

Response to Reviewer 2 Comments.

We would like to thank the reviewer for their constructive feedback and suggestions. We present our responses and the resultant changes to the original manuscript below. We have re-numbered the reviewers' comments (R1 or R2 C#1, C#2 etc.) and split some up (a, b, c etc.) in order to respond to individual points. Our responses are written below in blue 10pt text with changes indicated by bold type.

R2C#1: 'Comment on amt-2021-208', Anonymous Referee #2, 11 Sep 2021

The authors investigated the responses of heat treatments (namely dry- and wet-) on different types of atmospheric ice-nucleating particle (INP) proxies using their offline cold stage instrument. Based on their findings, they made some technical recommendations regarding the offline heat treatment study of INPs (L695-725). The study objective and hypotheses are valid. The reviewer generally agrees that different ice-nucleating materials respond to heating in various ways (e.g., L576 etc.). The authors' messages are clear (L639-640; L643-645) while some explanations sound speculative. The reviewer has some major and minor comments. Some re-organizations of sections seem necessary to improve the readability.

Major comments

Proteinaceous structures can be destroyed below boiling temperature (Steinke et al., 2016). For example, Szyrmer and Zawadzki (1997) found some known cell-free IN-active microbes (e.g., *Fusarium nuclei*) are stable only up to 60 °C. Other studies of IN active bacteria, fungi, and lichens have shown heat sensitivity at lower than 100 oC. The reviewer is missing the detailed discussion of what protein is (and what is not) denatured in different temperature ranges. It is somewhat temperature-dependent and perhaps employing ~ 80 °C for 10-30 min (i.e., Fig. A2) may be comparable to using truly boiling temperature for heat treatment?

We agree that proteinaceous INPs can be denatured and deactivated at well below boiling temperature. We did already acknowledge that, for example, fungal INPs are known to have higher heat resistance compared to bacteria (lines 603-604 of original manuscript) and we have now elaborated on this. **It now states in Section 2.1.2 (Biogenic sample selection rationale) that 'T Fungal INPs, have been found to have slightly higher heat resistance in wet mode compared to bacterial INPs, typically showing no reduction in INA with up to 60 °C of heating compared to 40 °C with bacterial INP but for both these it is eliminated by heating above 90 ° C. (Pummer et al., 2015; Pouleur et al., 1992; Fröhlich-Nowoisky et al., 2015),**

The motivation of using 100 °C (or near 100 °C) as the temperature for the wet heat treatment is primarily that maintaining a water bath at boiling temperature is more practically convenient than maintaining one at 60 or 80 for example, hence it is commonly used in the community. Another reason for using these conditions was that they are the representative of those used by previous workers performing heat tests on samples taken from the field.

R2C#1a. What is the minimum time for proteins to be denatured?

To section 2.3.1 (Wet heating methodology) we added:

“Samples of proteinaceous IN derived from lichen (Kieft 1988, Kieft and Ruscetti 1990, Moffat et al, 2015), fusarium fungi (Poleur et al. 1992) and psuedomonas syringae bacteria (Maki et al. 1974) all saw large deactivations after being heated in boiling water baths for less than 15 minutes, presumably due to denaturation. Therefore, it is presumed that 30 min of immersion under these conditions is sufficient to denature INP of proteinaceous origin.”

R2C#2. Fig. 1 and all associated discussions fit better in the results & discussion section rather than the materials & method section.

This figure has been moved to Section 3 (Results and discussion) prior to the sub-sections for each INP classes' results.

R2C#3. L338-358 & L466-467 & L478-479: Hydrolysis and dry-heating likely alter SSA and other physical properties of materials, which impact their ice nucleation abilities. Reporting SSAs of a subset of materials after wet- and dry-heating would clarify the authors' hypothesis given in these parts and strengthen the paper. Unless the authors can directly quantify the loss of active sites and/or the number of denatured proteins by a set of heat treatments, some arguments seemingly remain speculative

We reiterate the main focus of this paper was to empirically determine the sensitivity of ice-nucleating materials to heat, to test commonly used heat tests. That said, we also felt that the results have some fundamental mechanistic value and included the discussion accordingly. Our work clearly indicates future areas for research.

Regarding SSA measurements, we do not think a change in INA of minerals would necessarily correlate with a change in its total surface area after a heat treatment. We used a singular (active site) approach to conceptualise ice nucleation and there is physical evidence that immersion mode ice nucleation activity on quartz and K-feldspar occurs at specific active sites (Holden et al 2019). Therefore, the INA of these samples is contained within a very small proportion of the total surface area of the sample and any treatment that 'destroys' the active sites could be possible without a detectable change of SSA.

R2C#4. Sect. 2.3.2: The choice of 250 dC for dry-heating seems appropriate, but if the authors wish to do the apples-to-apples comparison of wet-heating vs. dry-heating, wouldn't it make more sense to use the same heating temperature and period for both heating methods? The authors state that “The dry heat test is a harsher treatment than wet heating...” in L369-370. Do the authors think the measurements with multi-temperatures could be a better procedure in dry heat tests (e.g., 100 dC vs. 250 dC etc.)? Perhaps, Amazonite microcline may have a different response to dry heat at 100 dC? SSA may be changing depending on the employed heat temperature?

Please refer to RC1## with regards to heating conditions and RC2## with regards to surface area changes.

R2C#5. The snapshot example of quartz in Fig. A1 is very nice. The reviewer wishes to see a similar dataset for wet-heat stable compounds (e.g., kaolinite and MCC). Does a similar trend hold for non-quartz samples?

Fig A1 shows the effect of heating Fluka Quartz, a wet heat sensitive sample, in different types of containers made of glass and plastic. This extra set of experiments was specific to quartz due to the

findings of Kumar et al., 2019 who found quartz INP suspensions may interact with glass containers in a way that reduces their INA, but not plastic containers. In light of this remarkable finding we wanted to eliminate this as the cause of the wet-heat deactivations seen with the quartz samples. Doing similar experiments with other types of INP would indeed be an interesting future study, but there is no indication from our or other people's experiments that there is a dependency on vessel type for other materials.

R2C#6. Sect. 2.2.: The used suspension concentration of 1% w/v for MCC etc. seems to exceed what is recommended in previous literature (e.g., Sect. 3.1. in Hiranuma et al., 2019). What is the rationale behind such a high concentration? Wouldn't such a high concentration cause some issues (e.g., flocculation of suspended particles)?

Please refer to RC1#6a where we addressed this issue. **We repeated experiments with MCC with lower concentrations of 0.1% and 0.01%. When plotted as $n_s(T)$ plots (Fig B1d), both runs agree well with the wet-dispersion parameterisation from Hiranuma et al 2015 which we have plotted.** Also, the results, which used the same protocol, agree with the μ -NIPI data in from Hiranuma et al 2019.

R2C#7. How do these high concentration n_s spectra compare to previous studies? The reviewer sees the K-feldspar reference spectrum in Fig. 2 but not for other materials the authors examined for this study.

To the $n_s(T)$ plots in Appendix B, Fig B1 we have added parameterisation spectra for quartz, Snomax, BPWW and MCC. Of these, Fluka Quartz at high concentration of 2.5% w/v is the only sample that appears to deviate from previous studies (Harrison et al., 2019) being about an order of magnitude higher, although this parameterisation not based on this specific quartz sample, rather on an amalgamation of several silica INP samples.

R2C#8. L648-670: Indistinguishable by the heat reaction itself but complementary mineralogy and composition analyses can distinguish these two populations.

We agree and have added **"Interpretation of results may be aided by identification of the mineral phases present in a sample using techniques such as XRD or SEM" in the 3rd paragraph of section 5 (Conclusions).**

R2C#9. Do the authors intend to argue the applicability of heating on environmental samples (i.e., the mixture of different compositions)?

Section 4 contains discussion of this issue, for example:

"Generally, marginal heat deactivations should be interpreted with caution and generally should not be attributed to the presence of proteinaceous ice-nucleating materials. This especially applies if heat deactivations have been used to calculate the ambient concentration of biological INPs in addition to identifying their presence."

R2C#10. L755-756: The reviewer thinks that online heating (i.e., INP measurement with a heating inlet etc.) could be a good alternative approach for the quantitative test. Perhaps, the offline heating tests can be done with a set of different temperatures? Include these points in P25 (a), (b), and (d)?

Online heating with a heated inlet would indeed be a valuable experimental technique, but we simply cannot access the short time scales (that the aerosol would spend in an inlet) with our set up. To explore this we would need to build a dedicated system.

Minor comments

R2C#11. What is the minimum detection limit of evaluation ice nucleation ability and/or efficiency of the cold stage for this study?

Data for blank runs (and handling blanks) are shown in each boxplot fig and in supplementary figure S3 and show T_{50} values that ranged from -27.4 to -25.6 °C. This is typical of background runs using 1 μ L ultrapure water droplets using the μ L-NIPI instrument (Whale et al., 2015). Adding the significance threshold of +/- 1.2 °C we introduce in section to the warmest background T_{50} value we can say that any sample run with a T_{50} value warmer than -24.4 °C is deemed as significantly above the limit of detection. All mineral samples, heated and unheated, fell above this threshold in terms of T_{50} . Previous studies which have used the μ L-NIPI instrument have used a more sophisticated background subtraction routine to determine whether signals are above detection limits (Umo et al. 2015, Wilson et al 2015). However, a relatively simple approach is justified here because of the relatively high concentrations of INPs we used, which ensured most runs produced freezing spectra well above blank levels.

R2C#12. L25-28: This sentence makes sense without the last few words (“, so long as ... K-feldspar”). This part sounds speculative. The reviewer suggests removing this part from the abstract. The importance of K-feldspar as an INP seems not the main focus of this study.

Reworded to the following at the end of the Abstract:

“We conclude that, while wet INP heat tests at (>90 °C) have the potential to produce false positives, i.e., deactivation of a mineral INA that could be misconstrued as the presence of biogenic INPs, they are still a valid method for qualitatively detecting proteinaceous biogenic INP in ambient samples **if** the mineral-based INA is controlled by K-feldspar. “

R2C#13. L57-59: Adding a discussion of the emission rates and atmospheric abundances of mineral dust and biogenic INPs might make this paragraph even more meaningful. Please consider providing some information to the reader.

We have added some extra background info on mineral and biogenic particle abundances in the 4th paragraph of the introduction

Atmospheric concentrations of ice-active bacteria, fungal spores and pollen grains are much smaller than mineral dusts (Hoose et al., 2010). Estimates of the mass of PBAPs emitted to the atmosphere annually range from low hundreds to ~1000 Tg (Hoose et al., 2010; Jaenicke et al 2005) compared to 1,000 - 3,000 Tg per year for mineral dust (Zender et al., 2004). However, the concentration of fragments of biogenic INPs may be much greater given the release of macromolecular INPs (Augustin et al., 2013; O’Sullivan et al., 2015) and their adsorption onto lofted soil dust (Schnell and Vali, 1976; O’Sullivan et al., 2016). Also the sources and atmospheric distribution of biogenic INPs are less well characterised compared to those of mineral dusts (Huang et al., 2021; Kanji et al., 2017), owing to the diversity of marine and terrestrial sources that may be subject to seasonal variations (Conen et al., 2015; Schneider et al., 2020; Šantl-Temkiv et al., 2019) or influenced by anthropogenic activities such as agricultural processes (Garcia et al., 2012; Suski et al., 2018; O’Sullivan et al., 2014).

R2C#14. L67-70: Tobo et al. (2019) shows soil dust has some contributions to it, too. This can be briefly discussed here?

We acknowledge this by adding the citation to the introduction, 5th paragraph:

“Arctic climate, as increasing surface temperatures may expose new terrestrial sources in thawing permafrost (Creamean et al., 2020), **newly exposed glacial outwash sediments (Tobo et al., 2019)**”

We also mention studies which inferred the presence of biological INP associated with soil dusts in the paragraph above:

“However the abundance of biogenic INPs of may be much greater than estimates of PBAP abundances suggest given the release of macromolecular INPs (Augustin et al., 2013; O’Sullivan et al., 2015) and their adsorption onto lofted soil dust (Schnell and Vali, 1976; O’Sullivan et al., 2016)”.

R2C#15. L287-293: Repetitive, and this part does not fit in the results and discussion section.

We included quite a lot of background material on the mineral samples we used and agree they do not belong in the results section, specifically reasons for including each individual sample, general properties and atmospheric relevance and literature references to past INA measurements.

Therefore, we have moved this background material to new sections:

- **2.1.1 Mineral sample selection rationale**
- **2.1.2 Biogenic sample selection rationale**
- **Supplementary section S1: Background information on classes of mineral INP**

This has considerably reduced the amount of text in the results section and improved readability.

R2C#16. L295-303: Better fits in the materials & method section.

See response to comment R2C15 above

R2C#17. L307-307 & L316-318: The authors can introduce a brief statement to guide the reader where the explanation of the observed results is given later in this section (L349-).

Added “**(we discuss the possible reasons for this later in this section) in the first paragraph of Section 3.1.1.**”

Removed the sentence on lines 316-318 as this is just repeating the previous sentence. It now flows straight into the explanation of the results.

R2C#18. L326-336: Fits better in the intro section.

See response to comment R2C15 above

R2C#19. L399-403: Fits better in the materials and methods section.

See response to comment R2C15 above

R2C#20. L405: Quartz presented before feldspar in Fig. 3a. Fig. 3b is not discussed until Page 17. The arrangement of Fig. and sub-sections seems a bit odd.

We have re-organised the figures as detailed above, including splitting up the boxplots with silica and plagioclase samples.

Fig 1 Representative heat responses ff plot
Fig 2 K-feldspar results: a) boxplot, b) ns plot of extended heating tests
Fig 3 Plagioclase feldspar results
Fig 4 Quartz results a) boxplot, b) ns plot of room temperature ageing tests
Fig 5 Clay results
Fig 6 Mineral dust analogues and calcite results a) boxplot b) ns plot for ATD with extended heating tests, c) ns plot for Calcite with room temperature ageing.
Fig 7 Biogenic INP results
Fig A1 Quartz heated in different types of container
Fig A2 Thermocouple measurements inside container during wet heating
Fig B1 Concentration dependence on heat responses: ns plots for a)–d) selected mineral samples; e)–h) biological samples
Fig B2 Temperature and duration dependence on heat responses: Boxplots for a) K-feldspar, b) Fluka Quartz, c) Snomax, d) birch pollen washing water
Fig S1: Fraction frozen plots for all mineral samples before and after standard heat tests
Fig S2: Fraction frozen plots for all biological samples before and after standard heat tests
Fig S3: Fraction frozen plot for background water and handling blanks

R2C#21. L430-431: Sounds speculative.

We agree with this since we are effectively extrapolating our results to those of Amelia Albite (Harrison et al., 2016) which is an atypical sample of plagioclase feldspar. We have therefore removed Lines 429-431.

R2C#22. L433-455: Fits better in the materials and methods.

See response to comment R2C#15 above

R2C#23. L504-521: Fits better in the materials and method section or SI.

L549-558: Fits better in the materials and method section or SI

L596-598: Fits better in the materials and method section or SI.

See response to comment R2C#15 above

R2C#24. L684-691: Sounds speculative

We disagree that this is speculative as there is enough evidence in the literature that grinding and milling increases the INA of some minerals (in the 5th paragraph of Section 4 Summary and implications for using INP heat treatments): “. The INA of quartz (Kumar et al., 2019a; Zolles et al., 2015; Harrison et al., 2019), hematite (Hiranuma et al., 2014) and also natural desert dusts (Boose et al., 2016) are increased by milling”. Moreover, it is important that there also be some discussion about the relevance of results seen in the laboratory to real processes in the environment.

R2C#25. L739-741: Showing the altered specific surface after wet heating can be more direct evidence. Accounting a different SSA may explain the alternation in IN ability of the material?

See response to comment R2C#3 above

R2C#26. Fig. S1: The axis text/numbers and legends are too small to see.

Reorganised so is now visible.

R2C#27. Fig. S3: Is this ns plot generated using the FF spectra data in Fig. S1(u)? It seems that Fig. S3 is missing the point above -12 dC for a non-treated sample. Or the authors did another set of measurements for generating Fig. S3? Please clarify.

The data point above $-12\text{ }^{\circ}\text{C}$ is part of the dry-heated sample and not included on Figure S3 (which is now moved to Figure 4b).

Technical comments

L3: Murray11

Done

L30: à the absence of ice nucleation active sites,

Done

L39 our models à atmospheric models

Done

L56: 2015b comes before 2015a

Done

L63: ice active à ice nucleation active

Done

L63-66: How small & how great? The reviewer suggests that the authors provide some quantitative information to the reader in this paragraph.

See response to R2C#13, **we have added quantitative info.**

L82: à characterize the ice-nucleating activity of

Done

L84: à known bacterial, fungal, and archaeal

Done

L250-274: "A" should be defined in L251, or the authors can introduce Eqn. 2 in L273.

In Section 2.4 (Ice nucleation measurements...) we have removed this equation and replaced with three separate ones (Eqns 1-3) which define $f_{ice}(T)$, $n_s(T)$ and $n_m(T)$.

L306: after a closing-parenthesis change “,” to “.”.

Done

L409:))

Done

L770: 1 mL or 1.5 mL? One figure says 1.5. What is the vendor and model number of the tube?

1.5 mL (Sarstedt Micro Tube 72.690) – added to Appendix A.

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