Dear Editor

Please find our responses to the referee comment reports below. The comments in Report #1 resulted in us editing the terminology used for our biological INP samples on the basis of their heat-resistance rather than composition. We appreciate their valid suggestions of alternative treatments such as autoclaving and denaturant treatment. However, we reiterate that the motivation of the study is to only assess treatments that involve heat for biological INP detection. In response to Report #2 we updated several figures to include handling blank data, discussed the filtration contamination issue and reported the data in the Supplementary Information.

Other than sections identified below where discussions have been added and minor edits made to text throughout the manuscript, no other substantial changes have been made. We sincerely thank the anonymous reviewers for their scrutiny and useful suggestions and we hope these improvements to the paper address their concerns.

We have re-numbered the reviewers' comments (Comment#1, Comment#2 etc.) and split some up (a, b, c etc.) in order to respond to individual points. Our author responses are written below in blue 11pt text.

Response to Report #1 (anonymous referee #3)

The authors compare dry and wet heating of ice nucleating particles (INP) and ice nucleating macromolecules (INM), some of which are biological or were eventually in contact with biological material. I agree with the general conclusion of the authors concerning inappropriate differentiation between inorganic and biological material by heating tests. I also agree that the manuscript has significantly been enhanced after the first round of discussion with two anonymous referees. Nevertheless, I would like to add some comments and concerns, which the authors might consider.

Comment #1a: I am not a biochemist, but I have learnt so far that dry heating is not a suitable way to denature proteins. The appropriate way is wet heating in an autoclave, which allows temperatures much higher than 100°C without falling dry of the sample. Otherwise, without water the proteins aggregate and can encapsulate and their structures can partially survive which might not be enough for biological activity but is enough at least for ice nucleation. I strongly encourage the authors to use or at least to recommend an autoclave for heating experiments and also to use other tests than only heating, e.g., incubation with subtilisin and urea, which is unfolding the protein structure and have proven the erasure of ice nucleation activity.

We have focussed the study solely on INP sample treatments that involve heat and rather than the use of chemical additives. Using autoclaving is, however, certainly a valid alternative method to 'wet' and 'heating' tests, at least for practical reasons as it does not involve adding chemicals to a sample and portable autoclaves could reasonably be deployed on field campaigns. We note that autoclaves typically use saturated steam at a temperature of about 120°C. This is somewhat harsher than our wet heating and before recommending this as a treatment we would need do a set of tests with all material tested here. We have already found, in some preliminary tests, that the activity of K-feldspar is strongly reduced when autoclaved, hence we suspect that it may not be useful in identifying biological and mineral ice nucleators. Conversely, dry heat offers the potential for separation of biological and mineral ice nucleators.

Comment #1b: The authors might also read and quote the respective literature form the fields of biochemistry.

To provide some extra context to the results we saw with dry heating Snomax at 'low' temperature we have added a discussion about the effect of water content on protein denaturing temperature with reference to the literature at the end of Section 3.2.

Major comment

Comment #2: My own experience with birch pollen washing water was that I was originally convinced that proteins can be excluded as INMs due to heating tests (Pummer et al. 2012), but only recently fluorescence spectroscopy and incubation tests proofed, that proteins are certainly involved in the ice nucleating mechanism (Burkart et al. 2021). Therefore, I would be extremely careful with statements correlating heating treatment tests with protein content if not other reliable methods have been chosen as well. In the data of this manuscript a downshift of the nucleation temperature due to heating of birch pollen washing water is indeed visible (1 °C), which however is small in comparison to Snomax samples.

We appreciate this being pointed out and in light of this we changed our classification of the biological INP samples from 'proteinaceous' and 'non-proteinaceous/polysaccharide' to 'heat-sensitive' and 'heat-resistant'. We also updated our description of the nature of birch pollen INP in Section 2.1.2 with appropriate references added and we also removed references throughout to pollen INP explicitly being polysaccharide based.

Minor comment

Comment #3 : In Table 1 the data set of Seifried et al., 2021 is missing, where heating of a bioaerosol sample at 98 °C for a duration of 1 hour has been investigated.

This paper has been added to Table 1.

Response to Report #2 (anonymous referee #1)

Comment #1: The authors substantially improved their manuscript and the quality of their data. I am concerned that the originally omitted handling blanks revealed contamination issues. The authors repeated a subset of their experiments for the birth pollen and noticed that in fact, birch pollen did not nucleate ice above their background after heating (consistent with (Pummer et al., 2012)). When I compare the first Figure 1 and the revised Figure 1, the changes are substantial, and in my opinion, require a discussion of contamination explicitly in the main manuscript. I am concerned that the authors replaced the data without any comments about how problematic contamination of their filter holder was. Let's make sure we let the community know the importance of handling blanks in ice nucleation, and that as a community we can improve in our analytical approach.

We added a short discussion in Section 2.2 to address this and also added the data showing the contamination from the stainless steel filter holder to Fig S3.

Here are my additional comments:

Comment #2: - Define blanks in Figure 1 – handling blanks that went through the setup should be used when possible.

To this figure we added the handling blanks for wet and dry heating and more clearly defined the blanks #1-3 as 'clean water blanks'. Blanks #4-5 were removed for clarity but remain in Fig S3.

Comment #3: - Figure 2 is improved, the blanks as box plots at the bottom are important to include. Define blank runs explicitly (through filter, through glassware, etc.) And rather than include #1-#5, the handling blanks can also be included.

The handling blanks and clean water blank data in Fig 1 have been added to Figs 2-7 in boxplot form. In addition, the filtration handling blank data has been added to Fig 7.

Comment #4:- Figure 4 – plot T50 as function of time immersed in solution

This has been added as Fig 4c.

Comment #5:- Again, to reiterate: I encourage the authors to be transparent about their discovery of the stainless steel filter contamination and the importance of handling blanks. I very much appreciate figure S3. And I recommend that the authors discuss the observation of contamination that led to substantial differences from the original Figure 1.

See reply to Comment #1.

Comment #6: On a more philosophical point, as scientists we have the responsibility to be our own harshest critics. If additional experiments are needed to clarify ambiguities and speculations, then there really shouldn't be pushback on validating our experiments. I know the work is hard and long, but I would argue that's what we signed up for as scientists: to be rigorous and trustworthy. Food for thought when addressing the following comment made by the authors: "To summarise, we sadly did not have the time and resources to carry out all of the experiments suggested by R1 and we believe many of them are more appropriate for a follow up study. However, their comments were extremely helpful in expanding the conclusions and particularly for identifying the issue with the filtration equipment (R1C##)."

We simply have to disagree on this point. There are limits to how much time people can spend on a project. We have made an important step forward in this paper and it opens up a great deal of future research on several fronts that should be tackled in the future. Also, this is already a long paper with lots of data.