



Microbial composition in seasonal time series of free tropospheric air and precipitation reveals community separation

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Abstract Primary biological aerosols are transported over large distances, are traveling in various media such as dry air masses, clouds or fog, and eventually deposited with dry deposition, especially for larger particles, or precipitation like rain, hail or snow. To investigate relative abundance and diversity of airborne bacterial and fungal communities, samples have been collected with a liquid impinger (Coriolis μ) from the top of Mount Sonnblick (3106 m asl, Austrian Alps) from the respective sources under a temporal aspect over four seasons over the year to include all climatic conditions. Bacterial and fungal

samples (16S rRNA and ITS) were sequenced using Illumina MiSeq paired-end sequencing, investigated for relative abundance by qPCR (16S rRNA and 18S rRNA) and ice nucleation activity. Results show that there is no stable free tropospheric air microbial community and air mass origin was different for the four sampling periods which exerted influence on the microbial composition of the atmosphere although a core microbiome could be identified consisting of 61 bacterial OTUs and eight fungal genera. Differentiation between seasons was stronger pronounced in air than in precipitation, with rain being most different and variable of precipitation types, indicating distinct forces driving microbial fate in the air. Microorganisms precipitated with snow, hail or rain or being transported by clouds differ in their species composition from free tropospheric air masses and do not mirror the air community structure. They were more diverse, distinct in composition, 16S:18S ratio and abundance from free-floating PBA. Hence, snow or cloud samples are not suitable proxies for free tropospheric air microbiome composition, since separation processes in aerosolization, transport and scavenging occur. The microbial composition of arriving precipitation or clouds represents only a part of the microbial air composition communities of the cumulative sources of origin. Relative abundance and composition of ice nucleation-active bacteria showed a higher share of relative % reads of known ice nucleation-active bacteria present in all wet phases compared to air. Results propose a separation of IN-

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active reads with higher shares occurring in precipitation. This study presents the first comparison of free tropospheric bacterial and fungal abundance and diversity in time series of air over several seasons in contrast to various precipitation forms in the free troposphere.

Keywords Free troposphere · 16SrRNA · 18SrRNA · ITS · Precipitation · Aerobiology

1 Introduction

Knowledge about and scientific interest in the composition and abundance of primary biological aerosols (PBA) (e.g., airborne bacteria and fungi) is rapidly increasing (180% increase in publications per year from 2000 to 2018, search term: “airborne bacteria”). Most aerobiology studies are restricted to ground-based sampling within the planetary boundary layer (PBL) or focus on extreme events such as heat events and dust storms (Fang et al. 2018; Jeon et al. 2011; Maki et al. 2017; Gat et al. 2017; Fierer et al. 2008; Bowers et al. 2013), civilization and health related questions, like disease spread (Park et al. 2014; Adams et al. 2015; Di Giulio et al. 2010; Kelley and Gilbert 2013; Hagan et al. 1995; Polymenakou 2012; Griffin 2007; Brown and Hovmøller 2002) or pollen events (Burge 2002; Damialis et al. 2015; Ziello et al. 2012; Clot 2003; Jäger 2000; Gioulekas et al. 2004; Sofiev et al. 2013; Hamaoui-Laguel et al. 2015). Microbial composition in air masses within the PBL is affected by ground emissions and mixing of air masses (Bowers et al. 2011; Carotenuto et al. 2017; Burrows et al. 2009a, b). Within the PBL (upper boundary around 100–1000 m above ground level, AGL), vertical mixing of air masses over mountainous terrain is driven by ground turbulence, thermal convection and dynamic forcing with strong diurnal and seasonal variations in elevation AGL and mixing force (Rotach et al. 2015). Above the PBL, laminar flowing air masses are stratified in the free troposphere (Wekker et al. 2015).

Studies focusing on free tropospheric air microbial communities often present data from single campaigns or few flights (Schmale and Ross 2015; Techy et al. 2010; Jimenez-Sanchez et al. 2018; Smith et al. 2018; Maki et al. 2017; Xia et al. 2013; Zweifel et al. 2012)

and have shown that microbes present in the free troposphere might originate from long-range travel (Maki et al. 2017; Smith and David 2012; Smith et al. 2011, 2012; Smith 2013; Brown and Hovmøller 2002; Burrows et al. 2009a, b). Air masses above and below PBL differ significantly in microbial composition. Free tropospheric habitats tend to have more Firmicutes (Smith et al. 2018; Smith 2013), Proteobacteria, Burkholderiales (DeLeon-Rodriguez et al. 2013) and extremophile yeasts, Saccharomycetes and Microbotryomycetes (Els et al. 2019). While the existence of either a stable (DeLeon-Rodriguez et al. 2013) or a highly variable (Zweifel et al. 2012) extremophile free tropospheric PBA community is disputed, generally few studies addressing the question were mainly performed over marine and oceanic regions (DeLeon-Rodriguez et al. 2013). Data on free tropospheric continental background PBA composition and variation are even more sparse due to numerous infrastructural challenges, like accessibility, need of aerial vehicles, suitable technologies for sufficient air sampling volume and challenging meteorological conditions. Thus, insight into seasonal variability and free-floating PBA composition compared to cloud and precipitation composition in free tropospheric conditions is limited.

Microorganisms are not only ubiquitous in dry air masses, but also in clouds (Amato et al. 2005, 2017; Joly et al. 2014; Evans et al. 2019; Fuzzi et al. 1997), snow (Amato et al. 2007a, b; Honeyman et al. 2018; Elster et al. 2007; Harding et al. 2011; Larose et al. 2010), hail (Temkiv et al. 2012; Vali 1971; Michaud et al. 2014) and rain (Christner et al. 2008a, b; Huffman et al. 2013). Their densities and diversity are higher in wet air masses and precipitation than in clear air due to source of moisture (Xia et al. 2013; Evans et al. 2019). Aerosolized microorganisms can act as cloud condensation nuclei (Bauer et al. 2003) and be incorporated in clouds. Clouds likely offer a more hospitable environment due to water availability and nutrients (Delort et al. 2017; Deguillaume et al. 2008; Bianco et al. 2016). Bacteria might be able to multiply (Sattler et al. 2001) and degrade substances (Lallement et al. 2018) in cloud droplets.

Microorganisms could even trigger their aerosolization and precipitation voluntarily by ice nucleation (IN) capacity as part of their life cycle, which was observed to express in enhanced concentrations of PBA and biological IN-activity in snow and

rain (Morris et al. 2014; Christner et al. 2008a, b; Huffman et al. 2013; Pouzet et al. 2017; Stopelli et al. 2015).

A range of processes, such as aerosolisation (Alsved et al. 2018), vapor to liquid phase change, particle coalescence, in-cloud circulation, temperature regime, cloud lifetime, cloud trajectories, nucleation properties and local air composition at the site of precipitation, effect on the microbial composition of precipitation (Möhler et al. 2007).

Recent publications used snow (Cáliz et al. 2018; Weil et al. 2017) or cloud sampling (Maki et al. 2017; Jiaxian et al. 2019) as a proxy for free tropospheric air microbial composition. However, the insights gained from precipitation and cloud studies above PBL might not directly depict the patterns in free tropospheric microbial communities.

Before being collected on the ground, precipitation travels through the PBL, where an inversion layer of accumulated particles is concentrated at the upper PBL (Brunet 2017). Scavenging impacts the microbial composition of droplets that pick up local PBA, but also release particles during transport and sedimentation (Jang et al. 2018; Huffman et al. 2013). Below-cloud scavenging of aerosol particles by snow is a known important mechanism of wet deposition (Paramonov et al. 2011), with differing effectivity for chemical components based on the forms of precipitation (Zhang et al. 2013; Zikova and Zdimal 2016). Thus, the scavenging effect might also be selective for PBA of certain properties (*e.g.*, size, hygroscopicity, density, sphericity and interface interactions) between precipitation types and thus foster a selection for specific PBA (Möhler et al. 2007).

This study presents the first comparison of free tropospheric bacterial and fungal abundance and diversity in time series of air over several seasons in contrast to various precipitation forms in the free troposphere. We further estimated the relative abundance and composition of ice nucleation-active bacteria in order to investigate whether the separation between air and precipitation forms in terms of ice nuclei can be observed on the molecular level, as it is observed on physical IN-effectivity.

2 Materials and methods

2.1 Study site

Samples were collected at the roof top terrace of Mount Sonnblick Observatory (3106 m asl.), Austria (47°3'14"N, 12°57'27"E, Fig. 1a, b). This location is operated as a meteorological observatory and is member of Global Atmosphere Watch of the World Meteorological Organisation (WMO). The site is exhaust neutral, supplied by electricity from the valley and equipped with an elevated air outlet to enable undisturbed air measurements. The vegetation in the valley consists of high alpine forest and shrubs that are dominated by pine (*Pinus cembra*, *Pinus mugo*, *Pinus silvestris*) and spruce (*Picea abies*, *Picea montana*). The closest settlement is the village Rauris village, which is 15 km away. The area within 100 km perimeter around the Sonnblick Observatory is sparsely populated.

In May, a complete snow cover was prevailing in the valley and on the mountain with high fresh snowfall (50–100 cm) before the sampling period, in August no snow cover was present at the mountain site or the valley, and in November and February a deep snow cover was present at the mountain site as well as at valley floor.

2.2 Air and precipitation sampling

A total of 17 snow, ten cloud samples, two rain and one hail sample were collected (Table 1). In total, 48 air samples comprising 288 m³ air were taken on four sampling campaigns in the months of May (2 to 5 May 2017, 13 samples, 78 m³), August (3 to 6 August 2017, 11 samples, 66 m³), November (27 November to 1 December 2017, 12 samples, 72 m³) and February (20 to 23 February 2018, 13 samples, 78 m³) approximately every 4 h. We used a liquid impinger (Coriolis μ , bertin Technologies, Montigny-le-Bretonneux, France) at a flow rate of 300L min⁻¹ for 10 min (*i.e.*, 3 m³ each) into separate sterile vials filled with 15 mL 0.2 μ m filtered, distilled water. Before each sampling for sequencing, one run was taken as decontamination purging. Two temporally close samples (*i.e.*, within the same hour) of three cubic meters each were pooled to account for low cell density in high elevations. Sampling could not always be conducted at the planned 4-hour interval, due to strong

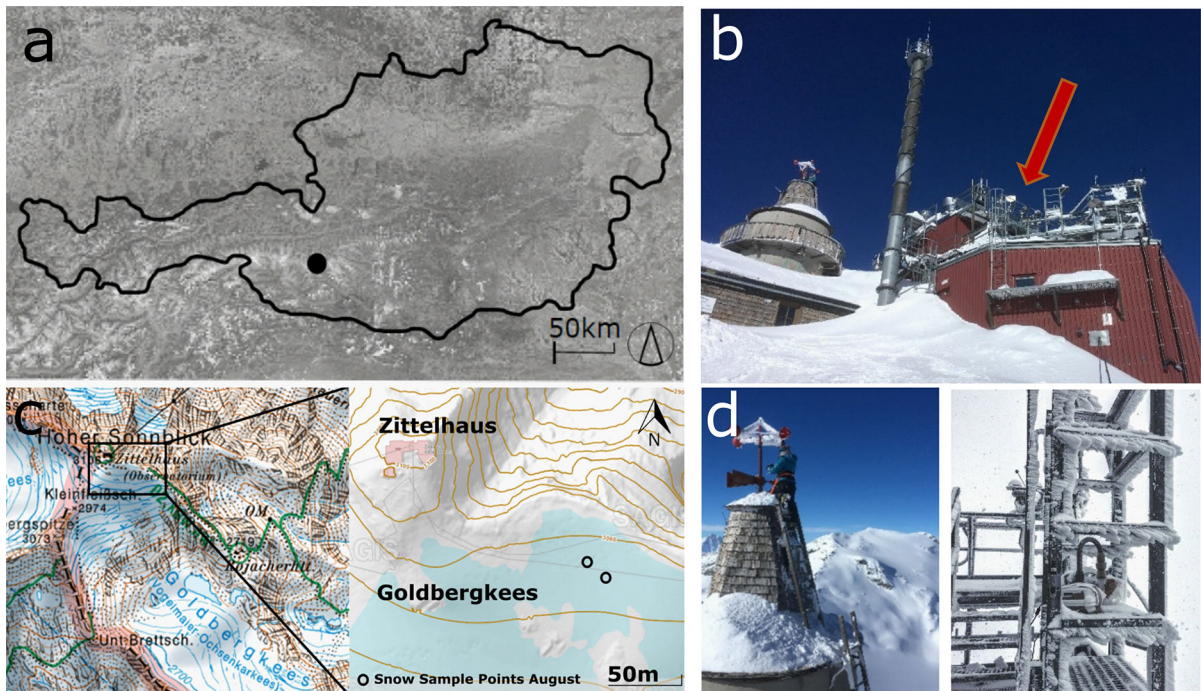


Fig. 1 **a** Location of mount Sonnblick in Austria, **b** location of the sampler on the roof terrace of the observatory, **c** location of August snow (slush) sample points, source: www.salzburg.gv.at/

sagisonline, **d** rime ice (supercooled atmospherical liquid water droplets frozen on surfaces) sampling from roof and railing

winds or precipitation events or too cold temperatures which hampered the functionality of the sampling device (compare Table 1).

Snow was sampled into sterile Nasco Whirl-Pak (Roth, Karlsruhe, Germany) after fresh snow fall with a stainless steel shovel (Roth, Karlsruhe, Germany). Before each sampling, we rinsed the shovel three times with 0.2 μm filtered milli-Q and turned it ten times in the snow, like the type of snow we would then sample, to precontaminate, usually this was conducted around 2 m apart from the actual sampling site. Thus, the idea of precontamination was to dispose non-site microbes and cover the shovel with the microbes we wanted to sample before the actual sampling. The shovel was kept in a sterile Nasco Whirl-Pak (Roth, Karlsruhe, Germany) for transport, to avoid contamination, and was washed three times with HCl (15%) and milli-Q before taken to the field. We did not use EtOH, as this can leave remnants that can then interfere with the aquatic chemistry measurements, we also conducted with these samples. Only the uppermost fresh snow layer was sampled.

In August, slush (melting snow and ice) was sampled from the upper section of the Goldbergkees, in approx. 500 m direct distance from the observatory (see map Fig. 1c). Cloud water (i.e., supercooled atmospherical liquid water droplets) was collected as rime ice frozen to the railing and meteorological devices on the roof terrace and tower and roof of the observatory (see picture for sampling illustration and locations in Fig. 1d) with a precontaminated stainless steel shovel (Roth, Karlsruhe, Germany) into sterile Nasco Whirl-Pak (Roth, Karlsruhe, Germany). Rime in direct contact with surfaces was not sampled. Rain was sampled as wet deposition in a WADOS (wet and dry only precipitation sampler, Kroneis GmbH, Vienna, Austria) as a 24-h cumulative sample at 10:00 h the 5.8.2017 and 6.8.2017, respectively. Hail was collected directly into sterile Nasco Whirl-Pak (Roth, Karlsruhe, Germany) during a strong thunderstorm event on the 5.8.2017 (20:00 h).

Table 1 Sampling dates and times of analyzed air samples and precipitation, times indicated in UTC + 1 (summer and winter time), samples were taken within one hour around indicated time

| May | | | | | | |
|-----------------|----------------|----------------|----------------|----------------|----------------|------------------------------------|
| 02.05.2017 | | | T1 13:00 h | T2 19:00 h | | Snow |
| 03.05.2017 | T3 07:00 h | T4 11:00 h | T5 15:00 h | T6 19:00 h | T7 23:00 h | Cloud |
| 04.05.2017 | T8 07:00 h | T9 11:00 h | T10 15:00 h | T11 19:00 h | T12 23:00 h | Cloud tower I, II Cloud terrace |
| 05.05.2017 | T13 07:00 h | | | | | Snow |
| August | | | | | | |
| 03.08.2017 | | | | T1 19:00 | | |
| 04.08.2017 | T2 07:00 h | T3 11:00 h | T4 15:00 h | | | |
| 05.08.2017 | T5 07:00 h | T6 13:00 h | T7 15:00 h | T8 19:00 h | | Slush snow I, II |
| 06.08.2017 | T9 07:00 h | T10 11:00 h | T11 15:00 h | | | Rain, hail |
| November | | | | | | |
| 27.11.2017 | | | | T1 19:00 h | T2 23:00 h | Rain |
| 28.11.2017 | T3 07:00 h | T4 11:00 | T5 15:00 h | T6 19:00 h | | Snow |
| 29.11.2017 | | T7 11:00 h | T8 15:00 h | | | Cloud, snow |
| 30.11.2017 | T9 07:00 h | T10 11:00 h | | T11 19:00 | | Cloud I, II, Snow |
| 01.12.2017 | T12 07:00 h | | | | | Snow |
| February | | | | | | |
| 20.02.2018 | | | T1 16:00 h | T2 19:00 h | | Cloud, snow I, II |
| 21.02.2018 | T4 07:00 h | T5 11:00 h | T6 15:00 h | T7 19:00 h | | Snow I, II, III Cloud, |
| 22.02.2018 | T8 07:00 h | T9 11:00 h | T10 15:00 h | T11 19:00 h | | Snow I, II, III, IV Cloud |
| 23.02.2018 | T12 07:00 h | T13 11:00 h | | | | |

2.3 DNA extraction and sequencing

Snow and cloud water were molten directly after sampling at room temperature (20 °C), and 1L of molten sample was filtered through a 0.2 µm

polycarbonate filter (47 mm, Isopore, Merck, Darmstadt, Germany) as soon as liquid, and then stored frozen at – 20 °C until DNA extraction. The air samples, collected in 15 mL solution of sterile distilled water, were stored at – 20 °C, until filtered

on a 0.2 µm polycarbonate filter (47 mm, Isopore, Merck, Darmstadt, Germany). DNA was extracted from the filters using DNeasy Power Water extraction kit (QIAGEN, Venlo, Netherlands) following the protocol provided with the kit, as this kit was stated to yield good results for aerobiological studies (Domergue et al. 2019). Amplification, library prep (MiSeq Illumina sequencing, 2 × 250 bp, Nextera XT Library Preparation Kit, Illumina, San Diego, USA) and sequencing were done with the Environmental Microbial Genomics group at the Laboratoire Ampère (ECL Lyon, University of Lyon, France). Community diversity was targeted: the V3-V4 region of the bacterial 16S rRNA SSU gene was amplified using 341F/785R (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21, Klindworth et al. 2013) primers, and the fungal internal transcribed spacer (ITS) regions were amplified with primer pair 5.8S_Fung/ITS4 targeting the ITS2 region (Taylor et al. 2016).

Raw sequences were submitted to NCBI BioProject database under Project ID PRJNA516816

(<http://www.ncbi.nlm.nih.gov/bioproject/516816>).

2.4 Bioinformatics and statistics

For 16S sequences, the base quality of the reads 1 and reads 2 was controlled using the functions `fastx_quality_stats` and `fastq_quality_boxplot_graph` of the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). PANDAseq (Masella et al. 2012) was used to assemble the read 1 and the read 2 using the RDP algorithm, a minimum and maximum length of the resulting sequence of 410 bp and 500 bp, respectively, a minimum and maximum overlap length of 20 bp and 100 bp, respectively. The resulting sequences were stripped out from the primers and annotated at the genus or family level by RDP Classifier (Wang et al. 2007) using the RDP 16S rRNA database and an assignment confidence cutoff of 0.6. This signifies that 60% of K-mers will match at the genus level. Singletons were removed.

For ITS sequences, forward and reverse reads were merged using `vsearch` (Rognes et al. 2016). Sequences were quality filtered and assembled in QIIME pipeline (Caporaso et al. 2010). Chimeras were removed using UCHIME (Edgar et al. 2011) with closed reference and de-novo approach. OTUs (operational taxonomic units) were assembled at 97% similarity using `vsearch` clustering algorithm at default settings (Rognes et al. 2016) and blasted against UNITE 7.2 database

(Kõljalg et al. 2013). Singletons were removed. Fungal OTUs were merged on genus level (i.e., 603 genera) for all analyses apart from alpha diversity, to account for species length polymorphisms in fungal ITS regions below genus level (Gomes et al. 2002).

Negative control bacterial genera resp. fungal OTUs from the kit and the sampling liquid were subtracted for all samples (see online supplementary material Table S10 and Fig. S1 for sequence statistics and rarefaction curves). Statistical analyses were done in *R* (R Core Team 2015) using the `phyloseq` (McMurdie and Holmes 2013), `vegan` (Oksanen et al. 2018) and `ggplot` (Wickham 2009) packages.

The alpha diversity Chao1 richness index S_1 was calculated as follows:

$$S_1 = S_{\text{obs}} + \frac{F_1^2}{2F_2}$$

where S_{obs} is the number of species in the sample, F_1 is the number of singletons, and F_2 is the number of doubletons.

The alpha diversity Shannon evenness-Index H' was calculated as follows:

$$H' = - \sum_i p_i \cdot \ln p_i \text{ mit } p_i = \frac{n_i}{N}$$

with p_i being the relative abundance of a certain species i of the total number of individuals N and n_i being the absolute number of individuals belonging to one species.

The data set was run both rarefied and untreated, which did not show differences in statistical analyses, but removed rare genera. To include otherwise removed genera and to present the data characteristics appropriately, relative abundance analysis was carried out on non-filtered data (Weiss et al. 2017). The fungal dataset was third-root transformed. Bray–Curtis distances for bacteria and fungi were calculated on datasets normalized to relative abundance (Weiss et al. 2017) and ordinated with non-metric multidimensional scaling (NMDS). The samples May_T12, May_5, May_T6 and May_T12 had to be excluded from the ITS analyses, as they had too few reads to calculate NMDS.

Pairwise PERMANOVA was conducted using the package “`pairwiseAdonis`” (Martinez Arbizu 2017); Bonferroni p value correction was applied as default for multiple corrections. Statistical parameters of ANOSIM (999 permutations), ADONIS (999

permutations), Kruskal–Wallis, Dunns PostHoc and PERMANOVA analysis are reported in the online supplementary data (Tables S5–S9).

SIMPER (Similarity Percentage), describing the species contribution of Bray–Curtis distance between two groups, was calculated in vegan (supplementary Tables S12–S18).

Obtained air and precipitation samples were blasted (blast version 2.7.1) against a nucleotide database of organisms containing the ice nucleation protein (search for “ice nucleation protein”), created from ncbi (www.ncbi.nlm.nih.gov), with a threshold of 97% similarity. The obtained reads were filtered for matches longer than 400 base pairs and merged on genus level.

2.5 Microbial enumeration

2.5.1 16S rRNA gene qPCR

The bacterial cell concentration was approximated by the number of 16S rRNA gene copies per cubic meter of air. The V3 region of the 16S rRNA gene was quantified using the Quantifast 2X SYBR Green dye (QIAGEN Venlo, Netherlands) and the following primer sequences: Eub 338f 5'-ACTCCTACGG-GAGGCAGCAG-3' as the forward primer and Eub 518r 5'-ATTACCGCGGCTGCTGG-3' as the reverse primer (Øvreås and Torsvik 1998) on a Rotor-Gene 3000 machine (QIAGEN, Venlo, Netherlands). The reaction mixture of 20 µl contained 10 µl of SYBR master mix, 2 µl of DNA and RNase-free water to complete the final 20 µl volume. The qPCR two-step program consisted of an initial step at 95 °C for 2 min for enzyme activation and then 35 cycles of 5 s at 95 °C and 20 s at 60 °C hybridization and elongation. A final step was added to obtain a denaturation from 55 °C to 95 °C with increments of 1 °C s⁻¹. The amplicon length was around 200 bp. PCR products obtained from DNA from a pure culture of *Escherichia coli* were cloned in a plasmid (pCRtm2.1-TOPO[®]-vector, Invitrogen, Carlsbad, USA) and used as a standard quantification with the Broad-Range Qubit Fluorometric Quantification (Thermo Fisher Scientific, Waltham, USA). Non-template controls were subtracted.

2.5.2 18S rRNA gene qPCR

The fungal cell concentration was approximated by the number of 18S rRNA gene copies per cubic meter of air. The region located at the end of the SSU 18S rRNA gene, near the ITS1 region, was quantified using the Quantifast 2X SYBR Green dye (QIAGEN, Venlo, Netherlands) and the following primer sequences: FR1 5'-AICCATTCAATCGGTAIT-3' as the forward primer and FF390 5'-CGATAACGAACGAGACCT-3' as the reverse primer (Chemidlin Prévost-Bouré et al. 2011) on a Rotor-Gene 3000 machine (QIAGEN, Venlo, Netherlands). The reaction mixture of 20 µl contained 10 µl of SYBR master mix, 2 µl of DNA and RNase-free water to complete the final 20 µl volume. The qPCR two-step program consisted of an initial step at 95 °C for 5 min for enzyme activation and then 35 cycles of 15 s at 95 °C and 30 s at 60 °C hybridization and elongation. A final step was added to obtain a denaturation from 55 °C to 95 °C with increments of 1 °C s⁻¹. The amplicon length was around 390 bp. PCR products obtained from DNA from a soil sample were cloned in a plasmid (pCRtm2.1-TOPO[®]-vector, Invitrogen, Carlsbad, USA) and used as a standard quantification with the Broad-Range Qubit Fluorometric Quantification (Thermo Fisher Scientific, Waltham, USA). Non-template controls were subtracted.

2.6 Meteorology

Meteorological and aerosol data were obtained from the Sonnblick Observatory repository. An aerosol backscatter profile from VAISALA CL51 ceilometer located at Kolm-Saigurn (the base of Mount Sonnblick at 1500 m asl.), and PBL heights were obtained from ZAMG (Zentralanstalt für Meteorologie und Geodynamik, Vienna). The mixing layer heights were calculated according to Lotteraner and Piringer (2016) (see online supplementary information Figs. S3–S6). The lagrangian model FLEXPART (Stohl et al. 2002) was used in backward mode to detect the most probable source regions for microorganisms which may have been transported with long-range air flows. The model output fields depict the regions with high source–receptor sensitivity due to the prevailing three-dimensional flow fields predicted by the ECMWF model for 120-h backcalculation (see online supplementary material Fig. S6 for model

Table 2 Mean meteorological values \pm standard deviation for the sample periods derived from hourly values (source: ZAMG)

| | Temperature (°C) | Humidity (%) | Precipitation (mm) | total Precipitation (mm) | Wind speed (m/s) | Wind Direction (dd) | maximum Mixing height (m AGL) | mean PBL (m AGL) | Pressure (hPa) |
|----------|---------------------|-------------------|-----------------------|--------------------------------|---------------------|--------------------------|----------------------------------|---------------------|-------------------|
| May | -6.90 ± 1.48 | 92.67 ± 4.79 | 0.10 ± 0.42 | 12.30 | 7.2 ± 3.22 | 197.49 ± 88 (SSW) | 1607 | 716 ± 453 | 692.3 ± 1.64 |
| August | 8.50 ± 2.90 | 91.47 ± 11.07 | 0.73 ± 1.81 | 70.30 | 6.17 ± 2.33 | 198.67 ± 84.94 (SSW) | 2438 | 748 ± 667 | 704.6 ± 1.54 |
| November | -12.80 ± 2.81 | 89.45 ± 3.01 | 0.16 ± 0.41 | 17.10 | 7.5 ± 3.04 | 188.18 ± 106.91 (S) | 819 | 349 ± 188 | 682.2 ± 5.22 |
| February | -14.40 ± 3.52 | 90.69 ± 1.45 | 0.00 ± 0.03 | 0.90 | 9.13 ± 1.88 | 111.25 ± 64.16 (ESE) | 766 | 261 ± 138 | 681.6 ± 1.58 |

output). Mixing layer height calculation and FLEX-PART backward calculation are described in the supplementary information of Els et al. (2019).

3 Results

3.1 Meteorology

Atmospheric temperatures were lowest during February and highest in August, with highest mean relative humidity in May (compare Table 2, compare Supplement Figure S3 for respective meteorological conditions at each sampling time).

Based on backward dispersion modeling results, air masses originated from different regions during each of the seasonal sampling campaigns (Supplement Figure S6). Samples collected in May were free tropospheric air mainly of maritime origin transported from the Atlantic over France to the Alpine region. Some transport from the Mediterranean region may have influenced the first sample collected on May 2, 2017. In August, the main source regions were identified as the Mediterranean Sea and North Africa and at the end of the sampling period, additional contributions from air masses traveling over Western Europe and the eastern Atlantic were observed. In November, the free tropospheric air was tracked back to the British Island and Iceland, even up to the northern Polar region. The sampling on November 11, 2017, was influenced by air that had been transported over Northern Spain, the Riviera and the Po Valley. In February, the air masses reaching the observatory during the sampling days had been influenced by flow from most of Europe and the Mediterranean area.

In the first days of May 2017, the core of a low-pressure system was centered over the Eastern Alpine region, resulting in cloudy conditions and strong snow fall in the morning of May 2, 2017 as well as in the night of May 4 to 5, 2017. In August, intermittent precipitation was observed during the sampling periods T1, T5 and T13 (Table 1). On August 3 to 6, 2017, a series of frontal passages dominated the meteorological conditions at Sonnblick Observatory. Heavy precipitation, thunderstorms and hail occurred during sampling, and mean wind speeds were lowest as compared to the other measurement periods.

For the period between November 27 and 30, 2017, weak pressure gradients dominated the weather

conditions in the Alpine area, resulting in low temperatures. In February, no precipitation was observed. While low-pressure gradients prevailed during the first day of this period, a cyclone over the Mediterranean caused an increase in wind speeds up to 14.8 ms^{-1} at the Sonnblick Observatory on February 21, 2018.

Samples collected in May, November and February were entirely in the free troposphere (Supplement Figure S4). A deep convective boundary layer reached above the mountain top on August 3 and 4, 2017, and the mixing heights determined from the ceilometer measurements indicated that the boundary layer air remained below the observatory on August 5 and 6, 2017.

Stable stratification of the valley boundary layer, characterized by low mixing heights, prevented significant transport of valley air to the mountain top in February and November. Mixing heights were low during these periods, most of the time below 400 m AGL (compare Supplement Figures S4 and S5).

3.2 Seasonality of bacterial and fungal communities

3.2.1 Abundance

The median of 16S rRNA gene abundance per m^3 air was highest in November ($1.14 \times 10^6 \text{ m}^{-3} \text{ air}$) and lowest in February ($2.67 \times 10^5 \text{ m}^{-3} \text{ air}$), with the biggest range (Coefficient of Variation CV: 2.47) and highest ($1.67 \times 10^7 \text{ m}^{-3} \text{ air}$) and lowest values ($1.18 \times 10^5 \text{ m}^{-3} \text{ air}$) in May. The smallest variation coefficient was found in August (0.58). Dunn pairwise comparison revealed significant difference for all groups except between May and February ($p = 0.054$) and May and August ($p = 0.44$) (Fig. 2a).

The highest median and absolute maximum, but lowest variation of 18S rRNA gene abundance per m^3 air was found in November (median: $5.1 \times 10^4 \text{ m}^{-3} \text{ air}$, max: $9.98 \times 10^4 \text{ m}^{-3} \text{ air}$, CV: 0.65), the lowest median and absolute value, but highest variation in May (median: $1.0 \times 10^2 \text{ m}^{-3} \text{ air}$, min: $6.13 \times 10^0 \text{ m}^{-3} \text{ air}$, CV: 2.45). Dunns pairwise comparison revealed significant difference between all groups but February and August ($p = 0.069$) (Fig. 2a).

The lowest median of the 16S to 18S rRNA gene ratio was found in November ($3.86 \times 10^1 \text{ 16S:18S m}^{-3} \text{ air}$). May had the highest median ratio

($4.02 \times 10^3 \text{ 16S:18S m}^{-3} \text{ air}$) and maximum value ($2.28 \times 10^5 \text{ 16S:18S m}^{-3} \text{ air}$), but also highest variation (2.31). The lowest ratio and variation coefficient was found in August ($9.74 \times 10^0 \text{ 16S:18 m}^{-3} \text{ air}$, CV: 0.72). Dunns pairwise comparison revealed significant differences between all but August and February ($p = 0.14$) (Fig. 2a). The seasonal abundance of 16S rRNA genes, 18S rRNA genes and their ratio was significantly different (Kruskal–Wallis $p < 0.001$).

3.2.2 Alpha diversity

Bacterial and fungal read counts per sample ranged from 13478 to 41882 and 568 to 34638, respectively, after quality filtering. The whole dataset comprised 1590 bacterial genera and 2573 fungal OTUs, which accounted for 963 bacterial genera and 1912 fungal OTUs (resp. 603 fungal genera) after removal of blanks.

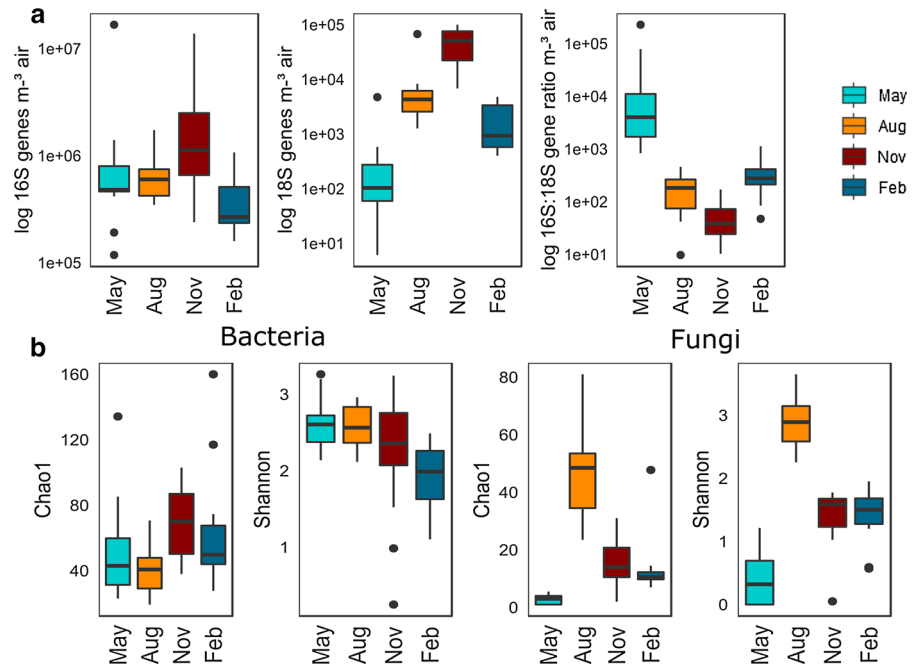
The overall median and mean of bacterial Chao1 (describing richness, Fig. 2b) were 48, with the lowest median in August (40.75) and the highest in November (70.06), and the lowest value in August (19.33) and the highest in February (160.00). November differed significantly from May ($p = 0.023$) and August ($p = 0.0368$), whereas in February significant differences to August were detected ($p = 0.0368$, Kruskal–Wallis $p = 0.03$). The highest variation was found in May (0.61) and the lowest in November (0.34).

The overall median of Shannon (describing evenness, Fig. 2b) alpha diversity index for bacteria was 2.37 and the mean 2.31, ranging from 1.99 (February) to 2.61 (May). The lowest value was found in November (0.25) and the highest in May (3.26). Variation was highest in November (0.39) and lowest in August (0.11). Shannon diversity differed significantly in February from all other months (Feb–May $p = 0.0006$, Feb–Aug $p = 0.0008$, Feb–Nov $p = 0.0247$, Kruskal–Wallis $p < 0.001$).

The highest fungal richness (median (48.5) and maximum value (81)) was observed in August, while the lowest value was recorded for May (median (3) and absolute value (1), variability (0.36)). Variability was highest in February (0.81). All values were significantly different from each other (Kruskal–Wallis $p < 0.001$) apart from November and February (Dunns $p = 0.3053$).

The highest fungal evenness (Shannon) median (2.89) and absolute value (3.65) was recorded in

Fig. 2 **a** Abundance of 16S, 18S and 16S to 18S ratio m^{-3} air per season, **b** alpha diversity measures Chao1 and Shannon for bacteria and fungi



August, and the lowest median occurred in May (0.32) and in November (0.05).

November and February did not differ significantly ($p = 0.4647$), but all other groups did (Kruskal–Wallis $p < 0.001$). Variability was highest in May (1.10) and lowest in August (0.15).

3.2.3 Beta diversity

Seasonal air bacterial communities were significantly different (ADONIS $p = 0.001$, $R^2 = 0.166$, ANOSIM $p = 0.001$, $R = 0.114$). They differed significantly between February and May (PERMANOVA adj. $p = 0.006$), February and August (PERMANOVA adj. $p = 0.006$), May and November (PERMANOVA adj. $p = 0.006$) and August and November (PERMANOVA adj. $p = 0.006$). February samples were the most variable and distinct as compared to the other communities. The February and November (winter) and May and August (summer) bacterial communities did not differ significantly. On phylum level, winter samples were most abundant in Firmicutes (Nov: 78.4%, Feb: 48.1%), whereas summer samples were abundant in Proteobacteria (May: 54.3%, Aug 49.4%), Bacteroidetes, Acidobacteria and Planctomycetes (Fig. 3c).

The highest value of unique air bacterial genera was reached in May, the lowest in August.

61 genera, i.e., 11% of the total bacterial genera, were common to all seasons (Fig. 3b). The most abundant of these common bacteria belonged to the genera *Geobacillus* (Bacilli), *Aminobacter* (Alphaproteobacteria) and *Schlesneria* (Planctomycetia) (see online supplement Tables S1 and S2).

Of all bacterial air genera, 54% were unique to one of the seasons. The most abundant genera only occurring in May and August were *GP2* (Acidobacteria), *Domibacillus* (Bacilli) and *Paludibacter* (Bacteroidia). Most abundant unique genera in winter samples were *Bifidobacterium* (Actinobacterium), *Vulcaniibacterium* (Gammaproteobacteria) and *Polaribacter* (Flavobacteria). SIMPER analysis (see supplement Tables S12 and S13) revealed *Geobacillus* abundance in February and November as the driver of differences between summer and winter samples. Further, high abundances in *Microbacterium*, *Tumebacillus* in winter and presence of *Aminobacter*, *Bosea*, *Burkholderia* and *Schlesneria* in summer differentiated samples.

Air fungal seasonal communities also significantly differed (ADONIS $p = 0.001$, $R^2 = 0.3075$, ANOSIM $p = 0.001$, $R = 0.5225$). All seasons differed from each other (PERMANOVA p .adj = 0.006, respectively); however, NMDS of Bray–Curtis distance (Fig. 3a) revealed that May samples were most dispersed, while August samples were most similar

and February and November samples had the highest overlap.

August samples were dominated Agaricomycetes, Lecanomyces and Leotiomyces, while May fungal communities were characterized by Eurotiomyces, Saccharomyces, Tremellomyces and Wallemiomycetes. Winter samples had a high amount of unidentified organisms, November was characterized by high abundance in Sordarimycetes (23.2%) and Tremellomyces (12.1%), while February featured many Agaricomycetes (34.6%).

Only 3% of the identified fungal air genera were common in all seasons. The most abundant were unidentified Ascomycota, unidentified Agaricomycetes, *Formitopsis* (Agaricomycetes), *Aspergillus* (Eurotiomyces) and *Trichaptum* (Agaricomycetes). The highest share of unique genera was found in August (53.9% of all identified fungal genera), with *Teloschistaceae* (Lecanomyces), *Gymnopus* (Agaricomycetes) and *Resupinatus* (Agaricomycetes) and range of Agaricomycetes identified as the most abundant. February contained the second most fungal genera detected in air (31.7% of all fungal genera detected), with *Exidia* (Agaricomycetes), *Talaromyces* (Eurotiomyces) and *Taphrina* (Taphrinomyces) as most abundant unique genera (supplementary. Table S2). SIMPER analysis (supplement Table S16) revealed *Phanerochaete*, *Formitopsis*, *Aspergillus* and *Wallemia* as driving fungal genera distinguishing May samples from all other seasons. August had characteristically high abundances of *Sacrogyne*, *Acarospora* and *Formitopsis*, while *Mycena*, *Saccharomyces*, *Extremus* were characteristic in February, and *Exophiala*, *Filobasidium* for November.

3.3 Abundance and diversity of bacterial and fungal communities in air and precipitation

3.3.1 Comparison of abundance in air and precipitation

The median of 16S rRNA gene abundance m^{-3} air (5.23×10^5) is within one order of magnitude of the abundance per mL cloud ($1.17 \times 10^5 \text{ mL}^{-1}$) or snow ($2.66 \times 10^5 \text{ mL}^{-1}$). Rain water ($2.29 \times 10^7 \text{ mL}^{-1}$) and hail ($1.88 \times 10^7 \text{ mL}^{-1}$) showed two orders of magnitude higher values (Fig. 4).

Minimum values of 16S rRNA genes mL^{-1} in precipitation were reached in snow ($1.87 \times 10^2 \text{ mL}^{-1}$); maximal concentration of 16S rRNA genes was present in rain ($4.28 \times 10^7 \text{ mL}^{-1}$). Variation was lowest in cloud (0.94) and higher in snow (1.67), but highest in air (2.18).

Air and precipitation 16S rRNA gene abundances were significantly different (Kruskal–Wallis $p = 0.001$), with cloud and air ($p.\text{adj.} = 0.0012$) and cloud and rain ($p.\text{adj.} = 0.0004$) differing strongly. Further hail vs. rain and snow vs. air, cloud, hail and rain differed in unadjusted testing.

The median of 18S rRNA gene abundance m^{-3} air ($3.01 \times 10^3 \text{ m}^{-3}\text{air}$) was lower than in cloud ($8.55 \times 10^4 \text{ mL}^{-1}$) and snow ($2.02 \times 10^4 \text{ mL}^{-1}$), much higher in hail ($1.46 \times 10^7 \text{ mL}^{-1}$) and lower in rain ($7.72 \times 10^2 \text{ mL}^{-1}$).

16S to 18S ratio (Kruskal–Wallis $p < 0.001$) differed in air and cloud ($p.\text{adj.} = 0.0001$), snow and air ($p.\text{adj.} = 0.0002$), and rain and snow ($p.\text{adj.} = 0.0161$). The highest median 16S:18S ratio occurred in rain (3.18×10^4), and a higher 16S:18S ratio in air (2.29×10^2) than in cloud (1.15×10^0), snow (2.81×10^1) and hail.

18S rRNA gene abundance in precipitation (Kruskal–Wallis $p = 0.01$) differed only in snow and air ($p.\text{adj.} = 0.0375$), but further in air and cloud, cloud and rain, hail and rain and rain and snow in unadjusted testing (supplement Tables S5, S8, S9).

3.3.2 Alpha diversity in air and precipitation

Chao1 for bacteria differed in air and cloud ($p.\text{adj.} = 0.0027$), air and snow ($p.\text{adj.} = 0.0000$), rain and snow ($p.\text{adj.} = 0.0038$) and cloud and rain ($p.\text{adj.} = 0.0254$) as well as overall (Kruskal–Wallis $p < 0.001$).

Bacterial Shannon evenness (Kruskal–Wallis $p < 0.001$) differed in air and cloud ($p.\text{adj.} = 0.0535$), air and snow ($p.\text{adj.} = 0.0000$), hail and snow ($p.\text{adj.} = 0.0464$).

Fungal Chao1 (Kruskal–Wallis $p < 0.001$) differed in cloud and air ($p.\text{adj.} = 0.0000$) and air and snow ($p.\text{adj.} = 0.0000$), further in cloud and rain, hail and rain and rain and snow in unadjusted testing. Fungal Shannon (Kruskal–Wallis $p < 0.001$) differed as well in air and cloud ($p.\text{adj.} = 0.0090$) and snow and air ($p.\text{adj.} = 0.0001$) and further in rain and cloud and rain and snow in unadjusted testing.

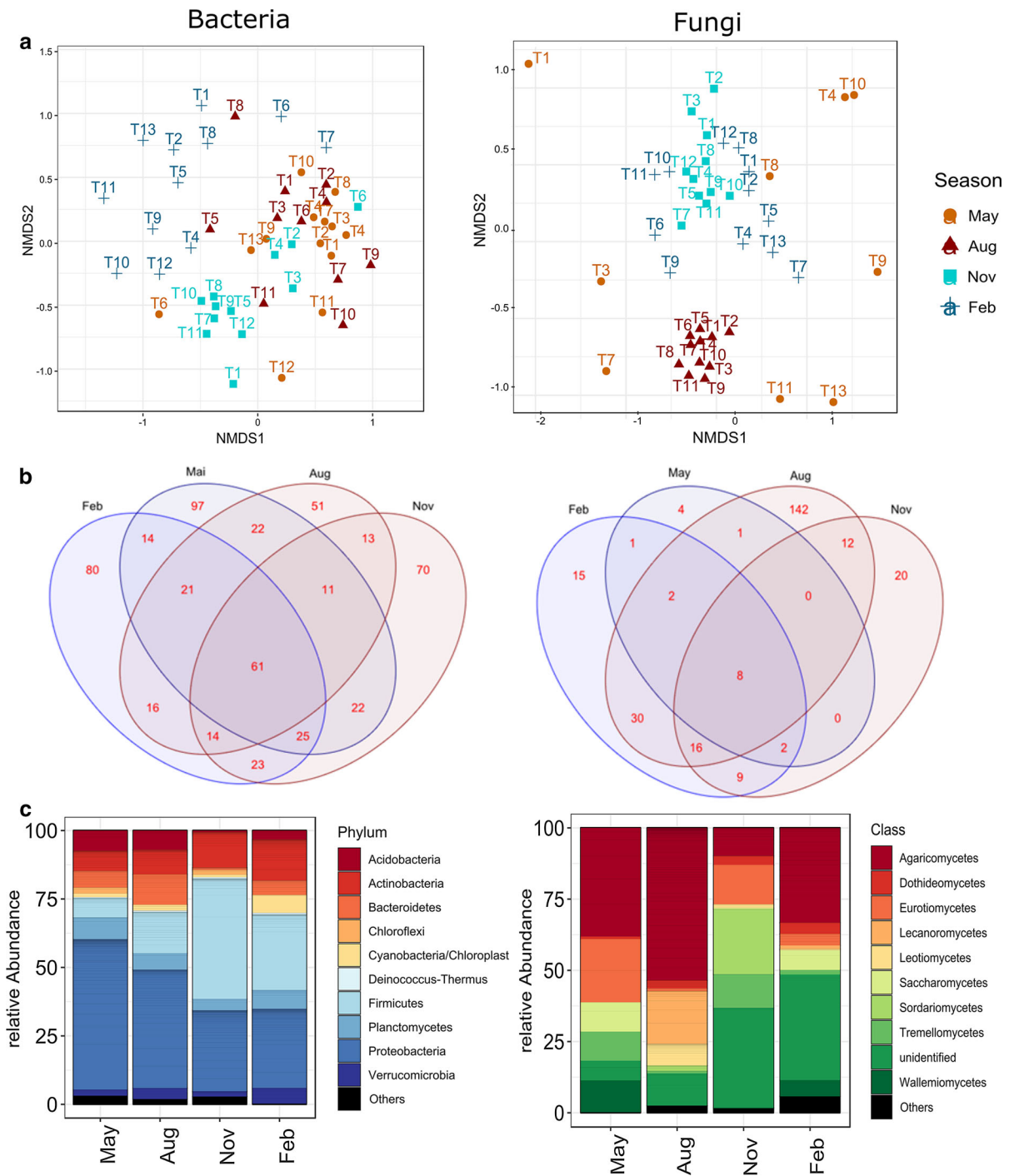


Fig. 3 **a** NMDS on Bray–Curtis distance on genus for bacteria and fungi for air composition by season, **b** Venn diagram of unique and common bacterial and fungal genera by season, **c** relative abundance (%) of top 10 most abundant bacterial phyla and fungal classes

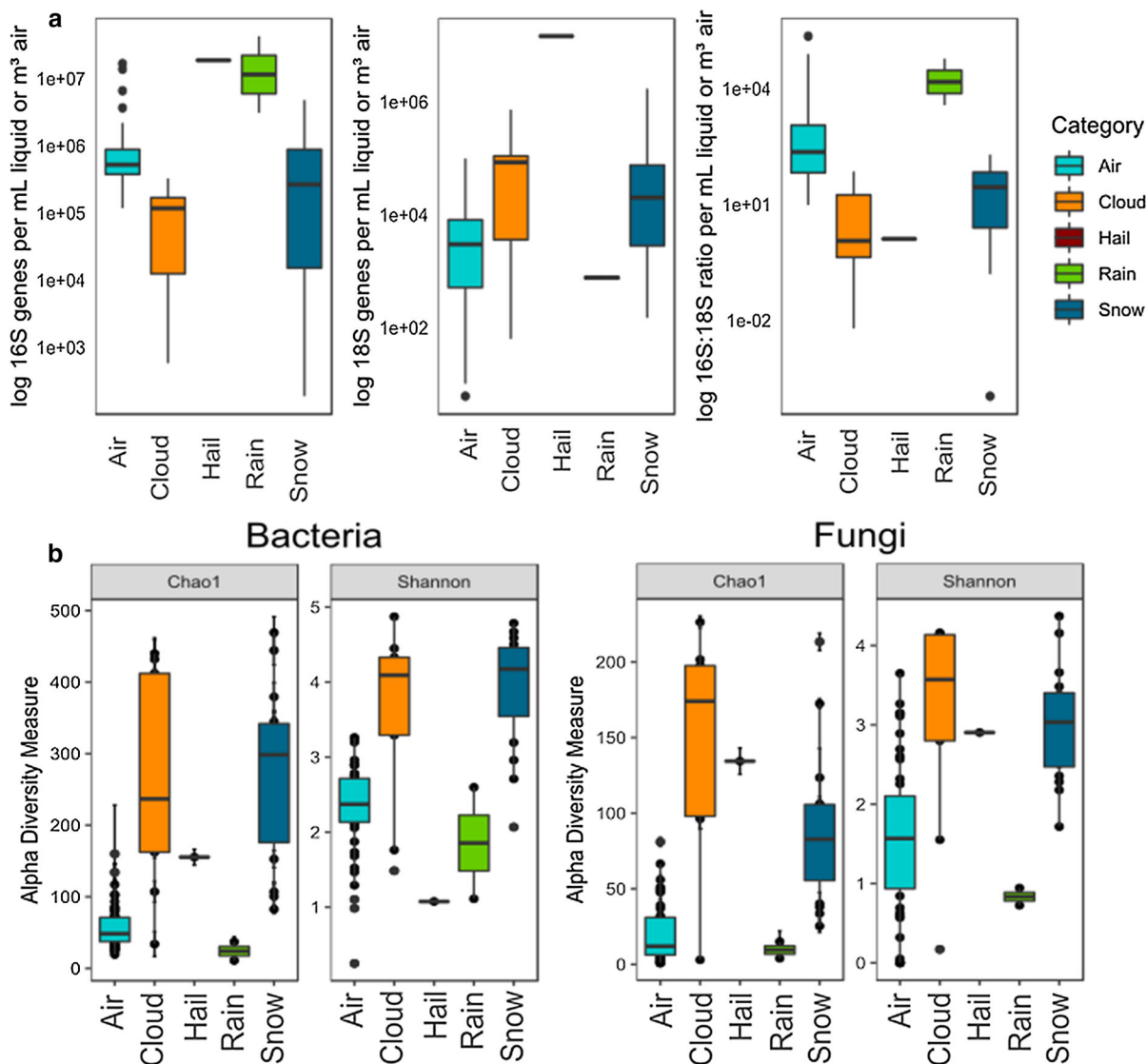


Fig. 4 **a** 16S rRNA genes, 18S rRNA genes and 16S:18S ratio m⁻³air and mL⁻¹ precipitation, **b** alpha diversity indices in air and precipitation for bacteria and fungi

3.3.3 Beta Diversity in air and precipitation

NMDS (Fig. 5a) revealed a strong clustering of bacterial communities of precipitation, with rain and one May cloud sample being most distinct. The February bacterial air samples are the most distinct from precipitation.

August fungal samples cluster closest together in air samples and closest to precipitation. Fungal precipitation samples cluster more densely than any season for air, apart from August rain samples and one May cloud sample, which are more distinct. Fungal

February and November air samples strongly overlap, while May samples are most scattered.

Bacterial air samples are significantly different from precipitation samples (ADONIS $R^2 = 0.1118$, $p = 0.001$, ANOSIM $R = 0.3004$, $p = 0.001$). Air and snow resp. cloud bacterial communities were significantly different (air–snow, air–cloud: PERMANOVA p .adj = 0.01), whereas air and rain did not differ significantly (PERMANOVA p .adj = 0.84), but hail did in unadjusted testing (PERMANOVA unadj. $p = 0.022$, p .adj = 0.22). Snow was significantly different from rain (PERMANOVA unadj. $p = 0.025$).

Also fungal air communities differed significantly from precipitation (ADONIS $R^2 = 0.1076$, $p = 0.008$, ANOSIM $R = 0.0697$, $p = 0.001$). Air and snow resp. cloud fungal communities differed significantly (air–snow, air–cloud: PERMANOVA p .adj = 0.01). Fungi in air and rain differed in single testing (PERMANOVA unadj. $p = 0.046$, adj. $p = 0.46$). Snow and rain fungi were significantly different (PERMANOVA adj. $p = 0.04$).

When compared pairwise for each season, bacteria in air were most similar to snow, rain and hail in August (PERMANOVA air–snow p adj. = 0.096, air–rain p adj. = 0.156, air–hail p adj. = 0.552) and snow and cloud in May (PERMANOVA air–snow p adj. = 0.054, air–cloud p adj. = 0.048). Cloud, snow, rain and hail were never significantly different, when compared against each other within one season. In the total dataset, rain and snow showed the biggest difference (PERMANOVA p non-adj. = 0.025).

Snow and cloud in cold seasons (November and February) appear similar in bacterial composition, with Acidobacteria (3.2–6.2%), Actinobacteria (18.6–40.7%), Bacteroidetes (5.5–22.0%) and Cyanobacteria (13.4–20.8%) being abundant, whereas air is dominated by Firmicutes (48.1%–78.4%). Proteobacteria make up a comparable share in air, cloud and snow in winter (11.2–27.2%). In May, air and cloud are more similar with high abundances in Proteobacteria (54.3% in air, 51.1% in cloud), while May snow features high abundances of Actinobacteria (53.8%) and Firmicutes (19.0%), while cloud and snow in May feature Cyanobacteria (7.8% in snow, 8.6% in cloud) that are very low in May air (0.9%, Fig. 6).

Air in August features also high numbers of Proteobacteria (49.4%) that are even higher in hail (88.8%). August snow is dominated by Bacteroidetes (47.4%), Proteobacteria (27.1%) and Actinobacteria (20.1%), with Firmicutes lacking, and the lowest abundances of Planctomycetes (0.6%), Chloroflexi (0.01%) and Cyanobacteria (2.7%) compared to snow from the other seasons.

Fungi show a similar pattern, with rain and snow being most different in the overall dataset (PERMANOVA p .adj. = 0.04) and precipitation never significantly differing within season comparison. Highest similarity occurred between air and snow in May (PERMANOVA p adj. = 1.0), but also all precipitations in August and did not differ significantly to air

(PERMANOVA snow p adj. = 0.102, rain p adj. = 0.066, hail p adj. = 0.468). Further, air and cloud in February were only significantly different in unadjusted testing (PERMANOVA p non-adj. = 0.028, p adj. = 0.084).

In fungi, the composition of cloud and snow is similar in winter samples, but different to air. In November, air is dominated by unidentified (35.5%), Sordariomycetes (23.2%) and Eurotiomycetes (14.1%). In February, air is dominated by Agaricomycetes (34.6%) and unidentified (39.9%). Clouds and snow in winter were dominated by Leotiomycetes (9.8% cloud, 16.8% snow) and Agaricomycetes in November (27.4% snow, 26.2% cloud), Dothideomycetes (9.9–35.8%), Eurotiomycetes (15.8–23.9%), Lecanomyces (9.2–26.5%), further unidentified (9.4–26.8%, Fig. 6).

In May, air featured in Wallemiomycetes (11.1%) and Saccharomycetes (10.4) that rarely occurred in any other air or precipitation sample and May cloud samples featured Tremellomycetes (36.4%) and Dothideomycetes (9.8%).

Snow in both summer samples was high in unidentified organisms. Rain and air featured high abundance of Agaricomycetes (59.9% in air, 55.5% in rain) in August. Rain was further rich in Sordariomycetes (37.2%), while hail was abundant in Dothideomycetes (36.5%) and Leotiomycetes (31.6%).

11.8% of bacterial genera and 9.7% of fungal genera were only found in the 288 m³ of sampled air and not in any of the precipitation samples (Fig. 5b). The share of common bacterial genera (12.4%) and fungal genera (11.6%) was similar. The ratios of unique and common bacterial and fungal genera between precipitation types display a comparable picture, with the biggest %-differences in shared genera between cloud, snow and air in bacteria (21.7%) compared to 13.3% in fungal genera, unique cloud fungi (15%) compared to unique cloud bacteria (9.1%), and bacteria shared between cloud and snow (18.2%) compared to fungi in cloud and snow (21.5%).

Most abundant in all sample types were *Geobacillus* (Firmicutes), *Aminobacter* (Proteobacteria) and *Herpetrichiellaceae* (Eurotiomycetes), *Fomitopsis* (Agaricomycetes) and

Hypholoma (Agaricomycetes) (Supplementary Tables S3 and S4).

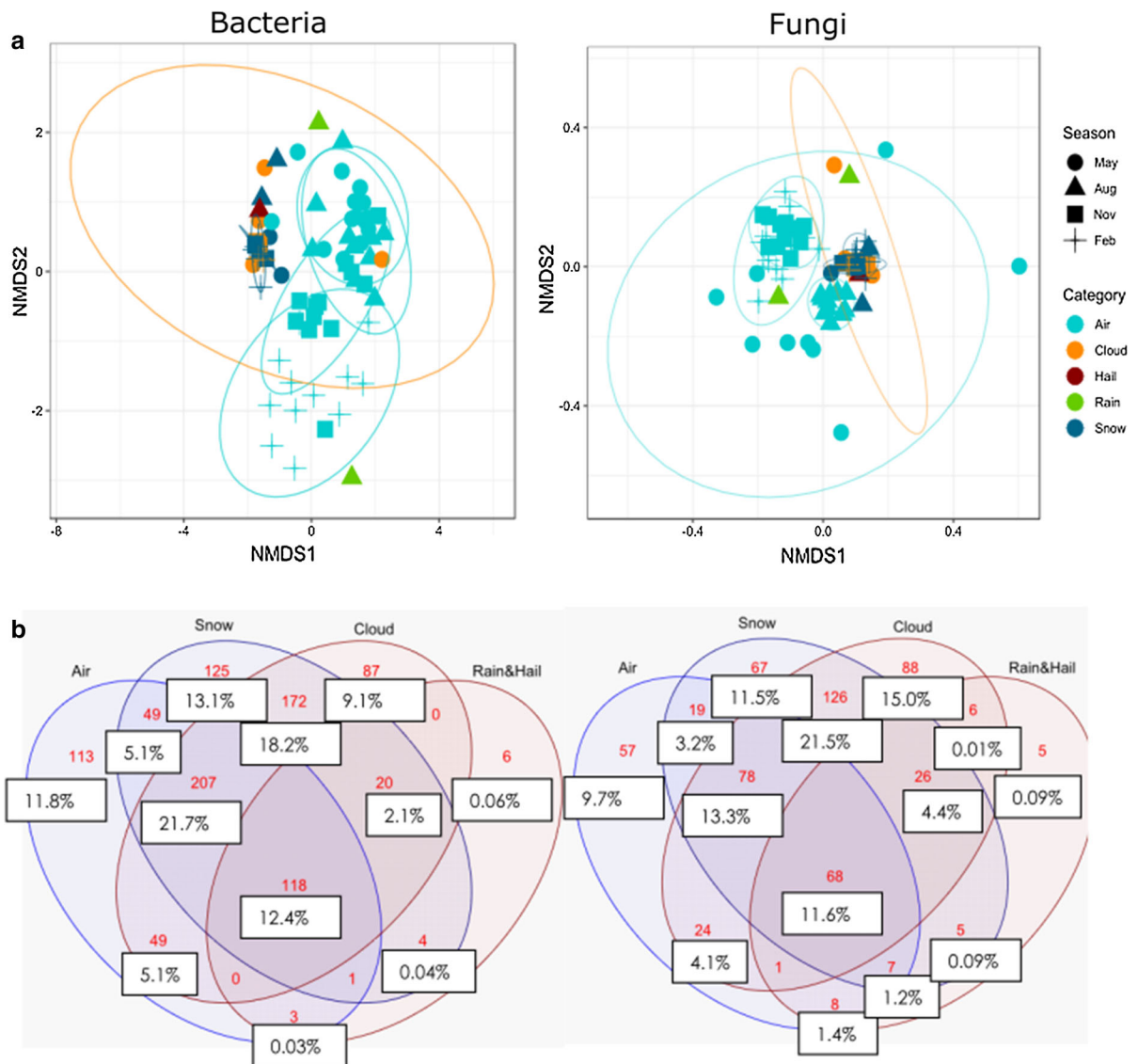


Fig. 5 a NMDS of Bray–Curtis distance of air and precipitation, b Venn diagram of common and unique bacterial and fungal genera

SIMPER analysis (supp Tables S1, S14, S15, S17, S18) revealed that the genera mostly driving differences between air and snow are *Geobacillus* and *Aminobacter* that are more abundant in air, and *Chlorophyta*, *Nakamurella* and *Ferruginibacter* that are more abundant in snow, on phylum level Firmicutes were dominant in air compared to Actinobacteria, Bacteroidetes and Cyanobacteria in snow.

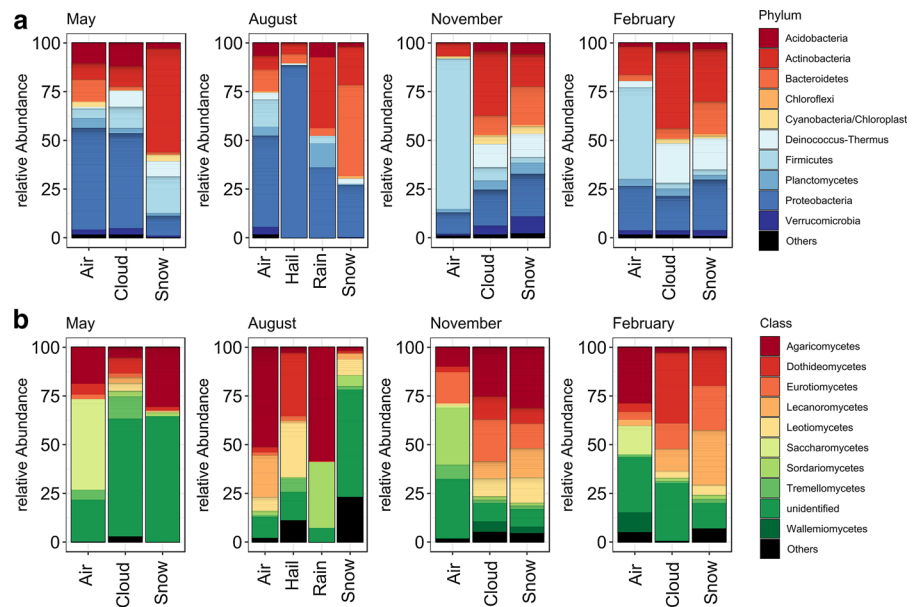
The difference between cloud and air was driven by *Geobacilli*, *Bosea* and *Aminobacter* that were more abundant in air and *Janthinobacterium* and *Chlorophyta* that were more abundant in clouds, on phylum

level Firmicutes were much more abundant in air, whereas Actinobacteria and Cyanobacteria were more abundant in clouds.

In rain, *Gp1* and *Dehalospirillum*, *Nakamurella* and *Aquisphaera* were more abundant than in air, and Actinobacteria and Acidobacteria were more abundant at the phylum level in rain than in the air.

In hail, *Janthinobacterium*, *Cytophagales* and *Subtercola* drove differences with air, and Proteobacteria was the dominant phylum as compared to Firmicutes, Actinobacteria and Planctomycetes for air samples.

Fig. 6 Top 10 **a** bacterial phyla and **b** fungal classes for air and precipitation by season



Most abundant unique fungal genera in air were *Stereaceae* (Agaricomycetes), *Curvularia* (Dothideomycetes) and *Corticium* (Agaricomycetes), while most abundant unique genera in snow comprised *Phaeosclera* (Dothideomycetes) *Lactiflus* (Agaricomycetes) and *Phaerophyscia* (Lecanoromycetes), and *Phaeomycoentrospora* (Dothideomycetes), *Bys-soloma* (Lecanoromycetes) and *Rhodospordiobolus* (Microbotryomycetes) in clouds.

For fungi, SIMPER analysis was conducted on class level, as too many unidentified taxa occurred on lower levels. Agaricomycetes were abundant in air compared to snow and cloud, whereas Eurotiomycetes and Dothideomycetes were more abundant in snow and cloud. Lecanoromycetes in snow and Tremellomycetes in cloud also drove differences with air.

Agaricomycetes and Sordariomycetes were more abundant in rain than in air, while Eurotiomycetes, Tremellomycetes and Lecanoromycetes drove the difference of air to rain. In hail, the biggest difference to air was dominated by the presence of Agaricomycetes, Eurotiomycetes and Sordariomycetes in air together with Dothideomycetes, Leotiomycetes and Arthoniomycetes in hail.

3.4 Ice nucleation-active bacteria

In total, there were 53 genera containing the ice nucleation protein (INP) detected in the dataset. In air

samples, the relative abundance of INP reads of the total reads per sample was lowest in November (1.56%) and highest in August (5.28%), but with high variation. In clouds, relative abundance of IN varied strongly between seasons displaying the highest value in the dataset (15.82%), whereas in May accounted for only 2.25% and in February 4.16%. In snow the mean IN abundance varied between 5.22% in May and 11.51% in August. Hail ranged at 7.04% and rain between 0 and 11% (Fig. 7a). The composition relative abundance of the most abundant INP-containing bacteria showed a similar pattern than the composition of most abundant phyla (discussed in Chapter 3.3, Fig. 6), with snow and cloud being similar in February and November, and more similarity of air and cloud in May, and high variability in August. (Fig. 7b)

NMDS (Fig. 8a) reveals that cloud and air have a higher overlap, than snow and air, with cloud at the interface between air and snow. The two rain samples are different in IN-composition. Hail clusters with snow and cloud. Most of the 53 identified genera containing the ice nucleation protein, present in the dataset, were associated with snow. The occurrence of ice nucleation-active reads is more similar in the frozen water samples (hail, snow) to the clouds, than air. However, the Venn diagram (Fig. 8b) reveals, that the most genera are shared between all of the samples

or air, snow and cloud, but by NMDS, indicating, that they are more abundant in snow and cloud.

The genera associated with higher abundance in air were *Corynebacterium*, *Pantoea*, *Enterobacteria*, *Micrococcus*, *Caulobacter*, *Paenibacillus*, *Acidovorax*. *Xanthomonas* and *Erwinia* were unique to air.

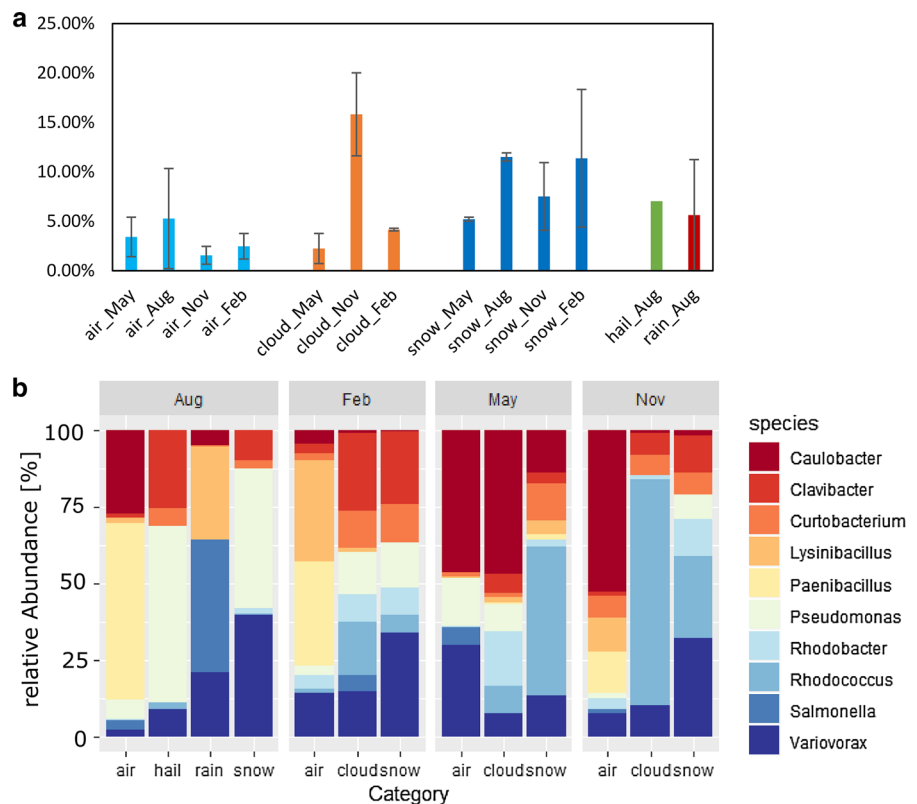
4 Discussion

4.1 Seasonal differences in air community composition

Seasonal variation and temporal vicinity of sample composition have been observed for near-surface airborne bacterial and fungal communities (Bowers et al. 2013; Fahlgren et al. 2010; Franzetti et al. 2011; Pickersgill et al. 2017), and even above PBL for bacteria (Bowers et al. 2012). These observations are consistent with our data, which showed that there was no stable free troposphere air microbial community during the year of sampling at Mount Sonnblick, although a core microbiome could be identified

consisting of 61 (11% of all air genera) bacterial and 8 fungal genera (3% of all fungal air genera). Many of these have previously been identified as indicator species for above PBL air compared to below PBL air (Els et al. 2019). Air mass origin was different for the four sampling periods. Differential source regions and transport have been shown to influence microbial composition of the atmosphere (DeLeon-Rodriguez et al. 2013; Innocente et al. 2017). Zweifel et al. (2012) also suggested annual variation, succession of species in airborne communities or random variation as explanatory mechanisms. One mechanism that might impact microbial composition in air communities is thermal convection and air mass mixing in the boundary layer. The lowest amount of unique bacterial genera was found in August, but the highest number of unique genera was found in May. In August the thermal convection and mixing was most active, with the highest mean boundary layer elevation and variation of the sampling dates (748 ± 667 m AGL). The height of PBL-mixing even reached up to the observatory for a short moment (between T3 and T4, compare supplement data Fig. S4), thus it is likely that

Fig. 7 **a** Relative abundance (%) of IN-active bacteria of the total number of 16S reads, **b** relative abundance of 10 most abundant IN-active genera



stronger, thus more spores were likely transported higher up in the atmosphere and injected to the free troposphere.

4.3 Different seasonal dispersal patterns for bacterial and fungal communities

Bacterial and fungal communities exhibited different patterns in changes in community structure throughout the year. Bacterial community structure differed significantly between summer and winter. Summer samples were dominated by ubiquitous, terrestrial associated genera like *Paludibacter* (Bacterioidetes), a gram-negative non-spore former involved in NH_4^+ oxidation (Zhang et al. 2018), *Domibacillus* (Firmicutes), a gram-positive, spore-forming, aerobic, non-motile, rod-shaped organism isolated from deep-sea sediment (Sun and Sun 2016), ocean sediment (Sharma et al. 2014), mangrove sediment soil and macroalgae (Verma et al. 2017) and *GPI* (Acidobacteria), found in alpine grassland on the Tibetan Plateau (Yuan et al. 2014). Winter samples were more characterized by extremophile but omnipresent bacteria like *Bifidobacterium* (Actinobacteria) that can withstand a high range of pH (from 8 to 2) (Charteris et al. 1998), *Vulcaniibacterium*, a thermotolerant Gammaproteobacteria isolated from a geothermally heated soil (Yu et al. 2013), and *Polaribacter* (Bacterioidetes), which has been found in several arctic habitats (Cuthbertson et al. 2017; Harding et al. 2011; Collins et al. 2010). Differences in the winter to the summer microbiome (in SIMPER analysis) were driven by changes in abundance in Firmicutes such as *Geobacillus* and *Tumebacillus*. *Geobacillus* is a known thermo-resistant, spore-forming, ubiquitous Firmicute (Zeigler 2014). *Tumebacillus* (aerobic, spore-forming, gram-positive, rod-shaped, chemolithoautotroph) has been found in high Arctic Canadian permafrost (Steven et al. 2008) and was identified as one of the most abundant lineages in Australian dry lake aerosols (Munday et al. 2016) and heavy-haze days in Beijing, China (Yan et al. 2018). Firmicutes are recurrently characterized as highly abundant in free tropospheric air masses and long-range transport air masses (Smith et al. 2011, 2012; David et al. 2018; Els et al. 2019) and were enhanced in free tropospheric fall samples (Bowers et al. 2012). They are known to withstand hostile, even extraterrestrial conditions, like heat, cold, radiation or

chemicals, by forming resistant endospores and are found in all investigated environments (Setlow 2006; Driks and Eichenberger 2016; Nicholson et al. 2000).

Fungi displayed a high seasonal variability with a range of different genera and phyla dominating the fungal composition at the different seasons. Sporulation strategy, dissemination adaption and lifestyle-dependent patterns are critical aspects for airborne fungal occurrence (Pickersgill et al. 2017). 53.9% of all fungal genera were unique to August air masses. These samples were dominated by a large group of lichenized fungi (Miadlikowska et al. 2014) and the largest non-lichenous fungal group Leotiomycetes (Wang et al. 2006), whereas xerophilic and psychrophilic Eurotiomycetes (Geiser et al. 2006), highly resistant, yeast forming Saccharomycetes (Mühlhausen and Kollmar 2014), yeast forming Tremellomycetes (Liu et al. 2015) and xerophilic Wallemiomycetes (Zalar et al. 2005) were dominant in May. Tremellomycetes were also abundant in November, together with plant pathogenic, endophytic, saprophytic, epiphytic and lichenolous Sordariomycetes (Maharachchikumbura et al. 2016). In February, a time when potentially low sporulation happens, air abundant Agaricomycetes dominated. High wind speed in February yielded the second highest number of genera occurring in one season (31.7%) and the communities were dominated by Agaricomycetes, which is ground and plant associated and known to form propagules being dispersed by the air and mainly connected to wet deposition (Hibbett et al. 2014; Prasher 2015). Additionally, compared to the other phyla, they are characterized by a relative small aerodynamic diameter which enables the spores to avoid faster sedimentation by dry deposition (Woo et al. 2018) and usually contribute a big share of airborne fungi (Pickersgill et al. 2017; Fröhlich-Nowoisky et al. 2012). However, it has to be taken in to account that the chosen molecular primer and amplification method can result in an over- or underrepresentation of certain fungal classes (e.g., here highly abundant Agaricomycetes) or phyla (Blaalid et al. 2013).

4.4 Comparison of community structure and abundance in air and precipitation

Microorganisms precipitated with snow, hail or rain or being transported by clouds differ in their species

composition from free tropospheric air masses and do not mirror the air community structure. They are distinct in composition, 16S:18S ratio, abundance and diversity from free-floating PBA. Clouds and snow generally had the highest diversity in both bacterial and fungal datasets, with atmosphere and rain being the least diverse.

Cloud bacterial communities were dominated by Proteobacteria, Actinobacteria and Bacteroidetes, which has also been observed in several cloud water studies from China, France, southern Africa and the USA (Jiaxian et al. 2019; Amato et al. 2017; Kourtev et al. 2011; Zhu et al. 2018; Evans et al. 2019). Fifteen of overall identified fungal genera were unique to clouds, whereas only 9% of bacterial genera were unique to clouds. The most abundant unique fungal cloud genera were *Phaeomycoentrospora* (Dothideomycetes), a large cosmopolitan genus of plant-pathogenic leaf and fruit fungi (Crous et al. 2013). Further abundant unique cloud genera were *Bysoloma* (Lecanomyces), a lichen with pantropical spread and scattered temperate localities that was only recently first described in Sweden (Hermansson and Thor 2004), and *Rhodospiridiobolus* (Microbotryomycetes), which was found in arctic air and is associated with elevated phospholipase and hemolytic activity (Kirtsideli et al. 2017). Microbotryomycetes contain a range of psychrophilic yeasts. Thus, for those abundant unique cloud genera, their likely source and part of their dissemination strategy are long-range transport.

In air as compared to snow, cloud, hail and rain especially differences in Bray–Curtis distance were driven on genus level by *Geobacillus* (Firmicutes) and *Aminobacter* (Alphaproteobacteria), an organism found in various terrestrial environments (McDonald et al. 2005) that supports legume nodulation (Maynaud et al. 2012) and is capable of herbicide degradation (Schultz-Jensen et al. 2014). A further driving organism was *Bosea* (Alphaproteobacteria) an amoeba-resisting bacteria which is associated with hospital water supply and intensive care ventilator-acquired pneumonia (Khamis et al. 2003; La Scola et al. 2003) and rhizosphere (Ahern et al. 2007). On phylum level, air was especially enhanced in Firmicutes. Hail was the only precipitation form not yielding significant differences to air at the respective season in unadjusted testing for bacteria and fungi in our dataset.

The bacterial abundance of fresh deposited snow, graupel or rain accounted for approximately 1×10^4 mL⁻¹ or higher in various samples (Amato et al. 2007a, b; Bauer et al. 2002; Carpenter et al. 2000; Sattler et al. 2001; Segawa et al. 2005) and 10^3 – 10^4 m⁻³ for bacterial density in air at remote sites (Bauer et al. 2002; Burrows et al. 2009a, b). This implies a higher density of microbes in precipitation at a range of 1:10⁶. PBA are more abundant in clouds than in the free atmosphere, and despite harsh conditions in clouds they might be more life-supporting due to the presence of water (Delort et al. 2017; Bauer et al. 2002). Clouds can highly vary in PBA abundance, with clouds formed during extreme events like desert dust uplift, containing higher than expected abundances (of 10^5 m⁻³ bacteria like particles) (Maki et al. 2017). Further, the median of 16S to 18S ratio was lowest in clouds, suggesting a higher capacity of cloud droplets to hold fungi in clouds in comparison with air, snow or rain. The result of lowest ratio of bacteria to fungi in clouds is in accordance with Bauer et al. (2002), whose counts result in a bacteria-to-fungi ratio of 4.4 for clouds, 5 for snow and 16.4 for air. Bacteria could provide a surface area for the condensation of water vapor on the surface of aerosolized particulate matter (Jiaxian et al. 2019). In rain, 16S:18S ratio was highest, suggesting a separation mechanism for fungal spores before rain forms.

Rain was high in 16S rRNA gene abundance but low in 18S abundance and in bacterial and fungal diversity. Air and rain were not significantly different in total bacterial air dataset, but in unadjusted testing in August dataset, fungi were significantly different in the total dataset.

Rain was the only precipitation form that yielded a significant difference when compared with another precipitation form in the total dataset, but it was not significant within the August dataset. However, the snow here was likely more influenced by melting, aging and aerial deposition. Rain was different to bacterial diversity in snow (unadjusted) and more significantly to fungi in snow.

Hail had the lowest 16S: 18S ratio, which might be due to the formation from cloud droplets that have also low 16S:18S ratio, where spores might possibly get stuck to ice surface during sudden freezing.

4.5 Seasonal effects related to temperature

A significant separation of air masses and precipitation samples was observed, and differences varied between seasons. In the winter seasons (November and February), the composition was similar in cloud and snow, but different to air, whereas in May bacterial composition of cloud and air was more similar, but pronouncedly different from snow. In May, the community of fungi in clouds featured high numbers of cold-adapted yeast and lichenous Tremellomycetes (Liu et al. 2015; Thomas-Hall et al. 2010) and plant-pathogenic Dothideomycetes (Schoch et al. 2009; Hane et al. 2007) that were less abundant in air or snow. These results indicate that cold environmental conditions lead to comparable bacterial and fungal composition of snow and cloud, but different composition of air, while in the warmer conditions of May, air and cloud water were more similar in bacterial composition. Here we found the highest number of shared genera of cloud and air in one season (8.6%) and highest total number of unique cloud genera (20.7%) and outstandingly high amount of unique fungi in May cloud (63.6%), with snow more different and a distinct fungal cloud composition. This indicates that warmer water vapor (but still negative ambient temperature in May around -6.9 °C) might have higher momentum and exchange or even promote a distinct composition by its living conditions. Also the composition of ice nucleation-active bacteria was similar in air and cloud in May with higher abundances of *Pseudomonas*, than in the other seasons, that can efficiently nucleate ice at a temperature of -7 °C or lower (Maki et al. 1974).

Bacteria in air were more similar to precipitation in August (especially hail and air) than in the other months. This could be due to higher temperatures and humidity, facilitating more thermodynamic momentum leading to exchange and incorporation of humid air into cloud formation, similar life conditions in humid air and fast, on-site precipitation rather than distant transport.

August snow was lacking Firmicutes and had a low abundance of Chloroflexi, Planctomycetes and Cyanobacteria compared to snow from the other seasons. Here melting and accumulation processes might have played a role, as the snowpack was percolated with melt and rain water, and sediment

form the molten snow pack and dry deposition was accumulated on the surface.

August fungal air samples were the closest to precipitation fungal samples. This might be due to the high abundance of fungi in August, but further also the higher humidity during this time, as fungal species actively discharge spores via liquid jets into the air, preferentially under humid conditions (Pringle et al. 2005; Elbert et al. 2007) and fungal variability being associated with humidity and rain frequency (Bowers et al. 2013). Fungal composition was shown to be significantly different in wet and dry deposition, suggesting taxon and size specific involvement in cloud condensation and precipitation (Woo et al. 2018).

4.6 Selection processes and potential drivers

The differences observed in community structure and abundance among the different sample types might be related to physical processes such as scavenging. In general, scavenging depends on the aerodynamic diameter, particle density but also chemical composition. Microbial cells can show hydrophilic or hydrophobic membranes which can alter their deposition substantially (Maria and Russell 2005). Cell surface hydrophobicity is often found in gram-negative bacteria (Krasowska and Sigler 2014). Rain or snow precipitation removes below-cloud aerosol particles and is a key process in aerosol chemical transport (Zhang et al. 2013). The scavenging coefficients or removal rates of particles are complex functions (Slinn 1983), but theoretical and experimental studies have shown that snow is a more efficient scavenger of particles than rain because of its larger surface area (Franz and Eisenreich 1998). The relative scavenging efficiency between snow and rain depends on particle sizes and precipitation intensity (Wang et al. 2014). For ultrafine and fine particles, the snow scavenging coefficient is predicted to be ~ 10 times larger than the rain scavenging coefficient at low precipitation rates (Wang et al. 2014). For rain, scavenging efficiency decreases with increasing particle size (Ardon-Dryer et al. 2015).

Clouds, fog and rain seem to represent media, where biological activity is significant due to the protection from desiccation and the potential use of chemical compounds in the aqueous solution (Deguillaume et al. 2008). Some bacteria can act as cloud

condensation nuclei and produce biosurfactants that increase droplet size and cloud lifetime, counteract desiccation and allow water scavenging and thus facilitate widespread dispersal (Ahern et al. 2007).

PBA originate from surface aerosolization and thus represent larger potential sources than precipitation. Stricter selective processes are occurring for PBA to reach clouds, survive and be precipitated.

Rain does not scavenge too many organisms, as the residence time in the atmosphere is lower in rain. Raindrops have a smaller surface and are potentially less active in atmosphere interactions. Snow, in contrast, acts more like a sponge.

Hail is a unique form of precipitation that allows direct characterization of particles present during atmospheric ice nucleation, as the nucleation event is captured in the hail stone embryo (Michaud et al. 2014). The embryo is then caught by updraught and cycling in a storm cloud while accreting supercooled water during hailstone formation and thus represents a vertical intersection of cloud microbial composition (Knight and Knight 2001; Michaud et al. 2014). Šantl-Temkiv et al. (2013) observe an enrichment in plant-associated bacterial groups (Gammaproteobacteria, Sphingobacteria, Methylobacteria). Hail is one of the most extreme environments to live (Šantl-Temkiv et al. 2013).

4.7 INP-containing bacteria are selected for precipitation

Another mechanism that might explain variation in community structure is the capacity of certain organisms to carry out ice nucleation (IN). IN-particles active at ≥ -10 °C deplete twice as rapidly in precipitating clouds than other particles at this size (Stopelli et al. 2015).

There was a higher share of relative % reads of known ice nucleation-active bacteria present in all wet phases compared to air. Within the wet phases, snow had the highest mean % reads, whereas clouds showed a big variation thereof. It seems that a separation of IN-active reads occurred with higher shares occurring in precipitation.

Regarding seasonality, within air masses, the highest share of IN-active reads was reached in August, when also strongest precipitation occurred and samples were taken during rain events. IN-

occurrence has been reported to increase during and after rain events (Huffman et al. 2013).

August precipitation was characterized by short but strong showers (compare supplement data Fig. S3) which have been observed to be significantly enhanced in IN in the first period of high intensity rain and hail, compared to continuous-type rain (Vali 1971). Ice nucleation-active bacteria were found to be relatively more abundant in rain than in air at the same site (Stephanie and Waturangi 2011). Christner et al. (2008a, b) report cumulative ice nuclei over several seasons to reach higher maximum values in rain (Louisiana rain 8–230 IN L⁻¹, Alberta rain 20–600 IN L⁻¹) compared to snow (mid-latitude snow 3–150 IN L⁻¹). Cloud IN varied between 1 and 200 mL⁻¹ at -10 °C at Puy de Dome (Joly et al. 2014), thus ranging one order of magnitude higher than in rain or snow.

Christner et al. (2008a, b) estimated 0.4% of DNA-containing cells in mid-latitude snowfall being IN-active at -7 °C to -4 °C, whereas in cloud water, estimates range between 0% and 1.5% IN-active bacteria at -10 °C of total bacteria (Joly et al. 2014). In air, numbers were reported as 0.001% (Garcia et al. 2012; Xia et al. 2013) and showed significantly higher numbers in foggy air than in clear air (Bowers et al. 2009; Xia et al. 2013). Petters and Wright (2015) describe a variation in IN-concentration between rain, cloud water, hail and snow of up to five orders of magnitude.

Abundant rainfall was reported to drive near-surface air IN-concentration with a positive correlation of rain intensity and IN-concentration, while strong snow fall had an attenuating effect on IN-concentration (Conen et al. 2017).

In comparison, the values we estimate here based on relative abundance of reads of INP containing bacteria yield substantially higher numbers of IN-bacteria in air, cloud and precipitation. This could impact terrestrial biodiversity, with microbes landing in new environments faster (Morris et al. 2014), as our results imply, when precipitating.

The observed separation for community composition between air, snow, cloud and wet precipitation was also observed for IN-active bacteria, with clouds seeming like a mediator between air and snow in IN-composition. Only a few genera were unique to one sample type, the discrimination by NMDS was due to changes in relative abundance between sample types,

which indicated an accumulation of most INP-containing bacteria in the wet phases, and only single species associated with air. *Erwinia* and *Xanthomonas* were unique in air in this dataset, though they are usually abundant in the environment, but also absent in other water-based IN-surveys (Du et al. 2017). Zweifel et al. (2012) found *Erwinia* and *Pantoea* in low frequencies, but recurringly in free tropospheric air samples.

The microbes present in clouds, snow and rain survived long-range transport, ice nucleation and cold cloud processes. Thus, it is likely that wet or snow deposited microbes will outperform their dry deposited counterparts in numbers and adaptations. As clouds often form far from the place where they precipitate and biological nucleators are frequently involved, their microbial composition is different from the present surface air microbiome.

4.8 Precipitation is no proxy for free troposphere air communities

Cáliz et al. (2018), who recently used snow as a proxy for air mass composition in the free troposphere, identified “airborne” Chytridiomycota as abundant in spring snow. The overall relative abundance of Chytridiomycota in all air samples was 0.01%, compared to 2.49% in snow and 6.15% in rain but only 0.13% in hail and 0.22% in clouds in our dataset, indicating scavenging snow and rain precipitation. Bacterioidetes, a phylum identified by Cáliz et al. (2018) as abundant in snow, was identified as the one of three phyla driving most differences between air and snow (5.67% air, 18.40% snow). While there is no doubt that microorganisms are aerosolized, incorporated in snow and then deposited (Harding et al. 2011), based on our dataset, selection at the phylum level occurs. Cyanobacteria were high in snow and cloud compared to air. They often contain a hygroscopic mucilaginous covering, that effects in density reduction and dynamic streamlining (Reynolds 2007), but might also absorb atmospheric moisture, why they become heavy and drop down (Sharma and Singh 2010). Air bacterial composition was found to be more similar to soil, decaying material, lichen and plant surface assemblages than to snow in a survey in Greenland, where snow featured higher abundances of

Beta- and Gammaproteobacteria and Bacterioidetes, while air was richer in Alphaproteobacteria, Cyanobacteria, Acidobacteria and Planctomycetes (Šantl-Temkiv et al. 2018).

Fog is known to have enriched concentrations of bacteria and yeast CFUs up to two orders of magnitude compared to clear air conditions, but no changes for mold CFUs (Fuzzi et al. 1997). Moreover, air samples derive from restricted altitudes, whereas the cloud samples can be addressed as a composite of this respective height including a thought black box from above. The composition of cloud chemistry and metabolically adapted cloud PBA community varies depending on the cloud origin (Deguillaume et al. 2014; Amato et al. 2017).

Evans et al. (2019) found that Shannon alpha diversity was greater in fog than clear conditions. Fog was more shaped by local sources than clear air, but also contained more remote marine signatures, indicating good conditions for long-range travel in clouds (Evans et al. 2019).

SIMPER analysis identified *Janthinobacterium* (Betaproteobacteria) and *Chlorophyta* (Chloroplast) as genera driving the difference between air and cloud and especially abundant in hail. *Janthinobacterium* in cloud water was found negatively correlated with SO₂, O₃, and particulate matter (PM₁₀ and PM_{2.5}) and positively correlated with K⁺ and Mg²⁺ (Jiaxian et al. 2019), which main sources are soil dust in big cloud droplets (Zhu et al. 2018). *Janthinobacterium* has antifungal properties (Rubio et al. 2018) and was found in high mountain lakes and Antarctica (Ahern et al. 2007) and alpine cryoconite, and is known for its psychrotolerant properties (Kim et al. 2012). It does fit the continental background location of Mount Sonnblick, where low concentrations of aerosol pollution markers (like SO₂ or high PM) would be expected (Deguillaume et al. 2014).

Chlorophyta (i.e., microalgae) have previously been found in cloud samples (Urbano et al. 2011). The reason for that might also be the higher retention capacity in droplets than in air for bigger size PBA. The aerosolization of chlorophyta is especially correlated with relative humidity, but also with temperature, sunshine and wind speed (Sharma and Singh 2010).

5 Conclusion

Significant differences were found in air microbial composition during different seasons, with pronounced summer and winter distinction in bacteria and strong discrepancies between all sampling events for fungi.

The season, thus temperature and humidity conditions, impact on free tropospheric microbial air composition and abundance, and should be kept in mind when interpreting single flight results. The differentiation between seasons was stronger pronounced in air than in precipitation, with rain being most different and variable of precipitation types, indicating distinct forces driving microbial fate in the air.

Microorganisms precipitated with snow, hail or rain or being transported by clouds differ in their species composition from free tropospheric air masses and do not mirror the air community structure. They are more diverse, potentially more viable due to the availability of water and as shown in this paper, distinct in composition, 16S:18S ratio and abundance from free-floating PBA.

Hence, snow or cloud samples are no suitable proxies for free tropospheric air microbiome composition, as used by several studies, due to the findings that separation processes in aerosolization, transport and scavenging occur. The microbial composition of arriving precipitation or clouds represents only a part of the microbial air communities of the cumulative sources of origin.

Air can travel thousands of kilometers, but it is even more important to consider what is migrating in clouds and wet air masses, respectively, as the precipitation of PBA is more likely than sedimentation from clear air masses. Thus, precipitation might even have a bigger impact in ecosystem input due to higher microbe density when considering the ratio of cell numbers in precipitation compared to air being $1:10^6$.

To understand global connectivity of bioaerosols, microbial migration and inoculation sources, a microbial air sampling network would be necessary, where precipitation cannot be used as a proxy but rather has to go alongside with air analyses.

With ongoing change in global water circulation, water availability and precipitation patterns this might have implications for microbial dispersal to ecosystems. Further, more continuous aerobiological

monitoring in the free troposphere will be needed to understand interacting factors and feedbacks.

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Data availability Raw sequences were submitted and are currently under evaluation at NCBI BioProject database under Project ID PRJNA516816 (<http://www.ncbi.nlm.nih.gov/bioproject/516816>).

Compliance with ethical standards

Conflicts of interests The authors declare that they have no conflict of interest.

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