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Atmospheric Chemistry and Physics Discussions

Interactive comment on "Ice nucleation by water-soluble macromolecules" *by* B. G. Pummer et al.

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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:

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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 simi-

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larities 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.,

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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 O3, 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 (NH4)2SO4 , 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

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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

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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,

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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

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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."

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"

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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

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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 MΩâĂćcm) 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"

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

p.24282, L.22: inserted "nm is the number of INMs per gram of mycelium" and deleted "is" after "fice".

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 fice 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 de-

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scribed 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 nm are compared to the nm 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

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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 nm 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 nm 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 (T50) was taken for interpretation."

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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 ho-

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mogeneous 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"

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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 nm(T) towards lower temperatures, and a T50 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."

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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

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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.,

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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

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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

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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

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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.,

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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 O3, 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 (NH4)2SO4 , 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

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"temperatures above which"; replaced "Introduction" with "Sect. 4.2"

Figure 2: diagrams were replaced; new figure caption: " Figure 2: nm(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 nm suggests that this method partly or fully destroyed the INMs. The absence of data points despite the listing in the figure legend indicates that nm 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.

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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 "aW" with "aw"

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:

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"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."

Interactive comment on Atmos. Chem. Phys. Discuss., 14, 24273, 2014.

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