Ice nucleation by water-soluble macromolecules

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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 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 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.

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 with a low molar mass 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 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. 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., 2015), from birch pollen (Pummer et al., 2012, Augustin et al., 2013), and from bacteria (Niedermeier 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) 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 θ and standard deviations σ from the 0.1 µm filtrate curves. This is done by determining values for θ and σ 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 θ and σ of *M. alpina* from comparable filtrates (Fröhlich-Nowoisky et al., 2015), 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. 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 (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 (ε_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

4.1 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

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., 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., 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., 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. 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.

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 are also present in the stratosphere (Griffin, 2004) and in cloud water samples (e.g. Vaitilingom et al., 2012, Joly et al, 2013). 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, which contains the INMs we described. 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.

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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 INs:	*R. 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 INs:	*Tipula*	+	+	+?	+	??	800 kDa	
	Dendroides	+	+	–?	+/–	??	>70 kDa	
	Vespula	+	+	–	??	<373 K	74 kDa	
	*Eurosta**	+	–	–	–	??	>100 µm	
Plant INs:	*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 INs. "T stability" shows the temperature above which they are denatured. A question mark indicates uncertainty. See Sect. 4.1 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., 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

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

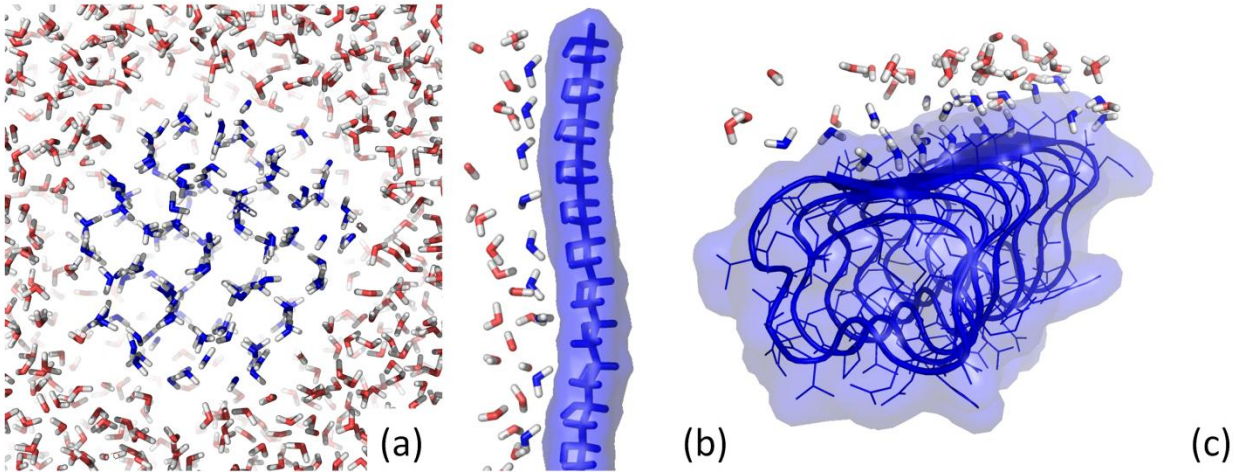

Figure 1: Visualization of water molecule ordering based on molecular model calculations (Sect. S2.1): homogeneous ice nucleation (a); heterogeneous ice nucleation by ordering of water molecules on a PVA strain, which is a 1D-template (b), and an antifreeze protein that has a similar sequence and structure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

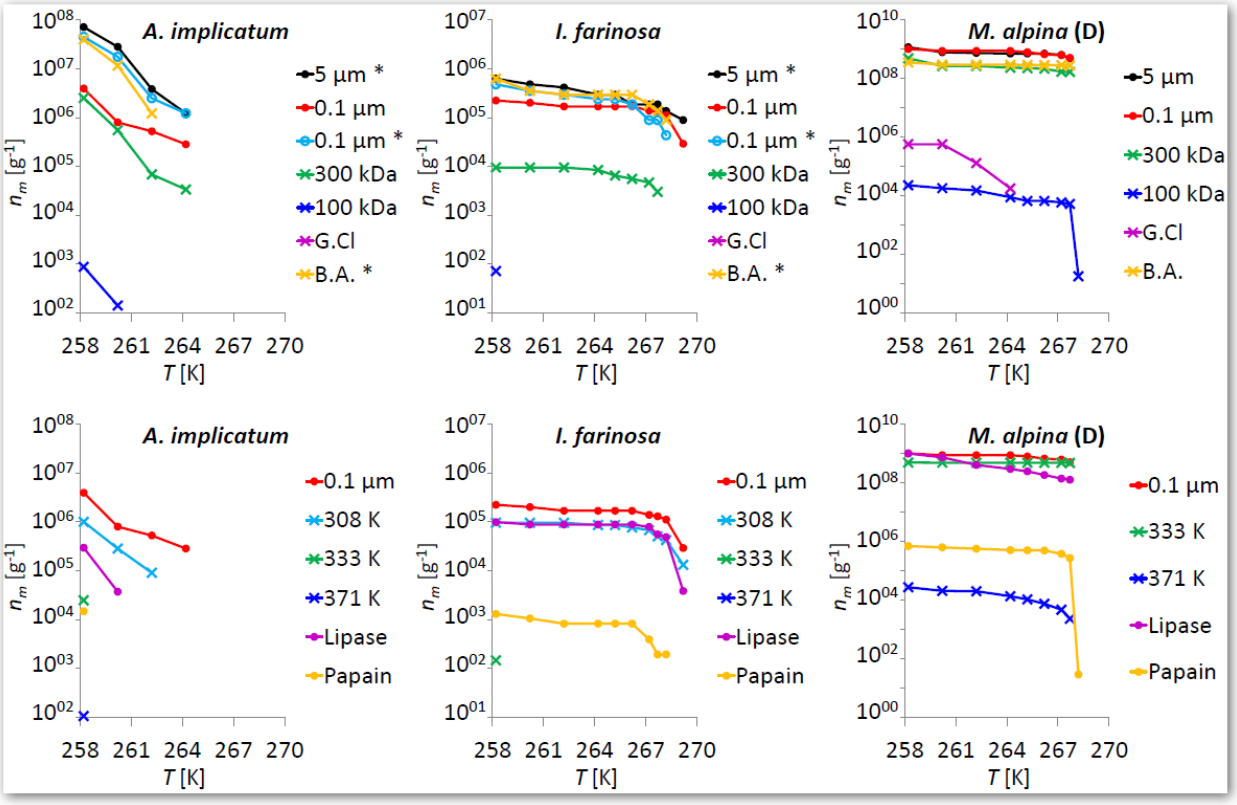

Figure 2: $n_m(T)$-curves for *A. implicatum*, *I. farinosa*, and *M. alpina* (subgroup D) INMs after different treatments. "G.Cl" stands for guanidinium chloride treatment, "B.A." for boric acid treatment. A reduction of n_m suggests that this method partly or fully destroyed the INMs. The absence of 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., 2015).
*) for *A. implicatum* and *I. farinosa*: these measurements were executed with the filtrates of another harvest, what explains the higher values in comparison to the other results.

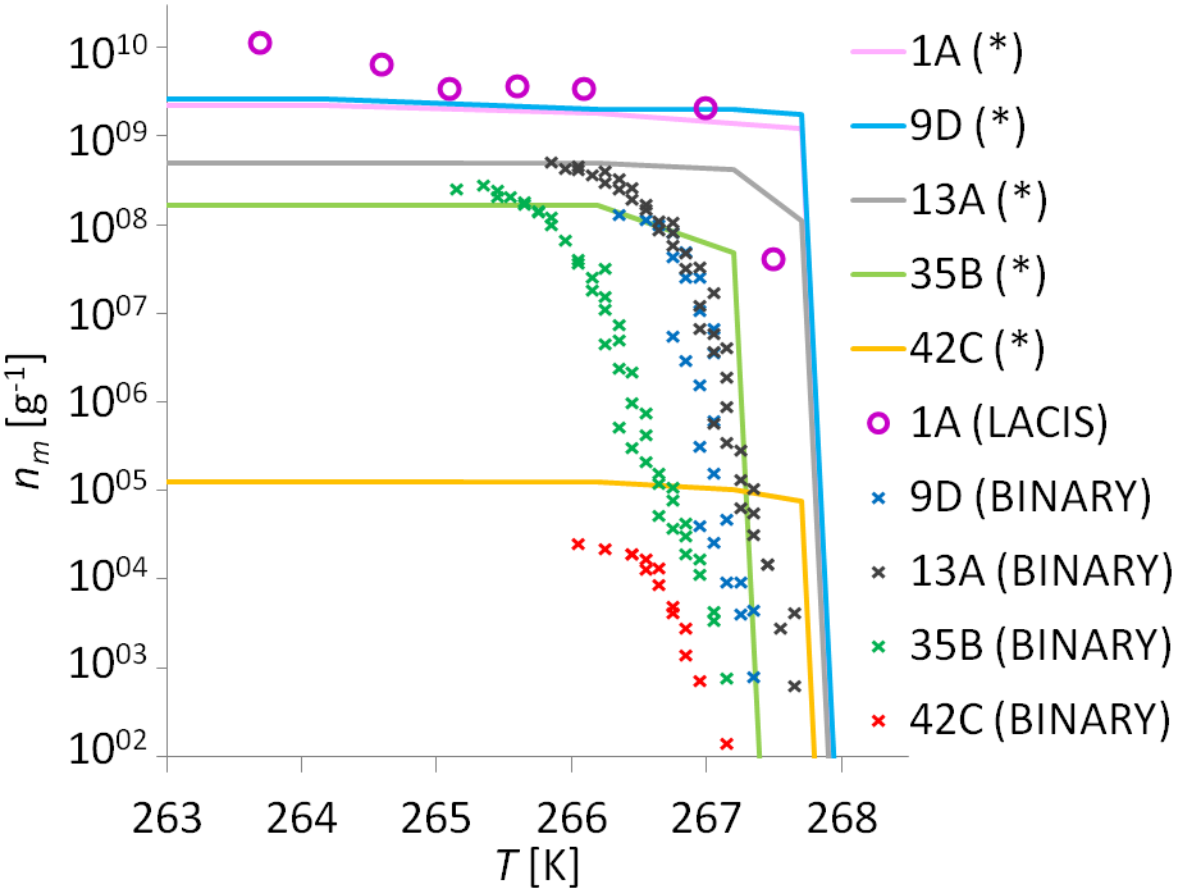

Figure 3: Comparison of ice nucleation curves of 0.1 µm filtrates from a few *M. alpina* strains. The number and letter combination labels the strain. The devices used for generating the respective curves are shown in brackets. "*" stands for the setup described in Fröhlich-Nowoisky et al. (2015).

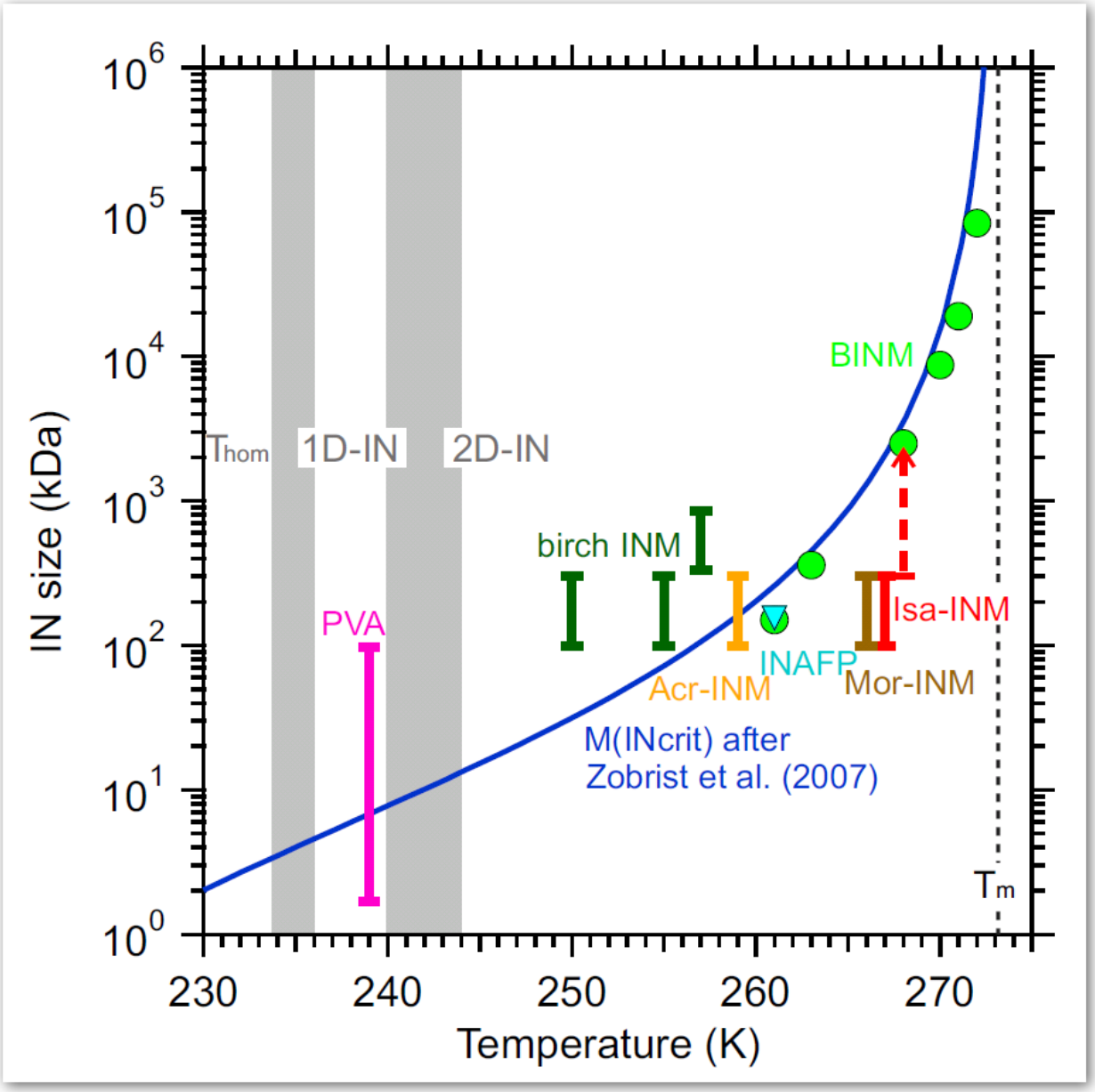

Figure 4: The dependence of the median freezing temperature on the size for different types of IN (colored dots). The blue curve is the calculated critical ice cluster size derived from *Classical Nucleation Theory* (Zobrist et al., 2007). The sources of the presented IN data are listed in Table 3. The 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.