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A Method for Collecting Atmospheric Microbial Samples From Set Altitudes for Use With Next-Generation Sequencing Techniques to Characterize Communities

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ABSTRACT: Dispersal of airborne microorganisms is an important ecological process, resulting in the distribution of bacteria to all habitats on Earth. Investigation of this process is limited by the ability to collect uncontaminated high-altitude microbial samples for use with next-generation sequencing approaches. Here, we describe the design of a Remote Airborne Microbial Passive sampling system. Troubleshooting experiments demonstrate that the samplers collect adequate DNA for bacterial 16S rRNA (ribosomal RNA) amplicon–based Mi-Seq sequencing at 2 and 150 m from the ground. When samplers are closed, they retain only a low number of sequences, and may be used as a negative control. We also demonstrate that the optimal amount of collection dishes to include in the sampler is 8, and that freezing collection dishes at −80°C is an alternative to immediate DNA extraction. Samplers may be used to address a variety of ecological and human health–related questions.

KEYWORDS: Atmosphere, sampler, microbe, bacteria, 16S rRNA, community

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Introduction

The atmosphere interconnects all terrestrial and marine habitats on Earth and is thought to be responsible for the wide dispersal of microorganisms across the Earth's surface. Once deposited on the surface, microorganisms are responsible for important steps in all major nutrient cycles, yet very little is known about the mechanisms that control atmospheric microbial dispersal to ecosystems.[1,2] This is because widespread sampling of microbial communities at many different altitudes throughout the atmosphere has not been conducted to address these questions. There are several reasons for this. First, the field of aerobiology is relatively new. We know that there are thousands of microorganisms in a liter of air[3] and that they are present throughout the atmosphere, even in the stratosphere[4]; microbial communities vary with meteorological events[5,6] and human activity[7] and some microbes are likely active while airborne.[8] However, there are still many unanswered questions about the ecology of these microorganisms. Next-generation sequencing techniques that can completely characterize microbial community composition through DNA or RNA-based approaches have only been available for the past decade and have only recently been applied to airborne samples.[9,10] In addition, next-generation approaches require sufficient microbial nucleic acid concentrations to assess community composition, which is difficult to acquire from low-biomass atmospheric samples.[2] As a result, most studies that assess airborne microbial communities use approaches that rely on samples collected close to the terrestrial surface.[7,11–20] Although information about the effect of land use, temporal dynamics, or environmental change on local air has been gained from near-surface studies, higher altitude samples are required to address questions about regional or global microbial dispersal patterns.

The physical difficulties involved in obtaining high-altitude samples are significant, and often scientists must work within specific flight restrictions to obtain samples. Another concern is that sufficient microbial biomass must be collected from the air samples to conduct next-generation sequencing. Finally, because these samples are typically low-yield, it is also important to ensure that samples are not contaminated from nontarget altitudes or other sources. Given these considerations, high-altitude microbial research has largely been limited to quantification of airborne particulates,[21–23] microscopic observation,[24,25] and cultivation-based studies.[4,26–30] To our knowledge, DeLeon-Rodriguez et al[5] conducted the only study that combined higher altitude tropospheric sampling with DNA-based molecular analyses of bacterial communities. In that study, samples were collected using a vacuum filtration system from the NASA DC-8 Airborne Science Laboratory jetliner; they found that bacterial cells constitute around 20% of the total particles 0.25 to 1 µm in diameter and were an order of magnitude more abundant than fungal cells.[5] Intriguingly, bacterial communities in the mid and upper troposphere originated from many different terrestrial sources—including soil,

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Ecological questions:

Is the atmosphere a microbial habitat or just a mechanism for dispersal?
Does altitude have a habitat filtration effect on airborne microbes?
Does urbanization have a homogenizing effect on airborne microbial communities?
What microbial functional pathways are in the air?

Human health questions:

How do microbes disperse within a built environment?
What pathogenic microorganisms are present in indoor and outdoor air?
Are bioterrorism agents present in the air?
Is antibiotic resistance transferred through air?

Figure 1. Many current scientific questions that relate to both microbial ecology and human health remain unanswered because airborne microbial sampling approaches have not been developed for use with next-generation sequencing technology.

freshwater, and plant material—but several bacterial community members were ubiquitous across all samples.[5] These results suggest that bacterial dispersal is inherently linked to higher atmospheric altitudes and provides evidence to support the hypothesis that the atmosphere may even serve as a native habitat for some microbial species.[2,8,19]

Although using a vacuum sampler from powered aircraft can provide the necessary microbial biomass to conduct these types of studies, they are limited in several ways.[5] First, relying on airplane flight times and patterns limits the ability for scientists to conduct fortuitous sampling at any location or altitude. Second, powered aircraft may introduce contaminants to the vacuum filtration system from exhaust and nontarget altitudes during ascent and descent. In contrast to powered-flight approaches, passive balloon-borne samplers could be more accessible and adaptable for scientists, but typical vacuum pump and liquid impinger-type samplers are too heavy for use with this type of approach. Access to a simple and lightweight sampling system can allow scientists to conduct comprehensive studies that use molecular approaches to examine bacterial, archaeal, and fungal communities from near-surface and high altitudes and address numerous questions related to both ecology and human health (Figure 1). Several types of passive samplers have been applied to airborne microbial sample collection and show promise for collecting sufficient microbial biomass for next-generation sequencing applications.[20,31]

In this study, we addressed these sampling challenges by developing a new Remote Airborne Microbial Passive (RAMP) sampling system. The system is similar in design to the aerosol sampler presented in Bryan et al[30] but is designed to passively collect sufficient quality and quantity DNA for molecular microbial community analysis using next-generation sequencing approaches. The RAMP samplers we designed are meant to be suspended from either moored or released balloon systems to enable sample collection from any specific target altitude for any length of time. Our RAMP samplers suit several engineering design requirements: (1) they are built from materials that can be decontaminated using ethanol and UV irradiation; (2) they weigh less than 2.7 kg (6 lbs), to adhere to Federal Aviation Administration payload regulations; (3) they are fitted with sensors to collect weather data associated directly with the samples, including temperature, barometric pressure, and relative humidity; (4) they open and close using a remote control operated by a researcher on the ground. Opening the RAMP sampler exposes a series of sterile collection dishes to the atmosphere while preventing contamination from nontarget altitudes during ascent, descent, and transportation. We designed a passive sampling method because they are lightweight and are more reliable to operate remotely than active samplers. Passive sampling also allows for collection of intact cells that may be used for culture- or microscopic-based approaches, in addition to molecular analyses, so it broadens the scope of use for the RAMP samplers.

We conducted several experiments to test the RAMP samplers and develop best practices for handling samples. When conducting field experiments, possibly in remote locations, it is crucial to know that samplers are collecting the desired biomass and are also preventing introduction of contaminants during transport to and from the lab and field site. First, we hypothesized that (1A) open RAMP samplers will collect sufficient bacterial biomass from the air to yield extracted DNA concentrations that can be used with next-generation sequencing approaches that characterize community composition and (1B) closed samplers yield only negligible amounts of microbial DNA, indicating that RAMP samplers prevent contamination during transport and collection dish attachment and removal. The RAMP samplers we designed can carry up to 16 passive collection dishes. We hypothesized that (2) RAMP samplers outfitted with 16 sterile collection dishes will accumulate a sufficient amount of DNA for next-generation sequencing and the use of fewer dishes will result in lower DNA yields. Finally, extracting DNA at field sites is challenging because researchers must transport large amounts of equipment, including a sterile biosafety hood, centrifuge, pipettes, and extraction chemicals. However, this effort may be necessary to determine the composition of airborne microbial communities most accurately. We hypothesized that (3) following collection, DNA extraction within 24 hours yields different

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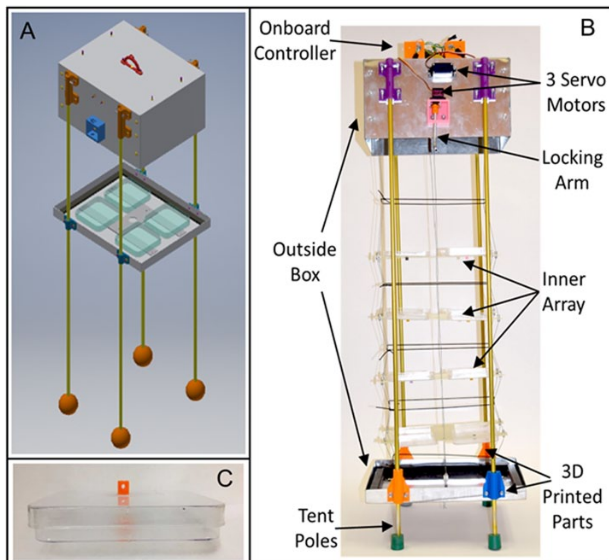

Figure 2. Remote Airborne Microbial Passive (RAMP) sampler design. (A) Computer-Aided Drawing (CAD) image of the RAMP sampler box showing array concept. (B) Photo of the RAMP sampler box open showing the features of the sampler. (C) Photo of a collection dish with a 3-dimensional (3D)-printed connector attached.

airborne bacterial communities from samples that are kept refrigerated for 1 week or frozen for 2 weeks. Finally, we demonstrated that the RAMP sampler system is capable of collecting samples from 150 m in the atmosphere and that those samples can be used with a next-generation sequencing approach to characterize bacterial community structure. The results of this work describe sampler design and best practices for sampling with a new type of airborne microbial sampler that can be used to collect samples for community analysis from higher altitudes than are typically investigated.

Materials and Methods

RAMP sampler system design

The RAMP sampler we designed holds 16 sterile collection dishes on an expanding array (Figure 2). The full system consists of the RAMP sampler box, an onboard computer, and a remote control box that sends commands to the onboard controller. The total mass of the RAMP sampler and the onboard controller box is 2.46 kg (5.44 lbs). The onboard computer box controls the opening and closing of the expanding array of collection dishes and records time and weather data. We constructed a total of 9 individual RAMP samplers for the troubleshooting experiments conducted here and for future studies.

Onboard controller. Electronics are housed in an onboard controller box. The RAMP sampler system uses an Arduino Mega 2560 microcontroller as its onboard computer with a stacked SD logger shield to record time and weather data (pressure, humidity, and temperature) obtained from a BME280 (Adafruit, New York, USA) sensor during sampling. The stacked boards communicate across a serial peripheral interface. We programmed the onboard computer to continuously record time and weather data when it is turned on. All other commands are executed only when a signal is received from a remote control, operated by a researcher from the ground. The remote is also operated with an Arduino Mega 2560 microcontroller. Communication between the RAMP sampler onboard computer and the remote occurs between the microcontrollers, each of which is outfitted with a wireless 433-MHz frequency communication chip (HC-12). We designed the remote so that the user presses a momentary button and the remote sends a unique signal that correlates with a unique command on a specific RAMP sampler. Thus, several individual samplers can be controlled by the same remote through the use of different commands. These commands begin and end the sampling operation, open and close the locking arms, and cause the crankshaft to reel up and down for 1 second. All Arduino code is included in the supplemental materials of this article.

The "begin sampling operation" commands 2 servo motors to rotate locking arms from a closed position where they prevent the sampling box from opening, to an open position. Once the locking arms are rotated, a continuous rotation servo motor connected to a crankshaft rolls down the expanding array for a specified time period to expose the collection dishes. When the RAMP sampler is opened, the onboard microcontroller performs a software reset that returns the processor to an idle state. This idle state awaits another HC-12 communication signal while collecting weather data and recording the time-stamped data to an SD card. The "end sampling operation" retracts the expanding array until a limit switch sends a signal to the onboard computer that the box is closed. Then, locking arms are rotated to a closed position. Two additional momentary buttons execute commands that are helpful for troubleshooting sampler box operation and in the event the box does not completely close or open. These commands open/close the locking arms and turn the crankshaft up/down for 1 second. All servo motors and onboard electronics are powered by 7.4-V 2-cell lithium ion polymer batteries.

Mechanical system. Complete drawings of the RAMP sampling system when it is open and closed are included in the supplemental materials. We constructed the RAMP sampler exterior from 0.8-mm-thick 3003 aluminum. We selected this material because it is lightweight, bendable, and easy to decontaminate with ethanol and UV irradiation. We constructed an expanding array of 4 layers of "H"-shaped G10/FR4 fiberglass board which holds the collection dishes for sampling (Figure 2A shows one of these layers). The aluminum and G10 board were water jet cut to ensure the custom boxes and arrays are identical. Each layer of G10 holds 4 collection dishes, so each RAMP sampler holds 16 collection dishes for microbial collection. Total microbial collection surface area is 1444 cm^2. The expanding array allows air to flow over the sterile collection

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dish surfaces while also allowing the sampler to compactly close and prevent contamination during transport. The collection dishes are secured to the G10 arrays with a small 3-dimensional (3D)-printed part that contains a hole where a cotter pin is used to lock the dishes in place (Figure 2C).

In the closed configuration, the sampler is 16.5 cm (6.5 in) tall, 29.2 cm (11.5 in) long, and 24.1 cm (9.5 in) deep, and the arrays rest on top of the collection dishes that are in the array below them or the bottom lid of the RAMP sampler. The sampler expands to 95.3 cm (37.5 in) tall when it is open for sampling. We included aluminum tent poles with blunt rubber stoppers on one end for safety, which keep the top and bottom lids of the sampler aligned so that it can seal closed when sampling is complete. The tent poles are held in place with 3D-printed tube collars, so the RAMP sampler cannot twist or slide. We created numerous 3D-printed parts using an Anet A8 3D printer, in addition to those previously discussed, for the RAMP samplers. These include attachments for servo motors, housings for electronics, and a handle from which the sampler hangs (Figure 2B). We used polylactic acid plastic filament for the 3D-printed parts because it will not deform at temperatures less than 60°C, is relatively strong, and prints easily with available 3D printers.

We designed the RAMP sampler to use 3 servo motors. One is a SpringRC High Torque Continuous Rotation (SM-S4315R) servo, and the other 2 are Tower Pro MG90S high-torque-limited-range servos. All servos have metal gearing and shafts and each servo requires a coupler to connect the shaft of the servo to an aluminum tube. The continuous rotation servo is used as the crankshaft motor. This servo can complete multiple rotations allowing the servo to extend and retract the expanding array based on the amount of time set in the onboard computer coding or the command that the onboard computer receives from the remote control. This servo has an aluminum coupler that is made from 1.3 cm (0.5 in) diameter 6064 aluminum rod. Holes in the sides of the coupler are used to make mechanical connections. The coupler connects the shaft of the servo to the 1.3 cm (0.5 in) diameter 3003 aluminum tube crankshaft. The limited rotation servos are used for the locking arms that have a 3D-printed plastic coupler connecting the servo shaft to a 0.64 cm (0.25 in) diameter aluminum tube. We bent this aluminum tube to provide a lock, so the bottom lid of the RAMP sampler remains sealed shut when not collecting samples. This is meant to prevent accidental contamination of the decontaminated box or collected samples during transport to/from a field site or while the target elevation is reached. We used a spring pin to make the connection between the locking arm servo motor coupler and the aluminum tubing.

We used uncoated stainless steel cable to connect the bottom lid of the RAMP sampler to the crankshaft in the top interior of the sampler. When the motor turns, the cable—which is wrapped around the crankshaft—uncoils, lowering the aluminum lid. The GR10 arrays are evenly spaced, 12.7 cm (5 in) apart, along the cable. When sampling is complete, the crankshaft servo motor reverses direction and wraps the cable around the crankshaft again. We used close cell foam to create a seal when the sampler is in the closed configuration, and the 2 locking servo motors rotate the aluminum arms under the bottom lid to secure it in place and prevent the lid from opening.

Sampler preparation and sample collection

We prepared all RAMP samplers and conducted all DNA extractions, sequencing, sequence analysis, and statistics according to the following standard procedures, except where changes were necessary to address our hypotheses. Differences are described in a later section. All experiments were conducted in December 2016 to January 2017, in Kalamazoo, Michigan, USA.

RAMP sampler preparation and sampler dish removal. We coated square sterile (9.5 cm × 9.5 cm) collection dishes (VWR International 60872-480, Radnor, PA, USA) with 200 µL of autoclave-sterilized silica gel (dimethicone, cyclomethicone, dimethicone/vinyl dimethicone crosspolymer). We applied the silica gel to both lids and bottoms of the collection dishes inside a UV-sterilized biosafety cabinet fitted with a HEPA (high-efficiency particulate air) filter. We spread the gel evenly across the surfaces using a sterile syringe and flocked swab (Puritan Diagnostics LLC 25-3406-H, Guilford, ME, USA). We then replaced collection dish lids onto the dish bottoms to maintain sterility. Outside the hood, we glued the small 3D-printed part described above (Figure 2C) onto the outside center of the top and bottom of each collection dish. To prepare the RAMP samplers, we decontaminated all surfaces inside and outside of the sampler by swabbing them with 70% ethanol using sterile gauze and then placed it in an upright UV decontamination chamber that was custom built for this project. The investigators responsible for decontamination and array preparation wore long-sleeve disposable plastic gloves that were also decontaminated with ethanol. While hanging in the chamber, we attached 16 open collection dishes to the 4 hanging H-arrays in the RAMP sampler. We then UV irradiated the open array and RAMP sampler for 15 minutes at a wavelength of 254 nm. Finally, we closed the samplers using the remote control from outside of the UV chamber. We repeated this process for each sampler and then conducted sampling for each troubleshooting experiment described in the next section. Within 24 hours of sampling, we decontaminated the outsides of the closed RAMP samplers by swabbing them with 70% ethanol and UV irradiated them for 10 minutes in the UV chamber. We then opened the boxes using the remote and removed the collection dishes while still in the chamber. We replaced the collection dish bottoms and tops to maintain sterility and then placed them back into plastic plate storage

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bags. The plates were refrigerated at 4°C until DNA extraction was completed.

DNA extraction and next-generation sequencing. We used one sterile flocked swab to remove airborne bacterial samples and silica gel from the surfaces of all 16 collection dishes used in each RAMP sampler. We extracted DNA from the swabs using a MoBio PowerWater DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following a modified version of the kit protocol to maximize DNA yields, with the following modifications: (1) the swab was placed directly into the PowerWater (PW) bead tubes instead of a filter, (2) 1.5 mL of solution PW1 was added to the bead tubes, (3) 360 μL of Solution PW2 was used, and (4) DNA was eluted in a final volume of 30 μL of Solution PW6 in the final step. For every round of extractions, we included a negative control, which consisted of a flocked swab dipped in autoclaved silica gel. All extracts were stored at −80°C prior to sequencing. We chose a next-generation sequencing approach that relies on the 16S rRNA (ribosomal RNA) gene, a phylogenetic marker that is present in all bacteria and is commonly used for community analyses. Amplicon preparation and Mi-Seq (Illumina, San Diego, CA, USA) sequencing was conducted at Michigan State University Genomics Core Facility. Bacterial 16S rRNA genes were polymerase chain reaction (PCR) amplified using the 515/806 primer pair, specific for the V4 hypervariable region.[32] A subset of PCR products was analyzed on a 1% agarose gel stained with ethidium bromide to ensure that samples contained sufficient DNA for amplification procedures. DNA libraries were normalized using the SequalPrep Normalization Plate Kit, 96 wells (Thermo Fisher Scientific, Waltham, MA, USA), and samples from each replicate plate were pooled into single wells. Pooled samples were quantified using a Kapa Biosystems qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA), and samples were normalized to an equal concentration. Each sample pool was loaded on an Illumina Mi-Seq flow cell v2 and sequenced using a 500 cycle (PE250) reagent kit. Bases were called using Real-Time Analysis (RTA) software v1.18.54, and RTA output was demultiplexed and converted to fastq files using Illumina Bc12Fastq v1.8.4. All fasta files have been deposited in the publicly available NCBI Sequence Read Archive under BioProject identification number PRJNA413697.

Following sequencing, we performed several data cleanup steps. We conducted primer sequence removal, quality filtering, and merged forward and reverse reads using PANDAseq version 2.8.[33] We excluded sequences from analysis if they contained ambiguous base calls, runs of greater than 8 identical bases, quality scores of less than 0.9 in a sliding scale of 0 to 1, fewer than 247 bases, more than 275 bases, or sequence overlap of less than 47 bases. Chimeric sequences were identified and filtered with QIIME v.1.9.1 using the vsearch algorithm.[34,35] We clustered the remaining sequences into operational taxonomic units (OTUs) using the pick_open_reference_otus.py script in QIIME, which selected open-reference OTUs via the vsearch algorithm and removed singleton sequences. We assigned taxonomy using the Ribosomal Database Project (RDP) classifier against the Silva version 128 reference database.[36,37] We removed any OTUs that were identified in the negative controls included with each DNA extraction, as well as any OTUs that were associated with archaea, chloroplasts, and mitochondria. We used unrarefied data sets to statistically compare airborne bacterial communities.

For all data sets, we determined (1) the total number of high-quality bacterial 16S rRNA sequences detected in each sample (ie, analogous to total abundance), (2) the total number of bacterial OTUs detected in each sample (ie, analogous to bacterial species richness), and (3) the phylogenetic diversity (PD) of each sample (ie, a combination of bacterial richness and evenness). We acknowledge that quantifying 16S rRNA bacterial sequences as a proxy for total abundance is subject to several biases because many bacteria carry more than one copy of the 16S rRNA gene and because of DNA extraction and sequencing biases inherent to these techniques, so this measurement should only be interpreted as the abundance of detected bacterial 16S rRNA genes and not total bacterial abundance. For these 3 univariate metrics, we used analysis of variance (ANOVA) to determine significant differences in the means ($\alpha = 0.05$). When ANOVA results were significant, we performed Tukey's Honest Significant Difference (HSD) post hoc analysis to determine which treatments differed from others. We used R statistical software (version 3.3.2) for all univariate statistical analyses.

When relevant, we also used multivariate approaches to examine differences between airborne bacterial communities from different treatments (ie, beta diversity). We used 2 different approaches to assess beta diversity using QIIME v. 1.9.1: (1) the unweighted UniFrac distance approach compares the presence/absence of OTUs in communities and (2) the weighted UniFrac distance approach compares the relative abundances of OTUs in communities.[38] Because both unweighted and weighted UniFrac distance matrices yielded similar results for all tests, we only present the results using the unweighted UniFrac distance matrix. We visualized multivariate community differences and similarities using principal coordinates analysis (PCoA). This ordination approach defines multivariate communities as single points and plots them on 2 to 3 axes based on how similar or different the communities are to each other. Finally, we conducted permutational ANOVA (permanova) tests to determine whether significant differences existed between communities associated with each treatment for each of our experiments described below. Multivariate statistical approaches in QIIME rely on the vegan 2.4-4 package developed for R.[39]

RAMP sampler troubleshooting experiments

To test our hypotheses and ensure that our RAMP samplers performed as expected, we conducted several experiments. First, we needed to ensure that the RAMP samplers collected sufficient airborne bacterial DNA for downstream extraction,

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Illumina Mi-Seq sequencing and community analyses. We hypothesized that open samplers collect sufficient bacterial biomass to yield extracted bacterial DNA suitable for amplification and sequencing. To test this, we followed the protocol described above and sampled using 2 RAMP samplers hung in an enclosed garage and 3 RAMP samplers hung in an outdoor yard. Samplers were within 2 m of the ground and remained open for 8 hours for both indoor and outdoor sampling efforts. We closed them remotely, transported them to the laboratory and conducted the procedures as described in the previous section.

In addition to collecting adequate samples for next-generation sequencing approaches, we also needed to ensure that the RAMP samplers remained decontaminated during storage and transport. We hypothesized that closed RAMP samplers would yield only negligible amounts of 16S rRNA sequences and would not become further contaminated during storage and transport. To test this hypothesis, 9 RAMP samplers were stored closed in an enclosed garage for 24 hours. We transported the closed samplers to the laboratory and conducted the procedures as described in the previous section.

Swabbing the sample from the 16 collection dish surfaces after sampling is a rate-limiting step in our RAMP sampler preparation process. We wanted to ensure that 16 dishes were necessary to collect sufficient bacterial DNA for downstream processing or whether similar amounts of bacterial sequences and diversity could be observed with fewer collection dishes. We hypothesized that 16 dishes would be required to collect sufficient amounts of DNA for community analyses, and that using fewer dishes would result in less high-quality sequences, fewer OTUs, and lower diversity. To test this, we prepared 8 RAMP samplers as described above and hung them in a line within 2 m of the ground, outside for 8 hours. Plates were removed from the samplers as described above and randomly assigned to 1 of 5 treatments: 16 dishes, 12 dishes, 8 dishes, 4 dishes, and 2 dishes. Using this design, there were 3 replicates for each treatment. We conducted the procedures described above for each treatment. In addition, we also examined collector's curves for each treatment, which plots OTU richness vs number of sequences observed (sampling effort) through iterative resampling of each data set. We conducted 10 iterations for every 10 sequences observed. We also examined shared (beta) diversity among the treatments using PCoA and permanova approaches.

Finally, for remote sampling locations, it is often a challenge to carry all the equipment necessary to conduct a DNA extraction immediately after sampling occurs. If possible, storing frozen or refrigerated samples for later extraction may be a better alternative. We hypothesized that storing samples for longer than one night would alter the bacterial communities in the samples and potentially produce inaccurate results. To test whether different storage conditions yielded different results, we prepared 9 RAMP samplers and hung them within 2 m of the ground in an outdoor yard. After 8 hours of sample collection, we remotely closed all samplers and transported them to the laboratory. All dishes were removed from the samplers. We refrigerated collection dishes from 3 RAMP samplers overnight at 4°C and extracted DNA within 24 hours. We also refrigerated collection dishes from 3 other RAMP samplers for 1 week and then extracted DNA from these samples. Finally, we froze collection dishes from another 3 RAMP samplers at −80°C for 2 weeks prior to DNA extraction. Once DNA was extracted, we followed the procedures described above. We also examined metagenomic profiles predicted from 16S rRNA sequences using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) version 1.1.1, implemented through the Langille Lab PICRUSt Galaxy Instance (http://galaxy.morganlangille.com/).[40]

Balloon-borne sampling using a RAMP sampler at 150 m. We launched a RAMP sampler to a target altitude of 150 m using a moored Helikite system. The RAMP sampler was opened remotely at 09:02 and closed at 15:00. Weather data were collected by the onboard sensors during the entire time of flight. Once the sampler was retrieved, we transported the sampler to the lab to remove the collection dishes and conducted the procedures for DNA extraction and sequence analysis as described in the previous section.

Results and Discussion

We constructed 9 RAMP samplers, as described in detail above. We tested the capacity of the RAMP samplers to collect adequate samples of airborne bacteria for use with 16S rRNA-amplicon–based Illumina Mi-Seq sequencing, a common next-generation sequencing approach used in microbial ecology studies. We also conducted several tests to inform best practices for sampler preparation and sample storage and tested the sampler at 150 m. The results of these experiments are discussed below.

RAMP samplers collect sufficient bacterial biomass for DNA extraction (H1A) and closed boxes remain decontaminated (H1B)

Conducting airborne bacterial community studies involves several inherent challenges that could significantly affect the results, such as low biomass, low bacterial sequence yields, and the possibility of contamination. To ensure that the RAMP samplers we designed collected adequate DNA for Mi-Seq sequencing, we conducted 2 sampling efforts in an indoor garage and in an outdoor yard. We note that these 2 tests inherently collected samples from different volumes of air, and that we did not correct for air volume sampled in the comparison, as our primary goal was to demonstrate that the samplers will provide sufficient sample for DNA extraction and next-generation sequencing. We also tested closed RAMP samplers to ensure that they contained negligible amounts of bacterial

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Table 1. A comparison of the average number (±95% confidence interval and min-max range) of high-quality bacterial 16S rRNA sequence reads, OTU richness, and phylogenetic diversity for open and closed RAMP samplers.

TREATMENT	N	16S RRNA SEQUENCES			RICHNESS			DIVERSITY		
		AVERAGE	MAX	MIN	AVERAGE	MAX	MIN	AVERAGE	MAX	MIN
Closed	9	606 ± 534[a]	2609	60	31 ± 11[a]	67	10	5 ± 0.74[a]	6	3
Open indoor	2	19290 ± 463[b]	19526	19054	502 ± 126[b]	566	437	23 ± 0.04[b]	23	23
Open outdoor	3	38636 ± 2772[c]	40172	35812	646 ± 50[c]	695	608	31 ± 1.5[c]	31	29

Abbreviations: OTU, operational taxonomic unit; rRNA, ribosomal RNA; RAMP, Remote Airborne Microbial Passive.
All treatments differed for all measurements; Tukey honest significant difference post hoc test significant differences are denoted with superscript letters ($\alpha = 0.05$).

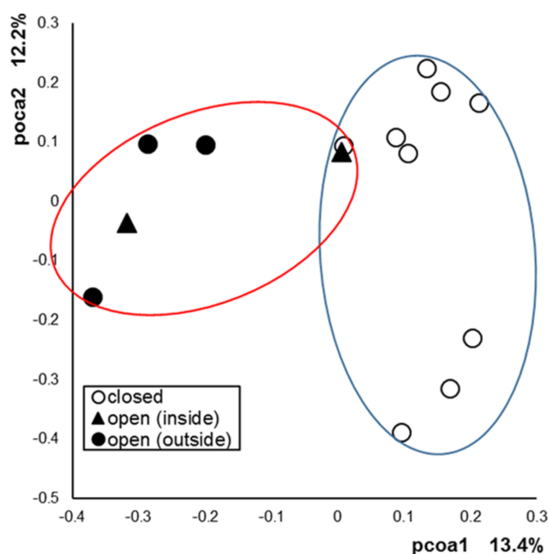

Figure 3. Principal coordinates analysis describing community variation between bacterial communities in closed RAMP samplers (○), samples from outdoor air (•), and samples from indoor air (▲). Closed (blue group) and open (red group) samplers yielded significantly different bacterial communities ($P = 0.001$). Indoor and outdoor air samples were not significantly different ($P = 0.17$). PCoA indicates principal coordinates analysis; RAMP, Remote Airborne Microbial Passive.

sequences. The results demonstrate that closed RAMP samplers contained 30× to 60× fewer high-quality sequence reads than open samplers used in the indoor and outdoor tests (Table 1). This resulted in much lower OTU richness and PD in closed samplers than in open samplers. In addition, RAMP samplers used outdoors collected 1× to 2× more high-quality sequences than those used indoors, which also resulted in higher OTU richness and PD in outdoor samples. We also compared the multivariate bacterial communities collected with each sampling effort (Figure 3). The communities obtained from the closed samplers differed significantly from those used to collect indoor and outdoor air samples (unweighted UniFrac, $P = 0.001$). The composition of indoor and outdoor air samples did not differ (unweighted UniFrac, $P = 0.17$). One indoor air community was very similar to the closed sampler communities (Figure 3), likely because this sample yielded a low amount of 16S rRNA sequences. The OTU richness in that indoor sample was 33 and the PD was 5.06. In comparison, the OTU richness in the closed RAMP sampler that had high community similarity with that indoor sample was 18 and the PD was 3.35.

These results demonstrate that RAMP samplers collect sufficient bacterial biomass from indoor and outdoor air for DNA extraction and use with a next-generation sequencing approach to characterize community structure. Results also demonstrate that the decontaminated closed RAMP samplers have extremely low levels of bacterial contamination in comparison with those used to collect air samples. This is a crucial design aspect because field sampling will require prior sampler preparation, decontamination, and transport to sampling sites. Despite the low number of sequences in the closed RAMP samplers, there were still some contaminant bacterial sequences associated with them. We expect that this issue is a common one across the entire field of microbial community ecology and could be a big consideration for studies where DNA yields are expected to be low. Although DNA extraction and PCR controls have become more common, controls that test for contaminants associated with the sampling equipment itself are rarely included in studies that use molecular approaches to describe microbial diversity. We explored this idea further, with the hypothesis that one or more closed RAMP samplers could be used as a negative control during each field sampling effort to identify contaminant OTUs and remove them from the data set. We suggest this type of approach for any microbial sampling effort where DNA yields are expected to be low.

We examined the OTUs present in closed RAMP samplers and categorized them according to their abundance in the open RAMP samplers. A total of 252 OTUs were shared between at least 1 of the 5 open samplers and at least 1 of the 9 closed samplers in this data set (Figure 4 and Supplemental Data). Of those, 93 OTUs were unique to the closed samplers (Category A) and were not present in the open samplers at all. If a closed RAMP sampler was used as a negative control, then these OTUs should be removed from the data set, but there would be no effect on the composition of the actual samples because they were not present in the samples. Another 29 OTUs were present in both open and closed samplers but were more prevalent in the closed samplers (Category B). Twenty OTUs were

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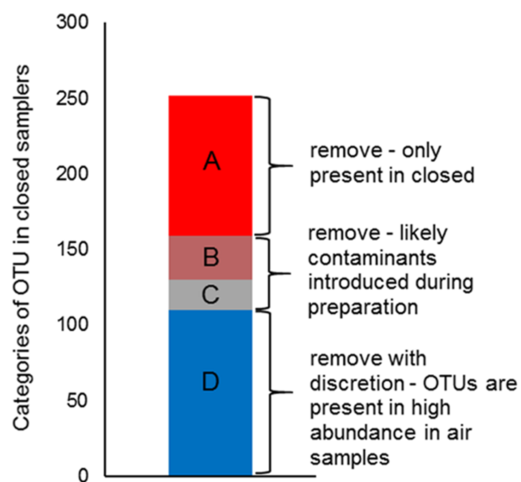

Figure 4. Depiction of the 4 categories of OTUs found in closed RAMP samplers and a description of how to use OTUs obtained from a closed RAMP sampler as a negative control for field studies. OTUs indicate operational taxonomic units; RAMP, Remote Airborne Microbial Passive.

equally present in the closed and open samplers (Category C). OTUs in Categories B and C are likely contaminants that were introduced during RAMP sampler preparation and were not removed by our decontamination procedures. Particularly, OTUs in the family *Bacillaceae* are of note in this category because they form spores and are impossible to inactivate without autoclaving. Even when they are autoclaved, *Bacillus* DNA that can be PCR amplified may remain, causing a possible bias in DNA extractions.[41] These OTUs may also have been introduced at low levels during plate preparation or sampler decontamination steps. Some notable examples within the families *Streptococcaceae*, *Staphylococcaceae*, *Micrococcaceae*, and *Corynebacteriaceae* can be human skin–associated taxa, which supports the idea that these contaminants are introduced by researchers as they prepared the samplers.[42–45] However, OTUs from the families *Sphingomonadaceae*, *Xanthomonadaceae*, and *Rhodobacteraceae*, which are typically of environmental origin, were also in these categories.[46–48] To be conservative, the OTUs in Categories B and C should also be removed from the data set because they are known to be at least equally prevalent in the closed sampler as in the open sampler.

Finally, 110 OTUs were present in both open and closed samplers but were more prevalent in the open samplers (Category D). The source of these OTUs is the most challenging to characterize. They may be contaminants that were introduced during collection dish preparation or decontamination, but they may also be contaminants that were introduced during storage, transport, or collection dish removal. Some of these taxa, such as *Pseudomonadaceae*, *Aerococcaceae*, and *Methylobacteriaceae*, are prevalent in air samples collected by other groups using different methods, so it is likely that they are a significant part of the airborne community and should only be removed from the data set with caution, or if they are known to be of human origin.[5,49–51] To test these recommendations, we removed subsets of OTUs we categorized as A-D from the samples we collected from indoor and outdoor air. When Categories A to C were removed, there was still no significant difference between indoor and outdoor air samples (unweighted UniFrac, $P = 0.20$). When Categories A to D were removed, there was again, no difference between indoor and outdoor samples (unweighted UniFrac, $P = 0.10$), but the P value is within $\alpha = 0.1$, suggesting that complete removal of Category D OTUs could influence the results. The strategy we propose for OTU removal should be tested and repeated with a higher sample size in future studies to avoid statistical error. Based on these results, we concluded that our RAMP samplers are functional and that closed RAMP samplers prevent most contamination. However, using a closed RAMP sampler during every sampling effort will provide a "field blank" negative control that can be used to remove known contaminant OTUs from the data set before analysis. This is in addition to using DNA extraction, PCR, and sequencing negative controls to correct for introduction of contaminants during preparation for sequencing. It is possible that including a second round of decontamination with a DNA removal spray (such as LookOut DNA Erase, Sigma-Aldrich, St. Louis, MO, USA) could remove some or all of this contamination. Design remedies for this issue could include redesigning the RAMP samplers to create a more thorough seal when closed or designing an array of collection dishes that can be autoclaved and inserted so that it is subject to more thorough sterilization.

Eight collection dishes provides a more diverse airborne bacterial sample than 16 collection dishes (H2)

We designed the RAMP sampler arrays to hold sixteen $9.5 \times 9.5\,cm^2$ collection dishes. This is because this is the maximum number of dishes that can be included while still maintaining a total payload weight of <6 lbs and adhere to Federal Aviation Administration regulations for balloon-borne payloads. We hypothesized that all 16 sampler dish surfaces were required to completely sample airborne communities and collect sufficient bacterial biomass for 16S rRNA-based amplicon sequencing. However, inserting plates into the RAMP sampler array is a time-consuming step and may also be a source of contamination. Therefore, we wanted to ensure that 16 was the optimal number of sampler dishes for future field experiments. We compared community results obtained from outdoor air sampling using 3 replicates each of 2-, 4-, 8-, 12-, and 16-plate treatments. Our results indicate that use of 8 plates yielded the highest average number of high-quality bacterial 16S rRNA high-quality sequences, bacterial OTUs and PD (Table 2). Tests using 12 and 16 plates exhibited a trend of yielding fewer bacterial 16S rRNA sequences, lower OTU richness, and lower PD than tests with 8 plates. This result is not significant because there is a high amount of error surrounding the averages. As expected, tests with 2 and 4 plates exhibited the lowest number of sequences, OTUs, and PD for

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Table 2. A comparison of the average number (±95% confidence interval and min-max range) of high-quality bacterial 16S rRNA sequence reads, OTU richness, and phylogenetic diversity for airborne samples extracted from 2, 4, 8, 12, and 16 collection dishes.

TREATMENT	N	16S RRNA SEQUENCES			RICHNESS			DIVERSITY		
		AVERAGE	MAX	MIN	AVERAGE	MAX	MIN	AVERAGE	MAX	MIN
2 plates	3	11 906 ± 13 472	23 953	2803	460 ± 521	668	244	29 ± 33	33	24
4 plates	3	12 704 ± 14 376	23 464	7272	449 ± 508	613	274	29 ± 32	33	21
8 plates	3	**41 159 ± 46 575**	**50 066**	**28 941**	**1076 ± 1218**	**1378**	**782**	**51 ± 58**	**62**	**37**
12 plates	3	22 548 ± 25 515	32 696	16 835	816 ± 923	1323	547	40 ± 46	58	29
16 plates	3	26 120 ± 29 557	50 272	3596	675 ± 764	1195	177	36 ± 41	55	16

Abbreviations: OTU, operational taxonomic unit; rRNA, ribosomal RNA.
No significant differences were detected using analysis of variance ($\alpha = 0.05$). Tukey honest significant difference P values ranged from .14 to .99 for all pairwise comparisons. The treatment using 8 plates yielded the highest number of sequences, OTU richness, and phylogenetic diversity among the treatments and is indicated in bold.

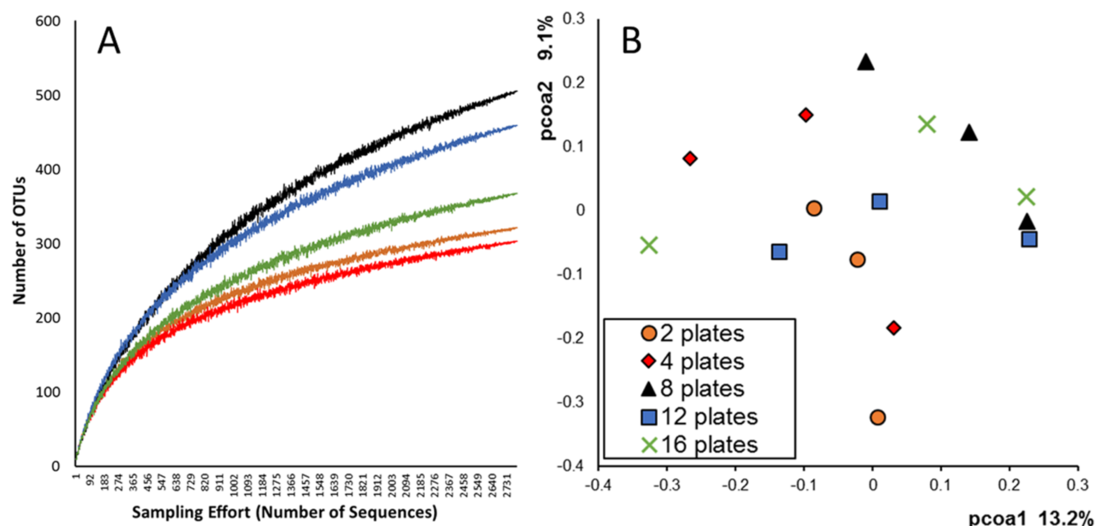

Figure 5. (A) Collector's curves indicating the average number of OTUs vs the average number of sequences observed for tests using 2, 4, 8, 12, and 16 collection dishes. Three replicates were included for each treatment. All collector's curves reach an asymptote, indicating complete sampling of the airborne community. On average, the greatest number of OTUs are present in the samples collected using 8 dishes. Average 95% confidence intervals (not shown) surrounding each curve are as follows: 2 plates = 54, 4 plates = 67, 8 plates = 111, 12 plates = 155, 16 plates = 145. (B) PCoA using unweighted UniFrac distances describing community similarity in the 5 number-of-plate treatments. There is no significant difference in community composition ($P = 0.134$). OTUs indicate operational taxonomic units; PCoA, principal coordinates analysis.

all tests. We standardized all samples to include exactly 2800 sequences per sample and examined collector's curves of observed OTUs vs number of sequences (Figure 5A). All curves approach an asymptote, indicating that bacterial diversity within the air samples was completely sequenced. The trend indicated by the averages described above remains obvious; the most amount of OTUs were present in the samples extracted from 8 plates, whereas fewer OTUs were observed in samples extracted from 2, 4, 12, or 16 plates. The error (95% confidence) surrounding the collector's curves largely overlapped but was lower with 2- and 4-plate treatments and higher with 8-, 12-, and 16-plate treatments. We also compared community composition of samples across the 5 treatments, using an unweighted UniFrac distance-based approach (Figure 5B). There was no significant difference in beta diversity among the 5 different plate treatments ($P = 0.134$).

The reason for this observation is likely due to the extraction technique. When we extracted DNA from the sampler dishes, the same flocked swab was used to remove the sample from all collection dishes. Then, the single swab was placed into the initial extraction tube for the bead-beating step. We noted that around halfway through swabbing 16 collection dishes, the swab procedure became less effective and the silica gel used to coat the plates completely encased the swab. Our results suggest that using this swab technique limits the amount of airborne bacterial diversity that will be collected by the sampler to a cutoff of 8 plates. There are 2 remedies to this issue. First, these results demonstrate that each RAMP sampler could be used to collect 2 technical replicate samples using 8 plates each. As there is a large amount of error surrounding the number of OTUs collected per air sample, this strategy provides further replication or a backup sample to ensure that a

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Table 3. A comparison of the average number (±95% confidence interval and min-max range) of high-quality bacterial 16S rRNA sequence reads, OTU richness, and phylogenetic diversity for airborne samples that were extracted from 16 collection dishes after exposure to 3 different storage conditions.

TREATMENT	N	16S RRNA SEQUENCES			RICHNESS			DIVERSITY		
		AVERAGE	MAX	MIN	AVERAGE	MAX	MIN	AVERAGE	MAX	MIN
Overnight at 4°C	3	36912 ± 2704.2[a]	38932	34274	650 ± 39.9[a]	699	617	32.5 ± 1.5[a]	34	31
1 week at 4°C	2	54110 ± 2494.1[b]	55382	52837	934 ± 147.6[b]	1041	828	42.9 ± 2.7[b]	45	41
2 weeks at −80°C	2	24971 ± 2745.9[c]	26372	23570	757 ± 14.6[a,b]	768	747	38.8 ± 1.8[a,b]	40	38

Abbreviations: OTU, operational taxonomic unit; rRNA, ribosomal RNA.
All storage condition treatments differed for all measurements; Tukey honest significant difference post hoc test significant differences are denoted with superscript letters ($\alpha = 0.05$).

sample is collected and sequenced correctly. Second, 2 separate flocked swabs could be used to obtain samples from 2 sets of 8 plates from each sampler. Then, both swabs could be placed together into the 5-mL bead-beating tube for downstream extraction. However, this would likely require an altered DNA extraction protocol beyond what we used here because it would require more physical extraction time and a larger volume of buffer during the bead-beating steps.

We note that we randomized the position of the different plates included in each of these 5 treatments within the sampler arrays. As this was the case, we did not have sufficient replication to determine whether there is a position-in-array effect in addition to a number-of-plates effect. It is possible that the aluminum sampler siding influences the volume of air sampled within the array so that some dishes collected more sample than others. Therefore, we add in the caveat that when these samplers are used, 8 plates from the left side of the array should be used for one extraction and 8 plates from the right side of the array should be used for the other extraction. This way, if the sampler itself influences sample collection, the bias is equally distributed across technical replicates. However, our results suggest that measurements comparing community beta diversity are not affected by this potential bias. Stated differently, overall community composition is the same, regardless of how many plates are used, but more of the rare members of the community are collected with 8 plates than with any other number of plates.

Storing samples at −80°C has minimal impact on community composition results (H3)

For remote sampling locations, it is a challenge to carry all the equipment necessary to conduct DNA extraction. If possible, storing samples for later extraction may be a better alternative. We hypothesized that storage of the collection dishes beyond 24 hours would significantly change the resultant communities, and that DNA extraction as soon as possible after sampling provides the most accurate representation of true airborne bacterial communities. In support of this hypothesis, the number of high-quality bacterial 16S rRNA reads differed significantly between samples that were stored at 4°C overnight, 4°C for 1 week, and −80°C for 2 weeks, prior to DNA extraction (Table 3). However, OTU richness and PD did not differ between samples stored overnight and frozen samples. Samples that were refrigerated for 1 week had significantly higher OTU richness and higher PD than samples stored overnight. This suggests that week-long refrigeration may cause shifts in airborne communities toward microorganisms that can tolerate cool temperatures. Certain taxa, such as those in the family *Polyangiaceae*, which are known to grow at low temperatures,[52] were highly prevalent in samples stored for 1 week and absent in samples stored overnight and stored frozen. Despite this, there was not a significant difference in community composition related to storage conditions (unweighted UniFrac, $P = 0.281$).

We used the 16S rRNA data to explore predicted functional pathways in samples stored at different conditions. Predicted pathways associated with protein synthesis (aminoacyl-tRNA biosynthesis, ribosome production), DNA synthesis (purine metabolism, pyrimidine metabolism), and gene regulation (transcription factor production) were all higher ($P > 0.05$) in samples that were frozen or stored overnight than those stored for 1 week. This suggests that pathways associated with DNA, RNA, and protein synthesis were negatively affected by week-long refrigerated storage. Together with the changes we observed in OTU richness and PD, this suggests that week-long refrigerated storage has significant impacts on airborne microbial communities and should be avoided to preserve the integrity of the community. However, immediate freezing and consistent storage at −80°C have little impact on the community beyond sequence loss and is a reasonable alternative for preserving samples when DNA cannot be extracted immediately.

Proof-of-concept test at 150 m

To demonstrate that the RAMP samplers we designed can be operated via remote control to collect airborne microbial samples from higher altitudes, we conducted a proof-of-concept test. We attached a sampler to a moored Helikite and collected sample for 6 hours. Following extraction and sequencing

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procedures, there were 11 951 high-quality bacterial 16S rRNA sequences in the sample, including 5116 bacterial OTUs. Predominant bacterial phyla in the community were *Firmicutes* (70%), *Proteobacteria* (17%), *Bacteroidetes* (7%), and *Actinobacteria* (5%). These phyla are typical of communities found in lower altitude samples, particularly from the Midwestern United States.[12] Onboard sensors also collected temperature, relative humidity, and barometric pressure data for the duration of the flight (Supplemental Data).

We note that this was a proof-of-concept test to determine whether the RAMP samplers could be used to collect sufficient DNA to conduct extraction and PCR-based approaches. When used for comparative studies in the future, calculations of air volume using wind speed measurements, co-located eddy covariance towers, or the HYSPLIT model should be used to correct for the volume of air sampled over time.[53]

Conclusions

Our experiments demonstrate that the novel RAMP sampling system design collects sufficient airborne bacterial biomass for use with next-generation sequencing approaches. We showed that RAMP samplers can be used for community analysis of air samples near the ground and using a balloon-borne method at an altitude of 150 m. The samplers are unique in several ways. First, because they are light enough to be used with balloon-borne samplers, this allows researchers greater flexibility in study design. RAMP samplers can be used to collect higher altitude airborne samples without relying on airplane flight schedules and costs and without introducing possible contaminants from powered aircraft. Typical sampling approaches for bioaerosols, including vacuum filtration and liquid impinger samplers, are both too heavy for this application and require battery power that may be unreliable at altitudes where they cannot be monitored by the researcher. It was beyond the scope of our study to conduct a comprehensive comparison of the RAMP samplers with vacuum filtration, liquid impinger, and other passive aerosol samplers, such as those described in Mhuireach et al[20] and Therkorn et al.[31] However, recent studies show that sampling method can influence the composition of airborne communities, so comparisons of results using multiple methods should be made with care.[54] In particular, if meta-analyses using publicly available sequence data are conducted in the future, it will be important to account for the differences that may introduced by sampling methods. Second, our samplers also collect atmospheric metadata (including pressure which can be used to determine altitude) that are measured and recorded in real time at the exact location that the sample is collected. These measurements can be used with the HYSPLIT model to calculate air volume sampled, as well as many other parameters that can be applied to specific projects.[53] Finally, samplers can be decontaminated easily and remain uncontaminated while closed. Although some contaminants may be introduced when collection dishes are inserted into and removed from the array, we suggest that a negative control sampler may be used to correct for these spurious OTUs during data analysis. This is an important consideration for all studies that use low-yield DNA applications to assess microbial community diversity patterns. In summary, the RAMP sampling system we developed provides an accessible method for collecting airborne microbial samples, allowing for further studies of atmospheric microbial communities that will address important unanswered questions about microbial dispersal through the air.

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Author Contributions

AMS, KMD, KDD, TVK, MMM, and KML conceived and designed the experiments; agree with manuscript results and conclusions. AMS and KMD analyzed the data. AMS wrote the first draft of the manuscript. AMS, KMD, KDD, and KML contributed to the writing of the manuscript. AMS, KMD, and KML jointly developed the structure and arguments for the paper; made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication, authors have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. The external blind peer reviewers report no conflicts of interest.

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