

Responses to referee #1

We thank referee #1 very much for the extensive review of our manuscript and the positive feedback. We also much appreciate the critical feedback and the suggestion to add a broader discussion of related literature. Addressing comments and suggestions clearly improved the quality of our manuscript.

The comments and our answers are listed below. The referee's comments are written in black, our responses in green.

Overview of the work:

The authors present a three-part study where they first developed a method to extract SPP from pollen grains, then measured the ice nucleating ability of SSP and its washing water, and finally, quantified the protein content of the INMs from the washing water. The authors investigated the ice nucleating ability of different extraction steps of the pollen grain, and found that the SPP themselves were not ice active. However, the authors determined that the washing of the SPP resulted in the isolation of ice nucleating macromolecules. This procedure is well explained and well-illustrated and the investigated work is clearly described. Furthermore, the authors used two methods, fluorescence spectroscopy and quantitative protein analysis assay, to determine the protein content of the ice nucleating macromolecules. This last chemical step is of particular value to the research field of biological ice nuclei. The work presented merits publication in Biogeosciences after minor revisions; my suggested revisions are focused on additional experimental details as well as on adding important literature context and comparisons to the results and discussion sections.

General feedback:

1. Additional details on the replicates and on the uncertainty of the ice nucleation results are necessary. Can the authors comment on the number of replicates necessary to generate Figures 4. What are the uncertainties associated with the immersion freezing technique and can the authors add appropriate error bars to their freezing data in Figure 4?

We thank the referee for this comment. Indeed, it is important to clarify the uncertainties of our ice nucleation assay. The temperature uncertainty of VODCA is 0.5 °C which was previously determined by Zolles et al., 2015. Furthermore, we determined the measurement uncertainty by calculating the counting error and performed a Gauß error propagation as described in Kunert et al., 2018. Errors larger than 100% were excluded in the graphs.

References:

Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., & Fröhlich-Nowoisky, J. (2018). Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples. *Atmospheric Measurement Techniques*, 11(11), 6327-6337.

Zolles, T., Burkart, J., Häusler, T., Pummer, B., Hitzemberger, R., & Grothe, H. (2015). Identification of ice nucleation active sites on feldspar dust particles. *The Journal of Physical Chemistry A*, 119(11), 2692-2700.

2. The work is well presented but lacks discussions on the comparison of the results with previously published work in the results sections and conclusion. For example, do the results presented corroborate (or not) the work by (Dreischmeier et al., 2017)? In the results section, can the authors elaborate on the comparison of their findings with work on fungal spores (Haga et al., 2014, 2013; Kunert et al., 2019)? Can the authors discuss their findings in terms of structural studies of INMs such as (Ling et al., 2018; Šantl-Temkiv et al., 2015)? The authors mention how the intine is composed of cellulose, yet there is interesting literature in ice nucleation on the relevance of cellulose which can be mentioned (see (Hiranuma et al., 2015, 2018)). Is there any connection with SPP and lignin (see (Bogler and Borduas-Dedekind, 2020))? In an atmospheric implication section, can the authors discuss if airborne ice nuclei of unknown origin could be attributed to SPPs (like in (Lloyd et al., 2020))? Are the INMs in this work forming nanogels (see (Xi et al., 2021))? In the opinion of the authors, is the field converging towards proteinaceous material is the most important INMs? If not, why? If so, why? Discussing these details will place the presented work in the greater context of the current literature on INMs and will allow future work to more effectively build upon the work presented.

We thank the referee for the feedback and agree that a deeper discussion regarding the chemical analysis of INMs will improve the manuscript. We extended chapter 3.3 Chemical Analysis not only with a deeper discussion but also with an additional experiment where proteins of sample B were unfolded using urea as a reagent and subtilisin as an enzymatic digestion tool (please see the details about the experiment in answer 1 to referee 2). Furthermore, we decided to remove the sub-headlines 3.3.1 and 3.3.2.

We added the following discussion to chapter 3.3 starting in line 268:

“However, in literature only Tong et al., 2015 deal with proteins as INMs in birch pollen. Still, the exact chemical composition of the INM from primary biological aerosol particles (PBAPs) is a matter of controversial discussion. When Pummer et al., 2012 discovered that ice nuclei are water soluble and can be extracted from pollen, they proposed that polysaccharides are the responsible moieties. This was supported by infrared and raman spectra and by size filtration experiments pointing to INMs larger than 100 kDa bearing carboxylate groups (Pummer et al., 2013, Dreischmeier et al., 2017). INMs with similar physical and chemical properties were also extracted from other PBAPs such as fungi

(Haga et al. 2013, 2014, Kunert et al. 2019). This led Hiranuma et al., 2015, 2018 to the conclusion that cellulose, which is a polysaccharide and part of PBAPs cell walls, might be responsible for the INA. But also for other polymers such as lignin and nanogels, which can take up water in their structures as well, a similar ice nucleation activity as for cellulose was found (Bogler and Borduas-Dedekind 2020, Xi et al., 2021). Structure and size are also crucial for bacterial ice nucleation proteins, where repeat numbers and oligomerization contribute in a seemingly independent manner to the nucleation mechanism (Ling et al., 2018, Šanti-Temkiv et al., 2015). In general, INMs in the atmosphere are manifold and found from different sources such as forests, deserts and marine environments (Lloyd et al., 2020).

In summary, our experiments come to a similar conclusion as Tong et al., 2015 pointing in the direction of proteins but are not a priori in contradiction with the other recent literature. The piece of puzzle could be a glycoprotein, which exhibits carboxylate functionalities, is larger 100kDa, can bind water in tertiary structures and displays degeneration and unfolding of its secondary structure due to heat treatment or reaction with enzymes. Also glycoproteins are known to be part of the metabolism of cells regulating freezing stress tolerance (Zachariassen and Kristiansen, 2000)."

References:

Tong, H. J., Ouyang, B., Nikolovski, N., Lienhard, D. M., Pope, F. D., & Kalberer, M. (2015). A new electrodynamic balance (EDB) design for low-temperature studies: application to immersion freezing of pollen extract bioaerosols. *Atmospheric Measurement Techniques*, 8(3), 1183-1195.

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(5), 2541-2550.

Pummer, B. G., Bauer, H., Bernardi, J., Chazallon, B., Facq, S., Lendl, B., ... & Grothe, H. (2013). Chemistry and morphology of dried-up pollen suspension residues. *Journal of Raman Spectroscopy*, 44(12), 1654-1658.

Dreischmeier, K., Budke, C., Wiehemeier, L., Kottke, T., & Koop, T. (2017). Boreal pollen contain ice-nucleating as well as ice-binding 'antifreeze' polysaccharides. *Scientific reports*, 7(1), 1-13.

Haga, D. I., Iannone, R., Wheeler, M. J., Mason, R., Polishchuk, E. A., Fetch Jr, T., ... & Bertram, A. K. (2013). Ice nucleation properties of rust and bunt fungal spores and their transport to high altitudes, where they can cause heterogeneous freezing. *Journal of Geophysical Research: Atmospheres*, 118(13), 7260-7272.

Haga, D. I., Burrows, S. M., Iannone, R., Wheeler, M. J., Mason, R. H., Chen, J., ... & Bertram, A. K. (2014). Ice nucleation by fungal spores from the classes Agaricomycetes, Ustilaginomycetes, and Eurotiomycetes, and the effect on the atmospheric transport of these spores. *Atmospheric Chemistry and Physics*, 14(16), 8611-8630.

Kunert, A. T., Pöhlker, M. L., Tang, K., Krevert, C. S., Wieder, C., Speth, K. R., ... & Fröhlich-Nowoisky, J. (2019). Macromolecular fungal ice nuclei in *Fusarium*: effects of physical and chemical processing. *Biogeosciences*, 16(23), 4647-4659.

Hiranuma, N., Möhler, O., Yamashita, K., Tajiri, T., Saito, A., Kiselev, A., ... & Murakami, M. (2015). Ice nucleation by cellulose and its potential contribution to ice formation in clouds. *Nature Geoscience*, 8(4), 273-277.

Hiranuma, N., Adachi, K., Bell, D., Belosi, F., Beydoun, H., Bhaduri, B., ... & Möhler, O. (2018). A comprehensive characterization of ice nucleation by three different types of cellulose particles immersed in water: lessons learned and future research directions.

Bogler, S., & Borduas-Dedekind, N. (2020). Lignin's ability to nucleate ice via immersion freezing and its stability towards physicochemical treatments and atmospheric processing. *Atmospheric Chemistry and Physics*, 20(23), 14509-14522.

Xi, Y., Mercier, A., Kuang, C., Yun, J., Christy, A., Melo, L., Maldonado, M. T., Raymond, J. A., and Bertram, A. K.: Concentrations and properties of ice nucleating substances in exudates from Antarctic sea-ice diatoms, *Environ. Sci. Process. Impacts*, 23, 323–334, <https://doi.org/10.1039/D0EM00398K>, 2021.

Ling, M. L., Wex, H., Grawe, S., Jakobsson, J., Löndahl, J., Hartmann, S., Finster, K., Boesen, T., and ŠantlâTemkiv, T.: Effects of Ice Nucleation Protein Repeat Number and Oligomerization Level on Ice Nucleation Activity, *J. Geophys. Res. Atmospheres*, 123, 1802–1810, <https://doi.org/10.1002/2017JD027307>, 2018.

Šantl-Temkiv, T., Sahyoun, M., Finster, K., Hartmann, S., Augustin-Bauditz, S., Stratmann, F., Wex, H., Clauss, T., Nielsen, N. W., Sørensen, J. H., Korsholm, U. S., Wick, L. Y., and Karlson, U. G.: Characterization of airborne ice-nucleation-active bacteria and bacterial fragments, *Atmos. Environ.*, 109, 105–117, <https://doi.org/10.1016/j.atmosenv.2015.02.060>, 2015.

Lloyd, G., Choularton, T., Bower, K., Crosier, J., Gallagher, M., Flynn, M., Dorsey, J., Liu, D., Taylor, J. W., Schlenczek, O., Fugal, J., Borrmann, S., Cotton, R., Field, P., and Blyth, A.: Small ice particles at slightly supercooled temperatures in tropical maritime convection, *Atmospheric Chem. Phys.*, 20, 3895–3904, <https://doi.org/10.5194/acp-20-3895-2020>, 2020.

Zachariassen, K. E., & Kristiansen, E. (2000). Ice nucleation and antinucleation in nature. *Cryobiology*, 41(4), 257-279.

Specific comments:

Title: I'm wondering if a title highlighting all three parts of the study (SPP extraction and INM isolation) may be more representative of the work. In addition, the major finding of the work is the chemical identification of proteinaceous material, which should also be mentioned in the title.

Would the authors consider revising their title along the lines of “Isolation of subpollen particles (SPP) and their ice nucleating ability: SPP are carriers of proteinaceous ice nucleating macromolecules”?

We thank the referee for the suggestion and decided to change the title. The new title now is: “Isolation of subpollen particles (SPP) of birch: SPP are potential carriers of ice nucleating macromolecules.”

Abstract:

Are the authors interested in citing the thunderstorm asthma literature to *motivate* their research? As it currently stands, the mention of thunderstorm asthma appears to be an afterthought in the introduction. I would recommend mentioning this context already in the abstract as well as move the introduction paragraph (lines 87-99) earlier. It's an arguably important motivation for the presented research. Recent reference (including refs therein): (Bannister et al., 2021)

We decided to mention thunderstorm asthma only as an afterthought, as the phenomenon of thunderstorm asthma is predominantly related to grass pollen. To our knowledge no case of thunderstorm asthma in relation to birch pollen has been reported.

Line 13: the authors state a gap in knowledge, “explanations on how these materials could distribute in the atmosphere are missing” but do not address this gap in their study. Perhaps this sentence can be reworded to address the gap indeed addressed here.

We reworded the sentence accordingly: “However, INM and SPP are not clearly distinguished. This has motivated the present study which focuses on birch pollen and investigates the relationship between pollen grains, INM and SPP.”

Line 16: what is meant by “loosely attached”? Van der Waals forces? Covalent bonds?

We could separate the INM from the SPP simply by rinsing the SPP with water. Due to the removability without reagents we believe that INMs are not covalently bonded. We decided to clarify the statement and reworded the sentence: “We show that INM are not bonded (i.e. can be washed off with water) to SPP.”

Introduction:

Lines 69-70: I would be interested to read a (Tong et al., 2015) discussion in the results section in more details.

We agree with the referee and added a discussion about the chemical analysis of INMs including a discussion about proteins (Tong et al., 2015) in chapter 3.3. starting in line 268 (please see the text in the comment above; point 2).

Lines 105-109: I would suggest also placing the emphasis of this work on the identification of proteinaceous material as the ice active INMs. The authors show clear chemical evidence of these types of molecules, and this finding is interesting and important.

We agree with the referee that the finding of proteinaceous material as the ice active INMs is important. To prove the findings, we conducted an additional enzymatic digestion experiment. At the end of the introduction in line 109 we also added a sentence: “To clarify the role of proteins in heterogeneous freezing we conducted a specific enzymatic digestion/ protein unfolding experiment.”

Methods:

Lines 116-118: add the date collection information.

We added the date of collection and the exact location of the birch tree. The sampling location has changed as we now refer to the pollen we used for the additional experiments (based on the suggestions of referee 2): “Freshly harvested pollen samples were collected from a birch tree at the Donaukanal (location birch tree: 48.237480, 16.362990) in Vienna. Collection took place on April 9, 2021.”

Lines 145-150: I wondered whether a figure with the described shapes could be helpful for the reader to visualize this calculation.

We think this is a good idea and decided to add a figure to visualize the calculation. In the text we also added the equation used to calculate the volume equivalent diameter.

“Based on the approximated shape the volume equivalent diameter d_v was calculated by equation:

$$d_v = s \sqrt[3]{\frac{1}{2} \left(\frac{3l}{s} - 1 \right)} \quad (1)''$$

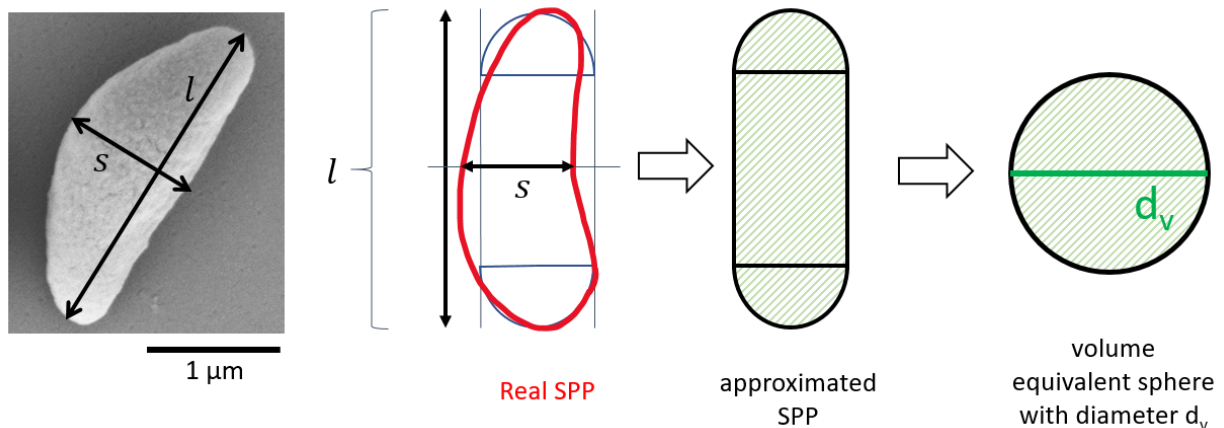


Figure 2: Approximation of the shape of an SPP and determination of the respective volume equivalent diameter d_v .

Line 155: can the formation of the emulsion be described in more detail?

The experimental procedure was published already in 2012 by Pummer et al., 2015 by Zolles et al., and 2018 by Felgitsch et al. To highlight the linkage, we added in line 155: “[...] a detailed description of the experiment is given in Felgitsch et al., 2018, Zolles et al., 2015 and Pummer et al., 2012.”

Furthermore, we described the preparation of the emulsion more clearly. We added in line 156 the mass percentage and the manufacturer/distributor: “(10 wt% lanolin, anhydrous, VWR Int., Radnor, PA, USA; 90 wt% paraffin, light, pure grade, AppliChem GmbH, Darmstadt GER)” and the information “by mixing with a pipette tip” to the sentence.

The improved description is stated as follows:

“INM content from birch pollen was quantified in immersion freezing mode by using the Vienna Optical Droplets Crystallization Analyzer (VODCA) setup (a detailed description of the experiment is given in Felgitsch et al., 2018, Zolles et al., 2015 and Pummer et al., 2012): An emulsion of 2 μl sample solution and 4 μl inert oil-mixture (10 wt% lanolin, anhydrous, VWR Int., Radnor, PA, USA; 90 wt% paraffin, light, pure grade, AppliChem GmbH, Darmstadt GER) is prepared by mixing with a pipette tip on a microscopic glass slide and transferred into a cryo-cell. Sample emulsions are cooled with a rate of $10\text{ }^{\circ}\text{C min}^{-1}$. The freezing process was monitored by videos at four different spots of each sample glass slide via a microscope camera (MDC320, Hengtech, GER).”

References:

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(5), 2541-2550.

Zolles, T., Burkart, J., Häusler, T., Pummer, B., Hitzemberger, R., & Grothe, H. (2015). Identification of ice nucleation active sites on feldspar dust particles. *The Journal of Physical Chemistry A*, 119(11), 2692-2700.

Felgitsch, L., Baloh, P., Burkart, J., Mayr, M., Momken, M. E., Seifried, T. M., ... & Grothe, H. (2018). Birch leaves and branches as a source of ice-nucleating macromolecules. *Atmospheric Chemistry and Physics*, 18(21), 16063-16079.

Lines 166-168: Can't dilution have an effect on the protein structure and thus its ice nucleating ability? How did the authors control for this issue?

We confirm that aggregation can influence the ice nucleation behavior as shown in Qiu et al., 2019 for example. However, investigating the aggregation of birch INMs is out of focus for this paper. To avoid this issue, we just state the cumulative nuclei concentration (CNC) values in the paper (instead of e.g. number concentration of proteins). CNC refers to the number of ice nucleating particles or macromolecules independent of their structural composition (e.g. composed of one single protein or an aggregate). To clarify, we added the following sentence to the text (line 164): "Note that one ice nuclei can also be an aggregate of more than one single molecule (Qiu et al., 2019)."

We like the idea of the referee to further investigate the ice nucleation of single molecules and aggregates from birch pollen. This will be part of a follow-up paper.

References:

Qiu, Y., Hudait, A., & Molinero, V. (2019). How size and aggregation of ice-binding proteins control their ice nucleation efficiency. *Journal of the American Chemical Society*, 141(18), 7439-7452.

Linea 167-168: Why was this diameter range chosen? Can a brief explanation be given?

This diameter range is relevant for atmospheric cloud droplets as described by Pruppacher and Klett, 2010. Therefore, the freezing behavior is similar to atmospheric ice nucleation. To clarify we added in line 169: "The selected size range is relevant for atmospheric cloud droplets." to the experimental section.

References:

Pruppacher, H. R., & Klett, J. D. (2010). Microstructure of atmospheric clouds and precipitation. In *Microphysics of Clouds and Precipitation* (pp. 10-73). Springer, Dordrecht.

Results:

Lines 199-200: How does this process affect the atmospheric relevance of the results in this manuscript? Can the authors elaborate?

In our experiments with fresh pollen grains we observed that particulate material is expelled from pollen grains in water. This process has also been documented in literature (e.g. Grote et al., 2003; already referenced in the manuscript). We have now added a video (<https://ucloud.univie.ac.at/index.php/s/FuF5SVBfqayb0ta>) and additional electron microscope images to illustrate the process of pollen rupture as it naturally occurs with fresh pollen grains. Insoluble SPP are clearly visible. In contrast, we do not find any insoluble SPP with commercial pollen, as commercial pollen has lost the ability to rupture and germinate. To mimic the natural process and to access the insoluble SPP we cracked the grains in the mixing mill. We again emphasize that such insoluble SPP are not obtained when commercial pollen grains are just mixed with water. In nature the process of pollen rupture is believed to occur (Schäppi et al., 1999, Grote et al., 2003; Taylor et al 2004, Hughes et al., 2020).

Line 210: “volume equivalent diameters” can be defined here.

We added a figure to visualize the calculation and defined the volume equivalent diameter in the figure (see comment 145-150).

Lines 237-240: good control experiment.

We thank the referee for this comment.

Lines 242-244: this sentence seems out of place? Move to the introduction?

We moved the sentences “Purified SPP are composed of starch and are contained in the pollen grains as energy storage units. Starch is a polysaccharide made of amylose and amylopectin (Hancock & Bryon 2000; Buléon et al., 1998) inside the amyloplast membrane (Matsushima et al., 2016)” to the introduction (starting in line 104).

Line 262: What is the mass percentage of the sample that is thought to contain these concentrations of proteins?

Unfortunately, we were not able to determine the dry mass of our samples due to low concentration and limited sample volume.

Figure 1: it's not clear in the caption where images of 1a and 1b are from? Could additional details be added? Which part of the figure is copyrighted?

We realized that the phrase ‘information for the drawing is taken from....’ is confusing and overcautious. We deleted the phrase. All three images were created by the authors.

Figure 2: very clear! Well done! Small addition: could the instrument be specified in the caption as well?

We thank the referee for this feedback. We added the name and details of the instrument in the caption: “Extraction process of SPP. Step 1: Entire pollen grains mixed with ultrapure water are crushed in a mixer mill (MM400, Retsch GmbH, Haan, GER). [...]”

Figure 3: additional details in the caption of how these distributions were generated would be useful.

We added details to the caption of Figure 3. The caption now reads:

Figure 3: a) Size distribution of SPP. The volume equivalent diameter d_v was calculated by equation 1 that is based on the approximated shape of an SPP. The d_v of 326 SPP was binned into 23 equidistant intervals between 0.2-2.5 μm . b) Aspect ratio of SPP.

Figure 4: Compelling data. Good job. Related to the general feedback, a discussion of replicates and uncertainties could be reported here in the caption and displayed on the graphs.

We thank the referee for these remarks. As mentioned above we added a statement regarding the uncertainty of the ice nucleation assay in chapter 2.4 and added error bars to the graphs.

Figure 5: Cool data!

We thank the referee for this positive feedback.

SI: the SI for this manuscript is quite short, and I wondered whether the authors might consider including the SI into the text (easier for the reader). For example, Figure S1 could be merged within Figure 2 of the main text.

We leave the control experiment in the SI but added the additional experiment to the main text.

Conclusion:

Line 274: can the authors make connections between the water-soluble component to dissolved organic matter? (see (Borduas-Dedekind et al., 2019; Knackstedt et al., 2018))

We thank the referee for this remark and added the following to line 273:

“The soluble INMs are easily extracted with water. In nature, INMs from the surface of birches are washed down by rain (Seifried et al., 2020) certainly ending up in soil and/or rivers (Borduas-Dedekind et al., 2019; Knackstedt et al., 2018).”

References:

Seifried, T. M., Bieber, P., Felgitsch, L., Vlasich, J., Reyzek, F., Schmale III, D. G., & Grothe, H. (2020). Surfaces of silver birch (*Betula pendula*) are sources of biological ice nuclei: in vivo and in situ investigations. *Biogeosciences*, 17(22), 5655-5667.

Borduas-Dedekind, N., Ossola, R., David, R. O., Boynton, L. S., Weichlinger, V., Kanji, Z. A., and McNeill, K.: Photomineralization mechanism changes the ability of dissolved organic matter to activate cloud droplets and to nucleate ice crystals, *Atmospheric Chem. Phys.*, 19, 12397–12412, <https://doi.org/10.5194/acp-19-12397-2019>, 2019.

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Technical comments:

In many instances throughout the text, a comma is missing to separate a clause from the sentence. Ex: lines 13, 15, 18, 19, 31 and so on. (English vs German syntax and comma usage)

We have carefully gone through the manuscript and added missing commas.

Line 48: “The solution is” should be “was”

Corrected.

References:

Bannister, T., Ebert, E. E., Williams, T., Douglas, P., Wain, A., Carroll, M., Silver, J., Newbiggin, E., Lampugnani, E. R., Hughes, N., Looker, C., Mulvenna, V., Csutoros, D., Jones, P. J., Davies, J. M., Suphioglu, C., Beggs, P. J., Emerson, K. M., Huete, A., and Nguyen, H.: A Pilot Forecasting System for Epidemic Thunderstorm Asthma in Southeastern Australia, *Bull. Am. Meteorol. Soc.*, 102, E399–E420, <https://doi.org/10.1175/BAMS-D-19-0140.1>, 2021.

Bogler, S. and Borduas-Dedekind, N.: Lignin’s ability to nucleate ice via immersion freezing and its stability towards physicochemical treatments and atmospheric processing, *Atmospheric Chem. Phys.*, 20, 14509–14522, <https://doi.org/10.5194/acp-20-14509-2020>, 2020.

Borduas-Dedekind, N., Ossola, R., David, R. O., Boynton, L. S., Weichlinger, V., Kanji, Z. A., and McNeill, K.: Photomineralization mechanism changes the ability of dissolved organic matter to activate cloud droplets and to nucleate ice crystals, *Atmospheric Chem. Phys.*, 19, 12397–12412, <https://doi.org/10.5194/acp-19-12397-2019>, 2019.

Dreischmeier, K., Budke, C., Wiehemeier, L., Kottke, T., and Koop, T.: Boreal pollen contain ice-nucleating as well as ice-binding “antifreeze” polysaccharides, *Sci. Rep.*, 7, <https://doi.org/10.1038/srep41890>, 2017.

Haga, D. I., Iannone, R., Wheeler, M. J., Mason, R., Polishchuk, E. A., Fetch, T., Kamp, B. J. van der, McKendry, I. G., and Bertram, A. K.: Ice nucleation properties of rust and bunt fungal spores and their transport to high altitudes, where they can cause heterogeneous freezing, *J. Geophys. Res. Atmospheres*, 118, 7260–7272, <https://doi.org/10.1002/jgrd.50556>, 2013.

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

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Hiranuma, N., Adachi, K., Bell, D., Belosi, F., Beydoun, H., Bhaduri, B., Bingemer, H., Budke, C., Clemen, H.-C., Conen, F., Cory, K., Curtius, J., DeMott, P., Eppers, O., Grawe, S., Hartmann, S., Hoffmann, N., Höhler, K., Jantsch, E., Kiselev, A., Koop, T., Kulkarni, G., Mayer, A., Murakami, M., Murray, B., Nicosia, A., Petters, M., Piazza, M., Polen, M., Reicher, N., Rudich, Y., Saito, A., Santachiara, G., Schiebel, T., Schill, G., Schneider, J., Segev, L., Stopelli, E., Sullivan, R., Suski, K., Szakáll, M., Tajiri, T., Taylor, H., Tobo, Y., Weber, D., Wex, H., Whale, T., Whiteside, C., Yamashita, K., Zelenyuk, A., and Möhler, O.: A comprehensive characterization of ice nucleation by three different types of cellulose particles immersed in water: lessons learned and future research directions, *Atmospheric Chem. Phys. Discuss.*, 1–82, <https://doi.org/10.5194/acp-2018-933>, 2018.

Knackstedt, K., Moffett, B. F., Hartmann, S., Wex, H., Hill, T. C. J., Glasgo, E., Reitz, L., Augustin-Bauditz, S., Beall, B., Bullerjahn, G. S., Fröhlich-Nowoisky, J., Grawe, S., Lubitz, J., Stratmann, F., and McKay, R. M.: A terrestrial origin for abundant riverine nanoscale ice-nucleating particles, *Environ. Sci. Technol.*, <https://doi.org/10.1021/acs.est.8b03881>, 2018.

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Responses to referee #2

We thank referee #2 very much for the detailed and critical feedback. Inspired by the comments and suggestions we have conducted additional measurements and rewrote sections of the manuscript to clarify statements. We believe that the quality of our manuscript has now greatly improved.

The comments and our answers are listed below. The referee's comments are written in black, our responses in green.

This is an interesting manuscript describing experiments regarding the preparation and release of molecular solutes and small subpollen particles (SPP) from birch pollen. Some of the results, for example the characterization of the SPP are novel, well described and interesting, while some other experimental procedures and results are described only poorly. Moreover, I am questioning some of the interpretation regarding the connection between SPP and ice nucleating molecules (INM), and whether the INM are proteinaceous or not.

The data presented in the current manuscript might be quite useful and, hence, may support publication. I do have, however, major concerns regarding some of the procedures and the interpretation of the experiments and their application to the atmospheric situation, see below. Moreover, in some places the manuscript is technically deficient, e.g. in the detailed description of procedures or results, sometimes making it hard to understand what the authors actually refer to. Also, the citations and reference list need more care.

Overall, I think that major revisions are certainly required, as outlined below, before the paper may become suitable for publication in Biogeosciences.

Scientific points:

1.) As far as I understand, cytoplasm generally contains also all kinds of soluble material such as proteins, lipids, polysaccharides, DNA, RNA and inorganic ions. Using the washing procedure in the extraction method described in Figure 2, I would assume that with more and more extraction volume in step 4, these solutes become more and more dilute in samples Cx. This is apparently the case for the INM. The authors also show a very slight decrease in CNC (meaning INM concentration) between samples A, B and C01. I am convinced one would see a very similar pattern with all other soluble materials, too, i.e. with proteins, with polysaccharides, with DNA, with ions etc. The authors of the current manuscript chose to investigate the content and concentration behavior of proteins using two methods (fluorescence and Coomassie staining), which is fine. And indeed, they found a similar concentration behavior for the proteins when comparing samples A, B, and C01. (They did not analyze whether proteins decrease in the same way as do INMs in samples Cx, though.) From the concentration correlation between INM and proteins in samples A, B, and C01, they infer that the INM may be proteins.

But what if the INMs belong to another substance class, for example DNA or polysaccharides? (The latter has been suggested in previous publications, also by the current authors.) I would assume to observe a similar correlation: The INMs are somewhat diluted between samples A, B and C01. In that case, the correlation between proteins and INMs would be fortuitous and would just indicate a small dilution of (any kind of) soluble material from sample A, to B, and C01. Or another thought experiment: if the authors had chosen to measure the concentration of DNA rather than that of proteins, wouldn't they have observed a similar concentration trend for DNA, even if the INMs had been the proteins? That would not directly imply that the INMs are DNA, wouldn't it? So from my standpoint, the correlation between INM concentration and protein concentration is not a proof that the INMs are proteins. This fact should be stated more clearly in the conclusions and abstract, to avoid a false interpretation by readers. The sentence in lines 280-281: 'We highlight the possibility that the ice nucleation activity of *Betula pendula* pollen is linked not only to polysaccharides (Pummer et al., 2015) but also to proteinaceous INM.' goes in the right direction, but may not be enough.

We agree with the referee that we would possibly observe a similar trend for other material from the cytoplasm (e.g. DNA, polysaccharides). However, we see a strong single signal in the fluorescence measurements. That is the first indication that proteins are present in our extracts and samples. This is confirmed with the Bradford assay. Still, we agree with the referee that there is no proof for the INM to be from proteinaceous origin.

To clarify we conducted an additional experiment with sample B. In this experiment the proteins of the sample were unfolded using urea as a reagent and subtilisin as an enzymatic digestion tool. The full experimental details were added to the manuscript (see new section 2.7 below).

We found that the unfolding procedure with urea decreases the ice nucleation ability significantly. Furthermore, the strongest reduction is observed for a combination treatment with subtilisin and urea (unfolding and digestion of amide bonds). The results are added to the manuscript and clearly indicate that proteins play an important role in the observed freezing behavior of the sample solution. However, proteins are not thought to be the only INM in the chemically broad cytoplasm.

We thank the referee for his or her concern regarding the statement that proteins are INMs in birch pollen extracts. However, we believe that the additional experiment supports the hypothesis that the majority of INMs are of proteinaceous nature.

We added the following to the paper:

We inserted a subsection in the methodology explaining the experiments' details (line 193):

“2.7 Enzymatic and chemical treatment

Sample B was treated with (i) subtilisin A, (ii) urea and (iii) a mixture of subtilisin A and urea. The enzyme subtilisin A is a protease which belongs to the Serine S8 Endoproteinase family (Hedstrom, 2002). It denatures proteins with a broad specificity by hydrolyzing peptide bonds

(Rawlings et al., 2010). Highly concentrated urea, in contrast, is a chaotropic reagent which does not cut covalent bonds but unfolds proteins by weakening the hydrogen bond system.

First, a 100 mM Tris buffer (Sigma Aldrich, 252859, St. Louis, MO, USA) with a pH of 8.4 (adjusted with a 2 M HCl) was prepared. Subtilisin A was dissolved in the Tris buffer with a resulting concentration of 2 mg ml⁻¹. Further, we prepared a 8 M urea solution (Sigma Aldrich, 33247, St. Louis, MO, USA) using the buffer as a solvent. For the incubation, we prepared four samples: 50 µl of sample B were mixed with (1) 210 µl Tris buffer (serving as a control sample, to monitor whether temperature changes the INA), (2) 10 µl subtilisin A and 200 µl Tris, (3) 10 µl Tris and 200 µl urea and (4) 10 µl subtilisin A and 200 µl urea. All samples had a total volume of 260 µl resulting in a Tris buffer concentration of 81 mM and 6.2 M urea. The samples were incubated at 37 °C (Heiz-Thermoshaker, Thermo Scientific, Waltham, WA, USA); after 1 h and 6 h, 2 µl sample aliquots were taken and diluted 1:10 with ultrapure water prior to ice nucleation measurements to decrease the freezing point depression of the buffer and urea. Chemicals used in the treatment did not show heterogeneous ice nucleation activity.”

Furthermore, we added the results in line 244:

“To further test the hypothesis whether proteins play a role in the ice nucleation activity we conducted an enzyme digestion/ protein unfolding experiment using subtilisin A and urea. Treating ice nucleation active samples with enzymes (e.g. Kozloff et al., 1991, Pummer et al., 2012, Felgitsch et al., 2019), chaotropic reagents (e.g. Pummer et al., 2012, Fröhlich-Nowoisky et al., 2015) or strong oxidizer (e.g. H₂O₂, Gute et al., 2020) to investigate the nature of ice nuclei has been performed in the past. The experiment conducted in this paper is inspired by a publication from Felgitsch et al., 2019 where they investigated the role of proteins in ice nucleation active extracts from perennial plants.

Results show that after incubating sample B for 1 h at 37 °C, the ice nucleation activity of samples containing urea decreased. For urea alone, 65 % of droplets remained to freeze heterogeneously (see Figure 6a). However, treating the sample with the combination of subtilisin A and urea led to an even stronger decrease in INA due to the unfolding activity of urea (50 % of droplets froze heterogeneously). Sample B in Tris (control sample) and the digestion with subtilisin A alone did not show a significant decrease of heterogeneous freezing activity after 1 h treatment. However, after 6 h incubation time, subtilisin A seems to slightly influence the INA and the freezing curve is dropping at around -22 °C (see Figure 6b). Nevertheless, the urea treatment decreased the ice nucleation ability even more. Further, the strongest influence of INA is clearly derived from the mixture of urea and subtilisin A. Again, this can be explained by the unfolding effect of urea in combination with the cleavage of peptide bonds by subtilisin A. In addition, the freezing onset temperatures of samples containing urea are shifted approx. 2 °C towards colder temperatures. This phenomenon is attributed to the freezing point depression induced by urea which is also visible in the homogenous freezing regime. The sensitivity of the sample to urea treatment indicates proteins to play a role in the ice nucleation activity. Unfolding using urea as a reagent and further cutting peptide bonds decreases the INA. This suggests that the secondary and primary structure is important for the proteins to act as INMs.”

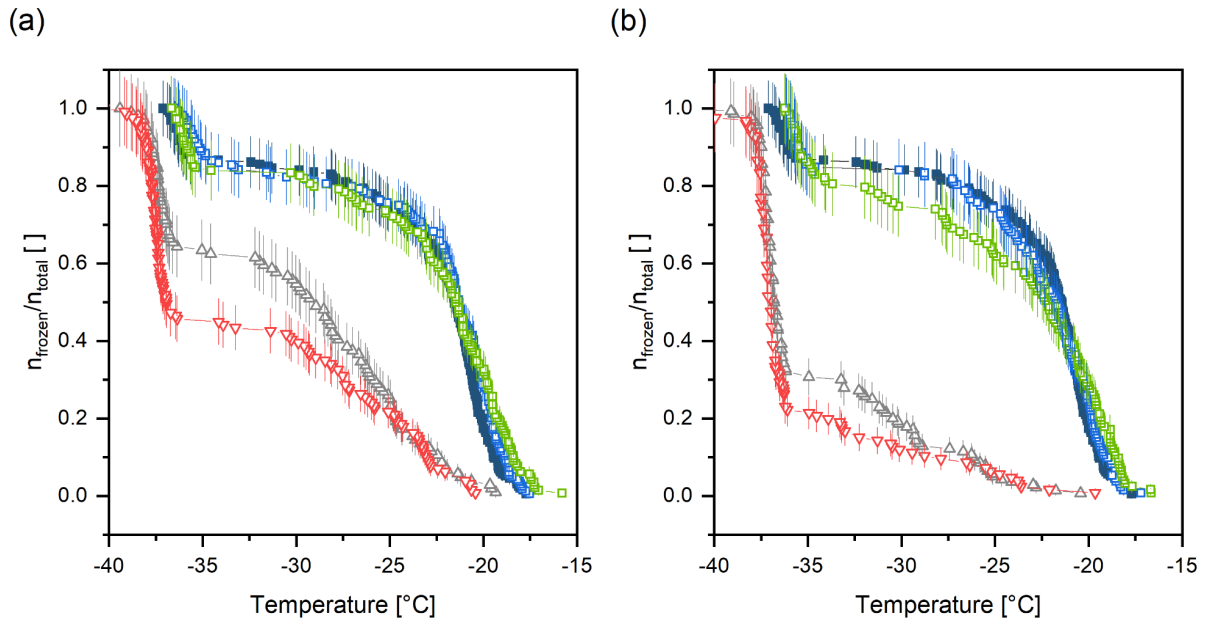


Figure 6. Results of the unfolding experiment and enzymatic digestion of extracted INMs (sample B) at 37 °C using subtilisin A (from *Bacillus licheniformis*) and urea after (a) 1 h and (b) 6 h incubation time. Filled, blue squares correspond to sample B in Tris buffer at room temperature prior to treatment and hollow, blue squares to the control sample. Green circles show the treatment with subtilisin A, grey triangles (cone up) with urea and red triangles (cone down) represent the treatment using a mixture of subtilisin A and urea. Vertical lines represent the counting error.

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Fröhlich-Nowoisky, J., Hill, T. C., Pummer, B. G., Yordanova, P., Franc, G. D., & Pöschl, U. (2015). Ice nucleation activity in the widespread soil fungus *Mortierella alpina*. *Biogeosciences*, 12(4), 1057-1071.

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2.) Lines 206-209: 'However, even after 1-hour ultrasonic treatment we did not find any ruptured pollen grains nor SPP (Figure S1). We believe that the usually applied extraction method, where pollen grains are only left in water and are then filtrated, do not actually yield SPP unless very fresh pollen grains are used. In this sense our method is unique and offers the possibility to study isolated SPP and gain further insight about the location of the INM within the pollen grain.'

These sentences suggest that in all previous studies on dried commercial pollen, SPP were not present in the washing water. Is this notion correct? Please make a clear statement. If so, doesn't this imply that the INM are NOT connected to the SPP as in previous studies INM were indeed found by simply washing the dried 'old' pollen. Please discuss in more detail.

We agree that this notion is correct. We rewrote section 3.1. to clarify statements and added a more detailed discussion including additional literature.

The new section now reads:

"The extraction process in this study differs from the usual approach in other studies (e.g. (Gute & Abbatt, 2020; Pummer et al., 2012; Steiner et al., 2015)) especially in one aspect: commercial or stored pollen grains do not germinate nor rupture, and therefore do not release insoluble SPP (starch granules) contained in the cytoplasm. In contrast, after fresh birch pollen grains had remained in water for ~ 1 day we found several grains with pollen tubes and SPP in the sample. This process has also been documented in literature (Grote et al. 2003). To ensure that insoluble SPP and material from inside the pollen grains is obtained we first crack the exine of the pollen grains. This is done with a mixer mill. As seen in Figure 2b the exine cracks and gives access to the pollen grain's interior including the starch granules. This step was necessary to mimic the natural behavior of fresh pollen grains.

Figure 4 shows fresh pollen grains that had been exposed to high relative humidity for 8 hours. The behavior of fresh pollen grains in water can also be seen in the linked video (<https://ucloud.univie.ac.at/index.php/s/FuF5SVBfqayb0ta>). In both cases particulate, insoluble material can be clearly observed. These insoluble SPP are likely to spread in the atmosphere due to pollen rupture or abortive germination (Schäppi et al. 1999, Grote et al. 2003; Taylor et al 2004, Hughes et al. 2020). With fresh birch pollen we find that both processes take place: pollen rupture (video) and abortive germination (Figure 4). Note that most of the insoluble SPP in Figure 4 are coated with amorphous material that is expelled by the pollen grain at the same time as the SPP. Only a few SPP seem "pure" (the interpretation, however, is limited by the resolution of the

electron microscope). Most of the amorphous material probably originates from the cytoplasm, but we cannot exclude that some of the material is also washed off the exine i.e. the pollen grain's surface.

We find that the ability to rupture is almost entirely lost when freshly harvested birch pollen grains were stored in the lab for a few days to weeks. The highest germination activity (i.e. most pollen grains germinated) was observed when fresh pollen grains were exposed to water on the very same day they were harvested. With commercially purchased pollen grains we did not find any germination activity and also no SPP. In addition, we also treated pollen grains mixed with water up to 1 hour in the ultrasonic bath to see if pollen rupture could be induced this way. However, even after 1-hour ultrasonic treatment we did not find any ruptured pollen grains nor SPP (Figure S1). We emphasize that the usually applied extraction methods, where pollen grains are only left in water and are then filtered, do not actually yield SPP unless very fresh pollen grains are used.

The usual extraction method likely yields only the most soluble components. For example, it is known that some highly soluble proteins (mostly allergenic ones) migrate to the pollen surface within seconds to minutes upon hydration even without pollen rupture (e.g. Vrtala et al. 1993). The exine of birch pollen contains microchannels that enable such an exchange (Diethart et al. 2007). Another study also documents the passage of proteins contained in the cytoplasm through the intact cell membrane (Hoidn et al. 2005). While insect pollinated plants produce pollen with a thick and sticky lipid-containing pollenkitt (coating of the exine) that functions as a barrier to water soluble components, this pollenkitt is almost entirely absent with pollen from wind pollinated plants such as birch pollen (Diethart et al. 2007).

Our extraction method is unique as it guarantees access to less soluble substances and might be closer approximation to the processes in the atmosphere than the usual applied extraction method. Our method offers as well the possibility to study the ice nucleation activity of isolated SPP for the first time and to investigate whether INM are connected to SPP.

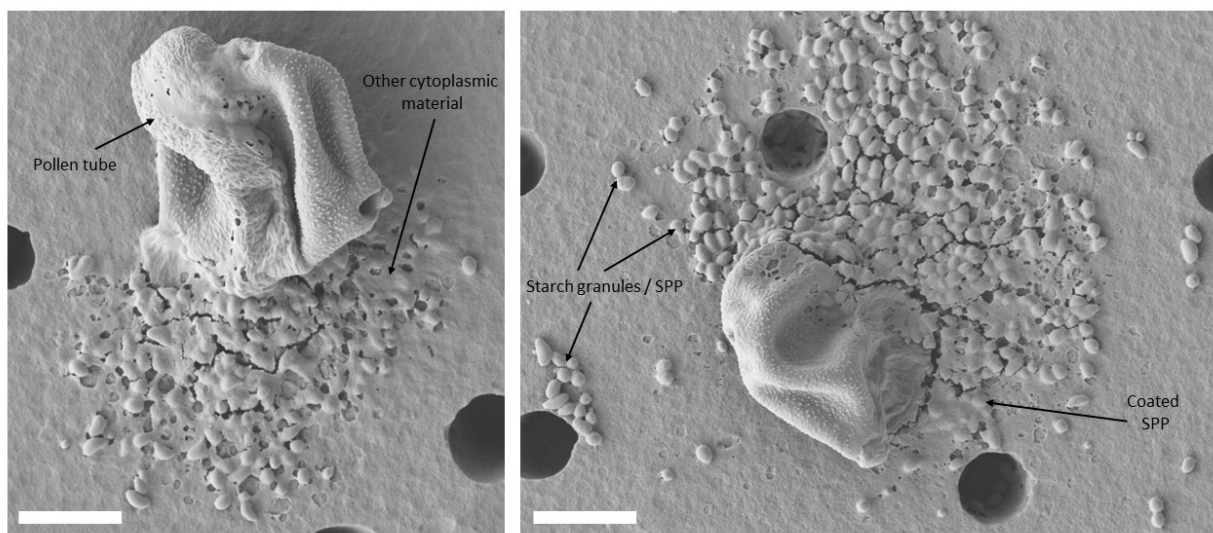


Figure 4: Germinated and ruptured pollen grains. Freshly harvested birch pollen grains were exposed to high relative humidity. Pollen grains were deposited on a polycarbonate nuclepore filter

(pore size: 8 μm) within an inline filter holder and humid air (95% relative humidity) was sucked through for 8 hours. This process can only be observed with fresh pollen grains. Commercial pollen grains remained intact after the same treatment. White scale bar is 10 μm .

Additional references:

Diethart, B., Sam, S., & Weber, M. (2007). Walls of allergenic pollen: Special reference to the endexine. *Grana*, 46(3), 164-175.

Hoidn, C., Puchner, E., Pertl, H., Holztrattner, E., & Obermeyer, G. (2005). Nondiffusional release of allergens from pollen grains of *Artemisia vulgaris* and *Lilium longiflorum* depends mainly on the type of the allergen. *International archives of allergy and immunology*, 137(1), 27-36.

Vrtala, S., Grote, M., Duchêne, M., Kraft, D., Scheiner, O., & Valenta, R. (1993). Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *International archives of allergy and immunology*, 102(2), 160-169.

3.) Lines 253-254: 'The signal correlates with heterogeneous ice nucleation of sample A, B and C01'. I am not sure I entirely understand what is meant by 'correlates' in this context. Simply, samples A, B, and C01 show fluorescence and they also show ice nucleation? Or the ice nucleation activity, namely CNC or T_50, correlates with the strength of the fluorescence signal? Please explain in more detail.

We thank the referee for her/his feedback. CNC values of samples A, B and C01 follow the same trend as the fluorescence intensity at the maximum. We changed the wording in lines 253-254 for clarification to the following: "The fluorescence intensity at the maximum decreases with decreasing CNC (Table 1)."

4.) Lines 271-272: 'In this study we develop an extraction method that gives access to the cytoplasmic material of pollen grains, even after the grains have lost the ability to germinate and rupture.'

While I applaud the authors for the realization and description of this extraction method for dried pollen, I am missing an analysis / a connection of the results presented here to the processes occurring in the atmosphere. The authors make a big point that the release of cytoplasmic material in fresh ('living') pollen is different from that of dried ones. How can they then make any quantitative conclusions and statements on free pollen and their release of ice nucleating material?

We observe that fresh pollen grains directly released from catkins germinate and rupture when immersed in water or exposed to high relative humidity (>95 %). To illustrate the process, we now also made a video of freshly collected birch pollen grains immersed in ultrapure water. It can be seen clearly that particulate material is expelled from the pollen grains. Additionally, we exposed fresh pollen grains to relative humidities above 90 % for several hours and also find ruptured

pollen grains. Looking at the material released by fresh pollen grains with an electron microscope we find particulate and amorphous material. We have now added electron microscope pictures of ruptured pollen grains to the manuscript. We again highlight that such subpollen particles are not found with commercial pollen grains. With our extraction method we aim to mimic the natural rupture process, and to specifically investigate the ice nucleation behavior of these insoluble subpollen particles that are omitted by commonly used aqueous extraction methods done with commercially purchased pollen grains. The main purpose was to explore the role of the insoluble SPP in ice nucleation activity. Insoluble SPP are not gained with commonly used extraction methods.

A proper quantitative comparison (e.g. of protein content or amount of SPP) of fresh/living pollen with commercial pollen is far beyond the scope of this study. This would involve many more additional steps, such as the proper purification of fresh pollen grains (from spores, plant debris etc.), the exact weighing of samples in all steps during the extraction process, and also an analysis of the storing effects of fresh pollen grains in the lab. For example, we have noticed that fresh pollen grains are less likely to rupture when stored in the lab for just a couple of days.

Moreover, in the sample preparation part (line 116: 'Freshly harvested pollen samples were collected from birch trees at the Danube Island in Vienna. '), the authors mentioned that they also collected fresh pollen in Vienna, but I did not see any comparative analysis or measurements of fresh with dried pollen. Why is that so? The authors could have made experiments with fresh pollen using the same SPP extraction procedure, using only steps 2-4, and then analyzing the filtrates in a similar manner. Why did they not do so?

We thank the referee for this comment. The goal of this work was firstly to define SPP, as there are many different definitions used in the literature. Further, we wanted to test the hypothesis whether SPP from birch pollen are ice nucleation active or not. We have observed that the behavior of fresh and commercial pollen in water and at high relative humidity differ. Fresh pollen rupture, germinate and release SPP, while commercial ones do not. We have now added a video and electron microscope images to better illustrate the behavior of fresh pollen. The lack of SPP with commercial pollen (when the usual extraction method is applied) is the most obvious difference between fresh and commercial pollen. However, fresh pollen is only available during a very short period of the year (roughly 2-6 weeks during the pollination season at a specific location). That is the reason why the scientific community uses mostly commercial pollen. In our study we wanted to draw comparisons to other studies and use a standardized sample (*Betula pendula*, Allergon, Sweden). We therefore developed an extraction method of SPP from commercial pollen and further focused our analysis on commercial pollen.

5.) Lines 281-285: 'INM and SPP are both contained in the cytoplasm. The abundance of INM suggests that INM and SPP might not naturally separate in the atmosphere. SPP could act as carriers of INM'

I was wondering whether the authors can really exclude that the observed INMs come from the outer part of the pollen. I again emphasize the fact that dried pollen release INM (as shown in previous publications), but not SPP (according to the authors' statements) contradicts the

statement that INM and SPP are both contained in the cytoplasm. If INM come from the cytoplasm AND are released even without rupture, do we need to consider two different types of INM then? Please elaborate.

We agree with the referee that we cannot entirely exclude that INMs come from the outer part of the pollen. At this point it is therefore not justified to distinguish between two different types of INM. We deleted the respective sentence in the conclusions.

6.) I still have not understood whether the amount of washing water given in Figure 4 (and Figure S2 in the supplement) refer to cumulative volume values or not. For example, for sample C01, 1 mL of washing water was used, and hence sample C01 has a total volume of 1 mL. What about sample C02? Was another 1 mL of washing water used (cumulatively the second mL) and the total volume is again 1 mL? Or were 2 mL of water used for sample C02, giving a total volume of 2 mL, and making it cumulatively 2-3 mL of waters used. Similarly, is sample C70 10 mL in total volume with the cumulative 60-70 mL of washing water used (there is a sample C60 given in the supplement)? Please explain more clearly.

Along the same lines, I am not sure how the dilution factor in equation 1 was applied to the different sample Cx solutions, and also to the samples A, B, and D. If you use different water volumes for extraction/preparing samples A, B, Cx, and D, shouldn't the CNC concentration be quite different? Or was that volume taken into account in the dilution factor? If yes, which solution is the reference? Solution C01?

After step 3 in the extraction process (see Figure 2a), the sample (retentate in the filter) was washed with 1 mL ultrapure water; the obtained filtrate was named C01. Next, the supernatant was washed again with 1 mL ultrapure water; the obtained filtrate was collected in a separate vessel and named C02. The number index of the samples Cx refers to the cumulative amount of ultrapure water used in the sequential washing procedure.

To clarify we changed the text in line 139: "The ice nucleation activity of each sample C fraction was tested for each rinsing step (note that the rinsing water was not pooled)." Furthermore, we changed the caption in Figure 2 to "(b) Freezing spectra of SPP washing solutions (C01, 1 mL water used to wash SPP, C02, second 1 mL fraction, etc.) [...]"

In general, all samples where we calculated the CNC value were diluted with ultrapure water prior to ice nucleation measurements to prevent an underestimation of INM concentration freezing at lower temperatures. Thus, samples which did not freeze partly homogeneously in the first measurement were diluted and re-measured (see Felgitsch et al., 2018). The results of CNC (diluted) measurements are shown in Figure 2a only. They should give an impression that the CNC value is not strongly influenced by filtration, i.e. the INMs pass every filter used in this study.

Reference:

Felgitsch, L., Baloh, P., Burkart, J., Mayr, M., Momken, M. E., Seifried, T. M., ... & Grothe, H. (2018). Birch leaves and branches as a source of ice-nucleating macromolecules. *Atmospheric Chemistry and Physics*, 18(21), 16063-16079.

Minor and technical points:

7.) Apparently, the citations and references have not been assembled very carefully and need to be revised. Here are some examples:

Line 34: 'Mikhail Sofiev, 2013' This is an incorrect citation (given name should be removed), probably due to the fact that the author list is incorrect, too, see below.

Lines 43-44: 'on a global scale (Corinna Hoose et al., 2010; C. Hoose et al., 2010).' Apparently, these are two different references. Please indicate them correctly and use correct citations, e.g. Hoose et al. 2010a; Hoose et al. 2010b.

Line 365: The reference to Gute & Abbatt is incomplete.

Lines 367-368: The reference to Gute et al is incomplete.

Lines 403-405: The author list is corrupted. The correct author list is: Mikhail Sofiev, Jordina Belmonte, Regula Gehrig, Rebeca Izquierdo, Matt Smith, Åslög Dahl, and Pilvi Siljamo

Some references are missing their doi.

We apologize for the mistakes in the reference list. We have now carefully checked the list and corrected all mistakes.

8.) Lines 48-49: 'The solution is then decanted and filtrated yielding what is called pollen washing water. The washing water is shown to induce ice formation at similar temperatures as the entire pollen grains.' The tense should be past, not present.

Corrected.

9.) Lines 90-91: 'For example, birch pollen grains were shown to germinate on leaves after light rain and release starch granules.' This sentence needs a reference.

We added the reference Schäppi et al. 1999 (already cited in the manuscript) to this sentence.

10.) Lines 155-56: Please provide more information on the emulsion preparation, i.e. the type of paraffin and the concentration (ratio) of the lanolin.

We added the corresponding mass concentrations and the provider/manufacturer in section 2.4, line 156: "[...] (10 wt% lanolin, anhydrous, VWR Int., Radnor, PA, USA; 90 wt% paraffin, light, pure grade, AppliChem GmbH, Darmstadt GER)" and the information "by mixing with a pipette tip" to the sentence to clarify the procedure.

11.) Lines 158-159: I could not find the number of droplets (typically) analyzed for each sample, A, B, Cx. Please provide this information.

We thank the referee for her/his comment. Every sample is measured four times (on four different spots). We observe about 20 droplets in the corresponding size range per spot. For clarification about the number of droplets counted in one freezing experiment we write the following in lines 167-168:

“The freezing process was monitored by videos at four different spots of each sample glass slide via a microscope camera (MDC320, Hengtech, GER). On each spot about 20 droplets in the corresponding size range are observed.”

12.) Lines 233-234 and Table 1: ‘only after 70 mL of washing the ice nucleation activity is entirely lost’ There are some data points at temperatures higher than -34°C , both in Figure 4 and Figure S2. Were they ignored in this statement? Also, in Table 1, the CNC values for sample D at -25°C and -34°C are given as zero. Again, I am surprised, because the $n_{\text{frozen}}/n_{\text{total}}$ ratio in Figure 4 and Figure S2 shows values slightly larger than 0. Please elaborate.

We thank the referee for this comment. We confirm that there are data points slightly above the background. Therefore, we elaborated the statement and change it to “After sample C10 the ice nucleation activity rapidly diminishes but only after 70 mL of washing homogeneous freezing was the dominant process in the experiment (99 % of the observed droplets froze homogeneously)”

13.) Figure 1: It is not clear to me whether the images shown in panels a) and b) and the sketch in panel c) are original to the current work, or whether they have been taken from the given references. I do not understand what is meant in the caption by ‘information for the drawing is taken from....’

The images and the sketch are original to the current work. We intended to point out that knowledge about the composition of a pollen grain was taken from the respective literature and is not original to the current work. This might be overcautious and to avoid confusion we deleted the notion “information for the drawing is taken from [...]”

Referee 1 stated: "Furthermore, the authors used two methods, fluorescence spectroscopy and quantitative protein analysis assay, to determine the protein content of the ice nucleating macromolecules."

I do not agree with the phrase "the protein content of the ice nucleating macromolecules". As far as I can see, the authors have shown that the soluble material released from the cytoplasm contains proteins, and quantified them, and that the cytoplasm also contains ice nucleating molecules. But they did not show that the proteins are the ice nucleating molecules. There is some concentration correlation between the proteins and the ice nucleating molecules, but I would argue that the same correlation would hold for any soluble molecules contained in the cytoplasm,

also those that were not analyzed regarding their chemical nature (e.g., polysaccharides, DNA etc.).

We agree with the statement of the referee. In order to make a clear connection to proteins we have conducted additional measurements as described above. These measurements strongly indicate that the majority of INMs is of proteinaceous origin. However, we cannot fully exclude that other substances also contribute to the ice nucleation activity