Referee #1:

We thank Referee #1 for his comments and suggestions concerning our manuscript. Below, we present our responses. We hope that the critical points were addressed sufficiently.

COMMENT: 1) The structure of the paper is not coherent. It is presented as a research article with an experimental part, results and discussion sections but it looks more like a review on the domain. Also the supplement part is unusual, closer to a patchwork of book chapters, the idea being to help some readers to understand the rest of the paper (see appendix of this text).

RESPONSE: Our main intention of this admittedly long theoretical part was to give an overview of the current knowledge about biological INs in the light of our hypothesis. Although there are reviews of biological INs, the angle of view has always been a different one. The long introduction has the purpose that all readers are on the same page, since many of our claims might lead to discord without the proper explanation for our point of view. Since we are part of an interdisciplinary community, where people have many different backgrounds, we preferred to explain too much over too little for the reader (mainly supplement S1). Although some of the messages might seem basic knowledge, their connection to heterogeneous ice nucleation might not be obvious for every reader without reading the secondary literature.
We cut the biological part of the introduction (p.24276, L.23 - p.24280, L.25) as short as possible and shifted the remains to the discussion section as chapter 4.2.

COMMENT: 2) The content, although interesting and informative, is not strong enough to be published in ACP. The experimental results and new data are limited in the paper; they only complement data published elsewhere, often by the same authors. One can compare this paper with the data published previously in ACP by the same authors, for instance Pummer et al. (2012, 12, 2541- 2550) or Augustin et al. (2013, 13, 10989-1103) on the same topic and which are much more consistent. Also the long supplementary information presents notably very basic definitions or discussions which are too general for being presented in such research paper (for instance the description of macromolecules could be part of any chapter of a biochemistry book for undergraduate students...).
I would suggest to completely rewriting this paper as a review. The new results and protocols could be shifted in the Supplement section, and the long discussions and information present in the actual supplementary part S1 should be deleted, incorporated in the review in a shorter form when possible (for instance the discussion about the motivation for the expression of biological INMs) or supported by references to general book chapters, reviews or other papers (for instance the descriptions of macromolecular chemistry, of basic physics of INA ...).
The authors may have two alternatives:

1) They could write a "mini-review" that takes into account the information presented in this paper, but in this case it should be published elsewhere, for instance in "Atmospheric research" or "Atmospheric environment". In my opinion this paper would be too short for a review in ACP when compared to other reviews on similar topics (see for instance of Hoose and Möhler, 2012)

C. Hoose, O. Möhler. Heterogeneous ice nucleation on atmospheric aerosols: a review of results from laboratory experiments. Atmospheric Chemistry and Physics 2012; 12(5):12531-12621. DOI: 10.5194/acpd-12-12531-2012

2) Alternatively they could write a deeper review that could include more aspects, for instance which would present in more details the atmospheric context, other macromolecules of interest for atmospheric sciences (HULIS, biosurfactants, EPS, SOAs, etc...) including CCN aspects. This new review could be then resubmitted to ACP. Finally they could "reuse" the information present in the Supplement section for writing a book chapter.

RESPONSE: Indeed, some of the primary data sets have already been published in other papers. We did include those data sets for comparison with the new data sets, which are partly building up on our former results; and to present the greater picture, which is the intention of our paper, which we constructed by combining all available data (e.g. Fig. 4, Table 1 and 3).
Therefore, the current manuscript does not fulfill the format of a review, since it contains new data and new deductions that have not been present in our former publications. To follow our claims, a lot of background information is needed. We therefore compressed this part as much as possible and shifted it into the discussion section (now chapter 4.2.).
The incorporation of other INMs, namely SOAs and HULIS, is indeed an excellent suggestion. Therefore, we inserted following paragraph after p.24280, L.19:

"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). 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)."

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.

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.

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.

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.

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.

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.

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.

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

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.

COMMENT: P 24282 Methods of characterization of INMs
Although basic methods such as heating or the use of guanidinium chloride, boric acid and enzymes are valid to determine to presence of proteins or saccharides in INMS they are limited and should be completed by more powerful analytical methods. Why did the authors not use NMR and/or mass spectroscopy, which are the common
tools to assess the structure of macromolecules? These techniques can be applied to
purified compounds and also on complex mixtures through to 2D NMR (1H-1H or 1H-13C) or LC-MS and Maldi-TOF MS. The idea is not necessarily to determine the exact structure but to determine the chemical functions present in the molecules. NMR for instance easily detect aromatic functions, sugar characteristic signals (anomeric 1H), amino acids signals, carboxylic or aldehyde functions etc.... These techniques are much more informative and reliable than those used in this manuscript and can give indications on structural motifs which are not polysaccharides or proteins, and with no a priori.

RESPONSE: The referee is right that these methods are powerful tools for characterization of biological samples. Therefore, investigations with different more sophisticated techniques are in progress.
The issue with MS and NMR is that the extracts are a complex mixture of many different molecules, and these methods do not discriminate between INMs and INA-negative material. Currently, our only chance is to either knock out certain groups of molecules, or purify the

sample, and then check for the change of INA in the freezing assay. It is crucial that in the end we have an aqueous sample that can be investigated in a freezing setup.
We already started with fractioning experiments (e.g. solid phase extraction) in order to narrow down the possible INM candidates further. However, even the individual fractions with relatively far fewer chemical species still contain a high diversity of them. Even the chemical species specific for the active fractions are currently too numerous to count. The isolation and identification of the INMs with these techniques will be an elaborate task with the proper combination of several isolation steps.

COMMENT: P 242887 Critical cluster size
Although Fig 4 presents interesting results, the discussion about PVA (p 24288) is long and rather useless; it is quite evident that this simple oligomer has nothing to do with a complex protein structure. The necessary molecular arrangement to make an ice crystal for different proteins has already been well described from models (see Garnham et al., 2011). Note that this paper, which is cited P 24277 line 5, should rather be cited when describing Fig 1c or within this paragraph. From this, it was expectable that PVA would remain a rather inefficient IN, whatever its size.

RESPONSE: It might be not evident for every reader, but the original version is admittedly too long. Therefore, we rewrote / shortened some lines:

p.24288, L.10-20: "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."

p.24288, L.25-29: "Summed up, randomly coiled INMs like PVA allocate only small, one-dimensional templates for ice nucleation (Fig. 1b) and therefore nucleate ice at rather low temperatures (Fig. 4)."

p.2489, L.5-7: deleted "Of course, the surface of these 2D-templates has to be properly functionalized in order to arrange the water molecules, or else they show no INA at all."

COMMENT: P 24292 line 6: the sentence should be changed to " At last, microorganisms were found in cloud waters....." .The reference Amato et al. 2007 could be changed to that of Vaïtilingom et al. (2012) which is more complete and recent.
M. Vaïtilingom, E. Attard, N. Gaiani, M. Sancelme, L. Deguillaume, A. I. Flossmann, P. Amato, A.-M. Delort. Long-term features of cloud microbiology at the puy de Dôme (France). Atmospheric Environment, 2012, 56, 88-100.

RESPONSE: We changed the reference and replaced "frequently found" with "present", since it can be safely assumed that microorganisms occur in precipitation, as our reference in the next line (Sattler et al., 2001) also states.

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

COMMENT: Finally the discussion about atmospheric impacts of INMS should be completed by considering data from the literature about biological IN in precipitations (snow, rain), aerosols or cloud samples. For instance Christner et al. (2008a, b) measured IN activity in precipitations on filtered samples, so such water soluble INMs might have been completely ignored. As a result the estimation of biological impact in ice nucleation process could be highly under-estimated. This is also true for modeling studies (see for instance Hoose et al., 2010) who considered only IN as whole cells, which again could underestimate the contribution of biological impact on precipitations. This list of examples is not exhaustive...

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, Proceedings of the National Academy of Sciences, 2008a, 105, 18854–18859.

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

C. Hoose, J. E. Kristjánsson, S. M., Burrows. How important is biological ice nucleation in clouds on a global scale? Environmental Research Letters, 2010; DOI:10.1088/1748-9326/5/2/024009

RESPONSE: We inserted the following paragraph after p.24293, L.5:

"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 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. The restrictions imposed on the methods due to the difficulty with grasping the molecular fraction might result in a severe underestimation of biological INs in the results."

Referee #2:

We thank Referee #2 for his comments and suggestions concerning our manuscript. Below, we present our responses. We hope that the critical points were addressed sufficiently.

COMMENT: I completely agree with RC C8428 that this paper reads like a review article in places. I cannot comment much on whether the data are only supplementary to other published works, rather than warranting publication outright. It seems there are potentially some new findings in the paper; however, as presented results were not explained coherently enough to be able to judge.

General
I enjoyed reading the introduction. I felt it gave a very nice introduction to the area. However, it is a little bit long for a research article and reads a little bit like a thesis in places. It is almost as if a table would help to summarise some of the findings for different sources of ice nucleating macromolecules.

RESPONSE: Indeed, the introduction may look like a mini-review, however, the rest of the paper does not fulfill this format, since our main drive is to present new data and ideas. The introduction has the purpose of helping our readers, which come from very different fields and backgrounds, to understand our motivations, thoughts and conclusions.
We shortened p.24276, L.23 - p.24280, L.25 and converted it into its own section in the discussion (now Sect. 4.2). A further shortening of the paragraph would lead to the loss of valuable information, which is essential for our arguments in the latter chapters. An overview of the central findings is shown in Table 1.

COMMENT: In general the whole of the Methods section 2.1 could be made much clearer. I found it hard to follow a lot of it. For a specific statement see, page 24282, lines 10-26 and next page lines 1-5. I struggled to understand what was being said here. What is the "proper dilution"? Also, please explain all the variables in Equation 1 and the meaning of them more clearly. nm for instance is not really defined. I would like to see an explanation of where the equation comes from.

RESPONSE: We rewrote several lines of the chapter. For a better overview, we only list the most relevant changes here, while we do not list minor exchanges of words:

p.24281, L.7-12: replaced with "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."

p.24282, L.10-12: expanded "To determine the IN concentration per gram of mycelium, the same setup and procedure as in Fröhlich-Nowoisky et al. (2014) 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)."

p.24282, L.22: inserted "n_m is the number of INMs per gram of mycelium" and deleted "is" after "f_{ice}".

The formula is a variation of the Vali formula, which assumes that the freezing of droplets is a first order reaction, since every droplet is isolated and freezes with a certain statistical probability. The additional factors scale the probability with the INM content per droplet.

p.24283, L.1-5: replaced with " 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)."

p.24283, L.10: inserted "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)."

p.24283, L.15-18: replaced with "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 (see e.g. Niedermeier et al., 2014). 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."

p.24283, L.19-22: expanded paragraph "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:"

Fröhlich-Nowoisky, J., Hill, T. C. J., Pummer, B. G., Franc, G. D., and Pöschl, U.: Ice nucleation activity in the widespread soil fungus Mortierella alpina, Biogeosci. Discuss., 11, 12697–12731, doi:10.5194/bgd-11-12697-2014, 2014.

COMMENT: In the next line (page 24283, line 6) the paper then talks about the soccer ball model; however, it is not clear how equation 1 relates to this treatment. This should be made clearer. The paper then talks about 2 extra methods using drop freezing array and the LACIS. There should be some justification as to why you use these different methods, to inform the reader why you are doing this.

RESPONSE: The respective equations describing the Soccer Ball Model are not reproduced in this text, as we feel that this is too detailed and not really needed. If you find this necessary, please let us know and we can add them. For the time being, the following was added

explicitly to the text (p.24283, L.10): "[…] 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)."

The extra methods were used to expand the data-set which were obtained with the 50 µl aliquots. The freezing array examined smaller droplets (about 1 µl), which reduced the background (see Fig. 3). Larger droplets/aliquots increase the probability of contamination of the sample due to ice nucleating matter included in the water (ever ultrapure water still carries a few ice nucleating entities in it, so that 50 µl). LACIS was included as this is the only one of the three methods which examines free floating droplets, i.e. data for ice nucleation is obtained without interference with any substrate. This was done to ensure that the substrates (a glass slide for the freezing array and the wells for the method used in Mainz) did not influence the results. We added to the text (p.24283, L.19): "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)."

COMMENT: Section 3 is very hard to follow. I tried to work my way through this section, but in the end I gave up trying. As an example of something that isn't clear see page 24288, line 27 "Consequently, the ice nucleation temperatures are maximum a few Kelvin above the homogeneous freezing temperature (see Fig.4)." Figure 4, however, does not appear to show anything about maxima in ice nucleation temperatures. Does it not just show that PVA nucleates ice at just lower than 240 K?

RESPONSE: We rewrote several of the paragraphs, the most important of which are listed below:

p.24285, L.1-3: replaced with "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."

p.24286, L.11-22: replaced with " Fig. 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."

p.24287, L.3-6: replaced with "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). "

p.24287, L.17: shifted up chapter 3.3 and deleted headline ("INA of BINM peptides")

p.24287, L.19-23: replaced with "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 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."

p.24288, L.4-7: replaced with "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."

p.24288, L.10-29: cut short and replaced with "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 rather low temperatures (Fig. 4)."

p.24289, L.16-21: replaced with "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."

COMMENT: Section 4.1 does not really present the findings in a coherent way. It is a discussion rather than conclusion. I would suggest that, if the authors still want to present a research article, a separate / concise conclusions section is needed to present the new ideas.

RESPONSE: We renamed chapter 4 "Discussion" instead of "Discussion / Conclusions" and added another chapter 5 "Conclusions"

COMMENT: A lot of Section 4.2 is pure speculation. I was hoping for a focussed conclusions section that explained the findings, perhaps in bullet points

RESPONSE: With section 4.2, we indeed speculate what implications our findings might have on the atmosphere, and want to encourage further field and model studies that put effort in answering these questions. We do not consider it too speculative, since we backed up all our claims with studies, or connected them with basic considerations (e.g. the build-up of a cell). To express stronger that we do not intend to forestall future studies addressing this topic, we renamed the chapter "Potential atmospheric effects" instead of only "Atmospheric impacts"

Following the suggestion, we added another chapter "Conclusions" with the following content:

"Even free water-soluble macromolecules are able to nucleate ice, since they are in the same size range as the ice embryos necessary for ice formation. 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."

COMMENT: Methods, line 6. You say the mycelium was scratched off and 10 mL of high-purity water was added. What was the weight percent of the mycelium in the water? For example in experiments with mineral particles this is often quoted (e.g. in the experiments of Murray et al, 2011). Was the mycelium a powder or solid lumps?

RESPONSE: Depending on the harvest, it ranged from 0.6% to 8.6%. For normalization, we divided by the dry mass of the mycelium in Eq. 1, which eliminates the influence of the weight percent on n_m.
The mycelium showed the consistency of cotton (*I. farinosa*) or lumps (*A. implicatum*).

COMMENT: What was the reason for autoclaving and how do you this will affect your results?

RESPONSE: It is a standardized procedure for denaturation of biological cells and molecules in order to rid the water of possible contaminants.

COMMENT: When you pass through the 0.1 micron filter, what do you estimate to be the mass loading?

RESPONSE: We never investigated it, since we were more interested in the fraction that passes the filter.

COMMENT: Page 24283, line 16. When you say CNT works perfectly, what is your metric for this statement? It should be justified.

RESPONSE: Thank you for pointing out that our choice of wording was not precise enough, here. We have changed the corresponding lines (L.15-18) accordingly, and they now are as follows: "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 (see e.g. Niedermeier et al., 2014). Here we apply it to describe the ice nucleation induced by INMs, and we were able to derive contact angle distributions such that the 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."

COMMENT: Page 24282, line 6 "no proteins" should be "not proteins"? There are a few typos throughout.

RESPONSE: We changed it as suggested.

COMMENT: Figure 2, font is too small. Legend is hard to interpret too, without delving into the text (i.e. it isn't explained in the figure caption what the different lines mean). This makes it very difficult to follow what is being said in Sectoin 3.1, lines 1-10.

RESPONSE: We split up each diagram into 2 separate diagrams with only the 0.1 µm curve as common reference. We updated the figure caption:
"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 in 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). The absence of a curve in a diagram means that no droplets were frozen at all.
*) 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."

COMMENT: Could the authors comment on the following point, which affects the key findings of the paper. On page 24285, line 20 you say that the filtrate are particle free, because you don't see particles in suspension. How do you know they are indeed particle free, and it is just that you can't see the small particles in suspension?

RESPONSE: We investigated the residues left in the water in former studies with SEM (e.g. Pummer et al., 2012, 2013), where the absence of larger structures was demonstrated. Additionally, the Vivaspin filters only allow very small (molecular) components pass through them.
It is a topic of debate where to draw the line between a molecule / particle in suspension and in solution for a macromolecule, since even a single dissolved molecule can be nanometers in size, and therefore be interpreted as a nanoparticle.
In our former papers, we described the INMs as "suspended", since it is in better agreement with the conventional view of IN. The formal definitions of where to draw the line, however, strengthen the perspective of viewing it as a solution. The criteria are e.g. the absence of optical effects (e.g. scattering) in the range of visible light and the long-term phase stability (no sedimentation, no effect of centrifugation). From a molecular point of view, a molecule is in solution, if it is fully covered in a hydration shell. This is also valid for water-soluble proteins, while insoluble proteins flock together and sediment. Therefore, proteins can be regarded as dissolved despite their size (e.g. Osborne, 1910, Macedo, 2005).
We also label our samples as "particle-free" to point out the difference to what is conventionally understood as particles: For mineral dusts, salts, soot, etc., a particle does not consist of a single formulaic unit, but of a large lattice respectively aggregate of a huge number of units (an elementary cell or an ion pair).

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

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

Pummer B.G., Bauer H., Bernardi J., Bleicher S., Grothe H. (2012): *Suspendable macromolecules are responsible for ice nucleation activity of birch and conifer pollen*; Atmospheric Chemistry and Physics 12, p.2541-2550

Pummer B.G., Bauer H., Bernardi J., Chazallon B., Facq S., Lendl B., Whitmore K., Grothe H. (2013): *Chemistry and morphology of dried-up pollen suspension residues*; Journal of Raman Spectroscopy 44, p.1654-1658

We inserted a further paragraph into chapter S1.1 in the supplement, p.2, L.24:

"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. 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."

COMMENT: Figure 3. I have the same issues with Figure 3 to Figure 2. Namely, the legend key and figure caption do not help me understand the figure. Is there really good agreement between the different methods? The 42C sample is coloured yellow for the MPIC method and

red for the BINARY method and there is a large difference in the results. In fact I couldn't follow the meaning of the legend at all.

RESPONSE: We now show the full acronyms of the setups in the legend instead of using a one letter abbreviation. The data measured with the setup described in Fröhlich et al. (2014), which has no acronym, was labeled with an asterisk. The figure caption was rewritten:
"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)."

APPENDIX: List of changes:

p.24275, L.4-16: we rewrote the 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 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. We combine 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."

p.24275, L.19-21: replaced with "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 (see Fig.1a). "

p.24275, L.25: deleted "as"

p.24276, L.2: replaced "surface of the IN" with "IN surface"

p.24276, L.10-11: replaced with "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)."

p.24276, L.12: inserted "a large" before "enough surface"

p.24276, L.14: inserted "More information about INMs is given in Sect. S1.1"

p.24276, L.14: deleted "organic"

p.24276, L.16-17: replaced with "Further information about the ice nucleation process is compiled in the supplement (Sect. S1.2, S1.3, and S1.4)." combined 2 paragraphs

p.24276, L.18: replaced "among a variety of organisms" with "in various forms of life"

p.24276, L.20: shifted "in almost all cases" to the end of the sentence, inserted "regarded"

p.24276, L.21-22: we expanded the statement to "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)."

p.24276, L.23: deleted "In the case of bacteria, it is a certain class of lipoglycoproteins."

p.24276, L.23 - p.24280, L.19: shifted; now chapter 4.2 ("Previous and recent findings on biological INMs")

p.24280, L.20-21: deleted "Fungi are abundant and diverse in the atmosphere (Fröhlich-Nowoisky et al., 2009, 2012). Therefore, their potential for atmospheric ice nucleation has to be regarded."

p.24280, L.22-25: replaced with " In this study, we 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), from birch pollen (Pummer et al., 2012), and from bacteria (Niedermeyer 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."

p.24281, L.6: inserted "either" before "a scalpel"

p.24281, L.7-12: replaced with "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."

p.24281, L.15: deleted "later"

p.24282, L.2: replaced "so" with "therefore"

p.24282, L.10-12: expanded "To determine the IN concentration per gram of mycelium, the same setup and procedure as in Fröhlich-Nowoisky et al. (2014) 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)."

p.24281, L.16: inserted "changes in" before "their INA"

p.24281, L.17: replaced "The change of INA" with "This"

p.24282, L.13: deleted 2x "then"

p.24282, L.19: replaced "so" with "therefore"

p.24282, L.20: deleted "see"

p.24282, L.22: inserted "n_m is the number of INMs per gram of mycelium" and deleted "is" after "f_{ice}".

p.24283, L.1-5: replaced with " 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)."

p.24283, L.10: inserted "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)."

p.24283, L.13-15: replaced with "[...] and added literature data for INMs from birch pollen (Augustin et al., 2013) and bacteria (Niedermeier et al., 2014)."

p.24283, L.15-18: replaced with "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 (see e.g. Niedermeier et al., 2014). 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."

p.24283, L.19-22: expanded paragraph "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:"

p.24283, L.23: replaced "A freezing droplet array called" with "A droplet freezing array termed"

p.24283, L.25-26: replaced with "A detailed description of the technique, the preparation of droplets, and the data acquisition and evaluation is given in Budke and Koop (2014)."

p.24284, L.6-8: replaced with "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."

p.24284, L.15-20: replaced with "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."

p.24284, L.24-26: replaced with "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."

p.24285, L.1-3: replaced with "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."

p.24285, L.4: renamed chapter "Ice nucleation experiments with bacterial INM peptides"

p.24285, L.6: replaced "Ps. syringae BINM" with "bacterial INM (BINM) of Pseudomonas syringae"

p.245285, L.16: deleted "/discussion"; chapter headline is "Results"

p.245285, L.24: deleted "see"

p.24286, L.5: inserted "approximately" before "100 to 300"

p.24286, L.11-22: replaced with "Fig. 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."

p.24286, L.24: inserted "for the trypsin test" after "medium"; deleted "however"

p.24286, L.25: deleted "at all"

p.24286, L.26: inserted "from the medium" after "formic acid"

p.24286, L.27: replaced "medium" with "respective measurement"

p.24287, L.3-6: replaced with "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). "

p.24287, L.10: replaced "would make" with "makes"

p.24287, L.13-14: replaced "critical cluster" with "ice embryo"

p.24287, L.17: shifted up chapter 3.3 and deleted headline ("INA of BINM peptides")

p.24287, L.18: expanded headline for chapter 3.2 ("Comparison with theoretical calculations of the ice embryo size")

p.24287, L.19-23: replaced with "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 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."

p.24287, L.25: replaced "critical cluster" with "ice embryo"

p.24288, L.4-7: replaced with "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."

p.24288, L.10-29: cut short and replaced with "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 rather low temperatures (Fig. 4)."

p.24288, L.29: inserted "long-range" before "confined"

p.24289, L.1-2: deleted 2x "see"

p.24289, L.5-7: deleted "Of course, the surface of these 2-D-templates has to be properly functionalized in order to arrange the water molecules, or else they show no INA at all."

p.24289, L.8: deleted headline ("INA of BINM peptides") and shifted chapter up to p.24287, L.17

p.24289, L.10: deleted "In view of Fig.4"

p.24289, L.16-21: replaced with "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."

p.24290, L.1: deleted "and conclusions"

p.24290, L.2: renamed chapter "Solubility of INMs"

p.24290, L.6-7: replaced with "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."

p.24290, L.13: deleted "in the supplement"

p.24290, L.14: inserted "protein" before "molecules", replaced "lower sugars" with "low molecular weight saccharides"

p.24290, L.16: replaced "be" with "act"; added "active" before "molecular surface"

p.24290, L.19: replaced "solution" with "solution's"

p.24290, L.26 - p.24291, L.2: replaced with "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 is a macroscopic interpretation of the molecular interaction between phases."

p.24291, L.6: replaced "be" with "cover"

p.24291, L.8-9: replaced "[...] or by a large hydration shell around these INMs that has to be added to the total IN mass." with "[...] or by the ability of forming a larger hydration shell that has to be taken into account."

p.24291, L.18: inserted parts from introduction (p.24276, L.23 - p.24280, L.19) as chapter 4.2., entitled "Previous findings on biological INMs". Cut it short and inserted paragraph about HULIS, SOA and organic acids. Full text:

"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., 2014). 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). 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) 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). 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)."

p.24291, L.19: replace headline "4.2. Atmospheric impacts" with "4.3. Potential atmospheric effects"

p.24291, L.23: replaced "that" with "which"

p.24291, L.25: inserted "however" before "the detection"

p.24291, L.25: inserted "despite their size" at the end of the sentence

p.24292, L.6: replace "frequently found" with "present"

p.24292, L.7: replace "Amato et al., 2007" with "Vaitilingom et al., 2012"

p.24292, L.10: deleted "furthermore"

p.24293, L.5: inserted discussion about modeling and a chapter for the conclusions:

"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 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 biological INs in the results.

5. Conclusions

Even free water-soluble macromolecules are able to nucleate ice, since they are in the same size range as the ice embryos necessary for ice formation. 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."

p.24293, L.18: inserted "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."

p.24293, L.19: deleted Amato et al. (2007)

p.24294, L.9: inserted "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."

p.24295, L.23: deleted Fröhlich et al. (2009)

p.24295, L.26: deleted Fröhlich et al. (2012)

p.24298, L.22: inserted "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."

p.24299, L.8: inserted "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."

p.24299, L.22: inserted "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."

p.24301, L.1: inserted "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."

p.24301, L.20: inserted "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."

p.24302, L.5: inserted "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."

p.24302, L.13: inserted "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." and "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."

p.24302, L.13: inserted "Wilson, T. W., Murray, B. J., Wagner, R., Möhler, O., Saathoff, H., Schnaiter, M., Skrotzki, J., 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."

p.24302, L.28: inserted "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."

Table 1: replaced "yes" and "no" with plus and minus; replaced "algae" with "different algae"; replaced "interrogation mark" with "question mark"; deleted "some" before "uncertainty"
deleted first "the" in figure caption; replaced "temperature about which" with "temperatures above which"; replaced "Introduction" with "Sect. 4.2"

Figure 2: diagrams were replaced; new figure caption: " 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 in 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 et al., 2014). The absence of a curve in a diagram means that no droplets were frozen at all.

*) 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."

Figure 3: format and labeling improved; new figure caption: "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 et al. (2014)"

List of changes in the supplement:

p.1, L.18: replaced "so" with "thereby"

p.2, L.5: inserted "it"

p.2, L.12: inserted "given"

p.2, L.25: inserted an additional paragraph to discuss insoluble particles vs. solution:

"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. 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."

p.3, L.17: replaced "critical cluster" with "ice embryo"

p.3, L.19: inserted "arranged" after "45000"; replaced "critical cluster" with "ice embryo"

p.4, L.6: replaced "therefore decreasing" with "and therefore decreases"

p.4, L.7: shifted "(INs)" before "and"

p.5, L.4: replaced "at" with "on"

p.5, L.20 - p.6, L.5: replaced all "a_W" with "a_w"

p.5, L.22: replaced "the" with "insoluble"

p.6, L.29: replaced "have" with "were"

p.7, L.6: replaced "are" with "is"

p.7, L.7: deleted "however"

p.8, L.10: replaced "Zolles, 2013" with "Zolles et al., 2015"

p.8, L.18-19: replaced ",which [...] its" with ". That [...] the"

p.8, L.26: inserted "(AFP)" after "protein"

p.9, L.8: replaced "in" with "by"

p.9, L.27: corrected format: "-1" is superscript

p.13, L.26-27: replaced Zolles et al. (2013) quote with:

"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."

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 is a foreign body in the water that functions as a template for arranging water molecules in an ice-like manner. It is often assumed that these INs 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~~We put in perspective that also dissolved single macromolecules can induce ice nucleation: They are several nanometers in size, which is also the size range of the necessary critical cluster. As the critical cluster size~~ is temperature-dependent, we see a correlation between the size of INMs~~such ice nucleating macromolecules~~ and the ice nucleation temperature. 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. We combine 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.~~Such ice nucleating macromolecules have been already found in many different biological species and are as manifold in their chemistry. Therefore, we additionally compare them to each other, based on a composition of former, recent and yet unpublished studies. Combining these data with calculations from *Classical Nucleation Theory*, we want to foster a more molecular view of ice nucleation among scientists.~~

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~~s~~ liquid, until temperatures as low as 235 K are reached~~ice nucleation takes place~~. 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). ~~Homogeneous ice nucleation (see Fig. 1a) is very unlikely, until temperatures as low as 235 K are reached.~~ 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 (~~see~~ Fig.1b+c). An impurity that possesses this ability is called ice nucleator (IN), or sometimes as 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 surface of the IN. 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 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). ~~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 so large that they allocate 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 organic compounds 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 various forms of life~~among a variety of organisms~~, including certain bacteria, fungi, algae, plants and animals. Studies to characterize the active sites of some of these organisms have revealed~~ in almost all cases~~ that they are biopolymers in almost all regarded cases. The chemistry of these INMs is as diverse as the range of species they represent: 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 (~~see~~ Table 1, Sect. 4.2). ~~In the case of bacteria, it is a certain class of proteins. The known bacterial INMs (BINMs) are fully sequenced and characterized (e.g. Abe et al., 1989), while more questions remain unresolved concerning the other biological INMs. In some cases, biological INMs of one type or species show more than one freezing temperature in an ice nucleation~~

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spectrum. This can be explained by the presence of different functional groups, different foldings or aggregation states, which also differ in their INA (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 motivations for certain species to produce them (see 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 (see 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 existence of such BINMs has been reported for several species of γ-Proteobacteria, such as a wide range of strains in the *Pseudomonas syringae* species complex (Lindow et al., 1982, Berge et al. 2014); *Ps. 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,); *Pant. ananatis* (Coutinho and Venter, 2009); *Xanthomonas campestris* (Kim et al., 1987); a *Pseudoxanthomonas* sp. isolated from clouds (Joly et al., 2013); and more. The efficacy of their INA depends on the strain, as well as on the cultural growth conditions, e.g. the amount of accessible 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, BINMs that have been isolated from the cell membrane show still 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, i.e. the whole bacterial cell which is about 1 µm long. In some cases, bacteria release cell free INs that are carried on particles that are only a small fraction of the size of the cell. This is the result of the formation of membrane vesicles, 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 *Pant. agglomerans* (formerly *E. herbicola*) (Phelps et al., 1986), *E. uredovora* (Kawahara et al., 1993), and *Ps. fluorescens* (Obata et al., 1993). For the production of BINM carrying vesicles by *Ps. syringae* and *viridiflava* special culture conditions are necessary (Obata et al., 1990b, Pooley and Brown, 1990). For *Ps. 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 contrast to the BINMs from the species described above, removal of the approximately 92 kDa of carbohydrates eliminated the INA. The antifreeze properties, however, were not affected (Xu et al., 1998).

INMs were also found in the kingdom of fungi. 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). Species that showed appreciable INA in laboratory studies include *Fusarium* sp. (Pouleur et al., 1992, Hasegawa et al., 1994, Tsumuki and Konno, 1994, Tsumuki et al., 1995, Richard et al., 1996, Humphreys et al., 2001), lichen mycobionts (Kieft, 1988, Kieft and Ahmadjian, 1989, Kieft and Ruscetti, 1990), rust fungi (Morris et al., 2013, Haga et al., 2013), *Mortierella alpina* (Fröhlich Nowoisky et al., 2014), *Acremonium implicatum* and *Isaria farinosa* (Huffman et al., 2013). The characterization of the last two INMs is a part of this study. 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), while the others are evidently proteins. The already characterized INMs from the lichen *Rhizoplaca chrysoleuca* (Kieft and Ruscetti, 1990), from *F. avenaceum* (Pouleur et al., 1992, Hasegawa et

al., 1994, Tsumuki and Konno, 1994), and from *M. alpina* (Fröhlich-Nowoisky et al., 2014) barely showed similarities with BINMs, apart from being proteinaceous. For example, they are more tolerant to stresses, have a different amino acid sequence, seem to have less to no lipid and carbohydrate functionalizing, and are extracellular, since they pass through filters with submicrometer pores. Only recently, a 49 kDa protein from *F. acuminatum* was suggested as being the INM (Lagzian et al., 2014). The study also suggests that posttranslational functionalization takes place in the native state and improves the INA, which is a new finding in comparison to former studies (Kieft and Ruscetti, 1990, Tsumuki and Konno, 1994, Fröhlich-Nowoisky et al., 2014).

INs were also found in extracellular fluids of multicellular organisms. The larvae of *Tipula trivittata* (a crane fly) carry an INA positive 800 kDa lipoprotein in their hemolymph, which shares a high similarity with the BINMs (Duman et al., 1985, Neven et al., 1989, Duman et al., 1991, Warren and Wolber, 1991). The hemolymph of the queens of *Vespula maculata* (a hornet) contains a 74 kDa hydrophilic INA protein (Duman et al., 1984), and the hemolymph of *Dendroides canadensis* (fire-colored beetle) larvae contains a cocktail of an INA protein, an INA lipoprotein and an antifreeze protein (Olsen and Duman, 1997). Most of the known animal INs are proteinaceous, although there are some exceptions, such as the calcium phosphate spherules and fat cells in the larvae of *Eurosta solidaginis* (a gall fly) (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 non-proteinaceous INs have also been found in plants such as the ones reported from the wood of *Prunus* species (drupes)(Gross et al., 1988), or the lignin in a waste water sample (Gao et al., 1999). Only few plant INs, like those of *Secale cereale* (winter rye, Brush et al., 1994), have been clearly identified as proteins. 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). All pollen with INA that were further investigated produce easily extractable INMs, but apart from that showed some differences from

~~each other. As it was confirmed by vibrational spectroscopy, the extracts of pollen contain saccharides, lipids, proteins, and in some cases carotenoids, but no signature of sporopollenin, which is the sturdy hydrophobic polymer building up the outer pollen wall (Pummer et al., 2013b). Birch pollen INMs have a size between 100 and 300 kDa, are tolerant to dry heat (up to 450 K), to high acid and guanidinium concentrations, as well as to several enzymes. Overall, they show typical non-protein and non-lipid behavior (Pummer et al., 2012).~~

~~Fungi are abundant and diverse in the atmosphere (Fröhlich-Nowoisky et al., 2009, Fröhlich-Nowoisky et al., 2012). Therefore, their potential for atmospheric ice nucleation has to be regarded. In this study, the INMs that were recently found in *A. implicatum* and *I. farinosa* were characterized and compared to other biological INMs, especially the recently characterized INA proteins in *M. alpina* (Fröhlich-Nowoisky et al., 2014). We also expand our knowledge about the chemistry of the birch pollen INMs (Pummer et al., 2012).~~

In this study, we 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), from birch pollen (Pummer et al., 2012), and from bacteria (Niedermeyer 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 a either 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~~Then 10 ml high purity water (18.2 MΩ·cm) was added, which was tapped from a water purification system (Thermoscientific™ Barnstead GenPure xCAD plus), autoclaved at 394 K for 20 min, and at last filtrated through a sterile 0.1 µm PES filter (Corning™). The suspension~~ 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®). Asmall aliquot of the 0.1 µm filtrate was saved for ~~later~~ INA tests. Further aliquots were exposed to different procedures, which are listed below, and then tested for changes in their INA. The~~ change of INA~~is 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~~so~~ blocks the site.
- Digestion with enzymes (Applichem®) for at a given incubation temperature: ~~Lipase~~Lipase for 1 hour at 308 K for fat digestion, ~~papain~~ 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~~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. (2014) were applied: Eeach 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). its proper dilution (which was determined by trial and error) according to Eq.(1). Then, 50 µl aliquots of the dilute were pipetted into 24–32 wells of a 96 well PCR tray (Axon™), which was then sealed with adhesive foil. The plate was then 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 so appear much darker. We calculated the IN concentration (number of INs per grams of mycelium) via a variant of the Vali formula (see 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} is 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).It is mentionable that the Vali formula has a slight inaccuracy, since it assumes that a freezing droplet contains only one IN. However, the distribution of INMs in the droplets follows Poisson statistics, so even at low concentrations some droplets may contain two or more INMs (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., 2014), and added literature data from birch pollen ~~for birch pollen INMs~~ (Augustin et al., 2013) and bacteria ~~BINMs~~ (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). 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.~~Although the concept of contact angles was originally developed for conventional ice nucleating particles, the application on INMs works perfectly. In fact, one can assume that from the mechanism, there is no difference between INA of a free INM and INA on a heterogeneous surface.~~

INA was also measured with two additional experimental techniques~~more systems~~. For both setups, 0.1 µm filtrates that were prepared as described at the top of this section were ~~properly~~ 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 ~~freezing~~ droplet freezing array ~~call~~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 ~~system, the preparation and the measurements~~ is given in Budke and Koop (2014).

(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 no proteins (Pummer et al., 2012), further procedures were carried out to characterize the birch pollen INMs. 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. ~~an aliquot was spiked with 0.75 M boric acid, left overnight at room temperature, which is known to esterify with sugars. In case that saccharides play a role, this treatment should alter the INA of the birch pollen INMs.~~ 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 ~~On the other hand, the INA assay preparation has a certain statistical uncertainty, which makes minor changes in INA~~ 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 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. ~~prepared as described: (i) 94 µl water added, (ii) 94 µl medium added, (iii) 94 µl medium and trypsin added, and all of them incubated for 18 h at 310 K.~~ Additionally, 100 µl water was treated like (iii). ~~Trypsin~~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. ~~To check, if the enzymatic treatment shifts the mass range of the birch pollen INMs, they were separated with a size exclusion chromatography column. Details about the sample preparation and separation are given in Sect. S2.2.~~

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~~INA of BINM peptides~~

A sample of the 16-amino acid peptide fragment which is the repetitive element in the bacterial INM (BINM) of *Pseudomonas syringae* ~~*Ps. syringae* BINM~~ 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). Thefrozen fractions of droplets with diameters of 20–50 µm were documented with the software Minisee© as a function of temperature.

3 Results ~~/ Discussion~~

3.1 Experimental characterization of INMs~~Characterization studies~~

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 (~~see~~ Table 1). The reduction of INA by ~~papain~~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 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 (~~see~~ 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. ~~In general, a good agreement can be seen between the data obtained with the different methods. However, it also becomes clear that onset temperatures, which were often reported in the past, do not properly describe the ice nucleation process. They are dependent e.g. on the detection limit of the different measurement methods used, and particularly for small IN concentrations, impurities or droplets which randomly contain a much more than average amount of ice nucleating material can influence these onset temperatures much. Hence, in the following, T_{50}, i.e. the temperature at which 50% of all droplets froze, will be used. For that value, however, also a note of caution should be given, as droplets with larger concentrations of similar IN will have higher freezing temperatures, due to an increased probability of freezing.~~

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. ~~However, t~~The addition of ~~trypsin~~Trypsin had no additional effect at all, which speaks against a proteinaceous nature of those INMs. It is most likely that it is the formic acid that decreases the INA in the medium, 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 no 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). ~~After applying the SEC column, the INMs were spread across the whole eluate. This might be explained by the glue-like behavior of the birch pollen extract (Pummer et al., 2013b), which causes adhesion to the packing material and therefore undermines the separation principle of the column.~~ 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 ~~would~~ 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 ~~cluster~~ 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.

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3.2 Comparison with theoretical calculations of the critical ice embryo size ~~Critical cluster size~~

In Fig. 4, we plot the experimentally determined molecular masses of INMs against the observed ice nucleation temperature. For comparison, ~~In the following, we will compare INMs, for which molecular mass and ice nucleation temperature were determined experimentally, with the critical water molecule cluster size, which depends on the temperature. For the latter,~~ we use the parameterization by Zobrist et al. (2007), which is based on *Classical Nucleation Theory*. The sources of the plotted data are specified in Table 3. ~~All available data are put together in Table 3 and Fig. 4.~~ 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 ~~cluster~~ 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. ~~From that we deduce that singular biological INMs which carry a suitable hydration shell are the perfect ice templates, but with the advantage that they do not randomly dissociate like ice embryos in homogeneous ice nucleation. This 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~~, like the wagons of a train~~. ~~As the backbone shows some flexibility, l~~Longer chains ~~will not be bolt upright sticks, but~~ fold into ~~more~~ compact three-dimensional structures. Without any further forces, polymers coil randomly~~, like a string of wool that tends to ravel~~. 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~~causes confinement~~. 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 ~~(e.g. hydrogen and disulfide bonds)~~, 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 ~~also~~ 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 (~~see~~ Fig. 1b) and ~~are~~ therefore nucleate ice at very low temperatures (Fig. 4)~~rather inefficient~~. Consequently, the ice nucleation

temperatures are maximum a few Kelvin above the homogeneous freezing temperature (~~see~~ Fig. 4). ~~On the other hand, molecules in confined geometries, like the BINM, allocate stable two-dimensional surfaces as ice nucleating templates (see Fig. 1c), which are larger and therefore nucleate at higher temperatures (see 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). ~~Of course, the surface of these 2D templates has to be properly functionalized in order to arrange the water molecules, or else they show no INA at all.~~

~~3.3 INA of BINM peptides~~

~~The examination shows that the 16 amino acid BINM peptide shows INA, when a certain concentration in solution is surpassed. In view of Fig. 4, 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 forms (e.g. parallel versus antiparallel β sheets), and consequently different INAs.~~

~~If the fractions of frozen droplets are plotted against the temperature, it can be seen that while the 10 mg/ml sample showed only homogeneous ice nucleation, the 30 mg/ml sample showed an initial freezing temperature of about 250 K, from which a broad flat slope ranged down to the homogeneous ice nucleation range. The variance of T_{50}, which ranges from 240 to 245 K in different experiments, 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 (see e.g. Fig. 2 and 3). Further investigations are in progress to measure the aggregates and get a better understanding of the process.~~

4 Discussion ~~/ Conclusions~~

4.1 ~~Basic physics of INA~~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~~as already stated, the template has to be of a certain size to make ice embryos that are large enough to grow~~. Consequently, particles that dissociate into low-molecular compounds 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; ~~see~~ Sect. S1.1), but single molecules are far larger than e.g. salt ions or low molecular weight saccharides~~lower sugars~~. Therefore, a deviation from the simplistic approach of Raoult's law is expectable. In this case, a soluble compound can also be an IN, if the 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 (~~see~~ 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 (~~see~~ Fig. 1). We therefore emphasize that a more molecular view on IN allows better understanding understanding of the process of heterogeneous ice nucleation. We see ~~the~~ a link between this molecular view and the macroscopic view that is necessary for developing atmospheric models. For example, the contact angle is a macroscopic interpretation of the molecular interaction between phases.~~atmospheric models in the contact angles.~~

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 be 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 a large hydration shell around these INMs that has to be added to the total IN mass. 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.

4.2 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., 2014). 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). 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) 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). 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.~~3~~2 Potential atmospheric effects~~Atmospheric impacts~~

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 ~~that~~ which are at least some micrometers in size are far too large to reach altitudes higher than a few kilometers. However, ~~T~~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 the stratosphere (Griffin, 2004). At last, microorganisms are frequently found in precipitation samples (e.g. Amato et al., 2007), 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).

~~Furthermore, b~~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 (~~see~~ 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.

Quantifying the atmospheric impact of fungi is even more difficult, as presumably 1 to 5million 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 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.

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Type	Organism	free?	protein?	?	lipid?	*T* stability	size (1 unit)	θ[°] ±σ[°]
BINMs:	*Ps. syringae*	–no	+yes	+yes	+yes	<313 K	150–180 kDa	34.1 ± 2.3
	E. herbicola	+yes	+yes	+yes	+yes	<313 K	150–180 kDa	
Fungal INMs:	*Rhiz. chrysoleuca*	+yes	+yes	–no	–no	>333 K	<0.22 µm	
	F. avenaceum	+yes	+yes	–no	–no	>333 K	<0.22 µm	
	A. implicatum	+yes	+yes	– ?no?	+yes	308–333 K	100–300 kDa	33.2 ± 2.3
	I. farinosa	+yes	+yes	– ?no?	–no	308–333 K	~300 kDa	24.6 ± 0.6
	M. alpina	+yes	+yes	– ?no?	–no	333–371 K	100–300 kDa	26.4 ± 1.1
	rust spores	???	???	+yes	???	~373 K	??	
Animal IN:	*Tipula*	+yes	+yes	+?yes ?	+yes	??	800 kDa	
	Dendroides	+yes	+yes	– ?no?	+/– both	??	>70 kDa	
	Vespula	+yes	+yes	–no	???	<373 K	74 kDa	
	*Eurosta**	+yes	–no	–no	–no	??	>100 µm	
Plant IN:	*Secale* leaves	???	+yes	+yes	+yes	<363 K	??	
	Prunus wood	–no	–no	???	???	313–323 K	??	
	Betula pollen	+yes	–no	+yes	–no	445–460 K	100–300 kDa	58.2 ± 4.6
	Lobelia fluid	yesyes	nono	yes?yes?	nono	>373 K	??	
	Opuntia fluid	yesye	nono	yesye	nono	??	<70 µm	

		~~s~~		~~s~~			
	different algae~~Algae~~	??	??	??	??	??	??

Table 1: ~~The c~~Chemical properties of some INMs. "T stability" shows the temperature about which the IN are denatured. A question ~~An interrogation~~ mark indicates some uncertainty. See Sect. 4.2 ~~Introduction~~ for the sources of these data. θ[°] $\pm\sigma$[°] 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~~Trypsin	30	258
medium + ~~trypsin~~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
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	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
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.

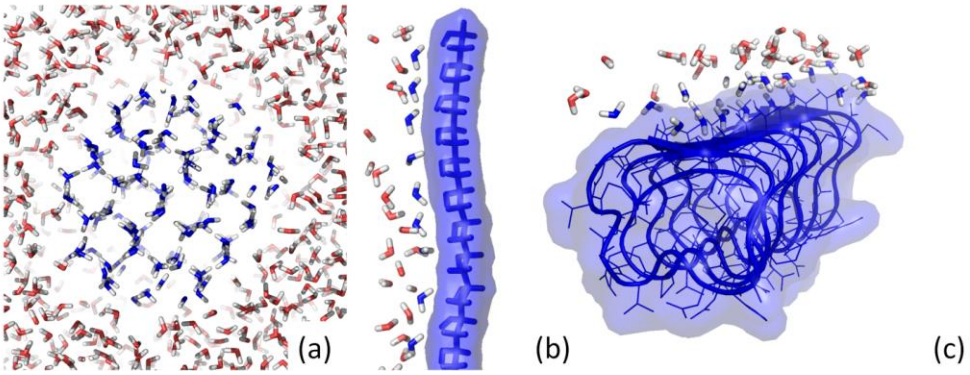

Figure 1: Visualization of water molecule ordering based on molecular model calculations (~~see~~ Sect. S2.1): homogeneous ice nucleation (a); heterogeneous ice nucleation by ordering of water molecules on a PVA strain, which is a 1D-template (b), and an antifreeze protein related to the BINMs, which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

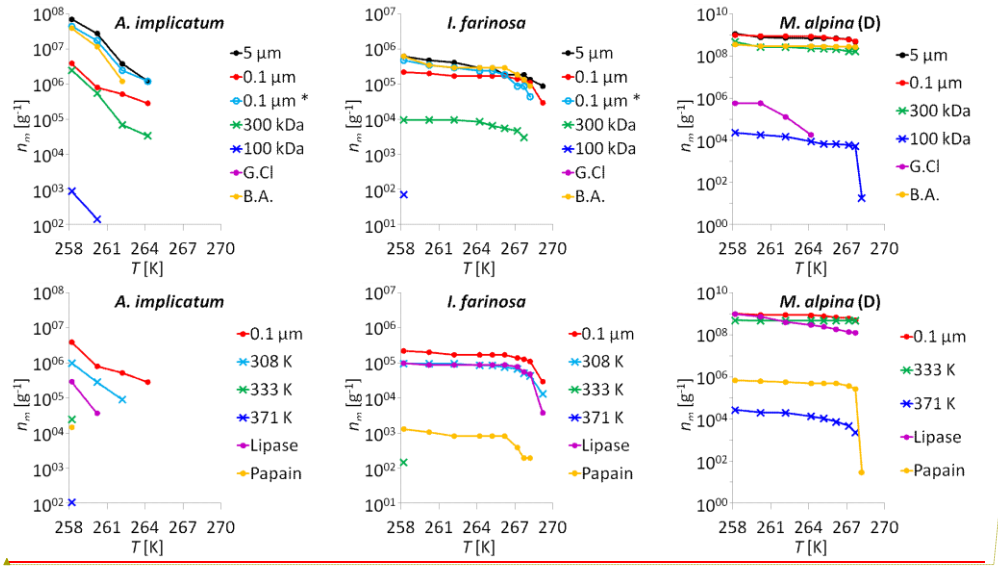

Figure 2: $n_m(T)$-curves for *A. implicatum*, *I. farinosa*, and *M. alpina* (subgroup D) INMs after different treatments. ~~Concentrations of *A. implicatum*, *I. farinosa*, and *M. alpina* (B,C,D) INMs after several treatments.~~ "G.Cl" stands for guanidinium chloride treatment, "B.A." for boric acid treatment. A reduction ~~in~~ 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. ~~The data point symbols o and x shall discriminate between different harvests.~~ For *M. alpina*, the data are the mean curves of all investigated strains of the phylogenetic subgroup~~s B, C, and~~ D (Fröhlich et al., 2014). ~~Subgroup A was ruled out due to its resistance against papain. The absence of a curve in a diagram means that no droplets were frozen at all.~~

*) 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.

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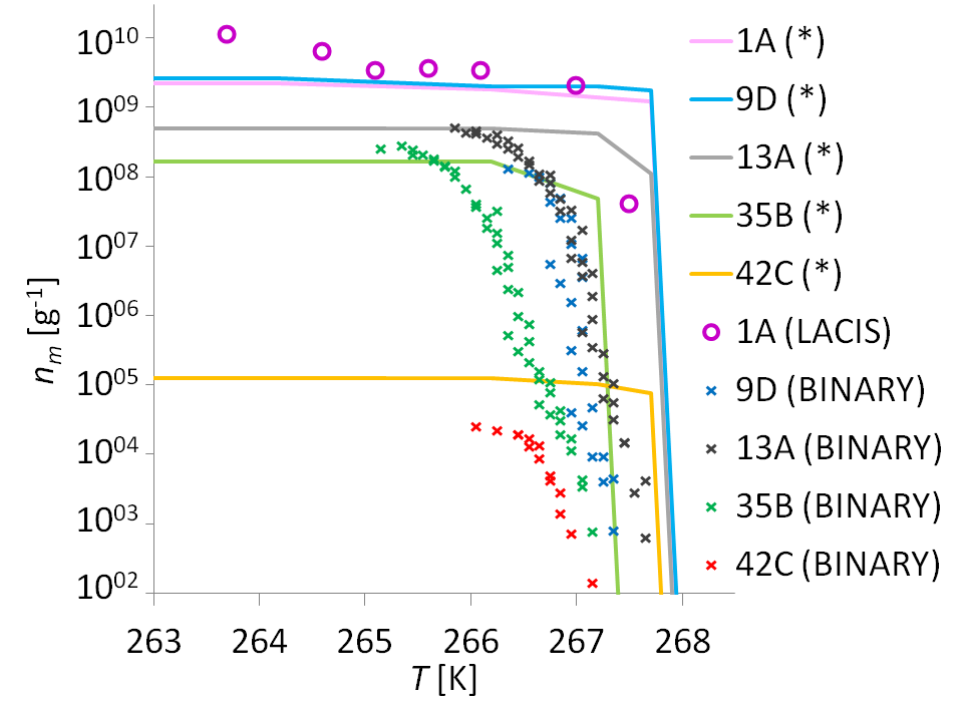

Figure 3: Comparison of ice nucleation curves of 0.1 µm filtrates from a few *M. alpina* strains. The number and letter combination labels the strain. The devices used for generating the respective curves are shown in brackets. "*" stands for the setup described in Fröhlich-Nowoisky et al. (2014). ~~measured with the droplet freezing assay at MPIC (M), LACIS (L), and BINARY (B).~~

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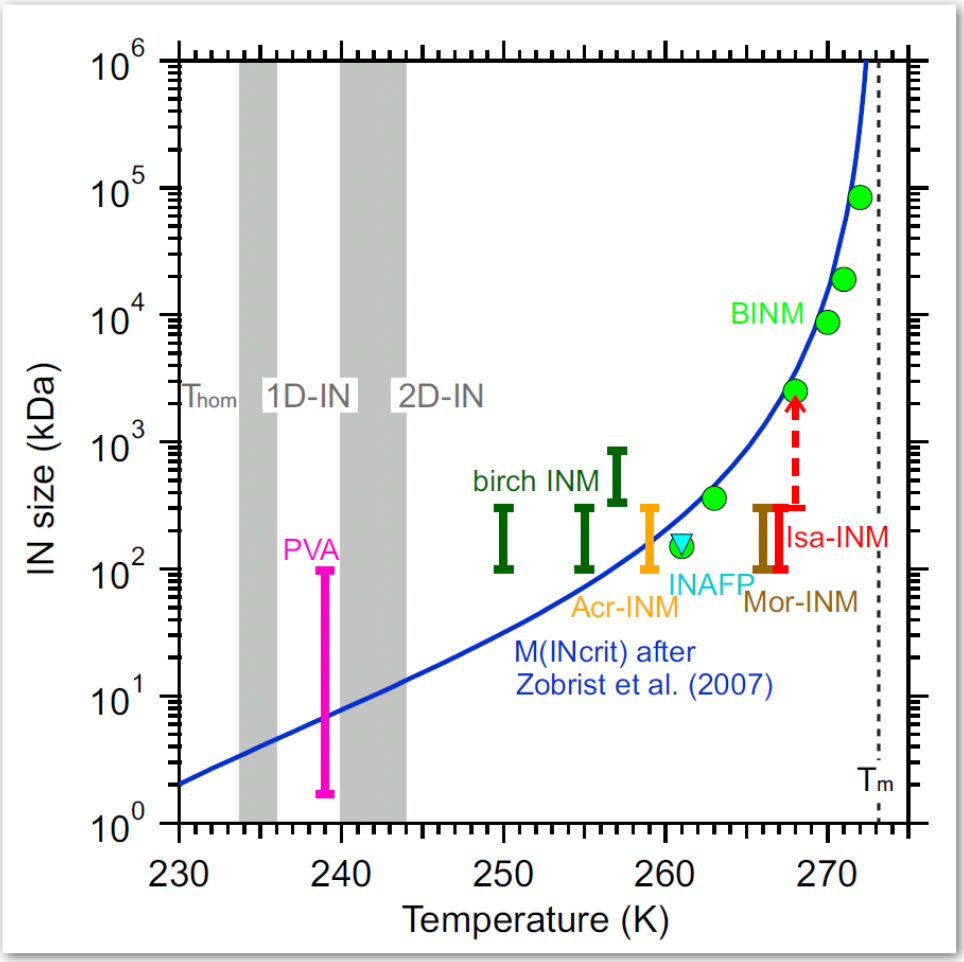

Figure 4: The dependence of the median freezing temperature on the size for different types of IN (colored dots). The blue curve is the calculated critical ice cluster size derived from *Classical Nucleation Theory* (Zobrist et al., 2007). The sources of the presented IN data are listed in Table 3. The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