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Effects of atmospheric conditions on ice nucleation activity of *Pseudomonas*

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Abstract. Although ice nuclei from bacterial origin are known to be efficient at the highest temperatures known for ice catalysts, quantitative data are still needed to assess their role in cloud processes. Here we studied the effects of three typical cloud conditions (i) acidic pH (ii) NO_2 and O_3 exposure and (iii) UV-A exposure on the ice nucleation activity (INA) of four *Pseudomonas* strains. Three of the *Pseudomonas syringae* strains were isolated from cloud water and the phyllosphere and *Pseudomonas fluorescens* strain CGina-01 was isolated from Antarctic glacier ice melt. Among the three conditions tested, acidic pH caused the most significant effects on INA likely due to denaturation of the ice nucleation protein complex. Exposure to NO_2 and O_3 gases had no significant or only weak effects on the INA of two *P. syringae* strains whereas the INA of *P. fluorescens* CGina-01 was significantly affected. The INA of the third *P. syringae* strain showed variable responses to NO_2 and O_3 exposure. These differences in the INA of different *Pseudomonas* suggest that the response to atmospheric conditions could be strain-specific. After UV-A exposure, a substantial loss of viability of all four strains was observed whereas their INA decreased only slightly. This corroborates the notion that under certain conditions dead bacterial cells can maintain their INA. Overall, the negative effects of the three environmental factors on INA were more significant at the warmer temperatures. Our results suggest that in clouds where temperatures are near 0 °C, the importance of bacterial ice nucleation in precipitation processes could be reduced by some environmental factors.

1 Introduction

In clouds, ice formation at temperatures above about −38 °C occurs via heterogeneous ice nucleation initiated by ice nuclei (IN). Without these IN, water droplets in clouds would remain liquid until −38 °C, the temperature of spontaneous freezing initiated by homogeneous ice nucleation. A large diversity of particles act as IN in the atmosphere: mineral dust, metallic particles, soot, and biological particles (e.g. DeMott et al., 2003; Möhler et al., 2007; Andreae and Rosenfeld, 2008). Among the biological particles, some bacteria are known as very efficient IN and notably some strains of *Pseudomonas syringae* which initiate freezing at temperatures as high as −2 °C (Orser et al., 1985). The ice nucleation activity (INA) of bacteria is due to a specific protein complex on the bacterial outer membrane that permits efficient arrangement of water molecules into ice embryos. This arrangement is orchestrated by the INA protein which is composed of a repeated octapeptide (Gurian-Sherman and Lindow, 1993). Aggregation of several ice nucleation-active protein modules leads to the formation of a protein complex whose size determines the level of INA: the larger the protein complex the higher the sub-zero temperature at which freezing is induced (Kozloff et al., 1991a; Turner et al., 1990). INA depends on the protein complex being held intact in the outer membrane of the bacteria. This condition is generally met by living cells, but dead cells can also have a high level of INA. This is the case for the commercial adjuvant for production

of artificial snow, Snomax®, a preparation of freeze-dried irradiated cells of *P. syringae*.

Ice nucleation active particles of biological origin have been detected in numerous environments (Bowers et al., 2009; Christner et al., 2008a; Christner et al., 2008b). INA bacteria have also been isolated from a range of substrates including plants (Gross et al., 1983; Kaneda, 1986), aerosols, precipitation (Lindemann et al., 1982; Morris et al., 2008) and from fresh water (Morris et al., 2008; Morris et al., 2010). Microbial species known to be ice nucleation active have been isolated from clouds (Sands et al., 1982; Amato et al., 2006) and their ice nucleation activity was reported in one case (Sands et al., 1982).

In clouds, there are particular environmental conditions such as light intensity and chemistry that could affect bacterial INA. However, quantitative data on such effects are lacking even though they could have considerable consequences for estimations of the impact of biological ice nucleation on cloud processes. Most of the data available on the effects of environmental conditions on bacterial INA are focused on (i) determining ice nucleation protein characteristics and structure (e.g. Turner et al., 1990; Kozloff et al., 1991a, b), (ii) methods to increase ice nucleation active biomass and efficiency for industrial applications in the food industry and in snow making (e.g. Nemecek-Marshall et al., 1993; Blondeaux et al., 1999) and (iii) limiting frost damage to plants (Anderson and Ashworth, 1986; Gurian-Sherman and Lindow, 1993; Kawahara et al., 2000). To our knowledge only the study by O'Brien and Lindow (1988) deviates from this tendency by analyzing the response of bacterial INA submitted to environmental conditions of the natural habitats of these bacteria. These authors observed that INA is sensitive to factors encountered on the plant surface and in particular those related to host plant genotype, light intensity and ambient humidity.

For non-biological ice nucleation particles, ample data are available about the influence of environmental conditions on their activity. For example, several studies have focused on the effects of sulfuric acid coating (Archuleta et al., 2005; Knopf and Koop, 2006; Eastwood et al., 2009), ammonium sulfate coating (Eastwood et al., 2009), organic coating (Kanji et al., 2008; Möhler et al., 2008), and ammonia gas (Salam et al., 2007) on the ice nucleation properties of particles. In certain studies (Kanji et al., 2008; Möhler et al., 2008) organic coating was found to deactivate ice nucleation, whereby a lower temperature is required to initiate ice formation. On the other hand, no common tendency was observed for other coatings and it was shown that effects of sulfuric acid coating depended on the IN material studied (Archuleta et al., 2005; Knopf and Koop, 2006; Eastwood et al., 2009). Therefore, our study aimed at evaluating the effect of key conditions in clouds on bacterial INA. We examined the effects of the pH of cloud water, UV light and reactive gas exposure on the INA of bacteria isolated from various environments including clouds.

To evaluate the influence of pH on INA, we took into consideration the range of pH observed in cloud water. At the summit of the Puy de Dôme, for example, clouds of oceanic origin have a less acidic pH (pH 5.0 to 6.5) than clouds from the north that are influenced by anthropogenic activities (pH 3.0 to 4.5) (database available at http://wwwobs.univ-bpclermont.fr/SO/beam/data.php; Marinoni et al., 2004). We hypothesized that acidification of cloud water can decrease the efficiency of bacterial INA probably via the denaturation of the tertiary structure of the ice nucleation protein and/or by an impact on bacterial metabolism.

To evaluate the role of important atmospheric chemical reactions on INA, bacterial strains were exposed to reactive gases. In heavily polluted urban areas there are high levels of NO_2 and O_3 in the atmosphere. It is known now that proteins can be subjected to oxidation and nitration when exposed to the gas mixtures of NO_2, O_3 and water vapor (Franze et al., 2005; Shiraiwa et al., 2011; Shiraiwa et al., 2012). In particular, tyrosine, an aromatic amino acid residue in proteins, can be efficiently nitrated (Walcher et al., 2003; Yang et al., 2010; Zhang et al., 2011). This amino acid residue is part of the water binding site of the ice nucleation protein conferring the tertiary structure with β-sheet folding at the glycine-tyrosine-glycine site (Gazit et al., 2002). In this way the nitration reaction may change the structure of this amino acid that is involved in the tertiary structure of the protein thereby leading to changes in its water-binding capacity and hence its INA efficiency. We exposed bacteria to synthetic gas mixtures of NO_2 and O_3 at relevant atmospheric concentrations and examined the impact on INA.

To evaluate the effect of UV light, bacterial strains were exposed to UV-A, the most important source of photons in clouds which can cause intense oxidative damage to a range of molecules in bacterial cells (Pourzand and Tyrrell, 1999). We hypothesized that UV-A could affect INA directly via denaturation of the ice nucleation protein exposed on the outer cell membrane or indirectly due to cell death.

2 Materials and methods

2.1 Bacterial strains

Bacterial strains used in this study originated from three different environments. Strains 13b-2 and 32b-74 of *P. syringae* were isolated from cloud water collected at the Puy de Dôme summit (France) in July 2004 and November 2009, respectively. Sampling conditions and isolation methods have been described by Amato et al. (2005) and Vaïtinlingom et al. (2012). The 16S rRNA partial gene sequences of strains 13b-2 and 32b-74 have been submitted to the GenBank database (accession numbers DQ512785 and HQ256872). Strain *P. syringae* CC0242 was originally isolated from diseased cantaloupe leaves as described for closely related strains from the same origin (Morris et al., 2000). Strain

CGina-01of *P. fluorescens* was isolated from ice melt, provided by C. Foreman (Montana State University), from Cotton Glacier in Antarctica. Snomax® is a commercial preparation containing a freeze-dried and irradiated preparation of a strain of *P. syringae* (Johnson Controls Inc, Milwaukee, USA). Before ice nucleation assays (described below), bacterial strains were cultured for 3 days at 17 °C on plates of King's medium B (KB) (King et al., 1954). Bacterial suspensions were prepared with the newly cultivated bacteria in sterile distilled water to obtain 5×10^8–5×10^9 cells mL^{-1}. The suspensions were incubated at 4 °C for at least 1 hour to allow full expression of the ice nucleation protein.

2.2 Bacterial ice nucleation activity

Bacterial INA was measured by an immersion freezing test (Vali, 1971). Thirty-two droplets of 20 µl of the initial suspension and then 32 droplets of 10 fold serial dilutions of the suspension (from 5×10^8 to 5×10^3 cells mL^{-1}) were distributed on a sterile aluminum plate and floated on a cooling bath (model F34-ED Julabo, Colmar, France). This serial dilution was performed to allow the calculations described below such that for at least one dilution series, some drops (at least 1) remained unfrozen.

The initial temperature was −2 °C and then reduced at 1 °C intervals until −10 °C. At each temperature step, the droplets were incubated for 8 min and at the end of each incubation step the number of frozen droplets was counted visually. The cumulative ice nuclei concentration per bacterial cell at each temperature was calculated according to the method described by Vali (1971) with the following equation: $c(T) = [\ln(N^0) - \ln(N^0 - N_x(T))]/A$ where $c(T)$ is the number of ice nuclei per bacterial cell active at temperature T, N^0 is the number of droplets tested, N_x is the number of frozen droplets at a given temperature T, and A is the number of bacteria per drop.

Therefore, for each bacterial strain, the level of the INA was determined by (i) the number of ice nuclei per cell and (ii) the highest temperature at which freezing was first observed.

To allow comparison of the INA of bacteria and the mineral compound montmorillonite, we calculated the ice nuclei concentration per surface area, assuming a bacterial surface of 5 µm^2 (considering bacteria as a cylinder of 1 µm in length and 0.5 µm of radius), and with 30 m^2 g^{-1} for montmorillonite (Mortazavi et al., 2008). We chose to present the comparison between bacteria and this mineral IN because the publication of Mortazavi et al. (2008) is, to our knowledge, the only study which has applied the immersion freezing assays to mineral compounds.
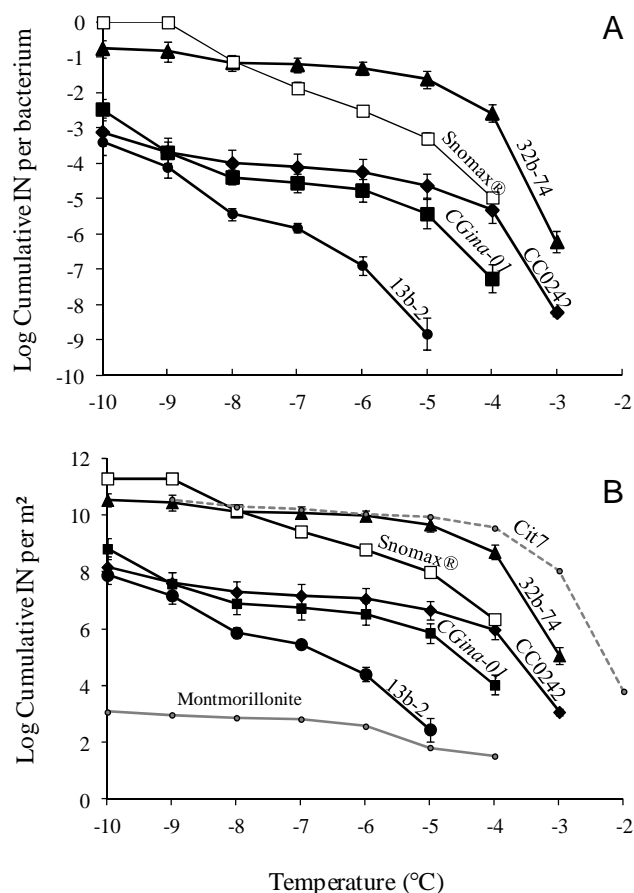
Fig. 1. Ice nucleation spectra of *Pseudomonas* strains in distilled water: *P. syringae* 32b-74 ▲ and 13b-2 ●, *P. syringae* CC0242 ♦, *P. fluorescens* CGina-01 ■, the commercial Snomax® □, *P. syringae* Cit7 (grey dotted line; data from Orser et al., 1985) and Montmorillonite (grey solid line, data from Mortazavi et al., 2008). A: log (cumulative IN per bacterium); B: log (cumulative IN per m^2). Errors bars indicate standard errors ($n = 6$).

Six replicates were used to calculate the ice nucleation spectra presented in Figs. 1 and 4 replicates for all the experiments concerning pH, NO$_2$/O$_3$, and UV exposure effects.

2.3 Exposure to acidic pH

For these experiments, instead of serially diluting in distilled water, the initial bacterial suspension was serially diluted in one of two acetate buffers. The buffer at pH 5.9 was prepared with 93 mL of sodium acetate at 0.2 M and 7 mL of acetic acid at 0.2 M. The buffer at pH 4.1 was prepared with 18 mL of sodium acetate at 0.2 M and 82 mL of acetic acid at 0.2 M. Since the original two buffers had different sodium cation concentration, NaCl was added to the pH 4.1 buffer in order to achieve about the same sodium ion concentration in both buffers.

At room temperature acetic acid is liquid and dissolves in water at all concentrations. The acetic acid/H$_2$O system

shows a eutectic temperature (ice/acetic acid) of approximately $-26.6\,°C$ at which point the solubility of acetic acid is about 59 wt %, corresponding to a molality of about 24 mol kg^{-1} (Dahms, 1896). For comparison, the maximum concentration of acetic acid in buffer 2 was only about 0.15 mol kg^{-1}. The solubility limit of aqueous sodium acetate solutions is actually determined by the solubility curve of its trihydrate (NaAc·3H$_2$O). At room temperature the solubility is about 5.7 mol kg^{-1} and at the eutectic temperature of $-18\,°C$ it is 3.7 mol kg^{-1} (Green, 1908). In contrast, the maximum concentration of sodium acetate in buffer 1 was only about 0.17 mol kg^{-1}. These calculations show that neither sodium acetate nor acetic acid could have precipitated from the buffer solutions in our experiments upon cooling. We also tested the freezing of buffer – without bacteria – adjusted to pH 4.1 and 5.9. For each pH, 40 drops were tested for freezing down to $-10\,°C$ under the same conditions used to determine the INA of bacterial suspensions. No drops froze under these conditions.

It is well established that solutes affect both homogeneous and heterogeneous ice nucleation through a reduction in the aqueous solution's water activity (Koop et al., 2000; Zobrist et al., 2008). This "colligative" effect on heterogeneous ice nucleation is different from that due to chemical, morphological, or other changes in the IN surface (e.g. the protein complex) induced by the presence of solutes. In order to discriminate between changes in the intrinsic IN properties and colligative effects, we have corrected the data for the colligative effect using a published protocol for estimating its magnitude (Koop and Zobrist, 2009).

First, we calculated the concentration of all ionic and non-ionic solute species introduced by adding the buffer stock solutions described above, also considering the relevant dissociation equilibrium of HAc (Ks = 1.7540×10^{-5}). Because the total molality of all solute species was less than 0.5 mol kg^{-1} in both cases, we assumed ideal behaviour resulting in a_w values of 0.99378 and 0.99160 for the pH 5.9 and pH 4.1/NaCl buffers, respectively. The observed freezing behaviour at these water activities was then corrected for the presence of solutes to yield the predicted freezing behaviour in pure water ($a_w = 1$), see Supplement A for details. For example, the cumulative number of IN per bacterium in a pH 5.9 buffer measured at $-3\,°C$ corresponds to the same cumulative number of IN per bacterium in water at $-2.34\,°C$. All corrections were smaller than $1\,°C$. The difference between the measured cumulative number of IN per bacterium in droplets with buffer corrected for solute effects and that measured separately in pure water without buffer is then attributed to the effect of pH owing to changes induced in the ice nucleating protein complex at the different pH.

2.4 Exposure to NO$_2$/O$_3$

An aliquot of 110 μL of the prepared bacterial suspension was introduced into a sterile syringe filter (cellulose acetate membrane, pore size 1.2 μm, 30 mm diameter, sterile, Whatman GmbH, Dassel, Germany). For each exposure experiment, 6 filters were prepared. They were then dried by a gentle stream of high purity N$_2$ for ~1–2 h. Two filters were kept in a sealed zip-lock bag without exposure to any synthetic gases as control samples to check the effect of exposure to gases with or without NO$_2$ and O$_3$. The other 4 filters were exposed to synthetic gas mixtures overnight (16–18 h). A schematic diagram of the experimental exposure setup is shown in Supplement B. The exposure setup was the same as described in Yang et al. (2010) except that a gas filter system was added upstream of two parallel syringe filters to obtain a synthetic gas mixture free of O$_3$ and NO$_2$. So for each exposure experiment we had 6 samples. Two were control samples without exposure to any synthetic gas. Two of them were exposed to gas mixtures of 108 ± 2.7 ppb (average value $\pm$ standard deviation) NO$_2$ and 110 ± 11.3 ppb O$_3$ at the relative humidity (RH) of ~50 %, the other two were exposed to filtered synthetic gas mixtures (major compositions: O$_2$ and N$_2$) without O$_3$ and NO$_2$ at a slightly reduced RH. After exposure, the filters were immediately washed-off with 1.1 mL of sterile high purity water. The suspension obtained was subsequently tested for its ice nucleation activity. To an aliquot of 100 μL of the remaining suspension, 5 μL of a 37 % (v/v) formaldehyde solution was added to fix bacterial cells for counting by flow cytometry.

2.5 UV exposure

An aliquot of 30 mL of the prepared bacterial suspension was placed in a crystallizer ($d = 15$ cm, $h = 10$ cm) with a Pyrex glass lid (thickness 3.3 mm, cut-off wavelength: 290 nm, transparent to UV-A). The crystallizer was surrounded by a plastic circle with 10 aeration tubes to avoid water vapour condensation. In the control experiment, the crystallizer was entirely covered with aluminum foil to avoid UV-A light. Both of the crystallizers were incubated at $17\,°C$, with gentle shaking (50 rpm) and with UV-A light mimicking the solar spectrum in the atmosphere (i.e. mainly UV-A from 315 to 400 nm, 33 J s^{-1} m^{-2}, with the lamp and solar spectra as described in Vaïtilingom et al., 2011). An aliquot of 1 mL of the incubated bacterial suspension was taken at 0 h, 4 h, 20 h and 42 h and the ice nucleation activity was measured.

2.6 Statistical analyses

One-way ANOVA and Tukey's tests were performed with the PAST software (Hammer et al., 2001) to determine if there were significant differences in the ice nucleation results obtained from different treatments.

3 Results

3.1 Bacterial ice nucleation activity

The bacterial strains collected from cloud water at the Puy de Dôme summit, *P. syringae* 13b-2 and 32b-74 showed marked INA. Both were collected from clouds originating from the French west coast with pH values of 5.2 and 5.5, respectively. Under our experimental conditions *P. syringae* 32b-74 initiated freezing at $-3\,°C$ and produced $10^{-0.75}$ IN per bacterium, i.e. 1 active ice nucleus per 5.7 cells at the coldest temperature tested, $-10\,°C$ (Fig. 1a). Strain 13b-2, initiated freezing at $-5\,°C$ and produced two orders of magnitude fewer ice nuclei than strain 32b-74 at $-10\,°C$ ($10^{-3.4}$ IN per bacterium, i.e. 1 active nucleus per 2.5×10^3 cells at $-10\,°C$) (Fig. 1a). Strain CC0242 of *P. syringae* isolated from the phyllosphere, strain CGina-01 of *P. fluorescens* from glacial melt and the Snomax® commercial product had freezing profiles intermediate to strains 32b-74 and 13b-2. Except for strain 32-b74, all these strains had less activity than that of strain Cit7 of *P. syringae* under the conditions used by Orser et al. (1985) (Fig. 1b). However, all of these strains had several orders of magnitude higher ice nucleation activity on a surface area basis than mineral dust e.g. montmorillonite at the temperature range between -3 and $-10\,°C$ (Fig. 1b, based on previously reported ice nucleation spectra of montmorillonite by Mortazavi et al., 2008). This mineral dust had $10^{3.1}$ ice nuclei per m^2 surface area active at $-10\,°C$ whereas strains 32b-74 and 13b-2 had $10^{10.4}$ and $10^{7.8}$ ice nuclei per m^2 surface area of cell, respectively (Fig. 1b).

3.2 Effects of acidic pH

Composition of pH buffers led to a reduction in ice nucleation temperature of around $0.7\,°C$ for the pH 5.9 buffer and of around 0.9–$1.0\,°C$ for the pH 4.1 buffer (Supplement C). The effect was slightly temperature dependent (i.e. a larger effect at a lower temperature) (Supplement C). Taking into account water activity corrections, the acidic pH 4.1 strongly reduced the IN number compared to pH 5.9 for the temperature range tested for strains 32b-74, 13b-2, CGina-01 and for Snomax® (Fig. 2). For *P. syringae* CC0242, IN number at pH 4.1 compared to pH 5.9 was not significantly different. Compared to pH 5.9, the temperature at which freezing began was decreased at pH 4.1 for all strains except *P. syringae* 32b-74 (Fig. 2).

Compared to distilled water, pH 5.9 led to a decrease in the IN number for *P. syringae* 32b-74, *P. fluorescens* CGina-01 at the highest temperatures, and for all temperatures for *P. syringae* CC0242 (Fig. 2). Compared to distilled water, pH 5.9 led to an increase in the IN number for Snomax® (Fig. 2).

3.3 Effects of NO$_2$/O$_3$ exposure

The number of viable cells of all strains was significantly reduced by gas exposure. Taking into account that dead cells could maintain an efficient ice nucleation protein, INA was calculated by dividing the number of IN by the number of viable cells counted at the beginning of the experiment. In order to check if cells were degraded by gas exposure, the total cell number was counted by flow cytometry. This confirmed that the total cell number of all strains was not significantly affected by gas exposure (data not shown).

In order to evaluate the effect of the exposure systems, comparisons of INA were made between bacteria exposed to synthetic air without NO$_2$/O$_3$ and bacteria not introduced in the exposure system. After exposure to synthetic air without NO$_2$ and O$_3$, the INA of strains 32b-74 and 13b-2 was not significantly different from that of bacteria (at the same concentration) not introduced into the exposure system (data not shown). For strains CC0242 and CGina-01, after exposure to synthetic air without NO$_2$ and O$_3$, the initial temperature of freezing decreased by $1\,°C$ and $2\,°C$, respectively (data not shown).

In order to evaluate the effect of NO$_2$/O$_3$ exposure, comparisons of INA were made between bacteria exposed to synthetic gas mixtures with and without NO$_2$/O$_3$. Exposure to NO$_2$/O$_3$ did not have a significant effect on the INA of strain 13b-2 (Fig. 3). For strain 32b-74, NO$_2$/O$_3$ exposure caused a decrease of $1\,°C$ of the temperature at which freezing initially occurred and a slight difference in IN number at $-4\,°C$ (Fig. 3). In contrast, the INA of CGina-01 was significantly affected by NO$_2$/O$_3$ exposure with a decrease of $2\,°C$ in the temperature at which freezing initially occurred (Fig. 3). For CC0242, two different patterns of response were observed between the four replicate trials. In two replicates, the initial temperature at which freezing occurred was not affected by NO$_2$/O$_3$ exposure whereas it was markedly decreased by $4\,°C$ in the other two replicates (Fig. 3).

3.4 Effects of UV-A exposure

The number of viable cells of strains 32b-74 and 13b-2 was significantly reduced after 42 h of UV-A exposure (Fig. 4). Viable cell number of *P. syringae* CC0242 began to decrease after 20 h of UV-A exposure. The number of cells of CGina-01 decreased significantly from the first sampling point i.e. after 4 h of UV-A exposure. Whereas the viable cell number of *P. syringae* 32b-74, 13b-2 and CC0242 decreased by a factor of 10 at the end of the UV exposure, the abundance of viable cells of *P. fluorescens* CGina-01 decreased by a factor of 27.

For all strains, UV-A exposure did not lead to any significant effect on INA at 4 h and 20 h of exposure (data not shown). At the end of the exposure, no effect of UV-A exposure on the temperature at which freezing initially occurred was observed in any of the strains tested (Supplement D).
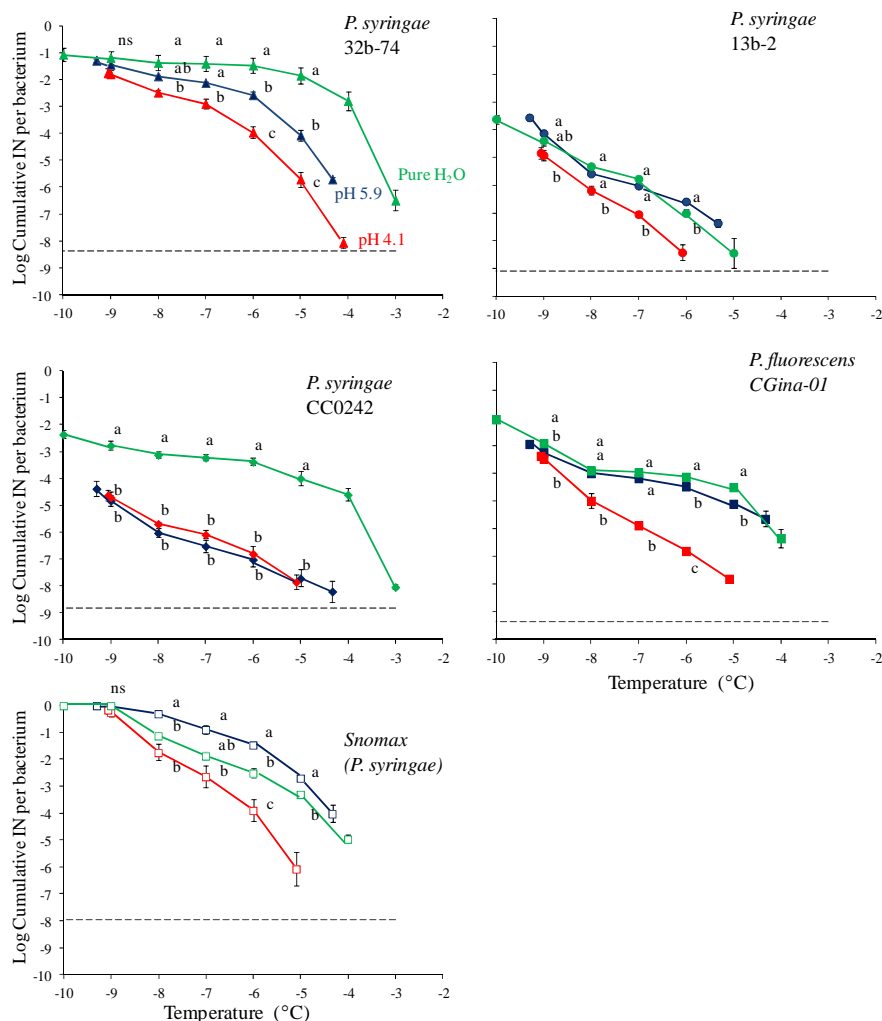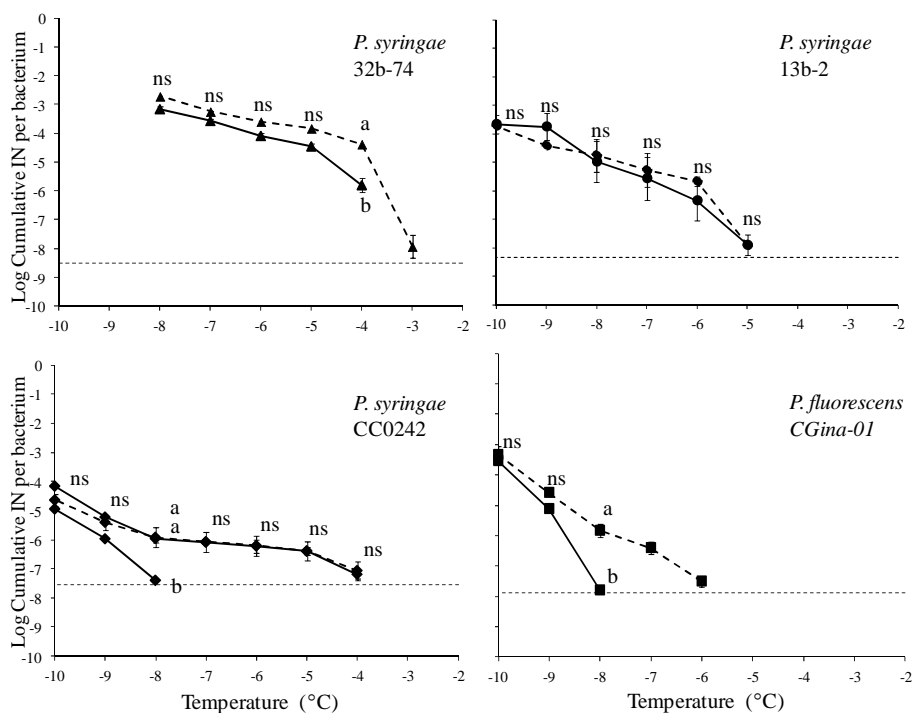
Fig. 2. Ice nucleation spectra of *Pseudomonas* strains submitted to different pH: pH = 4.1 (red), pH = 5.9 (blue) and in distilled water as a control (green). Data were corrected for water activity of the buffer and linearly interpolated. The horizontal line corresponds to the lower limit of detectable activity depending on the initial bacterial concentration of each experiment. Symbols are as in Fig. 1. At each temperature, values associated with different letters are significantly different ($p < 0.05$) based on Tukey's test. ns: no significant treatment effect. Errors bars indicate standard errors ($n = 4$).

INA of 13b-2 was not affected by 42 h of UV-A exposure (Supplement D). Conversely, the INA of 32b-74, CC0242 and CGina-01 increased after 42 h of UV-A exposure compared to unexposed control samples. Here, INA after 42 h of exposure was calculated by dividing the number of IN by the number of viable cells counted after 42 h of exposure. However, to take into account that dead cells could maintain an efficient ice nucleation protein, INA after 42 h exposure was also calculated by dividing the number of IN by the number of viable cells counted at the beginning of the experiment (Fig. 5). Consistent with the previous calculation, the INA of 13b-2 was not significantly affected by UV-A (Fig. 5). However, the INA of 32b-74, CC0242 and CGina-01 was slightly reduced by UV-A exposure.

4 Discussion

There has been a recent focus of atmosphere modeling on evaluation of the impact of biological ice nuclei on glaciation processes leading to precipitation (Hoose et al., 2010; Sesartic et al., 2011). However this approach is limited by quantitative data on abundance and activity of biological ice nucleators in the atmosphere. Here we reported not only ice nucleation spectra of different bacterial strains but also their response to three environmental conditions typically encountered in clouds. Our results demonstrate that among these conditions, acidic pH has a strong effect on bacterial INA but UV-A and NO_2/O_3 exposure have only little effects. Furthermore, the pH effects on INA are more evident at higher temperatures but by $-8°$ or $-10\,°C$ the effects are weaker.

Fig. 3. Ice nucleation spectra of *Pseudomonas* strains exposed to synthetic gases with (solid line) and without (dotted line) NO_2 and O_3. The horizontal line corresponds to the lower limit of detectable activity depending on the initial bacterial concentration of each experiment. Symbols are as in Fig. 1. Each symbol corresponds to the mean value of 4 replicates except for *P. syringae* CC0242 that was exposed to gases with NO_2 and O_3, where symbols correspond to the mean value of 2 replicates. At each temperature, values associated with different letters are significantly different ($p < 0.05$) based on Tukey's test. ns: no significant treatment effect. Errors bars are standard errors ($n = 4$, except for *P. syringae* CC0242 where $n = 2$).
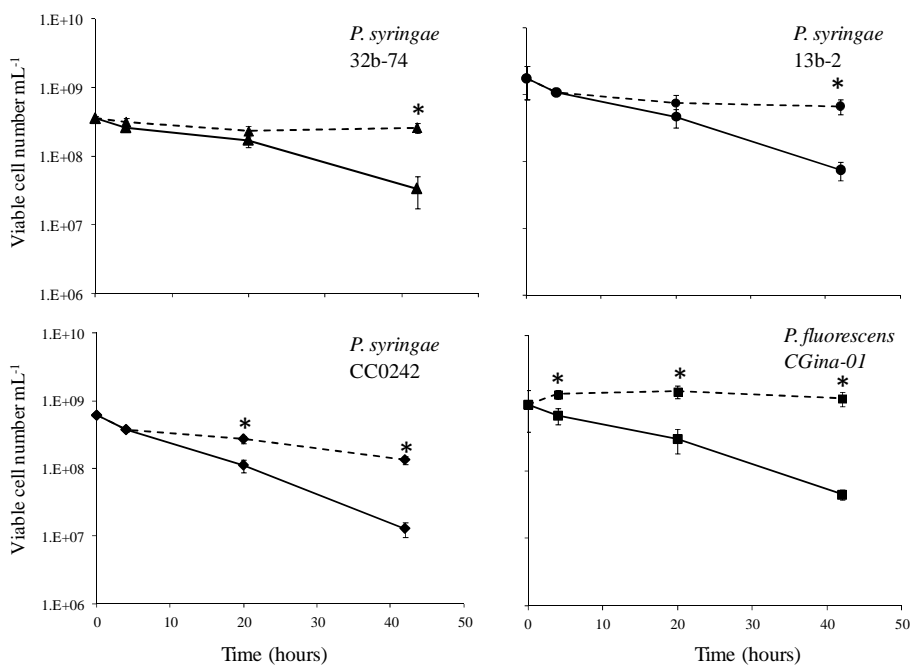
Fig. 4. Viable cell number (CFU) of the *Pseudomonas* strains after UV exposure (solid line) or darkness (dotted line). Symbols are as in Fig. 1. At each time point, values associated with an asterisk are significantly different ($p < 0.05$) based on Tukey's test. Errors bars are standard errors ($n = 4$).
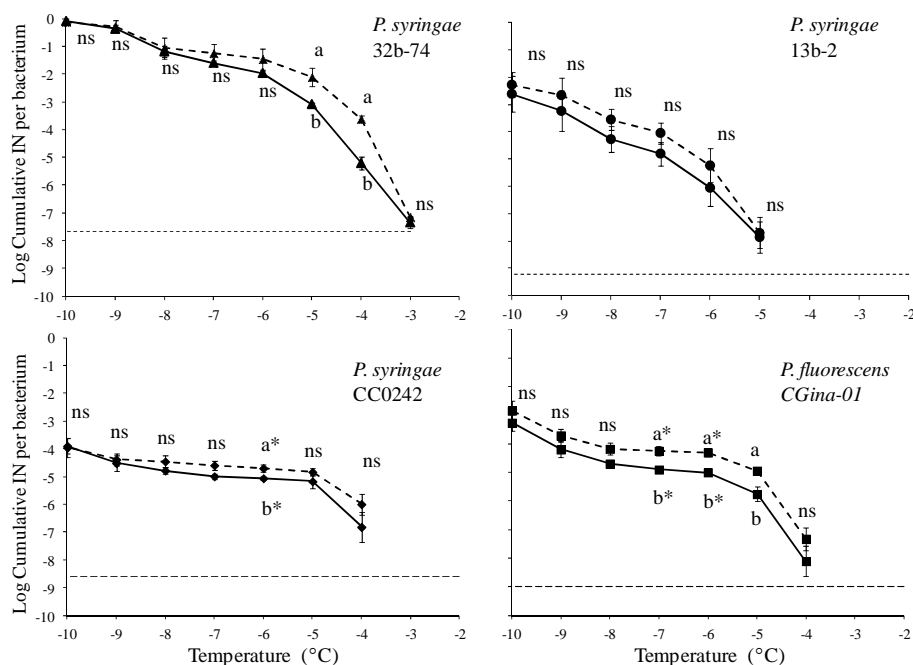
Fig. 5. Ice nucleation spectra of *Pseudomonas* strains submitted to 42 h of UV-A exposure (solid line) or darkness (dotted line). The horizontal line corresponds to the lowest value of detectable activity, which depends on the initial bacterial concentration of each experiment. Symbols are as in Fig. 1. INA was calculated by dividing the number of ice nuclei (IN) recorded after 42 h of UV-exposure by the viable cell number counted at the beginning of the experiment. At each temperature, values associated with different letters are significantly different at $p < 0.05$ and those associated with an asterisk are significantly different at $p < 0.1$ based on Tukey's test. ns: no significant treatment effect. Errors bars are standard errors ($n = 4$).

In this work we demonstrated that acidic pH reduced the INA of all the four strains studied and more intensely above $-8\,°C$. This effect of acidic pH on INA of *Pseudomonas* was previously studied by Kozloff et al. (1983) and Kawahara et al. (1995) whose results are consistent with ours. In addition, Turner et al. (1990) reported that effects of acidic pH were more intense for ice nuclei active at temperatures near $0\,°C$. They observed that the number of bacterial ice nuclei active at $-4\,°C$ was severely reduced by acidic pH whereas those active at $-9\,°C$ were almost not affected. Similarly, at a colder temperature ($-38\,°C$), no effects of acidic pH were observed on the IN properties of Snomax® (Chernoff and Bertram, 2010). As it has been proposed that ice nucleation efficiency is linked to the level of aggregation of the ice nucleation protein (Kozloff et al., 1991a) these results suggest that acidic pH acts via denaturation of the larger protein complexes. Indeed, bacteria expressing INA at the warmer temperatures carry larger protein complexes whereas at colder temperature smaller complexes are more abundant (Kozloff et al, 1991a). Then with acidic pH leading to denaturation of the protein complex, more effect will be observed on the larger complexes active at high temperatures and weaker effects will be observed on the smaller complexes active at cold temperatures. Furthermore, effects of acidic pH at the warmer temperatures were observed not only on the INA of living bacteria but also on the INA of the non-viable bacterial product Snomax®, thereby supporting the hypothesis that acidic pH affects the INA of bacteria probably by directly changing the ice nucleation protein structure. However, the effects of acidic pH were less intense on Snomax® than on living bacteria. This suggests that for living cells, acidic pH likely acts through a combination of direct changes of the ice nucleation protein structure and indirect effects via bacterial metabolism in reaction to acidic conditions.

To more clearly specify the effects of pH on INA, we took into account the effect on water activity of the solutes used as buffer. Indeed, as shown previously (Zobrist et al., 2008; Koop and Zobrist, 2009) a reduction in water activity is linked to a reduction in the INA of dusts and Snomax®. All our data were corrected for water activity of the pH buffer but, when subjected to pH 5.9, the INA of some strains differed more or less from that in distilled water. For Snomax®, a slight positive effect of this pH buffer on INA was observed. This increase is counterintuitive but could be linked to the complex nature of Snomax® which is a lyophilized product composed of bacteria but also culture medium residues and different wastes which can interact in a complex and still unexplained way with the pH buffer.

In contrast to the strong effects of acidic pH on INA, NO_2/O_3 exposure did not significantly influence the INA of strains *P. syringae* 32b-74 and 13b-2, which seem to indicate that the structure of the IN-inducing protein on the surface of the bacteria was not significantly changed by exposure to the reactive gases NO_2 and O_3. For *P. fluorescens* a reduction in the temperature at which freezing began was observed. It should be noted that the INA of this strain was already reduced by exposure to the synthetic gases without O_3 and NO_2. This fragility could account for the greater sensitivity of *P. fluorescens* to exposure to NO_2 and O_3 gases than of the *P. syringae* strains. The effect on strain CC0242 of *P. syringae* was more complicated than for the others strains: whereas the INA of two replicates was only slightly reduced, the INA of two others replicates was substantially affected by the reactive gas exposure. Variability among tests of INA was reported before. Mortazavi et al. (2008) have suggested that it is likely due to the ability of the ice-nucleating protein to aggregate. Despite the well-controlled conditions of preparation of bacteria for testing, some unidentified parameters may lead to differences in aggregation and thus to variations of INA.

Concerning UV-A exposure, we observed a significant loss of viability for all strains and especially for strain CGina-01 of *P. fluorescens*. Strikingly, this significant loss of viability was not associated with a decrease in INA (calculated with cell number at the beginning of the experiment) further validating the fact that under certain conditions, non viable cells can maintain INA and especially when cell integrity is maintained (Kozloff et al., 1991a). However, it should be noted that certain UV wavelengths can be damaging for INA. As shown by Anderson and Ashworth (1986) the highly lethal UV-C (almost absent in the atmosphere) caused a total loss of viability and a large reduction in INA, contrary to our observation of the effects of UV-A exposure.

5 Conclusions

Our results make a contribution to efforts to elucidate the impact of biological ice nucleators on cloud glaciation, but overall they illustrate the need for more in-depth quantification of the abundance and activity of INA organisms in the atmosphere. Firstly, it is interesting to note that two strains used here were isolated from cloud water providing direct evidence of their presence and viability in this environment. Furthermore, not all the *P. syringae* strains isolated in clouds were active as IN contrary to what was observed in snow and rain samples (Morris et al., 2008). This could suggest that INA is not necessarily involved in the incorporation of these bacteria in cloud water whereas it might be for incorporation into precipitation. Our results also illustrate that the ice nucleation activity of *Pseudomonas* is surprisingly robust in response to atmospheric conditions other than acidity. Hence the data presented here can contribute to efforts to model

cloud glaciation processes in that they suggest that the efficiency of some of the biological catalysts of ice formation in clouds might be reduced in clouds acidified by anthropogenic emissions. Such information can be incorporated in regional models but might be difficult to consider in global models. Exposure to other pollutants such as NO_2/O_3 seems to have a weaker effect. However, the differences in behaviour that we observed among strains and between the two species studied here suggest that the response to atmospheric conditions is rather strain-specific.

Acidic pH of atmospheric water droplets is a modern phenomenon due to the strong increase in emissions of SO_2 and NO_x from the 19th century, the beginning of the industrialization era (Smith et al., 2004; Dentener et al., 2006; Lamarque et al., 2010; Skeie et al., 2011). If initiating cloud glaciation processes is part of an adaptive survival and dissemination strategy for air-borne bacteria such as *P. syringae*, then it would be expected that the INA of these bacteria would not be significantly deteriorated by common conditions encountered in the atmosphere – and that likewise there could be selective pressures that will lead to eventual adaptation of *P. syringae* and other IN bacteria to these emerging conditions. Therefore, unlike mineral nucleators, it is feasible that, with continued exposure to atmospheric pollutants, biological ice nucleators in the atmosphere would adapt to the changing atmospheric conditions – and in particular chemical conditions – leading to enhanced efficiency of their INA. The challenge for cloud modelling will be to integrate these dynamic parameters.

Supplementary material related to this article is available online at: http://www.atmos-chem-phys.net/12/10667/2012/acp-12-10667-2012-supplement.pdf.

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Supplement A:

In Fig.1 we show a schematic picture that described the effects of solutes upon ice melting and nucleation. The green line indicates the ice melting point line (T_m) as a function of decreasing water activity (resulting from an increase in solute concentration), also frequently termed the colligative melting point depression line. In water activity space, this line is identical for any solute, and a numerical description is given in Koop and Zobrist (2009). For example, adding a buffer to pure water reduces the ice melting point temperature by the melting point depression ΔT_m. Similarly, solutes do also affect the heterogeneous ice nucleation temperature (T_{het}) specific for an IN. For example, $T_{het,w}$ in pure water is reduced by the addition of the buffer by the amount ΔT_{het} to the reduced $T_{het,b}$ in buffer. We can depict ΔT_{het} as the colligative effect of a solute upon heterogeneous ice nucleation. Moreover, it has been shown experimentally, that the corresponding blue line connecting all ice nucleation temperatures (T_{het}) for a specific concentration of IN is horizontally parallel to the green ice melting point line (Zobrist et al, 2008; Koop and Zobrist, 2009). The horizontal offset is specific to each IN and is usually termed $\Delta a_{w,het}$(IN). With this information, we can correct for the colligative effect of any solute and, hence, also any buffer, upon the heterogeneous ice nucleation temperature. We have applied this correction in the following way. First, we calculate the water activity of each buffer solution. This calculation was performed by determining the concentration of all ionic and non-ionic solute species introduced by adding the buffer stock solutions, including a consideration of the relevant dissociation equilibrium of HAc ($Ks = 1.7540 \times 10^{-5}$). Because the total molality of all solute species b_s was less than 0.5 mol kg^{-1} in both cases, we assumed ideal behaviour for calculating the water activity, a_w, of the solution after addition of a buffer. Hence, we can substitute the mole fraction of water, x_w, for water activity by $a_w = x_w = b_w/(b_w+b_s)$, where $b_w = 55.5093$ mol kg^{-1} is the molality of water in the solution. From these caluclations we obtain a_w values of 0.99378 and 0.99160 for the pH 5.9 and pH 4.1/NaCl buffers, respectively.

From the experimentally determined ice nucleation temperature in a given buffer $T_{het,b}$(exp) (solid red point in Fig. 2), we construct a T_{het} line (red dashed line) that is horizontally parallel in water activity space to the ice melting point line (T_m, green solid line). The intersection of this red dashed line with the y-axis at $a_w = 1$ (open red square) is then the hypothetical ice nucleation temperature of the IN in pure water $T_{het,wb}$(hyp), adjusted for the colligative effect (ΔT_{het}). This value can then be compared to the actual experimental ice nucleation temperature of the IN in pure water $T_{het,w}$(exp) (solid blue square). In case the presence of the buffer does not have any effect upon the IN apart from the colligative one, the experimental and hypothetical ice nucleation temperatures should be identical, i.e. $T_{het,wb}$(hyp) = $T_{het,w}$(exp) (middle panel in Fig. 2). If the IN becomes less active in the presence of buffer, for example by changing the ice nucleating protein complex, the hypothetical ice nucleation temperature should be below the experimental one, i.e. $T_{het,wb}$(hyp) < $T_{het,w}$(exp) (lower panel). Likewise, if the IN becomes more active, the hypothetical ice nucleation temperature should be higher than the experimental one $T_{het,wb}$(hyp) < $T_{het,w}$(exp) (upper panel).

We have made the corresponding correction for colligative effects of the buffer described here for each freezing temperature. For example, the cumulative number of IN per bacterium in a pH 5.9 buffer measured at -3 °C corresponds to the same cumulative number of IN per bacterium in water at -2.34 °C, i.e. the colligative correction was +0.66 °C. In summary, the temperature corrections were approximately plus 0.66-0.7 °C for the pH 5.9 buffer and approximately plus 0.89-0.94 °C for the pH 4.1 buffer. The difference between the measured cumulative number of IN per bacterium in droplets with buffer adjusted for the colligative solute effect and that measured separately in pure water without buffer is then attributed to the

effect of pH owing to changes induced in the ice nucleating protein complex at a different pH.

In order to allow a statistical comparison of data between both pH buffers and distilled water the cumulative number of IN per bacterium were required at the same temperature values. While the pure water data were available at temperatures with integer values of supercooling, those of the buffer data were not owing to the individual solute corrections. Therefore, we linearly interpolated the two nearest buffer data temperature values to obtain values at integer values of supercooling (e.g. the data at -2.34 °C and -3.34 °C were linearly interpolated to yield the value at -3.0 °C).

Fig. 1: Schematic picture for describing the colligative effect of solutes upon the ice melting point line (green line) and upon the heterogeneous ice nucleation temperature line (blue line) for an IN immersed in aqueous solutions; for details see text.
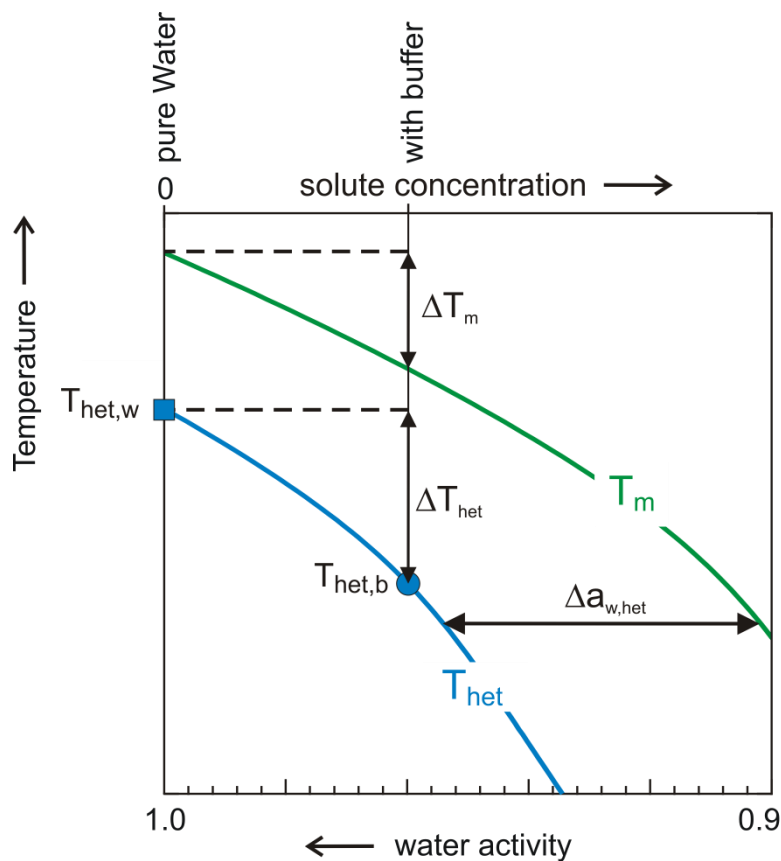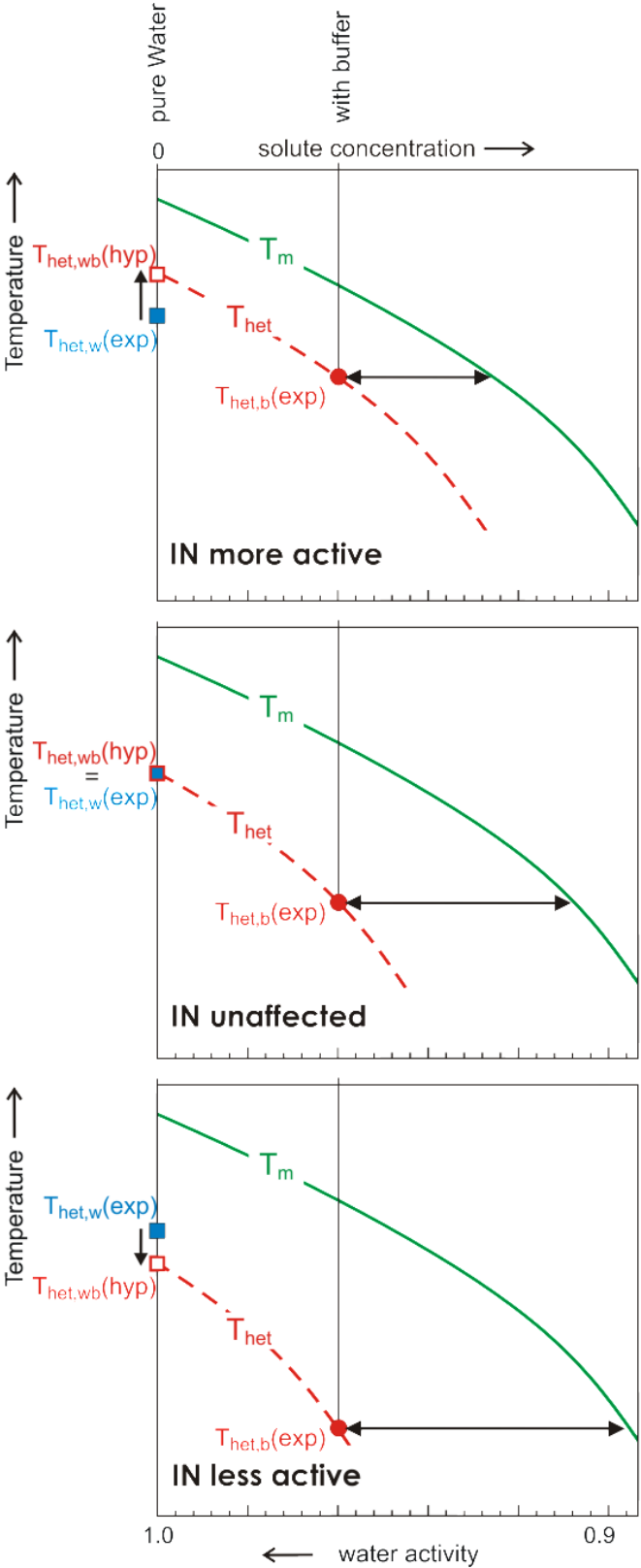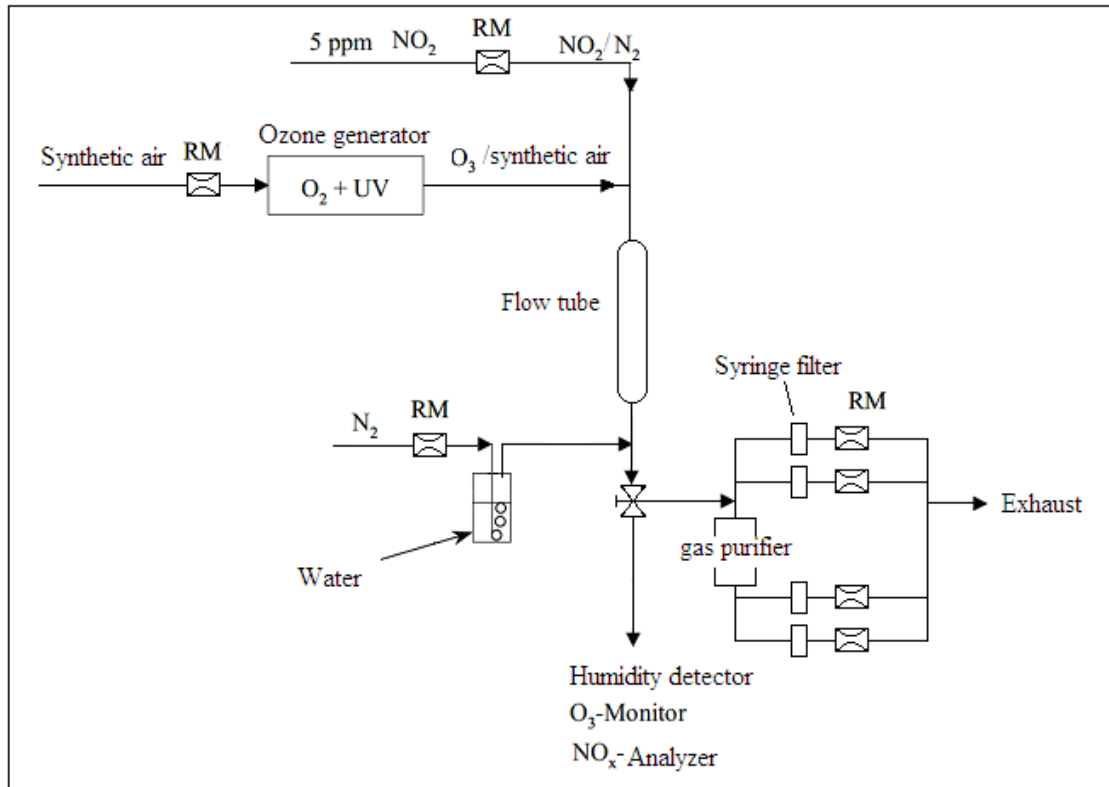
Fig. 2: Schematic picture for describing the approach taken in this work to adjust for the colligative effect of buffer upon the investigated heterogeneous ice nucleation temperatures (for details see text).

Supplement B: Diagram of the bacteria gas exposure system. RM stands for rotameter. Bacteria were loaded onto the syringe filter.

5 ppm NO_2 RM NO_2/N_2

Synthetic air RM   Ozone generator O_3 /synthetic air
$O_2 + UV$

Flow tube

Syringe filter   RM

N_2 RM

Exhaust

gas purifier

Water

Humidity detector
O_3-Monitor
NO_x- Analyzer

Supplement C: Ice nucleation spectra of *Pseudomonas* strains subjected to different pH: pH=4.1 (red), pH=5.9 (blue) and in distilled water as a control (green). For each pH, dotted lines correspond to original data whereas solid lines correspond to data corrected for water activity of the buffer. The horizontal line corresponds to the lower limit of detectable activity depending on the initial bacterial concentration of each experiment. Symbols are as in Figure 1. At each temperature, values associated with different letters are significantly different (p<0.05) based on Tukey's test. ns: no significant treatment effect. Errors bars indicate standard errors (n=4).
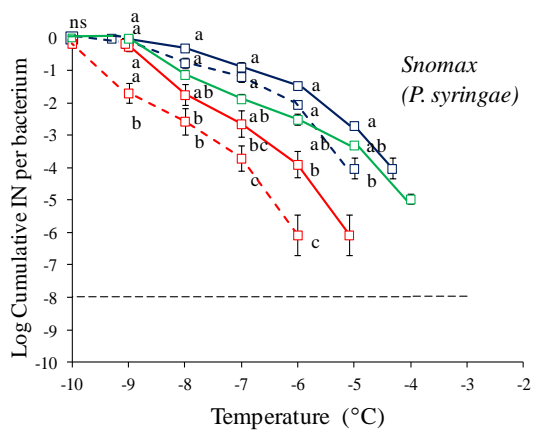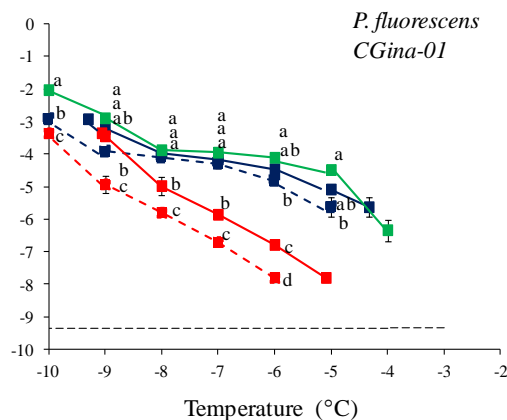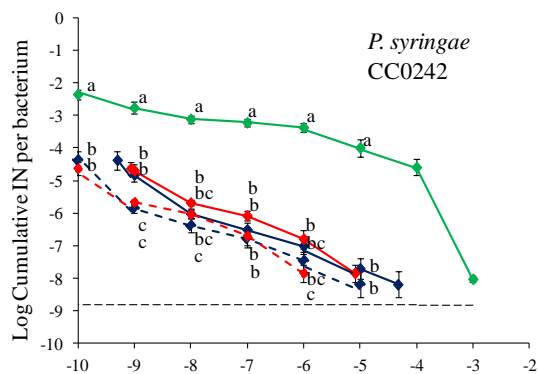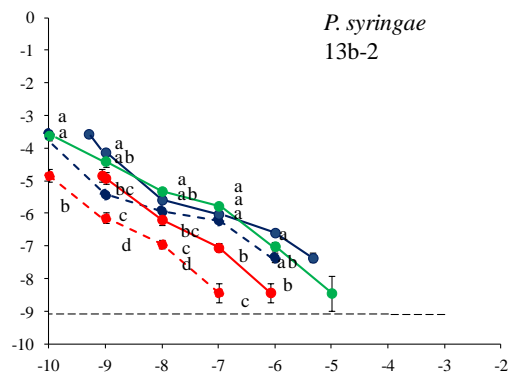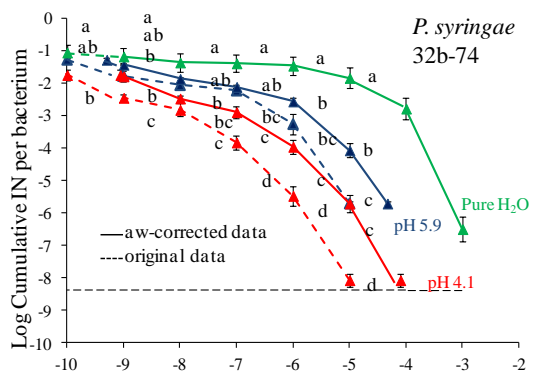
Supplement D: Ice nucleation spectra of *Pseudomonas* strains subjected to 42 h of UV-A exposure (solid line) or darkness (dotted line). The horizontal line corresponds to the lowest value of detectable activity, which depends on the initial bacterial concentration of each experiment. Symbols are as in Figure 1. INA was calculated by dividing the number of Ice Nuclei (IN) recorded after 42 h of UV-exposure by the viable cell number counted after 42h of UV exposure. At each temperature, values associated with different letters are significantly different (p<0.05) based on Tukey's test. ns: no significant treatment effect. Errors bars are standard errors (n=4).