# Survival and ice nucleation activity of bacteria as aerosols in a cloud simulation chamber

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**Abstract**

The residence time of bacterial cells in the atmosphere is predictable by numerical models. However, estimations of their aerial dispersion as living entities are limited by lacks of information concerning survival rates and behavior in relation to atmospheric water. Here we investigate the viability and ice nucleation (IN) activity of typical atmospheric ice nucleation active bacteria (*Pseudomonas syringae* and *P. fluorescens*) when airborne in a cloud simulation chamber (AIDA, Karlsruhe, Germany). Cell suspensions were sprayed into the chamber and aerosol samples were collected by impingement at designated times over a total duration of up to 18 hours, and at some occasions after dissipation of a cloud formed by depressurization. Aerosol concentration was monitored simultaneously by online instruments. The cultivability of airborne cells decreased exponentially over time with a half-life time of 250 ± 30 min (about 3.5 to 4.5 hours). In contrast, IN activity remained unchanged for several hours after aerosolization, demonstrating that IN activity was maintained after cell death. Interestingly, the relative abundance of IN active cells still airborne in the chamber was strongly decreased after cloud formation and dissipation. This illustrates the preferential precipitation of IN active cells by wet processes. Our results indicate that from $10^6$ cells aerosolized from a surface, one would survive the average duration of its atmospheric journey estimated at 3.4 days. Statistically, this corresponds to the emission of 1 cell that achieves dissemination every ~33 minutes per m² of cultivated crops fields, a strong source of airborne bacteria. Based on the observed survival rates, depending on wind speed, the trajectory endpoint could be situated several hundreds to thousands of kilometers from the emission source. These results should improve the representation of the aerial dissemination of bacteria in numeric models.

## 1. Introduction

Microorganisms are known to be dispersed into the atmosphere and disseminated over long distances (e.g., Bovallius et al., 1978; Brodie et al., 2007; Griffin et al., 2001; Smith et al., 2013, and review by Morris et al., 2014b). This has obvious implications for Human, animal and plant epidemiology as well as microbial ecology (Monteil et al., 2014; Morris et al., 2007, 2008; Ġantl-Temkiv et al., 2013). Moreover, some particular bacteria notably found in the atmosphere and clouds can induce heterogeneous ice formation (Cochet and Widehem, 2000; Joly et al., 2013; Lindemann et al., 1982), which probably affects cloud physics and potentially triggers precipitation (Möhler et al., 2007). All these aspects motivated the development of numerical models intended to describe and predict the aerial dispersion of microorganisms. For instance, Burrows et al. (2009a, 2009b) constrained a general atmospheric circulation model using data from the literature and estimates of concentrations and vertical fluxes of airborne microorganisms. They estimated that ~$10^{24}$ bacteria are emitted into the atmosphere each year at the global scale, with a residence time aloft between 2 and 10 days (~3 days on average) depending on emission sources and on meteorological conditions. Such a time span should allow microbial cells (*i.e.* particles of ~1 µm) to travel over hundreds or thousands of kilometers. However, it is not clear what fraction of the aerosolized microorganisms survive over this time scale, and if they maintain properties allowing interactions with atmospheric water.

Most studies aiming at predicting the death rate of airborne bacteria were carried out in the late 1960ôs and early 70ôs, with particular emphasis on the influence of temperature and relative humidity (Cox and Goldberg, 1972; Ehrlich et al., 1970; Lighthart, 1973; Wright et al., 1969). The ability of bacteria to survive as aerosols and the influence of abiotic parameters on survival were shown to strongly depend on the microorganism (Marthi et al., 1990). In experiments at constant temperature ranging from -18°C to 49°C, the survival rate

of *Mycoplasma pneumoniae*, *Serratia marcescens* and *Escherichia coli* decreased with increasing temperature, while this had no or little effect on the survival of *Bacillus subtilis* (Ehrlich et al., 1970; Wright et al., 1969). The highest survival rates were invariably observed at extreme low and high levels of humidity (Cox and Goldberg, 1972; Wright et al., 1969). Finally, carbon monoxide concentration was shown to have variable impacts on the viability of airborne bacteria, with protective or deleterious effects depending on humidity and on the species (Lighthart, 1973). Lighthart (1989) compiled these data and others to build statistical models describing the death rate of airborne bacteria based on aerosol age, temperature, Gram reaction and humidity. Survival rate was resolved by aerosol age, i.e. time after aerosolization, at more than 90%.

In a scientific context motivated by interrogations about cloud-microbes interactions, we studied bacteria originating from atmospheric samples and selected for their relevance to atmospheric questions, *Pseudomonas syringae* and *P. fluorescens*. Indeed, these bacteria are among the most frequent species recovered from natural clouds (Vaïtilingom et al., 2012), some strains are known plant pathogens (Berge et al., 2014) and some, including those investigated here, are ice nucleation (IN) active and have potential impacts on cloud microphysics and precipitation (e.g., Attard et al., 2012; Cochet and Widehem, 2000; Joly et al., 2013; Möhler et al., 2007; Sands et al., 1982). IN active bacteria were shown earlier to induce the formation of ice crystals within simulated clouds (Maki and Willoughby, 1978; Möhler et al., 2008). Here we aimed at examining the survival and IN activity of such typical bacterial aerosols in the atmosphere, using the AIDA (Aerosol Interactions and Dynamics in the Atmosphere) cloud chamber. Cell suspensions were sprayed in the chamber and the concentrations of airborne micron-sized particles, total and cultivable cells and ice nucleating particles (INP) were measured over time for up to several hours after aerosolization. The influence of cloud formation, and the presence of sulfates as surrogates for the presence of

anthropogenic aerosols were briefly approached and seemed to deeply alter cell survival and IN activity. The data presented could be used for improving the parameterization of numerical models describing the atmospheric dispersion of bacteria.

## 2. Material and Methods

### *2.1 Experimental setup and particle concentration measurements*

The AIDA 84-$m^3$ chamber at the Karlsruhe Institute of Technology was used in this study both as a static aerosol chamber in order to store and age the bacterial cell aerosols, and as an expansion cloud chamber in order to simulate cloud activation events and investigate the impact of fresh and aged IN active bacterial aerosols on cloud microphysics. The experiments were conducted during the BIO06 campaign in May 2011. Cell suspensions (see **section 2.2**) were sprayed into the chamber at the beginning of the experiments. The initial relative humidity inside the chamber was around 90% to 95% with respect to ice, thus the sprayed droplets quickly evaporated upon entering the chamber. The dried bacterial cell aerosols were then aged for up to 18 hours at the given chamber pressure, temperature and relative humidity, as summarized in **Table 1**. Aerosol samples were collected (see **section 2.3**) during this step of aerosol ageing in order to measure the airborne concentrations of total cells, the cultivable cell number fraction (**section 2.4**), and the IN activity of the material collected (**section 2.5**). Samples were systematically taken 30 min after spraying, and also after 120 min (2 h), 300 min (5 h), 420 min (7 h), 1020 min (17 h), and 1080 min (18 h).

During three experiments, aerosol samples for microbiological analyses were also taken after a cloud activation and evaporation cycle in the AIDA chamber. Such a cloud cycle in AIDA is initiated by reducing the chamber pressure within a few minutes from about 1000 hPa to 800 hPa by strong pumping. This pressure change simulates the conditions of an air parcel rising in the atmosphere at a vertical updraft velocity of up to a few m $s^{-1}$, which

induces a respective cooling of the air and an increase of the relative humidity. The expansion run starts at a relative humidity of about 90 to 95% with respect to ice, so that at start temperatures below 0°C the air in the cloud chamber first exceeds saturation with respect to ice, and then saturation with respect to liquid water. Depending on the temperature and the ice nucleation activity of the bacterial cells, some ice particles may already be formed in the regime between ice and water saturation. In all the experiments discussed here, water saturation was exceeded, so all bacterial cells acted as cloud condensation nuclei and were first immersed in supercooled cloud droplets before eventually targetting ice formation. After the pumping stopped at a pressure of about 800 hPa, the temperature started to increase due to heat flow from the warmer chamber walls, and the cloud droplets started to evaporate. After full evaporation of the cloud droplets, the chamber was re-pressurized using particle free synthetic air to atmospheric pressure. Aerosol samples were collected once the pressure inside the chamber was returned to ambient pressure. In one of the three experiments during which aerosol samples were collected for microbiological analyses after cloud evaporation, bacteria were sprayed as a suspension in $(NH_4)_2SO_4$ (50 g $L^{-1}$, or 0.38 M) (Exp. 12, **Table 1**), rather than deionized water, in order to generate sulfate aerosols and examine competition effects between sulfates and bacteria on cloud formation and ice nucleation. This also produced preliminary results about the potential impact of anthropogenic aerosols on the survival of airborne bacteria.

After each experiment, the chamber was cleaned by deep depressurization, and refilled with particle free air, so that the chamber was particle free at the beginning of the next experiment.

Aerosol concentration and size in the chamber were monitored during the experiments using a combination of a Scanning Mobility Particle Spectrometer (SMPS) and an Aerodynamic Particle Sizer (APS), both from TSI Incorporated, USA. The concentration of particles in the

size mode around 0.6 µm to about 5 µm is referred to here as **Cells$_{APS}$**; it corresponds to single intact bacterial cells and small agglomerates of cells.

*2.2 Bacterial strains and preparation of cell suspensions*

The following bacterial strains were used: *Pseudomonas syringae* 13b-2 and *P. syringae* 32b-74, both isolated from cloud water samples collected from the puy de Dôme Mountain in France [GenBank accession numbers of the 16S rRNA gene sequences: DQ512785 and HQ256872, respectively (Amato et al., 2007; Vaïtilingom et al., 2012)], and *P. fluorescens* CGina-01 isolated from Cotton Glacier in Antarctica [GenBank accession number FJ152549; (Foreman et al., 2013)]. These were all previously demonstrated to be IN active by droplet-freezing assays (Attard et al., 2012; Joly et al., 2013). *P. syringae* 32b-74 in suspension in deionized water at the concentration of ~$10^9$ cells mL$^{-1}$ nucleated ice at -3°C ; the frequency of IN active cells was > 2% at -4°C and > 4% at -6°C, which ranks this strain among the most efficient IN active bacteria described so far. The onset freezing temperature of *P. fluorescens* CGina-01 at similar cell concentration was -4°C, with a frequency of IN active cells 3 to 4 orders of magnitude lower than that of 32b-74. *P. syringae* 13b-2 nucleated ice at -4°C to -5°C, with a much lower activity (~$10^{-7}$ IN active cells per cell at -6°C).

Bacteria from stock suspensions were grown on Kingôs medium B agar (King et al., 1954) for two days at ambient room temperature (*i.e.* 22°C-25°C). Cells were then scrapped off agar using sterile plastic loops, suspended in sterile deionized water at a concentration of approximatively ~$10^9$ mL$^{-1}$, and incubated overnight at 4°C. In one experiment, cells were suspended in a solution of $(NH_4)_2SO_4$ (50 g L$^{-1}$, or 0.38 M) in order to examine the influence of sulfate coating. In each experiment, a volume of ~50 mL of the cell suspension was sprayed into the cloud simulation chamber (for details see Möhler et al., 2008). The actual cell concentration in the initial suspensions was later determined by flow cytometry (total cells)

and standard dilution platting (colony forming units; CFU), as described in **Section 2.4**. These were used for inferring the initial concentrations of total and cultivable cells airborne in the AIDA chamber, considering a volume of 84 $m^3$; these are referred to as **$Cells_{SUSP}$** and **$CFU_{SUSP}$**, respectively.

*2.3 Sampling from the cloud simulation chamber for microbiological analyses*

Sampling for microbiological analyses was performed using an ethanol-washed impinger (SKC Biosampler; Lin et al., 1999) rinsed several times with sterile deionized water and filled with ~20 mL of sterile deionized water just prior to use. Unexposed aliquots of the water used as the impingement liquid served as negative controls for ice nucleation assays and cell counts. In those controls, no ice nucleation event was detected within the temperature range investigated, and cell count was < 0.005% of the cell counts in samples. Sampling operations were performed at a constant air flow of 12.5 L $min^{-1}$ for 10 min periods using a membrane vacuum pump (KFC), with the inlet of the impinger connected to the inside of the chamber via a stainless steel sampling tube of 4 mm inner diameter. The exact volume of water contained in the sampler (~20 mL) before and after sampling was determined by weighting. It was used to relate the total and cultivable cell concentrations in the impingement liquid to their respective concentrations in the air in the AIDA chamber when equilibrated with atmospheric pressure, considering the volume of the impingement liquid and the sampling rate and time, and assuming 100% collection efficiency (Jensen et al., 1992). These are referred to as **$Cells_{IMP}$** and **$CFU_{IMP}$** throughout the manuscript.

*2.4 Total cells and colony counts*

The concentrations of cultivable and total cells in the impingement liquid were determined by two complementary methods. Cultivable cells were counted as colony forming units

(CFU). Twenty µL of 10-fold serial dilutions of the impingement liquid were spread on R2A medium (Reasoner and Geldreich, 1985) and incubated at 22-25°C for 2 to 3 days before counting the colonies formed. Total cells were counted by flow cytometry, on triplicate samples of 450 µL of the impingement liquid mixed with 50 µL of 5% glutaraldehyde (Sigma) (0.5% final concentration) and stored at -20°C. These were then mixed with one volume (500 µL) of Tris-EDTA buffer at pH 8.0 (10 mM Tris; 1 mM EDTA, final concentrations) and diluted in deionized water to a range of cell concentrations compatible with the analysis. Finally, 10 µL of the DNA specific fluorochrome SYBR-Green (100X concentration; Invitrogen) were added to the samples before incubation in the dark for at least 20 min then injection into the flow cytometer (Becton-Dickinson FACScalibur). Particles fluorescing at 530 nm when excited at 488 nm, *i.e.* labeled with SYBR-Green, were detected and counted by the cytometer. Counts were performed for 2 min or 100,000 events at a flow rate of about 90 µL $min^{-1}$. The exact flow rate was then measured for each series of measurements by weighting a water sample before and after a 20 to 30 minute run in the instrument. All solutions used for flow cytometry analyses were freshly filtered through polycarbonate syringe filters (0.22 µm porosity, Whatman) before use in order to prevent the presence of contaminating particles. In each sample, a population of particles unambiguously attributed to bacterial cells based on their intensity of fluorescence and side-scattering was detected. Finally, cultivability was calculated as the ratio between CFU and total cells counts.

*2.5 IN assays*

The concentration of ice nucleating particles ($C_{INP}$) in the collection liquid was assayed by the drop-freezing method described previously (Vali, 1971). Series of sixteen 0.2 mL microtubes containing 20 µL of the impingement liquid, undiluted or diluted 10-fold in distilled water, were placed in a cooling bath (Ecoline Staredition Lauda E200) and exposed

to decreasing temperatures from -2°C to -10°C with 1°C steps. The number of tubes containing aliquots still in the liquid phase was counted after exposition for 8 min at each temperature step, and $C_{INP}$ was calculated as:

$$C_{INP} = [\ln (N_{total}) - \ln (N_{liquid})]_T / V \times (1 / D_f)$$

with $N_{total}$ is the total number of tubes tested in a given dilution series (16), $N_{liquid}$ the corresponding number of tubes still liquid after 8 min at temperature T, V the volume of liquid in each tube (0.02 mL) and $D_f$ the dilution factor (1 or 10). $C_{INP}$ were finally normalized to the corresponding total cell concentrations measured by flow cytometry.

*2.6 Data analyses*

Exponential regression curves of the type $y = a*e^{(-bt)}$ were fitted to the data. As all the data were normalized to the first time point measured in the corresponding experiment (i.e. 30 minutes after spraying, time set as the ñtime zeroò for data analysis), *a* was equal to 1 and the concentration had its maximum value at t = 30 (time t being expressed in minutes). The time constant of this first-order decay equation is Ű= 1/b, b being the decay rate constant, and the half-life time $t_{1/2}$ , at which the concentration has decreased to half the start value, can be calculated as $t_{1/2} = \ln(2)/b$.

All statistical analyses were performed using PAST version 2.04 (Hammer et al., 2001).

## 3. Results and Discussion

### *3.1 Initial total and cultivable airborne cell concentrations*

A total of 9, 3 and 2 experiments were carried out in the cloud simulation chamber with the strains *Pseudomonas syringae* 32b-74, *P. fluorescens* CGina-01 and *P. syringae* 13b-2, respectively. The initial airborne total and cultivable cell concentrations inferred from in the initial cell suspensions (SUSP subscript), and the concentrations measured with the APS

(APS subscript) and from impinger samples (IMP subscript) 30 min and up to 1080 min (18 h) after aerosolization are presented in **Table 1**. Fifty mL of cell suspensions at concentrations ranging from $3.65\times10^{8}$ to $1.15\times10^{9}$ cells $mL^{-1}$ were sprayed in the chamber, corresponding to theoretical initial total airborne cell concentrations ($Cells_{SUSP}$) of 217 to 684 cells $cm^{-3}$ in the 84 $m^3$-chamber. The concentrations actually measured 30 minutes later by the APS ($Cells_{APS}$) and from impinger samples ($Cells_{IMP}$) were both significantly lower (t-test; $p < 10^{-6}$ and $p = 0.02$, respectively; $n = 13$) and ranged in average between 138 and 289 cells $cm^{-3}$ and between 258 and 451 cells $cm^{-3}$, respectively. At this time point, $Cells_{IMP}$ was significantly higher than $Cells_{APS}$ by a factor of $1.82 \pm 0.40$ in average (t-test; $p < 0.01$; $n = 13$), indicating the presence of cell aggregates in the ~1 µm aerosol population (it extended to about 5 µm at the beginning of the experiments). These were disrupted in the impinger during sampling and counted later as individual cells by flow cytometry (Terzieva et al., 1996). The presence of aggregates was also evidenced in the suspensions sprayed by the fact that the concentration of cultivable cell ($CFU_{SUSP}$) exceeded that of total cells ($Cells_{SUSP}$) (t-test; $p < 0.01$; $n = 13$), with particularly large deviations on CFU counts between technical replicates, and resulting in cultivability > 100%, and at some occasions > 1000% (see **Table 1**). Cell suspensions were prepared by scratching colonies from the surface of agar plates. Even though care was taken for homogenizing them, some heterogeneity probably persisted and resulted in the presence of cell clusters. However, it unintentionally mimicked bacterial aerosols in natural context, as most cultivable bacteria in the atmosphere were found associated with particles (Shaffer and Lighthart, 1997).

*3.2 Survival rate time dependence*

With the intention to take only into account cells already airborne and avoid any possible impact of the spraying process on cultivability, data analysis was restricted to t Ó 30 minutes

after aerosolization and data were normalized to the values measured at this experimental time point. This normalization also allowed the data to be cleaned by avoiding the large deviations on cultivable cell concentration and on cultivability rate in the initial suspensions ($CFU_{SUSP}$). Each individual absolute value of cultivability (*i.e.* not normalized by the cultivability measured at this time point) is plotted in **Figure S1**. The normalized temporal decay of airborne micron-sized particles ($Cells_{APS}$), total cells ($Cells_{IMP}$) and cultivable cells ($CFU_{IMP}$) concentrations was determined from experiments #7, #8, #10 and #11 (**Figure 1**). The concentration of particles in the 1 µm-mode ($Cells_{APS}$) decreased exponentially over time with a time constant Ű= 1260 ± 170 min (Pearsonôs $r$ = 0.992; n = 7). The concentration of airborne cells ($Cells_{IMP}$) decreased faster with a time constant Ű= 500 ± 120 min (Pearsonôs $r$ = 0.937; n = 9). The upper bound diameter of the $Cells_{APS}$ size mode, extending to approximately 5 µm at the beginning of the experiments, decreased to around 3 µm after 7 hours, and the cell-to-particle ratio ($Cells_{IMP}$/ $Cells_{APS}$) decreased from 1.82 ± 0.40 (n = 13) to 1.06 ± 0.06 (n = 2). These indicated that the cell clusters were progressively removed from the aerosol population by sedimentation. Cultivable cell concentration ($CFU_{IMP}$) decreased with a time constant Ű= 230 ± 10 min (Pearsonôs $r$ = 0.990; n = 9). This concentration therefore decreased about twice as fast as that of the concentration of total cells $Cells_{IMP}$ due to additional temporal loss of cultivability. The decay rate constant b for cultivability was ~0.28% $min^{-1}$, corresponding to a time constant Ű= 360 ± 40 min and a half-life $t_{1/2}$ = 250 ± 30 min (3.5 to 4.5 h) (Pearsonôs $r$ = 0.911; n = 9) **(Fig. 2**). This has to be regarded as the most conservative estimate (lower bound) for viability, as viable but non cultivable (VBNC) state is common in aerosolized cells (Heidelberg et al., 1997).

Despite the fact that the bacteria investigated here are non-spore-formers, they lost cultivability only 1.5 to 3 times faster than spores of *Bacillus subtilis* within the same temperature range, which decayed at rates of 0.19% and 0.10 % $min^{-1}$ at -29°C and 4 °C,

299 respectively (Ehrlich et al., 1970). Lighthart (1989) proposed a general time-dependent model
300 of biological decay (decrease of survival rate) for airborne bacteria by mixing experimental
301 data from several bacterial strains, including *Pseudomonas* species (**Figure 2**). This fits our
302 data with a Pearsonôs *r* of only 0.517 (n = 9), and we observed a much higher cultivability
303 than what would have been expected from this model, at least for the first 10 hours following
304 aerosolization. This implies that the *Pseudomonas* strains investigated here, which were
305 originally isolated from atmospheric samples, are more resistant as airborne than the
306 ñaverageò bacterium considered in this model; it could indicate that these strains are to some
307 extent adapted to atmospheric transport (e.g., Ġantl-Temkiv et al., 2012).
308
309 *3.3 Implications for airborne bacteria dissemination*
310 Assuming that bacteria have an aerodynamic diameter of about 1 µm, they have a low
311 sedimentation velocity on the order of $10^{-4}$ m $s^{-1}$ (Malcolm and Raupach, 1991). In addition,
312 such particles fall into the so-called ñscavenging gapò, and they have a particularly long
313 residence time in the atmosphere (Hobbs, 1993). Indeed, residence time was estimated to be
314 2.3 to 9.6 days in the case of single bacterial cells depending on the source ecosystem, with a
315 global mean of 3.4 days (Burrows et al., 2009a). Under our conditions, after 1 day airborne,
316 1.7% of the cells would still be cultivable. Based on these extreme and mean residence times,
317 between 0.009% and 1.22 × $10^{-15}$ % of aerosolized cells (0.0001% in average, *i.e.* 1/ $10^{6}$)
318 would survive the duration of their atmospheric journey until deposition. Statistically, this
319 implies that the emission of at least 11,000 cells is necessary, $10^{6}$ in average, to assure that
320 one survives the residence time and arrives at its endpoint by atmospheric dissemination.
321 Aerosolization, *i.e.* the transfer of cells from a solid surface or from a liquid to the air, is a
322 critical step. In nature, the drag forces created by wind on surfaces generate aerosols by
323 saltation/blasting phenomena (Grini et al., 2002) and result in increased amounts of airborne

microorganisms during high wind speed events (e.g., Lindemann and Upper., 1985). Splashing raindrops on surfaces colonized by microorganisms like plant leaves also lead to the aerosolization of living bacteria (Graham et al., 1977). From liquids, a well-know process of aerosolization is bubble-bursting (Blanchard and Syzdek, 1982). This is actually a phenomenon by which certain types of cells in a community are preferentially aerosolized, thus adding a new layer of complexity in the process of bacterial aerosolization as it results in dissimilarities between the microbial composition in the bulk liquid source and in the air above (Agogué et al., 2005; Fahlgren et al., 2015). The complexity of this phenomenon was probably not reflected in our experimental setup, with bacterial cells being sprayed from liquid suspensions. However, the results presented here only considered bacteria already aerosolized and avoided taking into account the aerosolization step. Hence, considering that the process of aerosolization did not affect subsequent survival rates as aerosol, we can place our results in natural atmospheric context. Plants are among the strongest natural sources of airborne bacteria identified, with emission fluxes around 500 CFU $m^{-2}$ $s^{-1}$ measured above bean and alfalfa fields (Lindemann et al., 1982). At such a rate, each $m^2$ of crop field would emit 1 cell capable of surviving its atmospheric transport every 33 minutes. In other words, 1 cell capable of disseminating alive would be emitted every second by a field of ~2000 $m^2$.

Once airborne, as a first approximation bacteria are passively transported horizontally at the speed of horizontal wind. So, for typical horizontal winds in the troposphere, *i.e.* ~2 to ~30 m $s^{-1}$ (not considering extreme events such as storms or cyclones), at the survival rate measured here, 50% of the cells emitted alive from a source would be transported about 30 to 600 km away, and 1% would reach the ground up to 4000 km away (**Figure 3**). There are indeed many observations of such long distance transport of living bacteria between distant ecosystems in nature (Bovallius et al., 1978; Hervàs et al., 2009; Hervàs and Casamayor, 2009; Comte et al., 2014).

*3.4 Impact of cloud processing*

The conditions investigated here (temperature between -20°C and 0°C and absence of light) can be considered relatively close to the conditions encountered in the high atmosphere during the night. It is probable that in nature, during the day, UV light has deleterious effect and increases mortality rates (Tong and Lighthart, 1997). In addition, cloud formation can alter viability, as shown in samples collected after expansion cooling (*i.e.* depressurization) experiments (experiments #6 and #9). Even though it is not statistically testable here, we noticed a strong decrease in the cultivability of *P. s.* 32b-74 and CGina-01 cells exposed to a cloud (see **Table 1, Figure 2 and Figure S1**). Fractions of only about ~12% and ~40% of the cells cultivable before expansion cooling remained cultivable after cloud dissipation for 32b-74 and CGina-01, respectively, compared to ~70% when the pressure was maintained constant. For cloud formation in the AIDA chamber, pressure was typically decreased at rates of 30 to 50 hPa $min^{-1}$ during expansion, and the associated cooling rates were typically 2 K $min^{-1}$ at the beginning of an expansion and below 0.5 K $min^{-1}$ towards the end of the expansion. Considering pressure and temperature changes with altitude of 10 hPa and 1 K every 100 m, these roughly correspond to uplifts of air masses of around 100 to 500 m $min^{-1}$ (1.7 to 8.3 m $s^{-1}$), which falls within the range of observations for convective precipitating clouds (Balsley et al., 1988). Our results suggest that the shifts in environmental conditions encountered by living cells transported upward, along with the osmotic shock and free radicals generated by water condensation and freezing (e.g., Stead and Park, 2000; Tanghe et al., 2003) probably alter airborne cell survival in clouds to a larger extent compared with non convective situations.

*3.5 Ice nucleation activity*

374 **Figure 4** shows freezing profiles of air samples collected by impingement from the
375 cloud chamber at different times after injection of *P. syringae* 32b-74 suspensions. Thirty
376 minutes after aerosolization, there were ~$2\times10^{-5}$ INP $cell^{-1}$ at -3°C (1 INP every ~50,000
377 cells) and ~$3\times10^{-3}$ INP $cell^{-1}$ at -5°C (1 INP every ~333 cells) on a per-total-cells ($Cells_{IMP}$)
378 basis. This is about one tenth the IN activity of cells in suspension for this strain (Joly et al.,
379 2013). Decreased IN activity in airborne bacteria compared with suspension was expected
380 from previous observations in cloud simulation chamber involving *P. syringae* (Maki and
381 Willoughby, 1978). No further significant loss of activity over time was observed at
382 temperatures Ò-4°C in aerosolized cells (ANOVA, 5% confidence level), *i.e.* the frequency of
383 INP $cell^{-1}$ did not vary with time after aerosolization. This confirmed that non-viable cells
384 retained IN activity, as previously reported (Kozloff et al., 1991).
385 In the natural atmosphere, phenomena such as coating may affect bacterial IN activity. For
386 example coating with sulfate was reported to decrease the IN activity of soot or Arizona Test
387 Dust particles, a material widely used in laboratory ice nucleation studies as a surrogate for
388 natural mineral aerosols (Cziczo et al., 2009; Möhler et al., 2005). However, sulfate coating
389 had no detectable impact on the IN activity of the commercial powder of lyophilized IN
390 active *P. syringae* cells Snomax (Chernoff and Bertram, 2010). In order to further investigate
391 the influence of sulfate coating, cells were suspended in a solution of ammonium sulfate
392 instead of water before being sprayed into the chamber (experiment #12, **Table 1**). Thirty
393 minutes after spraying, we found that the frequency of INP per cell had decreased markedly
394 compared to cells sprayed from water suspensions, especially at the warmest temperatures of
395 activity: the frequency of INP per cell was decreased by about 98.5%, 91% and 34% at -4°C, -
396 5°C, and -7°C, respectively (**Fig. 4**). In this particular bacterial strain, pH at values typical for
397 cloud water influenced by anthropogenic emissions (pH ~4) were also shown to be
398 responsible for a significant decrease of INA (Attard et al., 2012). Such observations show

399 that the IN activity of bacteria is clearly modulated by abiotic factors, and this must be kept in
400 mind when replacing experimentations into environmental context.
401 The capacity of cells of nucleating ice in the atmosphere is particularly relevant where
402 condensed water is present, i.e. in clouds. Using the AIDA chamber, it was shown previously
403 that some strains of *P. viridiflava* and *P. syringae* can act as INP in clouds at temperatures
404 around -10°C in the immersion-freezing mode (Möhler et al., 2008). Here, clouds were
405 formed in the chamber by expansion cooling in two experiments (experiments #6 and #9), and
406 aerosol samples were collected by impingement after cloud dissipation, when the pressure
407 inside the chamber was back to ambient pressure (**Table 1**). The onset ice formation
408 temperature of the impingement liquid was -6°C, compared to -3°C in samples not exposed to
409 cloud, and the frequency of INP per cell was decreased by three orders of magnitude (**Fig. 4**).
410 A possible ñdeactivation effectò of the IN activity of bacteria was already suggested from
411 equivalent experiments (Möhler et al., 2008). However, our results expressed on a per-cell
412 basis suggest that, more likely, IN active cells among a population of airborne bacteria were
413 more efficiently precipitated than others. This could explain the observed distribution of IN
414 active bacteria in natural air, clouds and precipitation: Stephanie and Waturangi (Stephanie
415 and Waturangi, 2011) observed that the proportion of IN active bacterial strains was higher in
416 falling rain water than in the air at the same location. In addition, whereas only 50% of the *P.*
417 *syringae* strains isolated from non-precipitating cloud water were IN active (8 strains) (Joly et
418 al., 2013), those isolated from freshly fallen snow by Morris et al. (Morris et al., 2008) all had
419 this capacity (47 strains).
420
421 **4. Conclusions**
422 In this work, we observed that the concentration and cultivability of cells aerosolized in the
423 AIDA cloud chamber decreased exponentially over time at constant rates. Aggregation

seemed to favor cell survival, but this was of course at the cost of the time span as airborne and so, in nature, of the potential distance of dispersion. Hence, for bacteria, aerial dissemination is clearly a compromise between the distance travelled (which decreases for large aggregates) and the chances of successful dissemination (which increases for large aggregates).

The survival rate determined here should provide basis to the existing numerical models describing the aerial dispersion of bacteria (Burrows et al., 2009a; Sesartic et al., 2012), in order to better predict their atmospheric transport as living entities. By focusing on time as the only explicative variable, we were able to explain quite well (Pearsonôs $r = 0.911$) the decrease of cultivability observed for *Pseudomonas syringae* and *P. fluorescens* in the AIDA chamber, although adjustments of the predictions in an environmental context could be made by integrating viability parameters as needed, like temperature, humidity, UV, or phenotypic traits. Some work in this direction has already been carried out (Attard et al., 2012; Lighthart, 1973; Lighthart et al., 1971; Smith et al., 2011; Tong and Lighthart, 1997), but more experiments would help building a more mechanistic viability model. In addition, these models are still weakened by the large uncertainties that remain concerning the input to be used, as there are still very few data available about the sources of airborne bacteria and the associated emission fluxes (e.g. Lindemann et al., 1982). These need to be documented for different surface types and meteorological situations.

Numerical simulations demonstrated that the impact of IN active bacteria on precipitation is probably negligible at the scale of the planet (Hoose et al., 2010; Sesartic et al., 2012). However precipitation patterns at regional scales have important socio-economic impacts and the underlying processes still need to be elucidated. We observed that the IN activity of airborne bacteria did not change over time for at least several hours after aerosolization. In nature, this is enough time for an IN active cell to be transported to high altitudes and get

incorporated into a cloud. Then, as suggested by others (Constantinidou et al., 1990; Möhler et al., 2008; Morris et al., 2008, 2014a), they can induce freezing of supercooled droplets, trigger precipitation and thus selectively prime their own redeposition. For a complete and accurate description of the transport of bacteria in the atmosphere, the partitioning of cells and in particular of IN active cells, between air, clouds and precipitation should be determined.

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**Table 1:** Detailed cell concentrations and cultivability for the different experiments carried out in the chamber, expressed as the mean of triplicate analyses ± standard error from the mean whenever available. (*P.s.*: *Pseudomonas syringae*; *P.f.*: *Pseudomonas fluorescens*).

| Exp # | AIDA BIO-06 Exp # | Strain | Initial characteristics[a] | | | Time after spraying (min) | Initial temperature and conditions of experiment | Airborne in the cloud chamber[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $Cells_{SUSP}$ $cm^{-3}$ | $CFU_{SUSP}$ $cm^{-3}$ | % cultivable $cells_{SUSP}$ | | | $Cells_{APS}$ $cm^{-3}$ | $Cells_{IMP}$ $cm^{-3}$ | $CFU_{IMP}$ $cm^{-3}$ | % cultivable $cells_{IMP}$ |
| 1ö | 2 | *P.s.* 32b-74 | 684 ± 64 | 3522 ± 2138 | 515% ± 316% | 30 | -5.2°C, no cloud | 138 | 293 ± 5 | 123 ± 63 | 42% ± 21% |
| 2ö | 4 | *P.s.* 32b-74 | 530 ± 59 | 1091 ± 169 | 206% ± 39% | 30 | 0.1°C, no cloud | 173 | 279 ± 232 | 328 ± 34 | 118% ± 99% |
| 3ö | 6 | *P.s.* 13b-2 | 474 ± 19 | 694 ± 172 | 147% ± 37% | 30 | -2.3°C, no cloud | 216 | 382 ± 8 | 314 ± 10 | 82% ± 3% |
| 4ö | 8 | *P.s.* 13b-2 | 474 ±19 | 694 ± 172 | 147% ± 37% | 30 | -17.8°C, no cloud | 204 | 448 ± 5 | 217 ± 61 | 49% ± 14% |
| 5ö | 10 | *P.f.* CGina-01 | 217 ± 34 | 1339 ± 107 | 616% ± 108% | 30 | -2.3°C, no cloud | 269 | 361 ± 49 | 249 ± 24 | 69% ± 11% |
| 13ö | 29 | *P.s.* 32b-74 | 491 ± 80 | 13591 ± 13980 | 2770% ± 2884% | 30 | -7.2°C, no cloud | 180 | 387 ± 22 | 227 ± 26 | 59% ± 8% |
| 14ö | 32 | *P.s.* 32b-74 | 491 ± 80 | 13591 ± 13980 | 2770% ± 2884% | 30 | -13.7°C, no cloud | 178 | 306 ± 19 | 195 ± 37 | 64% ± 13% |
| 7p | 15 | *P.s.* 32b-74 | 437 ± 82 | 754 ± 96 | 173% ± 39% | 30 | -1.4°C, no cloud | 214 | 436 ± 74 | 128 ± 21 | 29% ± 7% |
| | | | | | | 120 | | 184 | 291 ± 35 | 79 ± 11 | 27% ± 5% |
| | | | | | | 300 | | 155 | 171 ± 27 | 42 ± 4 | 25% ± 4% |
| | | | | | | 420 | | 141 | 143 ± 19 | 26 ± 3 | 18% ± 3% |
| 8p | 17 | *P.s.* 32b-74 | 525 ± 48 | 1349 ± 326 | 257% ± 67% | 30 | -2°C to -20°C, no cloud | 185 | 349 ± 24 | 237 ± 69 | 68% ± 20% |
| | | | | | | 120 | | ND | 261 ± 11 | 128 ± 15 | 49% ± 6% |
| | | | | | | 300 | | ND | 185 ± 13 | 69 ± 6 | 37% ± 4% |
| | | | | | | 420 | | 139 | 154 ± 15 | 46 ± 2 | 30% ± 3% |
| 10p | 22 | *P.s.* 32b-74 | 473 ± 90 | 1567 ± 757 | 332% ± 172% | 30 | -1.3°C, no cloud | 172 | 451 ± 59 | 276 ± 31 | 61% ± 11% |
| | | | | | | 1020 | | 92 | 101 ± 11 | 6 ± 1 | 6% ± 1% |
| 11p | 24 | *P.f.* CGina-01 | 303 ± 22 | 10700 ± 12584 | 3529% ± 4158% | 30 | -1.3°C, no cloud | 289 | 321 ± 36 | 242 ± 43 | 75% ± 16% |
| | | | | | | 1080 | | 121 | 82 ± 2 | 2 ± 0 | 3% ± 0% |
| 6ǂ | 12 | *P.f.* CGina-01 | 217 ± 34 | 1339 ± 107 | 616% ± 108% | 30 | -16.7°C, before cloud formed | 186 | 282 ± 36 | 264 ± 92 | 94% ± 35% |
| | | | | | | 150 | After cloud dissipation | ND | 221 ± 19 | 83 ± 29 | 38% ± 13% |
| 9ǂ | 19 | *P.s.* 32b-74 | 529 ± 103 | 13026 ± 12101 | 2464% ± 2339% | 30 | -19°C, before cloud formed | 158 | 258 ± 24 | 124 ± 7 | 48% ± 5% |
| | | | | | | 150 | After cloud dissipation | ND | 139 ± 19 | 8 ± 4 | 6% ± 3% |
| 12ǂ | 26 | *P.s.* 32b-74 | 340 ± 107 | 11161 ± 10312 | 3285% ± 3207% | 30 | -16.7°C, in the presence of ammonium sulfate | ND | 318 ± 24 | 10 ± 3 | 3% ± 1% |

[a] As inferred from the cell suspensions sprayed;

[b] As measured by aerosol particle sizer and from impinger samples.

ö Experiments intended to investigate the impact of fresh IN active bacterial aerosols on the microphysics of clouds generated in the AIDA chamber by expansion cooling; no sample for microbiological analyses was collected after cloud dissipation.

p Experiments intended to investigate the impact of ageing on the survival and IN activity of bacteria as aerosols; clouds were generated afterward for investigating their impacts on microphysics; no sample for microbiological analyses was collected after cloud dissipation.

ǂ Experiments intended to investigate the impact of clouds or sulfate coating on the survival and IN activity of bacterial aerosols.

670

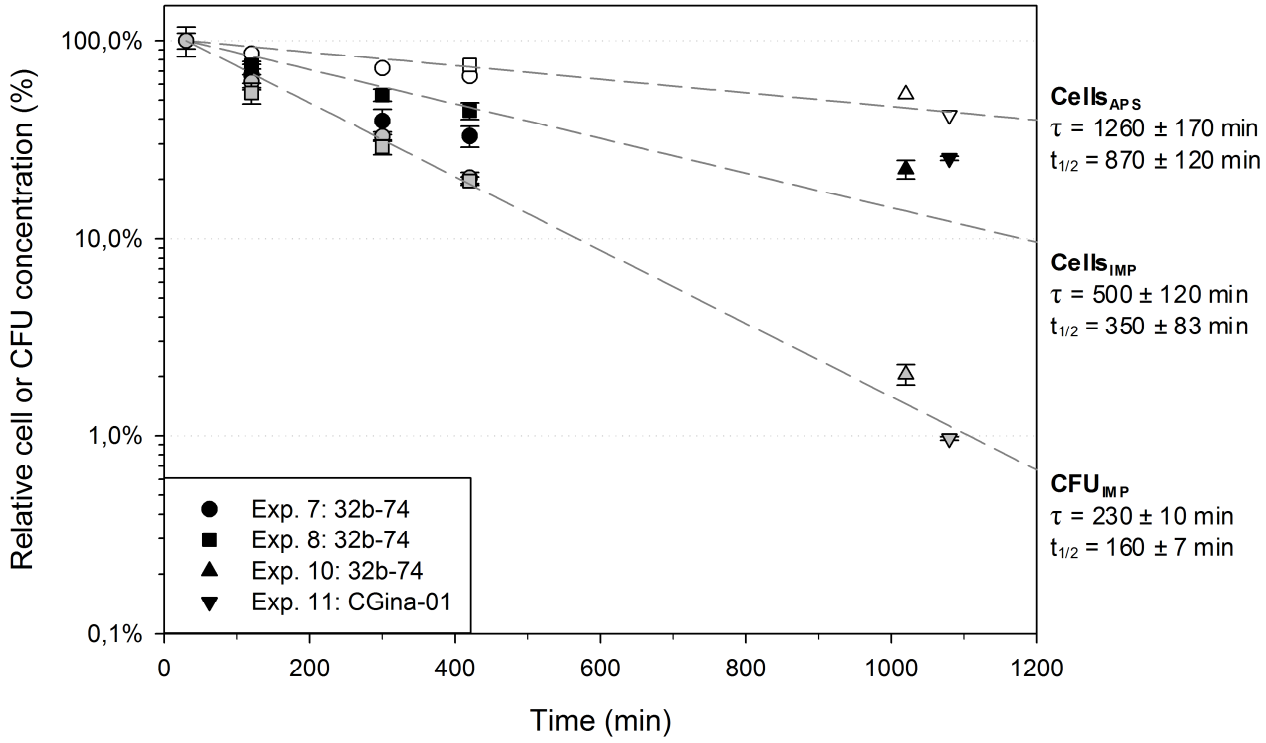
671 **Figure legends:**

672 **Figure 1**: Temporal evolution of total airborne cell concentration measured with the APS
673 ($Cells_{APS}$, open symbols) and total and cultivable cells concentrations measured from
674 impinger samples ($Cells_{IMP}$, black symbols, and $CFU_{IMP}$, grey symbols, respectively) of *P.*
675 *syringae* 32b-74 and *P. fluorescens* CGina-01 in the chamber, relative to the concentrations
676 measured 30 minutes after spraying cell suspensions. Error bars are standard deviations from
677 the mean of triplicate samples. The curves show fitted exponential temporal decays. For total
678 particles: Pearsonôs $r$ = 0.992. n = 7; for total cells: Pearsonôs $r$ = 0.937. n = 9; for cultivable
679 cells: Pearsonôs $r$ = 0.990. n = 9. Corresponding calculated time constants (Ű) and half-life
680 times ($t_{1/2}$) are indicated on the right of the figure (mean ± standard deviation).
681
682 **Figure 2**: Temporal evolution after aerosolization of the proportion of cultivable cells
683 (cultivability) in impinger samples in *P. syringae* 32b-74 and *P. fluorescens* CGina-01,
684 relative to the cultivability measured 30 minutes after spraying cell suspensions, in the
685 absence of cloud (black symbols) or after cloud formation and dissipation (open symbols).
686 Error bars are standard deviations from the mean of triplicate samples. The black dashed
687 curve shows fitted exponential temporal decay of cultivability in the absence of a cloud
688 (Pearsonôs $r$ = 0.911. n = 9). The corresponding calculated time constant (Ű) and half-life time
689 ($t_{1/2}$) are indicated on the right of the figure (mean ± standard deviation). Data using the
690 equation (5) from (Lighthart, 1989) are also plotted for comparison (dashed grey line); this
691 model valid for an ñaverageò bacterial strain fits our data with a Pearsonôs $r$ of 0.517 (n = 9).
692
693 **Figure 3**: Proportion of cultivable airborne cells associated with the distance reached from
694 their emission source for typical horizontal wind velocities (2. 5. 10 and 30 m $s^{-1}$, *i.e.* 7.2, 18,
695 36 and 108 km $h^{-1}$, respectively), relative to the respective initial cultivability, as inferred

from the data presented in **Figure 2**. The proportion of 0.0001% is reached in 3.4 days, the mean residence time of bacteria in the atmosphere estimated by Burrows et al. (2009a).

**Figure 4**: Cumulative frequencies of INP per airborne cell in *P. syringae* 32b-74 within the AIDA chamber 30 min, 7 h and 17 h after aerosolization in the absence of cloud (black symbols), 30 min after aerosolization in the presence of ammonium sulfate (grey symbols), and when the pressure inside the chamber was returned to ambient after cloud formation by expansion cooling (open triangles). Error bars are standard deviations from the mean of independent experiments, when available.

707 **Figure 1**

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710 **Figure 2**

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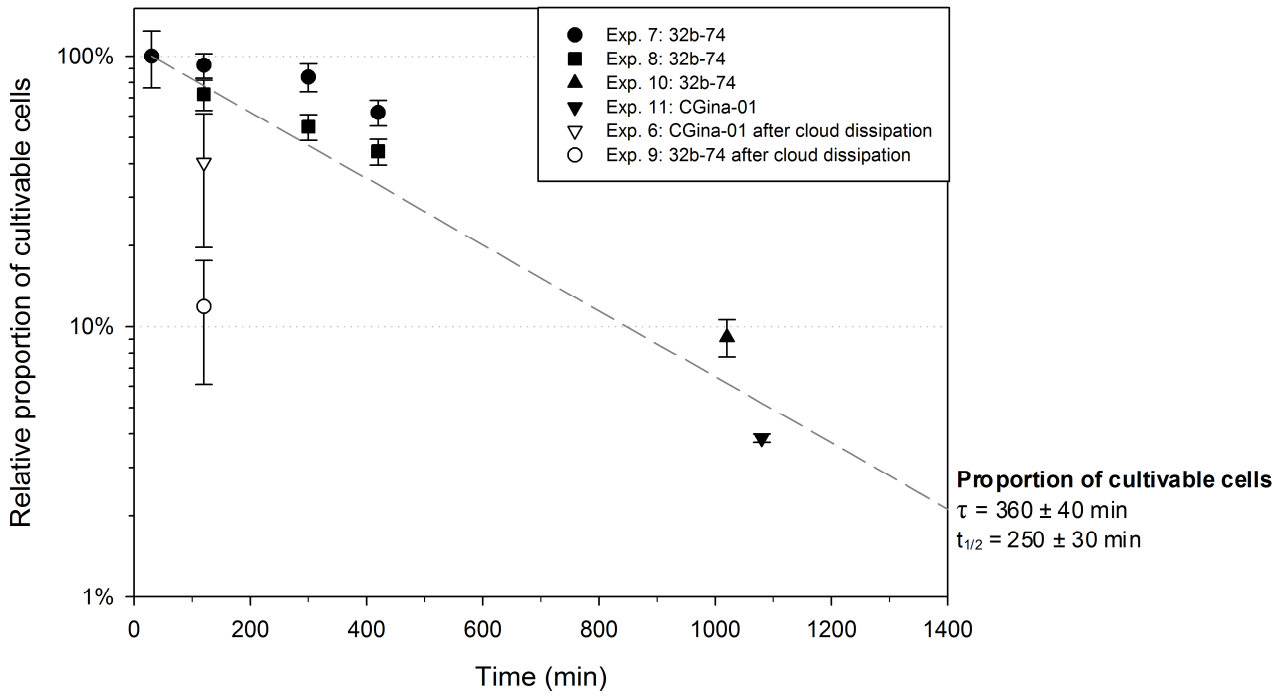


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718 **Figure 3**

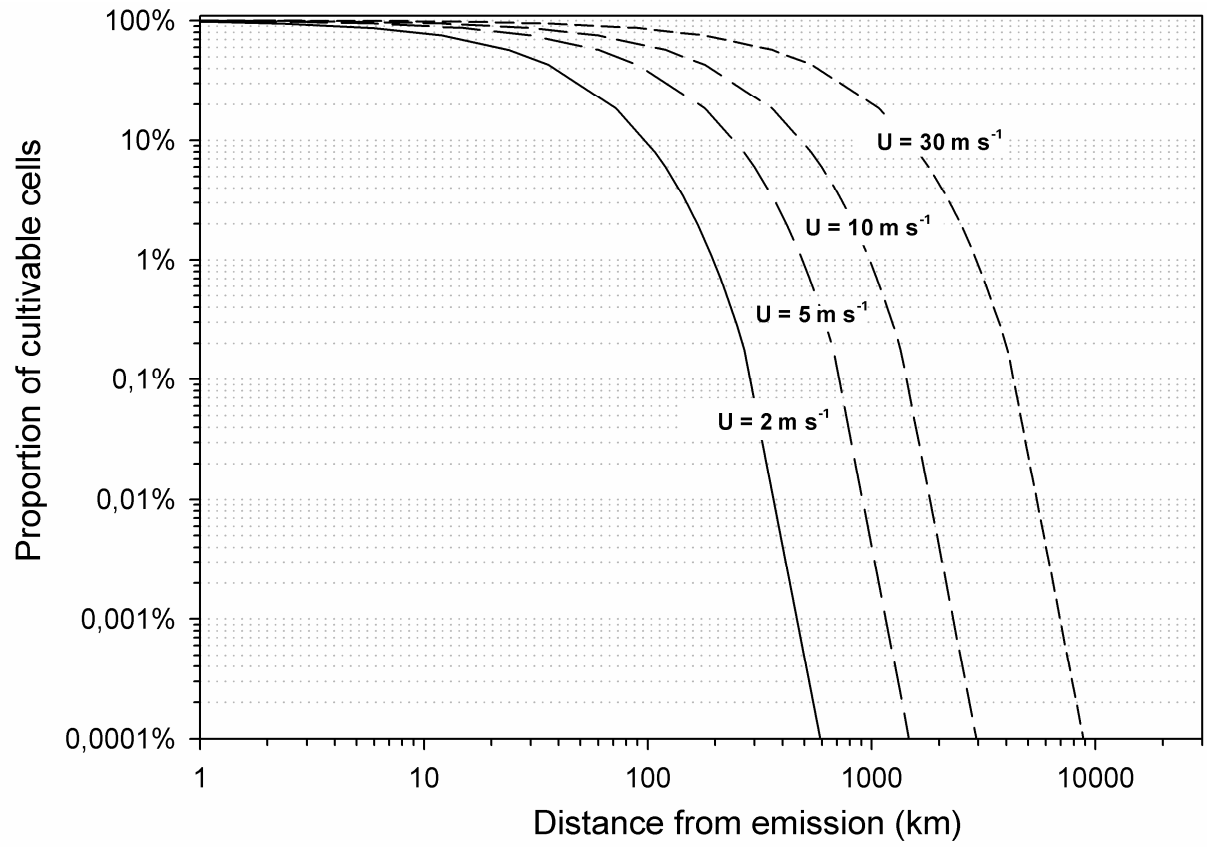


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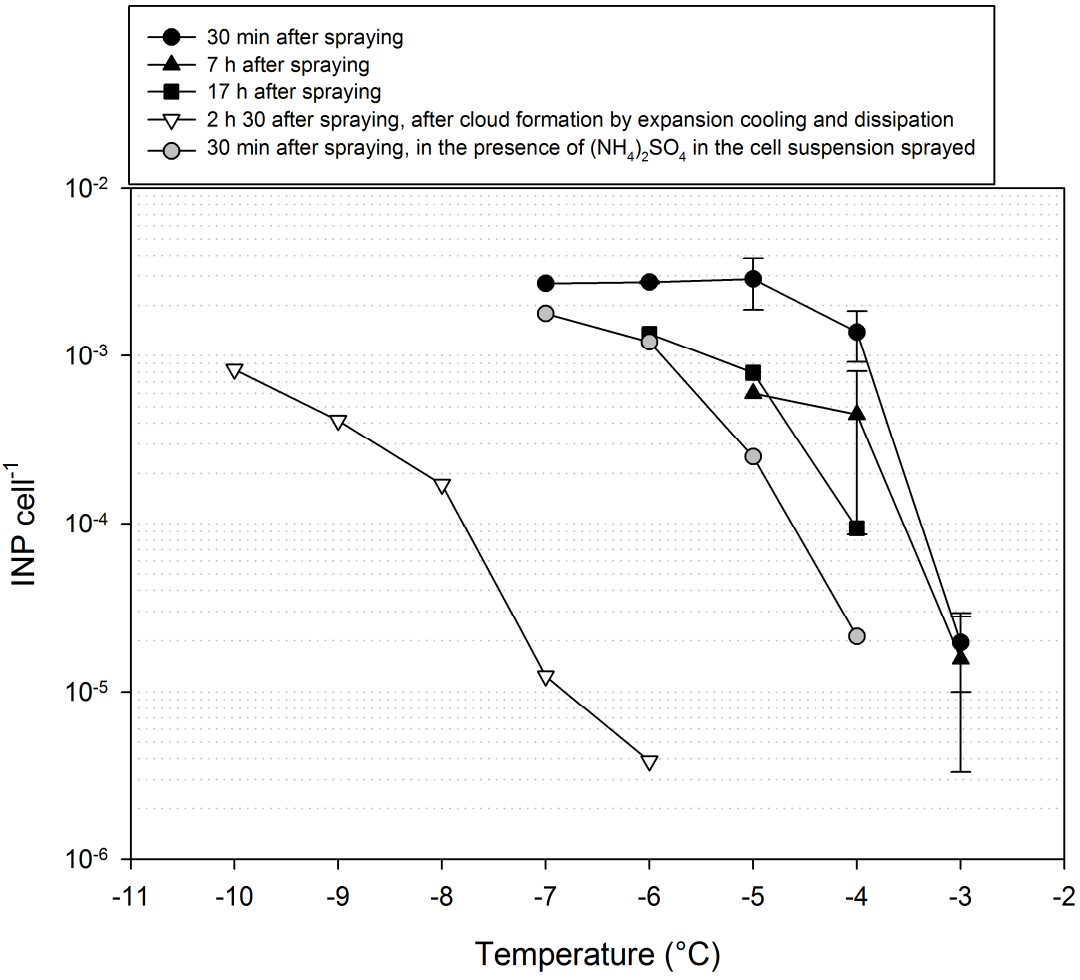
**Figure 4**


30 min after spraying
7 h after spraying
17 h after spraying
2 h 30 after spraying, after cloud formation by expansion cooling and dissipation
30 min after spraying, in the presence of $(NH_4)_2SO_4$ in the cell suspension sprayed
INP cell$^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
$10^{-5}$
$10^{-6}$
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
Temperature (°C)