MS amt-2018-230, Kunert et al.: Twin-plate ice nucleation assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples

We thank referee #2 for his comments, questions, and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. The comments and our responses are listed below.

Referee comment 1: Line 21, please be clear what does "deviations <0.5 K" mean and why it is high-precision temperature control.
Author's response: We recalculated our maximum total error and replaced "deviations < 0.5 K" by "uncertainty < 0.2 K". We included a new Figure S3, which shows the high-precision temperature control, by which TINA is operated.

Referee comment 2: Line 37, it is not clear why this method is suitable for high-throughput experiments and efficient analysis for field samples. I guess the authors don't mean in-situ field measurement? If I understood it right, as for the demonstration of the field sample in the manuscript, one should collect enough sample and extract them carefully to get different dilutions for nm(T) measurements.
Author's response: TINA is suitable for high-throughput experiments because the instrument enables the study of ice nucleation in 960 microliter range droplets in one experiment, which enables the analysis of many samples or dilution series with good statistics in a short period of time. This is demonstrated in Figure 10, for which aqueous extracts of two aliquots of an atmospheric aerosol filter sample were treated in three different ways. All treated samples and untreated controls were measured in six different dilutions to provide the full ice nucleation spectrum for each sample. Each dilution was measured in 96 droplets. All in all, 4608 droplets were measured for Figure 10, which correspond to six experiments performed by TINA. For each freezing experiment down to -30 °C, TINA takes about 45 min, which means 4.5 h of operation of TINA for Figure 10.

Referee comment 3: Line 52-53, why the IR detector is better for droplet freezing detection? Please provide more details.
Author's response: The infrared camera monitors the temperature of each droplet during cooling. As soon as a droplet freezes, latent heat is released and a sharp signal can be detected. We modified the last sentence of paragraph 2 in section 1, where we replaced "improved" by "efficient".

Referee comment 4: Line 79-80, the two biological INPs are very efficient IN, TINA could work very well with such efficient INPs. How about the situation when the unknown samples are less efficient especially when background freezing start contribute the freezing at e.g., -26C or higher.
Author's response: Our experiments with aqueous extracts of atmospheric aerosols confirm that TINA is suitable for freezing experiments of samples with unknown IN in a temperature range down to -25 °C. Below this temperature, background freezing needs to be considered.

Referee comment 5: Line 140, how the droplet temp. is calibrated? you only calibrate the sensors as described? Line 150, the sample temperature needs to be calibrated., not just the sensor. Where are the thermistors in the experimental setup? Depending the location, likely there will be thermal lag due to the plate thickness and different cooling rate. And is the temp. uniform cross such large sample plate? This could also contribute to the different freezing curves for different plates (or plate designs).

Author's response: We included thermocouple measurements in the individual wells of the sample holder blocks to correct for a temperature gradient within the two blocks. We added the following paragraph at the end of section 2.2. and included three new figures (Figure 4, S1, S2, S3) while renaming the existing. "To determine a potential temperature gradient of the sample holder blocks, two thermocouples (K type, 0.08 mm diameter, Omega) were positioned in various wells of multiwell plates (Figure S1a/b), each filled with 30 µL pure water (see Sect. 3.1). These thermocouples were connected to the thermocouple in the elevation of each sample holder block, and the temperature offset between sample holder block and wells was measured for a continuous cooling rate of 1 K min^{-1} (Figure S1c). Below -2 °C, the temperature offset between sample holder block and wells is nearly constant, in this example ~0.16 K and ~0.19 K. The measurement was performed in duplicates for all observed wells. Figure S2 shows the temperature gradient exemplarily for the 384-well sample holder block in a 2D interpolation based on all measurements.

To characterize the uncertainty of this measurement, the two thermocouples were placed in an ice water bath, and the sample holder block was cooled down to 2 °C, 1 °C, 0 °C, -1 °C, and -2 °C (T_{block}), while the difference between the ice water and the block temperature was monitored by the thermocouples (T_{diffTC}) (Figure S3). From these experiments, we obtained thermocouple uncertainties $\delta_{TC} < 0.05$ K ($\delta_{TC} = T_{block} + T_{diffTC}$).

Additionally, we used undiluted IN filtrate of *Mortierella alpina* 13A (see Sect. 3.2) as calibration substance, and a freezing experiment was performed as described for the biological reference materials (see Sect. 3.2). These results were used to compensate for the temperature gradient, and the thermocouple measurements were used to correct the temperature offset between gradient-corrected wells and thermistors. A correction matrix was calculated, and this matrix was used to correct subsequent freezing experiments. Figure 4 shows the results of the fungal IN filtrate measurement (a) before and (b) after correction. After correction, all fungal IN filtrate measurements showed a standard deviation of < 0.06 K (δ_{Morti}). From the calibration measurements, we obtained a total uncertainty estimate of $\delta_{total} < 0.2$ K ($\delta_{total} = \delta_{Thermistor} + \delta_{TC} + \delta_{Morti}$)."

Referee comment 6: Line 188, when using cooling rate of 1K/min, does the Wegener-Bergeron-Findeisen process affect the measurement?

Author's response: The individual droplets are in separate compartments and do not influence each other during the freezing experiment.

Referee comment 7: Line 256-257, please provide a brief description for the O3 and NO2 exposure experiment. Why such high concentration for both O3 and NO2 is used?

Author's response: In section 3.3, we included the following paragraph: "Briefly, O_3 was produced by exposing synthetic air to UV light (L.O.T.-Oriel GmbH & Co. KG, Germany), and the O_3 concentration was adjusted by tuning the amount of UV light. The gas flow was ~1.9 L min^{-1}, and it was mixed with N_2 containing ~5 ppmV NO_2 (Air Liquide, Germany). The NO_2 concentration was regulated by the addition of the amount of the ~5 ppmV NO_2 gas. The O_3 and NO_2 concentrations were monitored with commercial monitoring instruments (ozone analyzer: 49i, Thermo Scientific, Germany; NO_x analyzer: 42i-TL, Thermo Scientific). The gas mixture was directly bubbled through 1 mL of the Snomax® solution at a flow rate of 60 mL min^{-1} using a Teflon tube (ID: 1.59 mm). The Snomax® solution was exposed to a mixture of 1 ppm O_3 and 1 ppm NO_2 for 4 h, representing the exposure to an atmospherically relevant amount of about 200 ppb each for about 20 h. The exposure experiments were performed in triplicates. After exposure, the treated samples were serially diluted and the IN activity was measured as described for the Snomax® reference measurements."

Referee comment 8: Line 277, please provide the ice nucleation data for the field blank samples for comparison with ambient sample.

Author's response: We included the data for the blank sample in Figure 10.

Referee comment 9: Line 298-299, it is not clear why the decrease in IN activity after heat treatment is indication of the presence of biological IN.

Author's response: For clarification, we added the following sentence: "The concentration of IN active at temperatures above 263 K (-10 °C) was about 0.001 L^{-1}, but heat treatment led to a loss of IN activity above 263 K (-10 °C). Because the activity of known biological IN results from proteins or proteinaceous compounds (Green and Warren, 1985; Kieft and Ruscetti, 1990; Pouleur et al., 1992; Tsumuki and Konno, 1994), and proteins are known to be heat-sensitive, the results suggest the presence of biological IN."

Referee comment 10: Line 307-314, it is suggested to carefully evaluate the discussion and conclusion in this section. As showing in Fig. 8, there is less than one order of magnitude different between the samples after 5 um and 0.1 um filtration. This could be just within measurement uncertainty, which is showing in Fig. S1 and S2, for each three independent samples that there is about one order of magnitude variation at certain temperature range.

Author's response: We thank the referee for his suggestion. We carefully evaluated the results and rewrote the paragraph: "Filtration experiments did not affect the initial freezing temperature, but the concentration of biological IN decreased significantly. The results suggest the presence of many biological IN or agglomerates larger than 5 µm and of a few biological IN smaller than 0.1 µm. The cumulative number of IN active between 263 K (-10 °C) and 257 K (-16 °C) decreased up to two orders of magnitude upon filtration, but the IN concentration below 256 K (-17 °C) was not affected. The findings show that many IN active between 263 K (-10 °C) and 257 K (-16 °C) were larger than 5 µm, whereas IN active below 256 K (-17 °C) were smaller than 0.1 µm."

Referee comment 11: Fig. 4, please provide the detail description for freezing determination using IR camera.

Author's response: The infrared camera monitors the temperature of each droplet during cooling. As soon as a droplet freezes, latent heat is released and a sharp signal can be detected. At the end of section 2.3, the freezing determination using IR camera is explained: "Software analysis uses a grid of 96 and 384 points, respectively, where the grid point is set to the center of each well enabling to fit the dimensions of each plate under different perspective angles. The temperature is tracked for each well during the experiment. A self-written algorithm detects a local maximum shortly followed by a local minimum in the derivative of the temperature profile, which is caused by the release of latent heat during freezing. The software exports the data for each droplet in CSV format."

Additionally, we added the information about the resolution of the images to section 2.3 to specify the method: "The camera has a resolution of 206 x 156 pixels, and it takes ten pictures per second. These pictures are averaged to one picture per second."