Responses to Anonymous Referee #2

We thank the reviewer for his/her constructive comments that we address below point by point (responses are in italic, text additions to the revised manuscript are in blue).

The authors describe the ice nucleation active entities of biological materials and highlight the ice nucleation activity of proteins and viruses. The bulk freezing experiment DRINCZ is used to investigate 96 wells at the same time. Common proteins were screened; a particular focus was on ferritin in its iron-containing and iron-free modification. The authors conclude that ice nucleation activity seems to be a common feature of diverse proteins, irrespective of their function, but arising only rarely, most probably through defective folding or aggregation to structures that are ice nucleation active This paper is well-written and the topic fits into the journal Atmospheric Chemistry and Physics. The paper should be published after some changes, which are listed below:

1. Thoroughly describe the basic principles of proteinaceous ice nucleation in the introduction. How did other authors describe the correlation between the sizes of the proteins/aggregates and their ice nucleation activity? What are the differences between free proteins and those embedded in the outer membrane? Quote Pummer et al. 2015 and literature quoted within.

In Sect. 3.3 (Comparison with other ice-nucleating proteins), we discuss aspects of proteinaceous ice nucleation and relate it with the IN activity of the proteins screened in this study. Moreover, in response to the request by the reviewer, we add a more general description of proteinaceous IN activity to the introduction (page 3, lines 26 – 34 of the revised manuscript):

Taking surfaces that are large enough to host a critical ice embryo and have the ability to form hydrogen bonds to water molecules as requirements for IN activity, organic molecules with hydroxyl or carboxyl functionalities should potentially be able to induce freezing (Pummer et al., 2015). Indeed, microcrystalline cellulose has been found to nucleate ice up to -9°C (Hiranuma et al., 2015a). The IN activity from birch trees stems from macromolecules or aggregates of macromolecules which seem to involve polysaccharides (Pummer et al., 2012) and proteins (Tong et al., 2015; Felgitsch et al., 2018). Finally, ice-nucleating proteins expressed by *Pseudomonas* exhibit a repetition unit containing threonine amino acids with hydroxyl functional groups that are able to template ice. Aggregates involving only few of these proteins are water soluble and induce ice nucleation up to -7°C. Larger aggregates nucleate ice up to -2°C but require the intact outer cell membrane to be stable (Polen et al., 2016; Zachariassen and Kristiansen, 2000).

2. When mentioning the ice nucleation activity of Pseudomonas syringae, you might also explain the aging of P. syringae, which drops the freezing temperature by more than 5°C only due to storage in the dark at temperatures below 0°C (see e.g. Häusler et al. 2018). What are the reasons for the aging effect? Changing of size can be excluded at these conditions. How does this effect correlate to your findings?

A decrease of IN activity with sample storage time also occurs in some mineral dusts. In the case of quartz particles, we recently showed that nucleation sites generated by milling disappear with time (Kumar et al., 2019). Thus, chemically reactive sites may be relevant for ice nucleation. In case of biological nucleation sites, the presence of chemically reactive sites is less likely. Alternatively, sites available for hydrogen bonding to water molecules may disappear with time through intramolecular bonding within the protein. Also, alteration of hydrogen bonding patterns, may influence aggregation. In the revised manuscript, we discuss this point by adding this sentence to the discussion on page 14, lines 28 – 31:

Interestingly, the IN activity of Snomax® decreases with storage time indicating that the most efficient nucleation sites of Snomax® degrade with time (Polen et al., 2016; Häusler et al., 2018). This may be due to loss of free hydrogen bonding sites or disintegration of larger aggregates.

3. You might consider that aggregation is important not only between proteins but also between proteins and polysaccharides (e.g. cellulose). Please quote Felgitsch et al. 2018 and literature quoted within.

This is a good point. In the revised manuscript, we quote Felgitsch et al. (2018) on page 3, line 10 of the revised manuscript:

Moreover, IN activity has also been found in aqueous extracts of birch leaves and branches (Felgitsch et al., 2018).

Furthermore, we discuss the IN activity of macromolecules from birch trees on page 3, lines 29 – 31 of the revised manuscript:

The IN activity of birch tree extracts stems from macromolecules or aggregates of macromolecules which involve polysaccharides (Pummer et al., 2012) and proteins (Tong et al., 2015; Felgitsch et al., 2018) that may coaggregate.

4. P. syringae has large ice-templating sites, which most other proteins do not exhibit. Aggregation and defective folding will not generate such ice-templating sites. What kind of ice nucleation mechanism do you anticipate for the proteins in your study?

This is a very good question, which we address now in more detail in Sect. 3.3 on page 15 (lines 6 – 12).

Since the screened proteins all have characteristic freezing onset temperatures, their nucleation sites do not seem to be totally random but related to the protein structure. A templating effect may result from the pattern of hydrophilic and hydrophobic regions on alpha helices and beta sheets together with sites for hydrogen bonding responsible for the tertiary and quaternary structure. In misfolded proteins, these may be available to bind water molecules. Attached to ferritin are water molecules in inter-subunit interfaces through hydrogen bonds (Hempstead et al., 1997), which may be a starting point for ice embryos. Also, the outer protein shell features iron bonding sites (Massover, 1993), which may play a role in ice nucleation.

References

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