

Observations on the use of membrane filtration and liquid impingement to collect airborne microorganisms in various atmospheric environments

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Received: 19 March 2010 / Accepted: 5 June 2010
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Abstract The influence of sample-collection-time on the recovery of culturable airborne microorganisms using a low-flow-rate membrane-filtration unit and a high-flow-rate liquid impinger were investigated. Differences in recoveries were investigated in four different atmospheric environments, one mid-oceanic

at an altitude of ~ 10.0 m, one on a mountain top at an altitude of $\sim 3,000.0$ m, one at ~ 1.0 m altitude in Tallahassee, Florida, and one at ~ 1.0 m above ground in a subterranean-cave. Regarding use of membrane filtration, a common trend was observed: the shorter the collection period, the higher the recovery of culturable bacteria and fungi. These data also demonstrated that lower culturable counts were common in the more remote mid-oceanic and mountain-top atmospheric environments with bacteria, fungi, and total numbers averaging (by sample time or method categories) <3.0 colony-forming units (CFU) m^{-3} . At the Florida and subterranean sites, the lowest average count noted was 3.5 bacteria CFU m^{-3} , and the highest averaged 140.4 total CFU m^{-3} . When atmospheric temperature allowed use, the high-volume liquid impinger utilized in this study resulted in much higher recoveries, as much as $10\times$ greater in a number of the categories (bacterial, fungal, and total CFU). Together, these data illustrated that (1) the high-volume liquid impinger is clearly superior to membrane filtration for aeromicrobiology studies if start-up costs are not an issue and temperature permits use; (2) although membrane filtration is more cost friendly and has a 'typically' wider operational range, its limits include loss of cell viability with increased sample time and issues with effectively extracting nucleic acids for community-based analyses; (3) the ability to recover culturable microorganisms is limited in 'extreme' atmospheric environments and thus the use of a 'limited' methodology in these environments must be

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taken into account; and (4) the atmosphere culls, i.e., everything is not everywhere.

Keywords Bacteria · Fungi · Methods · Membrane filtration · Liquid impingement · Aeromicrobiology · Microbiology

1 Introduction

Research has shown that the concentration and diversity of atmospherically suspended microorganisms change with location and altitude (Pasteur 1861; Tyndall 1882; Wolf 1943; Gregory 1961; Smith et al. 2009). Microorganisms can be transported to various altitudes through a number of different means that include light- and heavy-wind events, fires, earthquakes, air traffic, volcanic activity, and impact events (Griffin 2004). A good example of the sheer volume of atmospheric microbial loading that can occur is that associated with desert-dust storms. It is currently believed that approximately 3 billion metric tons of desert dust moves some distance through our atmosphere each year carrying a microbiological load of ~ 1 million to ~ 1 billion bacterial cells per gram; that is $\sim 10^{21}$ to 10^{24} cells, and there are of course the other microbial types to consider, including protozoa, fungi, and viruses (Griffin 2007a). In larger-scale atmospheric events, such as those observed with large dust storms and volcanic eruptions, global-scale dispersion of microorganisms is common. Surprisingly, very diverse communities of bacteria and fungi have been shown to tolerate long-range transport in the atmosphere at near-surface altitudes, but it is clear in the peer-reviewed literature that very few microbial types can tolerate extended atmospheric suspension, particularly at altitudes above, in and beyond the upper troposphere (Griffin 2007a; Smith et al. 2009).

Microorganisms experience stress in the atmosphere due to UV exposure (pyrimidine dimerization), desiccation (dehydration via the movement of air over cell-wall surfaces), temperature (both low and high temperatures depending on the tolerance range of specific organisms), and atmospheric chemistry (humidity levels dependent on tolerance, oxygen radicals, etc.) (Gregory 1961; Mohr 1997; Dowd and

Maier 2000). Traits that are known to enhance survival are pigmented cell walls, genetic capability to resist UV damage (i.e., *Deinococcus* spp.), and the ability to produce spores. Spores are produced by a few genera of bacteria, such as *Bacillus* (one of the most common genera found in soils), and many fungi. Spores are egg-like vesicles covered with a thin layer of hydrophobic proteins that provide protection from physical stresses such as ultraviolet exposure, and desiccation (Dufrene 2000). Recent analyses of archived desert-dust samples collected by Schomburgk on Barbados in 1812 and by James (a colleague of Darwin) while aboard a ship off the west coast of Africa in 1838, demonstrated long-term survival (~ 194 and ~ 168 years, respectively) of various dust-borne spore-forming bacteria and fungi (Gorbushina et al. 2007). These data are great examples of ‘expect the unexpected’ when it comes to issues in microbial survival. Even at extreme altitudes (20 km), where stress exposure is significant, non-spore-forming microorganisms can be found that demonstrate variation in adaptation strategy (Griffin 2007b). Due to stress exposure experienced with atmospheric transport, a collection protocol that limits further cell stress is needed to ensure accurate determination of viable populations. For successful non-culture-based studies, the method of choice should limit loss of nucleic acids. The pros and cons of different collection methods that include membrane filtration, impaction, and impingement have previously been reviewed (Buttner et al. 1997; Griffin 2007a).

Membrane filtration, in which air is pumped through a porous membrane, is a widely used assay because of low expense, ease of use, and high-capture rate (Jensen et al. 1994; Buttner et al. 1997; Makino et al. 2001; Griffin 2007a). Filtration units are easily made in-house using readily available components such as metal or rigid polyvinyl chloride tubing and valves and small vacuum pumps. We have assembled numerous units housed inside plastic toolboxes or cases that can be powered by direct current via an inverter for use in remote locations. Membranes that can be used include cellulose-based nylon and glass fibers with pore sizes down to $0.02 \mu\text{m}$ (for viral capture) (Griffin et al. 2001). The disadvantages of filtration include desiccation of the microorganisms on the filter surface due to filtration rate and time (inadvertently selecting for spore-forming microorganisms), filter size (small-diameter filters can result in stacking of cells in high

particle-load-environments), inefficient extraction of nucleic acids from filter surfaces, and filter thickness (thin filters can result in nutrient shock) (Tobin et al. 1980; McFeters et al. 1982; Jensen et al. 1994; Buttner et al. 1997; Lin et al. 2000; Wang and Reponen 2001; Griffin et al. 2006).

Impingement of microorganisms into a liquid matrix is another assay used to collect atmospheric microorganisms (Terzieva et al. 1996; Buttner et al. 1997; Agranovski et al. 2005). A widely used liquid impinger is the AGI-30 (Ace Glass Inc., Vineland, NJ) that utilizes low-flow rates. Although research has shown that the AGI-30 is a low-cost and efficient method of collecting aerosolized microorganisms for culture- and non-culture-based analyses, the flow rates limit the analyses of large volumes of air, and the glass construction is susceptible to the rigors of field studies (Terzieva et al. 1996). High-flow-rate liquid impingers have been developed that allow the collection of low concentrations of microorganisms from the atmosphere and have been utilized for culture and non-culture analyses for the presence of bacteria, fungi, and viruses (Bergman et al. 2005). The benefit of using impingers is that the liquid matrix can be split for various analyses to include simultaneous culture of different nutrient sources, direct count, molecular, and cell-culture assays. The disadvantages of liquid impingement include the low-capture rate of some impingers, high cost (high-flow-rate impingers), loss of collection fluid to evaporation and violent bubbling (some high-flow-rate impingers are designed to monitor and compensate for loss via fluid reservoirs), capture efficiency of various impingement fluids, low-capture rate of bacteria and fungi due to the effects of contact chamber bounce and re-aerosolization, low-capture rate of viral-size particles, and loss of viability (i.e., viral inactivation due to bubbling or choice of collection solution) (Lin et al. 1997, 1999; Agranovski et al. 2004; Yu et al. 2009).

In this paper, we compare and contrast data obtained from the use of membrane filtration for aeromicrobiological analyses at four different locations. We focus in particular on the effect of filtration period at low-flow rates on the recovery of culturable bacteria and fungi. Additionally, we contrast aeromicrobiological membrane-filtration and liquid-impingement data obtained at two of the four locations.

2 Methods

2.1 Research sites

2.1.1 Tropical mid-Atlantic Ridge

Thirty-two membrane-filtration samples were collected from 6/15/03 to 6/30/03 while aboard the JOIDES *Resolution* drillship during Ocean Drilling Program (ODP) Leg 209 at $\sim 45^{\circ}\text{W}$ between $14^{\circ}43'\text{N}$ and $15^{\circ}44'\text{N}$ (Keleman et al. 2004). While on station, the *Resolution* was positioned with its bow to the east, into the trade winds. Samples were collected obtained from the bridge deck located near the bow at ~ 10.0 m above sea level between the hours of 0630 and 1900 and consisted of both short- (<1.3 h) and long-term (2.9–12.1 h) intervals. Flow rates for the samples ranged from ~ 1.9 to ~ 17.4 L min^{-1} . Additional samples were collected prior to 6/15/03 but are not included in this study because the short-term samples were not paired with long-term samples. For that complete data set, see Griffin et al. (2006). Atmospheric desert-dust concentration estimates were provided courtesy of the U.S. Navy Naval Aerosol Analysis and Predictions System (NAAPS) Global Aerosol Model, Naval Research Laboratory, Aerosol and Radiation Section, Monterey, CA.

2.1.2 Mount Bachelor Observatory, Bend, Oregon

Mount Bachelor Observatory is located atop Mount Bachelor (latitude 43.977517 , longitude -121.685974) in the first and second levels of the summit-chairlift building at an altitude of $\sim 3,000$ m. Thirty-six membrane and six liquid-impinger samples were obtained from 4/20/09 to 5/4/09. Membrane-filtration samples were collected continuously using both short- and long-term samples. Short-term membrane-filtration and liquid-impinger samples were collected between 0820 and 1530 h at flow rates of ~ 17.4 and ~ 230.0 L min^{-1} , respectively. Long-term membrane-filtration samples were taken between 1005 and 0955 (the following morning) at a flow rate of ~ 17.4 L min^{-1} . Both sample types were acquired through downward-facing intake lines (new unused swimming-pool vacuum-line tubing), which extended through an aluminum duct located on the second level of the Observatory. The liquid-impinger samples were

collected between 4/20/09 and 4/22/09, after which a drop in temperature below the impinger operational temperature ($>2.0^{\circ}\text{C}$) prevented use. Particle counts were obtained using an IQAir (Santa Fe Springs, CA) Particle Scan Pro.

2.1.3 US Geological Survey, Tallahassee, Florida

Twenty-eight membrane and seven liquid-impinger samples were collected from 6/30/09 to 7/17/09 between 0730 and 1700 h. Samples were recovered in an open courtyard atop a concrete patio table, located between two office buildings at latitude 30.477377 and longitude -84.294601 . Membrane-filtration samples were obtained at flow rates of 8.7 and 17.4 L min^{-1} over periods of 20, ~ 60 , and ~ 480 min. Liquid-impinger samples were acquired over a period of 20 min at a flow rate of $\sim 300.0 \text{ L min}^{-1}$.

2.1.4 Carlsbad Caverns National Park, New Mexico

Five membrane-filtration and two liquid-impinger samples were collected on 4/1/09 within the cave system (cave entrance at latitude 32.176246, longitude -104.441093) at the following locations: (1) Site A—next to audio tour sign 5 located at the cave entrance, (2) Site E—at the seating bench overlooking Iceberg Rock, ~ 33 m up from the NPS visitor emergency telephone, (3) Site H—in the Big Room at the Lower Cave overlook, (4) Lunch Room A (LRA)—in the lunch room eating area next to the lunch line cashier, and (5) Left Hand Tunnel ~ 33 m past the end of the public tour area, next to the rope-traverse pit. Membrane-filtration samples were obtained at all sites and liquid-impinger samples at sites E and LRA between the hours of 0915 and 1705. All membrane-filtration samples were taken over 40.0 min at a flow rate of $\sim 8.7 \text{ L min}^{-1}$. Liquid-impinger samples were collected over 30.0 min at a flow rate of $\sim 250 \text{ L min}^{-1}$.

2.2 Membrane-filtration assay

Membrane-filtration samples were obtained using a portable membrane-filtration apparatus (Fisher Scientific 110 V vacuum pump, product #13-310-485 and a PVC two-place-manifold, assembled in-house). Pre-sterilized filter housings containing 47-mm-diameter, 0.2- μm pore-size cellulose-acetate filter membranes were used to collect all air samples (Fisher Scientific,

Atlanta, GA, Catalog #09-74030G). The filter housings were removed from their respective sterile bags and placed on the analytical filter manifold. The housing lid was removed and vacuum applied using a vacuum pump. Filtration-flow rates ranged from ~ 8.7 to $\sim 17.4 \text{ L min}^{-1}$, and the total volume of air filtered ranged from $\sim 89.3 \text{ L}$ to $\sim 22,137.5 \text{ L}$. Use of the two-place manifold resulted in a normal flow rate of $\sim 8.7 \text{ L min}^{-1}$ when simultaneously collecting two samples or $\sim 17.4 \text{ L min}^{-1}$ when collecting a single sample. Data for all samples were normalized and is expressed as the numbers of CFU m^{-3} of air. All 139 samples were evaluated. To control for handling contamination (once each day), an additional filter housing was removed from its bag, placed on the manifold, and allowed to sit for 1 min without removing the lid or turning on the vacuum. After filtration, the housings and lids were replaced in their respective bags, sealed with tape, and immediately transported to the microbiology laboratory for processing. R2A medium (Fisher Scientific, Atlanta, GA) (Reasoner and Geldreich 1985) was utilized in the following manner for microbial culture. The sample filters were placed whole on R2A medium plates, sample side up, and were incubated in the dark at room temperature ($\sim 23^{\circ}\text{C}$). Room temperature was chosen as incubation temperature as we have observed that it limits plate overgrowth and has successfully been utilized for the culture of bacteria and fungi from samples collected at altitudes as high as 20.0 km. Bacterial and fungal CFU were enumerated at $\geq 96 \text{ h}$.

2.3 Liquid-impinger assay

Liquid-impinger samples were collected using an OMNI 3000 (Evogen, Inc., Kansas City, MO) and Evogen, Inc. sterile $1 \times \text{PBS}$ (phosphate-buffered saline) 10-mL sample cartridges. Filtration-flow rates ranged from ~ 250.0 to $\sim 311.5 \text{ L min}^{-1}$, and the total volume of air filtered ranged from $\sim 5,800.0$ to $\sim 14,640.0 \text{ L}$. Data for all samples were normalized and expressed as the numbers of CFU m^{-3} of air. Twenty-two samples were evaluated. After filtration, the $1 \times \text{PBS}$ sample cartridges were labeled, placed in Evogen, Inc. cartridge zip-lock bags, and transported to the field or primary (in the Tallahassee based study) microbiology laboratory for processing. Approximately 150.0 μL was removed from each $1 \times \text{PBS}$ 10 mL sample cartridge using a sterile 10-cc syringe

and needle. This was accomplished by opening the sealed cartridge lid and inserting the syringe needle through one of the rubber ports on the deep end of the cartridge. The aliquot was then dispensed into a sterile 1.5-mL microcentrifuge tube, and 100 μL was used for spread-plate technique on R2A medium. Samples were incubated in the dark at room temperature ($\sim 23^\circ\text{C}$) and enumerated at ≥ 96 h. For comparison to membrane filtration, incubation temperatures and times were matched to their respective membrane-filtration samples (study-site sample groups).

2.4 Statistical analyses

SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) was used for statistical analyses. Data were tested for normality using a One-Sample Kolmogorov–Smirnov Test. The Mann–Whitney U -test was utilized for CFU versus CFU data analyses as the Levene’s test for equality of variances P -value were less than 0.05 or the data were not normally distributed (Dytham 1999). Spearman’s rho or Pearson correlations were

used for rank analyses (CFU versus dust or particle concentrations).

3 Results

Table 1 lists the CFU and atmospheric dust data for samples collected over the tropical mid-Atlantic ridge while aboard the JOIDES *Resolution*. Of the samples collected with less than 80 min of filtration, four of 16 (25.0%) were positive for either fungal or bacterial CFU. Bacterial CFU were only detected in one sample at 23.7 CFU m^{-3} of air. Total CFU recovery ranged from 1.7 to 28.4 m^{-3} of air. CFU were only detected in these samples when atmospheric African desert-dust concentration estimates exceeded 20.0 $\mu\text{g m}^{-3}$ at 10.0 m above sea level, per the U.S. Navy Naval Aerosol Analyses and Predictions System (NAAPS) Global Aerosol Model (Honrath et al. 2004). Of samples collected with 174–726 min of filtration, 13 of 16 (81.3%) were positive for bacterial and/or fungal CFU. CFU recovery in these samples ranged from 0.1

Table 1 Mid-Atlantic membrane-filtration data

Date	Membrane filtration <80 min			Membrane filtration 174–726 min			NAAPS African desert-dust concentration estimates at 10 m above sea level in $\mu\text{g m}^{-3}$
	Bacterial CFU	Fungal CFU	Total CFU	Bacterial CFU	Fungal CFU	Total CFU	
6/15/03	0	0	0	0.2	1.0	1.2	11.9
6/16/03	0	0	0	0	1.3	1.3	15.3
6/17/03	0	1.7	1.7	0.5	0.7	1.2	21.4
6/18/03	0	0	0	0	0.6	0.6	23.7
6/19/03	0	4.9	4.9	0	0.7	0.7	32.9
6/20/03	0	0	0	0	0.3	0.3	20.1
6/21/09	0	0	0	0	0.1	0.1	14.7
6/22/09	0	0	0	0	0.2	0.2	5.3
6/23/09	0	0	0	0	0	0	7.2
6/24/09	0	0	0	0.1	0	0.1	7.4
6/25/09	0	0	0	0	0	0	6.3
6/26/09	0	0	0	0	1.3	1.3	10.1
6/27/09	0	0	0	0	0.5	0.5	24.8
6/28/09	23.7	4.7	28.4	0	3.2	3.2	45.7
6/29/09	0	11.2	11.2	0	0.2	0.2	21.8
6/30/09	0	0	0	0	0	0	17.3
Average	1.5	1.4	2.9	0.1	0.7	0.8	

Colony-forming units (CFU) expressed as cubic meters of air. Atmospheric desert-dust concentration estimates courtesy of the U.S. Navy Naval Aerosol Analysis and Predictions System (NAAPS) Global Aerosol Model, Naval Research Laboratory, Aerosol and Radiation Section, Monterey, CA

to 3.2 m^{-3} of air. The four samples where no CFU or less than 0.2 total CFU were detected were obtained during periods where the NAAPS model indicated that atmospheric African desert-dust concentrations were below an average of $10 \mu\text{g m}^{-3}$. Although the highest CFU recoveries occurred in lower-volume samples, statistical analyses demonstrated no significant difference between CFU recovery and the two volume groups for all categories (Mann–Whitney- U —Bacterial P -value = 0.30, Fungal P -value = 0.06, Total P -value = 0.11). Analyses of total CFU recovery and NAAPS dust concentration data revealed a stronger statistically significant relation in the low-volume samples (Spearman's rho P -value < 0.00, correlation coefficient = 0.64) than in the high-volume air samples (Spearman's rho P -value = 0.03, correlation coefficient = 0.49).

Table 2 lists the microbiological and particle-count data collected at Mount Bachelor Observatory. The two groups of membrane-filtration samples ranged from 1.0 to 5.2 m^3 of air for the low-volume samples and 17.7 – 25.1 m^3 of air for the high-volume samples. Low-volume CFU recovery m^{-3} of air ranged from 0.0 to 3.1 (avg. 0.8), 0.0–6.1 (avg. 1.3),

and 0.2–6.8 (avg. 2.1), for bacterial, fungal, and total CFU, respectively. High-volume CFU recovery m^{-3} ranged from 0.1 to 0.7 (avg. 0.3), 0.1–2.1 (avg. 1.1), and 0.2–2.8 (avg. 1.4), for bacterial, fungal, and total CFU, respectively. Although not statistically significant (Mann–Whitney- U , P -values of 0.18, 0.08, and 0.49), the average recoveries in the low-volume samples was on average 2.7, 1.2, 1.5 times higher than high-volume samples for bacterial, fungal, and total CFU, respectively. The peak daily recovery for CFU m^{-3} in the low-volume samples was 3.1, 6.1, and 6.8 in comparison with the high-volume peaks of 0.7, 2.1, and 2.8 for bacterial, fungal, and total CFU, respectively. Statistical analyses for CFU recovery versus particle count (for the 13 dates with particle data) demonstrated significant relation with fungal and total CFU in the low-volume samples (Pearson P -values of 0.04 and 0.01, respectively). Relation with the low-volume bacterial CFU (P -value 0.06) and with CFU categories in high-volume samples were not observed (P -values of 0.24, 0.09, and 0.06, bacterial, fungal and total, respectively). Liquid-impinger CFU counts m^{-3} were (bacterial, fungal, total, respectively) 42.3, 55.6, and 97.9 for samples

Table 2 Mount Bachelor Observatory, Bend, Oregon, membrane-filtration data

Date	Membrane filtration 60–300 min * (avg. of 2–3 samples per day, starting on 4/28/09)			Particle counts # $\times 10^6 > 0.3 \mu\text{m m}^{-3}$	Membrane filtration 1,020–1,440 min			Particle counts # $\times 10^6 > 0.3 \mu\text{m m}^{-3}$
	Bacterial CFU	Fungal CFU	Total CFU		Bacterial CFU	Fungal CFU	Total CFU	
4/20/09	1.1	1.1	2.2	9.2	0.3	2.1	2.4	11.2
4/21/09	1.3	2.0	3.3	11.0	0.1	1.7	1.8	8.2
4/22/09	0.6	1.5	2.1	7.5	0.1	1.4	1.5	9.7
4/23/09	0.5	0.8	1.3	6.2	0.3	1.1	1.4	ND
4/24/09	1.1	1.1	2.2	6.9	0.4	1.5	1.9	22.1
4/25/09	3.1	1.4	4.5	26.7	0.5	0.2	0.7	16.0
4/26/09	0.2	0.2	0.4	16.1	0.7	2.1	2.8	18.4
4/27/09	0.7	6.1	6.8	27.4	0.1	0.5	0.6	4.3
4/28/09	0.2	0	0.2	20.8	0.6	0.5	1.1	3.7
4/29/09	1.0	0.3	1.3	9.8	0.5	0.1	0.6	7.3
4/30/09	0.8	2.0	2.8	10.1	0.1	2.0	2.1	10.7
5/1/09	0	0	0	2.2	0.1	0.1	0.2	1.2
5/2/09	0.7	0.5	1.2	2.2	0.7	0.5	1.2	2.4
5/3/09	0	1.0	1.0	2.2	0.3	2.0	2.3	2.5
Average	0.8	1.3	2.1	11.3	0.3	1.1	1.4	9.1

Colony-forming unit (CFU) data expressed per cubic meter of air

Table 3 Tallahassee, Florida, membrane-filtration and liquid-impinger data

Date	Membrane filtration 20 min			Membrane filtration 60 min * (avg. of 2 samples)			Membrane filtration 420–480 min			Liquid impinger 20 min		
	Bacterial CFU	Fungal CFU	Total CFU	Bacterial CFU	Fungal CFU	Total CFU	Bacterial CFU	Fungal CFU	Total CFU	Bacterial CFU	Fungal CFU	Total CFU
6/30/09	57.1	114.3	171.4	11.4	42.9	27.2	3.8	4.2	8.0	982.8	431.0	1,413.8
7/1/09	5.7	85.7	91.4	25.7	40.0	32.9	4.2	16.3	20.5	17.0	1,158.4	1,175.4
7/2/09	34.3	114.3	148.6	17.2	50.5	33.9	2.2	9.9	12.1	0	1,256.1	1,256.1
7/7/09	22.9	85.7	108.6	11.4	35.3	23.4	0.4	13.5	13.9	16.3	2,291.3	2,307.6
7/8/09	5.7	62.7	68.4	15.3	22.0	18.7	3.6	18.1	21.7	65.3	1,223.5	1,288.8
7/9/09	45.7	62.7	108.4	81.0	170.0	125.5	9.1	31.6	40.7	81.1	2,013.1	2,094.2
7/10/09	108.6	177.1	285.7	31.5	82.9	57.2	1.4	20.1	21.5	164.2	1,428.6	1,592.8
Average	40.0	100.4	140.4	27.6	63.3	45.7	3.5	16.2	19.7	189.5	1,400.3	1,589.8

Colony-forming units (CFU) expressed per cubic meter of air

collected on 4/20/09 (avg. of two samples), 7.2, 7.2, and 14.4 for the sample collected on 4/21/09, and 14.5, 25.9, and 60.4 for the samples collected on 4/22/09 (avg. of 2 samples).

Table 3 lists the results of the membrane-filtration and liquid-impinger samples collected in Tallahassee, Florida. The three groups of membrane-filtration samples obtained with 20, 60, and 420–480 min of filtration show a decrease in recovery (bacterial, fungal, and total CFU) with an increase in filtration time, and in each sample group, bacterial CFU recovery was lower than fungal CFU. Bacterial CFU recovery was significantly different between the two short- and the long-filtration period groups (Mann–Whitney-*U*, *P*-value < 0.00 in both cases), but no significant difference was noted between the 20- and 60-min groups (*P*-value 0.46). All fungal CFU groups were significantly different with *P*-values of 0.02 (20 vs. 60 min), <0.00 (20 vs. 420–480 min), and <0.00 (60 vs. 420–480 min). Total CFU recovery showed a similar trend with *P*-values of 0.03 (20 vs. 60 min), <0.00 (20 vs. 420–480 min), and <0.00 (60 vs. 420–480 min). As Table 3 illustrates, the liquid-impinger CFU recoveries were higher for all categories of the membrane-filtration samples. While the impinger fungal and total CFU recoveries were significantly higher than the 20-min membrane-filtration samples (*P*-values < 0.00), bacterial CFU were not (*P*-value 0.48). In comparing liquid impingement recovery to 60-min and 420 to 480-min membrane-filtration

samples, impingement CFU was significantly higher for both fungal and total groups with all *P*-values < 0.00. In reference to bacterial CFU, liquid impingement CFU recovery was significantly higher than the 420 to 480-min samples (*P*-value 0.03), but not in comparison with the 60-min samples (*P*-value 0.28).

Table 4 lists the membrane-filtration and liquid-impinger data for the samples collected within the primary visitor cave system of Carlsbad Caverns National Park. The highest membrane-filtration counts for bacterial CFU occurred in the lunch room sample (11.8 CFU m⁻³) followed by 5.8 CFU m⁻³ at the cave mouth (Site A) and site H, 2.9 CFU m⁻³ at site E, and 0.0 CFU m⁻³ in Left Hand Tunnel. Fungal CFU were only detected in membrane-filtration samples at the cave mouth (11.8 CFU m⁻³), site E (5.8 CFU m⁻³), and in the lunch room (11.8 CFU m⁻³). Total membrane-filtration CFU ranged from 5.8 to 23.6 CFU m⁻³, with the highest counts occurring just within the cave mouth (17.6 CFU m⁻³) and in the lunch room (23.6 CFU m⁻³). Liquid-impinger samples were collected simultaneously at sites E and Lunch Room A, which produced total CFU counts of 64.1 and 205.2 m⁻³, respectively. For these two sites, impinger counts were 4.4× (bacterial CFU), 8.8× (fungal CFU), and 7.4× (total CFU) higher at site E than observed in the membrane-filtration samples. At the Lunch Room A site, all CFU categories were 8.7× higher than observed in membrane-filtration samples.

Table 4 Carlsbad Caverns membrane and liquid-impinger data

Date	Sample site	Membrane filtration 40 min at $\sim 8.7 \text{ L min}^{-1}$			Liquid impinger 30.0 min at $\sim 250 \text{ L min}^{-1}$		
		Bacterial CFU	Fungal CFU	Total CFU	Bacterial CFU	Fungal CFU	Total CFU
4/1/2009	Site A	5.8	11.8	17.6			
4/1/2009	Site E	2.9	5.8	8.7	12.8	51.3	64.1
4/1/2009	Site H	5.8	0	5.8			
4/1/2009	Lunch Room A	11.8	11.8	23.6	102.6	102.6	205.2
4/1/2009	Left hand tunnel	0	0	0			
Average		5.3	5.9	11.2	57.7	77.0	134.7

Colony-forming units (CFU) expressed as per cubic meter of air

4 Discussion

Two of the atmospheric sites utilized in this study, the tropical mid-Atlantic and Mount Bachelor sites, from a microbiological perspective can be classified as 'arduous environments.' In this case, an 'arduous environment' is defined as being one where only the more stress-resistant members of a foreign microbial community are culturable using standard nutrient agar and incubation regimes. Clouds of desert dust emanating off the northwest coast of Africa typically take 3–5 days to cross the Atlantic and impact air quality in the Caribbean and Americas. Given that the Bodele Depression is the primary source region for trans-Atlantic African dust (Koren et al. 2006), dust-borne microorganisms reaching the mid-Atlantic research site described in this study had probably been suspended in the atmosphere for a minimum of 48 h and thus had experienced prolonged stress due to UV exposure and desiccation (wind speeds averaged 5.6-m s^{-1} for the duration of the study). The atmospheric-particle load at this research site was strikingly visible on dust days, producing daytime haze and brilliant Saharan orange sunsets in relation to equally striking clear-condition days that were 'blue marble' in nature. Dust-cloud particles can provide UV shielding for dust-borne microorganisms; NASA has demonstrated a UV attenuation rate in these clouds of $\sim 50\%$. This shielding may significantly influence survival as demonstrated by the diverse culturable community of Saharan dust-borne bacteria and fungi that can be found at mid- and cross-Atlantic locations (Griffin et al. 2001, 2003, 2006). One stress-resistant trait that is readily visible is cell pigmentation. Clear or non-pigmented CFU are common and can be

numerous in environments where the microorganisms have not been in the atmosphere for extended periods of time as observed with atmospheric samples collected during dust storms in Mali, Africa (Kellogg et al. 2004). Clear or non-pigmented colonies are not common in UV stressed environments, and no such CFU were observed in any of the mid-Atlantic or Mount Bachelor samples. It is evident that pigmentation affords some degree of protection during atmospheric transport and that long range or extreme altitude atmospheric transport 'culls.' In the atmosphere, everything is not everywhere.

As demonstrated in Table 1, the highest mid-Atlantic recoveries occurred in the short-period samples. The average total CFU (bacteria and fungi) for the short-period samples was 2.9 CFU m^{-3} even though recovery only occurred on four of 16 days versus 0.8 CFU m^{-3} for the long-period samples where recovery was noted on 13 of 16 days ($3.7\times$ higher recovery in the short-period samples). This illustrates that on many days during which no bacteria or fungi were recovered with the short-period samples, there were low recoveries in the long-period samples. Although these data may indicate an obvious benefit of using longer-period sampling (selection for stress-resistant microorganisms or, at the very least, recovering something), the noted CFU may well have been an artifact of where the microbe(s) was (were) in the sample period (last few liters vs. the first few liters of a large volume sample = less filter surface desiccation stress) and thus is no indication of actual concentration as has been assumed by some who have utilized long-period sampling (Prospero et al. 2005).

These data trends were also noted at the Mount Bachelor site. At this high-altitude location,

the short- versus long-period differences in recovery of culturable bacteria and fungi were lower than those observed at the low-altitude mid-Atlantic site. Concentration differences in the short-period samples were 2.7 \times , 1.2 \times , and 1.5 \times higher than those observed in the long-period samples for culturable bacteria, fungi, and total CFU, respectively. This Mount Bachelor versus mid-Atlantic decrease in total CFU recovery may be due to differences in UV exposure experienced at the two different altitudes. This type of selective pressure may result in the recovery of more stress-resistant bacterial and fungal species at elevated altitudes, resulting in a decrease in differences in recovered CFU. UV-C measurements at Mount Bachelor over the first 7 days of the study averaged 0.22 mW cm⁻² (using a Lutron Electronic Enterprise, Co., Ltd., Taipei, Taiwan, UVC-254 Ultra-Violet Radiometer) versus a more ground-based observation of 0.14 mW cm⁻² (four day average of peak daily measurements as observed at the Tallahassee, Florida location). As observed with the mid-Atlantic data, CFU recovery was more likely with elevated concentrations of atmospheric particulates. The particle-count CFU relation was significant with fungal and total counts in the lower-volume samples at Mount Bachelor. This would be expected given the decrease in sample-period stress relative to the long-period samples and that fungal spores provide protection from desiccation and UV-related stress. The use of the OMNI-3000 impinger at the Mount Bachelor site clearly gave superior recoveries of bacteria and fungi. The average recoveries for bacteria, fungi, and total CFU m⁻³ were 26.7, 22.8, and 27.4 \times higher with the impinger than the membrane-filtration short-period assay, respectively.

Table 3 contains the Tallahassee, Florida, site data and demonstrates that this ground-level atmospheric environment is biologically rich in comparison with the more remote atmospheric environments found at the mid-Atlantic and Mount Bachelor locations. The between-building courtyard location is surrounded by trees, plants, and grass all of which provide a base for microbial growth and are significant sources of airspora. These data also demonstrate a decrease in recovered bacteria, fungi, and total CFU with increased sample period. Between 20- and 450-min sample periods, CFU recoveries m⁻³ dropped from averages of 40.0–3.5 for bacteria and 100.4–16.2 for fungi. From the \sim 20- to the \sim 60-min period

bacteria and fungi CFU recovery averages dropped 33.5 and 36.9%, respectively. From the \sim 20- to the \sim 450-min period bacteria and fungi CFU recovery averages dropped 91.3 and 83.9%, respectively. CFU recovery averages m⁻³ with the liquid impinger were 189.5 for bacteria and 1,400.3 for fungi, recoveries that dwarf those obtained with membrane filtration.

The Carlsbad Caverns CFU recoveries m⁻³ averaged 5.9 for both bacteria and fungi using a membrane-filtration sample period of 40 min. What is interesting here is that the highest recoveries (11.8 CFU m⁻³ for both bacteria and fungi) with membrane filtration occurred at the Lunch Room A site, a location within the cave system where individuals mingle at the gift stands and consume food purchased at the snack bar. With regard to total CFU m⁻³, recoveries at Site A near the mouth of the cave were 17.6 and dropped to 0.0 as the site locations progressed into the system with the exception of the Lunch Room A site (23.6 CFU). Again, as observed in samples obtained from the other sites outlined in this report, the average recoveries m⁻³ were superior when the impinger was used, $>10\times$ for all CFU categories, versus the membrane-filtration unit.

The data from the four research sites demonstrate that membrane filtration has its benefits and limitations. The primary benefit as stated is cost. The unit we utilized in this study was assembled using a small vacuum pump, PCV pipe, and a plastic tool-box as a housing. In all, the unit cost less than \$225.00 and can be powered using an AC or DC (with a DC to AC inverter) source. The unit is light, very portable, and very durable. In addition, pre-sterilized and individually packaged plastic funnels loaded with 0.2- μ m cellulose filters are readily available in lots of 50 at minimal cost (\$210.17, Thermo Scientific Nalgene Analytical Test Filter Funnels available through Fisher Scientific Catalog #09-740-30G). Utilization of a filter with a pore size in the range of 0.2 μ m ensures that all the bacteria and fungi in a given airmass are likely trapped on the filter surface, allowing non-culture-based studies to be conducted. Unfortunately, the inability to efficiently extract nucleic acids from filter surfaces has traditionally limited this application. The emergence of new extraction assays may allow the use of membrane filters for this approach. We recently had success with DNA extraction from membranes used to capture airborne microorganisms utilizing MOBIO's new

PowerWater DNA Isolation Kit (a kit designed for use in nucleic extraction from membrane filters used in aquatic studies). The limitations of membrane filtration are low recoveries of culturable microorganisms due to desiccation, the need to take multiple samples for different study types, the inability to accurately determine equivalent volume assayed if sectioning the filter, limitations associated with atmospheric viral studies, nutrient shock for culture-based studies when using ‘thin’ filters, and efficient extraction of both DNA and RNA from various filter types.

Although impinger use has been plagued with variation in capture efficiency, in particular issues associated with the finer particles (i.e., free unbound viruses), the impinger utilized in this study gave superior recoveries to those observed with the short-period membrane-filtration assay. Two significant benefits to use modern high-volume liquid impingers are the ability to rapidly sample large air masses and the ability to accurately split the impingement media for use in multiple studies (culture- and non-culture-based), and equate these splits accurately to a given volume of air. High-volume liquid impingers are much more expensive than ‘home-made’ membrane-filtration units, ~\$13K with supplies versus ~\$200, respectively, for this study, but do provide much more flexibility with regard to sample analyses as well as higher capture rates as demonstrated. The data outlined in this report drive home a point. With regard to recovering microorganisms from various atmospheric settings, methods have their limitations. Whereas the impinger utilized in this report outperformed the membrane-filtration unit, expect significant variation between those high-volume impingers that are currently available. Clearly, methods/comparison research is needed as well as an understanding of method limitations by those conducting aeromicrobiology research. Look in the scientific literature, back to the early nineteenth century, numerous atmospheric microbiology projects have utilized membrane filtration, and it should be obvious that previous concentration data reported in ‘like-method’ publications should be considered conservative at best.

Acknowledgments Appreciation is extended to Dr. Dan Jaffe of University of Washington at Bothell for support at Mount Bachelor Observatory and Dale Pate and Paul Burger of the U.S. National Park Service for assistance at Carlsbad Caverns National Park. Any use of trade, product, or firm

names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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