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ENVIRONMENTAL MICROBIOLOGY

Free Tropospheric Transport of Microorganisms from Asia to North America

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Abstract Microorganisms are abundant in the troposphere and can be transported vast distances on prevailing winds. This study measures the abundance and diversity of airborne bacteria and fungi sampled at the Mt. Bachelor Observatory (located 2.7 km above sea level in North America) where incoming free tropospheric air routinely arrives from distant sources across the Pacific Ocean, including Asia. Overall deoxyribonucleic acid (DNA) concentrations for microorganisms in the free troposphere, derived from quantitative polymerase chain reaction assays, averaged 4.94×10^{-5} ng DNA m^{-3} for bacteria and 4.77×10^{-3} ng DNA m^{-3} for fungi. Aerosols occasionally corresponded with microbial abundance, most often in the springtime. Viable cells were recovered from 27.4 % of bacterial and 47.6 % of fungal samples (N=124), with 49 different species identified by ribosomal DNA gene sequencing. The number of microbial isolates rose significantly above baseline values on 22–23 April 2011 and 13–15 May 2011. Both events were analyzed in detail, revealing distinct free tropospheric chemistries (e.g., low water vapor, high aerosols, carbon monoxide, and ozone) useful for ruling out boundary layer contamination. Kinematic back trajectory modeling suggested air from these events probably originated near China or Japan. Even after traveling for 10 days across the Pacific Ocean in the free troposphere, diverse and viable microbial populations, including presumptive plant pathogens *Alternaria infectoria* and *Chaetomium globosum*, were detected in Asian air samples. Establishing a connection between the intercontinental transport of microorganisms and specific diseases in North America will require follow-up investigations on both sides of the Pacific Ocean.

Introduction

Fronts and convective lofting can push microorganisms out of the boundary layer in the same way aerosols, dust, and other types of pollution gain altitude in the troposphere. Although airborne bacteria and fungi have been sampled from diverse locations around the world for hundreds of years, many questions remain about the nature of microbial "pollution"—particularly on the topic of global dispersion and how far microorganisms can travel from points of origin. Current knowledge of the sources, sinks, residence time, and distribution of atmospheric bacteria and fungi have been summarized elsewhere [8, 9, 17, 24, 54]. Atmospheric transport can impact life on the surface in many critical ways. Dobson et al. [10] hypothesized organic aerosols could have been essential prebiotic chemical reactors on early Earth, and once microbial life was underway, time aloft in the radiation-rich atmosphere probably accelerated natural selection and speciation [47]. Perhaps the persistence

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of microbial groups through extinction events and the cosmopolitan distribution of many species today could be explained, in part, by wind dispersal over geographic barriers [35, 54]. Recently, numerous diseases and allergens have been associated with the arrival of dust clouds [7, 20, 32, 45] and the influence of microorganisms on atmospheric chemistry, cloud formation, and precipitation is gaining attention [2, 50].

Seasonal reports published in the past decade [e.g., 13, 17, 43] have demonstrated that complex factors influence airborne microbial abundance, including: sampling site, weather, time of day, altitude and topography. Previous studies usually allude to global dispersion, but spreading patterns have never been systematically measured because of challenges associated with sampling in the free troposphere where most long range (>500 km) microbial transport occurs [53]. Several aerobiology research teams [6, 13, 38, 39] have sampled at isolated coastal locations with the objective to capture viable, airborne microbial populations that originated from distant continental sources. Field sites in these studies, however, were less than 5 m above sea level and the sampled populations may have included microorganisms dispersed in sea spray. In addition, back trajectory modeling of transport histories from lower elevation coastal areas is difficult because boundary layer air is heavily mixed and becomes untraceable over time [53]. Although earlier aerobiology surveys may have included bacteria and fungi from distant locations, boundary layer influences cannot be ruled out. Furthermore, the use of culture-based recovery methods in these studies [6, 13, 38, 39] would have only partially assessed microbial abundance since most microorganisms cannot be cultivated in the laboratory [8, 19].

Large deserts in Asia, including the Gobi, Takla Makan, and Badain Juran, are major sources of dust injected into the global atmosphere [31] and recent literature describes the concentration and diversity of Asian microorganisms mobilized by wind storms [23, 29, 33, 34, 52, 55]. Aerosols larger than microbes (up to 10 μm) routinely travel thousands of kilometers across the Pacific Ocean [5], leading some researchers [7, 20, 22, 31] to propose that Asian microorganisms, too, might be blown towards North America on prevailing westerly winds. Surprisingly, no study was found in the literature that documented the long-range dispersal of microorganisms downwind (i.e., east) of Japan. At the Mauna Loa Observatory in Hawaii, Mims and Mims [37] captured viable spores in ash thought to have originated from Asian fires; however, the authors did not address background levels of microorganisms in the air before the ash arrived. Smith et al. [46] might have gathered evidence of trans-Pacific microorganisms during a single high-altitude flight over the open ocean, but limited amounts of dust and atmospheric data prevented definitive statements about the source of sampled air.

In the spring and summer months, air traveling eastward from Asia gains height over the Pacific due to convection and warm oceanic conveyor belts [53]. Anywhere from 5 to 10 days later, it can arrive at the North American coastline and subside to lower elevations [27]. In this study, we test the hypothesis that microorganisms—like other types of free tropospheric pollution from Asia—periodically cross the Pacific Ocean and reach North America. The Mt. Bachelor Observatory (MBO) is a mountaintop atmospheric facility in central Oregon (43.98° N, 121.7 °W) that has been used to measure incoming Asian pollution since 2004 [15, 27, 53] and is an ideal location to test our hypothesis. The research station is positioned on the summit of an inactive volcano 2,763 m above sea level and is operated with cooperation from the Mt. Bachelor Ski Resort. Using a variety of atmospheric and chemistry data, the site has been shown to frequently (>50 %) sample air from the free troposphere with no recent contamination from the boundary layer or local emissions [15, 28, 40, 53]. Herein, we report results from the 2011 field season at MBO from 1 March to 20 May, including: (1) measurements of the abundance, diversity, and viability of airborne bacteria and fungi; (2) evidence for the long-range transport of microorganisms from Asia to North America during two separate events; (3) atmospheric data useful for understanding the origin and transport history of air from distant sources; and (4) aerobiology methods which permit molecular assays despite low densities of microorganisms in the free troposphere.

Materials and Methods

Operations at MBO

A specialized air sampling device (Fig. 1) was constructed to operate at the summit of MBO which frequently experiences extreme winter weather. Our apparatus had four separate copper intake pipes (1.5-cm inner diameter) each coupled with filters on a rotating cassette. The bulk of the apparatus was sheltered inside with only the intake pipes exposed to the outside environment through drilled holes on the uppermost level of the building. Intakes were unobstructed and faced in the general direction of prevailing winds. Sterile, 254 cm^2 polyethersulfone (PES) filters with 0.8-μm pores (PES009025, Sterlitech Corporation, Kent, WA) were placed inside individual plastic housing connected to the copper intake pipes via polyvinyl chloride joints. A high-volume vacuum pump (Windjammer model 117417-01, Ametek, Kent, OH) pulled ~0.5 m^3 min^{-1} of air through the filters (measured by an air velocity instrument, model FMA-905-VSR, Omega Engineering Inc., Stamford, CT). Prior to filter capture, the only other contact surface for the air was with the copper intake tubes. To minimize the

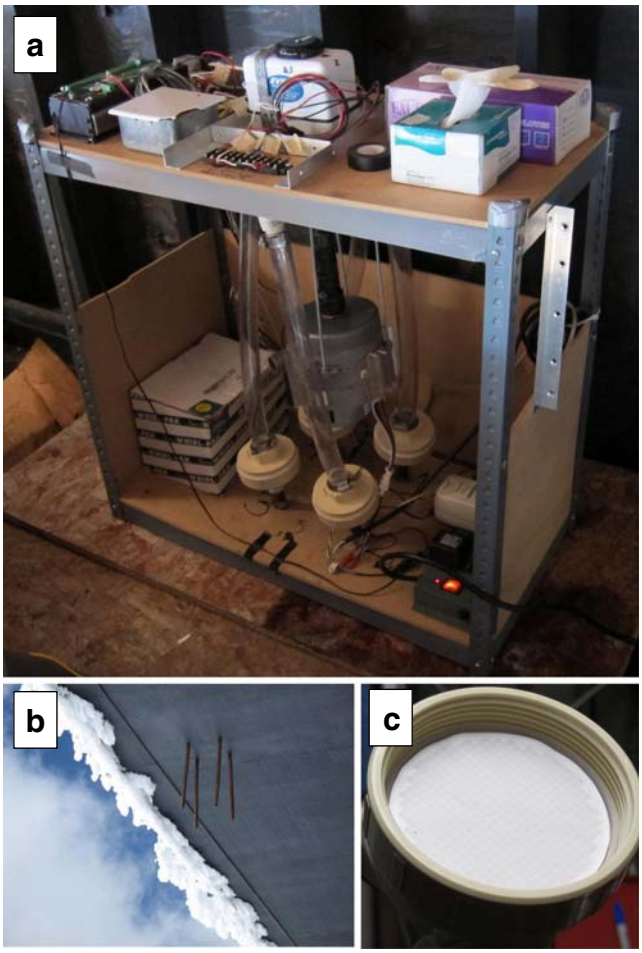

Figure 1 Air sampling apparatus at the Mt. Bachelor Observatory. **a** A high-vacuum pump, electronics board and four separate filter housings were connected to intake pipes through holes at the base of the device. **b** Copper intake pipes emerged outside the building, facing into prevailing winds. **c** Polyethersulfone filter membrane (254 cm^2, 0.8-μm pores) inside plastic housing after an air sampling period

possibility of contamination from air inside the building, the intake pipes were routinely washed with a bleach (NaOCl) solution. The sampler was controlled with a laptop by Loggernet™ software and a CR10x data logger (Campbell Scientific, Logan, UT) programmed to switch to a new filter position every 12 h, except for the fourth and final position which ran until manual shut down. This allowed for continuous air sampling even during poor weather conditions which sometimes prevented access to MBO. Thus, certain filters collected air for periods lasting longer than 12 h but most samples were of that exact duration. After the sampling interval, filters were removed from the apparatus using aseptic techniques and placed in separate sterile Whirl-Pak® bags (product B00736WA, Nasco, Modesto, CA), then transported on ice to a −80 °C freezer within 2 h. Fresh filters were quickly re-loaded into the sampler, leaving only a small gap (<45 min) in time coverage. Blank filters were periodically loaded and left in housing (with no air flow) to ensure sterility of the equipment, the building environment and the lag time prior to sampling.

Meteorological and atmospheric chemistry data were collected throughout the season to understand the composition and origin of arriving air. Measurements included concentrations of aerosols (dust), ozone (O_3), carbon monoxide (CO), water (H_2O) vapor mixing ratio, temperature, atmospheric pressure, wind speed, and direction. Sub-micron aerosol mass concentration (micrograms per cubic meter) was calculated from the sub-micron scattering data (at 530 nm) using a dry scattering efficiency of 3 m^2 g^{-1}. A detailed description of the instruments and calibrations used has been published elsewhere [28, 53]. Due to ski resort operations (e.g., snow clearing activities), artificial combustion sometimes contaminated air at MBO, but the periods were readily identifiable through short-lived spikes in aerosols and CO several orders of magnitude above baseline values. For sampling periods of particular interest, 10-day kinematic back-trajectories were calculated using the Hybrid Single-Particle Lagrangian Integrated Trajectory (HYSPLIT-4) model [11] available through the National Oceanic and Atmospheric Administration. Fischer et al. [16] has discussed the model inputs and error associated with using the Global Data Assimilation System meteorological archive for calculating back trajectories from MBO.

Imaging

In order to assess the distribution and morphology of particulates and microorganisms on air filters, samples from a period of variable dust concentration (18 to 20 April 2011) were imaged using a Zeiss EVO 60 environmental scanning electron microscope (SEM) with a tungsten filament (Carl Zeiss Microscopy, Peabody, MA). To prevent charges from building during high-magnification imaging, filters were first sputter-coated with a gold/palladium film (~220-Å thick) using a Denton Vacuum Desk IV system (Denton Vacuum, LCC, Moorestown, NJ) and Maxtek TM-400 Thickness Monitor (Maxtek, Inc., Cypress, CA). Sputter coating was performed at high vacuum (7 mtorr) for a 60-s interval with samples rotating at 10 rpm. Coated samples were immediately mounted onto the SEM stage for high-resolution imaging using carbon tape at a working distance of 11 mm.

Culturing

Microbe filters inside Whirl-Pak® bags were brought to room temperature in a laminar flow hood. Using sterile forceps and scissors, filters were cut into quarters, only one of which was used for microbial growth and recovery. The remaining pieces were returned to storage at −80 °C for use in other assays. Each quartered filter was halved to permit two different culturing approaches. The first approach put untreated samples

directly onto selective Difco™ R2A and potato dextrose agar (PDA) (Difco, Fisher Scientific, Pittsburg, PA). To encourage the recovery of spore-forming bacteria while preventing the growth of fungi that can quickly overtake mixed cultures, the second approach heat-shocked samples before placement onto solid media (again R2A and PDA). Heat shock treatment consisted of immersing filters in 5-ml sterile deionized H_2O and heating at 80 °C for 10 min. Plates from both cultivation approaches were wrapped with Parafilm® and incubated in the dark at 25 °C, given 14 days to shows signs of growth. Soon after initial colonies became visible, unique morphologies were sub-cultured and kept fresh until isolation.

Identification of Recovered Isolates

Bacterial sequencing of recovered isolates was performed with the MicroSeq® 500 16 S ribosomal RNA (rDNA) kit while fungal identification was performed with the MicroSeq® D2 large-subunit (LSU) rDNA kit (Applied Biosystems (ABI), Life Technologies Corp., Carlsbad, CA). The first 500 base pairs of the bacterial 16 S rDNA and the D2 region of fungal LSU rDNA genes were sequenced, analyzed and compared to a library using MicroSeq® ID Analysis Software Version 2.0. A working stock of deoxyribonucleic acid (DNA) was made at a 1:100 dilution, except for several samples with limited amounts of isolated DNA that required a 1:10 dilution instead. Otherwise, all downstream steps for polymerase chain reaction (PCR), purification, electrophoresis on the ABI 3130 Genetic Analyzer and sequencing were done according to the MicroSeq® kit protocol.

DNA Extraction from Filter Samples

DNA isolation for quantitative PCR (qPCR) experiments was achieved using MO BIO PowerWater® kits (product 14900-100-NF, MO BIO Laboratories, Inc., Carlsbad, CA) with a modified protocol to increase yield. For each sample, one quarter of a filter was placed inside a 5 ml PowerWater® Bead Tube containing 3 ml of Solution PW1. The tube was sealed and immersed in a 37 kHz sonicator (Elmasonic S 60/(H), Elma, Singen, Germany) for 30 min at 65 °C. Following the incubation, samples were vortexed for 15 min (product 12-812, Fisher Vortex Genie 2™, Fisher Scientific). All subsequent extraction steps followed the PowerWater® protocol, with solution volumes proportionally adjusted to match the initial volume of PW1. A centrifuge (product 5804, Eppendorf, Hauppauge, NY) and PowerVac™ manifold (product 11991, MO-BIO Laboratories, Inc.) were used, along with a wash of 800 μl of 100 % ethanol prior to the addition of Solution PW4. The resulting 100 μl DNA was then passed through a MiniElute® Reaction Cleanup Kit (product 28204, Qiagen Inc., Valencia, CA) to improve its quality, measured by a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). The final sample elution volume was 40 μl in Buffer EB.

Quantitative PCR

Primer sets and conditions were modeled after Fierer et al. [14] and are listed in Table 1. For each preparation, a standard 20-μl reaction volume contained: 5-μl extracted template, 10 μl of LightCycler® 480 DNA SYBR Green I Master Mix kit (contains FastStart DNA *Taq* polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, and $MgCl_2$), 40 nM of each forward and reverse primer for the bacterial assay or 250 nM of each primer for the fungal assay (Invitrogen, Carlsbad, CA), and 0.4 μl of Bovine Serum Albumin (for bacterial assay only). The remaining reaction volume was adjusted with sterile molecular-grade H_2O. All reagents were obtained from Roche Diagnostics Corp. (Indianapolis, IN) and reactions were performed in a 96-well semi-skirted plate with optical sealing foils. Amplification was conducted in a Roche LightCycler® 480 Real-Time PCR System. Annealing temperatures were experimentally optimized for bacteria and fungi. Each plate had triplicate reactions per sample, appropriate standards (bacterial or fungal) and sterile molecular-grade H_2O as the no template/reagent control. Fluorescence levels were recorded at the end of each amplification cycle, between 28 and 35 cycles, and reaction efficiencies ranged from 1.7 to 2.1. Melt curve analysis of the PCR products was conducted at the end of each assay to verify the quality and specificity of the fluorescence signal. A standard curve was generated by plotting the C_p value for a standard series of genomic DNA concentrations (20 ng μL^{-1} to 200 fg μL^{-1}) extracted from cultures of *Pseudomonas aeruginosa* for the bacterial assay and *Penicillium chrysogenum* for the fungal assay.

Statistical Analyses

Data were analyzed with the statistical program R version 2.14.1 (The R Foundation for Statistical Computing, Vienna, Austria, 2012). Meteorological values were averaged hourly and any errant signals (e.g., occasional anthropogenic emissions at MBO) were removed. Microbial concentrations were calculated across intervals (typically 12 h per filter sample) and the data set was divided into six smaller periods: 1–15 March; 15–29 March; 29 March to 11 April; 11–25 April; 25 April to 8 May; and 8–20 May. A multi-variable linear regression analysis was conducted to understand the relationship between airborne microbial abundance and meteorological variables (i.e., aerosols, CO, O_3, and H_2O vapor mixing ratio) for two specific events: 22–23 April and 13–15 May. At a 95 % confidence

Table 1 Reaction conditions and qPCR primers

Group	Amplicon length (bp)	Forward primer	Reverse primer	PCR conditions
Bacteria	~180	Eub338 (forward) 5′-ACTCCTACGGGAG GCAGCAG-3′	Eub518 (reverse) 5′-ATTACCGCGGCT GCTGG-3′	95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 20 s
Fungi	~300	5.8 s (forward) 5′-CGCTGCGTTCT TCATCG-3′	ITS1f (reverse) 5′-TCCGTAGGTGA ACCTGCGG-3′	95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s

level, the Wilcoxon test and *t* test were used to measure significant differences between means of independent variables.

Results

Figures 2 and 3 depict changes in airborne microbial concentration over time at MBO. Averages are summarized across six smaller periods in Table 2 along with meteorological data. The mean concentration of airborne bacteria and fungi over the entire sampling season was 4.94×10^{-5} ng DNA m^{-3} and 4.77×10^{-3} ng DNA m^{-3}, respectively. Fungal biomass consistently outnumbered bacterial biomass by two orders of magnitude, and the concentrations of each group varied significantly over time and in comparison to each other ($P<0.05$). Bacterial abundance was highly variable throughout the season, with peak concentrations (1.94 to 2.50×10^{-4} ng DNA m^{-3}) occurring across three different months. In contrast, fungi were not regularly detected until April but steadily increased thereafter, with concentration peaking on 14 May at 4.21×10^{-2} ng DNA m^{-3}. The period

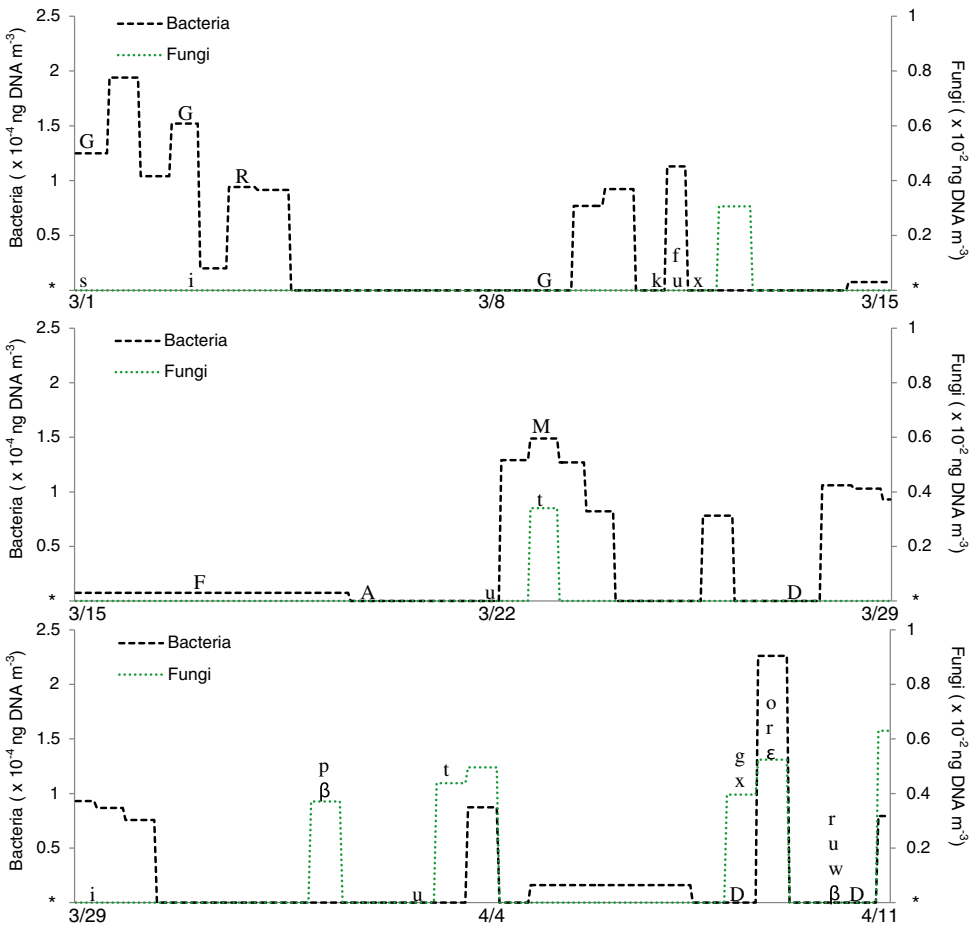

Figure 2 Microbial data from 1 March to 11 April. Values for bacteria and fungi are means of triplicate qPCR runs, time-averaged over the sampling interval. Note the difference in scale concentrations between groups. *Asterisks* represent the lower detection limit of the qPCR assay and letter codes correspond to recovered isolates listed in Tables 3 and 4

Table 2 Microbial and meteorological averages±standard deviation

Period	*N* (h)	Bacteria ($\times 10^{-5}$ ng NA m^{-3})	Fungi ($\times 10^{-3}$ ng DNA m^{-3})	Aerosols ($\times 10^{-1}$ μg m^{-3})	CO (ppbv)	O_3 (ppbv)	H_2O vapor (g kg^{-1})
1–15 March	315	3.93±5.75	0.130±0.610	1.20±2.43	138±6.36	47.0±3.91	2.91±0.488
15–29 March	336	0.251±4.84	0.122±0.632	0.251±0.963	137±11.9	35.9±8.45	2.70±0.451
29 March to 11 April	312	2.49±5.01	0.956±1.90	2.53±4.39	129±18.9	46.0±9.95	3.19±1.24
11–25 April	336	3.17±4.57	6.39±6.05	9.14±10.9	135±13.2	47.4±8.65	2.99±0.753
25 April to 8 May	312	2.65±4.04	6.02±6.16	4.62±5.56	128±8.70	51.5±7.95	2.66±0.856
8–20 May	307	9.06±6.24	10.6±10.5	8.47±0.0	121±16.1	46.8±6.19	3.46±0.684

with the highest concentration of both bacteria and fungi was 8–20 May. Bacteria were below the sensitivity limit of the qPCR assay in 52.4 % of the samples (N=124), while the month of March accounted for most of the failed fungi detections (in 44.4 % of the samples). All negative control filters (i.e., DNA extractions from blanks) were below the sensitivity limit of the qPCR assay. Figure 4a depicts the typical density of dust and microorganisms trapped on filter membranes, with a large distribution in the shape and size of captured particles. Fungal spores and bacteria were identifiable by size (1 to 10 μm) and shape, with the latter appearing in cocci, bacilli, and spirilla forms. Most microorganisms showed evidence of desiccation and were attached to the filter membrane independent of other particles (Fig. 4b, c), though clumping was also observed (Fig. 4d). Some particles and fragments of cells smaller than the filter membrane pore size (0.8 μm) were embedded deeper within the PES matrix. Viable bacteria and fungi were recovered from 27.4 and 47.6 % of samples (N=124), respectively, 1 to 7 days after incubation. The two cultivation treatments segregated samples: all bacterial were recovered from heat-shocked filters and all fungi were recovered from untreated filters. Some of the filters (28.2 %; N=124) had more than one viable microorganism. For both groups, viability was more common in the later months, with 8–20 May the period of highest frequency. Viability was not always correlated with overall microbial abundance—in fact, ten bacterial and nine fungal isolates were recovered from filters that were below the qPCR assay detection limit.

Best matches and frequencies of isolated microorganisms inferred through rDNA sequencing are listed in Tables 3 and 4, including a code which corresponds to sampling dates in Figs. 2 and 3. For bacteria, 18 unique species from six genera were recovered during the sampling season. *Bacillus megaterium* (N=8), *Bacillus pumilus/Bacillus safensis* (N=7) and *Bacillus simplex* (N=4) were the most commonly identified bacterial species. For fungi, 31 unique species from 26 genera were recovered. *Stromatinia narcissi* was the most commonly identified fungi (N=18), followed by *Cladosporium* sp./*Mycosphaerella aronici* (N=12), *P. chrysogenum* (N=10) and *Neosartorya fischeri* (N=9). Several bacterial and fungal isolates had <92 % rDNA sequence similarity (*Amphibacillus tropicus* and *Paenibacillus curdlanolyticus*; *Drepanopeziza populorum*, *Lophodermium pinastri*, and *Nigrospora oryzae*), making species identifications more uncertain in such instances. Microbial diversity increased significantly from March to May (P<0.05), with higher levels of species richness occurring in the latter half of the sampling season. The greatest amount of biodiversity was sampled on 22 April and 15 May (during two events discussed in greater detail later). Negative controls implemented in this study (blank filters and storage bags) did not result in microbial growth, and positive controls during sequencing methods for bacteria (*Escherichia coli* American Type Culture Collection (ATCC)=11,303) and fungi (*Saccharomyces cerevisiae* ATCC=18,824) confirmed the accuracy of techniques.

Table 3 Frequency and best match of bacteria recovered

Species	% Identity	Samples (*N*)	Figure code
Amphibacillus tropicus	90.6	1	A
Bacillus altitudinis	100	1	B
Bacillus atrophaeus	99.9	1	C
Bacillus megaterium	99.9–100	8	D
Bacillus mojavensis	100	1	E
Bacillus pumilus or *Bacillus safensis*	97.5–100	7	F
Bacillus simplex	100	4	G
Bacillus soli	97.9–98.3	2	H
Bacillus subtilis spizizenii DSM=15,029	100	1	I
Bacillus subtilis subtilis ATCC=6,051	100	2	J
Bacillus vallismortis	99.7	1	K
Brevibacillus choshinensis	99.9	1	L
Brevibacillus ginsengisoli	97.0–97.2	2	M
Paenibacillus alginolyticus	97.6	1	N
Paenibacillus borealis	95.7	1	O
Paenibacillus curdlanolyticus	88.8	1	P
Pseudomonas frederiksbergensis	100	1	Q
Streptomyces lienomycini	99.4	1	R

Table 4 Frequency and best match of fungi recovered

Species	% Identity	Samples (*N*)	Figure code
Allewia eureka	99.6	3	a
Alternaria infectoria	100	1	b
Alternaria longissima	99.7	2	c
Aspergillus parvathecius	99.9	1	d
Aspergillus niger ficuum DSM=932 or *Aspergillus phoenicis*	100	1	e
Arthrinium phaeospermum	93.0	2	f
Botryotinia sp. or *Botrytis* sp.	95.5–100	3	g
Chaetomium globosum CBS=145.38	98.9	1	h
Cladosporium sp. or *Mycosphaerella aronici*	100	12	i
Cryptococcus albidus	95.7	1	j
Cryptococcus dimennae	86.4–91.2	4	k
Dendrostilbella mycophila	93.6	1	l
Drepanopeziza populorum	85.5	1	m
Epicoccum nigrum	100	1	n
Lecythophora mutablis	97.3–97.7	6	o
Lophodermium pinastri	79.8	6	p
Malassezia restricta	99.7	1	q
Neosartorya fischeri	98.6	9	r
Nigrospora oryzae	89.1	1	s
Penicillium chrysogenum	100	10	t
Penicillium citrinum	99.8	4	u
Penicillium oxalicum	98.3	2	v
Phialocephala fortinii	96.4	1	w
Pithomyces atro-olivaceus	99.3	2	x
Pleospora papaveracea	99.7	1	y
Rhodotorula minuta	94.8	1	z
Sclerotinia sclerotiorum	99.7	1	α
Stromatinia narcissi	94.9–95.3	18	β
Sydowia polyspora	99.3–99.7	3	γ
Ulocladium sp.	100	1	δ
Venturia inaequalis	98.3	1	ε

Carbon monoxide, O_3, and H_2O vapor (also summarized in Table 2) were highly variable throughout the season, averaging 131.7 ppbv, 45.7 ppbv, and 2.98 $g kg^{-1}$, respectively. Means for these variables did not change significantly across the periods and concentrations were generally not a predictor for microbial abundance. Wind speed, temperature, relative humidity, atmospheric pressure, CO_2, Hg, and NO_X data were not included in this study but are available at http://www.atmos.washington.edu/jaffegroup/modules/MBO/. It was an unusually stormy period in the Pacific Northwest in 2011, with frequent precipitation and cloud cover. Average relative humidity and sub-micron aerosol concentrations for the spring were 88 % and 0.5 μg m^{-3}, compared to 78 % and 1.0 μg m^{-3} for the spring average from 2004 to 2010. From 1 March to 20 May, aerosol concentrations averaged 0.44 μg m^{-3} with values increasing significantly over the course of the season (Fig. 5). Initially, there was no correlation between fungi and aerosols (R^2= 0.087 for 11–25 April), but it steadily increased (R^2=0.13 for 25 April to 8 May) until the final time period which exhibited the strongest correlation between the variables (R^2=0.58 for 8–20 May). The relationship between aerosols and bacteria, in comparison, was weaker but still noteworthy from 15–29 March (R^2=0.22) to 8–20 May (R^2=0.24).

Elevated aerosol levels on 22–23 April and 13–15 May (highlighted in Fig. 5) were analyzed in detail along with changes in CO, O_3, and H_2O vapor concentration in Figs. 6 and 7. The 22–23 April event lasted nearly 48 h and was characterized by abrupt increases in aerosol, CO and O_3 levels along with a corresponding drop in H_2O vapor values. Multi-variable linear modeling measured a strong relationship between the atmospheric variables (R^2=0.92) and while overall microbial abundance (nanograms of DNA per cubic meter) was not higher than in the days preceding the event, the rise in number of cultured species was significant ($P<$ 0.05). In comparison, the 13–15 May event was longer-lasting and less sharply defined with a lower correlation between all atmospheric variables (R^2=0.44). However, the linear relationship between just microbial abundance, aerosols and CO was more substantial (R^2=0.75). Similar to the April event, microbial diversity was significantly higher than baseline values ($P<0.05$). Kinematic back trajectories over a range of altitudes were plotted to understand the transport history of air from these periods of interest. Air arriving to the site at 1600 Coordinated Universal Time (UTC) on 22 April showed transport from Asia (near China and Japan) at 2,700 and 3,200 m (Fig. 6). It took 10 days for the air to cross the Pacific Ocean in the free troposphere (reaching altitudes up to 6 km) before subsiding to MBO. Similarly, the trajectories plotted for air arriving at 0000 UTC on 13 May (Fig. 7) showed strong agreement, originating near Japan before traveling across the Pacific Ocean and reaching central Oregon.

Discussion

The objective of this study was to measure the concentration of microorganisms in the free troposphere—critical for understanding global dispersion, microbial biogeography, and the possibility of airborne bacteria and fungi traveling across the Pacific Ocean in an atmospheric bridge. By combining microbial observations with atmospheric chemistry, we closed a spatial/temporal gap acknowledged in the aerobiology literature [9, 54]. Overall, our seasonal data suggest that average microbial abundance in the free troposphere is low, consistent with earlier estimates by Spooner and Roberts [49]. Bacterial abundance levels at MBO averaged

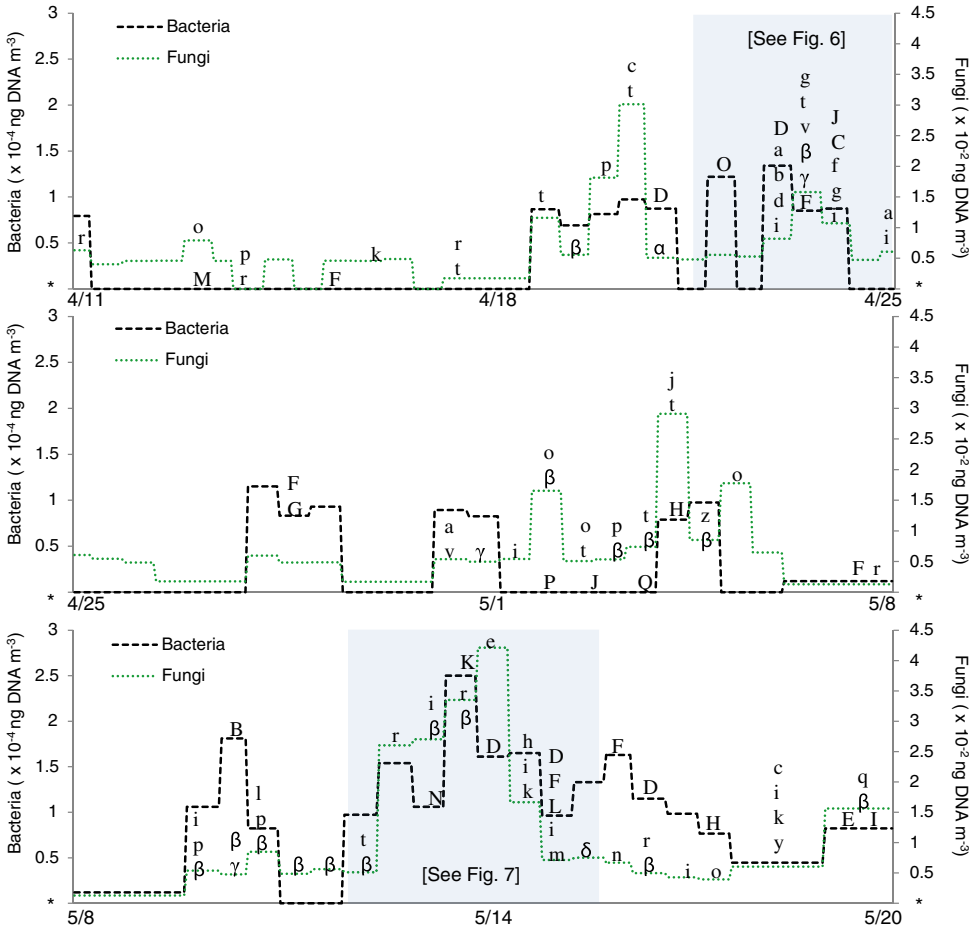

Figure 3 Microbial data from 11 April to 20 May. Values for bacteria and fungi are means of triplicate qPCR runs, time averaged over the sampling interval. Note the difference in scale concentrations between groups. *Asterisks* represent the lower detection limit of the qPCR assay and letter codes correspond to recovered isolates listed in Tables 3 and 4. Asian, long-range transport events are highlighted with *blue boxes*

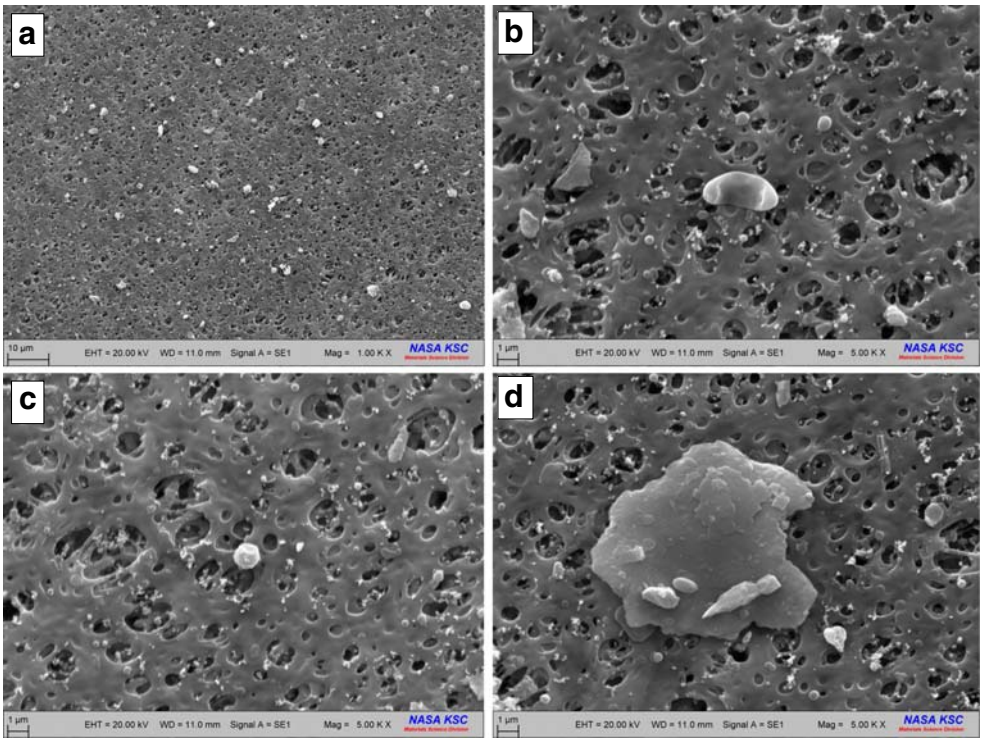

Figure 4 Scanning electron micrographs of PES filters used for capturing airborne microorganisms at the Mt. Bachelor Observatory. **a** Typical density and distribution of atmospheric particles and microorganisms which appear as bright forms against the darker filter background. **b** Desiccated, rod-shaped bacterium. **c** Fungal spore in the center of the micrograph. **d** Ovoid-shaped bacterial spore attached to a larger particle

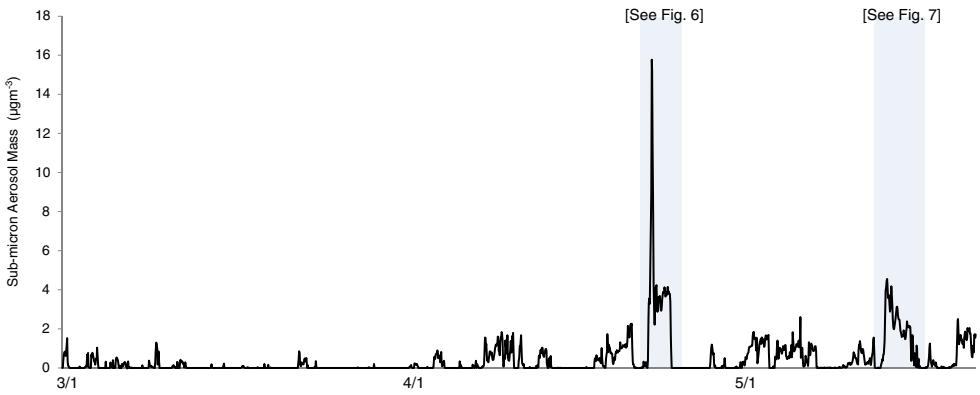

Figure 5 Seasonal dust measured by sub-micron aerosol mass concentration (micrograms per cubic meter). Stormy weather in earlier months kept dust levels low, but values increased towards springtime. Asian, long-range transport events are highlighted with *blue boxes*

49.4 fg DNA m^{-3}. This could represent 6–20 genomes m^{-3} assuming intact cells were sampled and considering bacterial genomes can range from 2–8 fg DNA (typically). While the relative amount of fungi was much greater than bacteria (in agreement with global flux projections [9, 17]), mass-based statistics can be misleading since fungal spores usually contain much more DNA than bacterial spores [18, 30]. It is possible that the ratio of whole airborne fungi and bacteria cells may be closer to 1:1, but the large variation in the amount of DNA between species and individual cells makes such calculations challenging.

All of the bacterial species recovered in this study (except *Pseudomonas frederiksbergensis*) are gram-positive, capable of forming endospores and commonly found in soils.

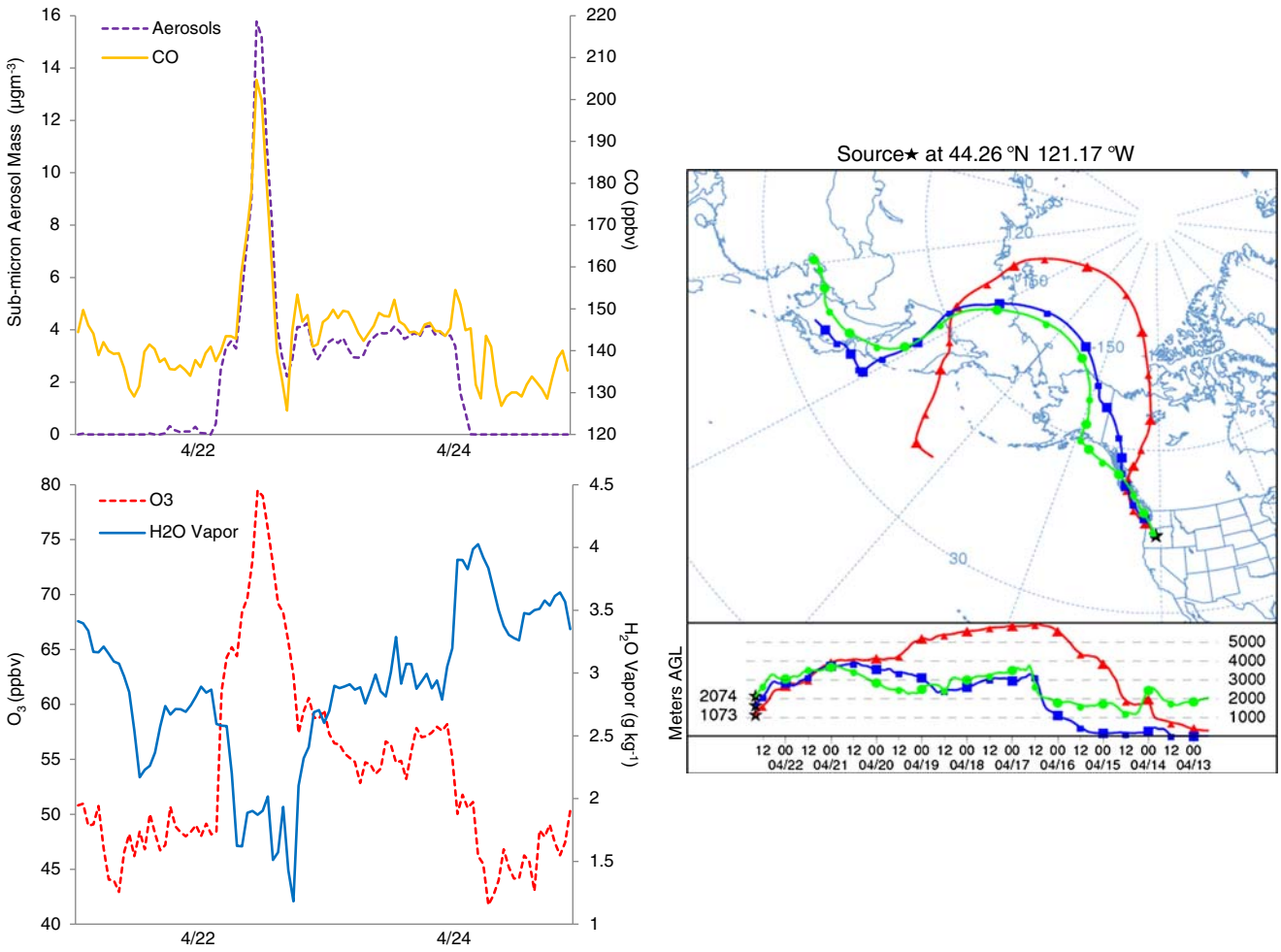

Figure 6 Profile of the 22–23 April long range transport event. Atmospheric data change significantly over the event ($P<0.05$) and exhibit free tropospheric signatures (i.e., dry, aerosol/CO/O_3-enriched air). Kinematic back trajectories ending at 1600 UTC were calculated at 2,200 m (*red line*), 2,700 m (*blue line*), and 3,200 m (*green line*) and suggested trans-Pacific transport from source regions near Asia

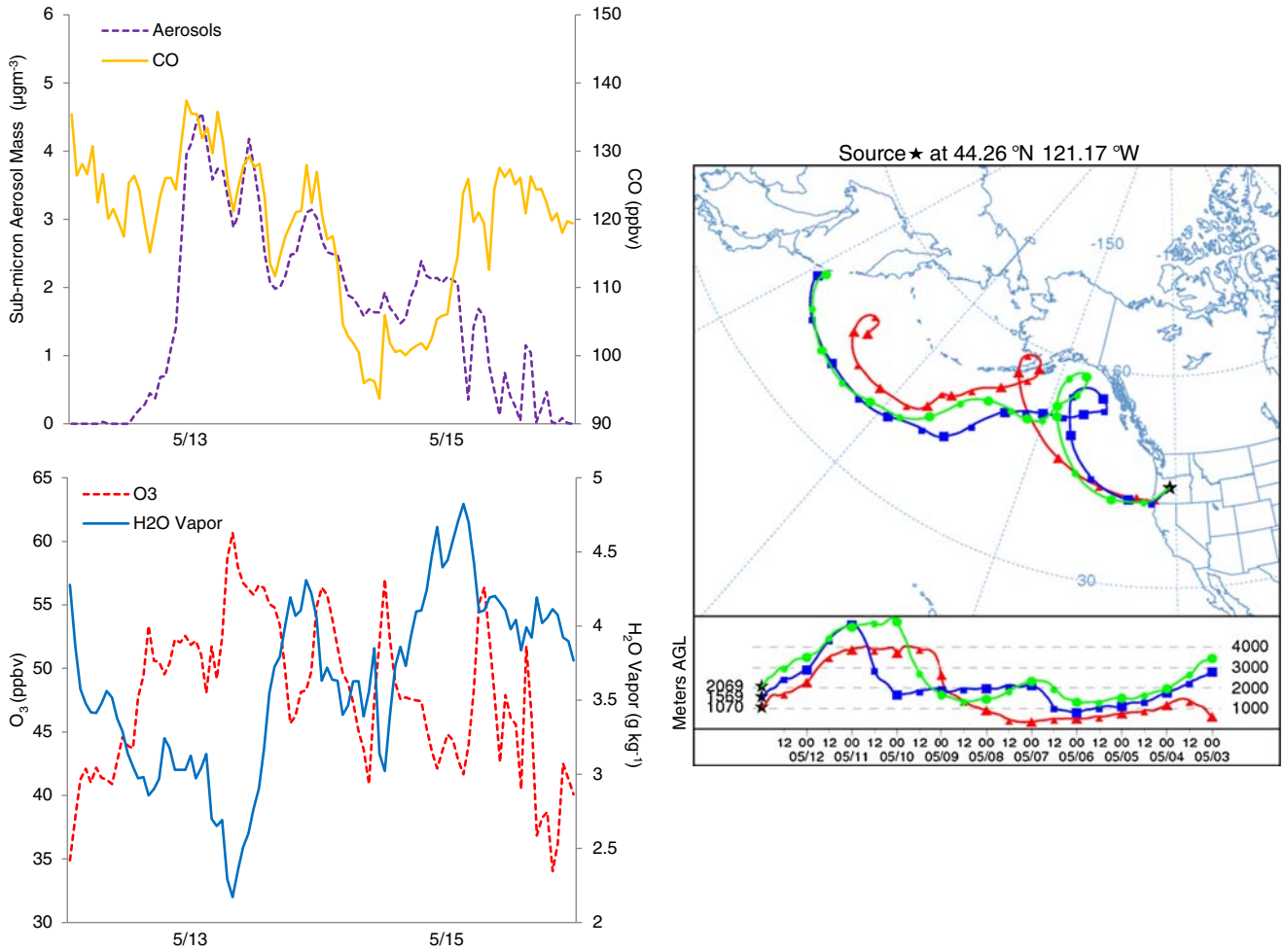

Figure 7 Profile of the 13–15 May long-range transport event. Atmospheric data change significantly over the event ($P<0.05$) and exhibit free tropospheric signatures (i.e., dry, aerosol/CO/O_3-enriched air). Kinematic back trajectories ending at 0000 UTC were calculated at 2,200 m (*red line*), 2,700 m (*blue line*), and 3,200 m (*green line*) m and suggested trans-Pacific transport from source regions near Asia

Surely cells of all types can be lofted into the atmosphere, but endospore formers may be uniquely capable of surviving atmospheric transport and desiccation on air sampling devices. Endospore formation in *Bacillus* sp. provides high resistance to ultraviolet radiation or desiccation [47] and the genus is quite common in the aerobiology literature [e.g., 26, 29, 33, 34, 52]. Similarly, the pigmentation of *Bacillus atrophaeus* and high guanine and cytosine content of *Streptomyces lienomycini* might protect DNA during radiation exposure in the atmosphere [48]. Two species, *Bacillus mojavensis and Bacillus vallismortis*, have been characterized by one of us in the past from desert soil samples [41, 42]. *Bacillus altitudinis* was captured during balloon experiments in the stratosphere by Shivaji et al. [44] and finding it again here suggests this species is well-suited for surviving high-altitude transport. *P. frederiksbergensis* was first isolated from coal gasification emission sites in Europe [1] and may be another species common to airborne communities. *A. tropicus* and *Brevibacillus ginsengisoli* have been sampled primarily from Russia [56] and South Korea [4], respectively, though the identity of the former was less certain based on rDNA sequence divergence (90.6 %). *Paenibacillus borealis* is often associated with forest humus [12] and could represent local influences on 21 April.

A broad range of fungi were recovered (26 genera), including molds, yeasts, and cup and sac fungi with diverse ecological distributions, mostly from soils and plant debris. Many of the sampled genera release spores in the spring time [32], explaining the fungal biomass "burst" measured in our study (beginning in April) and others [17]. Some of the 31 species recovered are presumptive allergens or plant pathogens (e.g., *A. infectoria*, *Alternaria longissima*, *Botryotinia* sp., *C. globosum*, *D. populorum*, *Epicoccum nigrum*, *L. pinastri*, *N. oryzae*, *Penicillium oxalicum*, *Pleospora papaveracea*, *Sclerotinia sclerotiorum*, *Sydowia polyspora*, and *Venturia inaequalis*) [49]. While the possibility of sampling local microorganisms was equally likely with bacteria, it may be easier to identify with fungi due to highly

specific ecological associations. For example, *Phialocephala fortinii* and *S. polyspora* are typically found on pine trees [49] which are abundant in the surrounding MBO wilderness. *L. pinastri* is another pine-associated fungus, but the rDNA identity match of the six isolated samples was low (79.8 %). Other possible contaminants include *Malassezia restricta* that is routinely found on the skin of animals [3].

Sampling at high altitude in the remote mountain wilderness created unique challenges which may have underestimated the actual abundance and diversity of microorganisms characterized in this study. For instance, air filters had to be frozen at −80 °C prior to recovery in the laboratory which may have killed a subset of culturable microorganisms (preferentially afflicting gram-negative bacteria more than gram-positive bacteria). To permit molecular methods, long-lasting sampling periods were necessary for capturing sufficient airborne biomass, but extended air flow can dehydrate cells (as observed in the SEM analysis) and select only the most resilient species to be recovered [38, 54]. Robust PES filters were used to withstand high air flow rates without tearing, however this made DNA extraction procedures more difficult since the dense membrane also trapped many particles and cellular fragments (<0.8 μm) deep inside its matrix. Extended bead beating, sonication and incubation with the lysing agent were needed to remove DNA and in doing so may have degraded its quantity and quality. Even with steps to maximize yield from samples, DNA quantities were still sometimes below the sensitivity limit for qPCR [25]. This explains why several bacterial and fungal isolates were recovered from samples with no measurable amplification signal. Future free troposphere studies using qPCR should employ even higher air pump flow rates and consider using liquid impingement to eliminate extraction inefficiencies associated with filter membranes [21].

Microorganisms and other biological particulates can act as cloud and ice condensation nuclei [2, 8], and the high incidence of storms during the sampling season at MBO could have led to a substantial fallout/washout of airborne cells, reducing overall microbial concentrations. When the weather improved later in the season, aerosol levels rose and so did microbial abundance. Once correlations became stronger, other atmospheric data were used to analyze the composition and origin of arriving air. Weiss-Penzias et al. [53] has described the approach for distinguishing between the North American boundary layer and free tropospheric, long-range transported air. The latter tends to be drier and O_3-enriched compared to the boundary layer. On average, daytime measurements reflected a mixture of boundary layer and free troposphere air whereas nighttime samples were mostly free tropospheric air with no local influence [40]. By focusing on two well-defined events with characteristic free tropospheric chemical compositions and coherent transport histories, we make an argument for sampling air from distant source regions. On 22–23 April the enhancement of O_3 from 50 to 80 ppbv and the depletion of H_2O vapor from 3 to 1 gkg^{-1} signaled the arrival of non-local, free tropospheric air. Simultaneously, aerosols jumped from 0 to almost 50 Mm^{-1} while CO rose almost 60 ppbv above baseline values. A similar (but less pronounced) pattern occurred on 13–15 May. In both cases, back trajectories showed transoceanic, free tropospheric transport beginning near China or Japan. Together, the chemical and meteorological data suggest local emissions were not the primary source of enhancements and depletions measured on 22–23 April and 13–15 May.

Richer levels of bacteria and fungi diversity coincided with the arrival of Asian air during the 22–23 April and 13–15 May events. We take a conservative approach identifying candidate Asian microorganisms. If a species was not recovered at any other time of the sampling season, it seems reasonable to suggest it traveled along with trans-Pacific free tropospheric pollution given the history of Asian emissions arriving at MBO [15, 27, 28] and other locations in North America [51]. The event on 22–23April delivered at least nine different species of fungi and four different species of bacteria and was the only occurrence in which *A. infectoria, Aspergillus parvathecius*, and *B. atrophaeus* were detected. Meanwhile the 13–15 May spike in diversity equaled that of April but delivered even more unique species, including: *Aspergillus niger ficuum*/*Aspergillus phoenicis* and *C. globosum*; *B. vallismortis*, *Brevibacillus choshinensis*, and *Paenibacillus alginolyticus*. *D. populorum* was also recovered but with less certainty based on rDNA sequence identity match (85.5 %). Pinpointing exact microbial emission source regions in Asia could soon be enabled (Smith et al., manuscript in preparation) through (1) DNA microarray data that can reveal many bacterial species ignored by culturing methods and (2) additional atmospheric transport analyses. Follow-up sampling seasons at MBO would be valuable given that overall biomass concentrations may have been reduced due to stormy weather and microbial extraction inefficiencies. For now, the detection of viable, presumptive plant pathogens (*A. infectoria* and *C. globosum*; and possibly *D. populorum*) in two separate Asian free tropospheric transport events has significant implications for epidemiology. While we are unaware of plant infections by these pathogens in North America, our results might inspire follow-up DNA fingerprinting studies [e.g., 36] to trace the relationship of pathogens on distant continents. With the health of ecosystems, agriculture, and human populations at stake, intercontinental aerobiology studies must ultimately work towards developing predictive models for disease dispersal.

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