**Review #1:**

In my previous review I had major concerns with the structure of this paper which was not coherent. It was presented as a research article with an experimental part, results and discussion sections but it looked more like a review on the domain. Also the supplement part was unusual, closer to a patchwork of book chapters, the idea being to help some readers to understand the rest of the paper. I did suggest to completely rewrite this paper as a review and to delete some basic information present in the Supplement part.
The authors did not completely take into account these remarks as they did not write a review. However they rewrote this paper in a form closer to that of a "classical article" by transferring most of the introduction part in the discussion. They also shortened the Supplement. I still believe that it is not the best choice and I would have really preferred a review. However, because the content is interesting anyway, I would accept this paper with minor revisions if the editor agrees with the final form of the manuscript.

Minor changes:

P 17 lines 14 to 16 "At last, microorganisms are present in precipitation samples (Vaïtilingom et al. 2012)... altitudes". This is not exact, they were found in cloud waters directly (and not precipitations). Also INA Bacteria were isolated from cloud waters (Joly et al. Ice nucleation activity of bacteria isolated from cloud water. Atmospheric Environment, 2013, 70, 392-400). So basically the whole sentence should be rephrased and this last reference added.

We changed p.18, L.10-13 to: "Cultivable microorganisms are also present in the stratosphere (Griffin, 2004) and in cloud water samples (e.g. Vaitilingom et al., 2012, Joly et al, 2013)."

Supplement:

S1.1 Macromolecular chemistry
This part is still too basic. I suggest making it even shorter and referring to text books for undergraduates. In addition the paragraph p2 line 11 to p3 line 4 "The solubility of a macromolecule…elementary cells" should be deleted.

we shortened this part by:

- deletion of p.1, L.21 - p.2, L.3 (until "stated in the manuscript"), replaced with "Polymers are a subgroup of macromolecules, which are built up by a chain of covalently linked small molecules."
- shortening p.2, L.5-6 to: "can be either random or in a well-defined manner"
- deletion of p.2, L.7-8 "and therefore a very distinct form. Common elements of protein folding are a-helices, b-sheets and b-helices."
- shortening p.2, L.11-19 to: "The solubility of a macromolecule depends on the chemistry of the macromolecule and the solvent. Based on the protein classification approach by T. B. Osborne (Osborne, 1910) biological matter is suspended and shaken in a certain solvent. Then the matter is centrifuged or filtrated off, thus removing particulate matter and yielding a transparent supernatant. Molecules that are extracted into the supernatant are considered to be soluble in that medium."
- deletion of p.2, L.19 ("Depending on") - L.25 ("glutelines")
- deletion of p.3, L.4-6 (everything from "Despite its size")

**Review #2:**

First of all I am satisfied that the authors have gone to a lot of effort to address my earlier comments, which centred around making the manuscript clearer and less like a review article in places. The review has been a useful process and clarified many aspects of the paper. There are

sections that contain a lot of detailed methods. This makes for a difficult read in places, but nevertheless some readers will find it useful. I have a few remaining comments below. The key one is the last specific comment, which may help put biological particles into context, although I may have misinterpreted the findings.

General

Abstract: much improved. However, can it be stated what the findings from the paper are? i.e. you mention you provide new data, but maybe it would be more substantive to say something like, "we argue that our data support this view of ice nucleation by macromolecules"? I am just thinking of some statement to link your paper to the data you've collected.

we added to the abstract:
p.2, L.12-14: "Our investigation on the fungal species Acremonium implicatum, Isaria farinosa, and Mortierella alpina shows that their ice nucleation activity is caused by proteinaceous water-soluble INMs."
p.2, L.17-19: "This has atmospheric implications, since many of these INMs can be released by fragmentation of the carrier cell and subsequently may be distributed independently. Up to now, this process has not been accounted for in atmospheric models."

Throughout: quite a few typos, gramma issues that should get picked up by copyediting.

In my opinion the discussion could be stronger. For example on line 27 of page 43 you state that contact angle is a macroscopic interpretation, but don't really develop this any further. You also state in several places that you want to develop a more molecular view of ice nucleation, but it is not clear what you mean by this. Afterall you are still using an nm approach, which is not really specific to a molecular view. The discussion then ends with some very speculative ideas.

we expanded the paragraph starting from p.12, L.27 (old version), respectively p.16., L.27 (new version):
"[...] process of heterogeneous ice nucleation. For example, the contact angle, which is useful in atmospheric models, is a macroscopic interpretation of the affinity between two phases: the ice embryo and the IN. Water molecules show a high affinity towards hydroxy (-OH) and amino (-$NH_2$) groups, since they form hydrogen bonds with them. The contact angle quantifies the outcome of the molecular interaction and allows comparison of different INs, but it does not allow to trace back the complex molecular structures that are responsible. If we understand which structures are the characteristics of INMs, it will make predictions of INA possible for any macromolecule with a known sequence. As an example, the already-mentioned TXT-sequences (Sect. 4.1) are one such element that foster INA, but there have to be others as well, since non-proteinaceous INs exist. Classification and precise characterization of the currently known INs might reveal other INA elements."
Note: We switched the positions of chapters 4.1 (solubility) and 4.2 (previous findings), since the text flows better this way.

Specific

Introduction: in reference to figure 1 you describe 1c as heterogeneous ice nucleation on an anti-freeze protein. I must be missing something subtle here. I would have thought anti-freeze inhibited ice nucleation. You also use the acronym BINMS in the figure, but the definition has been removed from the intro now.

We replaced the capture for 1c with: "and an antifreeze protein that has a similar sequence and structure as the bacterial INMs"
The intriguing detail here is that a certain class of antifreeze proteins is very similar to the bacterial INMs and also fixates water molecules in an ice-like manner, but the key difference is

that they are much smaller and so create only subcritical clusters that are too small to induce ice nucleation. Alternatively, the AFPs block the growth sites of the formed clusters and so prevent ice formation.

The introduction ends rather abrupt with reference to a table and no discussion of it. I think a sentence to lead into the next section would make the text flow better.

we added to the paragraph at p.3, L23: "They occur in several species of bacteria, fungi, plants and animals. Apart from being INMs, they are very diverse in their properties, like size or heat tolerance, as their diverse chemical nature suggests."

“some low-molecular organic compounds”? Not sure what is meant here

we rephrased it as "some compounds with low molar mass" throughout the text

When discussing the atmospheric implications I was wondering if you are able to put your measurements into perspective. It seems that the highest nm measured is about 1013 kg-1, which when multiplied by the size of a typical IN ~1x105 kDa as an upper estimate (or about 1.6x10-19 kg) is 1x10-6. Does this mean that only 1 in 106 of these particles would be IN?

It is correct that the mass of 100 kDa INMs as part of the whole fungal mass is - for these specific numbers - somewhere around 1.7 ppb. The assumption that therefore one in a billion particles is therefore INA-positive could be an approximation. But it has to be taken with care, since it is only exact if the whole fungal matter is portioned into 100 kDa fragments, which is in practice not possible. The reference whole fungal mass consists of cells and molecules of all kinds of sizes from small molecules (d < 1 nm) to macroscopic fungal tissue fragments (d > 1 mm). The latter are filtered off in the lab study, respectively are not elevated into cloud formation heights.
We added these calculations to the Results section, where we compare the different setups (p.9, L.26-29): "If we assume that the mass of 1 INM is 100 kDa or 1.7*10^-19 grams, and that the maximum number density we found was about 10^10 per gram (Fig. 3), the INMs amount to approximately 1.6 ppb of the total mycelium mass."

**List of significant changes:**

p.2, L.12-14: we added "Our investigation on the fungal species *Acremonium implicatum*, *Isaria farinosa*, and *Mortierella alpina* shows that their ice nucleation activity is caused by proteinaceous water-soluble INMs."

p.2, L.17-19: we added "This has atmospheric implications, since many of these INMs can be released by fragmentation of the carrier cell and subsequently may be distributed independently. Up to now, this process has not been accounted for in atmospheric models."

p.3, L-23-25: we added "They occur in several species of bacteria, fungi, plants and animals. Apart from being INMs, they are very diverse in their properties, like size or heat tolerance, as their diverse chemical nature suggests."

p.9, L.26-29: we added "If we assume that the mass of a single INM is about 100 kDa = $1.7 \cdot 10^{-19}$ grams and that the maximum number density we found was $n_m = 10^{10}$ per gram (Fig. 3), the INMs amount to approximately 1.7 ppb of the total mycelium mass."

chapters 4.1 and 4.2 switched places

p.15, L.27-30: we added "Alternatively, other organic compounds such as oxalic acid can act as an immersion IN in the crystalline state (Zobrist et al., 2006, Wagner et al. 2011) Also cellulose,

which is the most common biopolymer on Earth due to its ubiquity in plant cell walls, shows INA in the form of microcrystalline or fibrous particles (Hiranuma et al., 2015)."

p.16, L.28 - p.17, L.10: we changed the paragraph and expanded it: "[...] heterogeneous ice nucleation. For example, the contact angle, which is useful in atmospheric models, is a macroscopic interpretation of the affinity between two phases: the ice embryo and the IN. Water molecules show a high affinity towards hydroxy (-OH) and amino ($-NH_2$) groups, since they form hydrogen bonds with them. The contact angle quantifies the outcome of the molecular interaction and allows comparison of different INs, but it does not allow to trace back the complex molecular structures that are responsible. If we understand which structures are the characteristics of INMs, it will make predictions of INA possible for any macromolecule with a known sequence. As an example, the already-mentioned TXT-sequences (Sect. 4.1) are one such element that foster INA, but there have to be others as well, since non-proteinaceous INs exist. Classification and precise characterization of the currently known INs might reveal other INA elements."

p.18, L.10-13: we changed the text to "Cultivable microorganisms are also present in the stratosphere (Griffin, 2004) and in cloud water samples (e.g. Vaitilingom et al., 2012, Joly et al, 2013)."

p.18, L.29-31: we added "Recently, the presence of nanosized biological particles with INA were detected in precipitation (Santl-Temkiv et al., 2015) and soil (O'Sullivan et al., 2015)."

p.36: we changed the figure caption "[...] and an antifreeze protein that has a similar sequence and structure as the bacterial INMs [...]"

Supplement:

p.1, L.21 - p.3, L.6: was cut short to "Polymers are a subgroup of macromolecules, which are built up by small molecules that are covalently linked in a chain-like manner. Such a molecular chain will not stay linear, but will fold into a more compact form – especially if it contains hydrophobic elements in a hydrophilic surrounding or the other way round. This folding can be either random or in a well-defined manner. Proteins in their functional state usually have a very distinct folding. Protein chains that are not properly folded lack in most cases their functionality. Since the non-covalent forces holding the protein structure intact are usually weak, stress treatments lead to unfolding and therefore inactivation of the protein.

The solubility of a macromolecule depends on the chemistry of the macromolecule and the solvent. Based on the protein classification approach by T. B. Osborne (Osborne, 1910) biological matter is suspended and shaken in a certain solvent. Then the matter is centrifuged or filtrated off, thus removing particulate matter and yielding a transparent supernatant. Molecules that are extracted into the supernatant are considered to be soluble in that medium.
In the case of large molecules, it is disputable where to draw the line between solution and suspension. Per definition, a solution consists of a single phase, while a suspension consists of two phases with phase interfaces. If the particles sizes are close to the wavelength of visible light, a suspension shows light scattering, which makes it opaque. A solution, in contrast, shows neither light scattering, nor visible particles. Furthermore, a solution shows no phase separation over time, while sedimentation or agglutination lead to a progressive phase separation in time. Additionally, solutions cannot be separated by centrifugation. From a molecular point of view, a molecule in solution is fully covered with an energetically favorable hydration shell."

# Ice nucleation by water-soluble macromolecules

**B. G. Pummer[1], C. Budke[2], S. Augustin-Bauditz[3], D. Niedermeier[3,4], L. Felgitsch[5], C. J. Kampf[1], R. G. Huber[6], K. R. Liedl[6], T. Loerting[7], T. Moschen[8], M. Schauperl[6], M. Tollinger[8], C. E. Morris[9], H. Wex[3], H. Grothe[5], U. Pöschl[1], T. Koop[2], and J. Fröhlich-Nowoisky[1]**

[1] {Dept. Multiphase Chemistry, Max Planck Institute for Chemistry, Hahn-Meitner-Weg 1, D-55128 Mainz, Germany}

[2] {Faculty of Chemistry, Bielefeld University, Universitätsstraße 25, D-33615 Bielefeld, Germany}

[3] {Experimental Aerosol and Cloud Microphysics Dept., Leibniz Institute of Tropospheric Research, Permoserstraße 15, D-04318 Leipzig, Germany}

[4] {Dept. of Physics, Michigan Technological University, 1400 Townsend Drive, MI-49931 Houghton, Michigan, USA}

[5] {Inst. for Materials Chemistry, Vienna University of Technology, Getreidemarkt 9, A-1060 Wien, Austria}

[6] {Inst. for General, Inorganic and Theoretical Chemistry, University of Innsbruck, Innrain 80-82, A-6020 Innsbruck, Austria}

[7] {Inst. for Physical Chemistry, University of Innsbruck, Innrain 80-82, A-6020 Innsbruck, Austria}

[8] {Inst. for Organic Chemistry, Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innrain 80-82, A-6020 Innsbruck, Austria}

[9] {UR0407 Pathologie Végétale, Institut National de la Recherche Agronomique, F-84143 Montfavex Cedex, France}

Correspondence to: B. G. Pummer (b.pummer@mpic.de)

## Abstract

Cloud glaciation is critically important for the global radiation budget (albedo) and for initiation of precipitation. But the freezing of pure water droplets requires cooling to temperatures as low as 235 K. Freezing at higher temperatures requires the presence of an ice nucleator, which serves as a template for arranging water molecules in an ice-like manner. It is often assumed that these ice nucleators have to be insoluble particles. We point out that also free macromolecules which are dissolved in water can efficiently induce ice nucleation: The size of such ice nucleating macromolecules (INMs) is in the range of nanometers, which corresponds to the size of the critical ice embryo. As the latter is temperature-dependent, we see a correlation between the size of INMs and the ice nucleation temperature as predicted by *Classical Nucleation Theory*. Different types of INMs have been found in a wide range of biological species and comprise a variety of chemical structures including proteins, saccharides, and lipids. Our investigation on the fungal species *Acremonium implicatum*, *Isaria farinosa*, and *Mortierella alpina* shows that their ice nucleation activity is caused by proteinaceous water-soluble INMs. We combine these new ~~measurement~~ results and literature data on INMs from fungi, bacteria, and pollen with theoretical calculations to foster a chemical perspective of ice nucleation and water-soluble INMs. This has atmospheric implications, since many of these INMs can be ~~set free~~released by fragmentation of the carrier cell and subsequently may be distributed independently. Up to now, this process has not been accounted for in atmospheric models.


Formatiert: Schriftart: Kursiv

Formatiert: Schriftart: Kursiv

Formatiert: Schriftart: Kursiv

## 1 Introduction

Although ice is thermodynamically favored over liquid water at temperatures below 273.15 K, the phase transition is kinetically hindered. Consequently, supercooled droplets of ultrapure water stay liquid, until temperatures as low as 235 K are reached. The spontaneous self-assembling of water molecules in an ice-like arrangement, which is necessary for freezing to occur, is called homogeneous ice nucleation (Fig. 1a). At higher temperatures, catalytic surfaces which act as an ice-mimicking template are necessary. The process, in which water molecules are stabilized in an ice-like arrangement by an impurity, is called heterogeneous ice nucleation (Fig. 1b+c). An impurity that possesses this ability is called ice nucleator (IN), or sometimes ice nucleus. The driving force that causes ice nucleation activity (INA) is the interaction between the

partial charges on the H and O atoms in the water molecules and the properly arranged (partial) charges on the IN surface. Therefore, the IN has to carry functional groups at the proper position to be effective (Liou et al., 2000, Zachariassen and Kristiansen, 2000). In most cases it is not the whole surface of an IN that participates in ice nucleation, but only certain sections, which are known as "active sites" (Edwards et al., 1962, Katz, 1962).

The larger the active site of an IN, and the more fitting functional groups it carries, the more effective it stabilizes ice clusters, and so the higher the freezing temperature. Consequently, single molecules ~~of low molecular compounds~~ with a low molar mass ~~cannot~~ are not well suited to nucleate ice. In fact, soluble compounds consisting of ions, such as salts, or very small molecules, such as sugars and short-chained alcohols, cause a depression of the thermodynamic freezing point and the homogeneous ice nucleation temperature (Koop, 2004). However, if single molecules are so large that they allocate a large enough active surface, they are INs by themselves. Such ice nucleating macromolecules (INMs) are especially common among biological INs. More information about INMs is given in Sect. S1.1. Due to the same reason some ~~low molecular~~ compounds with low molar mass which show no INA in solution can act as IN, if they are crystallized in layers of a certain arrangement (Fukuta, 1966). Further information related to the ice nucleation process is compiled in the supplement (Sect. S1.2, S1.3, and S1.4). INA has been discovered in various forms of life, including certain bacteria, fungi, algae, plants and animals. Studies to characterize the active sites of some of these organisms have revealed that they are biopolymers in almost all regarded cases. The chemistry of these INMs is as diverse as the range of species they represent (Table 1, Sect. 4.1): Overall, proteins, higher saccharides and lipids, as well as hybrid compounds can play a role in INA, both as singular molecules as well as in aggregated form ~~(Table 1, Sect. 4.2)~~. They occur in several species of bacteria, fungi, plants and animals. Apart from being INMs, they are very diverse in their properties, like size or heat tolerance, as their diverse chemical nature suggests.

In this study, we chemically characterize the water-soluble INMs found in the fungal species *Acremonium implicatum* and *Isaria farinosa* and we compare the results with other recent studies of water-soluble INMs from the fungus *Mortierella alpina* (Fröhlich-Nowoisky et al., ~~2014~~2015), from birch pollen (Pummer et al., 2012, Augustin et al., 2013), and from bacteria (Niederme~~y~~er et al., 2014). We also discuss relevant key findings of related earlier studies on the INA of biological materials (e.g. Govindarajan and Lindow, 1988a). Combining these data

with calculations derived from *Classical Nucleation Theory* (Zobrist et al., 2007), we draw conclusions about the nature, sources, and potential atmospheric effects of biological INMs.

## 2 Methods

### 2.1 Characterization of new fungal INMs

The fungi *A. implicatum* and *I. farinosa* were cultivated on a plate of potato dextrose agar (VWR™), incubated at ambient temperature for 1–2 weeks, until the first mycelium was formed, and then left to grow at ~280 K for 2–3 months (*A. implicatum*) or 6–10 months (*I. farinosa*). The mycelium was scratched off with either a scalpel or an inoculating loop and put into a 15 ml Falcon tube. High-purity water (18.2 MΩ·cm) was tapped from a water purification system (Thermoscientific™ Barnstead GenPure xCAD plus), autoclaved at 394 K for 20 min, and filtrated through a sterile 0.1 µm PES filter (Corning™). Then 10 ml of the high-purity water were added to the mycelium in the tube, which was then shaken with a vortex device (VWR™ lab dancer) three times for 30 seconds and filtrated through a 5 µm PES syringe filter (Acrodisc®), yielding a transparent solution. A small aliquot of the 5 µm filtrate was branched off for INA measurement as described later in this section, while the rest was further filtrated through a 0.1 µm PES syringe filter (Acrodisc®). A small aliquot of the 0.1 µm filtrate was saved for INA tests. Further aliquots were exposed to different procedures, which are listed below, and then tested for changes in their INA. This provides information about the chemistry of the INMs. In all cases, not only the filtrates but also pure water samples which were treated the same way were tested as a negative reference.

- Filtration through size exclusion filtration tubes (Vivaspin® 500): 300 kDa and 100 kDa cutoff. The passage through a filter indicates that the molecules are smaller than the given cutoff.
- Exposure to heat for 1 hour: 308 K and 333 K, providing information about the thermal stability.
- Addition of 6.0 M guanidinium chloride (Promega®), which is a chaotropic reagent used for protein denaturation.
- Addition of 0.3 M boric acid (National Diagnostics®), which esterifies with saccharide OH groups and thereby blocks the site.

- Digestion with enzymes (Applichem®) for at a given incubation temperature: Lipase for 1 hour at 308 K for fat digestion, Papain for 5 hours at 296 K for protein digestion. For the latter, two more temperatures were investigated (5 hours at 308 K, 1 hour at 333 K), since its optimum temperature is about 338 K, but the investigated INMs turned out to be rather thermolabile. Conveniently, Papain still functions at far lower than its optimum temperature, but with lower reaction rates. In our case, the lowest investigated temperature was sufficient.

To determine the IN concentration per gram of mycelium, the same setup and procedure as in Fröhlich-Nowoisky et al. (2015~~4~~) were applied: Each sample was diluted with ultrapure water to an INM concentration where Eq. (1) gives finite results (the proper dilution was determined by trial and error). Then, 50 µl aliquots of the dilute were pipetted into 24–32 wells of a 96 well PCR tray (Axon™), which was sealed with adhesive foil. The plate was inserted into an isolated PCR-plate thermal block, which was tempered by a cooling bath (Julabo™ Presto A30). For recording a nucleation spectrum, the block was cooled to an initial temperature of 269.15 or 270.15 K. Then the block was further cooled in 0.5 to 2 K steps each 12 minutes. After each step, the number of frozen droplets was counted. They can be discriminated from liquid droplets, since they reflect the incident light differently, and therefore appear much darker. We calculated the IN concentration (number of INs per grams of mycelium) via a variant of the Vali formula (Eq.(1), Vali 1971):

$$n_m[g^{-1}] = -\ln(1 - f_{ice}) \cdot \frac{V_{wash}}{V_{drop}} \cdot \frac{F_{dil}}{m_{myc}} \quad (1)$$

$n_m$ is the number of INMs per gram of mycelium, $f_{ice}$ the fraction of frozen droplets, $V_{wash}$ the volume of water added for washing (10 ml in this study), $V_{drop}$ the droplet volume in the freezing assay (0.05 ml in this study), $F_{dil}$ the dilution factor of the extract and $m_{myc}$ the mass of the mycelium. For the formula to work, a proper dilution, where $0<f_{ice}<1$ is fulfilled, is necessary. In case of $f_{ice}=0$, the dilution is too high, and the formula gives $n_m=0$ as a result. In case of $f_{ice}=1$, the sample is too concentrated, since $n_m$ becomes infinite. We note that Eq. (1) assumes that each droplet contains the same number of IN, i.e. the mean number of IN. However, at very small concentrations, the distribution of INMs in the droplets follows Poisson statistics, so that even for a mean number of one INM per droplet some droplets may contain two or more INMs and

others no INMs at all. Without the use of Poisson statistics all of these would be counted as one in the analysis (Augustin et al., 2013).

To quantify the efficacy of the new-found INMs of *A. implicatum* and *I. farinosa* in comparison with others, we used the *Soccer Ball Model* (Niedermeier et al., 2011, 2014), which combines *Classical Nucleation Theory* with the assumption of a contact angle distribution to calculate mean contact angles $\theta$ and standard deviations $\sigma$ from the 0.1 µm filtrate curves. This is done by determining values for $\theta$ and $\sigma$ such that the measured values of $f_{ice}$ are reproduced by the model. The corresponding equation describing the contact angle distribution and the Soccer Ball Model are given explicitly in Niedermeier et al. (2014). Via a mass-to-size conversion table for proteins by Erickson (2009), we estimated the diameter of our INMs to be about 4 nm, which was used for the *Soccer Ball Model* parameterization. In comparison, we also calculated mean $\theta$ and $\sigma$ of *M. alpina* from comparable filtrates (Fröhlich-Nowoisky et al., 2015~~4~~), and added literature data for INMs from birch pollen (Augustin et al., 2013) and bacteria (Niedermeier et al., 2014). The concept of contact angles has, in the past, been applied for ice nucleating particles consisting of mineral dust, for which reasonable results were obtained (e.g. ~~Niedermeier et al., 2014~~Marcolli et al., 2007, Welti et al., 2012). Here we apply it to describe the ice nucleation induced by water-soluble INMs, and we were able to derive contact angle distributions such that all measured data can be reproduced by the *Soccer Ball Model*. More specifically, a contact angle distribution determined for a sample reproduced all measurements done for that sample, even if different concentrations, different cooling times or completely different measurement approaches, as those described in the following paragraphs, were used.

INA was also measured with two additional experimental techniques. For both setups, 0.1 µm filtrates that were prepared as described at the top of this section were diluted and applied. These two additional methods were included to expand the data to lower temperatures, which was possible due to the smaller droplet sizes these methods examined (BINARY droplet volumes are about 1 µl) and to ensure that a possible interaction between the examined droplets and the substrates did not influence the results (LACIS examined freely floating droplets). Resulting values for $n_m$ are compared to the $n_m$ derived from the conventional freezing droplet array. Those systems are:

(i) A droplet freezing array termed "Bielefeld Ice Nucleation ARraY" (BINARY), which consists of a 6x6 array of microliter droplets on a hydrophobic glass slide on top of a Peltier cooling stage. A detailed description of the technique, the preparation of droplets, and the data acquisition and evaluation is given in Budke and Koop (~~2014~~2015).

(ii) A vertical flow tube named "Leipzig Aerosol Cloud Interaction Simulator" (LACIS), which is described in detail in Hartmann et al. (2011). Basically, droplets are generated from the filtrate and dried. The residual particles are then size-selected, humidified to form uniform droplets and inserted into the tube, where they are cooled to the temperature of interest. The procedure was similar to that for the birch pollen washing waters described in Augustin et al. (2013).

## 2.2 Characterization of birch pollen INMs

To test the hypotheses that birch pollen INMs are polysaccharides and not proteins (Pummer et al., 2012), further procedures for characterization of the birch pollen INMs were carried out. Therefore, birch pollen extracts were prepared by suspending and shaking 10 mg/ml pollen in ultrapure water for several hours, and then vacuum filtering the suspension through a 0.1 µm PES filter (Corning™). The aqueous fraction was then exposed to different treatments, and $n_m$ was determined the same way as for the fungi, with 24 or 32 droplets per sample, at 258 K or 256 K. In all cases, reference samples without addition of the reagents were measured and defined as 100% INA. The results are listed in Table 2.

First, boric acid was added to an aliquot of fungal extract to a concentration of 0.75 M. The aliquot was left overnight at room temperature, as boric acid is known to esterify with sugars. This treatment should alter the INA of the birch pollen INMs, in case that saccharides play a role. However, since the esterification process does not necessarily affect all functional groups, the INA might be only partially eliminated. Since the INA assay preparation has a certain statistical uncertainty, minor changes in the INA are difficult to interpret. Therefore, we also investigated untreated birch pollen extracts as a reference. The same procedure was repeated with heating aliquots with and without boric acid to 343 K for 2 h to accelerate the esterification process.

To check if birch pollen INMs are indeed non-proteinaceous, three separate 100 µl aliquots were mixed with 94 µl of (i) water, (ii) medium without enzyme, (iii) medium with Trypsin, and incubated for 18 h at 310 K. Additionally, 100 µl water was treated like (iii). Trypsin is an enzyme that breaks down proteins, but demands a certain medium. For each sample an INA assay as described in Sect. 2.1 was run.

In addition, aliquots of the birch pollen extracts digested with Trypsin and medium before and after incubation were forwarded through a Size Exclusion Chromatography (SEC) column, and the different eluted fractions were tested for their INA. Details about the setup and the measurements are presented in Sect. S2.2. This way, we checked if the enzymatic treatment changed the mass range of the birch pollen INMs.

### 2.3 Ice nucleation experiments with bacterial INM peptides

A sample of the 16-amino acid peptide fragment which is the repetitive element in the bacterial INM (BINM) of *Pseudomonas syringae* was investigated for its INA. The peptide with the primary sequence GSTQTAGEESSLTAGY was obtained from PSL (Heidelberg, Germany) and purified chromatographically using a HiTrap Desalting column (GE Healthcare) with high-purity water (18.2 MΩ·cm) from a Milli-Q water purification system (Millipore). The yield of pure peptide was determined using a NanoPhotometer ($\varepsilon_0$ = 1490 $M^{-1}cm^{-1}$).

We measured peptide solutions with 10, 20, and 30 mg/ml via the oil immersion cryo-microscopic method, which is described in detail in Pummer et al. (2012). Therefore we prepared emulsions consisting of 45%wt aqueous peptide solution and 55%wt oil (paraffin-lanolin). The frozen fractions of droplets with diameters of 20–50 µm were documented with the software Minisee© as a function of temperature.

## 3 Results

### 3.1 Experimental characterization of INMs

The results of the chemical characterization of the fungal filtrates are composed in Fig. 2. The quantitative passage through the 0.1 µm pore size filters, yielding optically transparent, particle-

free filtrates, demonstrates that those INMs are cell-free and stay in solution, when they are extracted with water.

The initial freezing temperature was 269 K for *I. farinosa* and 264 K for *A. implicatum*. The calculated contact angles for *I. farinosa* and *M. alpina* are the highest, while the one of *A. implicatum* lies in the range of the BINM one (Table 1). The reduction of INA by Papain and by guanidinium chloride indicates that the INMs of both species are proteinaceous. Lipids seem to play a role in *A. implicatum*, but none in *I. farinosa*. Both were resistant against boric acids, making a contribution of carbohydrates to the INA unlikely. Both INMs are more heat sensitive than other fungal INMs, since they were already destroyed at 333 K. *A. implicatum* has a mass of approximately 100 to 300 kDa, since it quantitatively passes through the 300 kDa filter, but not through the 100 kDa filter. About 95% of *I. farinosa* INM were retained in the 300 kDa filter in comparison to the 0.1 µm filter, and the initial freezing temperature is shifted below 268 K. This suggests that there are larger, more active states of *I. farinosa* INMs and smaller ones active at lower temperatures.

Figure 3 shows the comparison between the data from BINARY, LACIS, and the droplet freezing array (Sect. 2.1). Each strain shows a relatively good overlap of the plateaus obtained with the different methods. Only when comparing the C-strain measurements, a difference in total $n_m$ can be seen, which, however, is less than one order of magnitude. The initial freezing temperatures are higher for the conventional droplet freezing array in Mainz in comparison with BINARY. This may indicate that the investigated INMs show a small time-dependence, which would lead to an increase in $n_m$ at lower temperature for the experiment with the larger cooling rate (i.e. BINARY), in agreement with the observations. From that it becomes evident that onset temperatures, which were often reported in the past, do not properly describe the ice nucleation process. They depend on the detection limit of the measurement method, as well as the INM content per droplet, and they are influenced by impurities or statistical outliers. Hence, the temperature at which 50% of all droplets froze ($T_{50}$) was taken for interpretation. If we assume that the mass of a single INM is about 100 kDa = $1.7 \cdot 10^{-19}$ grams and that the maximum number density we found was $n_m = 10^{10}$ per gram (Fig. 3), the INMs amount to approximately 1.7 ppb of the total mycelium mass.

The results of the birch pollen measurements, which are given in Table 2, suggest that both the medium for the Trypsin test and the boric acid led to a reduction in INA. The addition of Trypsin had no additional effect, which speaks against a proteinaceous nature of those INMs. It is most likely that it is the formic acid from the medium that decreases the INA in the respective measurement, since it esterifies with hydroxyls similar to the boric acid. This is consistent with the resistance against other proteases and guanidinium chloride (Pummer et al., 2012), and the lack of the spectroscopic signature typical for proteins in the most active eluates. Overall, we confirm that the birch pollen INMs are not proteins, but most likely polysaccharides. After the elution from the SEC column, small amounts of INMs were spread across all fractions of the eluate. This might be caused by the adhesion of the organic matter in the extracts to the column packing, what undermines the separation principle. The tendency for adhesion of organic matter from pollen was already investigated by Pummer et al. (2013b). Nevertheless, there was an unambiguous maximum in the 335 to 860 kDa fraction before and after digestion. This is the more intriguing, since we recorded the absorbance of the eluate at 280 nm via a UV detector, which is a quite reliable way to detect most proteins. However, the detector showed no signal when the INA maximum was eluted. This alone makes it very unlikely that the birch pollen INMs are proteinaceous. The discrepancy with the mass range stated by Pummer et al. (2012) could be explained by the slightly higher investigation temperatures, which was a necessity of the setup, which corresponds to a larger critical ice embryo or INM size. We suggest that the birch pollen INMs might be capable of forming aggregates that are larger, active at higher temperatures, but also less frequent. Consequently, they are overseen in INA assay devices with lower material loads per droplet, such as the oil immersion cryo-microscopy.

The examination shows that the 16-amino acid BINM peptide shows INA, when a certain concentration in solution is surpassed. This molecule should barely show INA, since its molecular mass is only 1.6 kDa and the number of fitting functional groups is limited to one TXT motif. However, these peptides tend to self-assemble into aggregates (Garnham et al. 2011), which consequently follow equilibrium of formation and decay. These aggregates may have different sizes and shapes, and consequently different INAs.

The 10 mg/ml sample showed only homogeneous ice nucleation. The 30 mg/ml sample showed an initial freezing temperature at about 250 K, a flat slope of $n_m(T)$ towards lower temperatures, and a $T_{50}$ between 240 and 245 K in different experiments. The variance is rather high, since the

aggregate formation seems to be very sensitive to the handling of the sample. This is in contrast to the typical biological INMs, which show a very steep slope at a given temperature and then reach a saturation plateau (e.g. Fig. 2 and 3). Further investigations are in progress to measure the aggregates and get a better understanding of the process.

### 3.2 Comparison with theoretical calculations of the critical ice embryo size

In Fig. 4, we plot the experimentally determined molecular masses of INMs against the observed ice nucleation temperature. For comparison, we show the theoretical parameterization of the critical ice embryo size by Zobrist et al. (2007), which is based on *Classical Nucleation Theory*. The sources of the plotted data are specified in Table 3. Apart from the fungal and birch pollen INMs investigated in our groups, we added BINM data by Govindarajan and Lindow (1988a), who already indicated the good agreement between aggregate size and critical ice embryo size. INA data of polyvinyl alcohol (PVA) were incorporated, since it also showed a slight INA in experiments (Ogawa et al., 2009). Its peculiarities are first that the formula is quite simple for a macromolecule, which is a sequence of $CH_2CHOH$-units, and second that the chain is rather randomly coiled. Therefore, the near-range molecular order is quite well defined, while the far-range order is merely statistical.

The data of birch pollen and fungal INMs are in appreciable agreement with the theoretical parameterization. We deduce that these free biological INMs which carry a suitable hydration shell mimic a theoretical ice embryo of the same size well enough to show the same INA. However, ice embryos of this size are almost impossible to form spontaneously, what explains the low temperatures that are necessary for homogeneous ice nucleation. In contrast, the biological INMs have a given shape, what explains their high INA.

In the case of PVA, we see that an increase in size does not lead to an appropriate increase in the freezing temperature. This can be easily explained by the different degrees of structure of biological macromolecules and technical homopolymers. Both PVA and BINMs consist of a sequence of monomers covalently linked to each other. Longer chains fold into compact three-dimensional structures. Without any further forces, polymers coil randomly. Therefore, confined geometries do not exceed the size of a few monomers, where it is the limited flexibility of the monomer-to-monomer bond that enforces certain geometries. Hence, an increase in the total

INM mass will not increase its INA. In contrast, intact proteins have a strongly determined folding, which is held together by intramolecular forces, and sometimes even forced on them by folding-supporting proteins. Therefore, a native protein's structure is stabilized in a certain geometry, as is the molecular surface. The unfolding of a biological macromolecule – a process called denaturation – changes many of its properties. This is also valid for the INA of INMs, and explains their deactivation by heat far below the temperatures where the covalent molecular bonds are broken. It is also responsible for the destruction of most INMs by the chaotropic guanidinium chloride. Summed up, randomly coiled INMs like PVA allocate only small, one-dimensional templates for ice nucleation (Fig. 1b) and therefore nucleate ice at very low temperatures (Fig. 4). On the other hand, molecules in long-range confined geometries, like the BINM, allocate stable two-dimensional surfaces as ice nucleating templates (Fig. 1c), which are larger and therefore nucleate at higher temperatures (Fig. 4). Also long-chained alcohols show appreciable INA, if they are crystallized in well-defined monolayers, depending on the chain length, the position of the OH group, and substitutions on the side chains (Popovitz-Biro et al., 1994).

# 4 Discussion

## 4.12 Previous findings on biological INMs

The already mentioned BINMs that have been found so far are a certain class of bacterial lipoglycoproteins that are fully sequenced and characterized (e.g. Abe et al., 1989). In some cases, biological INMs of one type or species show more than one freezing temperature in an ice nucleation spectrum. This variation in INA can be explained by the presence of different functional groups, foldings or aggregation states (e.g. Govindarajan and Lindow, 1988a, Augustin et al., 2013, Dreischmeier et al., 2014, this study). The presence of INMs seems to have certain advantages, which might be the motivation for certain species to produce them (Sect. S1.5).

The bacterial gene is highly conserved and codes for a 120 kDa β-helical membrane protein with many repeated octapeptides (Green and Warren, 1985, Abe et al., 1989, Kajava and Lindow, 1993, Schmid et al., 1997, Graether and Jia, 2001, Garnham et al., 2011). The INA induced by this protein also involves glycosides and lipids that stabilize it in the outer membrane of the

**Formatiert:** Tabstopps: Nicht an 1,24 cm

bacterial cell and assure its conformation for an optimum functioning (Kozloff et al., 1984, Govindarajan and Lindow, 1988a, Turner et al., 1991, Kawahara, 2002). With the side chains, the total mass of a single BINM is about 150–180 kDa (Table 1). It is assumed that the initiation point for ice formation is the amino acid sequence TXT in the repeated octapeptide, where T designates threonine and X any other amino acid. The OH groups of the two threonine moieties match the position of oxygen atoms in the ice lattice. Since a BINM contains several of these sequences at positions and distances that correspond to the ice lattice structure it can stabilize an ice embryo and so decrease the activation barrier for ice nucleation (Graether and Jia, 2001). As sequence modification studies on a structurally related antifreeze protein have shown, the loss of the TXT has a devastating effect on the interaction with water molecules, while other modifications have a much weaker impact (Graether et al., 2000).

The expression of BINMs is an exclusive property of certain bacterial species. It has been reported for a wide range of strains in the *P. syringae* species complex (Lindow et al., 1982, Berge et al. 2014), *P. fluorescens* and *borealis* (Fall and Schnell, 1985, Obata et al., 1987, Foreman et al., 2013), *Erwinia uredovora* (Obata et al., 1990a), *Pantoea agglomerans*, formerly called *E. herbicola* (Phelps et al., 1986,), *Pantoea ananatis* (Coutinho and Venter, 2009), *Xanthomonas campestris* (Kim et al., 1987), a *Pseudoxanthomonas* sp. (Joly et al., 2013), and more. The efficacy of their INA depends on both the strain and the cultural growth conditions, e.g. the available nutrients and the growth temperature (Rogers et al., 1987, Nemecek-Marshall et al., 1993, Fall and Fall, 1998). In most cases, these BINMs are aggregated and anchored in the outer cell membrane, where the strength of the INA depends on the aggregation state and the chemistry of the membrane (Govindarajan and Lindow, 1988a+b, Kozloff et al., 1991). However, free BINMs still show appreciable INA, although less than in the native state (Schmid et al., 1997). Since these complexes match the ice crystal lattice perfectly, these bacteria are the most active IN known at present.

These anchored aggregates of BINMs on the otherwise ice nucleation inactive cell surface are a demonstrative example of active sites on a larger IN, which is the micro-sized bacterial cell. In some cases, bacteria release their active sites carried on much smaller membrane vesicles. These are spherical pieces of the outer cellular membrane that are excised from the cell, a natural and common phenomenon in bacteria in general (Deatherage and Cookson, 2012). The expression of such vesicles with BINMs has been reported for *Pantoea agglomerans* / *E. herbicola* (Phelps et

al., 1986), *E. uredovora* (Kawahara et al., 1993), and *P. fluorescens* (Obata et al., 1993). *P. syringae* and *viridiflava* express such BINM-carrying vesicles only under certain growth conditions (Obata et al., 1990b, Pooley and Brown, 1990). For *P. putida*, the INA found in culture supernatants was associated with a 164 kDa lipoglycoprotein and had activity both as an IN and as an antifreeze protein. In this case, removal of the approximately 92 kDa of carbohydrates eliminated the INA, however, not the antifreeze properties (Xu et al., 1998).

INMs were also found in the kingdom of fungi (Kieft, 1988, Kieft and Ahmadjian, 1989, Kieft and Ruscetti, 1990, Pouleur et al., 1992, Hasegawa et al., 1994, Tsumuki and Konno, 1994, Tsumuki et al., 1995, Richard et al., 1996, Humphreys et al., 2001, Morris et al., 2013, Haga et al., 2013, Fröhlich-Nowoisky et al., ~~2014~~2015). Similarly to the bacteria, only a limited fraction of investigated strains showed INA, while the majority was inactive (Pouleur et al., 1992, Tsumuki et al., 1995, Iannone et al., 2011, Pummer et al., 2013a, Huffman et al., 2013, Fröhlich-Nowoisky et al., ~~2014~~2015). Fungal INMs can be divided into two subgroups, both of which differ from the BINMs. The INMs of rust fungi show properties of polysaccharide compounds (Morris et al., 2013). The already characterized INMs from *Rhizoplaca chrysoleuca* (Kieft and Ruscetti, 1990), *F. avenaceum* (Pouleur et al., 1992, Hasegawa et al., 1994, Tsumuki and Konno, 1994), and *M. alpina* (Fröhlich-Nowoisky et al., ~~2014~~2015) are evidently proteins, but show barely any other similarities with the BINMs. They are more tolerant to stresses, have a different amino acid sequence, seem to have less to no lipid and carbohydrate functionalizing, and are easily released from the cells. Only recently, a 49 kDa protein from *F. acuminatum* was suggested as being the INM (Lagzian et al., 2014).

Proteins and lipoproteins with INA were also found in extracellular fluids of insects like *Tipula trivittata* larvae (Duman et al., 1985, Neven et al., 1989, Duman et al., 1991, Warren and Wolber, 1991), *Vespula maculata* queens (Duman et al., 1984), and *Dendroides canadensis* larvae (Olsen and Duman, 1997). The only non-proteinaceous insect INs found up to date are phosphate spherules and fat cells in the larvae of *Eurosta solidaginis* (Mugnano et al. 1996). INs have also been detected in other animal taxa, e.g. amphibians (Wolanczyk et al., 1990) and mollusks (Aunaas, 1982, Hayes and Loomis, 1985, Madison et al., 1991, Lundheim, 1997), as well as in spider silk (Murase et al., 2001).

The fluid reservoirs of some succulent plants, namely *Lobelia telekii* and *Opuntia* species, contain polysaccharide INMs (Krog et al., 1979, Goldstein and Nobel, 1991, Goldstein and Nobel, 1994). Other reported non-proteinaceous plant INs are from the wood of *Prunus* species (Gross et al., 1988), or the lignin in a waste water sample (Gao et al., 1999). Among plant INs, only those of *Secale cereale* were identified as proteins (Brush et al., 1994). The pollen of some plant species showed appreciable INA in different lab studies, among which that of silver birch (*Betula pendula* or *alba*) was the most active one (Diehl et al., 2001, Diehl et al., 2002, von Blohn et al., 2005, Pummer et al., 2012, Augustin et al., 2013). The birch pollen contain easily extractable, very robust INMs, which are non-proteinaceous and most likely some type of polysaccharide (Pummer et al., 2012). The extracts were characterized via vibrational spectroscopy, which indicated that they contained sugar-like compounds, proteins, and other biological molecules, but no sporopollenin, which is the fabric of the outer pollen wall (Pummer et al., 2013b).

Other organic aerosols in the focus of ice nucleation research are Humic-Like Substances (HULIS) and Secondary Organic Aerosols (SOAs). They show certain similarities to the presented INMs, since they consist of a large variety of organic macromolecules that have undergone complex biochemical processing. Analogously, several exponents showed little to no INA in experiments, or even oppressed INA in mixed particles by blocking the active sites (e.g. Möhler et al., 2008, Prenni et al., 2009), while others showed appreciable INA. Certain HULIS standards (Wang and Knopf, 2011) and some SOAs (Wang et al., 2012, Schill et al., 2014) induced ice nucleation in the deposition and the immersion mode. The O/C-ratio of the latter did not affect the INA, although it influenced several other properties, such as the kinetics of the water uptake, in agreement with recent model simulations (Berkemeier et al., 2014). Among glassy aerosols composed of saccharidic components, some chemical species showed significant INA that might even compete with mineral dust INA in mid-latitude clouds (Wilson et al., 2012). Even a simple compound like citric acid shows INA when it is in the state of a glassy aerosol (Murray et al., 2010). Alternatively, other organic compounds such as oxalic acid can act as an immersion IN in the crystalline state (Zobrist et al., 2006, Wagner et al. 2011) Also cellulose, which is the most common biopolymer on Earth due to its ubiquity in plant cell walls, shows INA in the form of microcrystalline or fibrous particles (Hiranuma et al., 2015). The inorganic salt ammonium sulfate possesses INA in the crystalline state in both the immersion and

deposition mode, despite it being a highly soluble compound (Zuberi et al., 2001, Abbatt et al., 2006).

## 4.2. Solubility of INMs

In atmospheric science, INs are traditionally regarded as insoluble particles on the surface of which ice nucleation takes place. According to Raoult's law, soluble substances are expected to decrease the freezing point with increasing molar concentration. Furthermore, any ice nucleating template requires a certain size to be able to support a critical ice embryo that is large enough to grow into a macroscopic crystal. Consequently, particles that dissociate into molecules or ions with low molar mass in solution (e.g. NaCl, mono- and disaccharides) cannot act as IN. However, data by Pummer et al. (2012) showed that the ice nucleation active components of pollen have a mass between 100 and 300 kDa. This means, the INs have the size of single macromolecules. If these molecules are fully dissolved in water, one can regard them as being in solution and not in suspension. Many proteins are soluble in water (e.g. Osborne, 1910, Macedo, 2005; Sect. S1.1), but single protein molecules are far larger than e.g. salt ions or low molecular weight saccharides. Therefore, a deviation from the simplistic approach of Raoult's law is expectable. In this case, a soluble compound can also act as an IN, if the active molecular surface is large enough to stabilize ice embryos of critical size. The freezing point depression is expected to be rather weak for a dissolved >100 kDa molecule, because even a high mass concentration correlates with only a low molar concentration. The resulting small reduction of the solution's water activity is likely to affect the heterogeneous ice nucleation temperature only slightly (Sect. S1.4, Koop and Zobrist, 2009, Attard et al., 2012). Accordingly, certain macromolecules can act as IN in spite of being water-soluble, because the water-structuring effect over-compensates the colligative freezing point depression. Most molecules carry a well-defined hydration shell. In case of INMs, the geometry of water molecules in the hydration shell is supposedly similar to the geometry in an ice embryo, what triggers the freezing process (Fig. 1). We therefore emphasize that a more molecular view on IN allows a better understanding of the process of heterogeneous ice nucleation. ~~We see a link between this molecular view and the macroscopic view that is necessary for developing atmospheric models.~~ For example, the contact angle, which is useful in atmospheric models, is a macroscopic interpretation of the ~~molecular interaction~~ affinity between

two phases~~, which depends on the surface tension of~~: the ice embryo ~~growing on an~~and the IN. Water molecules show a high affinity towards hydroxy (-OH) and amino (-$NH_2$) groups, since they form hydrogen bonds with them. The contact angle quantifies the outcome of the molecular interaction and allows comparison of different INs, but it does not allow to trace back the complex molecular structures that are responsible. If we understand which structures are the characteristics of INMs, it will make predictions of INA possible for any macromolecule with a known sequence. As an example, the already-mentioned TXT-sequences (Sect. 4.1) are one such element that foster INA, but there have to be others as well, since non-proteinaceous INs exist. Classification and precise characterization of the currently known INs might reveal other INA elements.

As shown in Fig. 4, molecular size and INA exhibit a positive correlation. Deviations from the model line can be explained by different properties of different types of INMs. If molecules are larger than expected, like the birch pollen INMs, the active site might not cover the whole molecule, but just a small part of it. The INMs of *I. farinosa* and *M. alpina* seem to be too small. This can be either explained by spontaneous aggregation of several molecules after the filtration step, or by the ability of forming a larger hydration shell that has to be taken into account. Also, when data were derived from measurements in which droplets were examined which contain higher numbers of INM per droplet, the freezing temperature is shifted to higher temperatures, as can e.g. be seen when comparing data of birch pollen from Pummer et al. (2012) and Augustin et al. (2013). Very speculatively, one could try to go the other way and use experimentally determined freezing temperatures of IN, e.g. mineral dust and soot, to roughly estimate the size of their active sites. In combination with chemical and structural analyzing of the IN, one could try to identify which elements of these IN can be considered to be responsible for the INA. Considerations about the INA and active sites of mineral dust are given in Sect. S1.6.

### 4.3 Potential atmospheric effects

Apart from its cryobiological and evolutionary aspect, heterogeneous ice nucleation is of high importance for atmospheric research, since it causes cloud glaciation, and therefore impacts the global radiation budget (albedo) and initiates precipitation.

It is a common argument against the atmospheric INA potential of bioaerosols that whole cells which are at least some micrometers in size are far too large to reach altitudes higher than a few kilometers. However, the detection of cultivable microorganisms even in the mesosphere (Imshenetsky et al., 1978) shows that there have to be mechanisms that elevate intact cells to the higher atmosphere despite their size. As an example, the atmospheric turbulences caused by volcanic activity support a high- and far-range distribution of all kinds of aerosols (van Eaton et al., 2013). Furthermore, certain pollen (e.g. pine) and fungal spores (e.g. urediospores) are very buoyant, as they possess wing-like projections and other aerodynamic surface properties. Urediospores have been collected from the air at over 3 km above the ground level along with other microorganisms (Stakman and Christensen, 1946). Cultivable microorganisms ~~have also been collected from~~ are also present in the stratosphere (Griffin, 2004)~~. At last, microorganisms are present in precipitation~~ and in cloud water samples (e.g. Vaitilingom et al., 2012, Joly et al, 2013)~~, what indicates their presence at cloud formation altitudes~~. Even more intriguingly, some of these organisms are even able to proliferate in supercooled cloud droplets (e.g. Sattler et al., 2001).

Biological cells are not rigid spheres, but rather a composition of many different membranes, organelles and fluids, which further consist of many different molecules, ranging from water to small organic molecules and to biopolymers. Therefore, the release of molecular matter, as well as cell fragmentation, is common. Several studies detected molecular tracers from pollen grains and fungi in atmospheric fine particulate matter even in the absence of whole cells (e.g. Solomon et al., 1983, Yttri et al., 2007). In most cases, biological INMs are easily released from the producing cell (Table 1). Since a single primary biological particle can carry up to hundreds and thousands of INMs, and since the INMs are also much lighter, we expect their atmospheric concentration to be significantly higher as well. A possible mechanism of INM release is cell rupture caused by a rapid change in moisture. Scanning electron microscopy studies on wet pollen back up this idea by visualizing the release of organelles and organic matter (Grote et al., 2001, Grote et al., 2003, Pummer et al., 2013b). This explains why rainfall, which is expected to wash out aerosols, can indeed increase the concentration of allergens (Schäppi et al., 1999) or INs (Huffman et al., 2013) in the air. Recently, the presence of nanosized biological particles with INA were detected in precipitation (Santl-Temkiv et al., 2015) and soil (O'Sullivan et al., 2015).

Quantifying the atmospheric impact of fungi is even more difficult, as presumably 1 to 5 million fungal species exist (Hawksworth, 2001). Due to mutation and adaptation, every species consists of numerous strains, which differ in their INA (Tsumuki et al., 1995). Even if all studies are combined, it is only a minor fraction of all fungal species that have been tested for their INA. Furthermore, the expression of INMs is triggered by yet unknown conditions, which could be the availability of nutrients, the local climate or competition with other microorganisms. As a consequence, INA-positive strains can lose their activity when they are cultivated under laboratory conditions (Tsumuki et al., 1995, Pummer et al., 2013a). Therefore, more atmospheric IN counting and sampling will be necessary to understand the contribution of biological INA better.

Several former studies aimed at quantifying biological INs either by analyzing precipitation samples (Christner et al., 2008a+b), or by atmospheric modeling based on emission and deposition data (Hoose et al., 2010). In both cases, however, only whole cells were regarded. Christner et al. (2008a+b) filtered the particles of interest out of the samples, and so lost the molecular fraction, ~~to~~ which contains the INMs we described ~~belong~~. Hoose et al. (2010) did not include fragmentation or phase separation processes that can release molecular compounds from the carrier particles in the atmosphere. This might have led to an underestimation of the atmospheric relevance of biological INs.

## 5 Conclusions

Even free water-soluble macromolecules are able to nucleate ice, since they are in the same size range as the critical ice embryos. INMs can be diverse in chemical structure and origin, which may range from biopolymers in primary biological aerosols (proteins, saccharides, lipids, hybrid compounds), to secondary organic aerosol components (HULIS, etc.), to synthetic polymers (PVA).

The allocation of functional groups, as well as the confinement that keeps them in place, is essential for the efficacy of the INMs. An increase of the template size that can be realized by aggregation of single molecules leads also to an enhancement of the INA. In this study we have shown that the water-soluble INMs from the fungal species *A. implicatum* and *I. farinosa* are

proteins, and we have obtained additional evidence that the birch pollen INMs are polysaccharides without relevant protein content.

Water-soluble INMs are released by a wide range of biological species. They may be associated not only with primary biological aerosols but also with other atmospheric aerosol particles such as soil dust or sea spray. The potential effects of such INMs should be considered and pose an additional challenge in the quantification and assessment of the importance of biological ice nucleation in the atmosphere.

## Acknowledgement

We thank the Max Planck Society and the INUIT Research Unit (projects PO1013/5-1, KO2944/2-1, and WE4722/1-1; all for INUIT 1525) for project funding. We acknowledge M. Linden and D. Sebazungu for technical support, as well as Z. Dráb from Pharmallerga® for the donation of the birch pollen.

D. Niedermeier acknowledges financial support by the Alexander von Humboldt Foundation. H. Grothe and T. Loerting are grateful for support from the Austrian Science Fund FWF (projects P23027 and P26040). We thank the ESF for funding the workshops on ice nucleation within the research networking programme MicroDICE in the years 2013, 2014, and 2015.

**Formatiert:** Schriftart: (Standard) Times New Roman, 12 pt

**Formatiert:** Schriftart: (Standard) Times New Roman, 12 pt

**Formatiert:** Schriftart: (Standard) Times New Roman, 12 pt

## References

Abbatt, J. P. D., Benz, S., Cziczo, D. J., Kanji, Z., Lohmann, U., and Möhler, O.: Solid ammonium sulfate aerosols as ice nuclei: a pathway for cirrus cloud formation, Science, 22, 313, 1770-1773, doi:10.1126/science.1129726, 2006.

Attard, E., Yang, H., Delort, A.-M., Amato, P., Pöschl, U., Glaux, C., Koop, T., and Morris, C. E.: Effects of atmospheric conditions on ice nucleation activity of Pseudomonas, Atmos. Chem. Phys., 12, 10667-10677, doi:10.5194/acp-12-10667-2012, 2012.

Augustin, S., Wex, H., Niedermeier, D., Pummer, B., Grothe, H., Hartmann, S., Tomsche, L., Clauss, T., Voigtländer, J., Ignatius, K., and Stratmann, F.: Immersion freezing of birch pollen washing water, Atmos. Chem. Phys., 13, 10989-11003, doi:10.5194/acp-13-10989-2013, 2013.

Aunaas, T.: Nucleating agents in the haemolymph of an intertidal mollusc tolerant to freezing, Cell. Molec. Life Sci., 38, 1456-1457, 1982.

Berge, O., Monteil, C. L., Bartoli, C., Chandeysson, C., Guilbaud, C., Sands, D. C., and Morris, C. E.: A user's guide to a data base of the diversity of Pseudomonas syringae and its application to classifying strains in this phylogenetic complex, PLOS ONE, 9, e105547, doi:10.1371/journal.pone.010554, 2014.

Berkemeier, T., Shiraiwa, M., Pöschl, U. and Koop, T.: Competition between water uptake and ice nucleation by glassy organic aerosol particles, Atmos. Chem. Phys., 14, 12513–12531, doi:10.5194/acp-14-12513-2014, 2014.

Brush, R. A., Griffith, M., and Mlynarz, A.: Characterization and quantification of intrinsic ice nucleators in winter rye (Secale cereale) leaves, Plant Physiol.,104, 725-735, 1994.

Budke, C., and Koop, T.: BINARY: An optical freezing array for assessing temperature and time dependence of heterogeneous ice nucleation, Atmos. Meas. Tech. ~~Discuss.~~, ~~7~~8, ~~9137~~689 ~~–~~ ~~9172~~703, doi: 10.5194/amt-8-689-2015 ~~10.5194/amtd-7-9137-2014~~, ~~2014~~2015.

Burke, M. J., and Lindow, S. E.: Surface properties and size of the ice nucleation site in ice nucleation active bacteria: theoretical considerations, Cryobiol., 27, 88-84, 1990.

Christner, B. C., Cai, R., Morris, C. E., McCarter, K. S., Foreman, C. M., Skidmore, M. L., Montross, S. N., and Sands, D. C.: Geographic, seasonal, and precipitation chemistry influence on the abundance and activity of biological ice nucleators in rain and snow, Proc. Natl. Acad. Sci., 105, 18854–18859, 2008a.

Christner, B. C., Morris, C. E., Foreman, C. M., Cai, R. and Sands, D. C.: Ubiquity of biological ice nucleators in snowfall, Science, 319, 1214, 2008b.

Christner, B. C.: Bioprospecting for microbial products that affect ice crystal formation and growth, Appl. Microbiol. Biotech., 85, 481-489, 2010.

Coutinho, T. A., and Venter, S. N.: Pantoea ananatis: an unconventional plant pathogen, Molec. Plant. Pathol., 10, 325-335, 2009.

Deatherage, B. L., and Cookson, B. T.: Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life, Infect. Immun., 80, 1948-

1957, 2012.

Diehl, K., Quick, C., Matthias-Maser, S., Mitra, S. K., and Jaenicke, R.: The ice nucleation ability of pollen Part I: Laboratory studies in deposition and condensation freezing modes, Atmos. Res., 58, 75-87, 2001.

Diehl, K., Matthias-Maser, S., Jaenicke, R., and Mitra, S. K.: The ice nucleation ability of pollen Part II. Laboratory studies in immersion and contact freezing modes, Atmos. Res., 61, 125-133, 2002.

Dreischmeier, K., Budke, C., and Koop, T.: Investigation of heterogeneous ice nucleation in pollen suspensions and washing water, EGU2014-8653, 2014.

Duman, J. G., Morris, J. P., and Castellino, F. J.: Purification and composition of an ice nucleating protein from queens of the hornet, Vespula maculata, J. Comp. Physiol. B, 154, 79-83, 1984.

Duman, J. G., Neven, L. G., Beals, J. M., Olson, K. R., and Castellino, F. J.: Freeze-tolerance adaptations, including haemolymph protein and lipoprotein nucleators, in the larvae of the cranefly Tipula trivittata, J. Insect Physiol., 31, 1-8, 1985.

Duman, J. G., Wu, D. W., Wolber, P. K., Mueller, G. M., and Neven, L. G.: Further characterization of the lipoprotein ice nucleator from freeze tolerant larvae of the cranefly Tipula trivittata, Comp. Biochem. Physiol., 99B, 599-607, 1991.

Edwards, G. R., Evans, L. F., and La Mer, V. K.: Ice nucleation by monodisperse silver iodide particles, J. Colloid Sci., 17, 749-758, doi:10.1016/0095-8522(62)90049-1, 1962.

Erickson, H. P.: Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy, Biol. Procedures Online, 11, 32-51, doi:10.1007/s12575-009-9008-x, 2009.

Fall, A. L., and Fall, R.: High-level expression of ice nuclei in Erwinia herbicola is induced by phosphate starvation and low temperature, Curr. Microbiol., 36, 370-376, 1998.

Fall, R., and Schnell, R. C.: Association of an ice-nucleating pseudomonad with cultures of the marine dinoflagellate, Heterocapsa niei, J. Marine Res., 43, 257-265, 1985.

Foreman, C. M., Cory, R. M., Morris, C. E., SanClements, M. D., Lisle, J., Miller, P. L., Chin, Y.

C., and McKnight, D. M.: Microbial growth under humic-free conditions in a supraglacial stream system on the Cotton Glacier, Antarctica, Environ. Res. Lett., 8, 035022, doi:10.1088/1748-9326/8/3/0305022, 2013.

Fröhlich-Nowoisky, J., Hill, T. C. J., Pummer, B. G., Yordanova, P., Franc, G. D., and Pöschl, U.: Ice nucleation activity in the widespread soil fungus Mortierella alpina, Biogeosci. ~~Discuss.~~, 12~~1~~, 1057~~2697~~ -1071~~2731~~, doi:10.5194/bg~~d~~-12~~1~~-1057~~2697~~-2015~~4~~, 2015~~4~~.

Fukuta, N.: Experimental studies of organic ice nuclei, J. Atmos. Sci., 23, 191-196, 1966.

Gao, W., Smith, D. W., and Sego, D. C.: Ice nucleation in industrial wastewater, Cold Regions Sci. Tech., 29, 121-133, 1999.

Garnham, C. P., Campbell, R. L., Walker, V. K., and Davies, P. L.: Noveldimeric β-helical model of an ice nucleation protein with bridged active sites, BMC Struct. Biol., 11, 36, doi:10.1186/1472-6807-11-36, 2011.

Goldstein, G., and Nobel, P. S.: Changes in osmotic pressure and mucilage during low-temperature acclimation of Opuntia ficus-indica, Plant Physiol., 97, 954-961, 1991.

Goldstein, G., and Nobel, P. S.: Water relations and low-temperature acclimation for cactus species varying in freezing tolerance, Plant Physiol., 104, 675-681, 1994.

Govindarajan, A. G., and Lindow, S. E.: Size of bacterial ice-nucleation sites measured in situ by radiation inactivation analysis, Proc. Natl. Acad. Sci. USA, 85, 1334-1338, 1988a.

Govindarajan, A. G., and Lindow, S. E.: Phospholipid requirement for expression of ice nuclei in Pseudomonas syringae and in vitro, J. Biol. Chem., 263, 9333-9338, 1988b.

Graether, S. P., Kulper, M. J., Gagné, S. M., Walker, V. K., Jia, Z., Sykes, B. D., and Davies, P. L.: β-helix structure and ice-binding properties of a hyperactive antifreeze protein from an insect, Nature, 406, 325-328, 2000.

Graether, S. P., and Jia, Z.: Modeling Pseudomonas syringae ice-nucleation proteins as a β-helical protein, Biophys. J., 80, 1169-1173, 2001.

Green, R. L., and Warren, G. J.: Physical and functional repetition in a bacterial ice nucleation gene, Nature, 317, 645-648, 1985.

Griffin, D. W.: Terrestrial microorganisms at an altitude of 20.000 m in Earth's atmosphere,

Aerobiologia, 20, 135–140, 2004.

Gross, D. C., Proebsting, E. L., and Maccrindle-Zimmermann, H.: Development, distribution, and characteristics of intrinsic, nonbacterial ice nuclei in Prunus wood, Plant Physiol., 88, 915-922, 1988.

Grote, M., Vrtala, S., Niederberger, V., Wiermann, R., Valenta, R., and Reichelt, R.: Release of allergen-bearing cytoplasm from hydrated pollen: A mechanism common to a variety of grass (Poaceae) species revealed by electron microscopy, J. Allerg. Clin. Immunol., 108, 109-115, doi:10.1067/mai.2001.116431, 2001.

Grote, M., Valenta, R., and Reichelt, R.: Immunogold scanning electron microscopy of abortive pollen germination: how birch, hazel, and alder release allergenic particles into the atmosphere, Microsc. Microanal., 9, Suppl. S03, 402-403, doi:10.1017/S1431927603034020, 2003.

Gurian-Sherman, D., and Lindow, S. E.: Differential effects of growth temperature on ice nuclei active at different temperatures that are produced by cells of Pseudomonas syringae, Cryobiol., 32, 129-138, 1995.

Haga, D. I., Burrows, S. M., Iannone, R., Wheeler M. J., Mason, R. H., Chen, J., Polishchuk, E. A., Pöschl, U., and Bertram, A. K.: Ice nucleation by fungal spores from the classes Agaricomycetes, Ustilaginomycetes, and Eurotiomycetes, and the effect on the atmospheric transport of these spores, Atmos. Chem. Phys., 14, 8611-8630, doi:10.5194/acp-14-8611-2014, 2014.

Hartmann, S., Niedermeier, D., Voigtländer, J., Clauss, T., Shaw, R. A., Wex, H., Kiselev, A., and Stratmann, F.: Homogeneous and heterogeneous ice nucleation at LACIS: operating principle and theoretical studies, Atmos. Chem. Phys., 11, 1753-1767, doi:10.5194/acp-11-1753-2011, 2011.

Hasegawa, Y., Ishihara, Y., and Tokuyama, T.: Characteristics of ice-nucleation activity in Fusarium avenaceum IFO 7158, Biosci. Biotech. Biochem., 58, 2273-2274, 1994.

Hawksworth, D. L.: The magnitude of fungal diversity: the 1–5 million species estimate revisited, Mycol. Res., 105, 1422-1432, doi:10-1017/S0953756201004725, 2001.

Hayes, D. R., and Loomis, S. H.: Evidence for a proteinaceous ice nucleator in the hemolymph of the pulmonate gastropod, Melampus bidentatus, Cryo Lett., 6, 418-421, 1985.

Hiranuma, N., Möhler, O., Yamashita, K., Tajiri, T., Saito, A., Kiselev, A., Hoffmann, N., Hoose, C., Jantsch, E., Koop, T., and Muramaki, M.: Ice nucleation by cellulose and its potential contribution to ice formation in clouds, Nat. Geosci., ~~*accepted*~~, doi:10.1038/ngeo2374, 2015.

Hoose, C., Kristjánsson, J. E., and Burrows, S. M.: How important is biological ice nucleation in clouds on a global scale? Environ. Res. Lett., 5, doi:10.1088/1748-9326/5/2/024009, 2010.

Huffman, J. A., Prenni, A. J., DeMott, P. J., Pöhlker, C., Mason, R. H., Robinson, N. H., Fröhlich-Nowoisky, J., Tobo, Y., Després, V. R., Garcia, E., Gochis, D. J., Harris, E., Müller-Germann, I., Ruzene, C., Schmer, B., Sinha, B., Day, D. A., Andreae, M. O., Jimenez, J. L., Gallagher, M., Kreidenweis, S. M., Bertram, A. K., and Pöschl, U.: High concentrations of biological aerosol particles and ice nuclei during and after rain, Atmos. Chem. Phys., 13, 6151-6164, doi:10.5194/acp-13-6151-2013, 2013.

Humphreys, T. L., Castrillo, L. A., and Lee, M. R.: Sensitivity of partially purified ice nucleation activity of Fusarium acuminatum SRSF 616, Curr. Microbiol., 42, 330-338, doi:10.1007/s002840010225, 2001.

Iannone, R., Chernoff, D. I., Pringle, A., Martin, S. T., and Betram, A. K.: The ice nucleation ability of one of the most abundant types of fungal spores found in the atmosphere, Atmos. Chem. Phys., 11, 1191-1201, doi:10.5194/acp-11-1191-2011, 2011.

Imshenetsky, A. A., Lysenko, S. V., and Kazakov, G. A.: Upper boundary ofthe biosphere, Appl. Environ. Microbiol., 35, 1-5, 1978.

Jayaweera, K., and Flanagan, P.: Investigations on biogenic ice nuclei in the Arctic atmosphere, Geophys. Res. Lett., 9, 94-97, 1982.

Joly, M., Attard, E., Sancelme, M., Deguillaume, L., Guilbaud, C., Morris, C. E., Amato, P., and Delort, A. M.: Ice nucleation activity of bacteria isolated from cloud water, Atmos. Environ., 70, 392-400, 2013.

Kajava, A. V., and Lindow, S. E.: A model of the three-dimensional structure of ice nucleation proteins, J. Mol. Biol., 232, 709-717, doi:10.1006/jmbi.1993.1424, 1993.

Katz, U.: Wolkenkammeruntersuchungen der Eiskeimbildungsaktivität einiger ausgewählter Stoffe, Zeitschr. Angew. Math. Phys., 13, 333-358, 1962. (in German)

**Formatiert:** Englisch (USA)

**Formatiert:** Schriftart: Kursiv

Kawahara, H., Mano, Y., and Obata, H.: Purification and characterization of extracellular ice-nucleating matter from Erwinia uredovora KUIN-3, Biosci. Biotech. Biochem., 57, 1429-1432, 1993.

Kawahara, H.: The structures and functions of ice crystal-controlling proteins from bacteria, J. Biosci. Bioengineer., 94, 492-496, 2002.

Kieft, T. L.: Ice nucleation activity in lichens, Appl. Environ. Microbiol., 54, 1678-1681, 1988.

Kieft, T. L., and Ahmadjian, V.: Biological ice nucleation activity in lichen mycobionts and photobionts, Lichenol.,21, 355-362, 1989.

Kieft, T. L., and Ruscetti, T.: Characterization of biological ice nuclei from a lichen, J. Bacteriol.,172, 3519-3523, 1990.

Kim, H. K., Orser, C., Lindow, S. E., and Sands, D. C.: Xanthomonas campestris pv. translucens strains active in ice nucleation, Plant Disease, 71, 994-997, 1987.

Koop, T.: Homogeneous Ice Nucleation in Water and Aqueous Solutions, Zeitschrift für Phys. Chemie, 218, 1231-1258, doi:10.1524/zpch.218.11.1231.50812, 2004.

Koop, T., and Zobrist, B.: Parameterizations for ice nucleation in biological and atmospheric systems, Phys. Chem. Chem. Phys., 11, 10741-11064, doi:10.1039/b914289d, 2009.

Kozloff, L. M., Lute, M., and Westaway, D.: Phosphatidylinositol as a component of the ice nucleating site of Pseudomonas syringae and Erwinia herbicola, Science, 226, 845-846, doi:10.1126/science.226.4676.845, 1984.

Kozloff, L. M., Turner, M. A., Arellano, F., and Lute, M.: Formation of bacterial membrane ice-nucleating lipoglycoprotein complexes, J. Bacteriol., 173, 2053-2060, 1991.

Lagzian, M., Latifi, A. M., Bassami, M. R., Mirzaei, M.: An ice nucleation protein from Fusarium acuminatum: cloning, expression, biochemical characterization and computational modeling, Biotechnol. Lett., 36, 2043-2051, doi:10.1007/s10529-014-1568-4, 2014.

Lindow, S. E., Amy, D. C., and Upper, C. D.: Bacterial ice nucleation - a factor in frost injury to plants, Plant Physiol., 70, 1084-1089, 1982.

Liou, Y. C., Tocilj, A., Davies, P. L., and Jia, Z.: Mimicry of ice structure by surface hydroxyls and water of a β-helix antifreeze protein, Nature, 406, 322-325, 2000.

Lundheim, R.: Ice nucleation in the blue mussel (Mytilus edulis), Marine Biol., 128, 267-271, 1997.

Macedo, E. A.: Solubility of amino acids, sugars, and proteins, Pure Appl. Chem., 77, 559-568, doi:10.1351/pac200577030559, 2005.

Madison, D. L., Scrofano, M. M., Ireland, D. C., and Loomis, S. H.: Purification and partial characterization of an ice nucleator protein from the intertidal gastropod, Melampus bidentatus, Cryobiol.,28, 483-490, 1991.

Marcolli, C., Gedamke, S., Peter, T., and Zobrist, B.: Efficiency of immersion mode ice nucleation on surrogates of mineral dust, Atmos. Chem. Phys., 7, 5081-5091, doi:10.5194/acp-7-5081-2007, 2007.

Möhler, O., Benz, S., Saathoff, H., Schnaiter, M., Wagner, R., Schneider, J., Walter, S., Ebert, V., and Wagner, S.: The effect of organic coating on the heterogeneous ice nucleation efficiency of mineral dust aerosols, Environ. Res. Lett., 3, 025007, doi:10.1088/1748-9326/3/2/025007, 2008.

Morris, C. E., Sands, D. C., Glaux, C., Samsatly, J., Asaad, S., Moukahel, A. R., Goncalves, F. I. T., and Bigg, K. E.: Urediospores of rust fungi are ice nucleation active at > –10°C and harbor ice nucleation active bacteria, Atmos. Chem. Phys., 13, 4223-4233, 2013.

Morris, C. E., Conen, F., Huffman, J. A., Phillips, V., Pöschl, U., and Sands, D. C: Bioprecipitation: A feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere, Global Change Biol., 20, 341-351, 2014.

Mugnano, J. A., Lee, R. E., and Taylor, R. T.: Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larvae of the gall fly Eurosta solidaginis (Diptera, Tephritidae), J. Exp. Biol., 199, 465-471 (1996).

Murase, N., Ruike, M., Matsunaga, N., Hayakawa, M., Kaneko, Y., and Ono, Y.: Spider silk has an ice nucleation activity, Naturwissenschaften, 88, 117-118, 2001.

Murray, B. J., Wilson, T. W., Dobbie, S., Cui, Z., Al-Jumur, S. M. R. K., Möhler, O., Schnaiter, M., Wagner, R., Benz, S., Niemand, M., Saathoff, H., Ebert, V., Wagner, S., and Kärcher B.: Heterogeneous nucleation of ice particles on glassy aerosols under cirrus conditions, Nat. Geosci., 3, 233-237, doi:10.1038/ngeo817, 2010.

**Formatiert:** Schriftart: (Standard) Times New Roman, 12 pt

Nemecek-Marshall, M., LaDuca, R., and Fall, R.: High-level expression of ice nuclei in a Pseudomonas syringae strain is induced by nutrient limitation and low temperature, J. Bacteriol., 175, 4062-4070, 1993.

Neven, L. G., Duman, J. G., Low, M. G., Sehl, L. C., and Castellino, F. J.: Purification and characterization of an insect hemolymph lipoprotein ice nucleator: evidence for the importance of phosphatidylinositol and apolipoprotein in the ice nucleator activity, J. Comp. Physiol. B, 159, 71-82, 1989.

Niedermeier, D., Shaw, R. A., Hartmann, S., Wex, H., Clauss, T., Voigtländer, J., and Stratmann, F.: Heterogeneous ice nucleation: exploring the transition from stochastic to singular freezing behavior, Atmos. Chem. Phys., 11, 8767–8775, doi:10.5194/acp-11-8767-2011, 2011.

Niedermeier, D., Ervens, B., Clauss, T., Voigtländer, J., Wex, H., Hartmann, S., and Stratmann, F.: A computationally efficient description of heterogeneous freezing: A simplified version of the Soccer ball model, Geophys. Res. Lett., 41, 736-741, 2014.

Obata, H., Saeki, Y., Tanishita, J., Tokuyama, T., Hori, H., and Higashi, Y.: Identification of an ice-nucleating bacterium KUIN-1 as Pseudomonas fluorescens and its ice nucleation properties, Agric. Biol. Chem., 51, 1761-1766, 1987.

Obata, H., Takinami, K., Tanishita, J., Hasegawa, Y., Kawate, S., Tokutama, T., and Ueno, T.: Identification of a new ice-nucleating bacterium and its ice nucleation properties, Agric. Biol. Chem. 53, 725-730, 1990a.

Obata, H., Takeuchi, S., and Tokuyama, T.: Release of cell-free ice nuclei from Pseudomonas viridiflava with a Triton X-100/EDTA system and their ice nucleation properties, J. Ferment. Bioengineer., 70, 308-312, 1990b.

Obata, H., Tanaka, T., Kawahara, H., and Tokuyama, T: Properties of cell-free ice nuclei from ice nucleation-active Pseudomonas fluorescens KUIN-1, J. Ferment. Bioengineer., 76, 19-24, 1993.

Ogawa, S., Koga, M., and Osanai, S.: Anomalous ice nucleation behavior in aqueous polyvinyl alcohol solutions, Chem. Phys. Lett., 480, 86-89, 2009.

Olsen, T. M., and Duman, J. G.: Maintenance of the supercooled state in overwintering pyrochroid beetle larvae, Dendroides canadensis: role of hemolymph ice nucleators and antifreeze proteins, J. Comp. Physiol. B, 167, 105-113, 1997.

Osborne, T. B.: Die Pflanzenproteine, Ergebnisse der Physiologie, 10, 47-215, 1910. (in German)

O'Sullivan, D., Murray, B. J., Ross, J. F., Whale, T. F., Price, H. C., Atkinson, J. D., Umo, N. S., and Webb, M. E.: The relevance of nanoscale biological fragments for ice nucleation in clouds, Scientific Reports, 5, 8082, doi:10.1038/srep08082, 2015.

Phelps, P., Giddings, T. H., Prochoda, M., and Fall, R.: Release of cell-free ice nuclei by Erwinia herbicola, J. Bacteriol., 167, 496-502, 1986.

Pooley, L., and Brown, T. A.: Preparation of active cell-free ice nuclei from Pseudomonas syringae, Proc. R. Soc. Lond. B, 24, 112-115, 1990.

Popovitz-Biro, R., Wang, J. L., Majewski, J., Shavit, E., Leiserowitz, L., and Lahav, M.: Induced freezing of supercooled water into ice by self-assembled crystalline monolayers of amphiphilic alcohols at the air-water interface, J. Am. Chem. Soc., 116, 1179-1191, 1994.

Pouleur, S., Richard, C., Martin, J. G., and Antoun, H.: Ice nucleation activity in Fusarium acuminatum and Fusarium avenaceum, Appl. Environ. Microbiol., 58, 2960-2964, 1992.

Prenni, A. J., Petters, M. D., Faulhaber, A., Carrico, C. M., Ziemann, P. J., Kreidenweis, S. M., and DeMott, P. J.: Heterogeneous ice nucleation measurements of secondary organic aerosol generated from ozonolysis of alkenes, Geophys. Res. Lett., 36, L06808, doi:10.1029/2008GL036957, 2009.

Pummer, B. G., Bauer, H., Bernardi, J., Bleicher, S., and Grothe, H.: Suspendable macromolecules are responsible for ice nucleation activity of birch and conifer pollen, Atmos. Chem. Phys., 12, 2541-2550, doi:10.5194/acp-12-2541-2012, 2012.

Pummer, B. G., Atanasova, L., Bauer, H., Bernardi, J., Druzhinina, I. S., Fröhlich-Nowoisky, J., and Grothe, H.: Spores of many common airborne fungi reveal no ice nucleation activity in oil immersion freezing experiments, Biogeosci., 10, 8083-8091, doi:10.5194/bg-10-8083-2013, 2013a.

Pummer, B. G., Bauer, H., Bernardi, J., Chazallon, B., Facq, S., Lendl, B., Whitmore, K., and Grothe, H.: Chemistry and morphology of dried-up pollen suspension residues, J. Raman Spectroscopy, 44, 1654-1658, doi:10.1002/jrs.4395, 2013b.

Richard, C., Martin, J. G., and Pouleur, S.: Ice nucleation activity identified in some

pythopathogenic Fusarium species, Phytoprotection, 77, 83-92, 1996.

Rogers, J. S., Stall, R. E., and Burke, M. J.: Low-temperature conditioning of the ice nucleation active bacterium, Erwinia herbicola, Cryobiol., 24, 270-279, 1987.

Santl-Temkiv, T., Sahyoun, M., Finster, K., Hartmann, S., Augustin, S., Stratmann, F., Wex, H., Clauss, T., Nilsen N. W., Sörensen, J. H., Korsholm, U. S., Wick, L. Y., and Karlson, U. G.: Characterization of airborne ice-nucleation-active bacteria and bacterial fragments, Atmos. Environ., *in press*, doi:10.1016/j.atmosenv.2015.02.060, 2015.

Sattler, B., Puxbaum, H., and Psenner, R.: Bacterial growth in supercooled cloud droplets, Geophys. Res. Lett., 28, 239-242, doi:10.1029/2000GL011684, 2001.

Schäppi, G. F., Suphioglu, C., Taylor, P. E., and Knox, R. B.: Concentrations of the major birch tree allergen Betv1 in pollen and respirable fine particles in the atmosphere, J. Allerg. Clin. Immunol., 100, 656-661, 1997.Schill, G. P., De Haan, D. O., and Tolbert, M. A.: Heterogeneous ice nucleation on simulated secondary organic aerosol, Environ. Sci. Technol., 48, 1675-1682, doi:10.1021/es4046428, 2014.

Schmid, D., Pridmore, D., Capitani, G., Battistutta, R., Neeser, J. R., and Jann, A.: Molecular organization of the ice nucleation proteinInaV from Pseudomonas syringae, FEBS Lett., 414, 590-594, 1997.

Solomon, W. R., Burge, H. A., and Muilenberg, M. L.: Allergen carriage by atmospheric aerosol. I. Ragweed pollen determinants in smaller micronic fractions, J. Allerg. Clin. Immunol.,72, 443-447, 1983.

Stakman, E., and Christensen, C. M.: Aerobiology in relation to plant disease, Botan. Rev., 12, 205-253, 1946.

Tsumuki, H., and Konno, H.: Ice nuclei produced by Fusarium sp. isolated from the gut of the rice stem borer, Chilo suppressalis Walker (Lepidoptera: Pyralidae), Biosci. Biotech. Biochem., 58, 578-579, 1994.

Tsumuki, H., Yanai, H., and Aoki, T.: Identification of ice-nucleating active fungus isolated from the gut of the rice stem borer, Chilo suppressalis Walker (Lepidoptera: Pyralidae) and a search for ice-nucleating active Fusarium species, Ann. Phytopathol. Soc. Jpn., 61, 334-339, 1995.

**Formatiert:** Englisch (USA)

**Formatiert:** Englisch (USA)

**Formatiert:** Schriftart: Kursiv

Turner, M. A., Arellano, F., and Kozloff, L. M.: Components of ice nucleation structures of bacteria, J. Bacteriol., 173, 6515-6527, 1991.

Vaitilingom, M., Attard, E., Gaiani, N., Sancelme, M., Deguillaume, L., Flossmann, A. I., Amato, P., and Delort, A.-M.: Long-term features of cloud microbiology at the puy de Dôme (France), Atmos. Environ., 56, 88-100, 2012.

Vali, G.: Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids, J. Atmos. Sci., 28, 402-409, 1971.

van Eaton, A. R., Harper, M. A., and Wilson, C. J. N.: High-flying diatoms: Widespread dispersal of microorganisms in an explosive volcanic eruption, Geology, 41, 1187-1190, doi:10.1130/G34829.1, 2013.

von Blohn, N., Mitra, S. K., Diehl, K., and Borrman, S.: The ice nucleating ability of pollen. Part III: New laboratory studies in immersion and contact freezing modes including more pollen types, Atmos. Res., 78, 182-189, 2005.

Wagner, R., Möhler, O., Saathoff, H., Schnaiter, M. and Leisner, T.: New cloud chamber experiments on the heterogeneous ice nucleation ability of oxalic acid in the immersion mode, Atmos. Chem. Phys., 11(5), 2083–2110, doi:10.5194/acp-11-2083-2011, 2011.

Wang, B., and Knopf, D. A.: Heterogeneous ice nucleation on particles composed of humic-like substances impacted by $O_3$, J. Geophys. Res., 116, D03205, doi:10.1029/2010JD014964, 2011.

Wang, B., Lambe, A. T., Massoli, P., Onasch, T. B., Davidovits, P., Worsnop, D. R., and Knopf, D. A.: The deposition ice nucleation and immersion freezing potential of amorphous secondary organic aerosol: Pathways for ice and mixed-phase cloud formation, J. Geophys. Res., 117, D16209, doi:10.1029/2012JD018063, 2012.

Warren, G., and Wolber, P.: Molecular aspects of microbial ice nucleation, Molec. Microbiol., 5, 239-243, 1991.

Welti, A., Lüönd, F., Kanji, Z. A., Stetzer, O., and Lohmann, U.: Time dependence of immersion freezing: an experimental study on size selected kaolinite particles, Atmos. Chem. Phys., 12, 9893-9907, doi:10.5194/acp-12-9893-2012, 2012.

Wilson, T. W., Murray, B. J., Wagner, R., Möhler, O., Saathoff, H., Schnaiter, M., Skrotzki, J.,

**Formatiert:** Englisch (USA)

**Formatiert:** Englisch (USA)

**Formatiert:** Schriftart: (Standard) Times New Roman, 12 pt

Price, H. C., Malkin, Dobbie, S., and Al-Jumur, S. M. R. K.: Atmos. Chem. Phys., 12, 8611-8632, doi: 10.5194/acp-12-8611-2012, 2012.

Wolanczyk, J. P., Storey, K. B., and Baust, J. G.: Ice nucleating activity in the blood of the freeze-tolerant frog, Rana sylvatica, Cryobiol.,27, 328-335, 1990.

Xu, H., Griffith, M., Patten, C. L., and Glick, B. R.: Isolation and characterization of an antifreeze protein with ice nucleation activity from the plant growth promoting rhizobacterium Pseudomonas putida GR12-2, Can. J. Microbiol., 44, 64-73, doi:10.1139/w97-126, 1998.

Yttri, K. E., Dye, C., and Kiss, G.: Ambient aerosol concentrations of sugars and sugar-alcohols at four different sites in Norway, Atmos. Chem. Phys., 7, 4267-4279, doi:10.5194/acp-7-4267-2007,2007.

Zachariassen, K. E., and Kristiansen, E.: Ice nucleation and antinucleation in nature, Cryobiol., 41, 257-279, 2000.

Zobrist, B., Marcolli, C., Koop, T., Luo, B. P., Murphy, D. M., Lohmann, U., Zardini, A. A., Krieger, U. K., Corti, T., Cziczo, D. J., Fueglistaler, S., Hudson, P. K., Thomson, D. S. and Peter, T.: Oxalic acid as a heterogeneous ice nucleus in the upper troposphere and its indirect aerosol effect, Atmos. Chem. Phys., 6(10), 3115–3129, doi:10.5194/acp-6-3115-2006, 2006.

Zobrist, B., Koop, T., Luo, B. P., Marcolli, C., and Peter, T.: Heterogeneous ice nucleation rate coefficient of water droplets coated by a nonadecanol monolayer, J. Phys. Chem. C, 111, 2149-2155, 2007.

Zuberi, B., Bertram, A. K., Koop, T., Molina, L. T. and Molina, M. J.: Heterogeneous Freezing of Aqueous Particles Induced by Crystallized $(NH_4)_2SO_4$ , Ice, and Letovicite, J. Phys. Chem. A, 105, 6458–6464, doi:10.1021/jp010094e, 2001.

| Type | Organism | cell-free? | protein? | saccharide? | lipid? | *T* stability | size (1 unit) | $\theta$[°] $\pm\sigma$[°] |
|---|---|---|---|---|---|---|---|---|
| BINMs: | *P. syringae* | – | + | + | + | <313 K | 150–180 kDa | 34.1 ± 2.3 |
| | *E. herbicola* | + | + | + | + | <313 K | 150–180 kDa | |
| Fungal INMs: | *Rhiz. chrysoleuca* | + | + | – | – | >333 K | <0.22 µm | |
| | *F. avenaceum* | + | + | – | – | >333 K | <0.22 µm | |
| | *A. implicatum* | + | + | –? | + | 308–333 K | 100–300 kDa | 33.2 ± 2.3 |
| | *I. farinosa* | + | + | –? | – | 308–333 K | ~300 kDa | 24.6 ± 0.6 |
| | *M. alpina* | + | + | –? | – | 333–371 K | 100–300 kDa | 26.4 ± 1.1 |
| | rust spores | ?? | ?? | + | ?? | ~373 K | ?? | |
| Animal INMs: | *Tipula* | + | + | +? | + | ?? | 800 kDa | |
| | *Dendroides* | + | + | –? | +/– | ?? | >70 kDa | |
| | *Vespula* | + | + | – | ?? | <373 K | 74 kDa | |
| | *Eurosta** | + | – | – | – | ?? | >100 µm | |
| Plant INMs: | *Secale* leaves | ?? | + | + | + | <363 K | ?? | |
| | *Prunus* wood | – | – | ?? | ?? | 313–323 K | ?? | |
| | *Betula* pollen | + | – | + | – | 445–460 K | 100–300 kDa | 58.2 ± 4.6 |
| | *Lobelia* fluid | + | – | +? | – | >373 K | ?? | |
| | *Opuntia* fluid | + | – | + | – | ?? | <70 µm | |
| | different algae | ?? | ?? | ?? | ?? | ?? | ?? | |

Table 1: Chemical properties of some INMs. "T stability" shows the temperature above which they IN are denatured. A question mark indicates uncertainty. See Sect. 4.12 for the sources of these data. $\theta$[°] $\pm\sigma$[°] are the calculated contact angle distribution according to the *Soccer Ball Model*. *) Only the calcium phosphate spherules are regarded here, not the fat cells.

| Treatment | % INA | *T* [K] |
|---|---|---|
| none | 100 | both |
| none (ref) | <9 | both |
| boric acid | 15 | 256 |
| boric acid (ref) | 0 | 256 |
| 343 K | 29 | 256 |
| 343 K + boric acid | 3 | 256 |
| medium | 34 | 258 |
| medium + Trypsin | 30 | 258 |
| medium + Trypsin (ref) | 13 | 258 |

Table 2: An overview over the investigation on birch pollen extracts. The percentage is the relative number of INs in comparison to the untreated aliquot at a given temperature *T* [K]. Lines labeled with "(ref)" refer to reference measurements under the same conditions with pure water instead of extract.

| Type | Source | $m$ [kDa] | $T_{nuc}$ [K] |
|---|---|---|---|
| BINM (~560 units) | Burke and Lindow, 1990 | ~83700 | 272 |
| BINM (~130 units) | Govindarajan and Lindow, 1988a | ~19000 | 271 |
| BINM (~60 units) | Govindarajan and Lindow, 1988a | ~8700 | 270 |
| BINM (~20 units) | Govindarajan and Lindow, 1988a | ~2500 | 268 |
| crit. ice embryo | Zachariassen and Kristiansen, 2000 | 810 | 268 |
| *Isa*-INM (>1 units) | this study | >300 | 268 |
| *Isa*-INM (1 unit?) | this study | 100–300 | 267 |
| *Mor*-INM | Fröhlich-Nowoisky et al., ~~2014~~2015 | 100–300 | 266 |
| BINM (3 units) | Gurian-Sherman and Lindow, 1995 | ~360 | 263 |
| BINM (1 unit) | Govindarajan and Lindow, 1988a | ~150 | 261 |
| INAFP | Xu et al., 1998 | 164 | 261 |
| *Acr*-INM | this study | 100–300 | 259 |
| birch INM | this study | 335–860 | 257 |
| birch INM | Pummer et al., 2012 | 100–300 | 255 |
| birch INM* | Augustin et al., 2013 | 100–300 | 250 |
| PVA | Ogawa et al., 2009 | 1.7–98 | 239 |
| crit. ice embryo | Zachariassen and Kristiansen, 2000 | 1.26 | 233 |

Table 3: Overview over masses ($m$) and activation temperatures ($T_{nuc}$) of certain IN. *) $T_{nuc}$ here are $T_{50}$ of both the LACIS measurement with 800 nm particles and the oil immersion cryo-microscopy measurement with 5 µg/ml pollen.

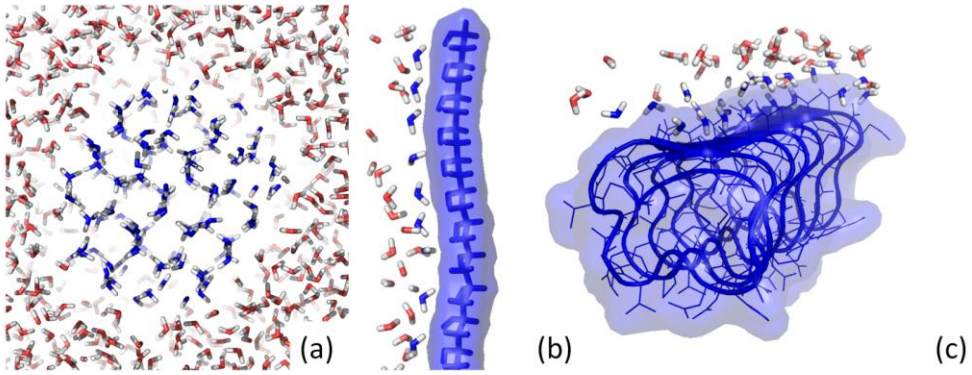


Figure 1: Visualization of water molecule ordering based on molecular model calculations (Sect. S2.1): homogeneous ice nucleation (a); heterogeneous ice nucleation by ordering of water molecules on a PVA strain, which is a 1D-template (b), and an antifreeze protein ~~related~~ that has a similar sequence and structure as~~to~~ the bacterial ~~B~~INMs, which is a 2D-template (c). Each image contains water molecules that are ordered (blue) and some randomly distributed water molecules (red).

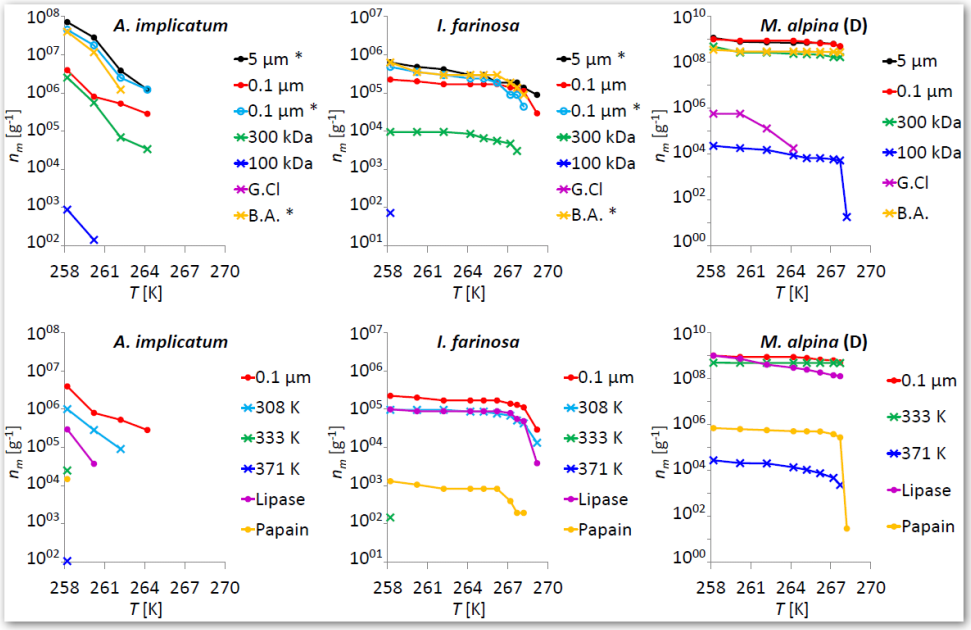


Figure 2: $n_m(T)$-curves for *A. implicatum*, *I. farinosa*, and *M. alpina* (subgroup D) INMs after different treatments. "G.Cl" stands for guanidinium chloride treatment, "B.A." for boric acid treatment. A reduction of $n_m$ suggests that this method partly or fully destroyed the INMs. The absence of data points despite the listing in the figure legend indicates that $n_m$ lied below the detection limit. For *M. alpina*, the data are the mean curves of all investigated strains of the phylogenetic subgroup D, which is the most representative (Fröhlich-Nowoisky et al., ~~2014~~2015).

*) for *A. implicatum* and *I. farinosa*: these ~~0.1 µm~~ measurements were executed with the filtrates of another harvest, ~~as were the 5 µm and the B.A. measurements,~~ what explains the higher values in comparison to the other results.

**Formatiert:** Schriftart: (Standard) Times New Roman, 12 pt

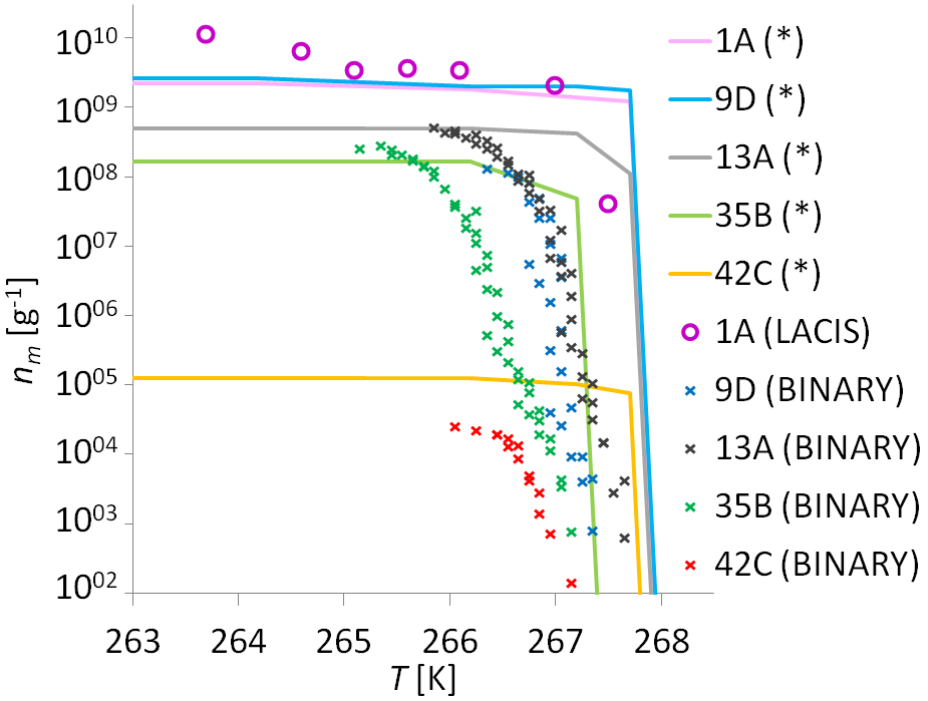


Figure 3: Comparison of ice nucleation curves of 0.1 µm filtrates from a few *M. alpina* strains. The number and letter combination labels the strain. The devices used for generating the respective curves are shown in brackets. "*" stands for the setup described in Fröhlich-Nowoisky et al. (~~2014~~2015).

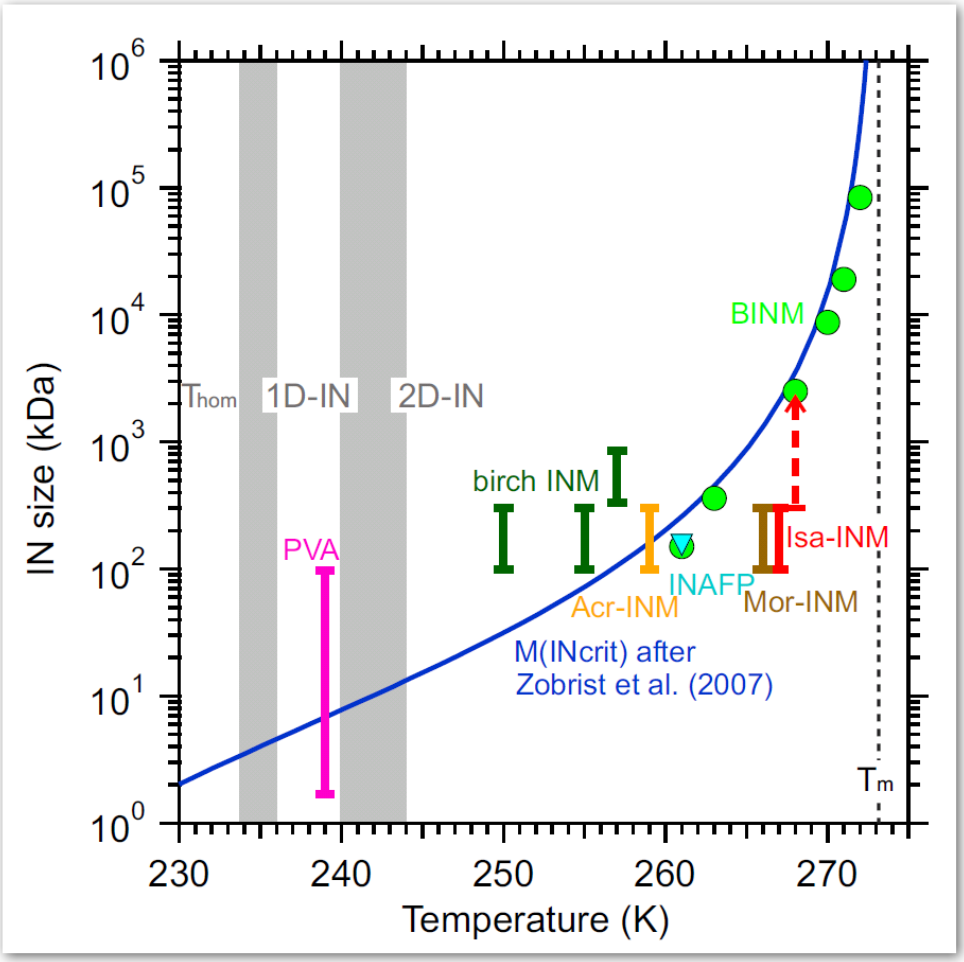


Figure 4: The dependence of the median freezing temperature on the size for different types of IN (colored dots). The blue curve is the calculated critical ice cluster size derived from *Classical Nucleation Theory* (Zobrist et al., 2007). The sources of the presented IN data are listed in Table 3. The graph further shows the region where we assume the domains where 1D- and 2D-templates act as IN. The grey areas mark the transition regions between the domains. The acronyms *Acr*, *Isa*, and *Mor* stand for the respective fungal species.

# Supplement:

# Ice nucleation by water-soluble macromolecules

**B. G. Pummer, C. Budke, S. Augustin-Bauditz, D. Niedermeier, L. Felgitsch, C. J. Kampf, R. G. Huber, K. R. Liedl, T. Loerting, T. Moschen, M. Schauperl, M. Tollinger, C. E. Morris, H. Wex, H. Grothe, U. Pöschl, T. Koop, and J. Fröhlich-Nowoisky**

Correspondence to: B. G. Pummer (b.pummer@mpic.de)

## S1 Theoretical considerations

### S1.1 Macromolecul~~ar chemistry~~es and solubility

Macromolecules are per definition molecules with a molecular mass of >10 kg/mol (Staudinger and Staudinger, 1954), which is equivalent to >10 kDa. In contrast to crystals or metals, which consist of subunits that are held together by non-covalent forces (e.g. ionic, metal or dipole bonds), each atom of a macromolecule is covalently bound to the rest of the molecule. Since covalent bonds are usually much stronger than non-covalent bonds, they stay intact in solution. In contrast, a sodium chloride crystal is broken down into single sodium cations and chloride anions and thereby loses its former structure. The variety of macromolecules ranges from inorganic (e.g. diamond, silicate) to organic (e.g. plastics) to biological (e.g. proteins, polysaccharides, sporopollenin, lignin) exponents.

Polymers are a subgroup of macromolecules, which are built up by a chain of covalently linked small molecules. ~~low-molecular-units that are stable molecules by themselves (which are called monomers) that are covalently linked in a chain-like manner. If the monomers have more than two functional groups that allow a covalent link, polymer chains can also be branched. The individual building units can either be all the same in the case of a homopolymer, or can be two or more different molecules. In the latter case, the sequence of the monomers determines the properties of the whole polymer. For example, PVA is a homopolymer built up only by vinyl~~

~~alcohol, while the monomers of proteins are 20 different amino acids. The frequency and sequence of these amino acids is responsible for the large variety of proteins that exist in nature. As already stated in the manuscript, s~~Such a molecular chain will not stay linear, but will fold into a more compact form – especially if it contains hydrophobic elements in a hydrophilic surrounding or the other way round. This folding can be either random ~~like in a ball of wool, or it can be~~ in a well-defined manner. Proteins in their functional state usually have a very distinct folding~~, and therefore a very distinct form. Common elements of protein foldings are α helices, β sheets and β helices~~. Protein chains that are not properly folded lack in most cases their functionality. Since the non-covalent forces holding the protein structure intact are usually weak, stress treatments lead to unfolding and therefore inactivation of the protein.

The solubility of a macromolecule depends on ~~both~~ the chemistry of the macromolecule and the solvent. ~~A practical classification of proteins according to their solubility is given by the~~ Based on the protein classification approach by T. B. Osborne ~~fractions, which were originally based on T. B. Osborne's analysis of cereal samples~~ (e.g. Osborne, 1910) ~~, which has been upgraded for application in modern biotechnology and food chemistry. A granular (resp. ground)~~ biological matter~~sample~~ is suspended and shaken in a certain solvent. Then the ~~biological~~ matter is centrifuged or filtrated off, thus removing particulate matter and yielding a transparent supernatant ~~free of turbidity~~. ~~Proteins~~ Molecules that ~~we~~are extracted into the supernatant are considered to be soluble in that medium. ~~Depending on the solvent, different proteins are extracted, which is the basis of this classification:~~

- ~~soluble in pure water: albumins~~
- ~~soluble in 10% NaCl solution: globulins~~
- ~~soluble in 70% ethanol: prolamins~~
- ~~soluble in diluted HCl: histones~~
- ~~soluble in NaOH: glutelins~~

In the case of large molecules, it is disputable where to draw the line between solution and suspension. Per definition, a solution consists of a single phase, while a suspension consists of two phases with phase interfaces. If the particles sizes are close to the wavelength of visible light, a suspension shows light scattering, which makes it opaque. A solution, in contrast, shows

**Formatiert:** Standard, Keine Aufzählungen oder Nummerierungen

neither light scattering, nor visible particles. Furthermore, a solution shows no phase separation over time, while sedimentation or agglutination lead to a progressive phase separation in time. Additionally, solutions cannot be separated by centrifugation. From a molecular point of view, a molecule in solution is fully covered with an energetically favorable hydration shell. ~~Despite its size, a protein is a single molecule, while insoluble suspended particles consist of either several molecules or a crystal lattice of elementary cells.~~

## S1.2 Basic physics of INA

At temperatures below the melting point (273.15 K at atmospheric pressure), ice is thermodynamically favored over liquid water. Nevertheless, the spontaneous freezing of liquid water that is supercooled below this point is statistically very unlikely, because the phase transition is kinetically hindered. To form ice, water molecules have to be arranged in a defined ice crystal structure instead of the more random orientation and translational degrees of freedom they have in a liquid. Due to energetic propitiousness, which comes from the crystallization energy, clusters of a few water molecules will tend to arrange in an ice-like structure in the liquid water body. These clusters, which are also known as ice embryos, however, are then ripped apart by their surface tension, so in supercooled water, there is equilibrium between formation and decay of ice embryos.

Crystallization energy is proportional to the volume of the ice embryo, and therefore to the radius cubed. In contrast surface tension is proportional to the surface, and therefore to the radius squared. The outcome of the battle between crystallization energy and surface tension depends on the value of the Gibbs Energy $\Delta G$, which is therefore a function of the radius $r$ (see Eq.(S1)), in other words the size of the water molecule cluster. $\Delta G(r)$ initially increases with $r$, then reaches a maximum $\Delta G^*$, which is equivalent to the activation energy of the process (see Eq.(S2)). After that, $\Delta G$ strongly decreases with $r$. Once the critical radius $r^*$ (see Eq.(S3)) is reached, meaning that the activation barrier $\Delta G^*$ is overcome, the ice embryo will grow unimpededly and subsequently catalyze the freezing of the entire supercooled body of water.

The critical ice embryo size in turn depends on the temperature, decreasing in size as the intensity of supercooling increases, or, in other words as the temperatures drop below 273.15 K. For example, 45000 arranged water molecules constitute the critical ice embryo size at 268 K,

while only 70 are required at 233 K (Zachariassen and Kristiansen, 2000). Furthermore, the probability of forming a cluster decreases with its size. Therefore, freezing becomes very unlikely at higher temperatures (so far we take only water molecules into account). This situation is the basis of why ultrapure water can be cooled down to temperatures about 235 K before it will eventually freeze. The manifestation of a critical ice embryo, which eventually leads to ice formation, is called ice nucleation. When only water molecules are involved, it is called homogeneous ice nucleation (see Fig. 1a).

$$\Delta G = 4\pi \cdot \gamma \cdot r^2 + \frac{4}{3}\pi \cdot \rho \cdot \Delta\mu \cdot r^3 \quad \text{(S1)}$$

$$\Delta G^* = \frac{16\pi \cdot \gamma^3}{3 \cdot \rho^2 \cdot (\Delta\mu)^2} \quad \text{(S2)}$$

$$r^* = -\frac{2 \cdot \gamma}{\rho \cdot \Delta\mu} \quad \text{(S3)}$$

$\Delta G$…Gibbs energy, $r$…cluster radius, $\gamma$… surface free energy, $\rho$…bulk density, $\Delta\mu$…phase transition chemical potential, $\Delta G^*$…activation energy, $r^*$…critical radius

The probability of freezing increases when water contains or comes in contact with structured surfaces that simulate ice and arrange water molecules in an ice-like manner. This stabilizes ice embryos, and therefore decreases the activation barrier in the manner of a catalyst. These ice-template structures are known as ice nucleators (INs) or ice nuclei, and the process they catalyze is known as heterogeneous ice nucleation (see Fig. 1b+c). The driving force of the arrangement of water molecules on IN surfaces is interaction between the partially charged ends of the water molecule and oppositely charged functional groups on the IN surface. This involves H-bonds between hydrogen atoms with partial positive charges and oxygen or nitrogen atoms with partial negative charges. Therefore, the IN has to carry functional groups at the proper position to be effective (Liou et al., 2000, Zachariassen and Kristiansen, 2000). In most cases only certain sections, which are known as “active sites”, participate in the INA, while the majority of the IN surface is inactive (Edwards et al., 1962, Katz, 1962).

The larger the active site of an IN, and the more fitting functional groups it carries, the more effective it stabilizes clusters, and so the higher the freezing temperature. Consequently, single molecules of low-molecular compounds cannot nucleate ice. In fact, soluble compounds consisting of very small molecules or ions, like salts, sugars or short-chained alcohols, cause a

freezing point depression. However, if single molecules are very large, they can allocate enough active surface to be INs by themselves. Such ice nucleating macromolecules (INMs) are especially common among biological INs. Due to the same reason some low-molecular organic compounds which do not induce ice formation in solution, can act as IN, if they are crystallized in layers of a certain arrangement (Fukuta, 1966).

### S1.3 INA modes

Throughout the manuscript we present the physics of ice nucleation mainly with regard to immersion freezing where the IN is inside a cooling water droplet. But in fact, three more modes of ice nucleation are defined. Immersion freezing is the most-investigated mode, and is suspected to be the dominant ice formation mechanism in mixed-phase clouds (Ansmann et al., 2009, Wiacek et al., 2010, de Boer et al., 2011). The other modes are contact, deposition and condensation ice nucleation. Contact ice nucleation means that the IN collides with a supercooled droplet, which freezes on contact. Deposition ice nucleation is adsorption of water vapor on the IN surface as ice, and condensation ice nucleation is condensation of water vapor as liquid layer on the IN, which then freezes at the same temperature. Deposition ice nucleation is somewhat different, since the water molecules from the gas phase have to be arranged, while in the other modes freezing occurs in the liquid phase. Consequently, some particles that have shown ice nucleation activity (INA) in the other three modes are inactive in the deposition mode (Diehl et al., 2001, Diehl et al., 2002). Condensation and deposition mode depend additionally on atmospheric pressure and humidity, which play no role, if ice nucleation occurs in pre-existing droplets. For condensation mode activity, the IN additionally has to carry hygroscopic functional groups, which also make it an efficient cloud condensation nucleus (CCN). Since all four modes are theoretical models, they are permanently under discussion. Debates go so far as to question not only the real-life relevance, but also the existence itself of some modes. For example, one could claim that a condensation IN is consecutively acting as a CCN and an immersion IN (Fukuta and Schaller, 1982, Wex et al., 2014). In light of this debate we focus only on immersion freezing.

### S1.4 Water activity

It is possible to view INA in the light of the water activity ($a_w$). The thermodynamic freezing and melting temperature of water ($T_m$), which is independent of insoluble INs, is a function of $a_w$. A reduction of $a_w$ due to the addition of solutes leads to a freezing point depression, as it is illustrated in Fig. S1. The effective freezing / ice nucleation temperature shows the same dependence on $a_w$, but is horizontally shifted relative to the $T_m(a_w)$-curve (Zobrist et al., 2008, Koop and Zobrist, 2009). The distance between the ice nucleation and melting curve at a given temperature is named $\Delta a_w$, which is the measure of the INA of a water sample. For example, for the homogeneous freezing on IN-free samples, $\Delta a_w$ is about 0.31±0.02 (Koop et al., 2000, Koop and Zobrist, 2009). The addition of IN in the water leads to a horizontal shift of the ice nucleation curve towards the melting curve, or a reduction in $\Delta a_w$. In the experiment, a nucleation spectrum of a water droplet ensemble with given INA and a given $a_w$ is like a vertical trajectory going through the phase diagram in Fig. S1 from top to bottom. Therefore, the ice nucleation temperature depends on both the present INs and $a_w$.

Instead of assigning a certain ice nucleation temperature to a sample, it is more accurate for stochastic, time-dependent INs to assign nucleation rate coefficients $J(T,a_w)$, which increase with decreasing $T$ and increasing $a_w$ (Knopf and Alpert, 2013). Therefore, one can add $J$ contour lines to Fig. S1, which show the same shape as the thermodynamic and the homogeneous freezing curve (Koop et al., 2000, Attard et al., 2012, Knopf and Alpert, 2013). This means that from the thermodynamic freezing line to the homogeneous freezing line we have a gradient of increasing $J$. Accordingly, cooling is a steady increase in $J$. This makes $J$ independent of the absolute freezing temperature, and therefore of the IN type.

### S1.5 Motivation for expression of biological INMs

There are several theories addressing the question of why some organisms produce IN. Overall, it is proposed that INA is a form of adaption for survival or enhanced fitness in cold environments. More than 80% of the total biosphere volume is exposed to temperatures below 278 K, thriving either in the oceans or in frosty regions (Christner 2010). Also in temperate climate zones, temperatures can regularly drop below the freezing point. The formation of ice crystals can pierce cell walls and membranes, which leads to loss of cell fluids. Consequently, adaptations for either avoiding or managing freezing make sense for the many species that are

exposed to such hostile conditions. The correlation between the INA of bacteria and the geographic latitude that was found by Schnell and Vali (1976) supports the idea of a selective advantage for organisms with INA in cold environments. For the γ-Proteobacteria the gene for the BINM most likely originates from the common ancestor of this class of bacteria and therefore has been part of the genome of these organisms for at least 0.5 to 1.75 billion years (Morris et al., 2014). To be maintained for this length of time, the gene is likely to be under positive natural selection because it confers a fitness advantage. The possible advantages that have been proposed are:

(i) Nutrient mining (Lindow et al., 1982): Highly active INMs were mainly found in plant pathogenic species (bacteria, *Fusarium*, rust fungi) or in lichen. By inciting the growth of ice crystals, these organisms can essentially "dig" into the substrate on which they are growing (mainly plant tissues, but also rocks in the case of lichens), thereby acquiring nutrients.

(ii) Cryoprotection (Krog et al., 1979, Duman et al., 1992): The INA of plants and animals, but possibly also of lichens, is protective against frost injury. Ice growth in organisms is dangerous, because it ruptures the sensitive cell membranes thereby damaging or killing the cells. If the ice is formed on a less sensitive location, such as outside of the cells (e.g. in intercellular fluids), the danger of frost injury is far lower. Forming ice on the INMs prevents further ice formation at other places – partly because of the change in water activity, but also due to the release of crystallization heat, which prevents a further temperature decrease. This might explain why most known biological INMs are extracellular (see Table 1), and why they are active at such high temperatures, where the heat of fusion is sufficient to warm the cells to survivable temperatures.

(iii) Water reservoir (Kieft and Ahmadjian, 1989): Ice crystals might serve as water storage in cold and dry environments. The form stability of ice and its low vapor pressure reduce the potential loss of water in comparison to the loss from liquid water droplets.

(iv) Cloud seeding to assure deposition (Morris et al., 2008, 2013a, 2013b): The lifecycles of some species involve long distance dissemination that takes them up into clouds but where they will not proliferate unless they return to Earth's surface. Particles that

attain cloud height are generally too small to deposit due to their own weight. Therefore, they require means of active deposition, such as precipitation that forms from ice initiated in clouds via ice nucleation.

(v) Incidental (Lundheim 2002): In some cases, INA was detected where it cannot be explained by any reason. In this case, the INA might be an accidental property of a bioparticle that has another function in the organism. For example, the low density lipoproteins in human blood show INA, although their purpose lies in fat metabolism.

Advantages (i) and (ii) might be distinguishable by the freezing temperature (Duman et al., 1992): Since (i) demands ice formation as soon as possible, and the formation of few large ice crystals, such INMs are active at a very high temperature. On the other hand, type-(ii)-INMs are active at lower temperatures, only before other parts of the organism would start freezing. Furthermore, less efficient IN favor formation of smaller, less sharp and damaging ice crystals than those formed by type-(i)-INMs.

### S1.6 Mineral dust IN

Apart from biological INMs, some types of mineral dust and soot have shown INA in different laboratory experiments (e.g. Murray et al., 2012), what might make them relevant for atmospheric ice formation.

Among mineral dust, potassium feldspar and fluorine phlogopite (a type of potassium micas) showed by far the highest INA (Shen et al., 1977, Atkinson et al., 2013, Augustin-Bauditz et al., 2014, Zolles et al., 2015). The reason for this higher accentuated activity compared to other closely related minerals is thought to be due to the potassium cations, whose hydration shell density matches that of ice. In contrast, the hydration shells of sodium and calcium ions are far tighter due to the higher ion charge density. So they likely disturb the ice-like water molecule arrangement, while potassium is neutral or supportive (Shen et al., 1977). It should be pointed out that this hypothesis is not valid for low molecular weight compounds. Soluble potassium salts (e.g. KCl, $KNO_3$, etc.) lead to a freezing point depression, as do salts with other cations. In the crystal lattice of feldspar the ions are fixed in a confined geometry that seems to match the ice crystal lattice. This probably causes the INA. Other ions with the same charge and the approximately same diameter, for example ammonium, might also have a favorable effect on the

INA. It is interesting to note that several studies suggest that traces of ammonium contaminants in silver iodine increase its INA (e.g. Corrin et al., 1964, Steele and Krebs, 1966, Bassett et al., 1970).

## S2 Details about methods

### S2.1 Molecular modeling

The insect antifreeze protein (AFP) from the beetle *Tenebrio molitor* was simulated (see Fig. 1c). The 8.4 kDa AFP is composed of 12-residue repeats and is stabilized by disulfide- bonds in the core of the protein. A defined structure of six parallel beta-sheets built up from the sequence TCT shows a high ordered surface to the water. The starting structure was taken from the *Protein Data Bank* (Liou et al., 2000), protonated with "prontonate3d" from the MOE2013.08 modeling package, and solvated in TIP4P-2005 water (Abascal and Vega, 2005) with 12 Å wall separation. Minimization and equilibration were performed according to Wallnoefer et al. (2010). Then 100 nanoseconds of NpT (isothermal and isobaric) molecular dynamics simulation at 220 K were recorded using an 8 Å cutoff for non-bonded interaction and the *Particle Mesh Ewald* algorithm for treating long-range electrostatics (Darden et al., 1993).

Water Analysis: Snapshots were taken every picosecond, and water density was estimated as described by Huber et al.(2013). Afterwards, the most likely water positions were extracted. During the simulation of 1EZG a very well structured first layer of water, which we colored blue, could be observed. Water less structured than the first layer was colored red.

### S2.2 Size exclusion chromatography

High-purity water (18.2 MΩ·cm) was taken from an ELGA LabWater system (PURELAB Ultra, ELGA LabWater Global Operations, UK). Ammonium acetate ($NH_4Ac$; ≥ 98%, puriss p.a.), DL-dithiothreitol (DTT; > 99%), iodoacetamide (IAM; ≥ 99%), 2,2,2-trifluoroethanol (TFE; ≥ 99%, ReagentPlus), ammonium bicarbonate ($NH_4HCO_3$; ≥ 99%, ReagentPlus), Trypsin from porcine pancreas (proteomics grade) and protein standard mix (15–600 kDa) were obtained from Sigma Aldrich, Steinbach, Germany. Formic acid (FA; > 99%, for analysis) was from Acros Organics, Geel, Belgium. Guanidinium chloride was from Promega, Madison, WI, USA.

The HPLC-DAD system consisted of a binary pump (G1379B), an autosampler with thermostat (G1330B), a column thermostat (G1316B), and a photo-diode array detector (DAD; G1315C) from Agilent Technologies (Waldbronn, Germany). Chemstation software (Rev. B.03.01, Agilent) was used for system control and data analysis. A size exclusion column (Agilent Bio SEC-3, 300 Å, 4.6 x 150 mm, 3 µm particle size) with exclusion limits of 5 kDa to 1.25 MDa was used for chromatographic separation. 50 mM $NH_4Ac$ in ultrapure water (pH 6.7) was used as the eluent. Isocratic analyses with a runtime of 10 min were performed at 303 K with a flow rate of 350 µL $min^{-1}$. After each measurement the column was flushed for 5 min with the same eluent before the next run. Absorbance was monitored at wavelengths of 220 and 280 nm. The sample injection volume was 40 µL. Sample fractions were collected at different retention time intervals corresponding to different molecular weight intervals as shown in Table S1. Molecular weights are calculated according to a protein standard mix with four calibration points ranging from 15 to 600 kDa. To get rid of the residues from the birch pollen extract, the column was cleaned after each work day with 6 M guanidinium chloride overnight, and then with pure water.

The protocol for the protein digestion was as follows: 5 µL of a 100 mM $NH_4HCO_3$ solution and 5 µL TFE were added to 100 µL of sample. Then 0.5 µL 200 mM DTT solution were added, the sample was briefly vortexed and then incubated for 1 h at 333 K to denature the proteins. After letting the sample cool to room temperature 2 µL of 200 mM IAM solution were added and the sample was allowed to stand for 1h in the dark (covered with aluminum foil) to alkylate the protein cysteine residues. The sample was allowed to stand for another hour in the dark after adding 0.5 µL 200 mM DTT solution to destroy excess IAM. Now 60 µL autoclaved water and 20 µL 100 mM $NH_4HCO_3$ solution were added to adjust the sample pH for digestion. Two microliters of 1 µg/µL Trypsin in 50 mM acetic acid was added and the sample was incubated at 310 K for 18 h. To stop the digestion 0.5 µL FA were added. The procedure for the treatment of samples and controls is given in Table 2.

## References (Supplement)

Abascal, J. L. F., and Vega, C.: A general purpose model for the condensed phases of water: TIP4P/2005, J. Chem. Phys., 123, 234505, doi:10.1063/1.2121687, 2005.

Abe, K., Watabe, S., Emori, Y., Watanabe, M., and Arai, S.: An ice nucleation gene of Erwinia

ananas, FEBS Lett., 258, 297-300, 1989.

Ansmann, A., Tesche, M., Seifert, P., Althausen, T., Engelmann, R., Fruntke, J., Wandinger, U., Mattis, I., and Müller, D.: Evolution of the ice phase in tropical altocumulus: SAMUM lidar observations over Cape Verde, J. Geophys. Res., 114, D17208, doi:10.1029/2008JD011659, 2009.

Atkinson, J. D., Murray, B. J., Woodhouse, M. T., Whale, T. F., Baustian, K. J., Carlslaw, K. S., Dobbie, S., O'Sullivan, D., and Malkin, T. L.: The importance of feldspar for ice nucleation by mineral dust in mixed-phase clouds, Nature, 498, 355-358, 2013.

Attard, E., Yang, H., Delort, A.-M., Amato, P., Pöschl, U., Glaux, C., Koop, T., and Morris, C. E.: Effects of atmospheric conditions on ice nucleation activity of Pseudomonas, Atmos. Chem. Phys., 12, 10667-10677, doi:10.5194/acp-12-10667-2012, 2012.

Augustin-Bauditz, S., Wex, H., Kanter, S., Ebert, M., Stolz, F., Prager, A., Niedermeier, D., and Stratmann, F.: The immersion mode ice nucleation behavior of mineral dusts: A comparison of different pure and surface modified dusts, Geophys. Res. Lett., 41, 7375-7382, doi:10.1002/2014GL061317, 2014.

Bassett, D. R., Boucher, E. A., and Zettlemoyer, A. C.: Adsorption studies on ice-nucleating substrates. Hydrophobed silicas and silver iodide, J. Colloid Interfac. Sci., 34, 436-446, 1970.

Corrin, M. L., Edwards, H. W., and Nelson, J. A.: The surface chemistry of condensation nuclei: II. The preparation of silver iodide free of hygroscopic impurities and its interaction with water vapor, J. Atmos. Sci, 21, 565-567, 1964.

Darden, T., York, D., and Pedersen, L.: Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems, J. Chem. Phys., 98, 10089-10092, doi:10.1063/1.464397, 1993.

de Boer, G., Morrison, H., Shupe, M. D., and Hildner, R.: Evidence of liquid dependent ice nucleation in high-latitude stratiform clouds from surface remote sensors, Geophys. Res. Lett., 38, L01803, doi:10.1029/2010GL046016, 2011.

Diehl, K., Quick, C., Matthias-Maser, S., Mitra, S. K., and Jaenicke, R.: The ice nucleation ability of pollen Part I: Laboratory studies in deposition and condensation freezing modes, Atmos. Res., 58, 75-87, 2001.

Formatiert: Englisch (USA)

Formatiert: Englisch (USA)

Formatiert: Englisch (USA)

Diehl, K., Matthias-Maser, S., Jaenicke, R., and Mitra, S.K.: The ice nucleation ability of pollen Part II. Laboratory studies in immersion and contact freezing modes, Atmos. Res., 61, 125-133, 2002.

Duman, J. G., Wu, D. W., Yeung, K. L., and Wolf, E. E.: Hemolymph proteins involved in the cold tolerance of terrestrial arthropods: antifreeze and ice nucleator proteins, Water and Life, Springer Berlin Heidelberg, ISBN-13: 9783540541127, 282-300, 1992.

Edwards, G. R., Evans, L. F., and La Mer, V. K.: Ice nucleation by monodisperse silver iodide particles, J. Colloid Sci., 17, 749-758, doi:10.1016/0095-8522(62)90049-1, 1962.

Fukuta, N.: Experimental studies of organic ice nuclei, J. Atmos. Sci., 23, 191-196, 1966.

Fukuta, N., and Schaller, R.C.: Ice nucleation by aerosol particles: Theory of condensation-freezing nucleation, J. Atmos. Sci., 39, 648-655, 1982.

Huber, R. G., Fuchs, J. E., von Grafenstein, S., Laner, M., Wallnoefer, H. G., Abdelkader, N., and Liedl, K. R.: Entropy from state probabilities: hydration entropy of cations, J. Phys. Chem. B, 117, 6466-6472, doi:10.1021/jp311418q, 2013.

Katz, U.: Wolkenkammeruntersuchungen der Eiskeimbildungsaktivität einiger ausgewählter Stoffe, Zeitschr. Angew. Math. Phys., 13, 333-358, 1962. (in German)

Kieft, T. L., and Ahmadjian, V: Biological ice nucleation activity in lichen mycobionts and photobionts, Lichenol.,21, 355-362, 1989.

Knopf, D. A., and Alpert, P. A.: A water activity based model of heterogeneous ice nucleation kinetics for freezing of water and aqueous solution droplets, Faraday Discuss., 165, 513-534, doi:10.1039/c3fd00035d, 2013.

Koop, T., Luo, B., Tsias, A., and Peter, T.: Water activity as the determinant for homogeneous ice nucleation in aqueous solutions, Nature, 406, 611-614, 2000.

Koop, T., and Zobrist, B.: Parameterizations for ice nucleation in biological and atmospheric systems, Phys. Chem. Chem. Phys., 11, 10741-11064, doi:10.1039/b914289d, 2009.

Krog, J. O., Zachariassen, K. E., Larsen, B., and Smidsrod, O.: Thermal buffering in Afro-alpine plants due to nucleating agent-induced water freezing, Nature, 282, 300-301, 1979.

Lindow, S.E., Amy, D.C., and Upper, C.D.: Bacterial ice nucleation - a factor in frost injury to

plants, Plant Physiol., 70, 1084-1089, 1982.

Liou, Y.C., Tocilj, A., Davies, P.L., and Jia, Z.: Mimicry of ice structure by surface hydroxyls and water of a β-helix antifreeze protein, Nature, 406, 322-325, 2000.

Lundheim, R.: Physiological and ecological significance of biological ice nucleators, Phil. Trans. R. Soc. Lond. B., 357, 937-943, doi:10.1098/rstb.2002.1082, 2002.

Morris, C. E., Sands, D. C., Vinatzer, B. A., Glaux, C., Guilbaud, C., Buffière, A., Yan, S., Dominguez, H., and Thompson, B. M.: The life history of the plant pathogen Pseudomonas syringae is linked to the water cycle, ISME Journal, 2, 321-334, 2008.

Morris, C. E., Sands, D. C., Glaux, C., Samsatly, J., Asaad, S., Moukahel, A. R., Goncalves, F. I. T., and Bigg, K. E.: Urediospores of rust fungi are ice nucleation active at > –10°C and harbor ice nucleation active bacteria, Atmos. Chem. Phys., 13, 4223-4233, 2013a.

Morris, C. E., Monteil, C. L., and Berge, O.: The life history of Pseudomonas syringae: linking agriculture to Earth system processes, Annu. Rev. Phytopathol., 51, 85-104, 2013b.

Murray, B. J., O'Sullivan, D., Atkinson, J. D., Webb, M. E.: Ice nucleation by particles immersed in supercooled cloud droplets, Chem. Soc. Rev., 41, 6519-6554, 2012.

Osborne, T.B.: Die Pflanzenproteine, Ergebnisse der Physiologie, 10, 47-215, 1910. (in German)

Schnell, R., and Vali, G.: Biogenic ice nuclei part I: Terrestrial and marine sources, J. Atmos. Sci., 33, 1554-1564, 1976.

Shen J. H., Klier, K., and Zettlemoyer A. C.: Ice nucleation by micas, J. Atmos. Sci., 34, 957-960, 1977.

Staudinger, H., and Staudinger, M.: Die makromolekulare Chemie und ihre Bedeutung für die Protoplasmaforschung; in Protoplasmatologia, 1, 1, 2-6, Springer-Verlag Wien GmbH, doi:10.1007/978-3-7091-2448-2, 1954.

Steele, R.L., and Krebs, F.W.: Characteristics of silver iodide ice nuclei origination from anhydrous ammonia-silver iodide complexes, part I, J. Appl. Meteorol., 6.1, 1966.

Wallnoefer, H. G., Handschuh, S., Liedl, K. R., and Fox, T.: Stabilizing of a globular protein by a highly complex water network: a molecular dynamics simulation study on factor Xa, J. Phys. Chem. B, 114, 7405-7412, doi:10.1021/jp101654g, 2010.

Wex, H., DeMott, P. J., Tobo, Y., Hartmann, S., Rösch, M., Clauss, T., Tomsche, L., Niedermeier, D., and Stratmann, F.: Kaolinite particles as ice nuclei: learning from the use of different kaolinite samples and different coatings, Atmos. Chem. Phys., 14, 5529-5546, doi:10.5194/acp-14-5529-2014, 2014.

Wiacek, A., Peter, T., and Lohmann, U.: The potential influence of Asian and African mineral dust on ice, mixed-phase and liquid water clouds, Atmos. Chem. Phys., 10, 8649-8667, doi:10.5194/acp-10-8649-2010, 2010.

Zachariassen, K. E., and Kristiansen, E.: Ice nucleation and antinucleation in nature, Cryobiol., 41, 257-279, 2000.

Zobrist, B., Marcolli, C., Peter, T., and Koop, T.: Heterogeneous ice nucleation in aqueous solutions: the role of water activity, J. Phys. Chem. A, 112, 3965-3975, 2008.

Zolles, T., Burkart, J., Häusler, T., Pummer, B., Hitzenberger, R., and Grothe, H.: Identification of ice nucleation active sites on feldspar dust particles, J. Phys. Chem. A, *accepted*, doi:10.1021/jp509839x, 2015.

| Elution time [min] | Mass range [kDa] |
|---|---|
| 2.8–3.5 | 335–860 |
| 3.5–4.5 | 50–335 |
| 4.5–5.2 | 13–50 |
| 5.2–6.0 | 5–13 |
| 6.0–7.5 | <5 |

Table S1: Sample fractions collected for INA tests and corresponding approximate molecular weights as estimated by calibration with standards. Although all fractions contained INMs, the first fraction contained the highest number concentration.

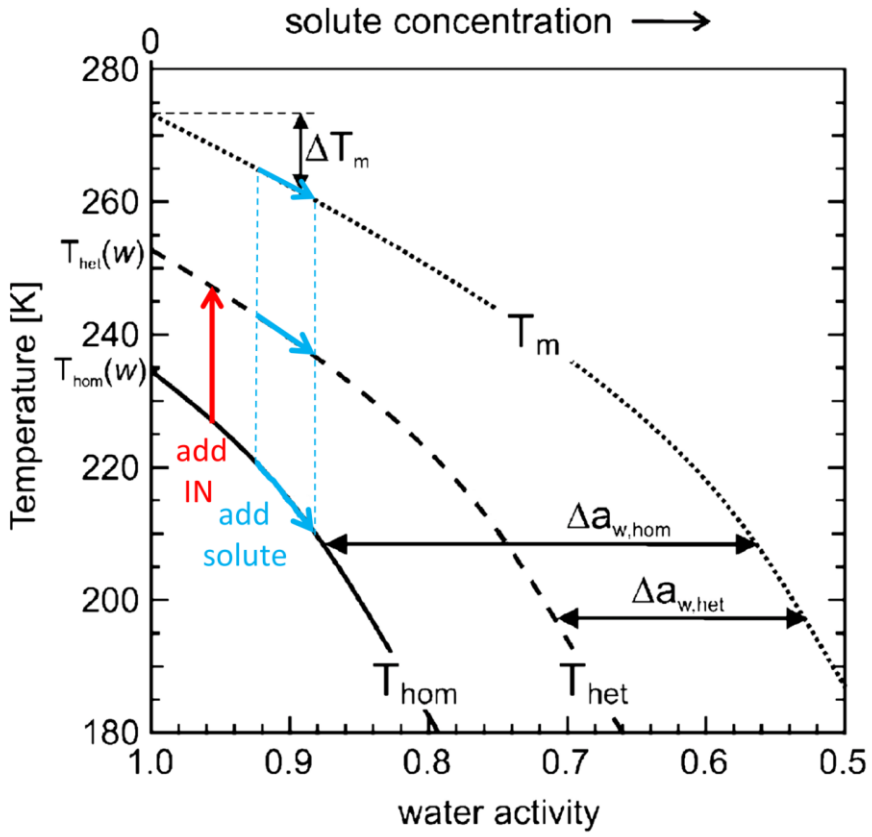


Figure S1: Correlation between $a_w$ and $T$, based on Koop and Zobrist (2009). The vectors show the impact of INs (red) and freezing point depressing solutes (blue).