

Referee #2:

We thank the reviewer for their comments and suggestions, which we have used to improve the manuscript. Below, we respond to each comment and describe the modifications implemented in the revision (highlighted in red in this response and the annotated manuscript for review).

Reviewer: The revised manuscript of Ingrid de Almeida Ribeiro et al. shows improvement from their previous submission. I am overall happy with their response in most aspects, but find they did not go far enough with text changes to major comments. I would consider acceptance only after following the comments are addressed.

Authors: We thank the reviewer for taking the time to review our revised manuscript. We are pleased to hear that they have noticed an improvement from our previous submission and appreciate their feedback on the text changes and comments in our manuscript.

Reviewer: i) In terms of theory, I think it is necessary to state the author's comment found in their responses, “We want to make clear here that the theoretical interpretation of these differential spectra (and their fully sampled underlying distribution of nucleation temperatures) is not a goal of the present study.” Often the authors claim a connection between their HUB models and theory/physical analysis/kinetic models/and nucleation mechanisms. Without this statement, there is too much confusion for a reader. The author's comment should be stated in the manuscript.

Authors: The paper has two clearly stated and explicit goals in the introduction:

“The first goal of the present study is to provide a strategy to optimize the sampling of drop-freezing experiments to derive interpretable differential spectra that is a good approximant of the underlying distribution of heterogeneous ice nucleation temperatures of the sample.”

“The second aim of our study is to map the cumulative freezing spectrum $N_m(T)$ into the differential spectrum $n_m(T)$, in terms of subpopulations that may correspond to different physical nucleation sites in the sample.”

Thus we interpret that the sentence the reviewer fears may confuse the readers is the one that indicates that the analysis performed with the methodology presented here can be interpreted with theory, models, etc. We now replace “further enables” by “could further enable” in the sentence that the reviewer found confusing and also refer the readers to two recent studies in which we do indeed use the results of HUB-backward together with classical nucleation theory for finite surfaces to predict the size of the IN in lichen and fungi:

“The determination of distributions obtained from the HUB-backward code **could further enable** the interpretation of the experimental ice nucleation spectra with size and structure of INs using nucleation theory, kinetic models, and molecular simulations. **For example, (Schwidetzky et al., 2023) illustrates the use of the distribution of freezing temperatures obtained with HUB-backward together with classical nucleation theory for finite surfaces to interpret the size of the IN of *Fusarium acuminatum*.**”

Reviewer: ii) The sentence in their abstract, l. 21-23, “The differential spectrum computed with HUBbackward is an analytical function that can be used to reveal and characterize the underlying number of IN subpopulations of complex biological samples”, is misleading. It is claimed by the authors in their response, it is not possible to know the subpopulations. Therefore, the HUB does not reveal and characterize the underlying number of IN subpopulations, the authors must prescribe them. I still find the authors overstate what the HUB does and does not do. I would like the authors to claim in the revised

manuscript, as they claim in their response and remove any mention that HUB reveals/characterizes subpopulations.

Authors: We stand by the claim that ““The differential spectrum computed with HUB-backward is an analytical function that can be used to reveal and characterize the underlying number of IN subpopulations of complex biological samples”. It is true that the user has to set the maximum number of subpopulations to be adjusted, but if the number is insufficient, the user will note the disagreement between the calculated and measured cumulative spectrum. If, instead, the proposed number of populations of freezing temperatures is too high, the user will notice that they are overlapped and not truly distinguishable.

Reviewer: iii) The added sentence, “However, the requirement of a model for the freezing rates and their distribution across the sample hinder their interpretability and accuracy at reproducing the experimental freezing curves, particularly in complex samples containing multiple populations.” I think would be highly contended by the Ben Murray group cited in the revised manuscript. They have used multi-component freezing rates across a sample and have accurately reproduced experimental freezing curves. I would remove this sentence on l. 59-61.

Authors: We appreciate the reviewer’s concern and remove the issue of accuracy from the the sentence and rewrite it as “These approaches require an analytical model for the freezing rates and their distribution across the sample.”

Reviewer: iv) In Sear (2013), Fig. 1A, a cumulative spectra/frozen fraction based on extreme value statistics is shown in an ice nucleation model. The authors of the manuscript in question make two claims in their response. 1) “note that –to our knowledge extreme-value statistics has not been previously used for the modeling of ice nucleation data, lines 77-93:” and 2) “To our knowledge, the fraction of frozen droplets and the cumulative spectra based on extreme value sampling have not been explored in any previous study.” I can agree the manuscript provides a unique perspective, but the claims 1) and 2) made by the manuscript authors are not correct. The title of Sear (2013) even states “...a model for ice nucleation”. Please remove these statements and similar and provide a better in-text description of the Sear (2013) citation and what was done previously for modeling ice nucleation using extreme value statistics.

Authors: Figure 1 A of Sear shows cumulative distribution functions obtained from the three generalized extreme value (GEV) distributions, i.e. Gumbel, Frechet and Weibull. These theoretical cumulative fractions obtained from one of these GEV correspond to the fraction of frozen droplets only at certain conditions that are not generally satisfied in experiments:

- The sampling of the droplets has to be complete. However, experiments typically sample tens to hundreds of droplets (and we show that depending on the sampling per dilution the same number of droplets may produce different results).
- The number of IN the droplets has to be extremely large and constant. However, in experiments there are fluctuations (that we model with the Poisson distribution) and –depending on the dilution- the number of IN per droplet spans all the range from reasonably concentrated to a few. Figure 2C shows that if the number of IN per droplet is not extremely large, extreme value sampling produces functions that are intermediate between the underlying distribution and the limit at infinite size; thus a GEV is only the limit for extremely concentrated solutions and we show in this study that sampling the more dilute concentrations is key to reconstruct the underlying distribution.

- As Sear himself notices in his paper, there is no analytical theory to predict the GEV of samples that combine distributions of nucleation temperatures (e.g. those of *Ps. syringae*, pollen, cholesterol, etc).

Our study builds on the same fundamental assumptions of Levine and Sear, but takes a numerical approach to the extreme value sampling that makes it suitable for a finite number of droplets, the full range of dilutions, and to model the outcome from underlying distributions of IN temperatures with multiple peaks. We now elaborate more on the work by Levine and Sear and what are the challenges they cannot address, which are the motivation for the approach in our work (lines 85-103):

“A central assumption of the singular freezing approximation is that the freezing of a droplet containing multiple INs is promoted by the IN with the highest nucleation temperature (Levine, 1950). The extreme-value sampling is apparent in the concentration dependence of $f_{ice}(T)$ in experiments (Marcolli et al., 2007; Budke and Koop, 2015; Kunert et al., 2018; Lukas et al., 2022). Using probability theory, Levine demonstrated that if the distribution of ice nucleation temperatures of the IN population follows an exponential distribution, then the sampling of droplet freezing temperatures corresponds to a Gumbel distribution, and the median freezing temperature T_{MED} of the droplets scales with the logarithm of the number (or total nucleating area) of IN per droplet (Levine, 1950). Sear more recently demonstrated that Levine’s approach is a particular solution for a generalized extreme-value problem, and used modern extreme value statistics to derive the scaling of T_{MED} with the number of IN sites per droplet for the three generalized extreme value distributions (GEV): Gumbel that would arise from an underlying IN distributions with exponential tails, Frechet from those with power law tails, and Weibull from those with an upper cutoff in the freezing temperature of the IN (Sear, 2013). However, there are limitations for the use of the analytical approaches of Sear and Levine for the interpretation of actual drop freezing data. First, the extreme value sampling results in one of the three GEV only in the limit of extremely large number of IN per droplet, while in experiments the sampling is typically performed over dilutions down to a few IN per droplet. There is no analytical formulation for the dependence of the extreme value distribution in the low to intermediate concentration regime. Second, the analytical theory assumes that the sampling is complete (i.e. the number of droplets is extremely large), while experiments are typically performed with tens to hundreds of droplets. Third, Sear notes that there is no general analytical theory to predict the GEV from a mixture of populations of nuclei with different temperature dependences (Sear, 2013). In this study we overcome these three limitations through a numerical implementation of extreme-value statistics for the modeling of drop-freezing experiments.”

Reviewer: v) “Underlying”: the authors must define the word, underlying, in their manuscript. There is too much risk of misconception that there are, in fact, different real populations. Their population number is assumed and distribution parameters are fitted in the manuscript. This is not “real”.

Authors: We understand the concern of the reviewer, and now clarify that these subpopulations are of freezing temperatures and do not necessitate a mapping to distinct physical entities (line 151-157):

“For generality, we represent $P_u(T)$ as a linear combination of normalized continuous distributions $P_i(T)$ that represent subpopulations of freezing temperatures:

$$P_u(T) = c_1 P_1(T) + c_2 P_2(T) + \dots + c_p P_p(T), \quad (2)$$

where p is the total number of subpopulations, $P_1(T), P_2(T), \dots, P_p(T)$ are normalized distribution functions, and c_1, c_2, \dots, c_p are their weights such that $\sum_{i=1}^p c_i = 1$. These subpopulations could correspond to different chemical, topographical or structural motifs in the IN samples, although chemically distinct species could also produce overlapped freezing signatures, and a single species could display a broad freezing range. Our formalism does not require a mapping of subpopulations of freezing temperatures to physical IN sites.”