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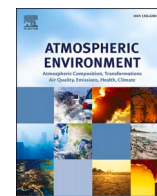
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Comparative analysis of bacterial diversity in clouds and aerosols

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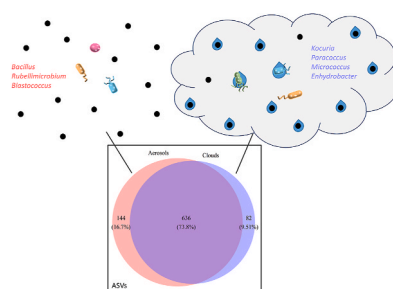
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HIGHLIGHTS

- Clouds harbor same bacterial taxa as clear situations.
- Certain taxa were found enriched in clouds, and conversely.
- Most differences were related to factors other than the presence of clouds.
- Replicate sampling is necessary in aerobiology to decipher environmental drivers.

GRAPHICAL ABSTRACT



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ABSTRACT

While the presence of condensed water in clouds could have influence on the diversity of bacteria circulating in the high atmosphere (multiplication or disruption of specific taxa), aerosols and clouds have been poorly studied comparatively in terms of microbiological content. Here we investigated the airborne bacterial diversity during cloudy and clear situations collected throughout the year at a high mountain site. Samples were collected using high flow rate impingers to examine total cell and DNA concentrations, as well as bacterial diversity by high-throughput sequencing. Overall very similar bacteria diversity was observed in clouds and clear air, with as high as 73.8% of the richness shared at the amplicon sequence variant (ASV) level. Bacterial diversity was more variable between sampling dates in the absence of cloud due to a wider range of meteorological conditions, but within single air masses, cloudy situations were more heterogeneous due to higher proportions of low-abundance taxa. Some taxa were found more abundant in clouds compared with clear situations, such as *Staphylococcus*, *Acinetobacter*, *Kocuria* and *Enhydrobacter*, or conversely, like *Bacillus*. Except for *Kocuria*, these could all be explained by concomitant factors other than the presence of cloud, i.e., season and altitude of the atmospheric boundary layer. This work emphasizes that airborne bacterial assemblages are driven by numerous interconnected environmental factors hardly distinguishable from each other. Replicated sampling help deciphering their respective roles, and such practice must be applied more widely in the field of aerobiology.

Abbreviations: ASV, Amplicon Sequence Variant; CCN, Cloud Condensation Nuclei; HFRI, High Flow Rate impinger; PUY, Puy de Dôme station; OPM, Opme station; ABL, Atmospheric Boundary Layer; NAP, Nucleic Acid Preservation buffer; rH, relative Humidity.

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1. Introduction

Bacteria emitted from a variety of surfaces (e.g., vegetation, soil, etc.) circulate in the atmosphere at typical concentration ranging from ~ 1 to $\sim 10^5$ cells m^{-3} of air (Burrows et al., 2009a). The airborne populations of bacteria are therefore highly diverse and variable over short spatial and temporal scales in relation with the influence of surfaces acting as sources, season, altitude, and meteorology (Bowers et al., 2011; Carotenuto et al., 2017; Tignat-Perrier et al., 2020).

When relative humidity in the air reaches and exceeds 100%, for instance in relation with the decrease of temperature at high altitude, water vapor condenses at the surface of aerosol particles (cloud condensation nuclei, CCN) and forms cloud droplets. Due to their large size ($\sim 1 \mu m$ or more), bacteria are considered excellent CCNs (Möhler et al., 2007) and wet processes largely contribute to their deposition (Aho et al., 2019; Morris et al., 2004; Péguilhan et al., 2021; Triadó-Margarit et al., 2019; Woo and Yamamoto, 2020), with eventually measurable impacts on surface ecosystems (Jalasvuori, 2020). Because they provide water and dissolved nutrients, the aqueous microenvironments formed by cloud droplets are thought to allow the multiplication of bacteria, with possible influence on the airborne microbial diversity (Ervens and Amato, 2020; Fuzzi et al., 1996; Sattler et al., 2001). Selective exclusion processes related with specific stresses such as osmotic shocks in clouds could also affect the composition of the airborne assemblages (Joly et al., 2015).

To date very few studies performed comparative microbiological analysis between the wet and dry phases of the atmosphere. In the Po Valley, Italy, airborne non-sporing culturable bacteria were found enriched by up to two orders of magnitude during fog events compared

with clear conditions (Fuzzi et al., 1996). Other studies focused on atmospheric deposits at high elevation sites (Els et al., 2019; Triadó-Margarit et al., 2019). They report a high similarity between the taxa of bacteria deposited by wet and dry processes, with up to 65% of OTUs in common, but a higher abundance of certain taxa in wet deposition, notably Oxalobacteraceae affiliated with *Noviherbaspirillum* and *Massilia* (Triadó-Margarit et al., 2019). At Mont Sonnblick (Austria, 3, 106 m a.s.l.), clear distinction could be made between the populations of bacteria deposited with snow, hail and rain and those circulating in free air. Wet deposits were more diverse and less sensitive to seasonal variations, and they harbored higher proportions of potential ice nucleation active taxa (Els et al., 2019b). These differences suggest modulations of the airborne bacteria diversity in relation with condensed water in clouds.

Here we investigated airborne bacteria during cloudy and clear situations at the high-altitude atmospheric station of puy de Dôme mountain (1,465 m a.s.l.). In total, more than 50 samples of clouds and aerosols were collected at 19 dates throughout the year using several high flow rate impingers (HFRi) deployed in parallel as replicates. Microbial cell concentrations and bacterial diversity were examined by flow cytometry and amplicon sequencing, and a comparative analysis between cloudy and non-cloudy situations was performed. The role of other environmental variables in explaining the observed variations, such as season and altitude of the atmospheric boundary layer, was also examined.

Table 1
Data related to sample acquisition.

Sample type and identifier	Sampling date (dd/mm/yyyy)	Season	Sampling site	Main geographical origin ^a	Sampling duration (h)	In/out ABL during sampling	Air temperature (°C) ^b	Relative humidity (%) ^b	Wind speed (m s ⁻¹) ^b
AEROSOLS									
20190712AIRPDD	12/07/2019	Summer	PUY	W	2.0	NA*	14.8	62	5.8
20190918AIRPDD	18/09/2019	Summer	PUY	NW	2.1	Out	11.3	67	4.4
20200206AIRPDD	06/02/2020	Winter	PUY	N	4.5	Out	4.7	20	6.0
20200518AIROPME	18/05/2020	Spring	OPME	NA*	5.1	In	15.2	24	NA*
20200610AIRPDD	10/06/2020	Spring	PUY	N	4.3	In	6.3	90	1.7
20200707AIRPDD	07/07/2020	Summer	PUY	NW	6.5	In	11.1	61	3.6
20200708AIRPDD	08/07/2020	Summer	PUY	NW	6.1	In	14.2	53	3.1
20200709AIRPDD	09/07/2020	Summer	PUY	N	6.0	In	20.3	48	3.4
20200922AIRPDD	22/09/2020	Fall	PUY	W	5.9	Out	12.4	78	1.0
20201118AIRPDD	18/11/2020	Fall	PUY	W	5.8	Out	14.1	41	6.4
20201124AIRPDD	24/11/2020	Fall	PUY	W	6.0	Out	8.6	50	3.4
Min	-	-	-	-	2.0	-	4.7	20	1.0
Max	-	-	-	-	6.5	-	20.3	90	6.4
Median	-	-	-	-	5.8	-	12.4	53	3.5
Mean	-	-	-	-	4.9	-	12.1	54	3.9
Standard error	-	-	-	-	1.6	-	4.4	21	1.8
CLOUDS									
20191022CLOUD	22/10/2019	Fall	PUY	S	6.4	Out	5.7	100	8.7
20200311CLOUD	11/03/2020	Winter	PUY	W	4.1	Out	5.0	100	7.4
20200717CLOUD	17/07/2020	Summer	PUY	NW	3.3	Out	10.1	100	1.6
20201016CLOUD	16/10/2020	Fall	PUY	NE	4.7	Out	1.1	100	1.8
20201028CLOUD	28/10/2020	Fall	PUY	W	6.0	Out	5.2	100	11.0
20201103CLOUD	03/11/2020	Fall	PUY	W	3.5	In	2.2	100	8.7
20201110CLOUD	10/11/2020	Fall	PUY	SW	3.1	Out	5.9	100	2.5
20201119CLOUD	19/11/2020	Fall	PUY	W	2.8	Out	0.3	100	7.7
Min	-	-	-	-	2.8	-	0.3	100	1.6
Max	-	-	-	-	6.4	-	10.1	100	11.0
Median	-	-	-	-	3.8	-	5.1	100	7.5
Mean	-	-	-	-	4.2	-	4.4	100	6.2
Standard error	-	-	-	-	1.4	-	3.2	0	3.7

NA*: No data available.

ABL: Atmospheric Boundary Layer.

^a From air masses backward trajectory plots over 72 h preceding sampling (<https://www.opgc.fr/data-center/public/data/copdd/trajectory>).

^b Average over the sampling period.

2. Materials and methods

2.1. Sampling procedure

Cloud and aerosol samples were collected at the top of puy de Dôme Mountain (PUY; 1,465 m a.s.l., 45.772° N, 2.9655° E; France) and, on one occasion, at Opme, a station located in the plain at 12 km from there (OPM; 680 m a.s.l., 45.7125° N, 3.090278° E; France) (Baray et al., 2020; Péguilhan et al., 2021). Throughout the manuscript, clouds are defined as situations where relative humidity (rH) was $\geq 100\%$ based on real-time measurements (Table 1); the presence of a cloud was also visually obvious during sampling. By opposition, the term “aerosol” is used as a reference to clear situations. The sample collected at OPM was considered a control for the “in the atmospheric boundary layer (ABL)” condition and local influence. For sampling, in general 3 HFRi (DS6, Kärcher SAS, Bonneuil-sur-Marne, France; $118 \text{ m}^3 \text{ h}^{-1}$; Santl-Temkiv et al., 2017) were deployed in parallel for $\sim 2\text{--}6 \text{ h}$, corresponding to $\sim 245\text{--}767 \text{ m}^3$ of air based on instrument specifications; at 3 occasions 2 or 4 replicates could be used. Volumes of either 850 mL or 1,700 mL of collection liquid were used for sampling during cloudy and clear situations, respectively, in order to account for the accumulation of liquid during wet situations, or evaporation otherwise. In the latter case, the volume was readjusted with sterile dH_2O every hour by weighing. HFRi were filled with nucleic acid preservation (NAP) buffer for DNA analyses and treated as replicates; here the entire volumes of liquids were filtered independently through $0.22 \mu\text{m}$ porosity (mixed ester cellulose filters, 47 mm) to recover microorganisms. DNA was extracted from filters using NucleoMag® DNA/RNA Water extraction kit (Macherey-Nagel, Hoerd, France), subjected to metabarcoded PCR (Supplementary Table 1) using the set of primers 515f-806r targeting the V4 region of the 16S rRNA gene of bacteria, and sequenced (Illumina Miseq 2*250bp) (see Supplementary Materials for more details on the methods). An additional HFRi sampler was filled with autoclaved ultrapure water or NaCl 0.9% solution for total cell counts (see Supplementary Materials).

The geographical origin of the air masses sampled and the fraction of time spent over sea or land at high ($>1 \text{ km a.g.l.}$) or low ($<1 \text{ km a.g.l.}$) altitude over the three days preceding sampling were recovered from 72-h backward trajectory plots, computed using the CAT trajectory model (Baray et al., 2020). In addition, each event was qualified as “In” or “Out” the atmospheric boundary layer (ABL) during the collection period, as in Péguilhan et al. (2021), based on ECMWF ERA5 data (Hoffmann et al., 2019). More details are provided as Supplementary Materials. Variables pertaining to sample collection are presented in Table 1 and Supplementary Fig. 1.

2.2. Bioinformatics and data analysis

The 4,834,814 raw sequences were processed using dada2 package (v 1.20.0) (Callahan et al., 2016), with functions filterAndTrim, learnErrors, dada, mergePairs, makeSequenceTable and removeBimeradenovo following authors guidelines, to obtain amplicon sequence variants (ASVs). For ASV affiliation, FROGS (Bernard et al., 2021; Escudé et al., 2018) was used with SILVA v138.1 as the reference database (Quast et al., 2013) (see Supplementary Table 2). Details on the methods are given as Supplementary Materials. Briefly, a total of 2,770 sequences from each sample were used for analyses, corresponding to a total of 862 ASVs. One of the replicate samples for aerosols (20190712AIRPDDK1) exhibited too low number of sequences (930) so this event was removed from the analysis of diversity. Due to rarefaction, bacterial richness was slightly underestimated in the richest samples, without affecting conclusions (Supplementary Fig. 2). Kruskal-Wallis and Mann-Whitney tests were used to compare bacterial genera distribution between cloud and clear situations. Permutation MANOVA were done to explore factors influencing the total bacterial diversity (environmental type, season, and ABL), using the ‘vegan’ R package (‘adonis2’ function) (see PerMANOVA results in

Supplementary Materials). Finally, regression analyses were performed (‘car’ R package) to determine which factors were linked with richness, cell and total DNA concentrations (see Regression analysis in Supplementary Materials) and the abundance of specific bacterial taxa. The aerosol event from Opme station was not included in the multifactorial analyses.

3. Results and discussion

Atmospheric samples were collected at 19 occasions from July 2019 to November 2020, of which 8 in the presence of a cloud at the sampling site. Details pertaining to sampling operations are presented in Table 1.

3.1. Biological contents in clouds and clear situations

Cell and DNA concentrations tended to be lower during cloudy than during clear situations, with medians of 5.46×10^2 versus 2.26×10^3 cell. m^{-3} of air, and 0.29 versus 0.69 ng DNA. m^{-3} of air, respectively, although not significantly due to large differences between sampling dates in both situations (Mann-Whitney test; p-value >0.05) (Table 2). This supports the absence of microbial multiplication processes in clouds that would be intense enough to have impact microbial biomass, in contrast to previous observations in the Po Valley fog on culturable bacteria (Fuzzi et al., 1996). It is conceivable that certain taxa developed without affecting significantly the total biomass. For instance, the bacteria culturable at 15°C in cloud water collected at PUY typically

Table 2

Airborne cell and DNA concentrations, and bacterial richness during clear and cloudy situations.

Sample name	Microbial cell concentration (cells. m^{-3}) ^a	Average total DNA concentration (ng. m^{-3}) ^a	Average richness
AEROSOLS			
20190712AIRPDD	9.82×10^3	0.07	NA [§]
20190918AIRPDD	6.05×10^3	0.11	298
20200206AIRPDD	NA [§]	0.26	133
20200518AIROPME	8.21×10^2	4.58	56
20200610AIRPDD	6.82×10^2	0.69	198
20200707AIRPDD	1.62×10^3	2.19	285
20200708AIRPDD	1.72×10^3	1.35	239
20200709AIRPDD	2.62×10^3	0.87	224
20200922AIRPDD	1.91×10^3	0.70	80
20201118AIRPDD	2.72×10^3	0.14	173
20201124AIRPDD	5.72×10^3	0.12	370
Minimum	6.82×10^2	0.07	56
Maximum	9.82×10^3	4.58	370
Median	2.26×10^3	0.69	211
Mean	3.37×10^3	1.01	205
Standard error	2.92×10^3	1.35	99
CLOUDS			
20191022CLOUD	4.95×10^3	0.24	136
20200311CLOUD	4.75×10^2	0.31	73
20200717CLOUD	1.20×10^2	0.33	125
20201016CLOUD	2.67×10^3	0.16	95
20201028CLOUD	3.67×10^3	0.16	130
20201103CLOUD	6.17×10^2	0.37	120
20201110CLOUD	3.90×10^2	0.38	171
20201119CLOUD	3.66×10^1	0.28	100
Minimum	3.66×10^1	0.16	73
Maximum	4.95×10^3	0.38	171
Median	5.46×10^2	0.29	122
Mean	1.62×10^3	0.28	119
Standard error	1.89×10^3	0.08	30
Aerosol-to-cloud comparison (p-value; Mann-Whitney test)	0.08	0.59	0.045*

NA[§]: no data available.

* significant p-value (<0.05).

^a Based on liquid water content (LWC; g/m^3) for clouds.

represented <1% of the total bacteria (Väntilgö et al., 2012).

Both clouds and aerosols harbored bacterial phyla generally associated with soil and plants and frequently reported in the atmosphere (Amato et al., 2017; Barberán et al., 2015; Bowers et al., 2012; Després et al., 2012; Lindemann et al., 1982; Tignat-Perrier et al., 2020). These include Proteobacteria (44% of the total sequences), Actinobacteria (19%), Firmicutes (11%) and Bacteroidetes (9%). At the genus level, bacteria affiliated with *Sphingomonas*, *Methylobacterium* (Alphaproteobacteria), *Pseudomonas* (Gammaproteobacteria), *Massilia* (Betaproteobacteria), *Hymenobacter* (Bacteroidetes) and *Bacillus* (Firmicutes) were among the most abundant (Supplementary Table 3).

Bacterial richness was more variable in clear air than in clouds depending on air masses, and it reached higher values (Table 2). This may attest of underlying phenomena leading microbial diversity in clouds to converge. Still, overall as many as 85% of the bacterial genera and 73.8% of the ASVs were shared between the two situations (Fig. 1A; Supplementary Fig. 3). These results are totally in line with previous finding at a high elevation site in Sierra Nevada, reporting high similarity in the affiliation of bacteria deposited by dry and wet processes, with up to 65% of the OTUs and 85% of the genera shared (Triadó-Margarit et al., 2019).

Several samplers were deployed in parallel at ca. 1 m from each other, and treated as replicates for biodiversity analyses. Clustering analysis based on bacterial diversity allowed distinguishing most air masses from each other thanks to replicates (i.e., the replicate samples from each event clustered together in most cases), even for samples taken on consecutive days. Distinctions were clearer in the absence of cloud (Supplementary Figs. 4 and 5), with the replicates of 9 out of 10 events clustering together, versus 3 out of 8 events in the presence of cloud, with a p -value <0.95. This is consistent with the fact that meteorological situations were more contrasted between sampling dates during clear conditions than in clouds (Table 1). During clear situations, 12%–47.4% of the bacteria genera detected (26.3% on average) were retrieved in triplicate samples, and 6.6%–44.5% were specific for one replicate. During cloudy situations, the proportion of taxa shared between all replicates dropped to 7.3%–11% (9.2% on average), and specific taxa represented up to 8.3%–37.7% of the richness (Supplementary Table 4). The variations observed illustrate the great heterogeneity of the atmospheric environment within single air masses. The presence of numerous low-abundance taxa likely from distant origins, in particular in clouds, was responsible for the high level of heterogeneity between replicates. Replicated sampling certainly helps attenuating the influence of low-abundance taxa on the dataset and better account for their scarcity. The most abundant taxa (>100 reads) were consistently retrieved between replicates, with low variability (CV from the mean < 100%) (Supplementary Fig. 6). The relative abundance of dominant taxa such *Sphingomonas*, *Pseudomonas*, and *Massilia* did not differ between cloudy and clear situations (Fig. 1B). Nevertheless, some were significantly more represented in clouds than in clear air, such as *Staphylococcus* (Fold change = 1.18; FC), *Acinetobacter* (FC = 2.36), *Paracoccus* (FC = 7.96), *Kocuria* (FC = 14.30), *Corynebacterium* (FC = 1.89), *Enhydrobacter* (FC = 5.22), *Micrococcus* (FC = 6.37), and conversely, such as *Bacillus* (FC = -0.60), *Rubellimicrobium* (FC = -0.52), and *Blastococcus* (FC = -0.65) (Kruskal-Wallis test, p -value <0.05; Fig. 1B; Supplementary Table 5). These are not issued from direct quantification data so the fold-change values indicated are not necessarily directly reflecting the actual change of taxa's cell numbers.

These taxa are commonly observed in clouds, and *Kocuria* and *Enhydrobacter* were identified among active groups (Amato et al., 2017), which is compatible with their multiplication. Furthermore, *Staphylococcus*, *Corynebacterium* and *Kocuria* were found more abundant in clouds than in the corresponding precipitation, evidencing higher abundance in the source cloud than in the air column (Péguilhan et al., 2021) and also supporting their potential multiplication in clouds.

Massilia and *Noviherbaspirillum* were reported enriched in precipitation compared with dry deposits in the Sierra Nevada (Triadó-Margarit

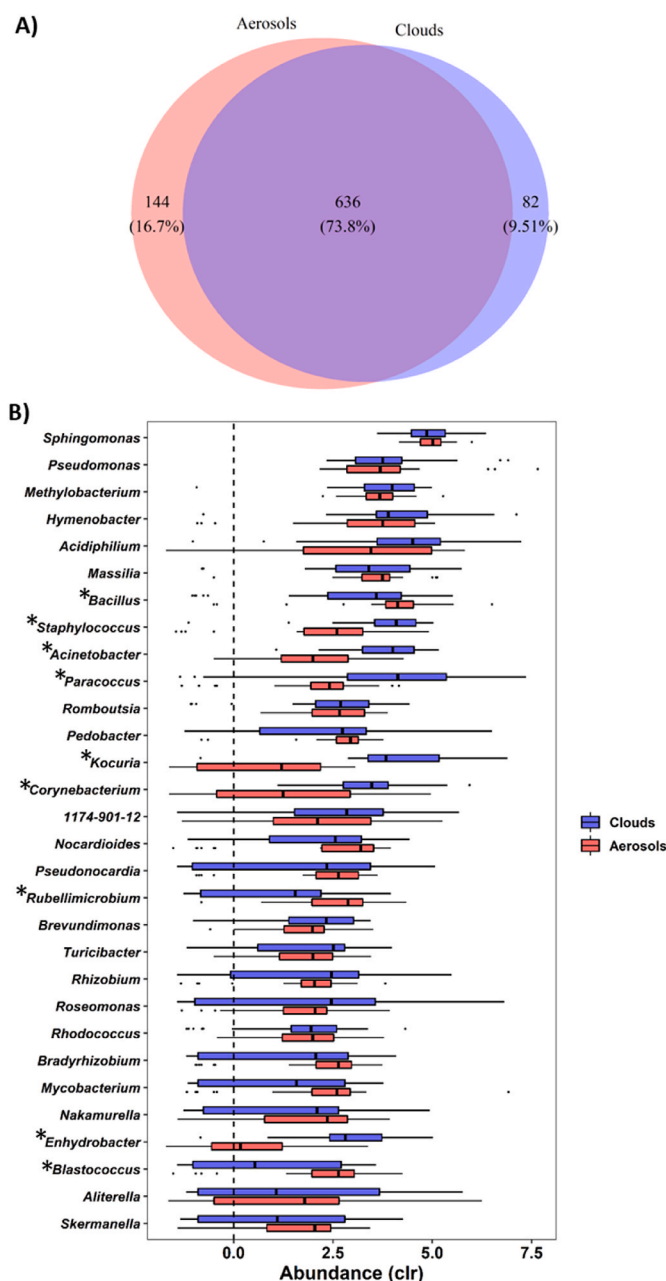


Fig. 1. Distribution of the 862 bacterial ASVs between clouds and aerosols (A) and of the 30 most abundant bacterial genera ordered by total abundance from top to down (B). Scale is represented in centered-log ratio (clr). An asterisk (*) indicates significantly different genus' abundance between cloud and clear air (Kruskal-Wallis test air; p -value <0.05).

et al., 2019), which is not confirmed here. In previous work we identified these two particular bacterial taxa among those more abundant in precipitation compared with clouds collected in parallel, rather indicating their scavenging from the air column by rainfall (Péguilhan et al., 2021). It is possible, in agreement with both observations, that their presence in the air was promoted by rainfall impacting the vegetation cover (Huffman et al., 2013).

Clouds provide aqueous conditions for airborne cells and likely better conditions for survival and development than dry situations (Fuzzi et al., 1996). Bacteria are known to be able to multiply in such drastic conditions as in clouds. For instance, numerous psychrophilic organisms have been cultured from clouds by our group over the last decades, and the chemical composition of natural cloud water was

evidenced to sustain microbial needs and potentially cell multiplication (Amato et al., 2007a, 2007b; Vaitilingom et al., 2012). In cloud water incubated under laboratory conditions, microbial generation rates of 3–20 days were measured at 0 °C, which is comparable to typical growth rates of phytoplankton in oceans (Sattler et al., 2001). Given the short time scale of clouds and the short residence time of bacterial cells in the atmosphere (~3–5 days; Burrows et al., 2009b), it was estimated that bacterial multiplication in cloud droplets should not significantly affect community structure (Ervens and Amato, 2020).

In turn, selective exclusion of certain taxa in clouds compared with dry situations could occur due to physical and biological factors, such as differential tolerance to osmotic and freeze-thaw shocks, and differential CCN activity in relation with cell size. The propensity of aerosol particles to act as CCN depends on their size (Kelvin effect) and capacity to attract water (Raoult effect). Bacteria cells are large aerosols (~0.1–2 µm) considered efficient CCN (Andreae and Crutzen, 1997; Bauer et al., 2003). In theory, a particle in that size range cannot avoid water condensation under supersaturated conditions, so similar diversity should be found based solely on such physical aspects. Nevertheless, larger cells sediment more rapidly than smaller cells, and may therefore have less possibility to escape the boundary layer and reach clouds. Our experimental set up did not allow distinguishing between bacteria integrated in cloud droplets and those eventually remaining excluded from condensed water in interstitial air. Whether or not certain bacteria could avoid or delay water condensation at supersaturated conditions remains to be investigated using specific methods.

Osmotic shocks and freeze-thaw cycles at high RH and in the presence of condensed water are known to alter the integrity of microbial cells (Christian and Ingram, 1959; Gutierrez et al., 1995). Some species of *Sphingomonas*, for example, have been reported to be particularly affected by osmotic shocks, while *Arthrobacter* and *Pseudomonas* species were more impacted by freeze-thaw cycles (Joly et al., 2015). However, these bacteria were equally distributed in clouds and clear situations in our study so no significant exclusion occurred.

3.2. Microbiological variables are driven by multiple interrelated environmental factors

Besides the presence of condensed water, environmental variables inherently connected with the presence of cloud at the sampling site could be pointed to explain the data, notably the season, the origin of air masses and the altitude of the atmospheric boundary layer (ABL) during sampling. While most of the air masses travelled at high altitudes (>1 km a.g.l.) over continental or marine environments before reaching the sampling site, based on 72-h backward trajectories (Supplementary Fig. 1), clouds were indeed collected mostly during the fall, from air masses originating from West (Atlantic Ocean), and in the free

troposphere (Table 1). On the other hand, aerosols were collected essentially during summer and fall, from air masses originating from West or North, and in half of the cases within the ABL. The observations were therefore likely the result of multiple factors intimately intertwined with each other.

The highest concentrations of DNA were observed in samples collected within the ABL, i.e., in the absence of cloud (Fig. 2). This is consistent with higher numbers of primary biological aerosol particles (fungal spores, pollen, plant and insect debris) circulating in this layer (Bryan et al., 2019). Microbial cell concentrations did not follow such trend and were often more abundant in the free troposphere than in the ABL, indicating very efficient transport processes from surfaces up to high altitudes. Similar observations were made at Mt Soonblich, along with higher proportions of bacteria respect to larger bioaerosols (fungi) at higher altitude (Els et al., 2019b). Consistently, the sample from OPM station, lower in altitude by ~800 m, exhibited the highest DNA concentration, but low microbial cell concentrations and low richness (Table 2). The latter may result from a narrower area of influencing sources due to lower altitude and different site configuration than at PUY. In previous work, precipitation collected at OPM was shown to carry lower richness than clouds collected in parallel at PUY, indicative of richer clouds than the air column underneath (Péguilhan et al., 2021).

To evaluate the extent of the different factors in explaining the distribution of biodiversity data, multivariate analyses were performed (see NMDS plot on Fig. 3, PERMANOVA results and Regression analysis in Supplementary Materials, and Table 3). The sample collected at OPM was removed from further multivariate analyses to avoid introducing bias linked with the influence of the sampling site. PERMANOVA results indicated that all factors (sample type, season and altitude relative to the ABL) were significant predictors explaining bacterial assemblages among samples, with significant relationships between sample type and ABL factors, and season and ABL factors (see PERMANOVA results in Supplementary Materials). Regression analysis provided more hierarchy among the factors, with sample type primarily explaining bacterial assemblages, followed by season (see Regression analyses in Supplementary Materials).

Thus, bacterial richness (number of taxa detected) was largely linked with the presence of clouds and season: it was most of the time lower in clouds than in clear air (119 versus 205 ASVs, respectively; Table 2) and lower in winter than in summer (~103 versus ~234 ASVs, respectively; Supplementary Fig. 7). The relative abundance of taxa including *Pseudomonas*, *Bradyrhizobium*, *Pseudonocardia*, *Acinetobacter*, *Enhydrobacter*, *Streptomyces*, *Micrococcus*, *Methylobacterium* and *Flavobacterium* could be strongly explained by season (Table 3). A seasonal effect on the airborne bacteria diversity at our sampling site was observed in earlier study (Tignat-Perrier et al., 2020), and related notably to variations in the vegetation cover at regional scale. Taxa such as *Bacillus* were found

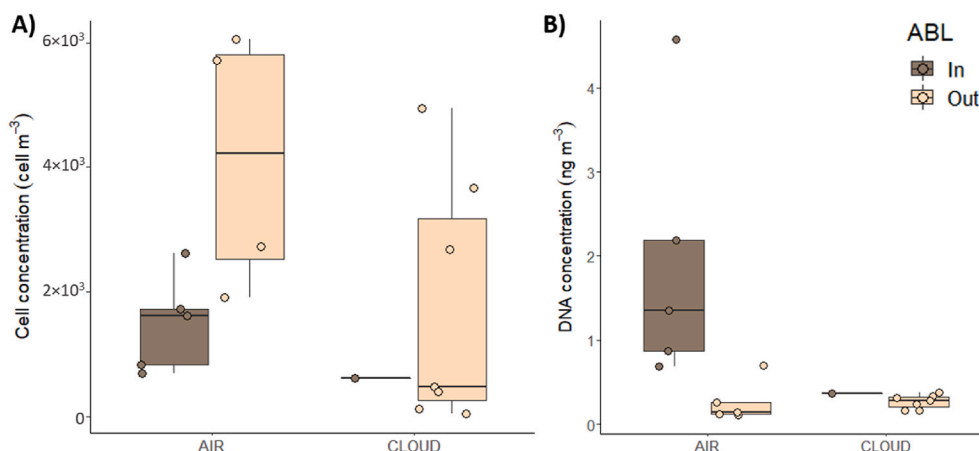


Fig. 2. Cell (A) and DNA concentrations (B) in the presence of cloud or in clear air, within ("In") or outside ("Out") the atmospheric boundary layer (ABL).

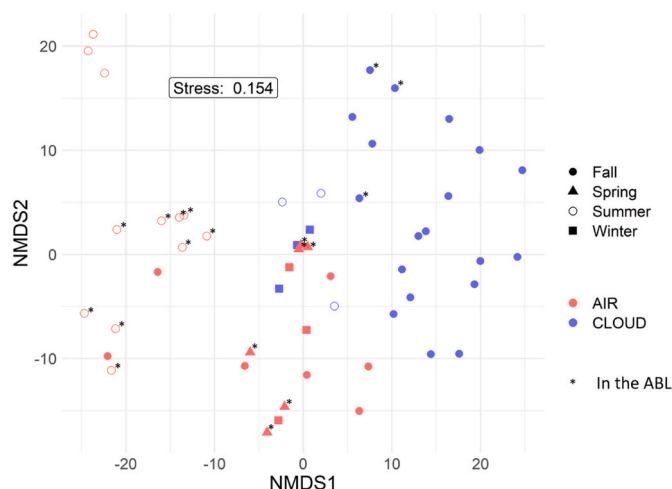


Fig. 3. Non-metric multidimensional scaling (NMDS) analysis of the biodiversity in clouds and clear atmosphere, based on the abundance of the total 862 ASVs (Euclidean distance), illustrating the entanglement between season, cloudy/dry meteorological situations, and altitude respect to the atmospheric boundary layer (ABL). All sample replicates are represented.

prevalent all throughout the year.

Several bacterial genera found differentially represented between cloudy and clear situations, whether enriched or depleted in clouds such as *Corynebacterium*, *Micrococcus*, *Enhydrobacter*, *Burkholderia* and *Bacillus* (Table 3), were more prevalent in the free troposphere than in the ABL suggesting distant origin. The variation in the relative abundance of *Staphylococcus* and *Leuconostoc*, more abundant in clouds, could be totally explained by the ABL factor alone. In previous work, *Bacillus* and *Staphylococcus* were reported viable at high altitudes in aerosols (Smith et al., 2018; Wainwright et al., 2003), and significantly more abundant in clouds compared to precipitation (Péguilhan et al., 2021). It was concluded that these bacteria could have adaptive attributes favourable for their survival during transport at high altitudes, such as low-GC content in the genome (Foerstner et al., 2005; Mann and Chen, 2010), and the ability to form spores for *Bacillus* (Smets et al., 2016; Smith et al., 2013). *Leuconostoc*, as a Firmicutes, also has a low-GC content, which

may favour its persistence outside of the ABL compared to other bacteria. The presence of pigments may also help bacteria such as *Staphylococcus*, *Corynebacterium*, and *Micrococcus* (e.g., carotenoids) to survive and persist in the free troposphere with potentially higher tolerance to UV exposure and low temperatures (Lighthart, 1997; Marshall and Wilmoth, 1981; Mueller et al., 2005; Sajjad et al., 2020). *Enhydrobacter* and *Burkholderia* are common taxa in the atmosphere; the reasons for their higher abundance in the free troposphere essentially reflects distant sources and cannot be further explained.

Finally, among dominant taxa, only the abundance of *Kocuria* could be explained solely by the presence of clouds. These micrococcaceae are widespread on Earth; they occupy various ecological niches among which some can be considered extreme, and are frequently responsible for disease to humans and animals (Savini et al., 2010). In the absence of alternative statistical explanation for its abundance in samples, and since they were reported among the taxa maintaining activity in clouds (Amato et al., 2017), we cannot exclude that these reproduce in clouds or better tolerate cloud conditions than others, which would favour their spread at large scale. Whether or not this may represent a threat to humans and animals remains to be elucidated.

4. Conclusions

In this study we observe no major alteration of the composition of airborne bacteria assemblages linked with the presence of clouds. Differential abundance of most taxa could be explained by concomitant factors such as season and altitude of the atmospheric mixing layer. Only in one taxon, *Kocuria*, abundance could be explained only by the presence of clouds. The presence and proliferation in clouds of these widespread bacteria could cause sanitary issues. Further research must be performed to evaluate such possibility.

The atmosphere is, by volume, the largest biome on Earth, but it remains probably the most obscure environment regarding microbial functioning. Bacteria have been demonstrated earlier to maintain activity in aerosols (Klein et al., 2016) and clouds (Amato et al., 2017), and adjust their metabolic functioning to respond to the presence of volatile organic compounds (Krumins et al., 2014) and, in clouds, to the many stresses encountered (Amato et al., 2019). Notwithstanding the fact that clouds are probably not environments where microorganisms, for most of them, can significantly multiply due to time limitations, they are likely to harbor specific microbial functioning and biological processes

Table 3

Summary of regression analyses for several relevant bacterial genera (non-exhaustive list). Relevant taxa were first selected based on their relative abundance (most abundant genera), and the Kruskal-Wallis test (significantly different abundance between clouds and clear situations), and only taxa influenced by at least one factor were retained. A positive value indicates a positive effect of the factor on genus' abundance compared to the opposite situation (or to summer for season), and a negative value a negative effect. Significant effects are highlighted in bold.

Genus	Abundance rank (/231)	Aerosol/Cloud difference (Kruskal-Wallis)	Factors	Type Cloud	Abl Out	Spring	Summer	Winter
<i>Acidothermus</i>	76	>air	-1.0318*		-0.6332	-2.0801*	0.9079	-0.4858*
<i>Acinetobacter</i>	12	>cloud	0.7735**		0.5288	0.1004	-1.6792***	-1.2355***
<i>Bacillus</i>	6	>air	-1.5426**		1.3174*	0.5469	1.1358	-0.8308
<i>Bradyrhizobium</i>	29	not sign.	-0.0082		-0.7796	0.5356	1.4087*	0.8919
<i>Burkholderia</i>	53	>cloud	1.9758***		1.1994*	-0.1739	-0.4163	-0.6352
<i>Corynebacterium</i>	14	>cloud	1.2458**		1.6367**	1.0670	-0.5910	0.3898
<i>Enhydrobacter</i>	30	>cloud	1.6995***		0.9681*	0.7601	-1.2090*	-1.6769**
<i>Flavobacterium</i>	35	not sign.	-0.8382		0.5150	0.0648	-1.6092**	1.2942
<i>Kocuria</i>	7	>cloud	2.9856***		0.7383	0.0794	-0.4300	-1.3082
<i>Lactobacillus</i>	218	not sign.	0.4140		0.4223	1.4954*	0.2117	0.5167
<i>Leuconostoc</i>	105	>cloud	0.6665		1.5954**	0.9643	0.8361	-0.7032
<i>Methylobacterium</i>	8	not sign.	-0.4302		-0.4034	-1.3324*	-1.1076**	0.1141
<i>Micrococcus</i>	22	>cloud	1.7571**		1.5269*	0.4997	-1.0200	-2.2885**
<i>Paracoccus</i>	5	>cloud	1.1558*		-0.4607	-1.0028	-0.8467	-3.2485***
<i>Pseudomonas</i>	3	not sign.	0.1333		-0.4001	0.4732	-0.6779*	3.0818***
<i>Pseudonocardia</i>	23	not sign.	-0.0197		-0.4456	0.3568	1.5762*	1.0738
<i>Roseomonas</i>	16	not sign.	-0.0090		1.5385*	1.7947	0.5622	0.3898
<i>Staphylococcus</i>	10	>cloud	0.5467		1.8684***	1.2246	-0.7413	0.0067
<i>Streptococcus</i>	24	>cloud	0.8681		0.4667	-0.1598	-1.4395	-1.9996*

Significance: 0.01 (*), 0.001 (**), 0 (***).

compared to the rest of the atmosphere; this has never been evaluated, despite the important implications for microbial ecology and atmospheric sciences.

There is a strong need for replication in aeromicrobiology, a field where such good practices are often neglected due to practical constraints. These issues have been raised in other environments such as soils (Ettema and Wardle, 2002) and water (Jones et al., 2012), but remain largely ignored in the atmosphere. In addition to strengthening the statistical analyses and allowing to decipher the explanatory variables of the observed bacterial diversity, sample replicates give information about the heterogeneity of the air masses at short scale. This approach also allowed determining the presence of numerous low-abundance taxa of distant origin, which largely contributed to variability within air masses. This also raises questions about the representativeness of data when replicate sampling is not performed.

CRediT authorship contribution statement

Raphaëlle Péguilhan: Formal analysis, Investigation, Writing – original draft. **Florent Rossi:** Investigation. **Olivier Rué:** Data curation. **Muriel Joly:** Formal analysis. **Pierre Amato:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Demultiplexed sequencing files were deposited to the European Nucleotide Archive and have the accession numbers ERR9924931 to ERR9924983.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atmosenv.2023.119635>.

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