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Quantification of ice nuclei active at near 0 °C temperatures in low-altitude clouds at the Puy de Dôme atmospheric station

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Abstract. The distribution, abundance and nature of ice nucleation active particles in the atmosphere are major sources of uncertainty in the prediction of cloud coverage, precipitation patterns and climate. Some biological ice nuclei (IN) induce freezing at temperatures at which most other atmospheric particles exhibit no detectable activity ($> -10\,°C$). Their actual contribution to the pool of IN in clouds remains poorly known, but numerical studies have suggested a probable significance of biological IN in atmospheric processes. In this study, cloud water was collected aseptically from the summit of Puy de Dôme (1465 m a.s.l., France) within contrasted meteorological and physico-chemical situations. Total and biological (i.e. heat-sensitive) IN were quantified by droplet-freezing assay between $-5\,°C$ and $-14\,°C$. We observed that freezing was systematically induced by biological material, between $-6\,°C$ and $-8\,°C$ in 92 % of the samples. Its removal by heat treatment consistently led to a decrease of the onset freezing temperature, by 3 °C or more in most samples. At $-10\,°C$, 0 to ~ 220 biological IN mL^{-1} of cloud water were measured (i.e. 0 to $\sim 22\,m^{-3}$ of cloud air based on cloud liquid water content estimates), and these represented 65 % to 100 % of the total IN. Based on back-trajectories and on physico-chemical analyses, the high variability observed resulted probably from a source effect, with IN originating mostly from continental sources. Assuming that biological IN were all bacteria, at maximum 0.6 % of the bacterial cells present in cloud water samples could have acted as IN at $-8\,°C$, 1.5 % at $-10\,°C$, and 3.1 % at $-12\,°C$. The data set generated here will help elucidate the role of biological and bacterial IN on cloud microphysics by numeric modelling, and their impact on precipitation at local scale.

1 Introduction

The formation of clouds and their evolution have global impacts on earth's climate. Within the last decade, considerable efforts have been made in order to identify and quantify the particles acting as ice nuclei (IN) in the atmosphere. Those particles are responsible for the heterogeneous nucleation of ice in supercooled clouds, leading to modifications of their radiative properties and initiating precipitation. At temperatures colder than about $-15\,°C$, feldspar particles were recently demonstrated to account for a great part to the pool of IN in mixed-phase clouds at a global scale (Atkinson et al., 2013). However, at warmer temperatures, most of the mineral aerosols as well as metallic and soot particles exhibit only very low or undetectable IN activity (INA), and the best ice nuclei candidates are biological (bacteria, fungi), or biogenic (macromolecules derived from living organisms, such

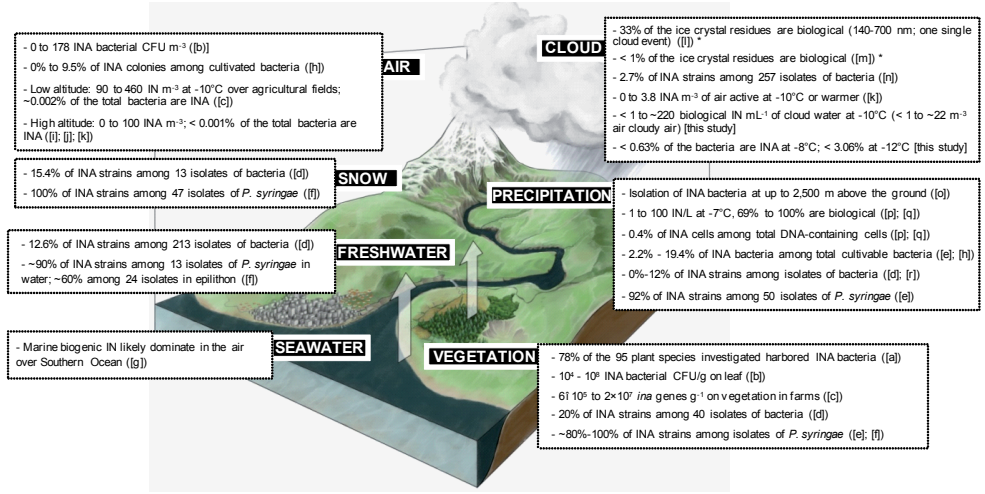

Figure 1. Schematic summarizing our current knowledge about the abundance of biological IN active at temperatures $\geq -10\,^{\circ}\mathrm{C}$ in the different environmental links of the water cycle. An asterisk indicates data relative to ice crystal residues in clouds at much colder temperatures. [a] Lindow et al. (1978); [b] Lindemann et al. (1982); [c] Garcia et al. (2012) ; [d] Maki and Willoughby (1978); [e] Constantinidou et al. (1990); [f] Morris et al. (2008) ; [g] Burrows et al. (2013); [h] Stephanie and Waturangi (2011); [i] Bowers et al. (2009); [j] Conen et al. (2012); [k] Xia et al. (2013); [l] Pratt et al. (2009); [m] Cziczo et al. (2013); [n] Joly et al. (2013); [o] Sands et al. (1982); [p] Christner et al. (2008a); [q] Christner et al. (2008b); [r] Šantl-Temkiv et al. (2009).

as proteins) (Conen et al., 2011; DeMott and Prenni, 2010). Hence, biological IN are thought to largely influence cloud evolution within the upper range of temperatures around freezing (e.g. Möhler et al., 2007). Among those, the most efficient natural IN described so far are bacteria, with representatives active at temperatures as "warm" as $-2\,^{\circ}\mathrm{C}$ (Maki et al., 1974); other very active biological IN different from bacteria were also detected in the air, but their exact nature remains unknown (Garcia et al., 2012). Specimens of INA bacteria have been recovered from all the compartments of the water cycle: freshwaters (Maki and Willoughby, 1978; Morris et al., 2008), clouds (Joly et al., 2013) and precipitation at high altitude (Sands et al., 1982) or closer to the ground (Constantinidou et al., 1990; Maki and Willoughby, 1978; Šantl-Temkiv et al., 2009; Stephanie and Waturangi, 2011). This supports the hypothetical concept termed "bioprecipitation" that such bacteria could participate to hydrological cycles by triggering precipitation (Morris et al., 2004).

Figure 1 summarizes our current quantitative knowledge about high-negative-temperature ($> -15\,^{\circ}\mathrm{C}$) IN in the atmosphere and in the environmental compartments of the water cycle. The main results of the present study are also indicated. Most plants harbour relatively large populations of epiphytic ice nucleation active (INA) bacteria (Constantinidou et al., 1990; Lindemann et al., 1982; Lindow et al., 1978; Maki and Willoughby, 1978; Morris et al., 2008), so the main source of atmospheric biological IN is probably vegetation (Pöschl et al., 2010). Recently, oceans were also cited as possible emitters of biogenic IN into the atmosphere (Burrows et al., 2013).

In the air at low altitude, Garcia et al. (2012) observed concentrations of 90 to 460 IN m^{-3} active at $-10\,^{\circ}\mathrm{C}$ over vegetated agricultural areas, most of which were classified as biological based on their sensitivity to heat. In this latter study, INA bacterial cells were estimated to represent only a small fraction of the total airborne bacteria ($\sim 0.002\,\%$) (Garcia et al., 2012). Nevertheless, some specimens of INA bacterial strains have been recovered by culture from atmospheric samples (e.g. Stephanie and Waturangi, 2011). At high altitude, notwithstanding their suspected importance in atmospheric processes, much less quantitative data of high-temperature IN is available. Their concentration there is in general much below $25\,\mathrm{m}^{-3}$, but it can vary drastically between < 1 and $\sim 100\,\mathrm{m}^{-3}$ within very short time frames (Bowers et al., 2009; Conen et al., 2012; Xia et al., 2013). Interestingly, the highest concentrations were observed at high relative humidity.

Airborne IN can be transported to regions very distant from the source of emission and affect rain patterns after being incorporated into clouds (Creamean et al., 2013). In the single orographic cirrus cloud event studied by Pratt et al. (2009), about half of the 46 ice crystals residues (140–700 nm in diameter; $-31\,^{\circ}\mathrm{C}$ ambient temperature) had a mass spectrometry signature typical of mineral dust, while about 33 % were biological particles. More recent and more extensive in situ observations of cirrus clouds at temperatures $< -30\,^{\circ}\mathrm{C}$ showed that biological particles are probably much more scarce among the solid residues of ice crystals (i.e. less than 1 %), but that rather mineral dust and metallic particles dominate (Cziczo et al., 2013). However, observing

ice crystal residues does not guarantee identifying the actual IN, and cirrus are high-altitude, very low temperature and non-precipitating clouds, so probably not the most appropriate environments for investigating high-temperature and biological IN. A quantitative study of high-temperature atmospheric IN at lower altitude was led at the Jungfraujoch summit in the Alps (3450 m a.s.l.); concentrations of 0 to 3.8 m^{-3} were measured when clouds were present on the site (Xia et al., 2013). Albeit, as emphasized by authors, their "precision was low" due to a limited air sample volume of less than 3 m^3.

Fresh snow and rain collected at different locations over the planet, from poles to sub-equatorial regions, carried ∼ 1 to ∼ 100 IN active at −7 °C per litre of water. Most were altered by heat treatment and were thus categorized as biological, and about half of these were probably bacteria (Christner et al., 2008a, b). INA bacteria were reported to be relatively more abundant in rainfall than in the air at a given site (Stephanie and Waturangi, 2011), which may indicate that INA bacteria are preferentially incorporated into rainfall than other bacteria.

Based on these studies, biological IN are undoubtedly present throughout the water cycle. They represent an important fraction of the pool of high subzero temperature IN where they were unambiguously quantified: in the air at low altitude, and in precipitation. However, our knowledge about their relative abundance in clouds is still scarce, which limits the evaluation of their impact on hydrological cycles using modelling approaches (Hoose et al., 2010; Phillips et al., 2008). As stressed by DeMott and Prenni (2010), it is technically not possible to provide any realistic concentration of airborne IN particles at the altitude of a cloud from measurements in precipitation, due to possible dilution/concentration effects and to non-nucleation particle scavenging. With the objective of providing quantitative data of IN concentration in clouds that could be utilized for modelling purposes, cloud water samples were collected throughout the year and under various meteorological situations from the summit of Puy de Dôme mountain in France (1465 m a.s.l.). Total and biological IN concentrations were measured by the droplet-freezing method (immersion freezing mode) between −5 °C and −14 °C. Data were then analysed against meteorological, chemical and biological variables, and maximum possible values of INA bacteria concentration were inferred.

2 Materials and methods

2.1 Cloud water sampling and meteorological measurements

Twelve random cloud events were sampled at the Puy de Dôme station (45°46′20″ N, 2°57′57″ E; 1465 m above sea level; see Figure S1 for localization) between June 2011 and October 2012. These events were visually classified as non-orographic, and care was taken to avoid precipitating clouds during collection at the sampling site. These events were identified as samples #76 to #87 following the numbering of cloud events sampled at Puy de Dôme since 2001, for which chemical and microbiological data sets are publicly available at http://wwwobs.univ-bpclermont.fr/SO/beam/data.php. Sampling operations were decided after visual estimation of cloud optical thickness at the sampling site. Cloud droplets were selectively collected using single-stage aluminum droplet impactors (cut-off diameter: ∼ 7 µm; Kruisz et al., 1992) sterilized by autoclave, as in Vaïtilingom et al. (2012) and previous studies from our group. Meteorological data were recorded continuously during sampling by the Observatory of the Globe of Clermont-Ferrand (OPGC)'s atmospheric station. The following parameters were considered in our analysis: temperature (T; Vaisala), relative humidity (RH; Vaisala), liquid water content (LWC; Gerber PVM-100), and cumulated precipitation downwind the sampling site (Fig. S1 in the Suppleemnt). For most samples, LWC was not available. Sample collection rates were not judged reliable enough for estimating LWC due to variations of collection efficiency with droplet size distribution or ice formation (Kruisz et al., 1992). So, in those cases, LWC was approximated from archive data by assigning the minimum, average or maximum value observed in clouds at Puy de Dôme (0.1, 0.3 or 0.6 g m^{-3}, respectively; Deguillaume et al., 2014) depending on the sample collection rate.

The global meteorological context was examined through 72 h back-trajectories of the air masses sampled using the HYSPLIT model (HYbrid Single-Particle Lagrangian Integrated Trajectory) with GDAS1 meteorological data archive and default settings (Draxler and Rolph, 2010) and satellite visible images of Europe and France from EUMETSAT, available for academic purposes at http://www.woksat.info/wwp.html.

2.2 Physico-chemical characterization and total cell counts

Cloud water samples were recovered either liquid or frozen onto the impaction plate depending on ambient temperature during sampling. For each sample, pH was measured (Consort multiparameters C830) and major inorganic and organic ions were examined by ion chromatography (Dionex DX 320 for anions and Dionex ICS1500 for cations).

Total bacteria were counted by epifluorescence microscopy on DAPI-stained samples as in Vaïtilingom et al. (2012). Directly after collection, samples were fixed by the addition of 2 % formaldehyde (final concentration; from 20 % stock solution prepared in phosphate buffer 0.1 M, pH 7.0), and incubated in the presence of 2.5 µg mL^{-1} of DAPI (4′,6-diamino-2-phenylindole) in the dark for at least 20 min before filtration on GTBP black filters (0.22 µm porosity; Millipore). Filters were then mounted on microscope slides and observed under UV-epifluorescence

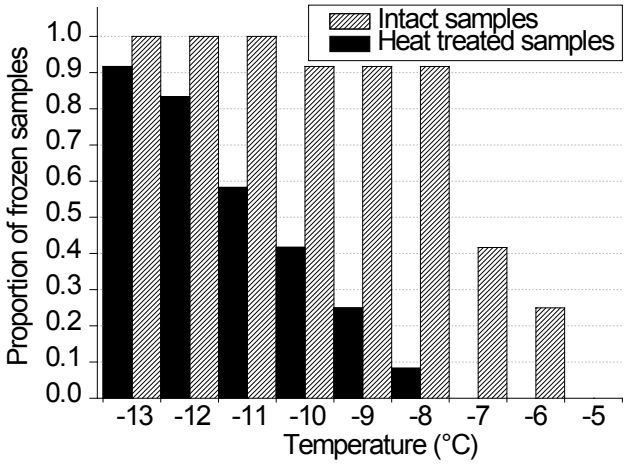

Figure 2. Cumulative proportion of cloud samples for which at least one freezing event was observed during IN assays, in the absence of treatment (shaded bars) or after heating at 95 °C for 10 min (black bars).

microscopy ($\lambda_{exc} = 365$ nm; $\lambda_{em} = 420$ nm) (Leica DM-IRB).

2.3 Droplet-freezing assays

The ice nucleation activity (INA) of the cloud water samples in the immersion freezing mode was determined within 2 h after collection following the well-tried droplet-freezing method (Vali, 1971). Thirty-two to 160 drops (Table 2) of 20 µL were distributed in 0.2 mL microtubes designed for high thermal conductivity and preventing aerial contamination and evaporation (Stopelli et al., 2014). These were placed in a cooling bath (Julabo F34-ED) at decreasing temperatures from −5 to −14 °C, with 1 °C intervals for 8 min. The tubes were visually inspected at the end of each temperature step, and those still liquid were counted. The concentration (mL^{-1}) of ice nuclei C_{IN} at the temperature T in the suspensions was calculated using the equation in Vali (1971): $C_{IN} = [\ln(N_{total}) - \ln(N_{liquid})]_T / V \times (1 / D_f)$, where N_{total} is the total number of droplets, N_{liquid} the number of droplets still liquid after 8 min at the temperature T, V the volume of the droplets assayed (mL) and D_f the dilution factor of the suspension. Under our experimental conditions, the quantification limits ranged from 1.6 to 173.3 IN mL^{-1} in the case where 32 droplets were assayed, and from 0.3 to 253.8 IN mL^{-1} in the case where 160 droplets were assayed. Negative controls consisted of ultrapure sterile water droplets, and these remained liquid over all the range of temperatures investigated.

2.4 Biological IN quantification

For each sample, the concentration of biological IN (INA_{bio}) was calculated as the difference between the concentration of IN measured in untreated sample (INA_{total}) and the concentration of IN measured after heating for 10 min at 95 °C (INA_{heated}), as in Christner et al. (2008a) and in Garcia et al. (2012). Heat denatures protein structures, so it eliminates at least a certain fraction of biological IN without altering non-biological material. When $[(INA_{heated})_{T-1} - (INA_{heated})_T]$ exceeded $[(INA_{total})_{T-1} - (INA_{total})_T]$, this calculation artificially led to a decrease in the concentration of INA_{bio} at $T-1$ compared to T, and values of INA_{heated} were corrected for being consistent with the values of INA_{total}. Following this rule, three values of INA_{heated} were corrected: −12 °C in sample #79, −10 °C in sample #82 and −11 °C in sample #86.

2.5 Statistical analyses

Principal component analysis was made using R software version 2.12.2 (R Core Team, 2011). Non-parametric tests (Pearson's rank correlation test, Mann–Whitney test) were performed as most data were not normally distributed and the number of samples was quite low (< 30), using PAST version 2.04 (Hammer et al., 2001).

3 Results and discussion

3.1 Main characteristics of the cloud water samples

Twelve cloud water samples were collected between 29 June 2011 and 10 October 2012 from cloud events lasting in total approximately 20 to 180 h at the Puy de Dôme, based on relative humidity measurements (Table 1; Fig. S1 in the Supplement). The meteorological context associated with each sampling period is presented in Fig. S1 in the Supplement. Most of the air masses sampled originated from the west (Atlantic Ocean) and travelled over different continental areas in Europe before reaching the Puy de Dôme, following different trajectories. Sampling operations were started about 5 to 110 h after clouds arrived at the sampling site. Cloud water was then collected for 1 h 10 min to 5 h 15 min, and after sampling the sampling site remained embedded in cloud for 5 to more than 160 additional hours.

Only clouds that were non-precipitating at the sampling site were collected, but on some occasions rainfall occurred in the vicinity. The amount of precipitation that fell at five sites downwind the sampling site around the sampling period of time was measured (see Fig. S1 in the Supplement and Table 1); most cloud events were not or slightly precipitating in this area, with less than 1 mm of rain accumulated considering the five rain gauges together. In contrast, it reached 1.6 and 7 mm for samples #76 and #77, respectively. Ambient temperature during sampling ranged from −1.5 to 13.3 °C, so some samples consisted of ice formed upon impaction on the collectors (samples #80 through #84); other samples were collected as liquid.

Bacteria concentration in the samples ranged between 1.65×10^3 and 3.37×10^4 mL^{-1}. The chemical composition varied greatly from one sample to another (Table S1 in the Supplement): pH ranged from 4.6 to 6.2, which are typical values for cloud water (e.g. Deguillaume et al., 2014). Ammonium (16.8 to 531.1 µM), sodium (0.6 to 145.7 µM), nitrate (1.0 to 126.0 µM) and sulfate (0.5 to 52.2 µM) dominated among inorganic ions, and formate was the most abundant dissolved carboxylic acid (3.2 to 109.6 µM). The chemical signature of the samples attested of mixed influences from oceanic and continental sources, the respective contributions to the global chemical composition of which were more or less marked depending on the origin of the air mass.

3.2 Quantification of total and biological ice nuclei

The total concentration of IN active between −5 °C and −14 °C was determined by droplet freezing assays. In 11 of the 12 cloud samples (92 %), the onset temperature of freezing (i.e. temperature at which the first droplet froze) was −8 °C or warmer. Only sample #87 started to freeze at colder temperature (−11 °C) (Table 2; Fig. 2). Ice initially formed due to the presence of 0.6 to 8.5 IN mL^{-1} (Table 2; Fig. 3a). Two samples (#81 and #83) were clearly outlying with much higher IN concentrations (∼ 70 mL^{-1} at −8 °C). Overall, the onset freezing temperature was significantly correlated with the concentration of IN in the sample at the warmest temperatures (Table S2; p value < 0.03, $0.66 < \rho < 0.79$ with IN concentrations at −8 and −9 °C). After correction for LWC (Fig. 3b), the concentration of IN per volume of cloud air ranged from 0.06 to more than 71.1 m^{-3} between −6 °C and −14 °C. This is in the range of concentrations typically observed in the air at high altitude (Fig. 1) (Bowers et al., 2009; Xia et al., 2013), and 1 order of magnitude lower than the concentrations measured at low altitude (Garcia et al., 2012). Rain and surface snow samples analysed using similar methods by Christner et al. (2008a, b) had total IN concentrations of about ∼ 1 to ∼ 300 per litre of water at −8 °C, i.e. 2 orders of magnitude fewer than in our cloud water samples. This probably resulted from the relative dilution of insoluble particles in precipitation compared to cloud water (Flossmann and Wobrock, 2010), and from differences in sample handling: Christner et al. (2008a, b) filtered samples for concentrating particles larger than 0.22 µm, so smaller IN particles were missed, among which some could have originated from bacteria (Phelps et al., 1986). In addition, it is possible that a fraction of IN particles was not recovered from the filters.

Heating samples for 10 min at 95 °C invariably decreased the highest temperature of freezing (Fig. 2), in general by 3 °C to 4 °C, and by 1 °C (sample #81) to more than 4 °C in samples #77 and #79, respectively (Table 2). This indicated that heat-sensitive IN (thereafter termed biological IN) were systematically responsible for freezing at the warmest temperatures. The proportion of biological IN in samples did not depend on the absolute total IN concentration (Table S2 in the Supplement; $p > 0.05$). As other IN were activated at lower temperature, the relative contribution of biological IN decreased with decreasing temperature, from 97 % to 100 % of the total number of IN active at −8 °C to as low as 77 % at −12 °C (Table 2). These are in accordance with observations of IN in the air (Garcia et al., 2012) and in precipitation (Christner et al., 2008a, b).

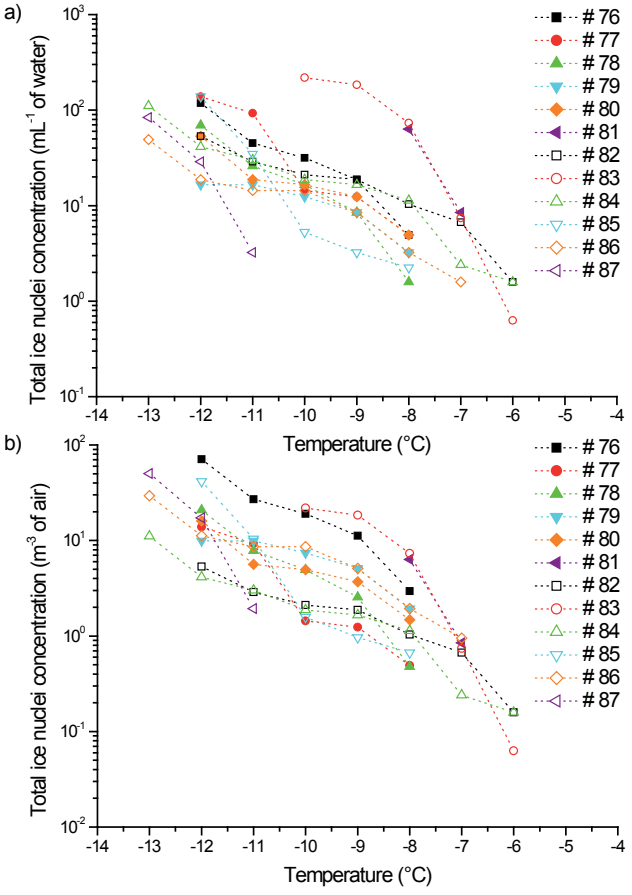

Figure 3. Cumulative concentration of total IN in the cloud samples. **(a)** per volume of water sample (mL^{-1}) and **(b)** per corresponding volume of cloud air (m^{-3}).

The average absolute concentrations of biological and non-biological IN are represented on Fig. 4. Since heat treatment does probably not inactivate every IN site of biological material such as fungi or pollen (Pummer et al., 2012), the concentrations of biological IN reported here should be seen as conservative (i.e. lowest possible) values. Clearly, non-biological (i.e. heat-resistant) particles contribution became significant only around −12 °C and colder. We examined the influence of the different variables measured on the IN content of our samples. Table S2 shows Spearman's correlation matrices (p values, ρ and n) linking the variables together. Among noticeable correlations, coldest sampling temperatures were linked with highest onset freezing temperatures (Table S2; Spearman's rank correlation test; $n = 12$,

Table 1. Main characteristics of the cloud events sampled. Samples recovered as ice formed upon impaction in the sampler are indicated in italic. See detailed ion composition in Table S1 in the Supplement.

Sample	Date	Sampling period (UTC)		Sampling duration (h)	Volume sampled (mL)	Cloud period (UTC)[a]		Cloud event duration (h)[a]	Time in cloud before sampling (h)[a]	Time in cloud after sampling (h)[a]	Precipitation accumulated in the vicinity (mL)[b]	Mean sampling temperature (°C)	Mean LWC during sampling (g m^{-3})	Bacteria concentration (mL^{-1})
		From	To			From	To							
# 76	29 Jun 11	6:30 a.m.	11:45 a.m.	5.25	> 200	28/06/11, 10:00 p.m.	30/06/11, 0:00 a.m.	26	8.5	12.3	1.6	11.5	0.6	n.d.*
# 77	7 Jul 11	1:50 p.m.	3:00 p.m.	1.17	15	07/07/11, 9:00 a.m.	08/07/11, 6:00 a.m.	21	4.8	15	7	12.0	0.1	n.d.*
# 78	20 Jul 11	7:30 a.m.	9:10 a.m.	1.67	47	19/07/11, 3:00 p.m.	23/07/11, 4:00 p.m.	97	16.5	78.8	0.2	8.3	0.3[c]	12355
# 79	7 Nov 11	1:00 p.m.	2:30 p.m.	1.50	193	06/11/11, 8:00 a.m.	08/11/11, 11:00 a.m.	51	29	20.5	0.4	7.0	0.6[c]	10825
# 80	*20 Jan 12*	*12:45 p.m.*	*3:00 p.m.*	*2.25*	*55*	*18/01/12, 11:00 p.m.*	*26/01/12, 0:00 a.m.*	*169*	*37.7*	*129*	*0*	*−0.4*	*0.3*[c]	*9980*
# 81	*23 Jan 12*	*1:00 p.m.*	*4:00 p.m.*	*3.00*	*53*	*18/01/12, 11:00 p.m.*	*26/01/12, 0:00 a.m.*	*169*	*110*	*56*	*0*	*−1.2*	*0.1*[c]	*33 724*
# 82	*19 Mar 12*	*12:10 p.m.*	*4:10 p.m.*	*4.00*	*45*	*17/03/12, 11:00 p.m.*	*21/03/12, 11:00 a.m.*	*84*	*37.2*	*42.8*	*0.2*	*−1.5*	*0.1*[c]	*1648*
# 83	*4 Apr 12*	*6:10 a.m.*	*9:20 a.m.*	*3.17*	*29*	*03/04/12, 11:00 p.m.*	*06/04/12, 12:00 p.m.*	*61*	*7.2*	*50.7*	*0.25*	*−0.4*	*0.1*[c]	*14 914*
# 84	*18 Apr 12*	*8:10 a.m.*	*12:15 p.m.*	*4.08*	*31*	*17/04/12, 6:00 p.m.*	*25/04/12, 6:00 a.m.*	*180*	*14.2*	*161.8*	*0*	*0.2*	*0.1*[c]	*3902*
# 85	25 Jun 12	1:35 p.m.	5:00 p.m.	3.42	66	25/06/12 01:00 a.m.	26/06/12 12:00 a.m.	35	12.6	19	0	13.3	0.3[c]	4474
# 86	13 Sep 12	7:50 a.m.	9:50 a.m.	2.00	75	12/09/12, 7:00 p.m.	13/09/12, 3:00 p.m.	20	12.8	5.2	0.8	6.0	0.6[c]	5199
# 87	10 Oct 12	8:40 a.m.	9:50 a.m.	1.17	70	08/10/12, 9:00 p.m.	11/10/12, 0:00 a.m.	63	35.7	26.2	0	9.4	0.6[c]	19 658

* n.d.: not determined. [a] Defined as RH > 95 % based on hourly average (see Fig. S1).
[b] Sum of precipitation accumulated at five rain gauge stations in the vicinity of Puy de Dôme (Royat, Farnette, Sayat, Trois Ponts and Blanzat) (see Fig. S1).
[c] Estimation from sample collection rate and Puy de Dôme data archive.

Table 2. Total IN concentration and proportion of heat-sensitive IN in the cloud water samples between −5 °C and −14 °C. Values below the detection limit are presented as "0" for visual clarity, and ">" indicates values higher than our quantification limit.

Sample	n =*	Onset freezing temperature (°C)	Onset freezing temperature after heat treatment (°C)	Decrease of onset freezing temperature by heat treatment (°C)	IN mL^{-1} [total *(% heat-sensitive)*]									
					Temperature (°C)									
					−5°C	−6°C	−7°C	−8°C	−9°C	−10°C	−11°C	−12°C	−13°C	−14°C
# 76	32	−8	−12	4	0 (– %)	0 (– %)	0 (– %)	4.9 (100 %)	18.7 (100 %)	31.6 (100 %)	45.0 (100 %)	118.4 (99 %)	n.d.	n.d.
# 77	32	−8	< −12	> 4	0 (– %)	0 (– %)	0 (– %)	4.9 (100 %)	12.3 (100 %)	14.4 (100 %)	92.8 (100 %)	138.6 (100 %)	n.d.	n.d.
# 78	32	−8	−11	3	0 (– %)	0 (– %)	0 (– %)	1.6 (100%)	8.5 (100%)	16.5 (100%)	26.1 (94%)	69.3 (88%)	n.d.	n.d.
# 79	32	−8	< −12	> 4	0 (– %)	0 (– %)	0 (– %)	3.2 (100%)	8.5 (100%)	12.3 (100%)	16.5 (100%)	16.5 (100%)	n.d.	n.d.
# 80	32	−8	−11	3	0 (– %)	0 (– %)	0 (– %)	4.2 (100 %)	12.3 (100 %)	16.5 (100 %)	18.7 (92 %)	53.4 (91 %)	n.d.	n.d.
# 81	32	−7	−8	1	0 (– %)	0 (– %)	8.5 (100%)	63.4 (97%)	> 173.3 (< 99 %)	> 173.3 (< 97%)	> 173.3 (< 95%)	> 173.3 (< 92 %)	n.d.	n.d.
# 82	32	−6	−9	3	0 (– %)	1.6 (100 %)	6.7 (100 %)	10.4 (100 %)	18.7 (74 %)	21.1 (66 %)	28.8 (70 %)	53.4 (77 %)	n.d.	n.d.
# 83	160	−6	−10	4	0 (– %)	0.6 (100 %)	7.4 (100 %)	73.2 (100 %)	184.4 (100 %)	219.1 (99 %)	> 253.8 (< 97 %)	> 253.8 (< 93 %)	n.d.	n.d.
# 84	64	−6	−9	3	0 (– %)	1.6 (100 %)	2.4 (100 %)	11.4 (100 %)	16.5 (90 %)	18.7 (92 %)	30.2 (95 %)	41.3 (88 %)	110.6 (66 %)	> 207.9 (< 55%)
# 85	160	−8	−12	4	0 (– %)	0 (– %)	0 (– %)	2.2 (100 %)	3.2 (100 %)	5.3 (100 %)	34.7 (100 %)	138.6 (99 %)	> 253.8 (< 91%)	> 253.8 (< 45%)
# 86	32	−7	−10	3	0 (– %)	0 (– %)	1.6 (100 %)	3.2 (100 %)	8.5 (100 %)	14.4 (89 %)	14.4 (89 %)	18.7 (83 %)	49.0 (93 %)	> 173.3 (< 56 %)
# 87	32	−11	−13	2	0 (– %)	0 (– %)	0 (– %)	0 (– %)	0 (– %)	0 (– %)	3.2 (100 %)	28.8 (100 %)	83.7 (72 %)	> 173.3 (< 46 %)
Median		−8	−10.5	3	0 (– %)	0 (– %)	0 (– %)	4.9 (100 %)	> 12.3 (100 %)	> 16.5 (100 %)	> 29.5 (< 96 %)	> 61.4 (< 92 %)	> 97.2 (< 82 %)	> 190.6 (< 51 %)
Min		−11	−13	1	0 (– %)	0 (– %)	0 (– %)	0 (– %)	0 (– %)	0 (– %)	3.2 (70 %)	16.5 (91 %)	49.0 (66 %)	> 173.3 (< 45%)
Max		−6	−8	> 4	0 (– %)	1.6 (100 %)	8.5 (100 %)	73.2 (100 %)	184.4 (100 %)	219.1 (100 %)	> 253.8 (100 %)	> 253.8 (100 %)	> 253.8 (93 %)	> 253.8 (< 56 %)

* Number of 20 µL droplets assayed by immersion freezing assays;
n.d.: not determined.

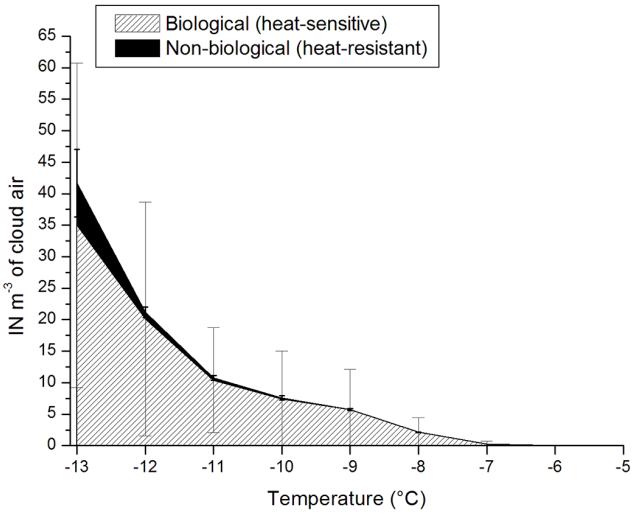

Figure 4. Mean cumulative concentrations of biological (heat-sensitive. shaded area) and non-biological (heat-resistant, black area) IN in clouds ($n = 12$) per volume of air. The sum of the two categories corresponds to the mean concentration of total IN. The lower bound was considered for values below the detection limit.

$p = 0.0361$, $\rho = -0.61$). Consistently, IN activity was higher in samples collected frozen than in samples collected as liquid: higher IN concentrations at the highest temperatures and warmer onset freezing temperature (Mann–Whitney test; medians $= -8$ and -6 °C, respectively). This result is quite surprising if, logically, one considers that the most active IN should be activated and precipitated first, and so that cold air masses should be depleted in highest temperatures IN compared to warmer air masses. Bigg (1996)'s observations of airborne IN in the Arctic indeed suggested that such a selection process occurs in the atmosphere. In our case, the minimum temperature during sampling was > -2 °C (Table 1), so likely still too warm for leading to any temperature partitioning of IN in the clouds sampled. So, despite the fact that the influence of freezing on further IN concentration measurements in our samples cannot be totally excluded, it is possible that the relationship observed results from a higher expression level of IN proteins by bacteria in the coldest clouds (Nemecek-Marshall et al., 1993).

Clouds which precipitated downwind the Puy de Dôme had globally a shorter lifespan at the sampling site (Table S2 in the Supplement). Despite the potential influence of IN on precipitation, no correlation was found here between IN concentrations and local rainfall.

Principal component analysis (PCA) revealed two different groups of IN depending on their temperature of activity, with a net separation between -10 °C and -11 °C (Fig. S2). This demonstrated differences in the origin of the two sets of IN and so probably in their nature as well. The clear positive correlation existing between $IN_{T \leq -11°C}$ and soluble inorganic ions concentrations supports their inorganic composition (Fig. S2). The concentrations of $IN_{T > -11°C}$, i.e. biological IN, and Ca^{2+} were positively correlated, while the trend toward chloride, which mostly originates from marine environment (Warneck, 1999) was negative (Table S2). These tend to situate the sources of biological IN on the continent, at the Puy de Dôme site, probably including both regional and more distant areas.

Considering cloud droplets as spherical, we propose an extrapolation of IN concentration per droplet based on the total IN concentration measured. Thus, for a population of cloud droplets distributed as a single mode of 20 µm in diameter, at the temperature of -8 °C there was a maximum of 1 IN every $\sim 3 \times 10^6$ droplets, and the median value corresponded to 1 IN every $\sim 5 \times 10^7$ droplets.

3.3 Estimation of the contribution of bacteria to biological IN

Joly et al. (2013) proposed an estimation of the concentration of INA bacteria in clouds based on laboratory results. It was proposed that between 0 and ~ 500 bacterial cells mL^{-1} could act as IN in cloud water at -10 °C. This very wide range needed clarification. In order to discriminate bacterial IN from other biological IN, Christner et al. (2008a, b) suggested treating samples with lysozyme. This was intended to alter bacterial cell wall and selectively eliminate bacterial IN. Lysozyme is indeed responsible for the lysis of peptidoglycans by hydrolysing the 1,4-β linkages between N-acetylmuramic acid and N-acetylglucosamine, so it is particularly active towards Gram-positive bacteria; its efficiency towards Gram-negative species is much less marked and it requires additional treatments incompatible with droplet freezing assays (Masschalck and Michiels, 2003; Repaske, 1956). So far, all INA bacteria described in literature including those encountered in clouds have been Gram-negative species (Cochet and Widehem, 2000; Joly et al., 2013). We verified lysozyme efficiency in altering INA of bacteria on two of our cloud samples and on laboratory cultures of INA Gamma-Proteobacteria (Gram-negative) isolated from cloud water (those reported in Joly et al., 2013): lysozyme had no effect on the freezing profiles (not shown). So, this treatment was finally judged not reliable enough here for suppressing specifically bacterial INA and it was not applied further.

In our samples, bacteria concentration ranged from 1.6×10^3 to 3.4×10^4 mL^{-1}, which is within the range of concentrations typically observed in cloud water at the Puy de Dôme site (Vaïtilingom et al., 2012) (Table 1). As expected, since only a small proportion of bacteria is actually IN active and that this can be very variable even within INA+ bacterial strains cultures (e.g. Joly et al., 2013), IN concentration did not vary with bacteria concentration. Rather, it was significantly correlated with most ion concentrations, particularly strongly with K^+ and NO_3^- (Table S2 in the Supplement), suggesting a similar source, i.e. continental origin. In order to provide an estimation of the possible proportion of

Table 3. Inferred maximum possible fraction of INA bacteria among total bacteria in the samples based on heat-sensitive IN concentrations and on total bacteria counts. A ">" indicate values higher than experimental quantification limit for heat-sensitive IN.

	IN/bacterial cell Temperature (°C)								
Sample	−6 °C	−7 °C	−8 °C	−9 °C	−10 °C	−11 °C	−12 °C	−13 °C	−14 °C
# 78	0.00 %	0.00 %	0.01 %	0.07 %	0.13 %	0.20 %	0.49 %	>0.49 %	>0.49 %
# 79	0.00 %	0.00 %	0.03 %	0.08 %	0.11 %	0.15 %	0.15 %	>0.15 %	>0.15 %
# 80	0.00 %	0.00 %	0.05 %	0.12 %	0.17 %	0.17 %	0.49 %	>0.49 %	>0.49 %
# 81	0.00 %	0.03 %	0.18 %	>0.51 %	>0.51 %	>0.51 %	>0.51 %	>0.51 %	>0.51 %
# 82	0.10 %	0.41 %	0.63 %	0.84 %	0.84 %	1.23 %	2.49 %	>2.49 %	>2.49 %
# 83	0.00 %	0.05 %	0.49 %	1.24 %	1.46 %	>1.66 %	>1.66 %	>1.66 %	>1.66 %
# 84	0.04 %	0.06 %	0.29 %	0.38 %	0.44 %	0.73 %	0.93 %	1.86 %	>2.95 %
# 85	0.00 %	0.00 %	0.05 %	0.07 %	0.12 %	0.77 %	3.06 %	>5.15 %	>5.15 %
# 86	0.00 %	0.03 %	0.06 %	0.16 %	0.25 %	0.25 %	0.30 %	0.88 %	>1.87 %
# 87	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %	0.02 %	0.15 %	0.31 %	>0.41 %
Mean	0.01 %	0.06 %	0.18 %	>0.35 %	>0.40 %	>0.57 %	>1.02 %	>1.40 %	>1.62 %

INA bacteria in our samples, biological IN concentration was normalized to bacteria concentration (Table 3). This has to be considered as an upper estimate as it obviously assumes only one IN site per cell, which is the most likely (Hartmann et al., 2013), and it ignores the fact that a certain but unknown fraction of biological materials other than bacteria could also have been inactivated by heat and contributed to the population of biological IN, such as cell fragments for example (Hartmann et al., 2013). At the temperature of −6 °C, a maximum of 0.1 % of the bacteria could have been responsible for freezing (sample #82). This proportion reached maxima of 1.24 % at −9 °C and 3.06 % at −12 °C (in samples #83 and #85, respectively), or about 200 INA cells mL^{-1}. In the air over vegetated areas, INA bacteria were estimated to contribute only ∼ 0.002 % of the total cells (Garcia et al., 2012), and this proportion falls to less than 0.001 % at high altitudes (Xia et al., 2013). In snowfall, comparable estimations gave a very similar fraction of 0.4 % of bacterial cells acting as IN between −4 °C and −7 °C (Christner et al., 2008a) (Fig. 1). In laboratory cultures of INA bacteria, the proportion of individual cells actually acting as IN largely depends on the strain. Except in some exceptionally efficient microorganisms for which this can reach up to more than 4 %, this is often around 1 % at −9 °C, and in general well below 0.1 % at −6 °C (Joly et al., 2013; Šantl-Temkiv et al., 2009; Yankofsky et al., 1981). So, at temperatures below −6 °C, the proportion of INA bacterial cells in clouds basically matched laboratory cultures of INA+ strains.

Low pH (i.e. pH ∼ 4) was shown to negatively impact bacterial INA (Turner et al., 1990). This suggested attenuation of bacterial IN efficiency in polluted clouds due to anthropogenic emissions responsible for acidification (Attard et al., 2012). Among the set of clouds investigated here, only sample #79, with a pH of 4.6, was clearly under influence of human emissions. Yet its freezing profile was not different from others, and on the whole we found no significant relationship between pH and total or biological IN concentrations (Spearman's correlation test; the p values ranged between 0.46 and 1 between −6 and −13 °C).

4 Conclusion

To our knowledge, this study constitutes the first quantitative data set of biological IN measured directly in cloud water. A basic but straightforward experimental set-up allowed us to determine that the concentration of total IN varies in general between ∼ 1 and ∼ 200 mL^{-1} at −10 °C. As previously observed in the air (Garcia et al., 2012) and in precipitation (Christner et al., 2008a), heat-sensitive material, i.e. biological particles, was systematically responsible for freezing at the warmest temperatures and largely dominated the population of IN particles at temperatures down to −11 °C. These data support the possibility that biological material could contribute to cloud evolution by triggering precipitation at temperatures close to 0 °C.

A certain proportion of the biological IN detected in the cloud water samples were likely bacterial cells. Some specimens were indeed previously recovered by culture from several clouds collected at that site (Joly et al., 2013). Assuming that the biological IN observed were all bacterial cells, between 0 % and about 1.5 % of the total bacteria were IN at −10 °C. This extends to much higher values than the proportion of around 0.001 % and 0.4 % proposed for air (Garcia et al., 2012; Xia et al., 2013) and precipitation, respectively (Christner et al., 2008a).

Our experimental procedure by conventional droplet freezing assay only allowed processing a limited number of samples, which also limited our conclusions. In addition, seasonality was not approached here. The development of online measurements is opening new perspectives in the prospection for atmospheric IN, and, in the near future, it should greatly help elucidate their role and environmental drivers

(e.g. Bundke et al., 2010; Huffman et al., 2013). Such estimates of in-cloud biological IN concentrations will allow the community of atmospheric scientists to explore, e.g. using cloud-resolving models, the extent to which these particles can contribute to cloud glaciation, to modification of cloud radiative properties and to regional precipitation patterns.

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