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## Quantification of antibiotic resistance genes (ARGs) in clouds at a mountain site (puy de Dôme, central France)

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1                   **Quantification of Antibiotic Resistance Genes (ARGs) in Clouds at a**  
2                   **Mountain Site (puy de Dôme, Central France)**

3  
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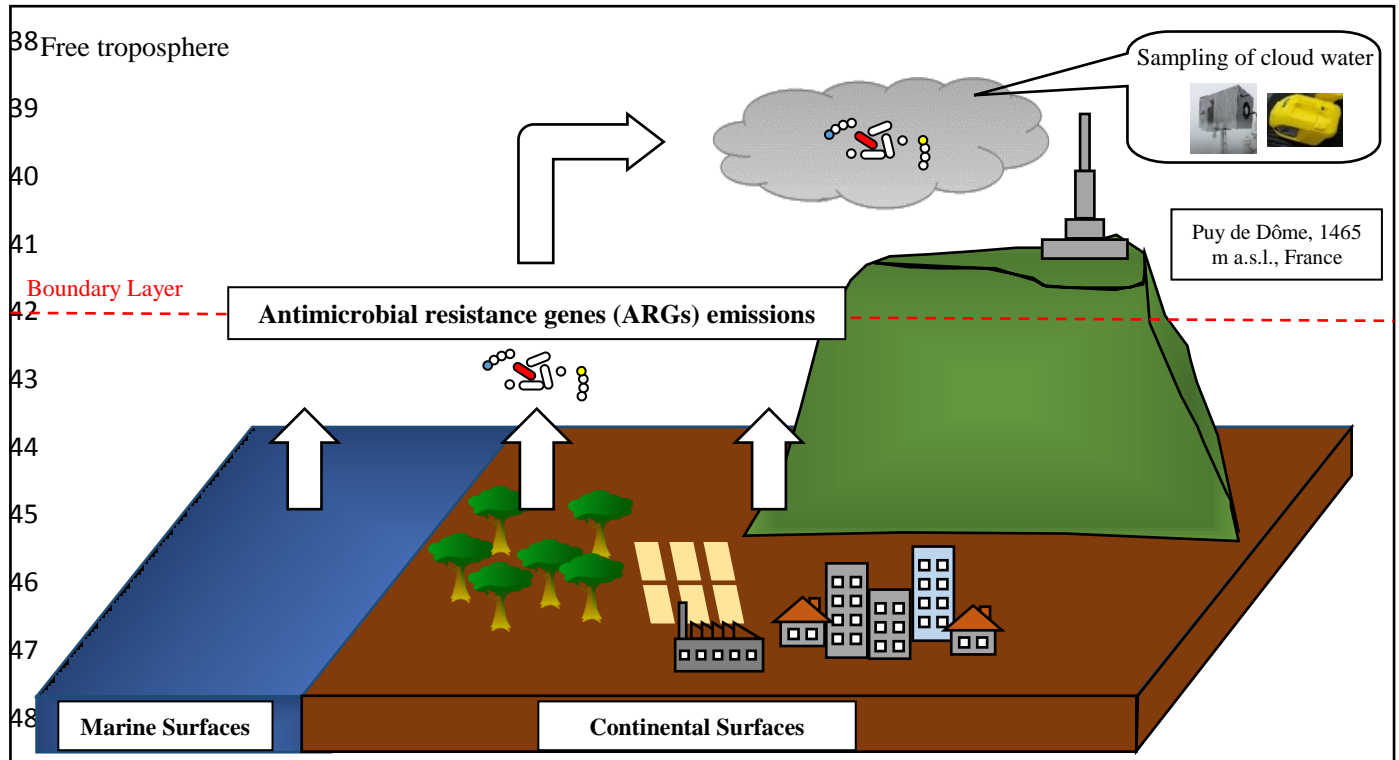
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34 **Keywords:** Clouds, Bioaerosols, Antimicrobial resistance genes (ARGs), Atmosphere

36 **Graphical abstract**

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53 **Highlights**

- 54 • Significant concentrations of ARGs are present in clouds in France
- 55 • The origin of air masses influenced the type and quantities of detected ARGs
- 56 • Veterinary use could be the main contributors of ARGs in the free atmosphere
- 57 • In such stressful environment, ARGs may provide selective advantage to
- 58 microorganisms

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## 63 **Abstract**

64 Antibiotic resistance in bacteria is becoming a major sanitary concern worldwide. The  
65 extensive use of large quantities of antibiotics to sustain human activity has led to the rapid  
66 acquisition and maintenance of antibiotic resistant genes (ARGs) in bacteria and to their  
67 spread into the environment. Eventually, these can be disseminated over long distances by the  
68 atmospheric transport. Here, we assessed the presence of ARGs in clouds as an indicator of  
69 long-distance travel potential of antibiotic resistance in the atmosphere. We hypothesized that  
70 a variety of ARGs can reach the altitude of clouds mainly located within the free troposphere.  
71 Once incorporated in the atmosphere, they are efficiently transported and their respective  
72 concentrations should differ depending on the sources and the geographical origin of the air  
73 masses. We deployed high-flow rate impingers and collected twelve cloud events between  
74 September 2019 and October 2021 at the meteorological station of the puy de Dôme summit  
75 (1,465 m a.s.l, France). Total airborne bacteria concentration was assessed by flow cytometry,  
76 and ARGs subtypes of the main families of antibiotic resistance (quinolone, sulfonamide,  
77 tetracycline; glycopeptide, aminoglycoside,  $\beta$ -lactamase, macrolide) including one mobile  
78 genetic element (transposase) were quantified by qPCR. Our results indicate the presence of  
79 29 different ARGs' subtypes at concentrations ranging from  $1.01 \times 10^3$  to  $1.61 \times 10^4$  copies  $m^{-3}$   
80 of air. Clear distinctions could be observed between clouds in air masses transported over  
81 marine areas (Atlantic Ocean) and clouds influenced by continental surfaces. Specifically,  
82 quinolones (mostly *qepA*) resistance genes were prevalent in marine clouds (54% of the total  
83 ARGs on average), whereas higher contributions of sulfonamide, tetracycline; glycopeptide,  
84  $\beta$ -lactamase and macrolide were found in continental clouds. This study constitutes the first  
85 evidence for the presence of microbial ARGs in clouds at concentrations comparable to other  
86 natural environments. This highlights the atmosphere as routes for the dissemination of ARGs  
87 at a large scale.

## 89 **Introduction**

90

91 Antibiotic resistance has been classified as one of the 10 biggest threats for human societies in  
92 the near future. In 2019, more than 4.95 million deaths were associated with antimicrobial  
93 resistance, and about 1.27 million were directly attributed to antibiotic-resistant bacterial  
94 infections <sup>1</sup>. If no action is taken, this number could rise to up to 10 million deaths per year by  
95 2050, making it the first cause of death worldwide and engendering cumulative global cost to  
96 the economy over 100 trillion USD <sup>2</sup>. The resistance to antimicrobial substances is a natural  
97 phenomenon that includes natural microbial evolution dynamics, and the extensive use of  
98 antibiotics to sustain growing human populations have greatly promoted it <sup>3</sup>. Each year, tens  
99 of thousands of tons of antibiotics are used for treating and preventing human, animal and  
100 plant diseases <sup>4</sup>, and at least a fraction can be released in the environment. Nowadays,  
101 antibiotics can be detected in waste water <sup>5</sup> and in the natural environment, from rivers <sup>6</sup> and  
102 sea water <sup>7,8</sup> to soils <sup>9</sup>, and therefore carrying antibiotic resistance can now be considered  
103 beneficial for bacteria in the environment. A recent report from the French national agency for  
104 sanitary, environment and work safety highlighted median total antibiotics concentrations in  
105 French rivers of 12 ng L<sup>-1</sup>, dominated by fluoroquinolones and sulfamethoxazole <sup>10</sup>. In  
106 hospital raw effluent and waste water treatment plants effluent, these concentrations were  
107 17.10<sup>3</sup> ng L<sup>-1</sup> and 300 ng L<sup>-1</sup> respectively. In soils, concentrations reaching up to 62 to 3.10<sup>3</sup>  
108 ng g of dw<sup>-1</sup> in sewage sludge were reported. Given such conditions, the natural environment  
109 is becoming a major actor in the emergence of antibiotic resistance genes (ARGs) in microbes  
110 naturally present in the environment.

111 So far, the main antibiotic resistance mechanisms described in bacteria include direct  
112 antibiotic inactivation through enzymatic digestion, and modifications of the biological targets

113 such as by methylation of the binding sites <sup>11</sup>. Other mechanisms prevent antibiotics from  
114 entering the cell (e.g. biofilm formation, reduced membrane permeability) or are responsible  
115 for their active evacuation (e.g. efflux pumps). These mechanisms are encoded by so-called  
116 antimicrobial resistance genes (ARGs), carried either by chromosomes or plasmids. In the  
117 latter case, these can be easily transferred horizontally between bacteria cells and species  
118 through genetic material exchanges (conjugation, etc).

119         ARGs dispersal is logically tightly linked with that of bacteria cells. ARGs can transit  
120 via microbes between ecosystems, and the atmosphere could act as a major route of their  
121 dispersal, in particular over long distances <sup>12,13</sup>. Although the conditions encountered in the  
122 atmosphere may severely limit bacterial survival, some amount of genetic material is  
123 undoubtedly dispersed by atmospheric circulations. Investigating the occurrence of ARGs in  
124 the air at high-altitude and clouds can thus provide valuable information regarding the long-  
125 distance spread of such functions. Main bacteria cell aerosolization mechanisms include  
126 mechanical disturbances, such as wind, raindrop impaction on surfaces, bubble-bursting, etc  
127 <sup>14-16</sup>. Once aerosolized, depending on air turbulence, airborne micro-organisms can eventually  
128 reach higher altitudes, travel over continents <sup>13,17</sup>, and can integrate the atmospheric water  
129 cycle, clouds and precipitation, before deposition <sup>18,19</sup>.

130         With the increase awareness that human health is closely related with the wider  
131 environment, studying ARGs dispersal in the atmosphere, its potential to spread over long  
132 distances and its relation with human activities could provide critical and valuable  
133 information. Hence, this study is a part of the wider “Discovery Frontiers” research program  
134 dedicated to the study of airborne dissemination of ARGs <sup>20</sup>. In compliance with the “One  
135 Health” interdisciplinary approach <sup>21,22</sup>, this research program incorporates assessment of  
136 several different indoor environments and their outside emissions, novel ARG tracking and

137 surveillance methods, selective culture approaches, and animal models to be incorporated into  
138 exposure and risk assessment models.

139 Here, we examined ARGs in clouds in order to investigate their capacity to be  
140 dispersed over long distances through the multiphase atmosphere. Clouds were sampled  
141 between September 2019 and October 2021 from a high altitude site in central France (puy de  
142 Dôme meteorological station, 1,465 m a.s.l.). For each event, total bacteria and a panel of 33  
143 ARGs subtypes were quantified by flow cytometry and qPCR, respectively, and interpreted in  
144 regards to the geographical origin of the corresponding air masses. The ARGs assessed were  
145 related to quinolone, sulfonamide, tetracycline, glycopeptide, aminoglycoside,  $\beta$ -lactamase  
146 and macrolide resistance and included one mobile genetic element (transposase). Overall, this  
147 study constitutes the first report of the presence of microbial ARGs in clouds and evidences of  
148 the atmosphere, and especially clouds, as pathways for the dissemination of antimicrobial  
149 resistance functions worldwide.

150

## 151 **Material and Methods**

### 152 **Study site, collection of clouds and Air Masses Backward Trajectories**

153 Cloud sampling was performed at the puy de Dôme (PUY) meteorological observatory  
154 (1,465 m a.s.l., 45.772° N, 2.9655° E - France) from September 2019 to October 2021. At  
155 each sampling occasion, two high flow rate impingers<sup>23</sup> (HFRi, DS6, Kärcher SAS,  
156 Bonneuil sur Marne, France) sampling at an air flow rate of  $\sim 2 \text{ m}^3 \text{ h}^{-1}$  were deployed from the  
157 roof platform of the station to collect the cloudy air, that were then used for qPCR analyses. In  
158 parallel, a cloud collector allowing to sample cloud droplets was deployed nearby<sup>24,25</sup> for  
159 total cell quantification using flow cytometry. Sampling were performed for 2 to 5  
160 consecutive hours depending on cloud events' duration. The HFRi were filled with 850 mL of

161 sterile nucleic acid preservation (NAP) buffer solution as the impaction liquid <sup>26</sup>. The volumes  
162 of cloud water recovered were estimated by weighting, assuming a cloud water density of 1.0.  
163 The NAP buffer solution was composed by 0.019 M of ethylenediaminetetra-acetic acid  
164 (EDTA) disodium salt dihydrate, 0.018 M of sodium citrate trisodium salt dihydrate, 3.8 M of  
165 ammonium sulfate, and H<sub>2</sub>SO<sub>4</sub> to pH 5.2. HFRi samplers were sterilized prior each sampling  
166 event by thorough rinses with 70% ethanol followed by 10 minutes of exposure to UV light.  
167 Field blanks were conducted before each sampling and consisted of 850 mL of sterile NAP  
168 buffer solution left in contact with the HFRi's tanks for several minutes before recovery. The  
169 cloud droplet impactor was rinsed with ultrapure water and sterilized by autoclave before each  
170 sampling.

171 The CAT three-dimensional (3D) forward/backward kinematic trajectory model was  
172 used to calculate the 72-hours backward trajectories of clouds' air masses. The model uses  
173 dynamic fields extracted from the ECMWF ERA-5 meteorological data archive  
174 (<https://www.ecmwf.int/en/forecasts/datasets/reanalysis-datasets/era5> <sup>27</sup>), allowing for the  
175 determination of air masses geographical origins and altitudes over the last 72 hours before  
176 sampling at the PDD meteorological observatory <sup>28,29</sup>. The model also provides the altitude  
177 relative to the planetary boundary layer (PBL) to estimate the influence of the underlying  
178 surfaces. In the present work, the ECMWF data used to calculate back-trajectories have a  
179 spatial resolution of 0.5° in latitude and longitude and a temporal resolution of 3 hours, and  
180 back-trajectory points have been calculated every 15 min.

181

## 182 **Sample Processing and DNA Extraction**

183 Cloud water samples collected with the HFRi in NAP preservation buffer were  
184 concentrated by individual filtration through 47 mm diameter mixed cellulose ester



185 membranes (MCE - 0.2  $\mu\text{m}$  porosity) within a laminar flow hood, rolled into 5 mL bead tubes  
186 (Macherey-Nagel) and added with 1.2 mL of lysis buffer (MWA1 - Macherey-Nagel) before  
187 being stored at  $-80^{\circ}\text{C}$  until further analysis. DNA extraction was carried out individually from  
188 each separate filter using the NucleoMag DNA/RNA Water kit for water and air samples  
189 (Macherey-Nagel), based on the manufacturer's instructions, with some minor modifications  
190 to match with the modifications made on the original protocol (use of 47 mm diameter filters  
191 instead of 25 mm, and larger volumes of lysis buffer): briefly, after the recommended 10 min  
192 of mechanical lysis and 30 s of centrifugation (30 s at 1,000 g), 450  $\mu\text{L}$  of the lysates was  
193 transferred into two different microtubes. DNA extraction was then performed as  
194 recommended, with final elution volumes of 50  $\mu\text{L}$  of RNase-free  $\text{H}_2\text{O}$  provided in the kit.

195

#### 196 **Quantification of Total Bacteria by Flow Cytometry**

197 The concentration of total bacteria in cloud water was determined from samples  
198 collected with cloud droplets impactors. Briefly, the cells contained in 40 mL of water  
199 samples were pre-concentrated into  $\sim 200$   $\mu\text{L}$  by ultrafiltration using vivaspin 20 (Sartorius, 10  
200 Kda molecular weight cut off), from two successive centrifugation steps of 20 mL at 4000 g  
201 for 16 min, and the addition of 1 mL of tris-EDTA buffer (1X), then transfer into 2 mL sterile  
202 microtubes. The exact concentration factor for each sample was obtained by weighting initial  
203 and final liquid volumes, considering a density of 1.0. Volumes of 200  $\mu\text{L}$  of the pre-  
204 concentrated samples were then stained with SYBR Green (1X final concentration,  
205 Invitrogen) for 15 minutes in the dark at room temperature, and total bacteria counts were  
206 performed using an LSR Fortessa Cell Analyzer flow cytometer (Becton Dickinson, Franklin  
207 Lakes, NJ). Cytograms were obtained by plotting SYBR green fluorescence intensity (488  
208 nm, voltage 350 V) over forward scattering (voltage 500 V) after 60 seconds of run, and flow

209 rates were measured before analyzes of samples by weighing of ultrapure water after 10  
210 minutes of run. All flow cytometry analyses were conducted within 4 to 12 hours of sampling.

211

## 212 **ARGs Quantification by qPCR**

213 A panel of 33 ARGs belonging to the main families of antibiotic resistance was  
214 assessed from samples collected with HFRi in NAP buffer:  $\beta$ -lactamases, quinolones,  
215 tetracyclines, macrolides, aminoglycosides, sulfonamides, vancomycins as well as one mobile  
216 genetic element (transposases). More details about the ARGs targeted, including the  
217 corresponding sets of primers, are described in supplementary material (Table 1SI<sup>30,31</sup>). PCR  
218 reactions and cycling conditions were identical for all target genes, and were as following: 4  
219  $\mu$ L of template DNA (diluted to 1/10), 10  $\mu$ L of IQ Green supermix (BioRad), 5.88  $\mu$ L of  
220 RNase-free H<sub>2</sub>O and 0.12  $\mu$ L of equimolarly mixed primers (50  $\mu$ M each) in 20  $\mu$ l final  
221 volume. Cycling conditions were set at 95°C for 3 min as initial denaturation step, followed  
222 by 40 cycles of 95°C for 10 s and 60°C for 30 s. The concentrations of each target gene in the  
223 samples were determined relatively to 10-fold dilution series of a 10<sup>6</sup> copies x  $\mu$ l<sup>-1</sup> mix of  
224 control plasmids (pUCIDT-AMP) including the target sequence. All qPCR reactions were  
225 performed in technical duplicates from the pool of DNA extracts from samples collected  
226 simultaneously. Field blanks were also processed under the same conditions. A Ct value  
227 threshold of 37 was used as the limit of quantification, and all amplification peaks were  
228 systematically checked based on the melting curves of the corresponding plasmid standard.

229

## 230 **Statistical Analyses**

231 All statistics were performed using R (R Core Team (2021)). Statistical differences between  
232 each family of ARGs considering all cloud events (n = 12) was assessed by the nonparametric

233 Kruskal-Wallis test followed by multiple pairwise comparison using Tukey contrast from the  
234 “nparcomp” function of the R package of the same name<sup>32</sup>. The relationships between the 4  
235 variables associated with the 72-hours backward trajectories (time spent within and above the  
236 planetary boundary layer, above continental and marine surfaces) and the relative  
237 concentrations of the different families of antibiotics quantified (quinolones,  $\beta$ -lactamases,  
238 vancomycin, tetracycline, sulfonamide, macrolide, aminoglycosides) including transposase in  
239 the samples were assessed using partial least square regression analysis (PLSR). The analysis  
240 was computed using the “PLS” function from the mixOmic R package<sup>33,34</sup> and all data were  
241 centered and reduced before analysis. Prior to the PLSR analysis, a hierarchical clustering was  
242 performed considering only the 72-hours backward trajectory parameters (Table 2 SI) to  
243 identify classes of cloud samples. The clustering involved Euclidean distances and was  
244 performed using the ward aggregation method. Statistical differences between clusters were  
245 assessed by multiple bootstrap resampling (100 bootstraps,  $P < 0.05$ ) using the “pvclust”  
246 function of the corresponding R package<sup>35</sup>. Based on these groups, 95% level confidence  
247 interval ellipses were drawn.

248

## 249 **Results**

### 250 **ARGs diversity and concentration in clouds**

251 Of the 33 ARGs subtypes assessed in our study on 12 independent cloud events, 29 were  
252 detected at least once, and 6 were observed in 75% (9) or more of the samples (*qnrB* – *qepA* -  
253 *aac(3)* – *ermB* – *tetO* and *vanA*); 4 of the targets were not detected in any of the samples (*tetS*  
254 – *blaGes* - *blaMOX* and *vanSA*, Table 3 SI). The total absolute concentration of the ARGs  
255 assessed, considering all clouds, averaged  $2.08 \times 10^4 \pm 6.40 \times 10^3$  copies mL<sup>-1</sup> of cloud water  
256 (mean  $\pm$  standard error of the mean), with values ranging from  $3.45 \times 10^3$  to  $6.47 \times 10^4$  copies

257 mL<sup>-1</sup> depending on samples (Table 1 and Figure 1A). This corresponded to an average  
258 concentration of  $5.42 \times 10^3 \pm 1.51 \times 10^3$  ARGs copies m<sup>-3</sup> of air ( $1.01 \times 10^3$  to  $1.61 \times 10^4$  copies m<sup>-3</sup>).  
259 The highest values were observed on the 03-Nov-20 and 10-Nov-20 and the lowest on the  
260 22-Oct-19 and 28 Apr-21. The concentration of every family of ARGs varied significantly  
261 between clouds (Kruskal-Wallis,  $P < 0.05$ ). However, quinolone resistance genes were by far  
262 the most prominent family (Kruskal-Wallis,  $P < 0.05$ ). Their absolute concentration ranged  
263 from  $2.69 \times 10^2$  to  $4.90 \times 10^4$  copies mL<sup>-1</sup> ( $1.15 \times 10^2$  to  $1.24 \times 10^4$  copies m<sup>-3</sup> of air respectively),  
264 with an average of  $1.19 \times 10^4 \pm 3.93 \times 10^3$  copies mL<sup>-1</sup> ( $3.18 \times 10^3 \pm 9.87 \times 10^2$  copies m<sup>-3</sup>). Based  
265 on the respective relative contributions of the different families of genes to the total pool of  
266 ARGs, two different patterns of samples could be observed (Figure 1B): half of clouds were  
267 characterized by limited proportions of resistance to quinolones (50% or less, 34% on  
268 average) and relatively high proportions of genes related with resistance to either macrolides  
269 (17-jul-20 and 10-Nov-20),  $\beta$ -lactamases (16-Oct-20 and 26-Oct-21), sulfonamides (28-Apr-  
270 21), or the three of them (22-Oct-19). The other half of samples displayed an overwhelming  
271 majority of quinolones resistance genes, reaching up to 88% (74% on average), and low  
272 proportions of all the other families. Interestingly, the vast majority of quinolone resistance  
273 was contributed by *qepA* (~98%), whereas *qnrB* represented only a minor fraction (< 2% on  
274 average), regardless of clouds. Concerning the other genes, aminoglycoside resistance and  
275 transposases were the lowest of all (Kruskal-Wallis,  $P < 0.05$ ) with average concentrations of  
276  $1.08 \pm 0.45$  and  $2.51 \pm 1.35$  copies mL<sup>-1</sup> respectively ( $0.26 \pm 0.11$  and  $1.14 \pm 0.76$  copies m<sup>-3</sup>).  
277 Their cumulated contribution to the total ARGs barely reached 0.05%, and there were many  
278 events where they could not be detected. The relative contributions of the other families of  
279 ARGs were, by decreasing importance:  $\beta$ -lactamases (~12%), sulfonamides (~11%),  
280 vancomycins (~9%), macrolides (~8%) and tetracyclines (~6%).

281 The total absolute bacteria number concentration averaged  $8.0 \times 10^3 \pm 2.9 \times 10^3$  cells per  $\text{mL}^{-1}$  of  
282 cloud water, with, as observed for ARGs, large variations between samples (Figure 2 and  
283 Table 1). Based on these data, each bacteria cell carried on average  $7.1 \pm 2.5$  ARGs.  
284 Nevertheless, no correlation could be observed between these two variables (Spearman  
285 correlation coefficient  $\rho = -0.23$ ,  $P = 0.5$ , figure 2), indicating that the total ARGs copies does  
286 not necessarily covariate with the total number of bacteria cells in cloud water. Specifically,  
287 there were  $>10$  times more ARGs copies than bacteria cells in the samples on the 10-Nov-20,  
288 19-Nov-20 and 13-Oct-21, and  $\sim 5$  times more in the samples collected on the 11-Mar-20, 03-  
289 Nov-20, 06-May-20, and 26-Oct-20. In contrast, in the samples collected on 22-Oct-19, 16-  
290 Oct-20, 28-Oct-20 and 28-Apr-21, bacteria cells were more abundant than ARGs copies by  
291 factors of up to one order of magnitude, with values that ranged between 1 ARG every 10  
292 bacteria to 1 ARG per bacteria at most.

293

#### 294 **Influence of the geographical origin of the air masses on the ARG and bacteria** 295 **abundances in clouds**

296 The clouds assessed originated from various geographical areas (Figure 1SI). Based on the  
297 time spent by air masses above continental or marine surfaces within the 72-hours preceding  
298 sampling, as well as their altitudes, 3 groups of samples could be distinguished (Figure 2SI  
299 and Table 2SI): “Continental” clouds (events 22-Oct-19, 17-Jul-20, 16-Oct-20, 10 Nov-20  
300 and 28-Apr-21), for which the air masses spent on average 70% of the time over continental  
301 surfaces, “Marine high” clouds (events 11-Mar-20, 03-Nov-20, 19-Nov-20 and 06-May-21),  
302 for which the air masses spent 85% of time over the Atlantic Ocean, mostly at high altitude in  
303 the free troposphere, and finally “Marine low” clouds (events 28-Oct-20, 13-Oct-21 and 26-  
304 Oct-21) for which the air masses spent most time (75%) above the Atlantic Ocean, for a large  
305 part within the planetary boundary layer (PBL).

306 Based on the relative abundance of the main families of ARGs (95% level confidence  
307 interval; Figure 3A), “Continental” clouds differed significantly and were more variable than  
308 “Marine” clouds (both “low” and “high”), whereas the two “Marine” types clouds did not  
309 significantly differ from each other. More precisely, quinolones resistance genes were more  
310 abundant in “Marine” clouds (Figure 3B), in particular “Marine high” clouds, whereas  
311 sulfonamide and tetracycline resistance genes appeared mostly associated with “Continental”  
312 surfaces, especially for air masses that travelled within the PBL. Macrolide resistance genes  
313 were associated with continental surfaces as well, but these were mostly prevalent in air  
314 masses that travelled at higher altitude. The abundances of  $\beta$ -lactamase, vancomycin and  
315 aminoglycoside resistance genes as well as transposases could not be explained by the  
316 geographical origin of the air masses using this classification.

317

## 318 **Discussion**

### 319 **ARGs reach high altitudes and clouds**

320 Detecting ARGs in clouds robustly evidences that they are transported over large  
321 geographical areas over the planet. Here, we monitored 33 different genes belonging to the  
322 main classes of antimicrobial resistance. This is standard in atmospheric studies<sup>36–38</sup>,  
323 although it is still non-exhaustive of the ARGs known. For this reason, most estimations of  
324 ARGs concentrations in the environment, including ours in clouds, likely underestimate the  
325 current situation. The ARGs known at the time of writing includes more than 6,500 ontology  
326 terms covering resistant mechanisms from approximately 2,923 antimicrobial resistance  
327 determinants<sup>39</sup>. Most have functions not directly associated with antibiotic resistance but with  
328 more general roles in stress response, for instance the increase of outer-membrane  
329 permeability to face osmotic variations, efflux pumps toward a large variety of toxic

330 substances<sup>40</sup>. Some antibiotic-degrading  $\beta$ -lactamases (ampC-type) were also found to be  
331 related with ancestral peptidoglycan-modifying activities. The atmosphere is a highly  
332 selective and stressful environment for microbial cells<sup>24</sup>; some of these genes and functions  
333 could therefore be favorable for survival<sup>41</sup>, and reinforce the capacity of bacterial ARGs to  
334 spread by atmospheric transport.

335 Our data evidence cloud's resistome. Numerous antibiotic resistance genes were found  
336 recurrent or more sporadic, at concentrations ranging from  $\sim 10^3$  to  $\sim 10^5$  gene copies  $\text{mL}^{-1}$ .  
337 Comparatively to other environments, this is 2 to 3 orders of magnitude lower than in rivers  
338<sup>42-44</sup> ( $6.2 \times 10^6$  to  $1 \times 10^8$  copies  $\text{mL}^{-1}$ ) and lakes<sup>45</sup> ( $4.4 \times 10^6$  to  $2.4 \times 10^7$  copies  $\text{mL}^{-1}$ ), and waste  
339 water effluents<sup>46</sup> ( $1.0 \times 10^3$  to  $1.0 \times 10^7$  copies  $\text{mL}^{-1}$ ) and sea in coastal areas ( $1.2 \times 10^4$  –  
340  $3.9 \times 10^5$  copies  $\text{mL}^{-1}$ ). Our observations are even higher than the concentrations reported in  
341 sea water<sup>47</sup> ( $21.1$  to  $8 \times 10^3$  copies  $\text{mL}^{-1}$ ). However, on a volumetric basis, it has to be kept in  
342 mind that water in clouds is distributed among droplets with micrometric radius, and cloud  
343 liquid water content (LWC) is typically on the order of  $\sim 0.1$  to  $0.8$  mL of water per  $\text{m}^{-3}$  of air.  
344 In this respect, clouds on the planet represent diluted environments since they are  
345 polydisperse medium, also composed of air. Additionally, direct comparisons of ARG  
346 quantification between independent studies are not necessarily relevant as the number and  
347 diversity of the targets may vary.

348

#### 349 **Spatial and temporal variability of ARGs in clouds and links with potential sources**

350 The abundance of antimicrobial resistance functions in the environment is likely variable in  
351 space and times in relation with the natural (seasonal) or anthropogenically induced  
352 fluctuations of biomass and biodiversity in ecosystems, meteorological conditions,  
353 atmospheric and oceanic circulation, etc. In the atmosphere, the microbial content is affected

354 by sources such as vegetation, so marked seasonal variations of the biodiversity are often  
355 observed, including the puy de Dôme sampling site <sup>48</sup>. Here, we did not observe such seasonal  
356 variations on the ARGs in clouds and their abundance. Many concurrent environmental  
357 factors influence the composition of cloud water (e.g. ionic concentrations), and although our  
358 dataset is relatively consequent regarding the difficulties of sampling, it remains too limited to  
359 clearly identify environmental trends such as seasonality, requiring long-term monitoring.

360 We could detect links between the geographical origin of the air masses and the relative  
361 abundances of the different ARG families in clouds, which attests of the heterogeneous  
362 distribution of ARGs in the environment. These can be related with differences of usage  
363 between countries and continents, as previously reported in waste water treatment plants <sup>46</sup>.  
364 Quinolones resistance genes were dominant in our study, in particular in air masses  
365 influenced by marine areas. This is consistent with literature as they were found to be the  
366 second most abundant in urban air samples from various cities around the Earth, although still  
367 far behind  $\beta$ -lactamases resistance genes <sup>38</sup>. However, their presence in the indoor-air of  
368 human facilities, including waste water treatment plants and many different animal farms,  
369 appears much lower <sup>49</sup>. While this partially traduces different detection methods, this could  
370 suggest that these systems may not be the main contributors of quinolones resistance in the  
371 atmosphere in comparison with natural sources. This is supported by metagenomics data from  
372 ocean samples collected by past TARA expeditions from 2009 to 2012 <sup>50</sup>, where an important  
373 proportion of quinolone resistance genes, in particular multidrug efflux pumps was observed  
374 and especially in coastal areas <sup>47</sup>. Similar trends have been observed in soils, where efflux  
375 pump resistance mechanisms represented nearly 60% of the total detected ARGs, out of 242  
376 tested <sup>51</sup>. In our study, the vast majority of quinolones were related with *qepA*, an exclusively  
377 plasmid-mediated efflux pump <sup>52</sup> from the superfamily of major facilitators, likely derived  
378 from soil  $\beta$ -proteobacteria strains' transmembrane transporters <sup>53,54</sup> and hence explaining its



379 occurrence in the environment . Its regulation involves the soxRS gene, a positive two-  
380 component regulator part of the superoxide stress response and triggered by various stresses,  
381 including damages to DNA, exposure to toxicants and reactive oxygen species <sup>52,54</sup>. This  
382 supports that the environment is not only an important reservoir of bacterial resistance but  
383 could promote their maintenance through environmental stresses, and that the atmosphere  
384 would act as vector at a large scale of these genes. Quinolone antibiotics, including  
385 fluoroquinolones, are broad spectrum synthetic antibiotics introduced in the early 1960s and  
386 widely used until the late 1980s <sup>55</sup>. Since the recommendation of their restriction in Europe  
387 emitted by the Pharmacovigilance Risk Assessment Committee (PRAC) in 2018, their  
388 utilization has plummeted down to ~6% in human health and ~2% in food production <sup>56</sup>.  
389 Notable molecules in this family of antibiotics includes ciprofloxacin, levofloxacin and  
390 norfloxacin, used against a large panel of Gram-negative (including *Pseudomonas*  
391 *aeruginosa*) and Gram-positive bacteria (including *Staphylococcus aureus*); their activity as  
392 antibacterial agents rely on their interaction with topoisomerase II (DNA gyrase), which  
393 prevents DNA replication. This class of molecules rapidly caused the emergence of resistance  
394 in bacteria <sup>57</sup>. It therefore makes sense to find quinolone resistance genes among the prevalent  
395 ARGs in the environment.

396 The other main classes of antibiotic resistance, namely  $\beta$ -lactamases, sulfonamides,  
397 vancomycin, macrolide and tetracycline, were relatively more abundant in “Continental”  
398 clouds. While this partly results from a relative decrease of the amounts quinolones resistance  
399 genes, it also suggests continental surfaces as their main sources. Table 2 summarizes  
400 antibiotic consumption for 29 countries in Europe for human and veterinary usages <sup>56</sup>.  
401 Veterinary usages contribute ~61.4% (6558 tons of active products) of the total antibiotics  
402 market, versus ~ 38.6% for human health (4122 tons) <sup>56</sup>. Most antibiotics related to human  
403 health are expected to be caught by waste water treatment systems, where removal efficiency

404 has been estimated to reach up to 76% of the inputs <sup>58</sup>, with variations depending on  
405 compounds <sup>59</sup>. If we omit the gene *qepA*, which we related to oceanic inputs as mentioned  
406 previously, we found a fairly good agreement between the relative abundances of  
407 antimicrobial functions observed in clouds and the proportions of the different families of  
408 molecules used in the various countries. For instance, the highest concentration of ARGs were  
409 observed on 10-Nov-20 in an air mass that flew over Spain. This country is the biggest  
410 purchaser of antibiotic products for veterinary purposes in Europe (~ 1,244 tons of active  
411 substance <sup>60</sup>). More local sources should also largely contribute to our observations.  
412 Accordingly, sulfonamides and macrolides, which represent 21.7 % and 12.3%, respectively,  
413 of the veterinary antibiotics sold in France <sup>61,62</sup>; accordingly, represented on average 21.5%  
414 and 13.9% of the total ARGs quantified in our samples. However, resistance to tetracycline  
415 was found at lower proportions than could be expected from such extrapolations. This may  
416 relate with seasonal variations, as observed in rivers with the lowest abundance of *tetA*, *tetC*  
417 and *tetM* during winter than the rest of the year <sup>63</sup>.

418

## 419 **The selective advantage of ARGs in clouds could promote their maintenance in the** 420 **environment**

421 There are increasing reports of the presence of multi-resistant bacteria in the environment, and  
422 this represents one of the most worrisome threats to human societies nowadays. In our work,  
423 although multi-resistance was not specifically addressed, we found ARGs at higher  
424 abundance than bacteria cell by a factor of 5 in ~60% (7/12) of the samples. As the samples  
425 were filtered through 0.22 µm porosity before analysis, viruses and cell-free DNA fragments  
426 (i.e., external DNA, bacteriophage-associated DNA, and membrane vesicle-associated DNA)  
427 were excluded. However, they are known to have critical roles in horizontal gene transfer and  
428 dispersal including ARGs <sup>66,67</sup>. Viruses for example, exhibit high proportions of ARGs in

429 their genome, with a strong prevalence of antibiotic efflux mechanism<sup>68</sup> Overall, these  
430 evidences the presence of multiple ARG copies in genomes and possibly multiple resistance  
431 combined together within single cells, and this was particularly true for “Marine” clouds.  
432 Such prevalence of antimicrobial resistance genes is most likely a combination of both  
433 intrinsic and acquired resistance, i.e. of ARGs carried by both chromosomes and plasmids; the  
434 methods used here do not allow distinguishing them.

435 Many ARGs such as the efflux pump *qepA* are involved in global stress regulation as  
436 already mentioned. We propose that these functions could participate to the survival of  
437 bacteria in clouds, which would in turn contribute to global spread. Clouds are indeed  
438 depicted as particularly harsh environments, selective for the most efficient multiple-stress  
439 response phenotypes<sup>69–71</sup>. Metatranscriptomics indicated the expression of biological  
440 responses to stress, including defense mechanisms like elevated transmembrane transports,  
441 binding activities and protein phosphorylation<sup>24</sup>. The selective advantage of carrying ARGs  
442 such as the efflux pump *qepA* in clouds could therefore contribute to their maintenance and  
443 spread in the environment, even in the absence of such antimicrobial molecules. A connection  
444 between antibiotic resistance acquisition and bacterial aerosolization was even recently  
445 established<sup>41</sup>. More specifically, the osmotic and desiccative stresses caused by aerosolization  
446 triggered an immediate response to antibiotic compounds targeting cell wall and protein  
447 synthesis (Cephalothin and Gentamicin) in *Escherichia coli*.

448

### 449 **Global ARGs Dispersal**

450 Based on our current dataset, that compiles 12 clouds with various geographical origins, we  
451 attempt here to provide an evaluation of the global contribution of clouds in antimicrobial  
452 genes dispersal (Table 3). The ARG concentrations observed in clouds were expressed as

453 copies  $\text{m}^{-3}$  of cloudy air, based on sampling times and sampler's air flow rate corrected for the  
454 altitude of puy de Dôme (1465 m). Considering an average volume of clouds within the first 6  
455 km above the Earth of  $4.66 \times 10^{17} \text{ m}^3$  <sup>72</sup> and assuming ARGs concentrations similar as in our  
456 study ( $5.42 \times 10^3$  copies of ARGs  $\text{m}^{-3}$  of air), we estimate that globally clouds carry in  
457 permanence  $\sim 2.53 \times 10^{21}$  copies of ARGs. Annually, this would represent fluxes of  $\sim 1.29 \times 10^{25}$   
458 to  $\sim 2.06 \times 10^{26}$  copies of ARGs transiting through clouds. Assuming a cloud precipitation rate  
459 of  $4.10 \times 10^{20} \text{ m}^3 \text{ year}^{-1}$  <sup>72</sup>, approximately  $\sim 2.2 \times 10^{24}$  copies would get precipitated to the Earth  
460 surface by wet processes every year, while the remaining fraction can be expected to  
461 evaporate and remain airborne. These estimations are based on a limited number of events  
462 collected at a very specific location, but there can be strong spatial and temporal variation as  
463 emphasized previously. Still, since we considered only clouds (i.e.  $\sim 15\%$  of the atmospheric  
464 volume) and did not account for below-cloud aerosol scavenging processes by precipitation,  
465 and that we did not account for all known ARGs, these probably represent low estimates of  
466 the total actual inputs of ARGs from the atmosphere.

467

## 468 **Conclusion**

469 In this study, we evidenced for the first time the presence of microbial ARGs in clouds at  
470 concentrations comparable to other natural environments. Using a unique dataset composed of  
471 12 events sampled over more than a year and originating from very different geographical  
472 origins, our results highlights that clouds' resistome can be viewed as a mosaic composed of  
473 antibiotic resistance from both distant origins and local landscapes. More specifically, a  
474 strong Atlantic Ocean signature was observed in most of the clouds, as shown by the  
475 predominance of the of *qepA* efflux pump, that decreased as the air masses flew over  
476 continental surfaces, to the benefit of antibiotic resistance in relation with the consumption of  
477 antibiotics in food-producing animals, such as  $\beta$ -lactamase, tetracycline and macrolide

478 resistance. Overall, our results point out clouds as a tremendous potential reservoir of ARGs  
479 within the environment, and highlights the high atmosphere as routes for the dissemination of  
480 ARGs at a large scale.

481

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497

## 498 **Conflict of Interest**

499 The authors declare no conflict of interest.

500

501 **Authors' contribution**

502 Caroline Duchaine, Nathalie Turgeon and Pierre Amato secured the funding. Caroline  
503 Duchaine and Pierre Amato designed the experimental setup and defined the goal of the  
504 study. Jean-Luc Baray and Laurent Deguillaume organized field sampling at the puy de Dôme  
505 station. Florent Rossi, Raphaëlle Péguilhan and Laurent Deguillaume sampled the clouds.  
506 Florent Rossi performed the ARGs monitoring, analyzed the results and produced the first  
507 draft of the manuscript. Jean-Luc Baray performed the atmospheric back-trajectories  
508 calculation and analysis. Marc Veillette and Nathalie Turgeon contributed significantly to the  
509 design of the qPCR and provided key information in data treatment and in the understanding  
510 of the results. All authors contributed significantly to the writing and the reviewing of the  
511 manuscript.

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702 **Quantification of Antibiotic Resistance Genes (ARGs) in Clouds at a**  
703 **Mountain Site (puy de Dôme, Central France)**

704 **Tables and Figures**

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**Table 1:** Concentrations of antimicrobial resistance genes (ARGs) and bacteria cells in cloud water (mL<sup>-1</sup> of water) in the 12 cloud events investigated. The concentrations of the main families of ARGs are displayed along with their means ( $\pm$  SEM; n=12) and median values. Statistical differences between families of ARGs are highlighted by the subscript (a > b > c > d, pairwise comparison,  $P < 0.05$ ). *N.D.* = not detected (Ct > 37) and *N.A.* = not acquired (missing value).

Cloud sampling date	Quinolones	Aminoglycosides	Macrolides	Sulfonamide	Tetracycline	$\beta$ -Lactamase	Vancomycin	Transposase	Total ARGs	Total Bacteria
	Gene copies ml <sup>-1</sup> of cloud water								Cell ml <sup>-1</sup> of cloud water	
22-Oct-19	1.46 10 <sup>3</sup>	2.69 10 <sup>-1</sup>	7.21 10 <sup>1</sup>	6.19 10 <sup>2</sup>	4.96 10 <sup>2</sup>	6.17 10 <sup>2</sup>	3.40 10 <sup>2</sup>	1.57 10 <sup>1</sup>	3.62 10 <sup>3</sup>	3.32 10 <sup>4</sup>
11-Mar-20	1.40 10 <sup>4</sup>	4.78 10 <sup>-1</sup>	2.14 10 <sup>2</sup>	9.40 10 <sup>2</sup>	8.44 10 <sup>2</sup>	2.92 10 <sup>-1</sup>	1.02 10 <sup>3</sup>	<i>N.D.</i>	1.70 10 <sup>4</sup>	3.18 10 <sup>3</sup>
17-Jul-20	1.81 10 <sup>4</sup>	2.6	1.21 10 <sup>4</sup>	2.30 10 <sup>3</sup>	1.19 10 <sup>3</sup>	1.19 10 <sup>1</sup>	2.06 10 <sup>3</sup>	<i>N.D.</i>	3.58 10 <sup>4</sup>	<i>N.A.</i>
16-Oct-20	1.71 10 <sup>3</sup>	<i>N.D.</i>	5.81 10 <sup>1</sup>	<i>N.D.</i>	2.43 10 <sup>1</sup>	3.50 10 <sup>3</sup>	8.50 10 <sup>2</sup>	<i>N.D.</i>	6.14 10 <sup>3</sup>	7.70 10 <sup>3</sup>
28-Oct-20	9.17 10 <sup>3</sup>	9.94 10 <sup>-1</sup>	1.82 10 <sup>2</sup>	9.39 10 <sup>2</sup>	9.58 10 <sup>2</sup>	6.79 10 <sup>1</sup>	1.76 10 <sup>3</sup>	<i>N.D.</i>	1.31 10 <sup>4</sup>	9.96 10 <sup>3</sup>
03-Nov-20	4.90 10 <sup>4</sup>	4.67	1.18 10 <sup>2</sup>	1.67 10 <sup>3</sup>	2.24 10 <sup>3</sup>	4.80 10 <sup>3</sup>	5.64 10 <sup>3</sup>	3.65	6.35 10 <sup>4</sup>	1.06 10 <sup>4</sup>
10-Nov-20	2.18 10 <sup>4</sup>	3.3	2.95 10 <sup>4</sup>	1.29 10 <sup>3</sup>	4.42 10 <sup>2</sup>	4.07	1.16 10 <sup>4</sup>	<i>N.D.</i>	6.47 10 <sup>4</sup>	5.53 10 <sup>3</sup>
19-Nov-20	5.74 10 <sup>3</sup>	1.78 10 <sup>-1</sup>	8.00 10 <sup>1</sup>	7.55 10 <sup>2</sup>	1.27 10 <sup>2</sup>	1.19 10 <sup>3</sup>	1.75 10 <sup>3</sup>	4.43	9.65 10 <sup>3</sup>	3.33 10 <sup>2</sup>
28-Apr-21.	2.69 10 <sup>2</sup>	6.14 10 <sup>-2</sup>	<i>N.D.</i>	2.24 10 <sup>3</sup>	9.39 10 <sup>2</sup>	<i>N.D.</i>	<i>N.D.</i>	2.60 10 <sup>-1</sup>	3.45 10 <sup>3</sup>	1.33 10 <sup>4</sup>
06-May-21	5.20 10 <sup>3</sup>	6.33 10 <sup>-2</sup>	<i>N.D.</i>	<i>N.D.</i>	2.05 10 <sup>2</sup>	<i>N.D.</i>	4.90 10 <sup>2</sup>	<i>N.D.</i>	5.89 10 <sup>3</sup>	1.19 10 <sup>3</sup>
13-Oct-21	1.32 10 <sup>4</sup>	3.36 10 <sup>-1</sup>	7.73 10 <sup>2</sup>	3.97 10 <sup>3</sup>	6.32 10 <sup>2</sup>	<i>N.D.</i>	1.23 10 <sup>3</sup>	6.12	1.98 10 <sup>4</sup>	1.36 10 <sup>3</sup>
26-Oct-21	3.00 10 <sup>3</sup>	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	8.23 10 <sup>1</sup>	3.58 10 <sup>3</sup>	<i>N.D.</i>	<i>N.D.</i>	6.67 10 <sup>3</sup>	1.63 10 <sup>3</sup>
Median	7.45 10 <sup>3</sup> <b>a</b>	3.03 10 <sup>-1</sup> <b>cd</b>	9.91 10 <sup>1</sup> <b>bc</b>	9.39 10 <sup>2</sup> <b>bc</b>	5.64 10 <sup>2</sup> <b>b</b>	3.99 10 <sup>1</sup> <b>bc</b>	1.12 10 <sup>3</sup> <b>b</b>	0 <b>d</b>	1.14 10 <sup>4</sup>	5.53 10 <sup>3</sup>
Mean $\pm$ SEM	1.19 10 <sup>4</sup> $\pm$ 3.93 10 <sup>3</sup>	1.08 $\pm$ 4.52 10 <sup>-1</sup>	3.60 10 <sup>3</sup> $\pm$ 2.56 10 <sup>3</sup>	1.23 10 <sup>3</sup> $\pm$ 3.39 10 <sup>2</sup>	6.82 10 <sup>2</sup> $\pm$ 1.80 10 <sup>2</sup>	1.15 10 <sup>3</sup> $\pm$ 5.08 10 <sup>2</sup>	2.23 10 <sup>3</sup> $\pm$ 9.54 10 <sup>2</sup>	2.51 $\pm$ 1.35	2.08 10 <sup>4</sup> $\pm$ 6.40 10 <sup>3</sup>	8.00 10 <sup>3</sup> $\pm$ 2.85 10 <sup>3</sup>

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723 Table 2: Proportions of the main families of antibiotics used over Europe (29 countries,  
724 human and veterinary usages; from JIACRA III (2021), and relative abundances observed in  
725 clouds (this study).

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Families of Antibiotics	Proportion of antibiotic usages in Europe		Relative abundance in clouds (this study)					
	Human Health	Food-producing animals	With <i>qepA</i>			Without <i>qepA</i>		
			Continental clouds	Marine clouds	All clouds	Continental clouds	Marine clouds	All clouds
$\beta$ -lactamases	54.1%	24.3%	14.8%	10.6%	12.3%	21.8%	21.4%	21.6%
Quinolones	5.9%	2.6%	32.0%	69.8%	54.0%	0.7%	3.7%	2.4%
Sulfonamides	3.5%	9.1%	18.1%	6.2%	11.1%	22.9%	20.5%	21.5%
Glycopeptides (vancomycin)	< 7%	< 1%	9.4%	8.7%	8.9%	14.6%	35.8%	26.3%
macrolides	6.1%	7.4%	16.5%	1.0%	7.5%	28.2%	3.7%	13.9%
tetracyclines	2.4%	30.5%	9.0%	3.6%	5.9%	12.1%	15.5%	14.0%
aminoglycosides	0.2%	4.6%	< 0.1%	< 0.1%	< 0.1%	< 0.1%	< 0.1%	< 0.1%

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731 Table 3: Global estimates of ARGs in clouds. Data for clouds (upper part of the Table) are  
732 estimates from Pruppacher and Jaenicke (1995).

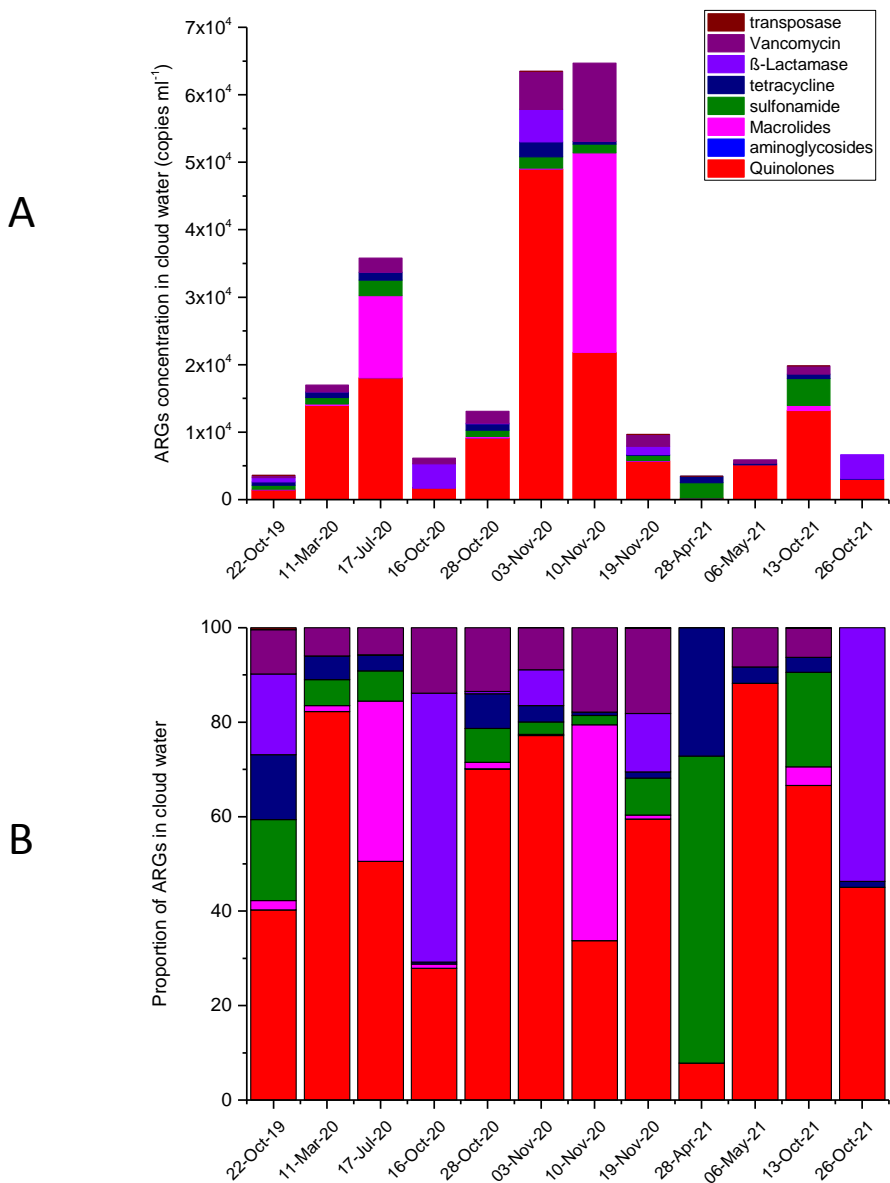
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	Average	min	max	Unit
Volume fraction of clouds in the first 6 km	15.53	-	-	%
Volume of the first 6 km of the atmosphere	$3.00 \cdot 10^{18}$	-	-	$\text{m}^3$
Total volume of clouds in the first 6 km	$4.66 \cdot 10^{17}$	-	-	$\text{m}^3$
Cloud precipitation rate	$4.10 \cdot 10^{20}$	-	-	$\text{m}^3 \text{ year}^{-1}$
ARGs concentration in clouds	$5.42 \cdot 10^3$	$1.01 \cdot 10^3$	$1.61 \cdot 10^4$	$\text{copies m}^{-3}$
ARGs transiting in clouds	$2.53 \cdot 10^{21}$	$4.71 \cdot 10^{20}$	$7.51 \cdot 10^{21}$	copies
Total ARGs transiting in clouds every year	$6.94 \cdot 10^{25}$	$1.29 \cdot 10^{25}$	$2.06 \cdot 10^{26}$	$\text{copies year}^{-1}$
Average precipitation rates of ARGs	$2.22 \cdot 10^{24}$	$4.14 \cdot 10^{24}$	$6.61 \cdot 10^{24}$	$\text{copies year}^{-1}$

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735 Figure 1: Concentrations of the different families of ARGs in the 12 clouds (A) and their  
 736 relative contributions to the pool of total ARGs (B).

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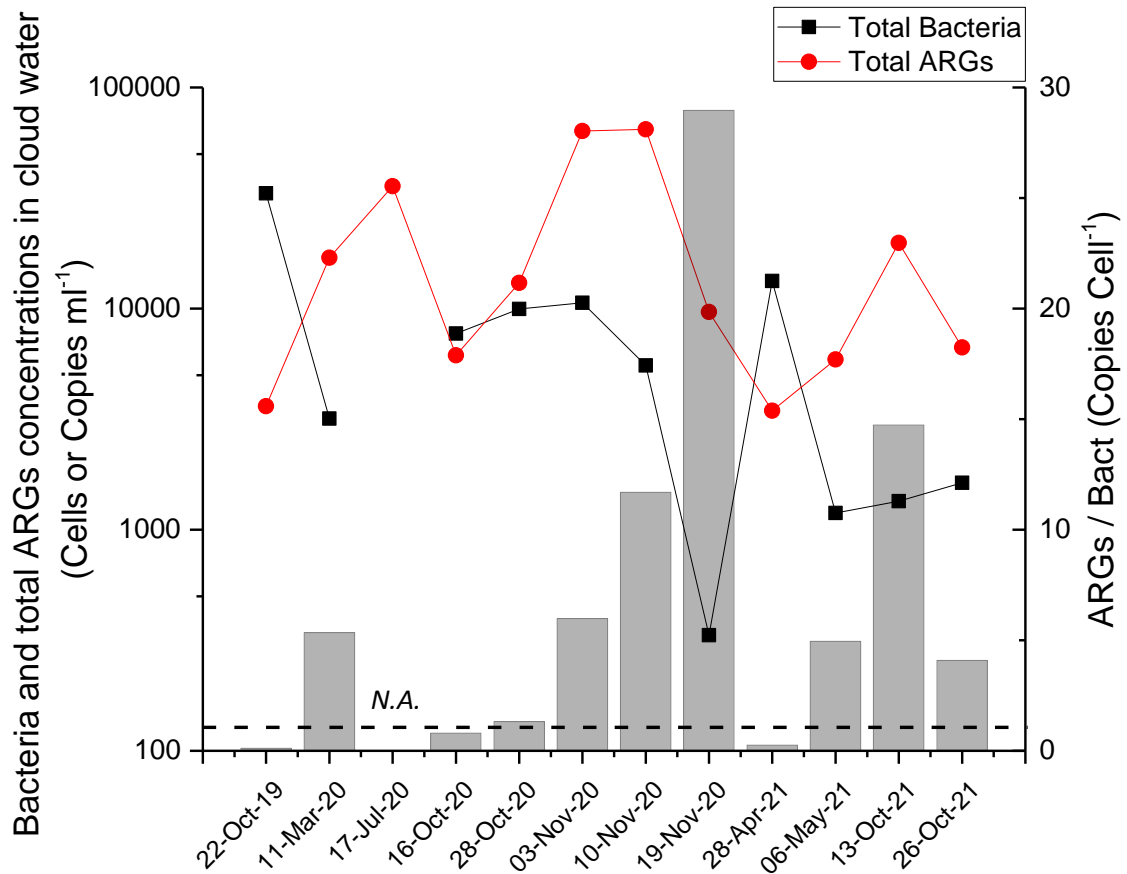




766 Figure 2: Total concentration of ARGs and bacteria in the 12 clouds (left axis; dots / squares  
 767 and lines) and the estimated numbers of ARGs normalized to that of bacterial cells (right axis;  
 768 grey bars). An ARGs per bacterial cells ratio of 1 is indicated by the dotted line. *N.A.* = not  
 769 available (missing value).

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783 Figure 3: Partial least square regression (pls) plot of the 12 cloud events based on  
 784 environmental variables. **(A)** Representation of individual clouds, where groups of samples  
 785 classified as “Continental” (red), “Marine high” (blue) and “Marine low” (grey) based on  
 786 backward trajectories are highlighted by ellipses at 95% confidence level. **(B)** Correlation plot  
 787 of the pls: predictors (origin and altitude of the air mass in the 72-hours preceding sampling)  
 788 are represented in black, and responses (families of ARGs) in blue.

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