We thank both referees for their encouraging comments and useful criticisms of our manuscript. Below we present, point-by-point, our response to the comments of each the referees.

RESPONSE TO COMMENTS OF REFEREE 1:

1. Most of the text in the two first paragraphs on page 4 is not directly related to the main thesis of the manuscript (e.g., discussion of inorganic IN particles) and thus can be deleted without loss of clarity.

The discussion about inorganic IN particles concerns the effect of coatings on IN. This information is important to understand the effect of chemical transformation in the atmosphere on ice nucleation activity. Such information is not available for biological particles. Therefore we feel that it is important to keep this text.

2. The description on the methods to account for the potential IN effect of the buffer of the medium used for pH testing (lines 0-15, page 8) is not very straightforward and could be re-written to be more accessible to the average reader.

The new text will be modified slightly to clarify. But more importantly, we propose to add a more detailed appendix (Appendix A, B) as supplementary material to give sufficient explanation. The contents of the appendix are presented at the end of this document.

Related to this, can Pseudomonas grow on the buffer (acetate) and thus alter the pH (or the growth is too slow to have an significant effect)?

Pseudomonas can grow in the buffer but the time of growth is too slow compared to the total time of the experiment. Strains of *Pseudomonas* show a lag period of at least 12 h before they begin to grow when introduced into a fresh medium. The time of our experiments was less than 30 min at ambient temperature. Furthermore, there is no nitrogen source in the buffer. Therefore, any possibility for growth would depend on the bacteria using their own nitrogen reserves (dead cells, for example).
However without growth, bacterial metabolism can alter the pH through ion pumping in or out of the cell. We checked this with a pH measurement of the medium before and after adding bacteria and we did not observe any significant pH change.

Finally, will it be easier to perform a control experiment where no bacteria are added to quantify the effect of IN of the buffer/medium alone?

The concentrations of sodium acetate (NaAc) and acetic acid (HAc) in the buffers are only very small (less than 0.2 mol/kg) and far below the solubility of the two compounds at room temperature and at low temperature. Hence, it is not possible that either of these solutes may precipitate during the experiments conducted here even at the lowest temperatures. We provide more information below (question 4 of the Reviewer n°2) and in the new appendix figures.

3. Line 6 page 11. I think the authors meant to write "for the temperature range tested" (as it is written is not grammatically correct).

We agree with the referee and we will change this sentence in the new version.

4. Page 14 and elsewhere. Could the authors provide a reasonable explanation why the effect of pH on the IN activity is temperature dependent? It might be related to the effect of temperature on the growth of the organisms (slowing down growth and thus IN production and activity).

We will take into account this remark and we will provide more information about the hypothesis concerning the temperature dependence of the effect of pH on the IN activity.
We will add precision in the section already mentioned, p11 l24-29:
"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."
We will add:
"Indeed, bacteria expressing IN activity at the warmer temperatures carry larger protein complexes whereas at colder temperatures 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 more abundant smaller complexes active at cold temperatures".

5. In general, it will be useful to mention how many biological replicates were performed for each experiment and what variation was observed among the replicates. Replicates are mentioned only for one set of experiments, I think, and in this case the variation may be considered too high to allow for robust conclusions to emerge, as the authors also indirectly implied.

We mentioned in each of the legends the number of replicates. In order to be clearer we will add this information in the material and methods section, that 6 replicates were used to calculate the ice nucleation spectra presented in Figure 1 and 4 replicates for all the experiments about the effects of pH and exposure to NO_2/O_3 and UV.

6. In the figures, the fonts on the axes could be enlarged to be easier to see. Not clear what "ns" means. The name of strain P. syringae cc0242 is not spelled out consistently; please use always capitalized "CC" or lower case "cc" in the text and figures.

We agree with the referee and we will enlarge the fonts and change "cc0242" to "CC0242".

RESPONSE TO COMMENTS OF REFEREE 2

1. Pg 9496, it is stated that bacteria suspensions were prepared in sterile water to obtain 5 x 10^8 to 5 x 10^9 cells per ml. How was this concentration determined?

To prepare bacterial suspensions before the drop freezing assays, Optical Density (OD) measurements at 600nm were conducted to know approximately but quickly how many bacterial cells were present in the sample. This explains why we can have different bacterial concentrations at the beginning of the experiments. However, in parallel, the exact bacterial concentration for each sample was determined by counting the CFU (colony forming units) on

culture medium in Petri dishes. In this case, results cannot be known immediately since bacterial growth is needed for CFU counting (~ 5days).

Also this is an entire order of magnitude in concentration. Differences within this concentration range could seriously impact the results and conclusions. Please explain if and how the impact of concentration was addressed?

Indeed, if we consider the number of frozen droplets, the initial number of cells matter. However, we presented here the INA which is the number of IN per cell (counted with the CFU method). Therefore, even if more or less cells were present in the initial bacterial suspension, this will not affect the INA value.

2. Pg. 9497. Of the numerous compositions of IN in the atmosphere, the authors choose to compare biological IN activity to that of a single mineral compound, montmorilonite. This is not an obvious or necessarily common mineral IN. This choice of comparison should be justified.

We agree with the reviewer and we will add more information in the material and methods section. We chose to present this mineral compound for two reasons. Firstly, our aim was to compare IN properties of mineral compounds with our data, so, we needed data acquired with the same method (drop freezing assays). The publication of Mortazavi et al. (2008) with the montmorillonite data is, to our knowledge, the only one which has applied the drop freezing assays to mineral compounds (montmorillonite and kaolinite). Secondly, our aim was to show the differences in IN properties between mineral and organic compounds. Then we focused on montmorillonte rather than the kaolonite which is a less efficient ice nucleator.

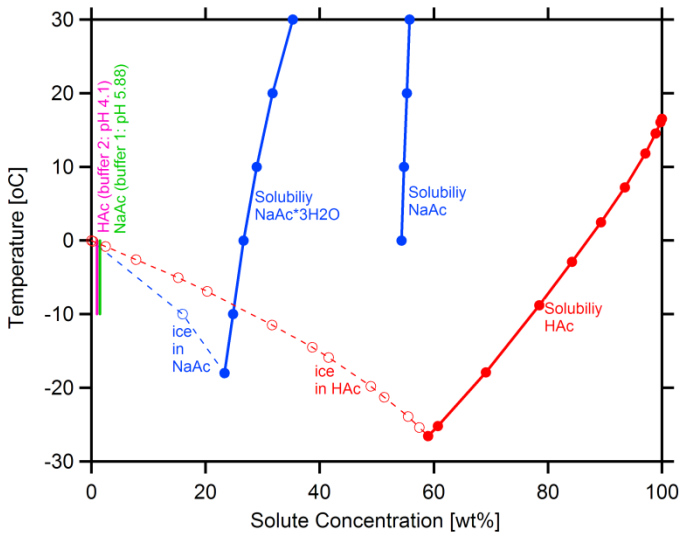
3. Bacteria were placed in distilled water which is likely to split apart the cells and may release proteins and other materials from within the cells. Were any control experiments in other solvents or the original glacier melt water or cloud water conducted? Do the authors have any way to access bacteria breakup and counting of fragments? This issue should be addressed through additional control experiments.

The first piece of evidence that there is not significant cell break-up is the stability of the bacterial population size during the experiment. The second piece of evidence comes from assays conducted to evaluate the need to use buffer rather than distilled water in testing ice nucleation activity of cells. We have observed that for the duration of time needed for ice nucleation experiments such as those conducted in this work, the activity of cell suspensions in distilled water is identical to that in a 0.1M phosphate buffer, for example. This is a good indication that cells in distilled water are not breaking up at a noticeable rate.
Finally our results show that INA is a quite robust activity; even when cells were not viable anymore the INA was not affected (for example, see the huge decrease in the number of viable cells after UV exposure and maintenance of INA). This suggests that even under some conditions that are unfavorable for cell viability, INA is not markedly affected.

4. "Testing the effects of pH is an interesting is a worthy experiment. However, orchestrating an appropriate experiment is a challenge. The authors choose to add sodium acetate and acetic acid to the samples to create the buffers. What is the solubility of acetic acid at temperatures below freezing? The solubility of some organic acids is

greatly reduced at colder temperatures. If this is the case with acetic acid, then the presence of acid itself may facilitate freezing."

The concentrations of sodium acetate (NaAc) and acetic acid (HAc) in the buffers are very low (less than 0.2 mol/kg) and far below the solubility of the two compounds at -10 °C. Hence, it is not possible that either of these solutes may precipitate during the experiments conducted here even at the lowest temperatures. We show the phase diagrams for HAc/H2O (in red) and NaAc/H2O (in blue) in the figure below (added here for information. We do not propose to include it in the revised manuscript). Note that for NaAc solutions the solubility is actually determined by Sodium Acetate Trihydrate (NaAc*3H2O), which shows a lower solubility than the anhydrous salt (NaAc), see blue lines in the figure. Also shown are the conditions at which the experiments of the current manuscript were performed, buffer 1 (pH 5.88) as a green line, and buffer 2 (pH 4.1) as a magenta line. As can be seen the concentrations of NaAc and HAc in the buffers are at least one order of magnitude smaller than the corresponding solubilities. Even at the lowest temperature in our experiments precipitation of sodium acetate and acitic acid will not occur.



We will add the following sentences to the manuscript.

At room temperature HAc is liquid and dissolves in water at all concentrations. The HAc/H2O 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 (Dahms, 1896). For comparison, the maximum concentration of HAc in buffer 2 was only about 0.15 mol/kg. The solubility limit of aqueous NaAc solutions is actually determined by the solubility curve of its trihydrate (NaAc*3H2O). At room temperature the solubility is about 5.7 mol/kg and it is 3.7 mol/kg at the eutectic temperature of -18 °C (Green, 1908). In contrast, the maximum concentration of NaAc in buffer 1 was only about 0.17 mol/kg. These calculations show that neither NaAc nor HAc could have precipitated from the buffer solutions in our experiments upon cooling.

References for the information concerning this comment that will be added to the manuscript:

A. Dahms, Nachträge und Bemerkungen zu der Arbeit über Gefrierpunkte binärer Gemenge, Ann. Phys. 296, 119–123 (1896)
W.F. Green, The "melting-point" of hydrated sodium acetate: solubility curves, J. Phys. Chem. 12, 655-660 (1908)

5: "Control experiments should be conducted in buffer solution only, without any bacteria."

The information provided in the response to the preceding comment is a very strong argument for the fact that the buffers without bacteria would not have frozen under the temperatures used in this study.

Nevertheless we tested the freezing of buffer – without bacteria – adjusted to pH 4.0 and 5.7. For each pH, 40 drops were tested for freezing down to -10°C under the same conditions used to determine the ice nucleation-activity of bacterial suspensions. No drops froze under these conditions.

6. "The authors say that they have corrected for colligative effects following Koop and Zobrist , 2009, but I do not think that that method is directly applicable to the solutes used in this study, and regardless, is an estimation, whereas running control freezing experiments would be direct evidence of the effect of the buffer chemicals."

Unfortunately, the referee did not provide any arguments for his/her statement that "the method by Koop and Zobrist 2009 is not applicable to the solutes used in this study". Hence, we cannot specifically address their criticism about our application of this method. The Koop and Zobrist 2009 method is generally applicable to ideal solutions and also to non-ideal solutions as long as these do not show significant temperature dependence in water activity for constant concentration solutions. This is normally obeyed for small water soluble organics and for electrolytes such as salts and small organic acids. For example, we compared published experimental freezing point depression data from the CRC Handbook of Chemistry and Physics (85^{th} edition) with calculations assuming ideal solution behavior for the very dilute buffer concentrations (i.e. assuming full dissociation of NaAc and no dissociation of HAc). The resulting absolute differences are less than 0.01 °C for HAc and less than 0.04 °C for NaAc, clearly showing how good these assumptions are.

As we indicated above, buffer solutions alone will not freeze at the temperatures we used in this study. This indicates that freezing in the buffer solutions with bacteria is indeed triggered by the bacteria and not by the solutes. Nevertheless, in order to determine the effect of pH on the IN ability of the bacteria the colligative effect of the buffer must be taken into account. This was done using the method by Koop and Zobrist (2009), which to our knowledge is the only method provided in the literature for this purpose. We have described the method more explicitly in the revised version of the manuscript (see also answer to referee 1). Note that the corrections are small, about 0.66-0.71 °C for the pH5.88 buffer and 0.89-0.95 °C for the pH4.1 buffer used here.

Additional appendix that will be added:

In Figure D 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}) (blue line in the figure) specific for an IN. For example, $T_{het,w}$ in pure water is reduced by the addition of the buffer by 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 = 1.7540×10^{-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.E), 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.E). 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).

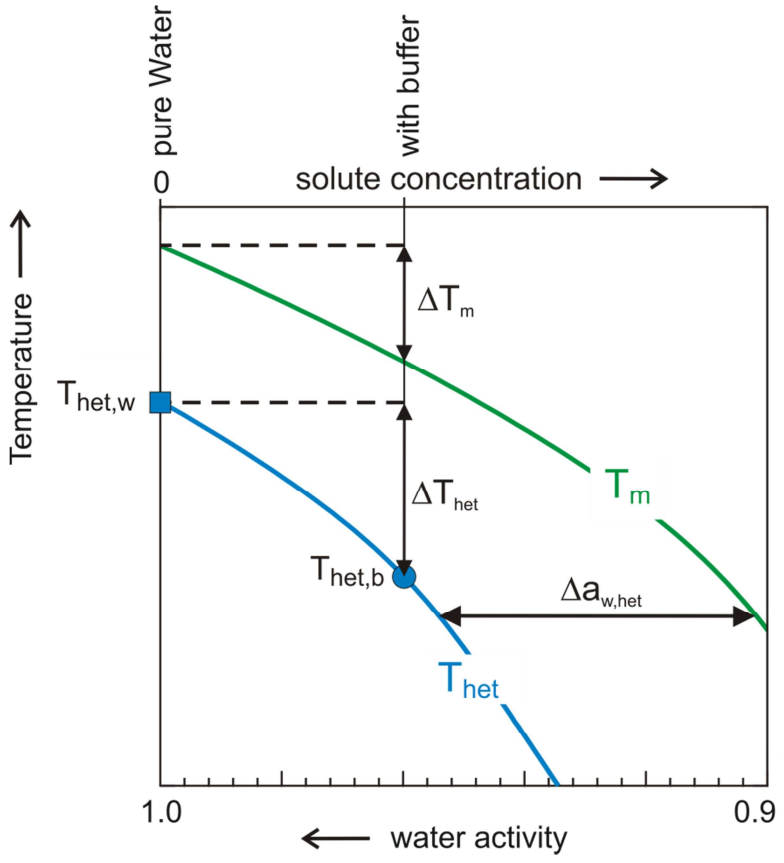


Fig. D: Schematic picture for describing the colligative effect of solutes upon the ice melting point and the heterogeneous ice nucleation temperature (for details see text).

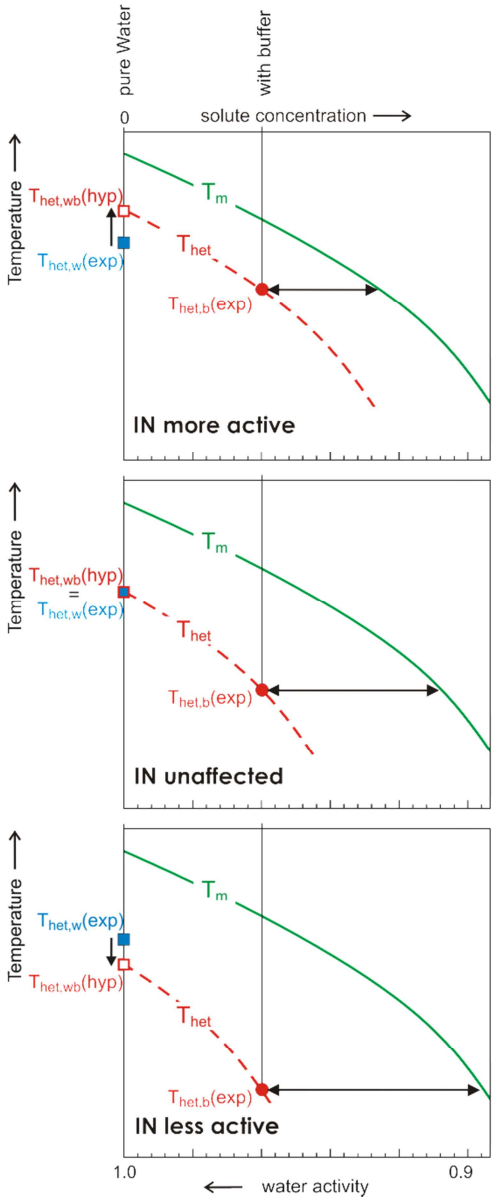


Fig. E: Schematic picture for describing the approach taken in this work to adjust for the colligative effect of buffer upon the investigated heterogeneous ice nucleation temperatures (for details see text).