



1 Ice nucleators have shorter persistence in the atmosphere than 2 other airborne bacteria

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9

10 **Abstract** Ice nucleation is a means by which the deposition of an airborne microorganism can be accelerated
11 under favourable meteorological conditions. Analysis of 56 snow samples collected at the high altitude
12 observatory Jungfraujoch (3580 m a.s.l.) revealed an order of magnitude larger dynamic range of ice nucleating
13 particles active at -8 °C (INPs_g) compared to the total number of bacterial cells (60 % was on average living).
14 This indicates a shorter atmospheric residence time for INPs_g. Furthermore, concentrations of INPs_g decreased
15 much faster, with an increasing fraction of water precipitated from the air mass prior to sampling, than the
16 number of total bacterial cells. Nevertheless, at high wind speeds (> 50 km h⁻¹) the ratio of INPs_g to total
17 bacterial cells largely remained in a range between 10⁻² to 10⁻³, independent of prior precipitation, perhaps
18 because of recent injections of particles in regions upwind. Based on our field observations, we conclude that ice
19 nucleators travel shorter legs of distance with the atmospheric water cycle than the majority of bacterial cells.
20 *Pseudomonas syringae*, a prominent ice nucleating bacterium, was successfully isolated from 3 of 13 samples
21 analysed. Colony forming units of this species constituted a minor fraction (10⁻⁴) of the numbers of INPs_g in
22 these samples. Overall, our findings expand the geographic range of habitats where this bacterium has been
23 found and corroborates theories on its robustness in the atmosphere and its propensity to spread and to colonise
24 new plants.

25 1 Introduction

26 The nucleation of ice in clouds is a process of primary relevance both for the radiative budget of clouds and for
27 the development of precipitation (Cantrell and Heymsfeld, 2005; Mülmenstädt et al., 2015; Murray et al., 2012).
28 Most ice nucleating particles (INPs) active at moderate supercooling in the atmosphere are of biological origin
29 (Murray et al., 2012). *Pseudomonas syringae* was the first organism found to produce an ice nucleation active
30 molecule (Maki et al., 1974) and to occur as an active biological INP in clouds (Sands et al., 1982). As it is also
31 a plant pathogen it received and continues to receive attention from biologists in the perspective of improving the
32 protection of crops from the spread of microbial diseases (Lamichhane et al., 2014; Lamichhane et al., 2015).
33 The combination of both roles, of an ice nucleator and of a plant epiphyte and pathogen, sparked the
34 bioprecipitation hypothesis (Morris et al., 2014; Sands et al., 1982). Part of the hypothesis is the idea that ice
35 nucleation activity contributes preferentially to the deposition of the organisms with this property helping them
36 to return to plant surfaces where they can proliferate (Morris et al., 2013). In fact, favouring the growth of ice
37 crystals could be a powerful and effective means for airborne microorganisms to reduce their residence time



aloft. Previous research has illustrated that bacterial strains capable of nucleating ice can deposit rapidly under laboratory simulated cloud conditions, corresponding to cold temperature regime and supersaturation (Amato et al., 2015). Modelling studies suggest that the atmospheric residence time of singularly airborne bacterial cells is highly reduced if such bacteria act as condensation and ice nuclei in clouds (Burrows et al., 2009). Furthermore, snowfall has been shown to enrich for the presence of ice nucleation active strains of *P. syringae* compared to a range of other environmental contexts including within clouds (Morris et al., 2008; Morris et al., 2013). Therefore, more direct evidence from field observations on the selective deposition of ice nucleation active microorganisms with precipitation is wanting.

Here we explore, through analysis of snow samples collected at a high altitude station, whether the capability to induce the formation and growth of ice crystals makes a discernible difference to the atmospheric residence time of INPs compared to the majority of bacterial cells, which are not INPs. We have sought to isolate the prominent ice nucleation active bacterium *P. syringae* at high altitude and compare its abundance with numbers of INPs. We have also carried out phylogenetic analysis in an attempt to identify potential sources of this bacterium in the atmosphere.

2 Materials and methods

2.1 Sampling, counting of bacteria and INPs

Fifty six snow samples were collected at the High Altitude Research Station Jungfraujoch (7°59'06'' E, 46°32'51'' N, 3580 m a.s.l., Switzerland) during 11 short sampling campaigns from March to September 2013 and May to October 2014. Samples were collected with a 0.1 m² Teflon-coated tin for periods of 1.5 to 8 hours depending on precipitation intensity. The tin was rinsed several times with 70 % ethanol and sterile Milli-Q water between samples to avoid cross-contamination. Snow samples were melted at room temperature (about 16 °C) and analysed immediately on site for concentration of INPs in a drop freeze assay with the LINDA device as described in Stopelli et al. (2014). The sample was adjusted to physiological conditions (9 ‰ NaCl) and two filters were prepared for later analysis of bacterial number concentration. For each filter, 20 mL of sample were passed through the active area (6 mm diameter) of a 0.22 µm black polycarbonate membrane (Whatman). The filter was rinsed with 3 mL of sterile phosphate-buffered saline (PBS) and the staining agents were added: 10 µL SYBR green (100x) for total cell count, and 10 µL SYBR green and 10 µL propidium iodide (in Milli-Q water, 1% wt/wt) to facilitate counting cells that are live (i.e with intact membranes) and dead or dying (with permeable membranes). After incubation in the dark for 10 min, the stains were filtered away and the filter column rinsed with 3 mL of sterile water. Once dry, the filters were put on glass slides, 15 µL of antifading agent (5 mL PBS, 5 mL glycerol, 10 mg p-phenyldiamine) was added for preservation in dark at -20 °C until analysis. Blanks were periodically prepared from the Milli-Q water used to rinse the tin. Blank counts were generally less than 10 % of sample counts. Filters were analysed with a fluorescence microscope (Leica DM2500) with a 100x ocular lens and an objective with 10x10 10 µm grids. Bacterial cells were recognised by shape and size. Each time, at least 10 fields and 300 cells were counted. In filters stained solely with SYBR green all cells were visible under blue light, while in filters treated with SYBR green and propidium iodide only living cells were counted.



2.2 Selective isolation and phylogenetic characterization of *Pseudomonas syringae*

Enough precipitation volume was available in 13 samples in a total of 15 samples collected in 2014 to study the presence of culturable *P. syringae*. The method employed for the selective isolation of *P. syringae* and its metabolic characterisation is described in Monteil et al. (2014) and in Morris et al. (2008). In addition to the above described procedure, the snow collecting tin was sterilised by dry heat (150 °C) for 20 min. Samples were concentrated 500 times by filtration of about 1 litre of melted snow across sterile 0.22 µm pore size polycarbonate filters and successive resuspension of the particles by stirring the surface of the filter into 2 mL of filtered precipitation water. The concentrated samples were dilution-plated on KBC, King's medium B supplemented with boric acid, cephalixin and cycloheximide, to isolate and calculate the abundance of *P. syringae*. Plates were incubated at 20–25 °C. Putative strains of *P. syringae* were purified on KB media (without the antibiotics and boric acid) where their production of fluorescent pigment could be observed under UV light (312 nm) and were then tested for the absence of arginine dihydrolase and for the absence of cytochrome C oxidase. Those that were negative for both the oxidase and arginine tests were suspended in sterile phosphate buffer and sent to the Plant Pathology Unit, INRA, Montfavet, France for the molecular identification using PCR primers specific for *P. syringae*: Psy-F: 5'-ATGATCGGAGCGGACAAG-3'; Psy-R: 5'-GCTCTTGAGGCAAGCACT-3' (Guilbaud et al., 2016). The strains confirmed to be *P. syringae* were also tested for their ice nucleating activity. After 3 days of growth on KB, suspensions of pure colonies corresponding to 10⁸ cells mL⁻¹ in 0.9 % NaCl were incubated 1 h in melting ice and subsequently tested for ice nucleation activity between 0 and -8 °C, with the freezing apparatus LINDA at 200 µL per tube (about 2·10⁷ cells in each tube). The capacity of strains of *P. syringae* to induce a hypersensitive response (HR), indicative of the presence of a functional type III secretion system used in pathogenicity by the strains, was determined on tobacco plants (*Nicotiana glauca*) by infiltrating bacterial suspensions of 48 h cultures at approximately 10⁸ cells mL⁻¹ into the leaves of the plant. After 24–48 h of incubation HR was revealed by the appearance of necrotic lesions at the site of infiltration.

Neighbour-joining phylogenetic trees of the strains of *P. syringae* isolated from Jungfraujoch were constructed on the basis of partial sequences of the citrate synthase housekeeping gene (*cts*) as previously described (Berge et al., 2014). Primers Cts-FP (forward): 5'-AGTTGATCATCGAGGGCGC(AT)GCC-3' and Cts-RP (reverse): 5'-TGATCGGTTTGATCTCGCACGG-3' were used for amplification and primer Cts-FS (forward): 5'-CCCGTCGAGCTGCCAAT(AT)TTGCTGA-3' for sequencing (Morris et al., 2010; Sarkar and Guttman, 2004). This analysis of partial *cts* gene sequences was performed as described previously using *P. syringae* reference strains (Berge et al., 2014), atmospheric strains (Amato et al., 2007; Joly et al., 2013; Vaitilingom et al., 2012) and the 24 strains from this study that were positive with the PCR specific for *P. syringae*. Alignment of sequences was made with DAMBE (version 5.6.8) and a Neighbour Joining tree was built with MEGA (version 5.05; Tamura et al., 2011).

2.3 Environmental parameters and statistics

The fraction of water vapour lost from an air mass prior to sampling was calculated from the relative abundance of ¹⁸O and ¹⁶O in precipitation, expressed as δ¹⁸O. More details on this calculation are provided in Stopelli et al, 2015. Wind speed was measured at Jungfraujoch by MeteoSwiss and data was stored as 10-minute averages.



1 Univariate and non-parametric statistics were carried out with PAST software version 2.17 (Hammer et al.,
2 2001).

3 **3 Results**

4 **3.1 Concentration variability of bacterial cells and INPs_g**

5 Concentrations of total bacterial cells in the 56 samples collected over the course of 18 months (excluding the
6 winter period between 2013 and 2014) were mostly between $3.0 \cdot 10^3$ (10th percentile) and $3.9 \cdot 10^4$ mL⁻¹ (90th
7 percentile), a range which is coherent with previous observations carried out on precipitation and cloud water
8 samples collected at several places around the world (Bauer et al., 2002; Joly et al., 2014; Šantl-Temkiv et al.,
9 2013; Sattler et al., 2001; Vaitilingom et al., 2012). Concentrations of INPs active at -8 °C or warmer (INPs_g)
10 (Fig. 1, data taken from Stopelli et al., 2016) presented a similar seasonal trend but with an order of magnitude
11 larger dynamic range (DR, i.e. the ratio between the largest and the smallest values) (10th percentile: 0.2 mL⁻¹;
12 90th percentile: 68.1 mL⁻¹).

13 The DR of atmospheric particles or molecules is largely determined by their atmospheric residence time: DR is
14 larger for species with shorter residence times (e.g. ²²²Rn) than for those with longer residence times (e.g. CO₂;
15 see for example Figure 2 in Griffiths et al., 2014). In fact, species with longer residence time have a higher
16 chance to mix and integrate more sources and over wider areas. Furthermore, a longer residence time leads to a
17 higher background concentration of a species in the atmosphere. Changing inputs due to changing source
18 strength, or changing losses due to changing sink strength, only make smaller differences to this larger pool.
19 Hence, the relatively small DR of bacterial cells compared to that of INPs_g suggests that bacterial cells have a
20 longer residence time in the atmosphere than INPs_g. This is likely to be due to the capacity of some bacteria and
21 other particles carrying a biological ice nucleation active fragment (O'Sullivan et al., 2015) to incite ice
22 formation and growth at -8 °C.

23 Surprisingly, the fraction of living cells among total bacterial cells was on average 0.6, with small standard
24 deviation (0.1), despite the harsh environmental conditions such as low temperature (sometimes down to -25 °C)
25 and intense solar radiation. The fraction of living cells was neither related to the fraction of water lost prior to
26 sampling nor to wind speed, and had no relation with the number of INPs_g. This finding does not exclude that
27 bacteria can constitute an important fraction of INPs_g. Rather, it suggests that i) the ice nucleating capability can
28 be retained beyond cellular death under atmospheric conditions (Amato et al., 2015; Möhler et al., 2007) and/or
29 ii) it can be linked not only to entire cells but also to cellular fragments or molecules released from cells
30 (O'Sullivan et al., 2016; Pummer et al., 2015).

31 In an earlier study we had already found evidence for INPs being more efficiently deposited from precipitating
32 clouds than the majority of particles larger than 0.5 µm (Stopelli et al., 2015). This evidence was based on the
33 comparison of INPs_g in precipitation with that of particles in air. In this work, by comparing number
34 concentrations of both INPs_g and bacteria in precipitation, we corroborate with field data the hypothesis that the
35 ability to foster the formation and growth of ice crystals increases the chance of an airborne particle to be
36 deposited. With an increasing fraction of water precipitated from air masses prior to arrival at the observatory,



1 the number concentration of INPs_g decreased much faster than that of bacteria (Fig. 2). Therefore, a more rapid
2 loss from the atmosphere with precipitation is one factor contributing to the greater dynamic range of INPs_g.
3 But, can we also find evidence for shorter time scales in the replenishment of their atmospheric concentrations?

4 3.2 The influence of wind speed on INPs_g and bacteria

5 Wind speed is an important factor associated with enhanced number concentrations of INPs_g in air masses at
6 Jungfraujoch (Stopelli et al., 2016). At high wind speeds ($> 50 \text{ km h}^{-1}$) numbers of INPs_g were 10^{-2} to 10^{-3} times
7 the number of bacterial cells in precipitation, independent of prior precipitation (Fig. 3, red symbols). At lower
8 wind speeds, the number of INPs_g decreased much more rapidly than the number of bacterial cells (blue
9 symbols). We interpret this observation in the following way. High wind speeds at Jungfraujoch could be
10 associated also with high wind speeds in source regions upwind (e.g.: Swiss Plateau, Po Valley) which promote
11 the aerosolisation of INPs_g and bacterial cells (Fig. 2; Lindemann and Upper, 1985). The atmospheric residence
12 time of total bacterial cells is longer than that of INPs_g. Therefore, their background number concentration is
13 relatively large and stable and it is not changed much by the additional cells aerosolised at high wind speeds in
14 the region. The shorter atmospheric lifetime of INPs_g means their background number concentration tends to be
15 smaller, relative to the emission strength of these particles. Consequently, numbers of INPs_g increase more
16 substantially above background values at high wind speed, than is the case for bacterial cells. Hence, the ratio of
17 INPs_g to bacterial cells remains large, even when a substantial fraction of water had already precipitated from
18 the air mass prior to sampling. At lower wind speeds, concentrations of INPs_g are not restored from regional
19 sources and we see more clearly the effect of a preferential washout of INPs_g relative to bacterial cells (blue
20 symbols in Fig. 3). The median ratio of INPs_g to bacterial cells we observed at Jungfraujoch was $6.6 \cdot 10^{-4}$, very
21 close to what Joly et al. (2014) had found over the course of a year in cloud water at Puy de Dôme ($5.5 \cdot 10^{-4}$).

22 3.3 Abundance and diversity of *P. syringae*

23 *Pseudomonas syringae* was successfully detected in three of the 13 samples analysed for the presence of this
24 bacterium. These samples had over 1000 bacterial cells mL^{-1} and more than 10 INPs_g mL^{-1} and were from
25 clouds that had precipitated less than 70% of their water vapor prior to sampling (Fig. 2). Two-thirds of the
26 strains (16/24), after culture in the laboratory, produced at least 1 cell in a suspension of $2 \cdot 10^7$ cells that was ice
27 nucleation active at temperatures warmer than $-8 \text{ }^{\circ}\text{C}$. The freezing onset temperature of all ice nucleation active
28 strains was warmer than $-5 \text{ }^{\circ}\text{C}$ and was $-2.1 \text{ }^{\circ}\text{C}$ for the most active strain (Table 1). The precipitation samples
29 from which these strains were isolated were characterised by a relatively warmer onset of freezing (median onset
30 temperature of $-5.0 \text{ }^{\circ}\text{C}$ for samples with *P. syringae* vs. $-6.8 \text{ }^{\circ}\text{C}$ for those without *P. syringae*, $p = 0.02$, Mann-
31 Whitney test). Although *P. syringae* was found in the samples with the largest numbers of INP_g and with the
32 warmest onset freezing temperature, only 2, 4, and 45 colony forming units (i.e. culturable cells) of *P. syringae*
33 were present per litre of sample. This corresponds to 2 orders of magnitude less than what has been found in
34 snowfall at lower altitudes (Monteil et al., 2014). We suspect that this can be due both to the preferential removal
35 of *P. syringae* with precipitation as soon as it develops at lower altitudes and to the larger distance of
36 Jungfraujoch from the sources where *P. syringae* entered into air masses. This possibly leads to longer exposure
37 of *P. syringae* to UV radiation and desiccation, reducing its culturability when collected at higher altitude. The



1 largest number concentration of colony forming units of *P. syringae* we found in snow water was 10^{-4} times the
 2 number of INPs_g in the same sample.

3 This is the first time this bacterium has been isolated at such altitudes (3580 m a.s.l.), and therefore this result
 4 expands the established limits for *P. syringae*'s dissemination and survival under atmospheric conditions.
 5 Sequencing of the *cts* gene for phylogenetic analyses was conducted for all 24 strains of *P. syringae* from culture
 6 plates from the precipitation samples (Table 1) to obtain insight into the possible origin of these strains. For all
 7 dates where *P. syringae* was isolated, the strains in each precipitation event were genetically diverse and
 8 represented a broad range of known phylogenetic groups (Table 1, Supplemental Fig. 1). This high diversity
 9 suggests that the process of entry of *P. syringae* into air masses moving up to Jungfraujoch involves either
 10 multiple events from a wide range of sources along the trajectory or a few entry events from common sources
 11 that harbor a high diversity of *P. syringae* that can readily be wafted into the air. Leaf litter, for instance, is one
 12 substrate that could have been a source of a high diversity of *P. syringae*, since it contains a high density and
 13 high genetic diversity of *P. syringae*, many of which are ice nucleation active, no matter the geographic origin
 14 and trajectory of the air masses (Berge et al., 2014; Monteil et al., 2012).

15 All but 3 of the 24 strains had functional type III secretion systems (hypersensitivity test). This suggests that they
 16 had potential to cause plant disease on some crops and illustrates the potential extent of spread of diseases
 17 caused by this pathogen.

18 4 Conclusions

19 Based on observations of INPs_g and bacterial cells in 56 precipitation samples collected at Jungfraujoch we
 20 have shown that ice nucleation activity at -8 °C and warmer temperatures contributes to shorter atmospheric
 21 residence times due to a greater probability for wet deposition. For bacterial cells that are disseminated via the
 22 atmosphere, this property is advantageous because, by enhancing deposition, the bacteria reduce the time of
 23 exposure of their cells to hostile conditions such as UV radiation, extreme cold temperatures and to drying. The
 24 preferential removal of INPs with precipitation can be delayed by high wind speed, which promotes the
 25 continuous uptake, mixing and transport of INPs over longer distances. Over half of the bacterial cells in
 26 precipitation that fell on Jungfraujoch were viable and among those *P. syringae* could be cultured from 3 of 13
 27 samples. But their concentration in precipitation was less than 50 culturable cells per litre of snow meltwater,
 28 about 10^4 times less than the concentration of INPs_g. Therefore, culturable *P. syringae* appeared as minor
 29 component of the ensemble of INPs_g collected in precipitation at Jungfraujoch. Nevertheless, the presence in
 30 snowfall on Jungfraujoch of a bacterium such as *P. syringae* sheds new light on the possibilities for this
 31 bacterium to survive journeys through the atmosphere and colonise new plants. At the same time, it opens
 32 exciting research perspectives. For *P. syringae* there are a wide array of techniques for the characterization of its
 33 phenotypic and genotypic variability and banks of strains and genomic data related to habitats and geographic
 34 origin. This makes *P. syringae* a powerful model for attempting to identify specific sources of INPs in
 35 precipitating air masses and for better defining the extent of their trajectories.

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1 5 Data availability

2 The data set for this paper is publicly available as Table in the Supplement.

3 6 Author contributions

4 Emiliano Stopelli carried out the field measurements at Jungfraujoch on the abundance of INPs, bacterial cells
5 and on the isolation of *P. syringae*, and together with Franz Conen analysed the data. Caroline Guilbaud did the
6 provided fundamental support in the isolation of *P. syringae* and did the phylogenetic analysis on the isolated
7 strains. Jakob Zopfi provided great help with the set up of epifluorescence microscopy technique. Christine
8 Alewell and Cindy E. Morris provided strong conceptual frameworks. Emiliano Stopelli wrote the manuscript
9 together with Franz Conen, with important contributions from all other co-authors.

10 The authors declare that they have no conflict of interest.

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1 **Table 1.** Diversity of strains of *Pseudomonas syringae* from fresh snow samples collected at Jungfrauoch.

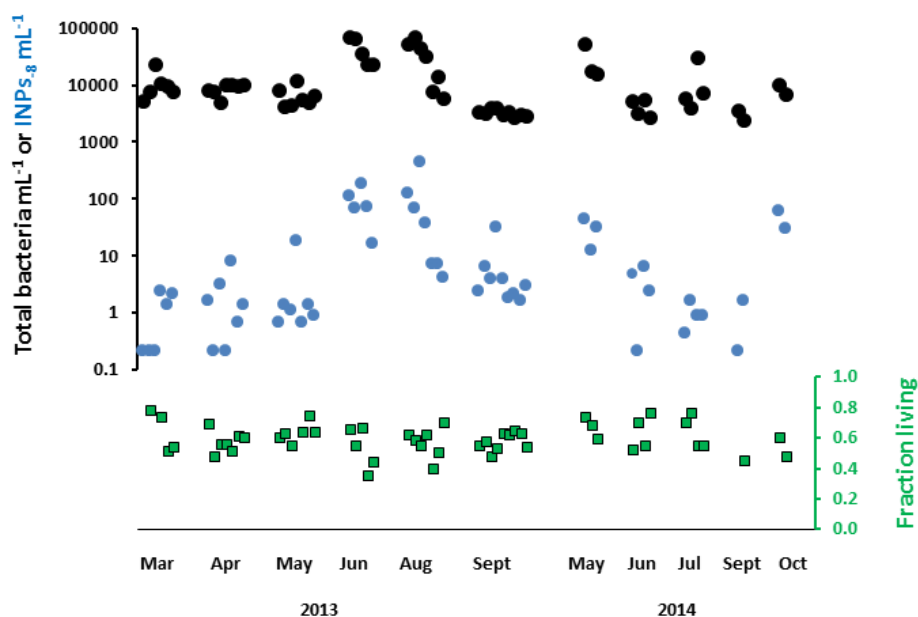
Strain	Date	Phylogroup ¹	INA ²	HR ³
JFJ-0007	22 May 2014	2	-4.2	+
JFJ-0008	22-23 May 2014	3	-	-
JFJ-0011	22-23 May 2014	5	-3.7	+
JFJ-0039	21-22 Oct 2014	1	-	+
JFJ-0045	21-22 Oct 2014	1	-	+
JFJ-0048	21-22 Oct 2014	1	-	+
JFJ-0061	21-22 Oct 2014	1	-	+
JFJ-0043	21-22 Oct 2014	2	-2.6	+
JFJ-0047	21-22 Oct 2014	2	-	-
JFJ-0050	21-22 Oct 2014	2	-3.6	+
JFJ-0052	21-22 Oct 2014	2	-2.7	+
JFJ-0054	21-22 Oct 2014	2	-2.2	+
JFJ-0055	21-22 Oct 2014	2	-2.2	+
JFJ-0058	21-22 Oct 2014	4	-	+
JFJ-0056	21-22 Oct 2014	7	-2.9	+
JFJ-0059	21-22 Oct 2014	7	-4.9	+
JFJ-0040	21-22 Oct 2014	10	-4.2	+
JFJ-0044	21-22 Oct 2014	10	-2.1	+
JFJ-0049	21-22 Oct 2014	10	-2.8	+
JFJ-0051	21-22 Oct 2014	10	-2.9	+
JFJ-0053	21-22 Oct 2014	10	-4.0	+
JFJ-0060	21-22 Oct 2014	10	-2.8	+
JFJ-0046	21-22 Oct 2014	13	-	-
JFJ-0057	21-22 Oct 2014	13	-	+

2
 3 ¹Full details of the phylogenetic situation of these strains compared to a range of reference strains is presented in
 4 the Supplemental Figure 1.

5 ²INA refers to ice nucleation activity of suspensions of $2 \cdot 10^7$ cells. The reported values are the freezing onset
 6 temperature.

7 ³Capacity to induce a hypersensitive reaction (HR) in tobacco indicative of the presence of a functional type III
 8 secretion system that is one of the fundamental traits usually required for pathogenicity of *P. syringae* to plants.

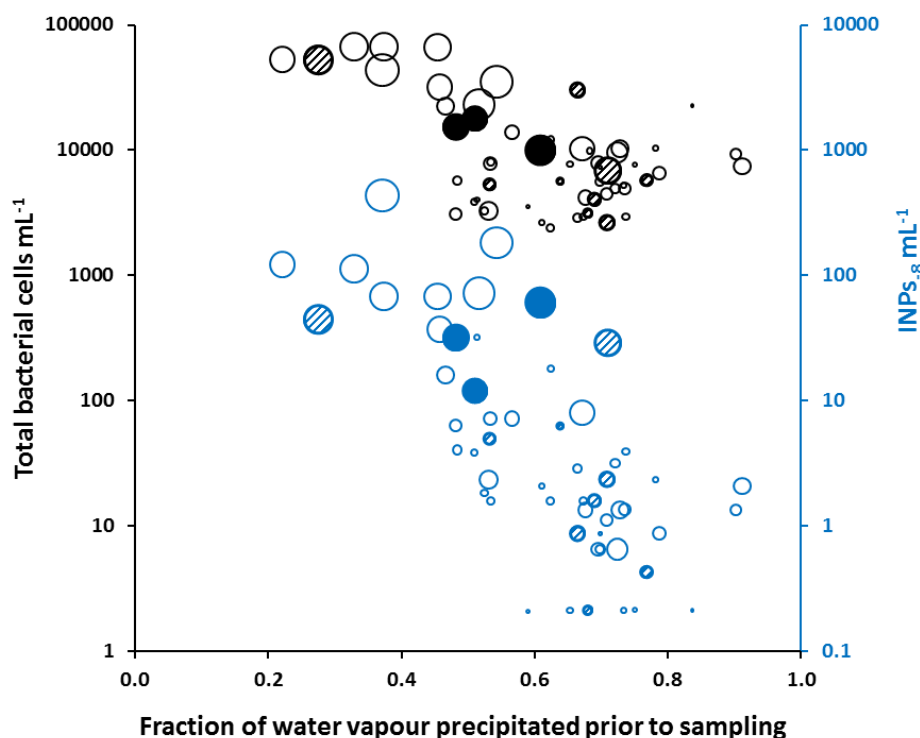
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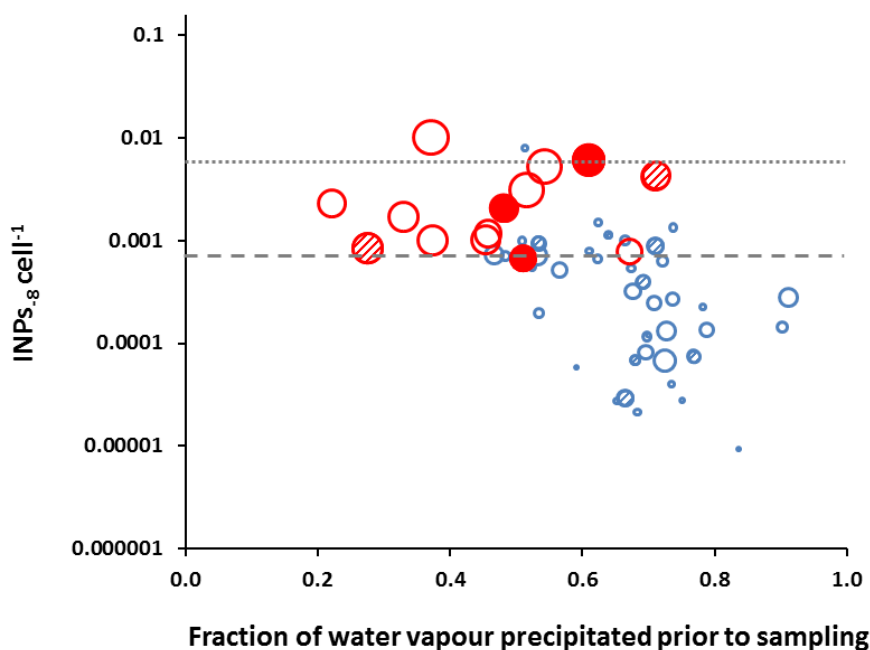
2 **Figure 1.** Number of total bacterial cells (black dots), fraction of living cells (green squares) and ice nucleating
3 particles active at -8 °C (INPs₋₈, blue, from Stopelli et al., 2016) in precipitation samples collected at the high
4 altitude research station Jungfraujoch (3580 m a.s.l.) during 11 sampling campaigns between March 2013 and
5 October 2014.

6



1
 2 **Figure 2.** Number of bacterial cells (black) and INPs_g (blue, from Stopelli et al., 2016) versus the fraction of
 3 precipitation lost from the air mass prior to arrival and sampling at the observatory. The width of the symbols is
 4 proportional to wind speed (minimum = 2 km h⁻¹, maximum = 89 km h⁻¹). Patterned symbols represent the 10
 5 fresh snow samples in which *P. syringae* was searched for, but not found. The three full symbols represent the 3
 6 samples where culturable *P. syringae* was found.

7



1

2 **Figure 3.** Ratio of INPs₈ to bacterial cells in precipitation samples collected at Jungfraujoch (3580 m a.s.l.). The
 3 width of symbols is proportional to wind speed (minimum = 2 km h⁻¹, maximum = 89 km h⁻¹). Red symbols
 4 correspond to values where average wind speeds > 50 km h⁻¹ during samplings and blue symbols correspond to
 5 data for lower wind speeds. Patterned symbols represent the 10 samples in which *P. syringae* was searched for,
 6 but not found. The three full symbols represent samples where culturable *P. syringae* was found. Dashed and
 7 dotted lines indicate median and maximum ratios observed in cloud water collected over the course of a year at
 8 Puy de Dôme (1465 m a.s.l.), 350 km west of Jungfraujoch (data from Joly et al., 2014).

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