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. H_2O_2, 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."

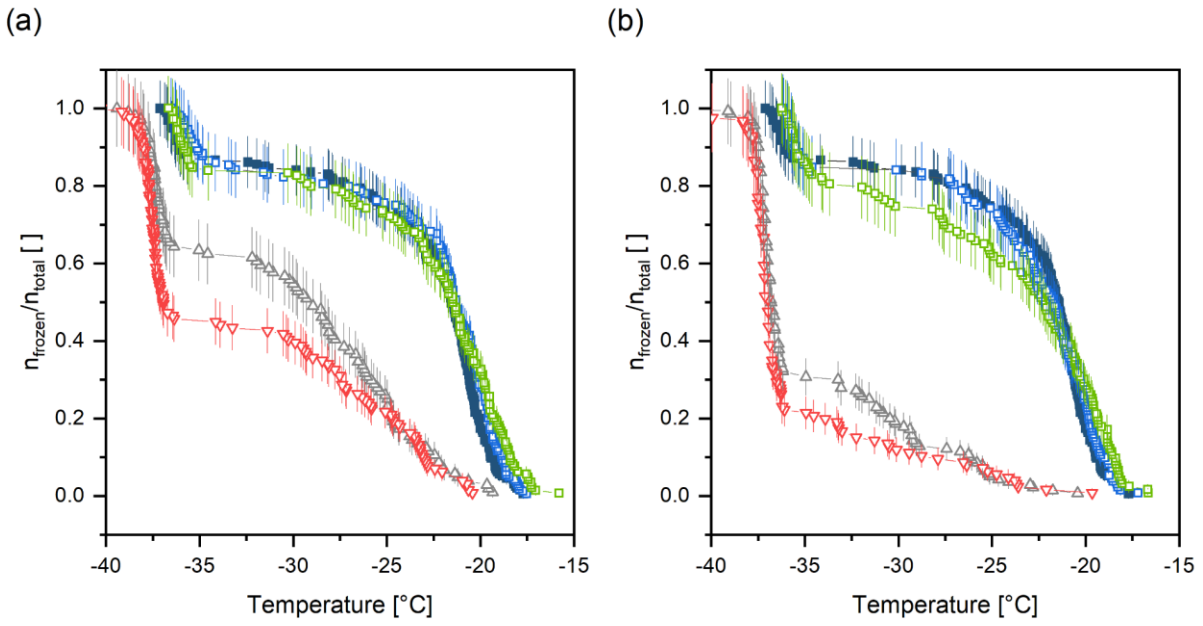

Figure 6. Results of the unfolding experiment and enzymatic digestion of extracted INMs (sample B) at 37 °C using subtilisin A (from Bacillus licheniformis) and urea after (a) 1 h and (b) 6 h incubation time. Filled, blue squares correspond to sample B in Tris buffer at room temperature prior to treatment 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.

References:

Hedstrom, L. (2002). Serine protease mechanism and specificity. Chemical reviews, 102(12), 4501-4524.

Rawlings, N. D., Barrett, A. J., & Bateman, A. (2010). MEROPS: the peptidase database. Nucleic acids research, 38, D227-D233.

Kozloff, L. M., Turner, M. A., & Arellano, F. (1991). Formation of bacterial membrane ice-nucleating lipoglycoprotein complexes. Journal of bacteriology, 173(20), 6528-6536.

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.

Felgitsch, L., Bichler, M., Burkart, J., Fiala, B., Häusler, T., Hitzenberger, R., & Grothe, H. (2019). Heterogeneous Freezing of Liquid Suspensions Including Juices and Extracts from Berries and Leaves from Perennial Plants. Atmosphere, 10(1), 37.

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.

Gute, E., David, R. O., Kanji, Z. A., & Abbatt, J. P. (2020). Ice Nucleation Ability of Tree Pollen Altered by Atmospheric Processing. ACS Earth and Space Chemistry, 4(12), 2312-2319.

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.

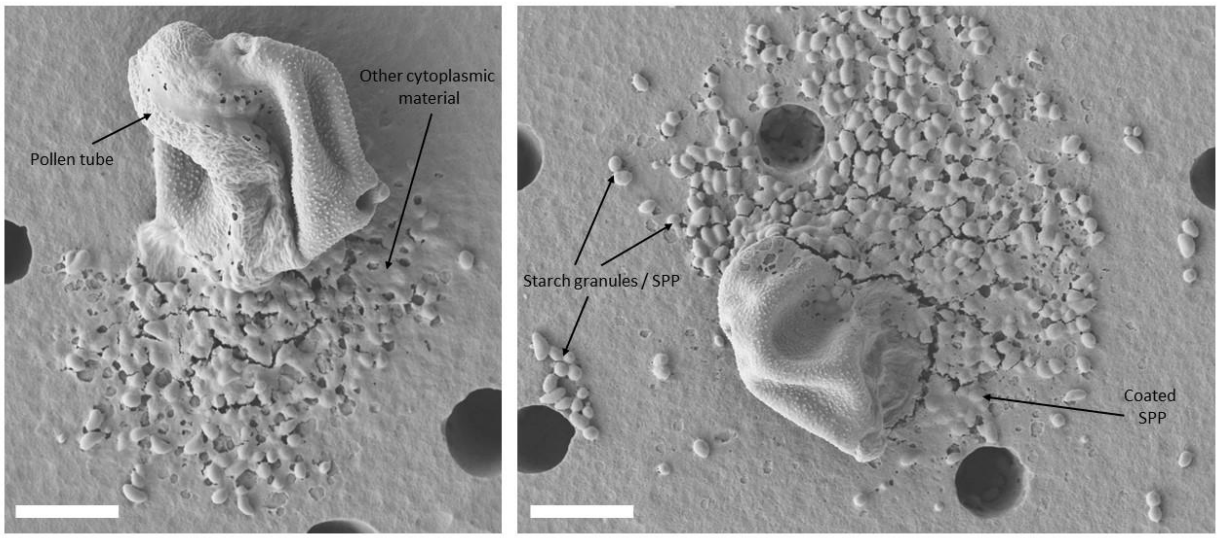

Figure 4: Germinated and ruptured pollen grains. Freshly harvested birch pollen grains were exposed to high relative humidity. Pollen grains were deposited on a polycarbonate nuclepore filter

(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_frozen/n_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 of birch pollen extracts.