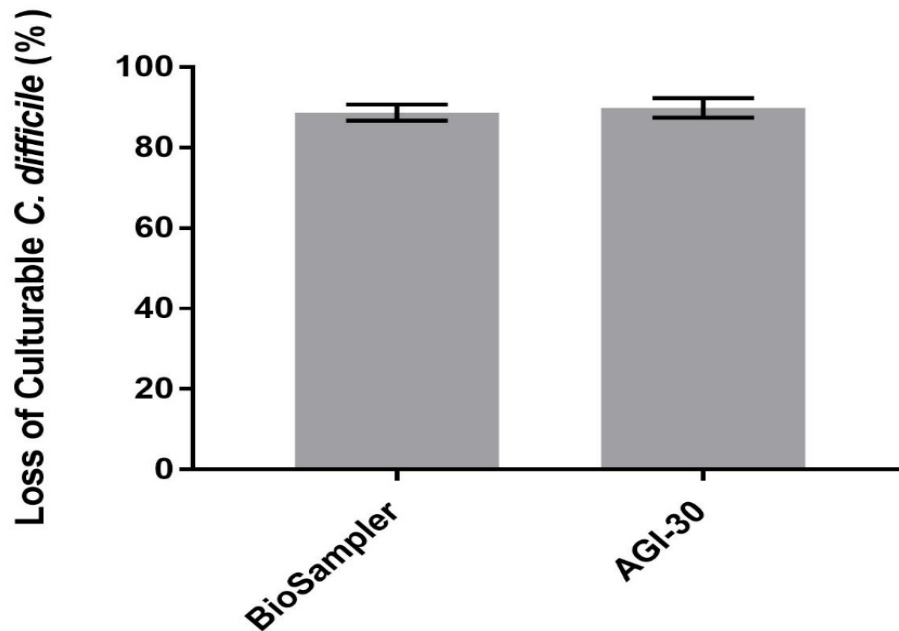


Figure 24: Mean Loss of Culturable *C. difficile* by Impinger Type. Error Bars Represent the 95% Confidence Interval

Impinger re-aerosolization losses

The potential influence of re-aerosolization on the observed decreases in culturable *C. difficile* in the impingers was assessed by placing MCE filters downstream of impingers seeded with *C. difficile* suspension and sampling particle-free air for 10 minutes. Detailed results of the 10 total trials are presented in Appendix C. *C. difficile* CFU on the MCE filters ranged from 0 to 4 CFU for the BioSampler™ and 18 to 41 CFU for the AGI-30. This represented median re-aerosolization losses relative to the original culturable concentration of 0.15% for the AGI-30 (range 0.08% - 0.35%) and 0.005% for the BioSampler™ (range 0.0% - 0.02%). As determined by the Shapiro-Wilk test, the re-aerosolization losses, in terms of the percentage of spores re-aerosolized, were non-normally distributed for the BioSampler™ ($p = 0.0085$), but normally distributed for the

AGI-30 ($p = 0.2657$). Results from the Mann-Whitney test comparing the re-aerosolization of the spores from the BioSampler™ and AGI-30 indicated significantly higher spore re-aerosolization from the AGI-30 than from the BioSampler™ ($p < 0.0001$). The median number of spores re-aerosolized was 0.5 CFU from the BioSampler™ and 22.5 CFU from the AGI-30. While the AGI-30 produced greater spore re-aerosolization compared to the BioSampler™, re-aerosolization losses for both instruments was negligible compared to total culturability lost during the 10 minute operating time. Raw data used to calculate decreases in culturable spores and used to determine total re-aerosolized spores from impinger operation are included in Appendix C.

Culturability loss in a seeded fritted glass bubbler comparing air and nitrogen

Twenty total trials were conducted with seeded fritted bubblers. Ten trials were conducted by supplying HEPA-filtered air to the fritted bubbler seeded with concentrated *C. difficile* spore suspensions, and another 10 trials supplying medical grade nitrogen to the bubbler. As determined by the Shapiro-Wilk test, the culturable spore losses over the 10 minute operating period were found to be normally distributed for both operation with filtered air ($p = 0.3904$) and operation with nitrogen ($p = 0.1450$). Distribution variances were also found to be similar using a folded F-test ($p = 0.1343$). The mean decreases in culturable spores in the fritted bubbler were 83.64% (95% CI: 77.12%, 90.16%) for operation with filtered air and 65.85% (95% CI: 54.83%, 76.87%) for operation with nitrogen. The mean decrease in culturable spores in a fritted bubbler was found by the two-sample t-test to be significantly higher when the bubbler

was operated with HEPA-filtered air than when operated with medical grade nitrogen ($p = 0.0060$). Mean culturability losses in the fritted bubbler operating with HEPA-filtered air and nitrogen are shown in Figure 25.

Total colonies collected on the exhaust MCE filter of the fritted bubbler ranged from 127 CFU to 273 CFU for air and from 112 CFU to 202 CFU for nitrogen. The mean re-aerosolized fraction of culturable spores initially present in the fritted bubbler was 2.62% (95% CI: 1.75%, 3.49%) for air and 1.72% (95% CI: 1.25%, 2.19%) for nitrogen. Results from the Shapiro-Wilk test found that the re-aerosolized fractions were normally distributed for operation with air ($p = 0.0888$) and nitrogen ($p = 0.3388$). A two-sample t-test failed to show a significant difference in the reaerosolized culturable spore fractions for a nitrogen and air fritted bubbler operation ($p = 0.0550$). Raw data used to calculate overall culturable spore fraction lost, remaining, and re-aerosolized for the fritted bubblers are included in Appendix C.

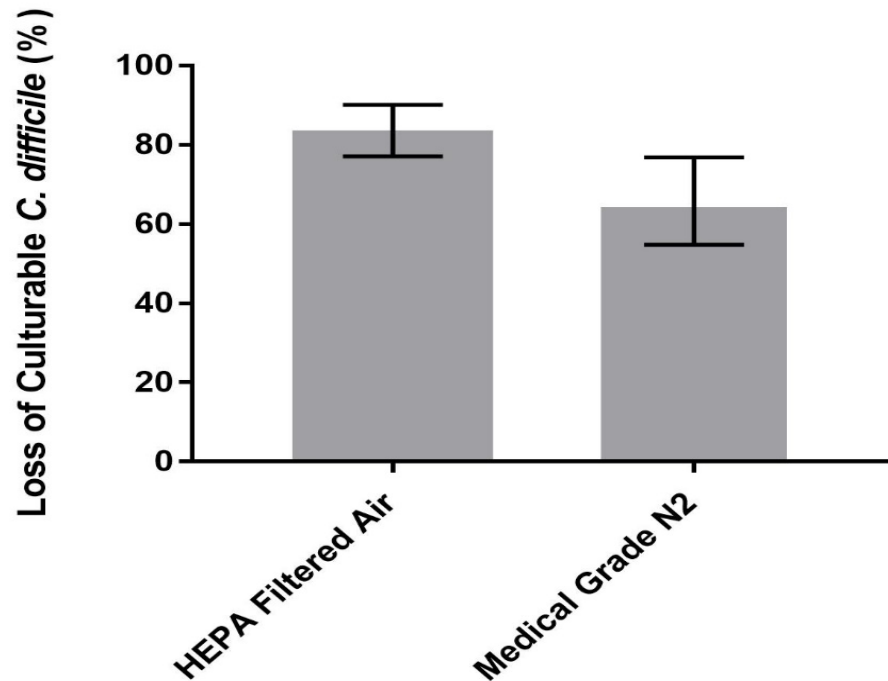


Figure 25: Mean Loss of Culturable *C. difficile* in a Fritted Bubbler Operating with HEPA-Filtered Air and Nitrogen. Error Bars Represent the 95% Confidence Interval.

Discussion

The high degree of *C. difficile* culturability losses from 10 minutes of seeded impinger operation was consistent with the poor impinger performance results from the air sampling comparison trials using *C. difficile* spores, but was not consistent with previous studies using other spore types, such as those on aerobic spore-forming and vegetative bacteria conducted by Kesavan (2010) that showed negligible losses for aerobic spores in both the AGI-30 and BioSampler™. The culturable spore losses in this study suggests that culturability loss in the liquid media after spore collection was a substantial source of impinger underperformance relative to the STA impactor. These culturability losses were also not consistent with the lack of culturability loss observed

during Collison nebulizer operation in Aim 1. Although some re-aerosolization from the impingers was observed, with significantly higher spore re-aerosolization from the AGI-30 than the BioSampler™, this was expected based on previous studies comparing the two impingers with *B. subtilis* (Lin, et al., 2000). However, the re-aerosolization observed for both impingers represented less than 1 percent of the total spores in the suspension. This suggests that re-aerosolization of culturable spores would not have significantly impacted the performance of the impinger or explain the high culturability losses from impinger operation.

A key difference in this and previous work with other spore types is that *C. difficile* is an obligate anaerobe. It may be that the observed culturability losses were at least in part due to oxygen toxicity stresses on the spores after capture, due to continuous aeration by the impinger jets. However, the potential role of mechanical stress due to fluid turbulence cannot be ruled out. Results of the fritted bubbler trials with air and oxygen suggest that both may be factors in culturability loss. These observations are consistent with those made in Aim 1 and suggest that conditions in an impinger during sampling are not conducive in maintaining culturability of collected *C. difficile* spore aerosol.

The fritted bubbler trials provided an initial exploration of the relative potential roles of post-capture oxygen toxicity and mechanical trauma in culturability loss. The bubbler was chosen because it provides high oxygenation with (presumably) less mechanical stress than the liquid impingers. Both nitrogen and oxygen operation produced a large degree of culturability losses, suggesting that oxygen toxicity due to

aeration, mechanical trauma due to turbulence, or their interaction were likely responsible for culturability losses.

The re-aerosolization of *C. difficile* spores from the fritted bubbler operating with both air and nitrogen was greater than that observed during impinger operation with air. Fritted glass bubblers have been used as an aeration source in previous studies and aeration has been shown to produce bioaerosols during the aeration of liquid media containing spore-forming aerobic bacteria, specifically *Bacillus niger* (Smith, 1968). However, this is the first study to show significant aerosol generation with *C. difficile* or any other anaerobic endospore-forming bacteria species. The observed spore aerosol production from fritted glass bubblers in this study suggests that *C. difficile* spore aerosol can be produced, under controlled conditions, by bubble scavenging and bursting. These observations support the suggestion made by previous studies including Johnson et al. (2013) that demonstrated that bubble bursting in flush toilets may produce aerosols in the size range of *C. difficile* spores. Aithinne et al. (2019) also suggested that bubble bursting may be responsible for some of the aerosol produced from flush toilets seeded with *C. difficile* spores.

Limitations

The seeded impinger and fritted glass bubbler trials were conducted for a single operation time, and observed culturability losses may not be representative of shorter operation times. These experiments were also limited by the use of a single liquid medium, i.e. sterile PBS. Although PBS does not affect culturability of *C. difficile* in storage and is a common liquid medium for impinger bioaerosol sampling for spores,

impingers operated with other liquid media may produce higher or lower culturability losses than those observed in this study. Further research would be needed to explore this question. A further limitation was that the trials were not conducted in a full-factorial randomized experimental design, so that main effects (oxygen, trauma) and their interaction could not be distinguished.

An additional limitation in the nitrogen fritted bubbler trials was the preparation of samples in an aerobic BSC hood environment. While exposure to oxygen during nitrogen bubbler operation would have been minimal, samples were collected and culture plates were prepared in the presence of air. Spores may have suffered culturability loss from air exposure while being transferred from the bubbler to the culture plate. This may partially explain the unexpectedly high culturability losses from nitrogen exposure in a fritted bubbler. While culturability losses were significantly lower with nitrogen than air, the degree of culturability loss may have been even lower had sample transfer and plating occurred in an anaerobic environment. Additional work in a totally oxygen-free environment would be required to further explore oxygen toxicity as a potential factor in culturability loss.

Aim 3. High-Volume Air Sampler Evaluation and Toilet Flush Demonstration

Comparison of high-volume air sampling in the collection of *C. difficile* spore aerosol

Fifteen trials were completed for each high-volume air sampler evaluated, with 60 total trials. High-volume air samplers included the DFU-1000, MB2, XMX/2L-MIL, and Biocapture-650. Resulting raw data are presented in Appendix D. Air flow rates were measured for the DFU-1000 and for the XMX using a hotwire anemometer (Model 9515,

TSI, Inc., Shoreview, MN). Measured mean flow rates were 919.7 L/min (95% CI: 889.4 L/min – 950 L/min) for the DFU-1000 and 699.8 L/min (95% CI: 684.0 L/min – 715.5 L/min) for the XMX/2L-MIL. A nominal flow rate of 200 L/min for the Biocapture-650, as provided by the manufacturer, was used in all calculations. The MB2 was calibrated by the manufacturer to be 100 L/min of air flow.

Based on the higher collection efficiency of the MCE filter versus the STA impactor, results for the high-volume air sampling trials are presented using the MCE filter as the reference air sampler. The results of Shapiro-Wilk tests show that the relative efficiencies were normally distributed for the DFU-1000 ($p = 0.9286$), Biocapture-650 ($p = 0.8656$), and XMX/2L-MIL ($p = 0.0576$). The mean relative efficiencies were 38.60% (95%CI: 31.09%, 46.11%) for the DFU-1000, 28.16% (95% CI: 24.44% - 31.89%) for the Biocapture-650, and 10.51% (95% CI: 9.02% - 11.99%) for the XMX/2L-MIL. The Shapiro-Wilk test indicated that the relative efficiencies for the MB2 were not normally distributed ($p = 0.0014$). The median relative efficiency of the MB2 air sampler relative to MCE filters was 1.44% (range 0.00% -13.02%). Due to the low number of *C. difficile* colonies collected by the MB2, positive hole correction did not substantially change the measured efficiencies. The DFU-1000, XMX/2L-MIL, and Biocapture-650 collected culturable *C. difficile* on all 15 trials, but the MB2 failed to collect culturable *C. difficile* spores in 4 of 15 trials. A summary of relative efficiencies for the four evaluated high-volume air samplers is shown in Figure 26.

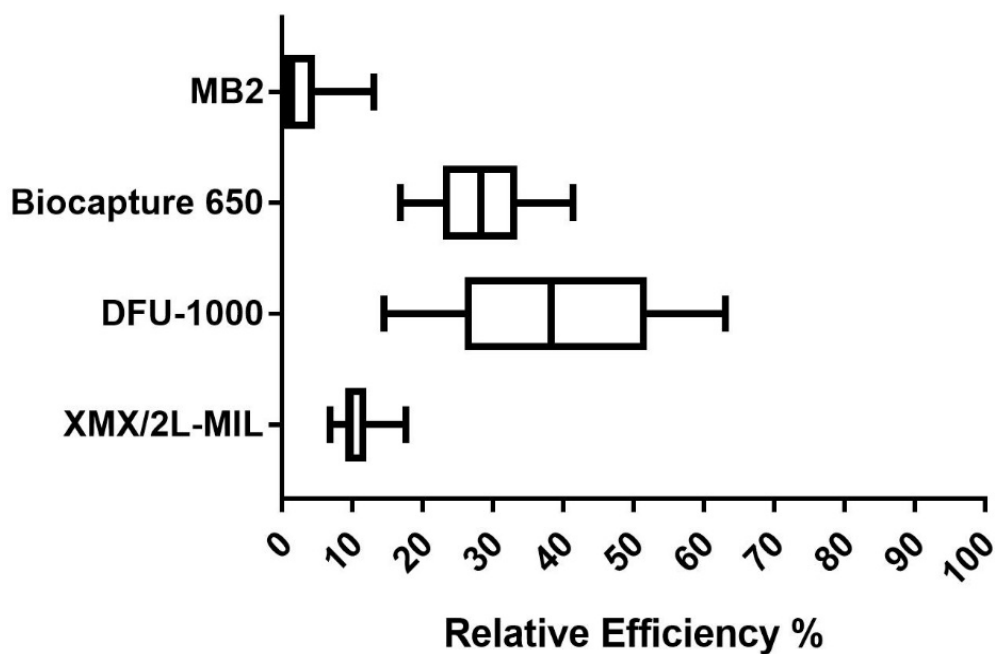


Figure 26: Efficiency of the MB2, Biocapture-650, DFU-1000, and XMX/2L-MIL relative to MCE filters (Minimum, Maximum, Median, 25th Percentile, and 75th Percentile values shown).

Since the relative efficiencies for the MB2 were not normally distributed, a multiple comparison analysis for the four high-volume air samplers was performed using a Kruskal-Wallis nonparametric ANOVA with post-hoc multiple pairwise comparison using the Bonferroni-Dunn test. The Kruskal-Wallis ANOVA for the relative efficiencies of the four high-volume air samplers showed a significant difference ($p < 0.0001$). The results of the Bonferroni-Dunn test are shown in Table 1 below.

Comparison	<i>p</i> -Value	Significance
DFU-1000 vs. Biocapture-650	> 0.9999	Not Significant
DFU-1000 vs. XMX/2L-MIL	< 0.0001	Significant
DFU-1000 vs. MB2	< 0.0001	Significant
Biocapture-650 vs. XMX/2L-MIL	0.0110	Significant
Biocapture-650 vs. MB2	< 0.0001	Significant
XMX/2L-MIL vs. MB2	0.3342	Not Significant

Table 1: Multiple Comparisons of High-Volume Air Samplers in the Collection of *C. difficile* spore Aerosol Using Kruskal-Wallis ANOVA with Bonferroni-Dunn Post-hoc Test

Based on these results, the four high-volume air samplers were divided into high and low efficiency categories for further analysis, with the DFU-1000 and Biocapture-650 categorized as high efficiency air samplers and the MB2 and XMX/2L-MIL categorized as low efficiency air samplers. Since the relative efficiencies of the DFU-1000 and Biocapture-650 were normally distributed, they may be separately compared with a t-test. Based on the results of the F-test, the variances of the two distributions were significantly different ($p = 0.0131$), thus requiring a Welch's two-sample t-test (Johnson, 2017, pp. 93-94). The Welch's two-sample t-test showed a significant difference ($p = 0.0145$) in the mean relative efficiencies of the DFU-1000 and Biocapture-650, with the DFU-1000 being the more efficient device. The mean relative efficiencies and 95% confidence interval for the Biocapture-650 and DFU-1000 as compared to MCE filters are shown in Figure 27.

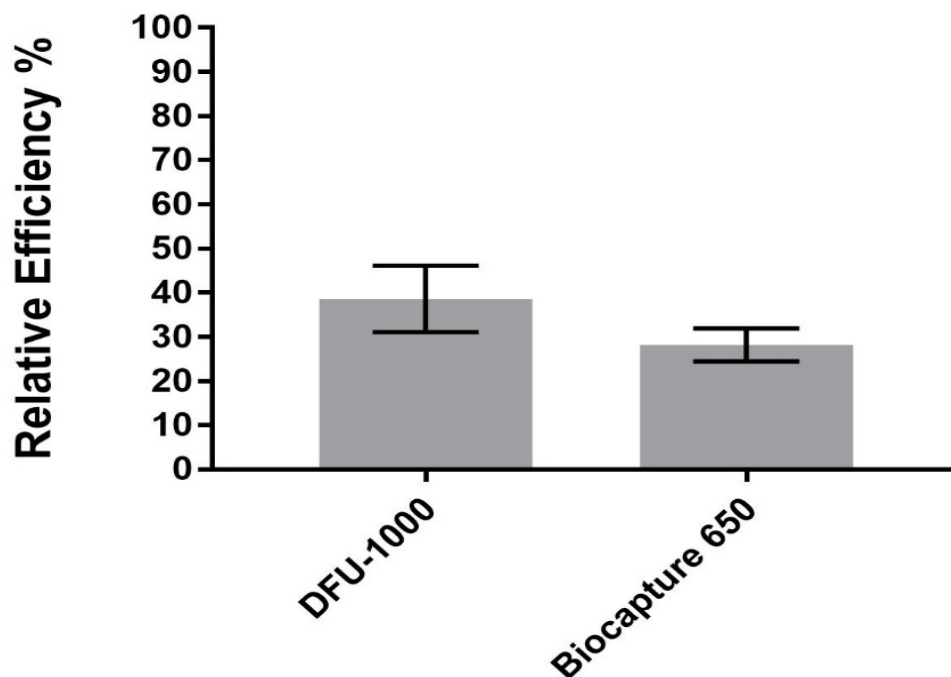


Figure 27: Mean Relative Efficiency of DFU-1000 and Biocapture-650. Error Bars Represent the 95% Confidence Interval

To compare the MB2 and XMx/2L-MIL, a Mann-Whitney Test was used due to the non-normal distribution of the MB2 relative efficiencies. The Mann-Whitney Test showed a significant difference between the median relative efficiencies of the XMx/2L-MIL and the MB2 ($p < 0.0001$), with the XMx/2L-MIL being the more efficient device. The median relative efficiency of the XMx/2L-MIL compared to the MCE filter was 9.76% (95% Median CI: 9.05% - 11.97%), whereas the median relative efficiency of the MB2 was 1.44% (95% Median CI: 0.00% - 4.65%). Additionally, the MB2 failed to collect culturable *C. difficile* in 4 out of 15 trials. During these trials the MCE filter collected culturable *C. difficile* spores ranging from 31 to 41 CFU, thus demonstrating multiple non-detection results with the MB2 during trials when aerosols of culturable *C.*

difficile spores were present in the chamber. Collection efficiencies including median and 95% confidence intervals of the XMX/2L-MIL and MB2, relative to the MCE filter, are shown in Figure 28.

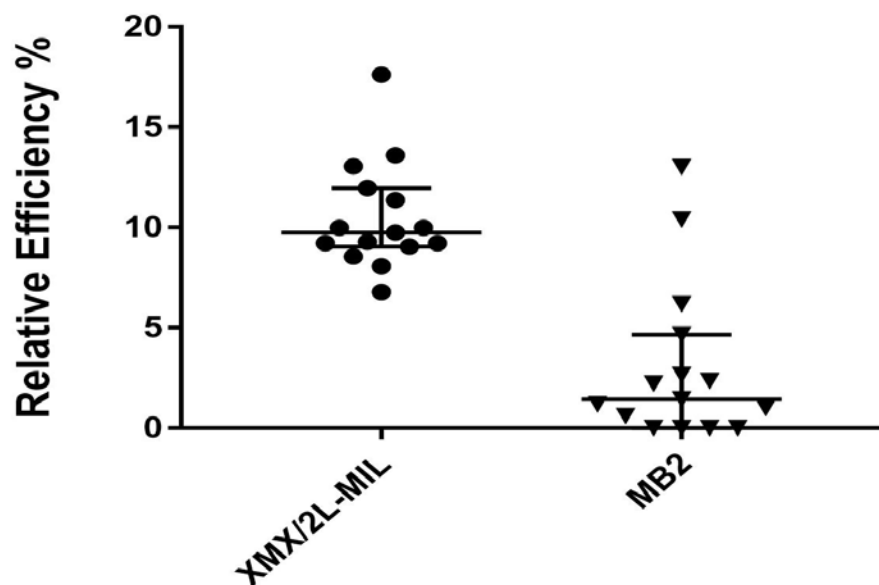


Figure 28: Median Relative Efficiency vs. MCE Filter for XMX/2L-MIL and MB2 Air Samplers. Error Bars Represent the 95% Confidence Interval for the Median

Comparison of the MCE filter and slit-to-agar impactor in the large aerosol chamber

Paired MCE and STA impactor samples were collected in all 60 high-volume air sampler trials. Using the MCE filter as the reference, the mean relative efficiency of the STA impactor was 77.08% (95% CI: 72.53% - 81.62). The 60 measured relative efficiencies were normally distributed as shown by the Shapiro-Wilk test ($p = 0.9822$). The STA impactor performance relative to the MCE filter was compared using Student's t-test and showed that there was a significant difference between the MCE filter and the impactor ($p < 0.0001$), which is in agreement with the air sampling comparison

performed in the 75 L ATC in Aim 1. Raw data used to calculate and compare the efficiencies of the STA impactor relative to the MCE filter are included in Appendix D.

Discussion

The significantly higher relative efficiency of the DFU-1000 compared to the Biocapture-650, XMX/2L-MIL, and MB2 was an unexpected result when compared to previous comparative studies using other bioaerosols. Previous studies comparing the XMX/2L-MIL and DFU-1000 showed that the DFU-1000 collected significantly less aerosolized MS2 coliphage virus than the XMX/2L-MIL (Cooper, et al., 2014). Extraction of collected aerosol from the filter and desiccation of collected viral particles during sampling were suggested as the cause of underperformance of the DFU-1000 in the collection of MS2 virus aerosol. The observed relative efficiency of the DFU-1000 in this study, however, further supports the observations in Aim 1, in which filtration was a more efficient air sampling method than liquid impingement and impaction. Both the XMX/2L-MIL and the MB2 subject collected aerosols to high velocity air jets, so that desiccation may be a factor in their observed performance with *C. difficile*. Thus, the results of this study suggest that samplers employing high velocity jets, including jet-to-plate impactors or a virtual impactor, may produce substantial culturable spore losses.

The observed lower relative collection efficiency of the STA impactor for the 60 experimental trials in the LAC further supports the observed efficiencies in Aim 1, with the MCE filter collecting significantly more culturable *C. difficile* spore aerosol than the STA impactor. This comparison in the LAC allowed for the STA impactor to directly sample the air in the chamber as opposed to sampling through the inlet tube required

when using the smaller ATC in the Aim 1 trials. This eliminated potential aerosol losses from the sampling tube, which had been estimated to be less than 2 percent. STA impactor efficiencies (compared to the MCE filter) observed in the 60 trials in the LAC were similar to those seen in the ATC in Aim 1, suggesting that the STA impactor would be expected to underperform the MCE filter in the collection of *C. difficile* regardless of operating configuration.

The observed underperformance of the MB2 relative to the DFU-1000, XMX/2L-MIL, and Biocapture-650 was unexpected. A similar instrument to the MB2, the Microbio MB1, was used on pig farms to air sample for *C. difficile* by Keessen et al. (2011). Keessen recovered *C. difficile* in multiple environmental air samples using the MB1. However, agricultural environments are known to have high concentrations of bioaerosols with typical total bioaerosol concentrations ranging from 10^4 to 10^{10} CFU/m³ (Hinds, 1999, p. 396). A bioaerosol sampling study on pig farms found mean total bioaerosol concentrations of 9.3×10^5 CFU/m³ for air samples collected at 5 different farms (Mackiewicz, 1998). The results of this study, which demonstrated a very low collection efficiency for the MB2, suggest that the *C. difficile* spore aerosol measured by Keessen may have significantly underestimated the actual *C. difficile* present. Another explanation for the differences between the MB2 results in this study and those reported by Keessen may be due to the particle characteristics of the *C. difficile* spore aerosol in different environments. As discussed in Aim 1, this study used relatively pure *C. difficile* spore suspensions in sterile water. Environments with higher aerosol concentrations, such as those in agriculture, more readily form agglomerates, whereby individual small particles collide with each other to form larger particles (Hinds, 1999, p. 260).

Increased particle coagulation can result in a larger particle size for *C. difficile* containing particles, thus increasing the capture efficiency of an impactor air sampler. Additionally, the *C. difficile* spores collected by Keessen could have been adhered to other environmental media such as soil and feces. This spore encasement may provide some protection from sampling stresses allowing spores to be less susceptible to the oxidative and mechanical sampling stresses produced by the jet to plate operation of the MB2 compared to *C. difficile* spore aerosol produced from a pure suspension.

Absolute limits of detection are difficult to estimate for biological air sampling equipment; however, a single organism or CFU of an organism must be collected in a sample to produce a positive result. The data from this study may be used to estimate a minimum required sampling time for a given concentration. For a concentration of 1 CFU/m³ and where the MCE filter was assumed to be 100% efficient and operating at 25 L/min, a 40 minute sample would be required to ensure detection. In contrast, a DFU operating at 920 L/min with a relative efficiency of 38.6% would require a sampling time of only approximately 3 minutes to ensure detection. This brief example, based on the relative efficiencies calculated in this study, demonstrates the detection capability of a high-volume air sampler, even with a relatively low efficiency, in detecting the presence of *C. difficile* at low concentrations. Best (2010) demonstrated that peak air concentrations of *C. difficile* in patient care environments rarely exceeded 1 CFU/m³ (Best, et al., 2010); therefore, for short duration samples, use of a high-volume air sampler, even with an efficiency below 50%, would ensure the greatest probability of detection.

Although the observed relative efficiency of the Biocapture-650 was significantly lower than the DFU-1000, there are specialized settings where the Biocapture-650 may be an effective high-volume air sampler. The Biocapture-650 had a lower observed variance in relative efficiencies than the DFU as measured by the F-test. This lower variance in efficiency may be advantageous in settings where sufficient aerosols of *C. difficile* are present to be detectable by the Biocapture-650 and where comparison studies are necessary such as control evaluation or for comparing different aerosol generating processes in healthcare. The Biocapture-650 and MB2 are small, lightweight, battery-powered air sampling instruments, whereas the XMX/2L-MIL and DFU-1000 are not. Due to power supply, weight, and space limitations on aircraft, air sampling with the DFU-1000 may not be possible during aeromedical patient evacuation and the results of this study suggest that the Biocapture-650 could be used as a substitute sampling method if necessary. The results of this study suggest that the Biocapture-650 would be the preferable lightweight, battery-powered air sampling instrument compared to the MB2.

Limitations

The evaluation of high-volume air sampling equipment was limited by equipment availability. The XMX/2L-MIL, DFU-1000, and Biocapture-650 were available from the United States Air Force and the MB2 was a relatively inexpensive commercially available sampler. Other high-volume air samplers have been used for healthcare acquired infection air sampling, including wet walled cyclones, and may be effective for *C. difficile* air sampling. The results of this study were limited to air sampling *C. difficile*

for culture analysis. Culture analysis was the only method that was economical for this project. Air sampling with subsequent analysis using quantitative PCR may produce different results than those obtained in this study.

Aim 4: Demonstration of high-volume air sampling equipment in the collection of *C. difficile* spore aerosol produced by toilet flushing

The DFU-1000 air sampler was selected for this experiment based on its superior performance in the aerosol collection trials described above. Twelve total trials using a Flushometer toilet seeded with *C. difficile* were conducted as previously described. Three individual toilet water samples were collected for each trial: prior to flush 1, following flush 3, and following flush 6. Median bowl water concentration immediately after spore seeding and prior to flush 1 was 5.30×10^4 CFU/mL (range 4.00×10^4 to 9.00×10^4 CFU/mL). This was similar to the target concentration of 10^4 to 10^5 CFU/mL.

Spores were cultured from all bowl water samples collected. As indicated by the Shapiro-Wilk test, the bowl water concentrations prior to flush 1 ($p = 0.0332$), following flush 3 ($p = 0.0459$), and following flush 6 ($p = 0.0053$) were non-normally distributed. Median bowl water concentration following flush 3 was 12.90 CFU/mL (range 7.11 to 30 CFU/mL). Median bowl water concentration following flush 6 was 3.30 CFU/mL (range 1.71 to 8.40 CFU/mL). The mean decreases in the concentration of spores in the toilet water between the initial water sample collected prior to flush 1 and the water sample collected after flush 3 was 99.98% (range 99.96% to 99.99%). The mean bowl water concentrations with ranges of the 12 samples collected is shown in Figure 29.

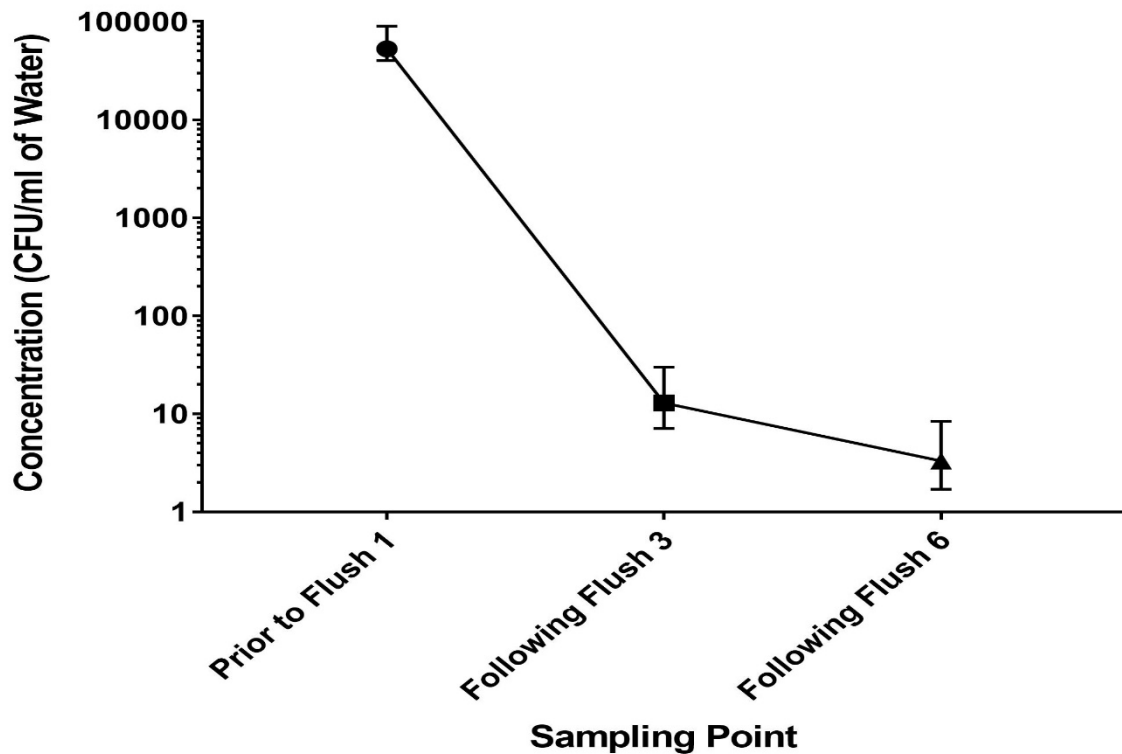


Figure 29: Median Toilet Bowl Water Concentrations by Sampling Point with Range Indicated

Two sets of air samples were collected by a single DFU-1000 and two MCE filters in each two-part trial. The samples in the first part were collected starting immediately following flush 1 and stopping 15 minutes after flush 3. The second set of samples started immediately following flush 4 and stopped 15 minutes after flush 6. The MCE filters were operated at a mean flow rate of 25.8 L/min (95% CI: 25.8 L/min – 25.9 L/min). The DFU-1000 was operated at a mean air flow rate of 920 L/min (95% CI: 889 L/min - 950 L/min). The total air volume sampled in each 3-flush series was therefore approximately 43.7 m³. The volume of the LAC was approximately 5 m³, so this sampling rate ensured complete sampling of the air in the chamber surrounding the

toilet and a virtual census of the droplet nuclei spore aerosol produced by the toilet (assuming negligible surface deposition).

Based on the results from Shapiro-Wilk tests, the number of CFU collected by the two MCE filters ($p = 0.0780$ for the pooled results) and by the DFU ($p = 0.4770$) were normally distributed, as were the total CFU collected by all air samplers ($p = 0.5490$). During the sampling period following flushes 1 through 3, a mean of 2.8 CFU (95% CI: 1.9, 3.8 CFU) were collected by the two MCE filters, whereas the DFU-1000 collected a mean of 30.1 CFU (95% CI: 19.6, 40.6 CFU). A mean total count of 32.9 CFU (95% CI: 22.1, 43.8 CFU) was collected by all samplers during the flush 1 - flush 3 sampling period.

A chart with the *C. difficile* collected by the pooled MCE filters, the DFU-1000, and the total *C. difficile* collected for air samples collected in the flush 1 – flush 3 sampling period is shown in Figure 30. In spite of its lower capture efficiency relative to MCE filters, the DFU-1000 collected approximately 10 times as many culturable spores as the two MCE filters combined.

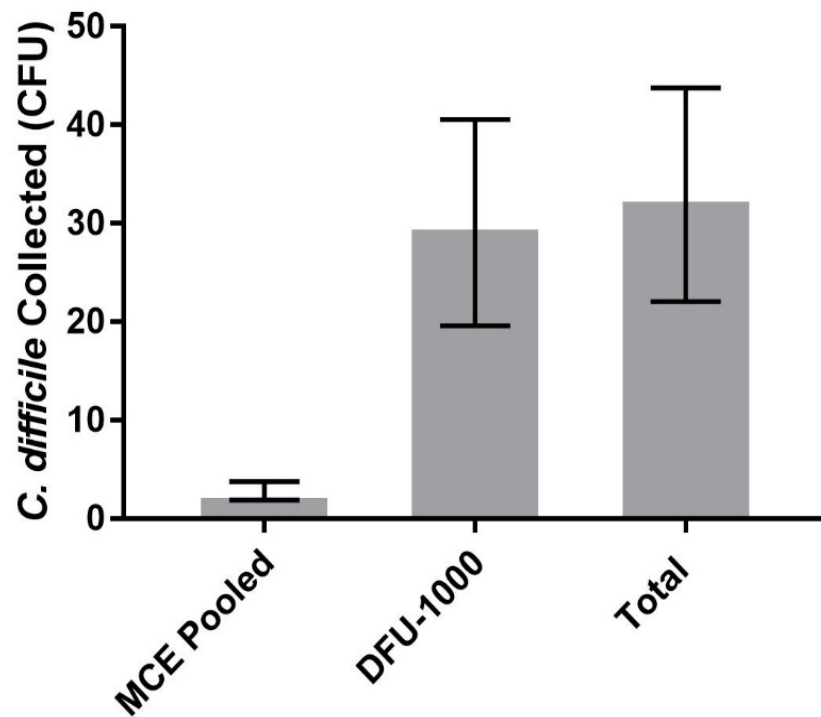


Figure 30: Mean *C. difficile* Aerosol (in CFU) Collected During Flush 1 - 3 Sampling Period. Error Bars Represent the 95% Confidence Interval

The mean number of spores collected in these toilet flush trials can be used with the relative efficiency of the DFU from Aim 3 to determine a total number of spores produced by toilet flushing. The mean relative efficiency of the DFU was 38.60%. Each sample collected by the DFU was corrected using this efficiency to determine a mean corrected count for the DFU of 77.93 CFU (95% CI: 50.82, 105.00 CFU). These corrected counts were added to the pooled number of spores collected by the MCE filters for each of the 12 trials. The mean corrected total number of spores collected in the flush 1 - 3 series (assuming 100% capture efficiency for the MCE filters) for all instruments combined (1 DFU, 2 MCE filters) was 80.77 CFU (95% CI: 53.28, 108.3 CFU).

In the second set of air samples collected in the sampling period starting after flush 4 and continuing 15 minutes after flush 6, the DFU collected a mean of 2.17 CFU (95% CI: 1.36 - 2.97 CFU). Using the relative efficiency of 38.60%, this can be corrected to a mean collection of 5.63 CFU (95% CI: 3.54 - 7.73 CFU). The counts of *C. difficile* spores (in CFU) collected by the DFU-1000 were found to be normally distributed based on the results of the Shapiro-Wilk test ($p = 0.4493$). The mean corrected total counts for all flushes (1 - 6) were 86.47 CFU (95% CI: 58.48 - 114.4 CFU).

The DFU-1000 collected culturable *C. difficile* in all 12 of the samples for the flush 1 - 3 period (100%), and in 11 out of 12 samples for the flush 4 – 6 period, or for approximately 92 percent of the samples collected over the flush 4 - 6 period. In comparison, the MCE filters collected culturable *C. difficile* in 18 out of 24 samples (75%) over the flush 1-3 period and in only 1 of the 24 samples (approximately 4%) during the flush 4 – 6 sampling period. This frequency distribution of detectable versus non-detectable *C. difficile* by instrument can be analyzed using a Fisher's Exact Test. The Fisher's Exact Test for the flush 1 - 3 sampling period was not significantly different for the DFU-1000 and the MCE filters ($p = 0.0793$). However, the Fisher's Exact Test shows that the frequency of detectable *C. difficile* aerosol during the flush 4 - 6 sampling period was significantly higher with the DFU-1000 than with the MCE filters ($p < 0.0001$). Raw data used to determine toilet bowl water concentrations and the number of spores collected from toilet flushing in each trial is included as tables in Appendix E.

Discussion

The observations made in this demonstration support earlier observations made by Best (2012) and Aithinne et al. (2019) suggesting that toilets contaminated with *C. difficile* spores are capable of disseminating significant amounts of airborne spores. Using an STA impactor, Aithinne et al. (2019) estimated that approximately 90 airborne spores were generated during the first three flushes from a flushometer toilet seeded with *C. difficile*. This compares to the estimated 80.77 CFU from air samples collected with the DFU-1000 and MCE filters in this work.

Earlier studies by Aithinne et al. (2019) using the lower-volume STA impactor infrequently detected *C. difficile* spore aerosol after the third toilet flush (Aithinne, et al., 2019). In contrast, this study using a the high-volume DFU-1000 demonstrated consistent production of *C. difficile* spore aerosol after the third flush. This further demonstrated the application of high-volume air sampling equipment in the detection of extremely low concentration *C. difficile* spore aerosol. The census sampling allowed by the DFU-1000 under these experimental conditions was capable of collecting virtually all spores produced, thus allowing consistent detection even at very low levels.

Similar to previous studies, large decreases in toilet water concentration did not produce decreases in spore aerosol production of a similar magnitude. In this study, the starting bowl water concentration decreased by approximately 3 orders of magnitude (99.9%) between the sample collected prior to flush 1 and the sample collected prior to flush 3. However, the number of spores collected from the air decreased by approximately 1 order of magnitude (90%). This was consistent with bowl water clearances observed by Johnson et al. (2013) which demonstrated that toilet

aerosol generation is not proportional to bowl water concentrations and that significant aerosols can be generated even after a significant reduction in bowl water concentration. Airborne droplet nuclei spore bioaerosol was consistently generated throughout at least 6 flushes after toilet contamination. The results of this study support previous findings by Aithinne (2019) and Best (2012) that a toilet will continue to disseminate significant numbers of spores for prolonged periods after initial use and that toilet cleaning must thoroughly eliminate contaminated water and contaminated toilet bowl surfaces to acceptably reduce the risk of spore dissemination from toilets.

Limitations

Demonstration of air sampling equipment in laboratory-based toilet flushing was limited in direct applicability to conditions in the field. For example, the laboratory demonstration used spore suspensions in water versus fecal spore suspensions in water as would be expected in field sampling. It is not known if individual spores are common after shedding in environmental settings or if spores primarily remain adhered to other particles in environmental settings. The aerosol properties and environmental survivability of spores suspended in water may therefore be significantly different for environmental aerosols compared to laboratory generated aerosols. Additionally, a single type of toilet was used in this study. While the toilet used in this study is identical to toilets used at the University of Oklahoma Medical Center, it may not be representative of toilets in healthcare facilities elsewhere.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

This study evaluated eight air sampling methods in the collection of non-toxigenic *C. difficile* spore aerosol as a surrogate for toxigenic *C. difficile*. Low-volume methods included slit-to-agar impaction, two types of liquid impingers, and filtration using MCE filters. High-volume methods included virtual impaction with subsequent filtration of the concentrated aerosol, jet-to-agar plate impaction, filtration using felt filters, and solid surface centrifugal impaction with subsequent transfer into liquid. Observed sampling efficiencies were highest using low-volume MCE filtration. Impingers were particularly ineffective, with very low relative sampling efficiencies, and failed to consistently detect *C. difficile* under the experimental conditions used. Impingers were found to inhibit culturability of *C. difficile* spores by over 88% during 10 minutes of operation. Oxidative and mechanical stresses were identified as potential causes of *C. difficile* spore culturability losses in the impingers. Military and commercial high-volume air samplers capable of sampling up to 950 L/min, including the DFU-1000, XMX/2L-MIL, Biocapture-650, and Microbio MB2, were evaluated for sampling efficiency relative to the low-volume MCE filter method. For high-volume air sampling, the DFU-1000 which employed filtration using 1 µm pore sized polyester felt filters, produced the highest relative efficiency out of the high-volume methods evaluated. Finally, the DFU-1000 and low-volume MCE filter methods were demonstrated in the collection of aerosols produced from flushing a toilet inoculated with *C. difficile* spores. Consistent with

previous studies, the DFU-1000 results showed that flush toilets are capable of producing *C. difficile* aerosol sufficient to result an increased infection risk to susceptible individuals. Also consistent with previous studies, the DFU-1000 conclusively demonstrated that toilets continue to consistently produce *C. difficile* aerosol for multiple flushes following initial contamination. The DFU-1000 was shown to be effective in the detection of low concentration *C. difficile* aerosols produced from repeated toilet flushing with a detection frequency of greater than 90% under experimental conditions representative of a small restroom. This demonstration shows that while low-volume MCE filtration was the most efficient, simplest, and perhaps least expensive *C. difficile* air sampling method evaluated, high-volume air sampling systems with lower efficiency may be more effective in detecting very low concentration aerosols of *C. difficile* (as in healthcare settings).

Major Findings

This study demonstrates the importance of conducting a laboratory evaluation for air sampling equipment and methods for *C. difficile* prior to their application in a field environment. Previous studies did not evaluate air samplers prior to their use and researchers were therefore unaware of confounding effects such as culturable spore losses due to sampling stress. Three of the eight evaluated air sampling methods including the AGI-30 impinger, BioSampler impinger, and MB2 jet to plate impactor failed to detect the presence of *C. difficile* spore aerosol even when presence was confirmed by a comparative side-by-side method. These air sampling methods are

commonly used in the collection of bioaerosols but were found to be ineffective for the detection or quantification of *C. difficile* spore aerosol.

To date, no study has investigated or demonstrated the susceptibility of *C. difficile* or other anaerobic endospore-forming bacteria to sampling stresses. Prior to this study, *C. difficile* spores were known to be resistant to prolonged exposure to air, environmental desiccation, and heat. This study demonstrated that aerosolized *C. difficile* spores are highly susceptible to stresses produced by air sampling and that collected spores are rendered unculturable by multiple air sampling methods including jet-to-plate impaction, virtual impaction, and especially liquid impingement. The results of this study further suggest that aerobic bacterial endospores such as *Bacillus thuringiensis*, as used by Troup (2017), are not effective surrogate organisms for *C. difficile* in aerosol trials. Non-toxigenic *C. difficile* or another species of *Clostridia* should instead be used as a surrogate organism for the evaluation of air sampling methods.

Impingers were shown to be particularly ineffective for air sampling *C. difficile*, with near-total culturability losses during relatively short (10 minute) operating times. These observations sharply contrast earlier studies on culturable spore losses in aerobic endospore-forming bacteria. Sampling efficiencies and sample retention were shown to be significantly lower for *C. difficile* spores than for aerobic spore-forming bacteria and aerobic vegetative bacteria.

Filtration was shown to be an effective sampling method for both low-volume and high-volume air sampling methodologies. Air sampling *C. difficile* spores using low-volume MCE filtration was shown to be more efficient than the low-volume STA impaction methods used by Best et al. (2010) and Aithinne et al. (2019). This suggests

that sampling with the MCE filter would produce more representative air concentration data than an STA sampler. Additionally, air sampling using the high-volume DFU-1000 was shown to effectively sample a large volume of *C. difficile* spore aerosol in a short period of time, providing superior detection capability compared to lower-volume methods for the same sample duration in spite of its lower relative efficiency. This suggests that the DFU-1000 would be a preferred air sampler for the detection of spore aerosol in a healthcare environment.

Toilet flushing was shown to produce sufficient airborne dissemination of *C. difficile* to present a contamination risk to adjacent surfaces and areas. Due to the relatively low infectious doses estimated for susceptible patients of 1 CFU/cm², multiple flushes from a contaminated toilet could quickly contaminate nearby areas with enough spores to cause infection. This would occur during cases where the infected patient was using the toilet multiple times a day.

Implications and Applications

These results demonstrate that air sampling methods and equipment should be evaluated in the laboratory prior to field use. Several commonly-used air sampling methods were found to be highly ineffective in the collection of *C. difficile* spore aerosol and could not be expected to collect reliable data in the field. This may partially explain why numerous air sampling trials prior to Roberts (2008) failed to recover *C. difficile* from the air. This study also showed that these and other methods that subject collected samples to oxidative and mechanical stresses may not be reliable in assessing airborne concentrations of *C. difficile* spores, especially at low

concentrations. The demonstration of MCE filter air sampling as an effective means of *C. difficile* spore air sampling provides a widely available and low-cost tool to occupational and environmental health professionals. Previous air sampling studies in healthcare settings used low-volume wet-walled cyclones and slit-to-agar impaction that required highly specialized and expensive equipment that is not widely available, whereas MCE requires only inexpensive and widely available equipment and supplies that are common in occupational and environmental health practice. The MCE filter used in this study allows the sampling media (a filter) and the pump to be separate thus removing the need for inlet tubes and reducing aerosol losses from the tubes. This allows the sampling media to be placed closer to the aerosol source. For example, a noise reducing enclosure could be used for the pump and connected to the MCE filter which could be placed in close proximity to the patient or patient care process being evaluated. This addresses a key limitation in air sampling in close proximity to a CDI patient. Previous air sampling near a patient conducted by Best (2010) required the transfer of air sample to the collection media via a 2 meter long tube and the placement of the air sampler beneath the foot of the patient's bed. While this study only evaluated MCE filters at one flow-rate, 25.5 L/min, MCE filter sampling can be used with a variety of air sampling pumps and flow rates based on pore size and surface area. This allows more customization for operation requirements at a particular location due to noise, size constraints, or power supply. The use of MCE filters for air sampling also does not require agar use on-site, thus preventing the potential contamination or spoilage of agar during transport, set-up, or sample collection. An example of a pump enclosure with an MCE filter cartridge attached is shown in Figure 31.



Figure 31: MCE Filter with Pump in Noise Dampening Enclosure

Finally, this study demonstrated that filter air sampling at both low- and high-volume air flows is an effective sampling method for *C. difficile* and provides a scalable sampling method that should enable the detection of low-concentration *C. difficile* spore aerosol in healthcare settings. The high-volume DFU-1000 is capable of being fitted with a portable noise dampening enclosure to minimize nuisance noise and to allow for easy movement in patient care areas (i.e. rooms and hallways). A DFU-1000 inside of an example noise dampening enclosure is shown in Figure 32.



Figure 32: DFU-1000 in Noise Reducing Enclosure

Suggestions for Future Research

Expanded Filter Evaluation

This study demonstrated the effectiveness of MCE filters for low-volume *C. difficile* air sampling applications and of polyester felt filters for high-volume air sampling. A wide range of filters have been used to sample for other bioaerosols including PTFE filters and gelatin filters. These additional filter types should be evaluated for *C. difficile* air sampling in future studies.

Impinger Parameter Evaluation

This study investigated *C. difficile* culturability losses in impingers under one set of conditions. Impingers may not exhibit the same losses under all conditions including sampling time, flow rate, and type of liquid media used. Evaluating these parameters

may allow impinger air sampling of *C. difficile* in certain settings. Furthermore, this study was limited to only *C. difficile*, and should be repeated for other *Clostridia* species including *C. perfringens*, a significant cause of food contamination, and *C. sporogenes*, a potential biosafety level 1 surrogate for infectious species of *Clostridia*. A repeat of this study's experiments using toxigenic *C. difficile* aerosolized from a suspension more representative of real-world conditions, such as from stool suspension, would be technically difficult but worthwhile.

Oxidative Stress Trials in a Strict Anaerobic Environment

Additional studies on oxidative stresses as a cause of culturability loss in impingers should be repeated in a strict anaerobic environment. This study observed a significant difference caused by oxidative stress during fritted glass bubbler trials, however losses using medical grade nitrogen were still unexpectedly high. It is possible that oxidative stress occurred during the sample preparation for the nitrogen fritted bubbler trials. Sample preparation and plating occurred in an aerobic environment (in a BSC hood). Since this study suggested that oxidative stress is likely a significant cause of *C. difficile* spore culturability loss, which may be associated with preparation and analysis as well as air sampling, a repeat study should be performed under strict anaerobic environment conditions. These anaerobic conditions in future trials should be maintained for all stages of the trial including sample collection and preparation of culture plates.

Hospital Evaluation using High-Volume Air Sampling for Specific Patient Care

Processes

This study identified air sampling methodologies effective for air sampling under controlled laboratory conditions. However, as discussed, these laboratory conditions may not be fully representative of a patient care environment. Therefore, air sampling using the high-volume and low-volume methodologies identified in this study should be completed in a patient care environment. In addition, these air sampling methods should be used to identify specific patient processes responsible for *C. difficile* aerosol generation including bedding changes, toilet flushing, bathing, waste processing, and laundry servicing.

Conclusions

Numerous studies have demonstrated that the aerosolization of *C. difficile* spores may contribute to the transmission of CDI in healthcare settings. While these previous studies were occasionally able to isolate *C. difficile* from the air in both field and environmental settings, the reliability and effectiveness of air sampling equipment in *C. difficile* air sampling was not well developed or understood. This is the first study to evaluate and demonstrate bioaerosol air sampling methodologies for the collection of *C. difficile*. *C. difficile* endospores, despite their demonstrated ability to survive environmental stresses, were found to be highly susceptible to sampling stresses. Oxidative and/or mechanical stresses from air sampling, especially during liquid impingement, appear likely to influence culturable spore recovery and may provide an explanation for the failure of earlier studies to isolate *C. difficile* from the air in patient

care environments. Effective, economical, and widely available filtration-based methodologies will enable more widespread sampling in healthcare settings. While further study is needed to fully understand the role that aerosol dissemination of *C. difficile* has in the transmission of CDI in healthcare environments, the air sampling filtration methods assessed in this study should enable these future studies and allow for further risk assessment of airborne *C. difficile* spores.

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APPENDIX A

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A comparison of air sampling methods for *Clostridium difficile* endospore aerosol

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Abstract The airborne dissemination of *Clostridium difficile* (*C. difficile*) endospores (spores) in healthcare environments is documented in multiple studies. Once airborne, spores have the potential for transport on air currents to other areas. This study compared the methods in the collection of *C. difficile* spore aerosol. This study determined the relative efficiency of commonly used bioaerosol air sampling methods when characterizing airborne *C. difficile* spore concentrations. Air samplers evaluated in this study were the AirTrace slit-to-agar impactor, AGI-30 impinger, SKC BioSampler impinger, and a 47-mm mixed cellulose ester (MCE) filter cassette. Non-toxigenic *C. difficile* spores were nebulized into an enclosure

contained in a biological safety cabinet. Side-by-side air samples were drawn from the enclosure. The slit-to-agar impactor, successfully used in previous studies to collect airborne spores, served as the reference method. Relative efficiency for the 47-mm MCE filter cartridge was higher than the slit-to-agar impactor (mean 136.6%, 95% CI 124.7–148.5%). Efficiencies of the impingers were similar and were low (mean 4.13%, 95% CI 2.27–5.99%). Impingers failed to maintain culturability of *C. difficile* spores during sampling. This study is the first to compare the efficiencies of commonly used bioaerosol sampling methods to collect airborne *C. difficile* spores. Filter air sampling provided the greatest collection of airborne spores. Slit-to-agar air sampling may underestimate the number of airborne spores present. Impinger air sampling could significantly underestimate the actual number of airborne *C. difficile* spores present or fail to detect airborne spores.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the United States Air Force.

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1 Introduction

1.1 Background

Healthcare-acquired *Clostridium difficile* infection (CDI) is a significant burden to US healthcare facilities

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and their patients, with an estimated 453,000 incident CDI and 29,000 deaths in 2011 (Lessa et al. 2015). Of these nearly half million cases, an estimated 293,300 were healthcare-associated with 107,600 hospital onset, 104,400 nursing home onset, and 81,300 post-outpatient care onset. CDI is the leading cause of gastroenteritis-associated death, causing 14,000 deaths in 2007 alone (Hall et al. 2012), was responsible for an estimated \$4.8 billion in excess healthcare costs for acute care facilities in 2008 (Dubberke and Olsen 2012), and has become one of the most common healthcare-acquired infections in the USA (Magill et al. 2011).

CDI patients experience multiple episodes of watery diarrhea each day, and both *C. difficile* bacterial endospores (spores) and vegetative cells are shed in stool. Spore concentrations in symptomatic CDI patients may be from 10^4 to 10^6 spores per gram of stool (Kim et al. 1981; Naaber et al. 2011), and vegetative cell concentrations may be an order of magnitude more abundant (Jump et al. 2007). Whereas vegetative cells die off rapidly in the environment on dry surfaces (Jump et al. 2007), spores are resistant to environmental degradation and will survive in the environment for long periods of time (Gerding et al. 2008). The spores are infectious, and CDI transmission is believed to be primarily via hand contact with the patient and contaminated environmental surfaces during patient care (McDonald et al. 2018).

Environmental contamination of CDI patient care rooms and adjacent areas with *C. difficile* spores is a recognized contact transmission risk factor, which has been demonstrated in numerous studies conducted since the 1980s (Kim et al. 1981; McDonald et al. 2018). Culturable spores have been collected from surfaces in treatment rooms with symptomatic patients, asymptomatic patients, and even patients with no evidence of *C. difficile* colonization. Recent clinical practice guidelines for CDI prevention and control include: accommodate CDI patients in a private room with a dedicated toilet if possible, or cohort CDI patients if insufficient rooms are available; gown and glove upon entry to a CDI patient room and during patient care; perform hand hygiene before and after CDI patient contact; continue precautions for at least 48 h after diarrhea has resolved; perform terminal room cleaning with a sporicidal agent; and consider daily cleaning with a sporicidal agent during outbreaks or in hyper-endemic settings (McDonald

et al. 2018). Because CDI is only considered a contact transmission risk, negative pressure isolation in an engineered Airborne Infection Isolation Room is not included.

These practice guidelines implicitly assume that spore contact risk is limited to the patient, fomites in the patient room, and room surfaces; however, several studies have shown that *C. difficile* spores are intermittently aerosolized during patient care activities such as bedding changes, toilet flushing, and patient feeding or meal delivery. These spores can remain airborne for extended periods (Aithinne et al. 2018; Best et al. 2010, 2012; Roberts et al. 2008). These “droplet nuclei” spore aerosols could then travel with air currents and may contaminate environmental surfaces remote from the patient room. This airborne transport and deposition may provide additional, and likely unsuspected, opportunities for contact transmission. However, few studies have been successful in isolating *C. difficile* from air samples in healthcare environments (Roberts et al. 2008; Best et al. 2010, 2012).

Commonly used bioaerosol sampling methods include liquid impingement (e.g., SKC BioSampler and AGI-30 impingers), filtration (e.g., mixed cellulose ester and gelatin filters), and direct-to-agar impaction (e.g., MB2 and N6 multi-hole impactors, AirTrace slit-to-agar impactor) (Willeke and Macher 1999). Filtration is the simplest of the air sampling methods. A filter with support pad is placed in a cassette that is connected to a calibrated air sampling pump. The air flow rate may be selected from within a range of allowable values determined by the filter media and filter area. Filters may be extracted into a liquid for subsequent spread plating on agar-filled culture dishes, or placed directly on the agar surface. Gelatin filters will dissolve into the agar, whereas mixed cellulose ester (MCE) filters must absorb agar media in sufficient amounts to support colony development. Liquid impingers direct a high-velocity jet of particle-laden air onto the surface of collection fluid. The jet is forced to change directions abruptly, and the particles’ inertia causes them to strike the liquid and be captured. Whereas the AGI-30 has a single nozzle oriented normally to the liquid surface, the BioSampler has three nozzles oriented at an angle to the liquid surface. The angled nozzles are intended to reduce stress on captured organisms and minimize re-aerosolization of captured organisms from the

collection fluid. After sampling, an aliquot of the collection fluid (diluted as necessary) may be spread onto agar-filled culture dishes; alternatively, the fluid may be filtered and the filter was then placed on agar for incubation. The slit-to-agar impactor directs a high-velocity stream of air through a narrow slit and impinges it onto the surface of an agar-filled culture dish, much like a knife preparing to cut into the radius of a birthday cake. The sheet of air must make an abrupt 90° turn, causing entrained particles to impact on the agar due to their inertia. The agar plate is slowly rotated under the inlet slit, so that particles will deposit over the agar surface area covered by the moving line of impingement. Collection plates from jet-to-agar or slit-to-agar impactors are directly incubated. To date, no studies have been reported that compared the relative performance of these methods when sampling for airborne *C. difficile* spore aerosol, and only one of these (slit-to-agar impaction) has been successful in detecting airborne *C. difficile* in a healthcare environment (Best et al. 2010).

Characterization of airborne *C. difficile* spore concentrations is needed to fully perform risk assessments for the transport of *C. difficile* in healthcare environments. It is therefore of interest to determine which sampling methods are most efficient for determining *C. difficile* spore aerosol presence in healthcare environments so that strategies can be developed for quantifying spore aerosol generation sources and rates during patient care, characterizing spore migration patterns in the care environment, and developing more effective strategies for minimizing transmission risk from patient contact with aerosol-transported spores.

1.2 Goals of this investigation

Our goal was to determine the relative efficiency of inexpensive and commonly used bioaerosol air sampling methods compared to the more expensive (but known to be successful) slit-to-agar method when characterizing airborne *C. difficile* spore concentrations. We compared, under controlled conditions, the relative capture efficiencies of commonly used collection devices based on liquid impingement, slit-to-agar impaction, and filtration. Our hypothesis was that the sampling efficiencies of filter- and impinger-based sampling methods would be comparable to those of the more expensive slit-to-agar method successfully used in previous studies.

2 Methods

2.1 Spore suspensions

All experiments were performed using a non-toxigenic strain of *C. difficile* (ATCC 700057, Microbiologics, St Cloud, MN). Spore suspensions were prepared after the method of Aithinne et al. (2018). Briefly, source organisms were placed in 500 mL of brain–heart broth and incubated anaerobically at 37 °C for 10 days. This extended incubation time ensured depletion of broth nutrients, resulting in sporulation. The resulting spore suspension was heat-shocked at 80 °C for 20 min to remove any remaining vegetative cells. The spore suspension was then mixed and separated into 50-mL aliquots to be centrifuged for 15 min at 5000g. For each aliquot, supernatant broth was decanted, and the pelleted spores were re-suspended in sterilized water and were pelleted again via centrifugation for 15 min at 5000 g. The washing step was repeated three more times, and the final suspensions were refrigerated at 4 °C until needed.

2.2 Air samplers

The bioaerosol samplers evaluated were the AGI-30 liquid impinger (Acc Glass, Vineland, NJ), BioSampler liquid impinger (SKC, Eighty-Four, PA), Air-Trace rotating plate slit-to-agar impactor (Particle Measuring Systems, Boulder, CO), and mixed cellulose ester (MCE) filters in conductive cassettes (MilliporeSigma, Burlington, MA) (Fig. 1). As previously noted, the liquid impingement and filtration-based devices are commonly used for bioaerosol sampling (Willeke 1999). MCE filters were included both because they are commonly used and because Xu et al. (2013) found that airborne bacteria in indoor environments could be cultured from MCE filters placed directly on ChromAgar® media. MCE filters were also shown to be compatible with ChromAgar media for enumerating *C. difficile* spores in water when placed directly onto the media surface (Aithinne et al. 2018). Gelatin filters were excluded due to their tendency to dry out during sampling (Macher and First 1984) and because its potential effect on *C. difficile* culturing on ChromAgar® was unknown. The rotating plate slit-to-agar impactor was included as the reference device because of its success in isolating airborne

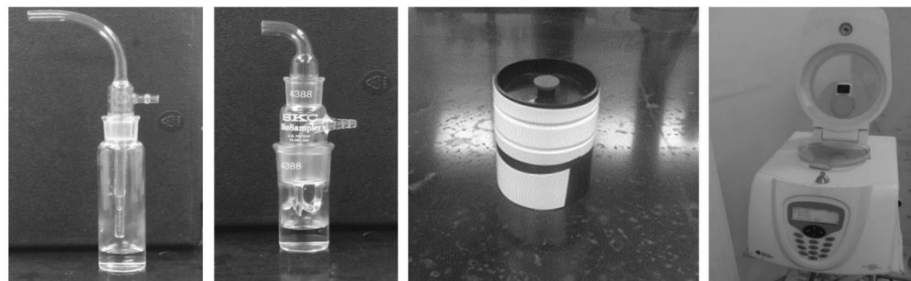


Fig. 1 Air samplers used in the study included (left–right) the AGI-30 impinger, BioSampler impinger, 47-mm MCE filters in conductive cassettes, and AirTrace slit-to-agar rotating plate impactor (not to scale)

C. difficile in recent studies (Best et al. 2010, 2012; Aithinne et al. 2018).

Low suspension concentrations were expected, so in this work we chose to filter the undiluted impinger liquid to maximize our detection limit and minimize potential variability due to inhomogeneous suspensions. Distilled water or phosphate-buffered saline is typically used as the collection fluid. PBS was selected as the impinger fluid due to its demonstrated effectiveness at preserving *C. difficile* spores in long-term storage up to 56 days without the loss of viability (Freeman and Wilcox 2003). The total colonies counted divided by the total volume of air sampled provides an estimate of the average air concentration during the sampling period, expressed as colony-forming units per cubic liter of air sampled (CFU/L). Both impingers were sampled at $12.5 \text{ L/min} \pm 10\%$ and were filled with 20-mL sterile PBS (Sigma-Aldrich, St. Louis, MO) per manufacturer recommendations. Under these conditions, both types of impingers have been shown to have an absolute capture efficiency for particles $1 \mu\text{m}$ aerodynamic diameter of approximately 80–90% for the AGI-30 when sampling $1.1\text{-}\mu\text{m}$ *Bacillus cereus* spores (Grinshpun et al. 1997) or $1.0\text{-}\mu\text{m}$ inert particles (Willeke et al. 1998) and approximately 96% for the BioSampler sampling $1.0\text{-}\mu\text{m}$ inert particles (Willeke et al. 1998). Larger size particles up to $4 \mu\text{m}$ size have similar or higher absolute capture efficiencies for both types of impingers (Kesevan et al. 2010). The physical size of *C. difficile* spores is in the range at approximately $1\text{--}1.5 \mu\text{m}$ length and $0.5\text{--}0.7 \mu\text{m}$ diameter (Snelling et al. 2010). With a dry spore density of approximately 1.42 g/mL (Tisa et al. 1982), this

results in an orientation-averaged aerodynamic diameter in the range of $1.42\text{--}1.87 \mu\text{m}$ (Johnson et al. 1987).

The AirTrace impactor was sampled at a measured air flow rate of $25.5 \text{ L/min} \pm 10\%$. Under these conditions, the impactor has a high sampling efficiency for particles of *C. difficile* spore size (Hinds 1999).

We used 45-mm -diameter, $0.45\text{-}\mu\text{m}$ pore-size MCE filters (MilliporeSigma, Burlington, MA) that allowed sampling at the same rate as the slit-to-agar impactor, i.e., 25.5 L/min . After sampling, filters were placed directly onto agar-filled culture plates for incubation and counting, with average air concentrations again expressed in CFU/L. This technique of direct placement of an MCE filter onto agar was used in the collection of environmental bacteria by Xu et al. (2013). Post-sampling, the conductive filter cartridges were decontaminated with a 10% sodium hypochlorite bleach solution, rinsed, and reused with fresh filters. To verify effective decontamination, a blank filter was incorporated in each day of trials.

Clostridium difficile-selective ChromAgar® chromogenic agar (ChromAgar, Paris, France) was used in the impactor plates and in the plates used to culture air sample filters and filters used to recover spores from impinger liquid. This agar contains antibiotics to inhibit other organisms, as well as a reagent that causes the *C. difficile* colonies to fluoresce under ultraviolet light. The media also wicks efficiently into MCE filters when they are placed on the agar surface, which is necessary for the filter culture technique. All plates were anaerobically incubated at 37°C for 24 h,

and colonies were counted under 365-nm UV illumination per manufacturer specifications.

2.3 Experimental apparatus for side-by-side air sampling

University of Oklahoma Health Sciences Center Institutional Biosafety Committee approval was obtained before conducting any experiments with *C. difficile*. Side-by-side air sampling with liquid impingers, the slit-to-agar impactor, and MCE filters was conducted in an aerosol containment chamber. A 75-L volume transparent plastic aerosol containment chamber was constructed, into which *C. difficile* spore aerosol could be generated using a 3-jet Collision MRE-type air jet nebulizer (Model CN-24, Mesa Labs, Butler, NJ). The chamber was placed within a Type II biological safety cabinet (BSC) to provide secondary containment and HEPA-filtered air for the containment chamber. Aerosol was sampled from the chamber by multiple samplers simultaneously, providing side-by-side comparisons. Due to space limitations, experiments were conducted in blocks with the slit-to-agar impactor and the two impingers as one combination, and the slit-to-agar impactor and two MCE filter cassettes as the other combination. The slit-to-agar impactor served as the “reference sampler” against which the impingers and filters were compared.

The impingers or filter cassettes could be placed inside the chamber, but due to its size the slit-to-agar impactor had to be placed outside the chamber with a 85-cm-long, 12.5-mm ID Tygon® 3606 tube conducting aerosol from the chamber to the instrument’s inlet. This setup is similar to that used in previous air sampling studies by Best et al. (2012) utilizing a slit-to-agar impactor with air sample supplied by a Tygon 3606 inlet tube. The potential for losses to tubing walls and bends was assessed through penetration calculations and found to be negligible (1%) for the tubing size and flow conditions used in this work. The impingers, or the MCE filters, were placed to either side of the impactor tube inlet. A 47-mm MCE filter cassette was placed in line between the impinger exhaust and the air pump to capture any spores sampled but not captured, or captured and re-aerosolized. The impinger samplers were operated at 12.5 L/min $\pm$ 10% air flow rate and the impactor at 25.5 L/min $\pm$ 10%. The filter samplers were operated

at 25.5 L/min to match the flow rate of the slit-to-agar impactor. Total air sampled from the chamber was thus approximately 53 L/min for the impinger trials and 76.5 L/min for the filter trials. The nebulizer air flow rate to the chamber was 10 L/min, so an additional 43 L/min of HEPA-filtered makeup air was drawn into the chamber from the BSC interior via relief holes in the chamber walls for the impinger trials and 66.5 L/min for the filter trials. Aerosol generated into the chamber was mixed by an 80-mm-diameter circular air fan. Uniform aerosol distribution across sampling points was verified in a series of nine trials using three MCE filter cassettes placed at different locations in the chamber, for which one-way ANOVA on these side-by-side measures showed no significant difference in indicated concentration at the three sampling locations (data not shown). Nevertheless, impinger location left or right of center for the two impinger types was alternated between impinger trials.

Four different AGI-30 samplers and three BioSamplers were used over the series of trials. The sampler flow rates were verified to be within 10% of their 12.5 L/min design flow. After each impinger trial, the impinger collection fluid was filtered through a 47-mm-diameter, 0.45- μ m pore-size MCE filter, which was then placed directly onto a 65-mm-diameter ChromAgar® culture plate and anaerobically incubated at 37 °C for 24 h. The agar readily wicked into the filter matrix. Due to the low concentrations of culturable spores in the impingers, this census method provided a lower limit of detection than would be possible by surface plating a 0.1-mL aliquot. Filters from air filtering sample trials were also placed directly on ChromAgar® plates for culturing.

Sampling efficiencies were assessed relative to the slit-to-agar impactor, which has been shown to be capable of culturing *C. difficile* from healthcare environment air samples (Best et al. 2010). Air sampling time in all trials was 10 min, beyond which excessive impinger fluid losses might occur. For each trial, *C. difficile* air concentrations indicated by the two impingers or by the MCE filters were divided by the concentration indicated by the slit-to-agar impactor to provide measures of relative sampling efficiency.

2.4 Evaluation of impinger culturability retention and re-aerosolization

To evaluate the retention of culturable *C. difficile* spores by impinger air samplers, AGI-30s and BioSamplers were seeded with concentrated spore suspensions in PBS. Concentrations ranged from 500 to 1200 CFU/mL and the impingers drew HEPA-filtered, particle-free air through the sampler for 10 min at 12.5 L/min $\pm$ 10%. 0.1-ml aliquots of the impinger fluid, collected before and after the impinger operation, was spread onto a ChromAgar[®]-filled culture plate and anaerobically incubated for 24 h. Culturability retention was determined as a ratio of the number of culturable organisms in the impinger before and after impinger operation, as calculated from the colony counts, plated volume, and fluid volumes before and after operation.

2.5 Assessment of impinger re-aerosolization potential

We conducted an additional experiment to assess re-aerosolization, which is known to occur to some extent in liquid impingers due to fluid agitation (Grinshpun et al. 1997; Kesevan et al. 2010). We placed an MCE filter cassette in line between a BioSampler impinger and its air pump, seeded the impinger fluid with concentrated spore suspension at concentrations ranging from 500 to 1200 CFU/mL, and drew HEPA-filtered, particle-free air through the sampler for 10 min at 12.5 L/min $\pm$ 10% as before. Spore concentrations in the impinger fluid were assessed by surface plating dilutions of 0.1-mL aliquots taken immediately after seeding, and again after the 10-min air flow period. Downstream air filters were cultured on agar plates as before. Liquid volumes in the impingers were measured at initial seeding and again after impinger operation. Total culturable organisms present before and after impinger operation were calculated from the colony counts, plated volumes, and pre- and post-operation fluid volumes.

3 Results

Example of slit-to-agar and filter culture plates is shown in Fig. 2. *C. difficile* colonies were well defined and easily identified under UV illumination. No non-

fluorescent colonies of other types were apparent on the plates, thus verifying the absence of contamination in the test chamber.

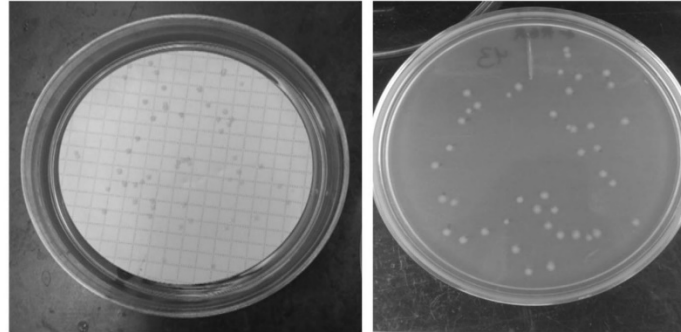
Nine sets of slit-to-agar versus impinger trial data and ten sets of slit-to-agar versus air filter trial data were collected. Slit-to-agar plate counts ranged from 30 to 73 (mean 49, median 49) for the slit-to-agar versus filter trials, while air filter counts ranged from 31 to 107 (mean 67, median 64). Slit-to-agar plate counts ranged from 67 to 150 (mean 108, median 106) for the slit-to-agar versus impinger trials, while impinger counts ranged from 0 to 4 for the AGI-30 and 0 to 5 for the BioSampler. Total counts for the two impingers ranged from 0 to 9 (mean 4, median 4).

Total CFU counts on the in-line exhaust filters for the two impingers ranged from 3 to 14 (median 9), which together sampled at approximately the same rate as the slit-to-agar impactor. This total was less than 10% of the culturable spores drawn into the two impingers, as estimated from corresponding slit-to-agar samples. This suggested, consistent with previous studies (Grinshpun et al. 1997; Willeke et al. 1998), that only minor penetration and re-aerosolization losses may have occurred in the impingers.

All slit-to-agar (reference) colony counts were nonzero, so when calculating relative efficiency a zero value in the numerator indicated an observed relative efficiency less than 1 over the slit-to-agar count. However, these are censored values. Six of the 18 impinger relative efficiency values had zero colonies (3 from each impinger type), so we compared impinger efficiencies first by using the nonparametric Mann–Whitney *U* test. The test showed no significant difference in the impinger relative efficiencies. We then assigned the values of $\frac{1}{2}$ the LOD for each censored data point, i.e., $\frac{1}{2}$ of 1/(slit-to-agar count) for the trial and compared the impinger mean efficiencies using the two-sample *t* test after assessing normality. The *t* test also failed to show a significant difference in the impinger relative efficiencies ($p > 0.05$), and the data were pooled for the two impingers. The pooled collection efficiency was then 4.1% (95% CI 2.27–5.99%) relative to the slit-to-agar sampler.

For the trials in which two MCE filters sampled side-by-side, with each's location randomized across trials, the lack of a position-related difference was verified by conducting a paired-sample *t* test. The pair differences for the ten filter trials were approximately normally distributed as shown by the Shapiro–Wilk

Fig. 2 Representative MCE filter (left) and slit-to-agar impactor plate (right)



test ($p = 0.45$). The paired-sample t test failed to show a significant positional difference in the paired filter measures ($p > 0.05$), indicating good mixing in the chamber, and the filter measures were pooled. In contrast to the extremely low impinger relative efficiencies, pooled sampling efficiency values for MCE filters relative to the slit-to-agar sampler ranged from 89.0 to 189.6%. Mean relative sampling efficiency, compared to the slit-to-agar impactor, was 136.6% (95% CI 124.7–148.5%). Sample means and standard errors of the mean (SEM) are shown in Fig. 3.

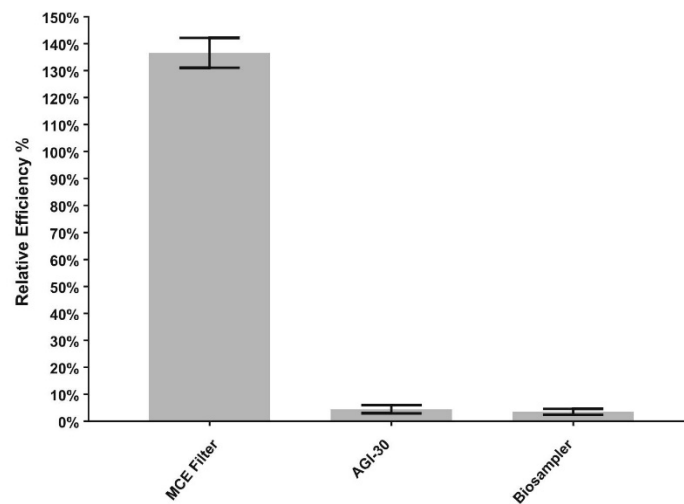
In the 20 trials to assess the potential for re-aerosolization of spores from impinger suspensions,

with 10 trials per impinger type, the culturable spore concentrations were seen to decrease by a mean of 89.96% for the AGI-30 and 89.51% for the BioSampler. While some CFUs were seen on the in-line filters placed downstream of the impingers, these accounted for less than 0.35% of the spores in the original suspension.

4 Discussion

These results demonstrate substantial and surprising differences in the sampling efficiencies of liquid

Fig. 3 Air sampler efficiencies (mean $\pm$ SEM) relative to the slit-to-agar impactor



impinger, slit-to-agar impactor, and MCE filter sampling methods when sampling airborne *C. difficile* spores under these conditions. The impingers were particularly ineffective. This suggested that *C. difficile* spores drawn into the impingers were either not captured in the liquid, were captured but became re-aerosolized by fluid turbulence, or were captured but rendered non-culturable by the collection sampling conditions. However, in-line filters on impinger samples demonstrated only minor penetration through these devices. *C. difficile* spores are approximately 1.4–1.9 μm in orientation-averaged aerodynamic size, and particles of this size are collected with high efficiency in all four collectors used. Our experiments demonstrated that spores were indeed captured in the impingers and were not re-aerosolized in significant numbers; rather, spores were rendered non-culturable by the impinger operation.

Sampling stresses that might affect culturability include osmotic shock, oxygen toxicity, desiccation, and mechanical stresses. All of these are known to affect vegetative organisms during air sampling (Kesevan et al. 2010) but are not generally associated with effects on bacterial endospores. Indeed, spores of most types, including *C. difficile*, have been shown to survive in the environment for long periods while subjected to desiccation, light exposure, atmospheric oxygen, and temperature extremes (Kim et al. 1981; Edwards et al. 2016). The extremely poor recovery of *C. difficile* spores from impinger fluid in these trials is in sharp contrast to the recovery of *Bacillus atrophaeus* spores, used as a surrogate for anthrax spores, when sampled using liquid impingers (Kesevan et al. 2010). The influences of each of these stressors on *C. difficile* culturability during air sampling by impaction, impingement, or filtration are an area deserving of additional research.

5 Study limitations

The scope of this study was necessarily limited to a selection of the air sampling methods commonly used for the bioaerosol assessment. We examined the *C. difficile* spore bioaerosol sampling efficiency of the two most commonly used liquid impinger bioaerosol samplers, one type of impactor and one type of filter medium (MCE). We used one impinger fluid (sterile PBS), one sampling duration, and one set of culturing

conditions. We also used a non-toxigenic strain of *C. difficile* as a surrogate for toxigenic *C. difficile* strains and prepared and aerosolized the spores in essentially pure suspensions. Whether naturally occurring toxigenic organisms aerosolized from other media (e.g., when flushing a contaminated toilet) and sampled and cultured by other methods would have similar susceptibility to sampling stresses is unknown. The particular mechanisms affecting the culturability of captured *C. difficile* spores, and whether other endospore types might be affected, are also unknown. These and associated questions will require further study.

6 Conclusions

This study was the first to compare air sampling devices and methodologies in the sampling of a laboratory-generated aerosol of *C. difficile* spores. Surprisingly, impinger bioaerosol sampling as conducted in this work was not effective for characterizing airborne *C. difficile* spore concentrations. The two impingers had equivalent performance, which was extremely poor compared to both the slit-to-agar impactor and MCE filters. Therefore, impinger bioaerosol sampling could routinely fail to detect the presence of aerosolized *C. difficile* as demonstrated by the absence of *C. difficile* in 6 of 18 impinger trials despite the presence of numerous colonies on the slit-to-agar plate. Of the three methods, filter-based sampling using MCE filters with direct culture yielded the highest estimates of airborne spore concentrations and indicated that air sampling of *C. difficile* by slit-to-agar impaction, the most practiced method, may actually underestimate airborne concentration and thus fail to fully assess the airborne transport risk in healthcare settings. Filtration-type air samplers are far more widely available to health and safety professionals and industrial hygienists. Air sampling via filtration is also less costly and much simpler than slit-to-agar sampling in terms of equipment requirements, consumables use, and sample storage. The mechanism by which *C. difficile* spores were damaged by impinger operation, and the rate at which damage occurs for captured spores are presently unknown. However, similar damage has not been observed in *B. atrophaeus* endospores. Additional research in each of these areas is needed. This study also demonstrated the

effectiveness of *C. difficile*-selective chromogenic agar in the analysis of air samples using multiple air sampling media.

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Appendix B

Air Sampler Comparison Data (Aim 1)

Location refers to sampler location with the aerosol test chamber (i.e. center, left, right)

Collison Nebulizer Survivability Data

Trial #	Concentration at Time = 0 min (CFU/ml)	Concentration at Time = 10 min (CFU/ml)	Concentration Difference $C_{10min} - C_{0min}$ (CFU/ml)
1	770.0	1000.0	230.0
2	1030.0	910.0	-120.0
3	940.0	1090.0	150.0
4	680.0	1030.0	350.0
5	960.0	1130.0	170.0
6	730.0	1190.0	460.0
7	900.0	970.0	70.0
8	1060.0	920.0	-140.0
9	760.0	970.0	210.0

Collison Nebulizer *C. difficile* Survivability Analysis (Paired t-test From Prism v.7)

Column B vs. Column A	Time 10 vs. Time 0
Paired t test	
P value	0.0476
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.338 df=8
Number of pairs	9
How big is the difference?	
Mean of differences	153.3
SD of differences	196.8
SEM of differences	65.6
95% confidence interval	2.07 to 304.6
R squared (partial eta squared)	0.4058

Low Volume Comparison Data: Slit-to-Agar Impactor and MCE Filter

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
1	STA	Center	25.5	10	255	73	0.29	Reference
1	MCE Filter	Left	25.4	10	254	97	0.38	133.40%
1	MCE Filter	Right	25.5	10	255	107	0.42	146.58%
2	STA	Left	25.5	10	255	66	0.26	Reference
2	MCE Filter	Center	25.4	10	254	95	0.37	144.51%
2	MCE Filter	Right	25.5	10	255	82	0.32	124.24%
3	STA	Center	25.5	10	255	35	0.14	Reference
3	MCE Filter	Left	25.4	10	254	31	0.12	88.92%
3	MCE Filter	Right	25.5	10	255	57	0.22	162.86%
4	STA	Right	25.4	10	254	54	0.21	Reference
4	MCE Filter	Center	25.6	10	256	80	0.31	146.99%
4	MCE Filter	Left	25.5	10	255	68	0.27	125.43%
5	STA	Right	25.4	10	254	43	0.17	Reference
5	MCE Filter	Center	25.6	10	256	53	0.21	122.29%
5	MCE Filter	Left	25.5	10	255	60	0.24	138.99%
6	STA	Left	25.4	10	254	67	0.26	Reference
6	MCE Filter	Center	25.5	10	255	69	0.27	102.58%
6	MCE Filter	Right	25.6	10	256	77	0.3	114.03%
7	STA	Center	25.5	10	255	54	0.21	Reference
7	MCE Filter	Left	25.5	10	255	69	0.27	127.78%
7	MCE Filter	Right	25.4	10	254	55	0.22	102.25%
8	STA	Left	25.5	10	255	36	0.14	Reference
8	MCE Filter	Center	25.5	10	255	55	0.22	152.78%
8	MCE Filter	Right	25.4	10	254	68	0.27	189.63%
9	STA	Right	25.5	10	255	33	0.13	Reference
9	MCE Filter	Left	25.5	10	255	51	0.2	154.55%
9	MCE Filter	Center	25.4	10	254	48	0.19	146.03%

Low Volume Comparison Data: Slit-to-Agar Impactor, BioSampler™, and AGI-30

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
1	STA	Center	25.9	10	259	110	0.425	Reference
1	AGI-30	Left	11.7	10	117	2	0.017	4.02%
1	BioSampler	Right	12.7	10	127	0.5*	0.004	0.93%
2	STA	Right	25.9	10	259	101	0.39	Reference
2	AGI-30	Center	11.7	10	117	4	0.034	8.77%
2	BioSampler	Left	12.5	10	125	3	0.024	6.15%
3	STA	Left	25.9	10	259	67	0.259	Reference
3	AGI-30	Right	11.7	10	117	4	0.034	13.22%
3	BioSampler	Center	11.8	10	118	2	0.017	6.55%
4	STA	Center	25.5	10	255	74	0.29	Reference
4	AGI-30	Right	11.7	10	117	0.5*	0.004	1.47%
4	BioSampler	Left	12.5	10	125	0.5*	0.004	1.38%
5	STA	Left	25.5	10	255	94	0.369	Reference
5	AGI-30	Center	11.7	10	117	0.5*	0.004	1.16%
5	BioSampler	Right	11.8	10	118	1	0.008	2.30%
6	STA	Right	26.3	10	263	147	0.559	Reference
6	AGI-30	Center	11.8	10	118	4	0.034	6.06%
6	BioSampler	Left	12.5	10	125	0.5*	0.004	1.09%
7	STA	Left	26.3	10	263	150	0.57	Reference
7	AGI-30	Right	11.8	10	118	4	0.034	5.94%
7	BioSampler	Center	11.8	10	118	5	0.042	7.43%
8	STA	Center	25.5	10	255	106	0.416	Reference
8	AGI-30	Left	12	10	120	0.5*	0.004	1.00%
8	BioSampler	Right	12.8	10	128	4	0.031	7.52%
9	STA	Right	25.5	10	255	126	0.494	Reference
9	AGI-30	Left	12	10	120	1	0.008	1.69%
9	BioSampler	Center	13	10	130	1	0.008	1.56%

*Censored Value: 1/2 Limit of Detection (0.5 CFU) Assigned

Low Volume Comparison Trial Analysis: Slit-to-Agar, MCE Filter, BioSampler™, and AGI-30 (Descriptive Statistics from Prism v.7)

	Biosampler™	AGI-30	MCE Filter
Number of values	9	9	18
Minimum	0.93	1	88.92
Median	2.3	4.02	136.2
Maximum	7.52	13.22	189.6
Mean	3.879	4.814	134.7
Std. Deviation	2.931	4.162	24.34
Std. Error of Mean	0.9772	1.387	5.737
Lower 95% CI of mean	1.626	1.615	122.6
Upper 95% CI of mean	6.132	8.014	146.8
95% CI of median			
Lower confidence limit	1.09	1.16	122.3
Upper confidence limit	7.43	8.77	147
Shapiro-Wilk normality test			
W	0.7957	0.8691	0.9784
P value	0.0182	0.1201	0.9322
Passed normality test (alpha=0.05)?	No	Yes	Yes
P value summary	*	ns	ns
One sample t test			
Theoretical mean	100	100	100
Actual mean	3.879	4.814	134.7
Discrepancy	-96.12	-95.19	34.66
95% CI of discrepancy	-98.37 to -93.87	-98.39 to -91.99	22.55 to 46.76
t, df	t=98.37 df=8	t=68.6 df=8	t=6.041 df=17
P value (two tailed)	<0.0001	<0.0001	<0.0001
Significant (alpha=0.05)?	Yes	Yes	Yes
Wilcoxon Signed Rank Test			
Theoretical median	100	100	100
Actual median	2.3	4.02	136.2
Discrepancy	-97.7	-95.98	36.2
Sum of signed ranks (W)	-45	-45	165
Sum of positive ranks	0	0	168
Sum of negative ranks	-45	-45	-3
P value (two tailed)	0.0039	0.0039	<0.0001
Significant (alpha=0.05)?	Yes	Yes	Yes

Low Volume Comparison Trial Analysis: (Paired t-test BioSampler™, and AGI-30 From Prism v.7)

Column B vs. Column A	AGI-30 vs. Biosampler™
Paired t test	
P value	0.4958
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.7135 df=8
Number of pairs	9
How big is the difference?	
Mean of differences	0.9356
SD of differences	3.934
SEM of differences	1.311
95% confidence interval	-2.088 to 3.959
R squared (partial eta squared)	0.05983

APPENDIX C

Aim 2: Impinger Culturability Loss Data and Analysis

Impinger Culturable Fraction Loss Data: AGI-30 and BioSampler™

Instrument	Concentration initial (CFU/mL)	Volume Initial (mL)	N Initial (CFU)	Concentration Final (CFU/mL)	Volume Final (mL)	N Final (CFU)	Culturable Fraction Remaining (%)	Culturable Fraction Lost (%)
AGI-30	1155	20	23100	82.5	17.97	1483	6.42	93.58
AGI-30	1155	20	23100	105.0	18.05	1895	8.20	91.80
AGI-30	1155	20	23100	117.5	17.98	2113	9.15	90.85
AGI-30	577.5	20	11550	62.5	18.13	1133	9.81	90.19
AGI-30	577.5	20	11550	107.5	18.15	1951	16.89	83.11
AGI-30	577.5	20	11550	85.0	18.13	1451	13.34	86.66
AGI-30	577.5	20	11550	45.0	18.02	811	7.02	92.98
AGI-30	820	20	16400	65.0	18.00	1170	7.13	92.87
AGI-30	820	20	16400	90.0	18.10	1629	9.93	90.07
AGI-30	820	20	16400	120.0	17.89	2147	13.09	86.91
BioSampler	820	20	16400	107.5	18.25	1962	11.96	88.04
BioSampler	827.5	20	16550	62.5	18.34	1146	6.93	93.07
BioSampler	827.5	20	16550	85.0	18.24	1550	9.37	90.63
BioSampler	827.5	20	16550	77.5	18.30	1418	8.57	91.43
BioSampler	827.5	20	16550	95.0	18.21	1730	10.45	89.55
BioSampler	827.5	20	16550	87.5	18.43	1613	9.74	90.26
BioSampler	640	20	12800	92.5	18.34	1696	13.25	86.75
BioSampler	640	20	12800	85.0	18.19	1546	12.08	87.92
BioSampler	640	20	12800	100.0	18.30	1830	14.30	85.70
BioSampler	640	20	12800	112.5	18.25	2053	16.04	83.96

Culturable Fraction Loss Analysis for AGI-30 and BioSampler™ (Descriptive Statistics from Prism v7)

	BioSampler™ Culturable Fraction Lost (%)	AGI-30 Culturable Fraction Lost (%)
Number of values	10	10
Minimum	83.96	83.11
25% Percentile	86.49	86.85
Median	88.8	90.52
75% Percentile	90.83	92.9
Maximum	93.07	93.58
Mean	88.73	89.9
Std. Deviation	2.784	3.367
Std. Error of Mean	0.8802	1.065
Lower 95% CI of mean	86.74	87.49
Upper 95% CI of mean	90.72	92.31
Shapiro-Wilk normality test		
W	0.9858	0.9038
P value	0.9885	0.2411
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns

**Impinger Culturable Fraction Loss Analysis for AGI-30 and BioSampler™
(Unpaired, Two-Sample t-test From Prism v7)**

Column B vs. Column A	AGI-30 Culturable Fraction Lost (%) vs. BioSampler™ Culturable Fraction Lost (%)
Unpaired t test	
P value	0.4078
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.8477 df=18
How big is the difference?	
Mean ± SEM of column A	88.73 ± 0.8802, n=10
Mean ± SEM of column B	89.9 ± 1.065, n=10
Difference between means	1.171 ± 1.381
95% confidence interval	-1.731 to 4.073
R squared (eta squared)	0.03839
F test to compare variances	
F, DFn, Dfd	1.463, 9, 9
P value	0.5800
P value summary	ns
Significantly different (P < 0.05)?	No

Culturable Fraction Loss Data: Fritted Bubbler, Air and Medical Grade Nitrogen

Intake Gas	Concentration initial (CFU/mL)	Volume Initial (mL)	N Initial (CFU)	Concentration Final (CFU/mL)	Volume Final (mL)	N Final (CFU)	Culturable Fraction Remaining	Culturable Fraction Lost
Air	862.5	10	8625	80.0	9.04	723	8.38	91.62
Air	862.5	10	8625	60.0	9.06	544	6.30	93.70
Air	535.0	10	5350	135.0	9.18	1239	23.16	76.84
Air	535.0	10	5350	92.5	9.20	851	15.91	84.09
Air	535.0	10	5350	140.0	9.19	1287	24.05	75.95
Air	535.0	10	5350	42.5	9.07	385	7.21	92.79
Air	1045.0	10	10450	337.5	9.26	3125	29.91	70.09
Air	1045.0	10	10450	75.0	9.23	692	6.62	93.38
Air	1045.0	10	10450	170.0	9.24	1571	15.03	84.97
Air	1045.0	10	10450	307.5	9.19	2826	27.04	72.96
Nitrogen	685.0	10	6850	337.5	8.94	3017	44.05	55.95
Nitrogen	685.0	10	6850	335.0	8.86	2968	43.33	56.67
Nitrogen	727.5	10	7275	472.5	9.01	4257	58.52	41.48
Nitrogen	727.5	10	7275	447.5	8.85	3960	54.44	45.56
Nitrogen	727.5	10	7275	225.0	8.89	2000	27.47	72.53
Nitrogen	727.5	10	7275	145.0	8.95	1298	17.84	82.16
Nitrogen	1142.5	10	11425	200.0	8.91	1782	15.60	84.40
Nitrogen	1142.5	10	11425	427.5	8.88	3796	33.23	66.77
Nitrogen	1142.5	10	11425	227.5	8.88	2020	17.60	82.40
Nitrogen	1142.5	10	11425	377.5	8.90	3360	29.41	70.59

**Culturable Fraction Loss Analysis for Fritted Glass Bubbler with Air and Nitrogen
(Descriptive Statistics from Prism v7)**

	Air Culturable Fraction Lost (%)	Nitrogen Culturable Fraction Lost (%)
Number of values	10	10
Minimum	70.09	41.48
25% Percentile	75.2	53.35
Median	84.53	68.68
75% Percentile	92.94	82.2
Maximum	93.7	84.4
Mean	83.64	65.84
Std. Deviation	9.119	15.41
Std. Error of Mean	2.884	4.873
Lower 95% CI of mean	77.12	54.82
Upper 95% CI of mean	90.16	76.86
Shapiro-Wilk normality test		
W	0.884	0.9239
P value	0.1450	0.3906
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns

**Culturable Fraction Loss Analysis for Fritted Glass Bubbler with Air and Nitrogen
(Unpaired, Two-Sample t-test From Prism v7)**

Column D vs. Column B	Nitrogen Culturable Fraction Lost (%) vs. Air Culturable Fraction Lost (%)
Unpaired t test	
P value	0.0056
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.143 df=18
How big is the difference?	
Mean $\pm$ SEM of column B	83.64 $\pm$ 2.884, n=10
Mean $\pm$ SEM of column D	65.84 $\pm$ 4.873, n=10
Difference between means	-17.8 $\pm$ 5.662
95% confidence interval	-29.69 to -5.902
R squared (eta squared)	0.3544
F test to compare variances	
F, DFn, Dfd	2.855, 9, 9
P value	0.1340
P value summary	ns
Significantly different (P < 0.05)?	No

Re-aerosolization Loss Data: AGI-30 and BioSampler™

Instrument	Concentration initial (CFU/mL)	Volume Initial (mL)	N Initial (CFU)	<i>C. difficile</i> on Filter (CFU)	Re-aerosolized Fraction (%)
AGI-30	1155	20	23100	18.0	0.08
AGI-30	1155	20	23100	30.0	0.13
AGI-30	1155	20	23100	19.0	0.08
AGI-30	577.5	20	11550	41.0	0.35
AGI-30	577.5	20	11550	20.0	0.17
AGI-30	577.5	20	11550	23.0	0.20
AGI-30	577.5	20	11550	30.0	0.26
AGI-30	820	20	16400	22.0	0.13
AGI-30	820	20	16400	20.0	0.12
AGI-30	820	20	16400	35.0	0.21
BioSampler	820	20	16400	1.0	0.01
BioSampler	827.5	20	16550	0.0	0.00
BioSampler	827.5	20	16550	0.0	0.00
BioSampler	827.5	20	16550	0.0	0.00
BioSampler	827.5	20	16550	4.0	0.02
BioSampler	827.5	20	16550	2.0	0.01
BioSampler	640	20	12800	0.0	0.00
BioSampler	640	20	12800	0.0	0.00
BioSampler	640	20	12800	3.0	0.02
BioSampler	640	20	12800	1.0	0.01

Re-aerosolization Loss Data: Fritted Bubbler, Air and Medical Grade Nitrogen

Intake Gas	Concentration initial (CFU/mL)	Volume Initial (mL)	N Initial (CFU)	<i>C. difficile</i> on Filter (CFU)	Re-aerosolized Fraction (%)
Air	862.5	10	8625	156.0	1.81
Air	862.5	10	8625	203.0	2.35
Air	535.0	10	5350	127.0	2.37
Air	535.0	10	5350	217.0	4.06
Air	535.0	10	5350	273.0	5.10
Air	535.0	10	5350	187.0	3.50
Air	1045.0	10	10450	237.0	2.27
Air	1045.0	10	10450	143.0	1.37
Air	1045.0	10	10450	175.0	1.67
Air	1045.0	10	10450	174.0	1.67
Nitrogen	685.0	10	6850	148.0	2.16
Nitrogen	685.0	10	6850	140.0	2.04
Nitrogen	727.5	10	7275	190.0	2.61
Nitrogen	727.5	10	7275	202.0	2.78
Nitrogen	727.5	10	7275	120.0	1.65
Nitrogen	727.5	10	7275	116.0	1.59
Nitrogen	1142.5	10	11425	124.0	1.09
Nitrogen	1142.5	10	11425	148.0	1.30
Nitrogen	1142.5	10	11425	112.0	0.98
Nitrogen	1142.5	10	11425	116.0	1.02

Re-aerosolization Loss Analysis for Fritted Bubbler, Air and Medical Grade Nitrogen (Descriptive Statistics from Prism v7)

	Fritted Bubbler Air Re-aerosolized Fraction (%)	Fritted Bubbler Nitrogen Re-aerosolized Fraction (%)
Number of values	10	10
Minimum	1.37	0.98
25% Percentile	1.67	1.073
Median	2.31	1.62
75% Percentile	3.64	2.273
Maximum	5.1	2.78
Mean	2.617	1.722
Std. Deviation	1.215	0.6536
Std. Error of Mean	0.3841	0.2067
Lower 95% CI of mean	1.748	1.254
Upper 95% CI of mean	3.486	2.19
Shapiro-Wilk normality test		
W	0.8656	0.9178
P value	0.0888	0.3388
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns

APPENDIX D

Aim 3: High Volume Air Sampling Comparison Data and Analysis

High Volume Comparison Trial Data: MB2

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
1	STA	Left	25.9	15	388.5	29	0.075	69.37%
1	MCE Filter	Right	25.4	15	381	41	0.108	Reference
1	MB2	Center	100.0	15	1500	0	0.000	0.00%
2	STA	Left	25.9	15	388.5	24	0.062	75.92%
2	MCE Filter	Right	25.4	15	381	31	0.081	Reference
2	MB2	Center	100.0	15	1500	0	0.000	0.00%
3	STA	Left	25.9	15	388.5	36	0.093	103.84%
3	MCE Filter	Right	25.4	15	381	34	0.089	Reference
3	MB2	Center	100.0	15	1500	0	0.000	0.00%
4	STA	Right	25.7	15	385.5	27	0.070	81.18%
4	MCE Filter	Center	25.5	15	382.5	33	0.086	Reference
4	MB2	Left	100.0	15	1500	3.04*	0.002	2.35%
5	STA	Right	25.7	15	385.5	37	0.096	94.13%
5	MCE Filter	Center	25.5	15	382.5	39	0.102	Reference
5	MB2	Left	100.0	15	1500	4.06*	0.003	2.66%
6	STA	Right	25.7	15	385.5	39	0.10	75.88%
6	MCE Filter	Center	25.5	15	382.5	51	0.13	Reference
6	MB2	Left	100.0	15	1500	2.03*	0.00	1.01%
7	STA	Center	25.9	15	388.5	34	0.09	77.24%
7	MCE Filter	Left	25.3	15	379.5	43	0.11	Reference
7	MB2	Right	100.0	15	1500	2.03*	0.00	1.19%

*Adjusted Value for Positive Hole Correction

High Volume Comparison Trial Data: MB2 (Continued)

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
8	STA	Center	25.9	15	388.5	36	0.093	83.73%
8	MCE Filter	Left	25.3	15	379.5	42	0.111	Reference
8	MB2	Right	100.0	15	1500	1.01*	0.001	0.61%
9	STA	Center	25.9	15	388.5	27	0.069	64.33%
9	MCE Filter	Left	25.3	15	379.5	41	0.108	Reference
9	MB2	Right	100.0	15	1500	0	0.000	0.00%
10	STA	Right	26.0	15	390	16	0.041	78.46%
10	MCE Filter	Center	25.5	15	382.5	20	0.052	Reference
10	MB2	Left	100.0	15	1500	8.16*	0.005	10.40%
11	STA	Center	26.0	15	390	17	0.044	92.63%
11	MCE Filter	Left	25.5	15	382.5	18	0.047	Reference
11	MB2	Right	100.0	15	1500	9.19*	0.006	13.02%
12	STA	Center	26.0	15	390	26	0.067	73.14%
12	MCE Filter	Left	25.6	15	384	35	0.091	Reference
12	MB2	Right	100.0	15	1500	3.04*	0.002	2.23%
13	STA	Right	26.0	15	390	24	0.062	84.40%
13	MCE Filter	Center	25.6	15	384	28	0.073	Reference
13	MB2	Left	100.0	15	1500	5.08*	0.003	4.65%
14	STA	Left	26.0	15	390	22	0.056	60.17%
14	MCE Filter	Right	25.6	15	384	36	0.094	Reference
14	MB2	Center	100.0	15	1500	2.03*	0.001	1.44%
15	STA	Left	26.0	15	390	34	0.087	88.10%
15	MCE Filter	Right	25.6	15	384	38	0.099	Reference
15	MB2	Center	100.0	15	1500	9.19*	0.006	6.19%

*Adjusted Value for Positive Hole Correction

High Volume Comparison Trial Data: DFU-1000

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
1	STA	Left	25.8	15	387.0	11	0.028	67.42%
1	MCE Filter	Right	25.3	15	379.5	16	0.042	Reference
1	DFU	Center	920.0	15	13800.0	134	0.010	23.03%
2	STA	Left	25.8	15	387.0	9	0.023	35.30%
2	MCE Filter	Right	25.3	15	379.5	25	0.066	Reference
2	DFU	Center	920.0	15	13800.0	132	0.010	14.52%
3	STA	Left	25.8	15	387.0	14	0.036	72.26%
3	MCE Filter	Right	25.3	15	379.5	19	0.050	Reference
3	DFU	Center	920.0	15	13800.0	179	0.013	25.91%
4	STA	Right	25.9	15	388.5	7	0.018	45.77%
4	MCE Filter	Center	25.4	15	381.0	15	0.039	Reference
4	DFU	Left	920.0	15	13800.0	141	0.010	25.95%
5	STA	Right	25.9	15	388.5	9	0.023	80.24%
5	MCE Filter	Center	25.4	15	381.0	11	0.029	Reference
5	DFU	Left	920.0	15	13800.0	207	0.015	51.95%
6	STA	Right	25.9	15	388.5	10	0.026	65.38%
6	MCE Filter	Center	25.4	15	381.0	15	0.039	Reference
6	DFU	Left	920.0	15	13800.0	208	0.015	38.28%
7	STA	Center	25.9	15	388.5	8	0.021	39.69%
7	MCE Filter	Left	25.7	15	385.5	20	0.052	Reference
7	DFU	Right	920.0	15	13800.0	196	0.014	27.38%

High Volume Comparison Trial Data: DFU-1000 (Continued)

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
8	STA	Center	25.9	15	388.5	18	0.046	89.31%
8	MCE Filter	Left	25.7	15	385.5	20	0.052	Reference
8	DFU	Right	920.0	15	13800.0	253	0.018	35.34%
9	STA	Center	25.9	15	388.5	16	0.041	122.13%
9	MCE Filter	Left	25.7	15	385.5	13	0.034	Reference
9	DFU	Right	920.0	15	13800.0	243	0.018	52.22%
10	STA	Center	25.9	15	388.5	9	0.023	88.61%
10	MCE Filter	Left	25.5	15	382.5	10	0.026	Reference
10	DFU	Right	920.0	15	13800.0	192	0.014	53.22%
11	STA	Center	25.9	15	388.5	12	0.031	73.84%
11	MCE Filter	Left	25.5	15	382.5	16	0.042	Reference
11	DFU	Right	920.0	15	13800.0	205	0.015	35.51%
12	STA	Left	25.9	15	385.5	9	0.023	59.53%
12	MCE Filter	Right	25.6	15	382.5	15	0.039	Reference
12	DFU	Center	920.0	15	13800.0	252	0.018	46.57%
13	STA	Left	25.7	15	385.5	8	0.021	49.61%
13	MCE Filter	Right	25.5	15	382.5	16	0.042	Reference
13	DFU	Center	920.0	15	13800.0	239	0.017	41.40%
14	STA	Left	25.7	15	385.5	10	0.026	82.68%
14	MCE Filter	Right	25.5	15	382.5	12	0.031	Reference
14	DFU	Center	920.0	15	13800.0	273	0.020	63.06%
15	STA	Right	25.9	15	388.5	9	0.023	73.55%
15	MCE Filter	Center	25.4	15	381.0	12	0.031	Reference
15	DFU	Left	920.0	15	13800.0	194	0.014	44.63%

High Volume Comparison Trial Data: XMX/2L-MIL

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
1	STA	Center	25.9	15	388.5	17	0.028	48.84%
1	MCE Filter	Right	25.3	15	379.5	14	0.042	Reference
1	XMX/2L-MIL	Left	697.0	15	10455.0	33	0.010	8.56%
2	STA	Center	26.1	15	391.5	20	0.023	78.47%
2	MCE Filter	Right	25.6	15	384.0	25	0.066	Reference
2	XMX/2L-MIL	Left	697.0	15	10455.0	68	0.010	9.99%
3	STA	Center	26.1	15	391.5	7	0.036	76.29%
3	MCE Filter	Right	25.6	15	384.0	9	0.050	Reference
3	XMX/2L-MIL	Left	697.0	15	10455.0	32	0.013	13.06%
4	STA	Center	26.1	15	391.5	9	0.018	88.28%
4	MCE Filter	Right	25.6	15	384.0	10	0.039	Reference
4	XMX/2L-MIL	Left	697.0	15	10455.0	22	0.010	8.08%
5	STA	Center	26.1	15	391.5	5	0.023	98.08%
5	MCE Filter	Right	25.6	15	384.0	5	0.029	Reference
5	XMX/2L-MIL	Left	697.0	15	10455.0	24	0.015	17.63%
6	STA	Left	26.0	15	390.0	12	0.026	56.04%
6	MCE Filter	Center	25.5	15	382.5	21	0.039	Reference
6	XMX/2L-MIL	Right	697.0	15	10455.0	39	0.015	6.79%
7	STA	Left	26.0	15	390.0	7	0.021	85.82%
7	MCE Filter	Center	25.5	15	382.5	8	0.052	Reference
7	XMX/2L-MIL	Right	702.0	15	10530.0	22	0.014	9.99%

High Volume Comparison Trial Data: XMX/2L-MIL (Continued)

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
8	STA	Right	29.8	15	447.0	18	0.046	102.28%
8	MCE Filter	Left	25.4	15	381.0	15	0.052	Reference
8	XMX/2L-MIL	Center	697.0	15	10455.0	38	0.018	9.23%
9	STA	Right	29.8	15	447.0	14	0.041	85.23%
9	MCE Filter	Left	25.4	15	381.0	14	0.034	Reference
9	XMX/2L-MIL	Center	702.0	15	10530.0	35	0.018	9.05%
10	STA	Right	29.8	15	447.0	9	0.023	54.79%
10	MCE Filter	Left	25.4	15	381.0	14	0.026	Reference
10	XMX/2L-MIL	Center	697.0	15	10455.0	46	0.014	11.97%
11	STA	Right	30.5	15	457.5	14	0.031	90.74%
11	MCE Filter	Left	25.7	15	381.5	13	0.042	Reference
11	XMX/2L-MIL	Center	702.0	15	10530.0	33	0.015	9.29%
12	STA	Right	30.5	15	457.5	6	0.023	84.26%
12	MCE Filter	Left	25.7	15	381.5	6	0.039	Reference
12	XMX/2L-MIL	Center	697.0	15	10455.0	15	0.018	9.22%
13	STA	Left	30.5	15	457.5	5	0.021	60.19%
13	MCE Filter	Center	25.7	15	381.5	7	0.042	Reference
13	XMX/2L-MIL	Right	702.0	15	10530.0	26	0.017	13.60%
14	STA	Left	30.5	15	457.5	21	0.026	76.34%
14	MCE Filter	Center	25.5	15	382.5	23	0.031	Reference
14	XMX/2L-MIL	Right	702.0	15	10530.0	72	0.020	11.37%
15	STA	Left	30.5	15	457.5	20	0.023	69.67%
15	MCE Filter	Center	25.5	15	382.5	24	0.031	Reference
15	XMX/2L-MIL	Right	697.0	15	10455.0	64	0.014	9.76%

High Volume Comparison Trial Data: Biocapture-650

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
1	STA	Center	30.4	15	456.0	40	0.088	106.09%
1	MCE Filter	Left	25.8	15	387.0	32	0.083	Reference
1	Biocapture	Right	200.0	15	3000.0	87	0.029	35.07%
2	STA	Center	30.4	15	456.0	36	0.079	92.58%
2	MCE Filter	Left	25.8	15	387.0	33	0.085	Reference
2	Biocapture	Right	200.0	15	3000.0	86	0.029	33.62%
3	STA	Left	30.5	15	457.5	12	0.026	56.61%
3	MCE Filter	Right	25.9	15	388.5	18	0.046	Reference
3	Biocapture	Center	200.0	15	3000.0	34	0.011	24.46%
4	STA	Left	30.5	15	457.5	12	0.026	56.61%
4	MCE Filter	Right	25.9	15	388.5	18	0.046	Reference
4	Biocapture	Center	200.0	15	3000.0	30	0.010	21.58%
5	STA	Right	30.1	15	451.5	34	0.075	59.24%
5	MCE Filter	Center	25.7	15	385.5	49	0.127	Reference
5	Biocapture	Left	200.0	15	3000.0	75	0.025	19.67%
6	STA	Center	30.3	15	454.5	27	0.059	104.10%
6	MCE Filter	Left	25.7	15	385.5	22	0.057	Reference
6	Biocapture	Right	200.0	15	3000.0	71	0.024	41.47%
7	STA	Center	30.3	15	454.5	30	0.066	77.11%
7	MCE Filter	Left	25.7	15	385.5	33	0.086	Reference
7	Biocapture	Right	200.0	15	3000.0	79	0.026	30.76%

High Volume Comparison Trial Data: Biocapture-650 (Continued)

Trial #	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colony Count (CFU)	Measured Concentration (CFU/L)	Relative Efficiency
8	STA	Center	30.4	15	456.0	25	0.055	96.82%
8	MCE Filter	Left	25.9	15	388.5	22	0.057	Reference
8	Biocapture	Right	200.0	15	3000.0	57	0.019	33.55%
9	STA	Left	30.4	15	456.0	29	0.064	98.83%
9	MCE Filter	Right	25.9	15	388.5	25	0.064	Reference
9	Biocapture	Center	200.0	15	3000.0	64	0.021	33.15%
10	STA	Left	30.3	15	454.5	28	0.062	93.89%
10	MCE Filter	Right	25.4	15	381.0	25	0.066	Reference
10	Biocapture	Center	200.0	15	3000.0	62	0.021	31.50%
11	STA	Left	30.2	15	453.0	20	0.044	68.61%
11	MCE Filter	Right	25.9	15	388.5	25	0.064	Reference
11	Biocapture	Center	200.0	15	3000.0	48	0.016	24.86%
12	STA	Right	30.2	15	453.0	23	0.051	89.66%
12	MCE Filter	Center	25.9	15	388.5	22	0.057	Reference
12	Biocapture	Left	200.0	15	3000.0	42	0.014	24.72%
13	STA	Right	30.2	15	453.0	13	0.029	69.68%
13	MCE Filter	Center	25.9	15	388.5	16	0.041	Reference
13	Biocapture	Left	200.0	15	3000.0	35	0.012	28.33%
14	STA	Right	30.2	15	453.0	22	0.049	62.89%
14	MCE Filter	Center	25.9	15	388.5	30	0.077	Reference
14	Biocapture	Left	200.0	15	3000.0	39	0.013	16.84%
15	STA	Right	30.2	15	453.0	31	0.068	88.62%
15	MCE Filter	Center	25.9	15	388.5	30	0.077	Reference
15	Biocapture	Left	200.0	15	3000.0	53	0.018	22.88%

High Volume Comparison Trial Analysis (Descriptive Statistics from Prism v7)

	XX/2L- MIL	DFU- 1000	Biocapture- 650	MB2
Number of values	15	15	15	15
Minimum	6.79	14.52	16.84	0
25% Percentile	9.05	25.95	22.88	0
Median	9.76	38.28	28.33	1.44
75% Percentile	11.97	51.95	33.55	4.65
Maximum	17.63	63.06	41.47	13.02
Mean	10.51	38.6	28.16	3.05
Std. Deviation	2.688	13.56	6.732	3.969
Std. Error of Mean	0.6942	3.5	1.738	1.025
Lower 95% CI of mean	9.017	31.09	24.44	0.8521
Upper 95% CI of mean	11.99	46.11	31.89	5.248
95% CI of median				
Lower confidence limit	9.05	25.95	22.88	0
Upper confidence limit	11.97	51.95	33.55	4.65
Shapiro-Wilk normality test				
W	0.8856	0.9754	0.9705	0.7666
P value	0.0576	0.9286	0.8656	0.0014
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	No
P value summary	ns	ns	ns	**

High Volume Comparison Trial Analysis (Non-Parametric, Kruskal-Wallis Multiple Comparison Test From Prism v7)

Table Analyzed	High Vol Comparison MCE Ref
Kruskal-Wallis test	
P value	<0.0001
Exact or approximate P value?	Approximate
P value summary	****
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	4
Kruskal-Wallis statistic	49.04
Data summary	
Number of treatments (columns)	4
Number of values (total)	60

High Volume Comparison Trial Analysis on XMX/2L-MIL, MB2, DFU-1000 and Biocapture-650 (Non-Parametric, Dunn's Post-Hoc Test from Prism v7)

Number of families	1			
Number of comparisons per family	6			
Alpha	0.05			
Dunn's multiple comparisons test	Mean rank diff.	Significant	Summary	Adjusted P Value
XMX/2L-MIL vs. DFU-1000	-27.67	Yes	****	<0.0001
XMX/2L-MIL vs. Biocapture-650	-19.87	Yes	*	0.0110
XMX/2L-MIL vs. MB2	12.2	No	ns	0.3342
DFU-1000 vs. Biocapture-650	7.8	No	ns	>0.9999
DFU-1000 vs. MB2	39.87	Yes	****	<0.0001
Biocapture-650 vs. MB2	32.07	Yes	****	<0.0001
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1
XMX/2L-MIL vs. DFU-1000	21.67	49.33	-27.67	15
XMX/2L-MIL vs. Biocapture-650	21.67	41.53	-19.87	15
XMX/2L-MIL vs. MB2	21.67	9.467	12.2	15
DFU-1000 vs. Biocapture-650	49.33	41.53	7.8	15
DFU-1000 vs. MB2	49.33	9.467	39.87	15
Biocapture-650 vs. MB2	41.53	9.467	32.07	15

High Volume Comparison Trial Analysis on XMX/2L-MIL and MB2 (Non-Parametric Mann-Whitney Test from Prism v7)

Table Analyzed	High Vol Comparison MCE Ref
Column D vs. Column A	MB2 vs. XMX/2L-MIL
Mann Whitney test	
P value	<0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,D	323 , 142
Mann-Whitney U	22
Difference between medians	
Median of column A	9.76, n=15
Median of column D	1.44, n=15
Difference: Actual	-8.32
Difference: Hodges-Lehmann	-8.08
95.47% CI of difference	-9.62 to -6.39
Exact or approximate CI?	Exact

**High Volume Comparison Trial Analysis on DFU-1000 and Biocapture-650
(Welch's t-test from Prism v7)**

Table Analyzed	High Vol Comparison MCE Ref
Column C vs. Column B	Biocapture-650 vs. DFU-1000
Unpaired t test with Welch's correction	
P value	0.0145
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=2.67 df=20.51
How big is the difference?	
Mean $\pm$ SEM of column B	38.6 $\pm$ 3.5, n=15
Mean $\pm$ SEM of column C	28.16 $\pm$ 1.738, n=15
Difference between means	-10.43 $\pm$ 3.908
95% confidence interval	-18.57 to -2.295
R squared (eta squared)	0.2579
F test to compare variances	
F, DF _n , DF _d	4.056, 14, 14
P value	0.0131
P value summary	*
Significantly different (P < 0.05)?	Yes

Appendix E

Aim 4: High Volume Air Sampling Demonstration in the Collection of *C. difficile* Spore Aerosol from Toilet Flushing

Toilet Bowl Water Concentration Data

Trial #	Sample	Filtered Volume (ml)	CFU on Filter	Dilution Factor	Water Concentration (CFU/ml)	Fraction Remaining from Start	Fraction Remaining from Previous
1	Pre-Flush	50	24	1.00E-05	48000.0	1.00E+00	1.00E+00
1	Post Flush 3	50	9	1.00E-02	18.0	3.75E-04	3.75E-04
1	Post Flush 6	50	35	1.00E-01	7.0	1.46E-04	3.89E-01
2	Pre-Flush	50	25.0	1.00E-05	50000.0	1.00E+00	1.00E+00
2	Post Flush 3	45	43.0	1.00E-01	9.6	1.91E-04	1.91E-04
2	Post Flush 6	50	13.0	1.00E-01	2.6	5.20E-05	2.72E-01
3	Pre-Flush	50	23.0	1.00E-05	46000.0	1.00E+00	1.00E+00
3	Post Flush 3	45	43.0	1.00E-01	9.6	2.08E-04	2.08E-04
3	Post Flush 6	50	20.0	1.00E-01	4.0	8.70E-05	4.19E-01
4	Pre-Flush	50	24.0	1.00E-05	48000.0	1.00E+00	1.00E+00
4	Post Flush 3	45	50.0	1.00E-01	11.1	2.31E-04	2.31E-04
4	Post Flush 6	45	77.0	1.00E+00	1.7	3.56E-05	1.54E-01
5	Pre-Flush	50	38.0	1.00E-05	76000.0	1.00E+00	1.00E+00
5	Post Flush 3	50	15.0	1.00E-02	30.0	3.95E-04	3.95E-04
5	Post Flush 6	50	14.0	1.00E-01	2.8	3.68E-05	9.33E-02
6	Pre-Flush	50	45.0	1.00E-05	90000.0	1.00E+00	1.00E+00
6	Post Flush 3	45	76.0	1.00E-01	16.9	1.88E-04	1.88E-04
6	Post Flush 6	50	15.0	1.00E-01	3.0	3.33E-05	1.78E-01

Toilet Bowl Water Concentration Data (Continued)

Trial #	Sample	Sample Volume (ml)	CFU on Filter	Dilution Factor	Water Concentration (CFU/ml)	Fraction Remaining from Start	Fraction Remaining from Previous
7	Pre-Flush	50	45.0	1.00E-05	90000.0	1.00E+00	1.00E+00
7	Post Flush 3	45	44.0	1.00E-01	9.8	1.09E-04	1.09E-04
7	Post Flush 6	50	13.0	1.00E-01	2.6	2.89E-05	2.66E-01
8	Pre-Flush	50	26.0	1.00E-05	52000.0	1.00E+00	1.00E+00
8	Post Flush 3	45	84.0	1.00E-01	18.7	3.59E-04	3.59E-04
8	Post Flush 6	50	17.0	1.00E-01	3.4	6.54E-05	1.82E-01
9	Pre-Flush	50	42.0	1.00E-05	84000.0	1.00E+00	1.00E+00
9	Post Flush 3	45	76.0	1.00E-01	16.9	2.01E-04	2.01E-04
9	Post Flush 6	50	18.0	1.00E-01	3.6	4.29E-05	2.13E-01
10	Pre-Flush	50	27.0	1.00E-05	54000.0	1.00E+00	1.00E+00
10	Post Flush 3	45	66.0	1.00E-01	14.7	2.72E-04	2.72E-04
10	Post Flush 6	50	42.0	1.00E-01	8.4	1.56E-04	5.73E-01
11	Pre-Flush	50	30.0	1.00E-05	60000.0	1.00E+00	1.00E+00
11	Post Flush 3	45	44.0	1.00E-01	9.8	1.63E-04	1.63E-04
11	Post Flush 6	50	16.0	1.00E-01	3.2	5.33E-05	3.27E-01
12	Pre-Flush	50	20.0	1.00E-05	40000.0	1.00E+00	1.00E+00
12	Post Flush 3	45	32.0	1.00E-01	7.1	1.78E-04	1.78E-04
12	Post Flush 6	50	18.0	1.00E-01	3.6	9.00E-05	5.06E-01

Toilet Flush Air Sampling Data

Trial #	Flush Series	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colonies Collected (CFU)
1	Flush 1-3	DFU	Center	920	45	41400.0	42
1	Flush 1-3	MCE Filter	Left	25.7	45	1156.5	2
1	Flush 1-3	MCE Filter	Right	25.6	45	1152.0	4
1	Flush 4-6	DFU	Center	920	45	41400.0	1
1	Flush 4-6	MCE Filter	Left	25.7	45	1156.5	0
1	Flush 4-6	MCE Filter	Right	25.6	45	1152.0	0
2	Flush 1-3	DFU	Center	920	45	41400.0	25
2	Flush 1-3	MCE Filter	Left	25.7	45	1156.5	1
2	Flush 1-3	MCE Filter	Right	25.8	45	1161.0	0
2	Flush 4-6	DFU	Center	920	45	41400.0	1
2	Flush 4-6	MCE Filter	Left	25.7	45	1156.5	0
2	Flush 4-6	MCE Filter	Right	25.8	45	1161.0	0
3	Flush 1-3	DFU	Center	920	45	41400.0	28
3	Flush 1-3	MCE Filter	Left	25.8	45	1161.0	0
3	Flush 1-3	MCE Filter	Right	26	45	1170.0	2
3	Flush 4-6	DFU	Center	920	45	41400.0	3
3	Flush 4-6	MCE Filter	Left	25.8	45	1161.0	0
3	Flush 4-6	MCE Filter	Right	26	45	1170.0	0
4	Flush 1-3	DFU	Center	920	45	41400.0	23
4	Flush 1-3	MCE Filter	Left	25.8	45	1161.0	2
4	Flush 1-3	MCE Filter	Right	25.9	45	1165.5	3
4	Flush 4-6	DFU	Center	920	45	41400.0	2
4	Flush 4-6	MCE Filter	Left	25.8	45	1161.0	0
4	Flush 4-6	MCE Filter	Right	25.9	45	1165.5	1

Toilet Flush Air Sampling Data (Continued)

Trial #	Flush Series	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colonies Collected (CFU)
5	Flush 1-3	DFU	Left	920	45	41400.0	6
5	Flush 1-3	MCE Filter	Center	25.8	45	1161.0	1
5	Flush 1-3	MCE Filter	Right	25.9	45	1165.5	0
5	Flush 4-6	DFU	Left	920	45	41400.0	0
5	Flush 4-6	MCE Filter	Center	25.8	45	1161.0	0
5	Flush 4-6	MCE Filter	Right	25.9	45	1165.5	0
6	Flush 1-3	DFU	Left	920	45	41400.0	67
6	Flush 1-3	MCE Filter	Center	25.8	45	1161.0	0
6	Flush 1-3	MCE Filter	Right	25.9	45	1165.5	3
6	Flush 4-6	DFU	Left	920	45	41400.0	3
6	Flush 4-6	MCE Filter	Center	25.8	45	1161.0	0
6	Flush 4-6	MCE Filter	Right	25.9	45	1165.5	0
7	Flush 1-3	DFU	Left	920	45	41400.0	19
7	Flush 1-3	MCE Filter	Center	25.7	45	1156.5	1
7	Flush 1-3	MCE Filter	Right	25.7	45	1156.5	1
7	Flush 4-6	DFU	Left	920	45	41400.0	2
7	Flush 4-6	MCE Filter	Center	25.7	45	1156.5	0
7	Flush 4-6	MCE Filter	Right	25.7	45	1156.5	0
8	Flush 1-3	DFU	Left	920	45	41400.0	15
8	Flush 1-3	MCE Filter	Center	25.8	45	1161.0	2
8	Flush 1-3	MCE Filter	Right	25.8	45	1161.0	1
8	Flush 4-6	DFU	Left	920	45	41400.0	3
8	Flush 4-6	MCE Filter	Center	25.8	45	1161.0	0
8	Flush 4-6	MCE Filter	Right	25.8	45	1161.0	0

Toilet Flush Air Sampling Data (Continued)

Trial #	Flush Series	Instrument	Instrument Location	Flow Rate (L/min)	Sample Time (min)	Volume Sampled (L)	Colonies Collected (CFU)
9	Flush 1-3	DFU	Right	920	45	41400.0	20
9	Flush 1-3	MCE Filter	Left	25.9	45	1165.5	1
9	Flush 1-3	MCE Filter	Center	25.8	45	1161.0	1
9	Flush 4-6	DFU	Right	920	45	41400.0	4
9	Flush 4-6	MCE Filter	Left	25.9	45	1165.5	0
9	Flush 4-6	MCE Filter	Center	25.8	45	1161.0	0
10	Flush 1-3	DFU	Right	920	45	41400.0	42
10	Flush 1-3	MCE Filter	Left	25.8	45	1161.0	3
10	Flush 1-3	MCE Filter	Center	25.7	45	1156.5	0
10	Flush 4-6	DFU	Right	920	45	41400.0	4
10	Flush 4-6	MCE Filter	Left	25.8	45	1161.0	0
10	Flush 4-6	MCE Filter	Center	25.7	45	1156.5	0
11	Flush 1-3	DFU	Right	920	45	41400.0	29
11	Flush 1-3	MCE Filter	Left	26	45	1170.0	0
11	Flush 1-3	MCE Filter	Center	25.8	45	1161.0	3
11	Flush 4-6	DFU	Right	920	45	41400.0	2
11	Flush 4-6	MCE Filter	Left	26	45	1170.0	0
11	Flush 4-6	MCE Filter	Center	25.8	45	1161.0	0
12	Flush 1-3	DFU	Right	920	45	41400.0	43
12	Flush 1-3	MCE Filter	Left	26.1	45	1174.5	1
12	Flush 1-3	MCE Filter	Center	25.9	45	1165.5	2
12	Flush 4-6	DFU	Right	920	45	41400.0	1
12	Flush 4-6	MCE Filter	Left	26.1	45	1174.5	0
12	Flush 4-6	MCE Filter	Center	25.9	45	1165.5	0

Toilet Flush Air Sampling Analysis Flush Series 1-3 (Descriptive Statistics from Prism v7)

	MCE Pooled	DFU-1000	Total
Number of values	12	12	12
Minimum	1	6	7
25% Percentile	2	19.25	21.25
Median	3	26.5	29
75% Percentile	3	42	47.25
Maximum	6	67	70
Mean	2.833	30.08	32.92
Std. Deviation	1.467	16.47	17.09
Std. Error of Mean	0.4234	4.754	4.934
Lower 95% CI of mean	1.901	19.62	22.06
Upper 95% CI of mean	3.765	40.55	43.78
Shapiro-Wilk normality test			
W	0.876	0.9383	0.9438
P value	0.0780	0.4770	0.5490
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns

Toilet Flush Air Sampling Analysis Flush Series 1-3 Comparing Total Aerosol Collection from DFU-1000 and MCE filters (Welch's t-test from Prism v7)

	Data Set-A
Table Analyzed	Data 1
Column B vs. Column A	DFU-1000 vs. MCE Pooled
Unpaired t test with Welch's correction	
P value	0.0001
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=5.71 df=11.17
How big is the difference?	
Mean $\pm$ SEM of column A	2.833 $\pm$ 0.4234, n=12
Mean $\pm$ SEM of column B	30.08 $\pm$ 4.754, n=12
Difference between means	27.25 $\pm$ 4.773
95% confidence interval	16.77 to 37.73
R squared (eta squared)	0.7447
F test to compare variances	
F, DFn, Dfd	126, 11, 11
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes

Toilet Flush Air Sampling Analysis Flush Series 4-6 Comparing Detection Frequency between DFU-1000 and MCE Filters (Descriptive Statistics from Prism v7)

P value and statistical significance Test	Fisher's exact test		
P value	<0.0001		
P value summary	****		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	Yes		
Data analyzed	Detect	Non-Detect	Total
MCE	1	24	25
DFU-1000	11	1	12
Total	12	25	37
Percentage of row total	Detect	Non-Detect	
MCE	4.00%	96.00%	
DFU-1000	91.67%	8.33%	
Percentage of column total	Detect	Non-Detect	
MCE	8.33%	96.00%	
DFU-1000	91.67%	4.00%	
Percentage of grand total	Detect	Non-Detect	
MCE	2.70%	64.86%	
DFU-1000	29.73%	2.70%	