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 #1 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 answers are listed below.

Referee comment: The authors should stress what is the scientific innovation in their instrument given the very recent paper of Harrison et al. (2018), which was mentioned shortly in the end of the introduction section.

Author's response: TINA studies 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. The cooling system allows a fast and high-precision temperature control over a wide temperature range at variable cooling rates. The infrared detection is an efficient method to doubtlessly determine freezing events, which was first applied to droplet freezing assays by Zaragotas et al. 2016. As discussed by Grothe 2018 (doi:10.5194/amt-2018-177-RC3, 2018), the authors of Harrison et al. 2018 attended several workshops and conferences and also have been the organizers for one conference, where our Twin-plate ice nucleation assay (TINA) with infrared detection was presented and discussed, so that our new setup was well-known to them.

Referee comment: Also, why infrared detector enables improved detection over other methods?

Author's response: The infrared detector 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. For clarification, we modified the last sentence of paragraph 2 in section 1, where we replaced "improved" by "efficient".

Referee comment: The ability of high-throughput experiments was mentioned repeatedly in the manuscript, and it will be valuable contribution if the authors could use their existing data to show if this ability is important.

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 five different dilutions to provide the full ice nucleation spectrum for each sample. Each dilution was measured in 96 droplets, and every sample consisted of two aliquots. 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: I also wonder why error bars are lacking from all data and figures.

Author's response: The uncertainty of the temperature sensor was used as the error of the temperature and was added into the figure captions. The error of the IN number concentrations was calculated using the counting error and the Gaussian error propagation, and error bars of the IN number concentrations were added into all figures.

The section 2.4 was optimized and the following paragraph was included:

"Assuming ice nucleation as a time-independent (singular) process, the number concentration of IN ($\frac{\Delta N_m}{\Delta T}$) active at a certain temperature (T) per unit mass of material is given by Eq. (1) (Vali, 1971a).

$$\frac{\Delta N_m}{\Delta T}(T) = -\ln\left(1 - \frac{s}{a - \sum_{i=0}^{j} s}\right) \cdot \frac{c}{\Delta T} \qquad ; 0 \le j \le a \tag{1}$$

$$\text{with } c = \frac{V_{wash}}{V_{drop}} \cdot \frac{d}{m} \tag{2}$$

where s is the number of freezing events in 0.1 K bins (ΔT), a is the number of all droplets, m is the mass of the particles in the initial suspension, V_{wash} is the volume of the initial suspension, V_{drop} is the droplet volume, and d is the dilution factor of the droplets relative to m. The measurement uncertainty ($\delta\frac{\Delta N_m}{\Delta T}(T)$) was calculated using the counting error of s plus one digit and the Gaussian error propagation (Eq. (3)).

$$\delta\frac{\Delta N_m}{\Delta T}(T) = \sqrt{\left(\frac{1}{1 - \frac{s}{a - \sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{\sqrt{s+1}}{a - \sum_{i=0}^{j} s}\right)^2 + \left(\frac{1}{1 - \frac{s}{a - \sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{s \cdot \sqrt{\sum_{i=0}^{j} s+1}}{\left(a - \sum_{i=0}^{j} s\right)^2}\right)^2} \tag{3}$$

The cumulative IN number concentration ($N_m(T)$) is given by Eq. (4).

$$N_m(T) = -\ln\left(1 - \frac{\sum_{i=0}^{j} s}{a}\right) \cdot c \qquad ; 0 \le j \le a \tag{4}$$

The error of the cumulative IN number concentration ($\delta N_m(T)$) was calculated using Eq. (5).

$$\delta N_m(T) = \sqrt{\left(\frac{c}{1 - \frac{\sum_{i=0}^{j} s}{a}} \cdot \frac{\sqrt{\sum_{i=0}^{j} s+1}}{a}\right)^2} \tag{5}$$

According to the above equations, the uncertainty is proportional to the number of frozen droplets per temperature bin. In the freezing experiments described below, the lowest number of freezing events and largest uncertainties were obtained at the lower and higher end of each dilution series (Poisson distribution). Data points with uncertainties ≥100% were excluded (overall less than 6% of the measurement data)."

Specific comments:

Referee comment: Line #26: It is stated that there is a good agreement with literature data. Where was this shown or detailed in the manuscript?

Author's response: In section 3.2, we discussed the results of our experiments with Snomax®, which are shown in Figures 7 and S4. "These findings are in accordance with the results of Budke and Koop (2015)." Here, we replaced "in accordance" with "in good agreement".
In the same section, we also discussed the results of our experiments with *Mortierella alpina*, which are shown in Figures 8 and S5. "The cumulative number of IN and the initial freezing temperature of 268 K (-5 °C) are in good agreement with the literature (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015)."

Referee comment: Line #76: I think it is confusing: up to 10 K min-1 or more?

Author's response: We tested our setup with continuous cooling rates of up to 10 K min^{-1}, but it is possible to run the setup at higher cooling rates. But it has to be considered, that for each cooling rate a new correction matrix has to be generated. For clarification, we deleted "or more".

Referee comment: Line #83: Is this the correct place to introduce the similar approach by Harrison et al.?

Author's response: We deleted the sentence "Very recently, a similar approach for droplet freezing experiments with IR detection has been presented by Harrison et al. (2018), investigating K-feldspar, NX-illite, and atmospheric aerosol samples." at the end of section 1, and we modified in section 1: "Infrared (IR) detectors enable efficient detection of droplet freezing (Harrison et al., 2018; Zaragotas et al., 2016)."

Referee comment: Line #94: Here it is not clear if the plates are commercial product or self-designed? If commercial, manufacture details should be specified.

Author's response: We added the following sentence: "For each experiment, new sterile multiwell plates are used (96-well: Axon Labortechnik Kaiserslautern, Germany, 384-well: Eppendorf, Hamburg, Germany)."

Referee comment: Line #137: It confused me that it was cooled to 218.2 K and heated from 220.7 K?

Author's response: To clarify this, we modified the sentence: "The temperature within the bath was cooled down from 303.2 K to 218.2 K (30.0 °C to -55.0 °C) in 5 K steps, warmed to 220.7 K (-52.5 °C), and raised again from 220.7 K to 300.7 K (-52.5 °C to 27.5 °C) in 5 K steps."

Referee comment: Section 2.2: So what is the temperature uncertainty of TINA and how was it propagated?

Author's response: The temperature uncertainty of TINA is 0.2 K. We added the following sentence at the end of section 2.2: "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: Line 144: I think it is still not clear at this point what is the temperature gradient you refer to. I would first defined that.

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 four 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 δ_{total} < 0.2 K ($\delta_{total} = \delta_{Thermistor} + \delta_{TC} + \delta_{Morti}$)."

Referee comment: Line #151: please clarify why do you mention here Zaragotas et al. (2016).
Author's response: We deleted the sentence "In contrast, Zaragotas et al. (2016) used infrared camera, which was calibrated only once by the company, to measure the accurate temperature of each droplet."

Referee comment: Line #152: I think it would be nice if you will add the plate temperature at the different images.
Author's response: We thank the referee for this suggestion, and we added the plate temperature at the different images.

Referee comment: Line #157: what is the resolution in which images are taken?
Author's response: 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."

Referee comment: Line #182: Are those new plates? or the same plates described earlier in the text?
Author's response: We changed the sentence as follows: "For background measurements, 3 µL aliquots of autoclaved and filtered pure water were pipetted into new sterile multiwell plates by a liquid handling station."
Moreover, we added this information in section 2.1.: "For each experiment, new sterile multiwell plates are used (96-well: Axon Labortechnik Kaiserslautern, Germany, 384-well: Eppendorf, Hamburg, Germany)."

Referee comment: Line #209: Please add a reference to this claim.
Author's response: We assume that different plates from different manufactures can lead to differences in freezing because of the production process and well shape, but cannot add a specific reference. For clarification we changed the sentence as follows: "The 96-well plates were obtained from a different manufacturer than the 384-well plates."

Referee comment: Line #235: Is this correct? Class A only seen for high suspension concentrations.
Author's response: We thank the referee for this comment. We removed the following text: "These differences result from three different classes of IN with different activation temperatures as described by Turner et al. (1990). Based on this classification, the Snomax® sample contains a large number of class A and C IN, but only a few IN of class B. These findings are in accordance with the results of Budke and Koop (2015). Below 259 K (-14 °C), a flat plateau arises where no IN are active." and we included the following sentence: "These findings are in good agreement with the results of Budke and Koop (2015)"

Referee comment: Line #302: per liter air? Or liter water.

Author's response: The IN concentration was calculated per liter air, which passed the filter during sampling. We included the following sentence: "All IN concentrations were calculated per liter air."

Technical corrections:

Referee comment: Line #97: Fig. 1b should be describes before Fig. 1c.
Author's response: Changed as suggested.

Referee comment: Line #165: add "is" after Vdrop, and m, and etc..
Author's response: We modified the sentence.

Referee comment: Line #206: You can remove 'respectively'.
Author's response: This has been removed.

Referee comment: Line #209: 'showed' and not 'show'. Also found in other places in the text.
Author's response: We replaced it in several places in the text.

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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 four 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."

List of changes

All changes have been marked in the revised version of the manuscript using track-changes. The most relevant changes are listed below.

Author list:

The order was changed from "Anna T. Kunert[1], Mark Lamneck[2], Frank Helleis[2], Mira L. Pöhlker[1], Ulrich Pöschl[1], and Janine Fröhlich-Nowoisky[1,*]" to "Anna T. Kunert[1], Mark Lamneck[2], Frank Helleis[2], Ulrich Pöschl[1], Mira L. Pöhlker[1,*], and Janine Fröhlich-Nowoisky[1,*]"

The following author was added to the corresponding authors:
Mira L. Pöhlker

Figures:

All figures of the manuscript and the supplementary material containing experimental data have been modified regarding data processing and error bars. Figure 4 was added to the manuscript. Figure 6a and 6b have been divided into two separate figures (Figure 7a and 8a), and differential IN spectra were added (Figure 7b and 8b). All subsequent figures have been renamed. In the supplementary material, the following figures have been added: S1, S2, S3, while renaming the existing.

Section 2.2:

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 four 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 $\delta_{\mathrm{total}} < 0.2$ K ($\delta_{\mathrm{total}} = \delta_{\mathrm{Thermistor}} + \delta_{\mathrm{TC}} + \delta_{\mathrm{Morti}}$)."

Section 2.4:

As requested by the referees, we included a detailed error calculation and added error bars to all figures. The error of the IN number concentrations was calculated using the counting error and the Gaussian error propagation. The total uncertainty estimate was used as the error of the temperature and was added into the figure captions. Additionally, we calculated the differential IN number concentration and plotted the corresponding spectrum.

The former section 2.4. was replaced by "Assuming ice nucleation as a time-independent (singular) process, the number concentration of IN ($\frac{\Delta N_{\mathrm{m}}}{\Delta T}$) active at a certain temperature (T) per unit mass of material is given by Eq. (1) (Vali, 1971a).

$$\frac{\Delta N_{\mathrm{m}}}{\Delta T}(T) = -\ln\left(1 - \frac{s}{a - \sum_{i=0}^{j} s}\right) \cdot \frac{c}{\Delta T} \qquad ; 0 \le j \le a \tag{1}$$

$$\text{with } c = \frac{V_{\mathrm{wash}}}{V_{\mathrm{drop}}} \cdot \frac{d}{m} \tag{2}$$

where s is the number of freezing events in 0.1 K bins (ΔT), a is the number of all droplets, m is the mass of the particles in the initial suspension, V_{wash} is the volume of the initial suspension, V_{drop} is the droplet volume, and d is the dilution factor of the droplets relative to m. The measurement uncertainty ($\delta\frac{\Delta N_{\mathrm{m}}}{\Delta T}(T)$) was calculated using the counting error of s plus one digit and the Gaussian error propagation (Eq. (3)).

$$\delta\frac{\Delta N_{\mathrm{m}}}{\Delta T}(T) = \sqrt{\left(\frac{1}{1 - \frac{s}{a - \sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{\sqrt{s+1}}{a - \sum_{i=0}^{j} s}\right)^2 + \left(\frac{1}{1 - \frac{s}{a - \sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{s \cdot \sqrt{\sum_{i=0}^{j} s+1}}{\left(a - \sum_{i=0}^{j} s\right)^2}\right)^2} \tag{3}$$

The cumulative IN number concentration ($N_{\mathrm{m}}(T)$) is given by Eq. (4).

$$N_{\mathrm{m}}(T) = -\ln\left(1 - \frac{\sum_{i=0}^{j} s}{a}\right) \cdot c \qquad ; 0 \le j \le a \tag{4}$$

The error of the cumulative IN number concentration ($\delta N_{\mathrm{m}}(T)$) was calculated using Eq. (5).

$$\delta N_{\mathrm{m}}(T) = \sqrt{\left(\frac{\mathrm{c}}{1 - \frac{\sum_{i=0}^{j} s}{a}} \cdot \frac{\sqrt{\sum_{i=0}^{j} s+1}}{a}\right)^2} \tag{5}$$

According to the above equations, the uncertainty is proportional to the number of frozen droplets per temperature bin. In the freezing experiments described below, the lowest number of freezing events and largest uncertainties were obtained at the lower and higher end of each dilution series (Poisson distribution). Data points with uncertainties ≥100% were excluded (overall less than 6% of the measurement data)."

Section 3.2:

We added additional differential IN spectra for the reference materials and rewrote the discussion:

"Three independent experiments with Snomax® showed reproducible results (Fig. S4), and, therefore, droplets of the same dilution were added to a total droplet number of 288. The obtained results were plotted in a cumulative and a differential IN spectrum (Fig. 7). The cumulative IN number concentration represents the total number of IN active above a certain temperature. The cumulative IN spectrum showed two strong increases, around 270 K (-3 °C) and around 265 K (-8 °C). These findings are in good agreement with the results of Budke and Koop (2015). The differential IN number concentration was calculated according to Vali (1971a), and it represents the number of IN active in a particular temperature interval. The differential IN spectrum showed a similar shape as the cumulative IN spectrum with a distinct plateau between 268 K and 266 K (-5 °C and -7 °C) and two slight maxima, around 269 K (-4 °C) and around 264 K (-9 °C). This indicates the presence of highly-efficient IN, active at a temperature of approximately 269 K (-4 °C), and less-efficient IN, active around 264 K (-9 °C). The fact that the less-efficient IN appeared in higher dilutions implies that they occur in higher concentrations than the highly-efficient IN. The presence of further IN with lower freezing temperatures and low concentrations cannot be excluded."

"The cumulative and the differential IN spectra showed similar shapes with one maximum around 267 K (-6 °C), indicating the presence of one type of IN, which is highly-efficient."

Section 3.3:

We included the following paragraph to provide a brief description for the O_3 and NO_2 exposure experiments:

"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."

We rewrote the results:

"The results showed that gas exposure affected the IN activity of Snomax® (Fig. 9). High concentrations of O_3 and NO_2 reduced the cumulative number of IN from Snomax® between one and two orders of magnitude, while exposure to synthetic air showed smaller effects."

Section 3.4:

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."

We removed the following paragraph from the manuscript, because the outliers were excluded based on the introduced error calculation.

"The results of both, the untreated and the heated filter extracts, showed a few outliers. These were probably caused by single large particles or aggregates larger than 5 µm, which were statistically distributed over the different dilutions. These particles nucleated at warmer subfreezing temperatures than the other IN within a dilution, so they are overestimated due to their efficient nucleation. This hypothesis is supported by the fact that the filtered filter extracts did not contain any outliers."

Twin-plate ice nucleation assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples

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Gelöscht: , Ulrich Pöschl[1],

Abstract. For efficient analysis and characterization of biological ice nuclei under immersion freezing conditions, we developed a Twin-plate Ice Nucleation Assay (TINA) for high-throughput droplet freezing experiments, in which the temperature profile and freezing of each droplet is tracked by an infrared detector. In the fully automated setup, a couple of independently cooled aluminum blocks carrying two 96-well plates and two 384-well plates, respectively, are available to study ice nucleation and freezing events simultaneously in hundreds of microliter range droplets (0.1-40 µL). A cooling system with two refrigerant circulation loops is used for high-precision temperature control (uncertainty < 0.2 K), enabling measurements over a wide range of temperatures (~272-233 K) at variable cooling rates (up to 10 K min^{-1}).

The TINA instrument was tested and characterized in experiments with bacterial and fungal ice nuclei (IN) from *Pseudomonas syringae* (Snomax®) and *Mortierella alpina*, exhibiting freezing curves in good agreement with literature data. Moreover, TINA was applied to investigate the influence of chemical processing on the activity of biological IN, in particular the effects of oxidation and nitration reactions. Upon exposure of Snomax® to O_3 and NO_2, the cumulative number of IN active at 270-266 K decreased by more than one order of magnitude. Furthermore, TINA was used to study aqueous extracts of atmospheric aerosols, simultaneously investigating a multitude of samples that were pre-treated in different ways to distinguish different kinds of IN. For example, heat treatment and filtration indicated that most biological IN were larger than 5 µm. The results confirm that TINA is suitable for high-throughput experiments and efficient analysis of biological IN in laboratory and field samples.

Gelöscht: gradient

Gelöscht: deviations <

Gelöscht: 5

Gelöscht: -270

Gelöscht: and more

Gelöscht: samples

Gelöscht: concentration

Gelöscht: around 269

Gelöscht: (-4 °C, “class A”)

Gelöscht: about two orders of magnitude, while the concentration of IN active around 265 K (-8 °C, “class C”) decreased by about

Gelöscht: experiments

Gelöscht: highly efficient

Gelöscht: smaller than 0.1 µm, and many IN active between 263 K (-10 °C) and 256 K (-14 °C) were heat-resistant and

1 Introduction

Clouds and aerosols still contribute the largest uncertainty to the evaluation of the Earth's changing energy budget (Boucher et al., 2013). Thus, the understanding of the contribution of atmospheric aerosols in cloud processes is of fundamental importance. Atmospheric ice nucleation is essential for cloud glaciation and precipitation, thereby influencing the hydrological cycle and climate. Ice can be formed via homogeneous nucleation in liquid water droplets or heterogeneous nucleation triggered by particles serving as atmospheric ice nuclei (IN) (Pruppacher and Klett, 1997).

A wide range of droplet freezing assays and instruments have been developed and applied for the analysis of IN in immersion freezing experiments (e.g., Budke and Koop, 2015; Fröhlich-Nowoisky et al., 2015; Häusler et al., 2018; Murray et al., 2010; O'Sullivan et al., 2014; Stopelli et al., 2014; Tobo, 2016; Vali, 1971b; Whale et al., 2015; Wright and Petters, 2013; Zaragotas et al., 2016). Most of the available assays and instruments, however, are limited to the investigation of small droplet numbers and use optical detection systems in the UV/Vis wavelength range.

Infrared (IR) detectors enable efficient detection of droplet freezing (Harrison et al., 2018; Zaragotas et al., 2016). Upon the phase change of water from liquid to solid, latent heat is released resulting in a sudden temperature change of the droplet, which can be detected by IR video thermography. In 1995, Ceccardi et al. (1995) used IR video thermography as a new technique to non-destructively study ice formation on plants by visualizing the changes in surface temperature. Wisniewski et al. (1997) evaluated the IR video thermography under controlled conditions and determined it as an excellent method for directly observing ice nucleation and propagation in plants. Since then, IR video thermography was used in a range of studies investigating freezing in plants (e.g., Ball et al., 2002; Carter et al., 1999; Charrier et al., 2017; Fuller and Wisniewski, 1998; Hacker and Neuner, 2007; Pearce and Fuller, 2001; Sekozawa et al., 2004; Stier et al., 2003; Wisniewski et al., 2008; Workmaster, 1999). Further applications of IR video thermography are investigations of cold thermal stress in insects (Gallego et al., 2016), monitoring of freeze drying processes (Emteborg et al., 2014), as well as detection of ice in wind turbine blades (Gómez Muñoz et al., 2016) and helicopter rotor blades (Hansman and Dershowitz, 1994). Freezing of single water droplets in an acoustic levitator has also been successfully observed by IR video thermography (Bauerecker et al., 2008).

Here, we introduce a Twin-plate Ice Nucleation Assay (TINA) for high-throughput droplet freezing experiments, in which the temperature profile and freezing of each droplet is tracked by an infrared detector. In the fully automated setup, a couple of independently cooled

Gelöscht: (e.g., Budke and Koop, 2015; Fröhlich-Nowoisky et al., 2015; Häusler et al., 2018; Murray et al., 2010; O'Sullivan et al., 2014; Stopelli et al., 2014; Tobo, 2016; Vali, 1971b, 1971a; Whale et al., 2015; Wright and Petters, 2013; Zaragotas et al., 2016). Most of the available assays and instruments, however, are limited to the investigation of small droplet numbers and use optical detection systems in the UV/Vis wavelength range. As shown by Zaragotas et al. (2016), however, infrared (IR) detector enable improved detection of droplet freezing.

Gelöscht: infrared (

Gelöscht:)

Gelöscht: gradient

aluminum blocks are available to study ice nucleation and freezing events in nearly 1000 microliter range droplets simultaneously. The instrument was developed in the course of the INUIT project over the last three years, in which it has been presented and discussed at several conferences and workshops (Kunert et al., 2016a, 2016b, 2017a, 2017b, 2018). We use the bacterial IN Snomax® and the IN-active fungus *Mortierella alpina* as biological test substances to investigate heterogeneous ice nucleation. Moreover, TINA is applied to investigate the effect of O_3 and NO_2 exposure on the IN activity of Snomax®. Furthermore, aqueous extracts of atmospheric aerosols are treated in different ways and are analyzed for different kinds of IN.

2 Experimental setup

2.1 Technical details

The core of the Twin-plate Ice Nucleation Assay (TINA) are two independently cooled, customized sample holder aluminum blocks, which have been shaped for multiwell plates with 96 and 384 wells, respectively. In each cooling block, two multiwell plates can be analyzed simultaneously. The maximal droplet volume in the 96-well block is 250 µL, and the minimal droplet volume is 0.1 µL, which is the limit of our liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany). For each experiment, new sterile multiwell plates are used (96-well: Axon Labortechnik Kaiserslautern, Germany, 384-well: Eppendorf, Hamburg, Germany). As shown in Fig. 1, the design of the two sample holder blocks is basically identical, but the detailed construction varies slightly. Both blocks consist of two parts, a trough and a cap, which are screwed together and sealed with an O-ring. But, for the 96-well block (Fig. 1a), the cap is at the top (Fig. 1b), and the trough is at the bottom (Fig. 1c), whereas, for the 384-well block (Fig. 1d), the trough is at the top (Fig. 1e) and the cap is at the bottom (Fig. 1f). Two openings with Swagelok adapters for cooling liquid are placed next to each other, and the cooling liquid flows in a small passage around an elevation in the middle of the trough.

The customized sample holder blocks are cooled with a silicon-based cooling liquid (SilOil M80.055.03, Peter Huber Kältemaschinenbau AG, Offenburg, Germany) tempered by an external high-performance refrigeration bath circulator (CC-508 with Pilot ONE, Peter Huber Kältemaschinenbau AG), which can supply temperatures down to 218 K (-55 °C). Both sample holder blocks can be operated in parallel and independently from each other by use of two self-developed mixing valves and cooling loops (Fig. 2). This allows either the cooling of two different droplet freezing assays at the same time, or the observation of 960 droplets in one experiment. The mixing of a cold and a warm loop of cooling liquid for each block enables a fast and precise adjustment of the sample holder block temperatures without being dependent

Gelöscht: carrying two 96-well and two 384-well plastic plates, respectively,

Gelöscht: A cooling system with two refrigerant circulation loops is used for high-precision temperature control (deviations <0.5 K), enabling measurements over a wide range of temperatures (~233-270 K (~ -50 °C to -3 °C)) at variable cooling rates (up to 10 K min^{-1} and more).

Gelöscht: Very recently, a similar approach for droplet freezing experiments with IR detection has been presented by Harrison et al. (2018), investigating K-feldspar, NX-illite, and atmospheric aerosol samples.

Gelöscht:) and the cap is at the top (Fig. 1b

Gelöscht: due to the usage

on the cooling rate of the refrigeration bath circulator itself. In each experiment, the refrigeration bath circulator is cooled down 5 K below the coldest temperature, which is projected for the experiment, while no mixing of warm and cold cooling liquid occurs. By changing the position of the mixing valves for a defined period of time, cold and warm cooling liquids are mixed together, so that the desired temperatures within the two blocks are reached. Two pumps (VPP-655 PWM Single Version, Alphacool International GmbH, Braunschweig, Germany) ensure the continuous circulation of cooling liquid through each block independently from the position of the mixing valves. Figure 3 is a schematic illustration of the overall setup of TINA.

2.2 Temperature control and calibration

Within each sample holder block, the temperature is measured with two temperature sensors, a NTC thermistor in the cooling liquid stream (TH-44033, resistance: 2255 Ω/298 K, interchangeability: ±0.1 K, Omega Engineering GmbH, Deckenpfronn, Germany) and a customized sensor with a NTC thermistor (10K3MRBD1, resistance: 10000 Ω/298 K, interchangeability: ±0.2 K, TE Connectivity Company, Galway, Ireland) and a thermocouple (K type, 0.08 mm diameter, Omega), which were glued together in a 1/8 inch Swagelok® pipe, placed in the elevation. With further thermocouples connected to this reference, this offers the possibility to measure temperature differences between the NTC thermistor and arbitrary points simultaneously. Another NTC thermistor (10K3MRBD1, resistance: 10000 Ω/298 K, interchangeability: ±0.2 K, TE Connectivity Company) monitors the temperature behind each mixing valve. Temperature control within the entire system is achieved by a self-developed microcontroller-based electronic system. The analog input unit is equipped with a 24 Bit Low Noise Delta-Sigma ADC (ADS1256), which assures the required accuracy to process the resolution of the used thermistors. All thermistors had been calibrated with a reference thermometer (2180A, Fluke Deutschland GmbH, Glottertal, Germany; 0.01 K resolution, system uncertainty δ_{Fluke}: ± 0.08 K at 223 K and ± 0.07 K at 273 K). Therefore, all thermistors were bound together with a PT100 sensor of the reference thermometer, and the bundle was placed inside a brass cylinder filled with cooling liquid. The cylinder was placed inside the cooling bath of the refrigeration bath circulator. The temperature within the bath was cooled down from 303.2 K to 218.2 K (30.0 °C to -55.0 °C) in 5 K steps, warmed to 220.7 K (-52.5 °C), and raised again from 220.7 K to 300.7 K (-52.5 °C to 27.5 °C) in 5 K steps. Each step was kept for 30 min to equilibrate the temperature, while the resistance of all thermistors and the temperature measured by the reference thermometer were monitored. For the

Gelöscht: in the elevation.

Gelöscht: : 0.01 K, maximum

Gelöscht: error

Gelöscht: , Fluke Deutschland GmbH, Glottertal, Germany

conversion of the measured resistance of the thermistors into temperature, cubic spline interpolation was used ($\delta_{Ipol} < 0.01$ K). We obtained the thermistor calibration uncertainty $\delta_{Thermistor} < 0.09$ K ($\delta_{Thermistor} = \delta_{Fluke} + \delta_{Ipol}$).

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 $\delta_{total} \leq 0.2$ K ($\delta_{total} = \delta_{Thermistor} + \delta_{TC} + \delta_{Morti}$).

2.3 Infrared video thermography

Droplet freezing is determined by a distinct detection system, where the temperature profile of each single droplet is tracked by infrared cameras (Seek Thermal Compact XR, Seek Thermal Inc., Santa Barbara, CA, USA) coupled to a self-written software. The camera has a resolution of 206 x 156 pixels, and it takes ten pictures per second. These pictures are averaged to one

Gelöscht: gradient

Gelöscht: This

picture per second. The concept enables a doubtless determination of freezing events because freezing of supercooled liquid releases energy, which leads to an abrupt rise in the detected temperature of the observed droplet, as discussed earlier (Sect. 1). This detection system uses the IR video thermography only to determine freezing events, while the proper temperature is monitored by thermistors. Figure 5 is a sequence of infrared camera images showing 384 droplets during cooling and freezing (red circles). 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.

2.4 Data analysis

Assuming ice nucleation as a time-independent (singular) process, the number concentration of IN ($\frac{\Delta N_m}{\Delta T}$) active at a certain temperature (T) per unit mass of material is given by Eq. (1) (Vali, 1971a).

$$\frac{\Delta N_m}{\Delta T}(T) = -\ln\left(1 - \frac{s}{a - \sum_{i=0}^{j} s}\right) \cdot \frac{c}{\Delta T} \quad ; 0 \le j \le a \qquad (1)$$

with $c = \frac{V_{wash}}{V_{drop}} \cdot \frac{d}{m}$ (2)

where s is the number of freezing events in 0.1 K bins (ΔT), a is the number of all droplets, m is the mass of the particles in the initial suspension, V_{wash} is the volume of the initial suspension, V_{drop} is the droplet volume, and d is the dilution factor of the droplets relative to m. The measurement uncertainty ($\delta\frac{\Delta N_m}{\Delta T}(T)$) was calculated using the counting error of s plus one digit and the Gaussian error propagation (Eq. (3)).

$$\delta\frac{\Delta N_m}{\Delta T}(T) = \sqrt{\left(\frac{1}{1-\frac{s}{a-\sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{\sqrt{s+1}}{a-\sum_{i=0}^{j} s}\right)^2 + \left(\frac{1}{1-\frac{s}{a-\sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{s \cdot \sqrt{\sum_{i=0}^{j} s+1}}{\left(a-\sum_{i=0}^{j} s\right)^2}\right)^2} \qquad (3)$$

The cumulative IN number concentration ($N_m(T)$) is given by Eq. (4).

$$N_m(T) = -\ln\left(1 - \frac{\sum_{i=0}^{j} s}{a}\right) \cdot c \quad ; 0 \le j \le a \qquad (4)$$

The error of the cumulative IN number concentration ($\delta N_m(T)$) was calculated using Eq. (5).

Gelöscht: In contrast, Zaragotas et al. (2016) used infrared camera, which was calibrated only once by the company, to measure the accurate temperature of each droplet. Figure 4

Gelöscht: gradient

Gelöscht: n_m)

Gelöscht: $n_m(T) = \frac{-\ln(1-f_{ice})}{V_{drop}} \cdot \frac{d}{m}$ (1)

Gelöscht: f_{ice}

Gelöscht: fraction of frozen droplets at

Gelöscht: particular temperature, V_{drop}

Gelöscht: droplet volume

Gelöscht: To simplify

$$\delta N_{\mathrm{m}}(T) = \sqrt{\left(\frac{\mathrm{c}}{1 - \frac{\sum_{i=0}^{j} s}{a}} \cdot \frac{\sqrt{\sum_{i=0}^{j} s + 1}}{a} \right)^2} \quad (5)$$

According to the above equations, the uncertainty is proportional to the number of frozen droplets per temperature bin. In the freezing experiments described below, the lowest number of freezing events and largest uncertainties were obtained at the lower and higher end of each dilution series (Poisson distribution). Data points with uncertainties ≥100% were excluded (overall less than 6% of the measurement data).

Gelöscht: analysis, freezing events were merged in 0.1 K bins.

3. Freezing experiments

The fully automated TINA setup was tested and characterized for immersion freezing experiments with pure water droplets, as well as Snomax® and IN filtrate of the fungus *Mortierella alpina* as biological reference substances. Moreover, TINA was used to study the effect of O_3 and NO_2 exposure on the IN activity of Snomax®. Furthermore, TINA was applied to atmospheric aerosol samples.

3.1 Pure water

Pure water was obtained from a Barnstead™ GenPure™ xCAD Plus water purification system (Thermo Scientific, Braunschweig, Germany). The water was autoclaved at 394 K (121 °C) for 20 min, filtered three times through a sterile 0.1 µm pore diameter sterile polyethersulfone (PES) vacuum filter unit (VWR International, Radnor, PA, USA), and autoclaved again.

For background measurements, 3 µL aliquots of autoclaved and filtered pure water were pipetted into new sterile multiwell plates by a liquid handling station. Therefore, four (96-well plate) and eight (384-well plate) different water samples were pipetted column-wise distributed into the plates. In total, six columns per sample were apportioned over the two twin-plates, i.e., 48 droplets per sample in 96-well plates, and 96 droplets per sample in 384-well plates. The plates were placed in the sample holder blocks and were cooled down quickly to 273 K (0 °C), and, as soon as the temperature was stable for one minute, in a continuous cooling rate of 1 K min^{-1} further down to 238 K (-35 °C).

Gelöscht: 96-well

Gelöscht: (Axon Labortechnik, Kaiserslautern, Germany) and 384-well plates (Eppendorf), respectively,

As the phase transition from liquid water to ice is kinetically hindered, supercooled water can stay liquid at temperatures down to 235 K (-38 °C), where homogeneous ice nucleation takes place. This is only true for nanometer-sized droplets because the freezing temperature is dependent on droplet volume and cooling rate, and the classical nucleation theory predicts a homogeneous freezing temperature of about 240 K (-33 °C) for microliter

volume droplets using a cooling rate of 1 K min^{-1} (Fornea et al., 2009; Murray et al., 2010; Pruppacher and Klett, 1997; Tobo, 2016). However, several studies reported average freezing temperatures for microliter volume droplets of pure water at significantly higher temperatures because of possible artifacts (e.g., Conen et al., 2011; Fröhlich-Nowoisky et al., 2015; Hill et al., 2016; Whale et al., 2015). To our knowledge, only two studies reported an average homogeneous freezing temperature of 240 K (-33 °C) for microliter volume droplets and a cooling rate of 1 K min^{-1}, using hydrophobic surfaces as contact area for the droplets (Fornea et al., 2009; Tobo, 2016). Providing microliter droplets free of suspended IN and surfaces free of contaminants is difficult, so that the temperature limit below which freezing cannot be traced back to heterogeneous IN needs to be determined individually for each setup.

Our results showed that most pure water droplets froze around 248 K (-25 °C) in 96-well plates (Fig. 6a) and around 245 K (-28 °C) in 384-well plates (Fig. 6b). The 96-well plates were obtained from a different manufacturer than the 384-well plates. All in all, these freezing temperatures are substantially above the expected temperatures for homogeneous nucleation of microliter droplets, but they are in accord with the results of Whale et al. (2015).

3.2 Biological reference materials

The performance of TINA was further assessed using Snomax® as a bacterial IN-active reference substance (e.g., Budke and Koop, 2015; Hartmann et al., 2013; Möhler et al., 2008; Turner et al., 1990; Ward and DeMott, 1989) and IN filtrate of the well-studied IN fungus *Mortierella alpina* (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015).

Snomax® was obtained from SMI Snow Makers AG (Thun, Switzerland), and a stock solution was prepared in pure water with an initial mass concentration of 1 mg mL^{-1}. This suspension was then serially diluted 10-fold with pure water by the liquid handling station. The resulting Snomax® concentrations varied between 1 mg mL^{-1} and 0.1 ng mL^{-1}, equivalent to a total mass of Snomax® between 3 µg and 0.3 pg, respectively, per 3 µL droplet.

Each dilution was pipetted column-wise distributed over the twin-plates as described before in 96 droplets into 384-well plates by the liquid handling station. Two plates at a time were placed inside the 384-well sample holder block, and the plates were cooled down quickly to 273 K (0 °C), and, as soon as the temperature was stable for one minute, in a continuous cooling rate of 1 K min^{-1} further down to 253 K (-20 °C).

Three independent experiments with Snomax® showed reproducible results (Fig. S4), and, therefore, droplets of the same dilution were added to a total droplet number of 288. The obtained results were plotted in a cumulative and a differential IN spectrum (Fig. 7). The

Gelöscht: have

Gelöscht: warmer subfreezing

Gelöscht: due to

Gelöscht: show

Gelöscht: 247

Gelöscht: 26

Gelöscht: 244

Gelöscht: 29

[1] verschoben (Einfügung)

Gelöscht: 5a

Gelöscht: 5b), respectively. This discrepancy can have different explanations. First, the

Gelöscht: are

Gelöscht: Second, the different well shape leads to an altered shape of the droplet, which could influence its freezing abilities at very low temperatures.

Gelöscht: show reproducible results (Fig. S1). Therefore, droplets of the same dilution were added to a total droplet number of 288 (Fig. 6a). The data show two strong increases in the cumulative number of IN, one around 269 K (-4 °C) and one around 265 K (-8 °C), interrupted by a slightly increasing plateau between 268 K and 266 K (-5 °C and -7 °C). These differences result from three different classes of IN with different activation temperatures as described by Turner et al. (1990). Based on this classification, the Snomax® sample contains a large number of class A and C IN, but only a few IN of class B. These findings are in accordance

cumulative IN number concentration represents the total number of IN active above a certain temperature. The cumulative IN spectrum showed two strong increases, around 270 K (-3 °C) and around 265 K (-8 °C). These findings are in good agreement with the results of Budke and Koop (2015). The differential IN number concentration was calculated according to Vali (1971a), and it represents the number of IN active in a particular temperature interval. The differential IN spectrum showed a similar shape as the cumulative IN spectrum with a distinct plateau between 268 K and 266 K (-5 °C and -7 °C) and two slight maxima, around 269 K (-4 °C) and around 264 K (-9 °C). This indicates the presence of highly-efficient IN, active at a temperature of approximately 269 K (-4 °C), and less-efficient IN, active around 264 K (-9 °C). The fact that the less-efficient IN appeared in higher dilutions implies that they occur in higher concentrations than the highly-efficient IN. The presence of further IN with lower freezing temperatures and low concentrations cannot be excluded.

The analysis of different IN active within a wide temperature range was only possible with the measurement of a dilution series. TINA enables the simultaneous measurement of such a dilution series with high statistics in a short period of time.

Mortierella alpina 13A was grown on full-strength PDA (VWR International GmbH, Darmstadt, Germany) at 277 K (4 °C) for 7 months. Fungal IN filtrate was prepared as described previously (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015) and contained IN from spores and mycelial surfaces. It was serially diluted 10-fold with pure water by the liquid handling station. The experiment was performed as described above.

For test measurements with fungal IN, IN filtrate of three different culture plates from *Mortierella alpina* 13A was measured, and the results were reproducible (Fig. S5). The cumulative number of IN per gram mycelium only varied between one order of magnitude, which is a good achievement for a biological sample, and droplets of the same dilutions were added to a total droplet number of 288. A cumulative IN spectrum (Fig. 8a) and a differential IN spectrum (Fig. 8b) were plotted. The cumulative number of IN and the initial freezing temperature of 268 K (-5 °C) are in good agreement with the literature (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015). The cumulative and the differential IN spectra showed similar shapes with one maximum around 267 K (-6 °C), indicating the presence of one type of IN, which is highly-efficient.

3.3 Ozonized and nitrated samples

Gelöscht: Below 259 K (-14 °C), a flat plateau arises where no IN are active.

Gelöscht: due to

Gelöscht: 269

Gelöscht: S2

Gelöscht: varies

[1] nach oben verschoben: 6b).

Gelöscht: 267.7

Gelöscht: 5.

To study the effect of O_3 and NO_2 exposure on the IN activity of Snomax®, an aliquot of 1 mL of a 1 mg mL^{-1} suspension of Snomax® in pure water was exposed in liquid phase to gases with or without O_3 and NO_2 as described in Liu et al. (2017).

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.

The results showed that gas exposure affected the IN activity of Snomax® (Fig. 9). High concentrations of O_3 and NO_2 reduced the cumulative number of IN from Snomax® between one and two orders of magnitude, while exposure to synthetic air showed smaller effects.

Snomax® contains IN proteins of the bacterium *Pseudomonas syringae*. Attard et al., (2012) found no significant or only weak effects of exposure to ~100 ppb O_3 and ~100 ppb NO_2 on the IN activity of two strains of *P. syringae*, and a variable response of a third strain, suggesting a strain-specific response.

3.4 Air filter samples

Total suspended particle samples were collected onto 150 mm glass fiber filters (Type MN 85/90, Macherey-Nagel GmbH, Düren, Germany) using a high-volume sampler (DHA-80, Digitel Elektronik AG, Hegnau, Switzerland) operated at 1000 L min^{-1}, which was placed at the roof of the Max Planck Institute for Chemistry (Mainz, Germany). There, a mix of urban and rural continental boundary layer air can be sampled in central Europe. The filter was taken in April 2018, and the sampling period was seven days, corresponding to a total air volume of approximately 10,000 m^3. Filters were pre-baked at 603 K (330 °C) for 10 h to remove any biological material, and blank samples were taken to detect possible contaminations. All filters

Gelöscht: A dilution series of the treated samples was measured as described for the Snomax® reference measurements.

Gelöscht: The results show that high concentrations of O_3 and NO_2 reduce the concentration of IN active around 269 K (-4 °C, "class A") about two orders of magnitude, while the concentration of IN active around 265 K (-8 °C, "class C") decreased by about one order of magnitude (Fig. 7). The cumulative number of IN per unit mass is slightly reduced by the exposure to synthetic air, which can be explained by a small loss of IN within the system during exposure.

Gelöscht: MPIC,

were packed in pre-baked aluminum bags, and loaded filters were stored at 193 K (-80 °C) until analysis.

An aerosol and a blank filter were cut with a sterilized scissor into aliquots (~1/16), and the exact percentage was determined gravimetrically. For reproducibility, two filter sample aliquots of each filter were extracted. Each filter sample aliquot was transferred into a sterile 50 mL tube (Greiner Bio One, Kremsmünster, Austria), and 10 mL of pure water was added. The tubes were shaken horizontally at 200 rpm for 15 min. Afterwards, the filter was removed, and the aqueous extract was tested for IN activity. To further characterize the IN, the effects of filtration and heat treatment were investigated. Therefore, aliquots of the extract were treated as follows: (i) 1 h at 371 K (98 °C), (ii) filtration through a 5 µm pore diameter filter (Acrodisc, PES, Pall, Germany), (iii) filtration through a 5 µm and a 0.1 µm pore diameter filter (Acrodisc).

Each solution (96 aliquots of 3 µL) was pipetted column-wise into 384-well plates by the liquid handling station. The plates were cooled down quickly to 273 K (0 °C), and, as soon as the temperature was stable for one minute, in a continuous cooling rate of 1 K min^{-1} further down to 243 K (-30 °C).

Each solution of the two aliquots of each filter was measured separately, and droplets of the same solution were added to a total droplet number of 192 (2 x 96 droplets) (Fig. 10, S6). All IN concentrations were calculated per liter air.

The untreated filter extract showed IN activity at relatively high temperatures with an initial freezing temperature of 267 K (-6 °C). 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. The concentration of IN between 263 K (-10 °C) and 257 K (-16 °C) increased about two orders of magnitude and in a sudden increase another two orders between 257 K (-16 °C) and 256 K (-17 °C). The IN concentration below 256 K (-17 °C) increased continuously up to about 500 L^{-1}, but heat treatment reduced the IN concentration of up to one order of magnitude below 256 K (-17 °C). 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

Gelöscht: Filters

Gelöscht: aerosol and blank

Gelöscht: S3). For better clearness, data of different dilutions were averaged for each treatment (Fig. 8).

Gelöscht: warm subfreezing

Gelöscht: 10^{-3}

Gelöscht:), which indicates

Gelöscht: 255

Gelöscht: 18

Gelöscht: continuously

Gelöscht: 255

Gelöscht: 18

Gelöscht: 254

Gelöscht: 19

Gelöscht: A maximum

Gelöscht: of 10^2 L^{-1} was reached around 250

Gelöscht: 23

Gelöscht:),

Gelöscht: maximum

Gelöscht: 10^1 L^{-1}at 250

Gelöscht: 23

Gelöscht: was

Gelöscht: about half an order of magnitude, at which

Gelöscht: 0.1

Gelöscht: filtration showed

Gelöscht: slightly bigger effect.

Gelöscht: concentration

Gelöscht: 256

Gelöscht: 17

Gelöscht: about

Gelöscht: 5 µm

Gelöscht: and 0.1 µm filtration reduced

Gelöscht: slightly more. The maximum IN concentration of 10^2 L^{-1} around 250

Gelöscht: 23

Gelöscht: upon filtration. The results suggest that there were highly efficient biological IN smaller than 0.1 µm and other biological IN or agglomerates of the same biological IN with different sizes. Moreover, the

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.

4 Conclusions

The new high-throughput droplet freezing assay TINA was introduced to study heterogeneous ice nucleation of microliter range droplets in the immersion mode. TINA provides the analysis of 960 droplets simultaneously or 192 and 768 droplets in two independent experiments at the same time, enabling the analysis of many samples with high statistics in a short period of time. Moreover, an infrared camera-based detection system allows to reliably determine droplet freezing. The setup was tested with Snomax® as bacterial IN, and IN filtrate of *Mortierella alpina* as fungal IN. For these reference materials, both, the initial freezing temperature and the cumulative number of IN per gram unit mass, were in good agreement with the literature, which demonstrates the functionality of the new setup.

TINA was applied to study the effect of O_3 and NO_2 exposure on the IN activity of Snomax®, where high concentrations of O_3 and NO_2 reduced the IN activity significantly. Atmospheric aerosol samples from Mainz (Germany) were analyzed for IN activity to show the applicability of TINA for field samples. Here, the results suggest that most of the biological IN were larger than 5 µm. Moreover, 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. The results confirm that TINA is suitable for high-throughput experiments and efficient analysis of biological IN in laboratory and field samples.

Gelöscht: 256

Gelöscht: 17

Gelöscht: between

Gelöscht: and 250 K (-23 °C)

Gelöscht: Most of the IN active between 263 K (-10 °C) and 259 K (-14 °C) were heat-resistant. ¶
The results of both, the untreated and the heated filter extracts, showed a few outliers. These were probably caused by single large particles or aggregates larger than 5 µm, which were statistically distributed over the different dilutions. These particles nucleated at warmer subfreezing temperatures than the other IN within a dilution, so they are overestimated due to their efficient nucleation. This hypothesis is supported by the fact that the filtered filter extracts did not contain any outliers.

Gelöscht: mycelium

Gelöscht: accordance

Gelöscht: we found highly efficient

Gelöscht: and heat-resistant IN larger than 5 µm.

Author Contributions. A.T.K., M.L., and F.H. developed the instrument. A.T.K., U.P., J.F.-N. conceived and designed the experiments. A.T.K. performed the experiments. M.L.P. wrote the code to process the data and did the error calculation. All authors discussed the data and contributed to the writing of the manuscript.

Competing Interests. The authors declare that they have no conflict of interest.

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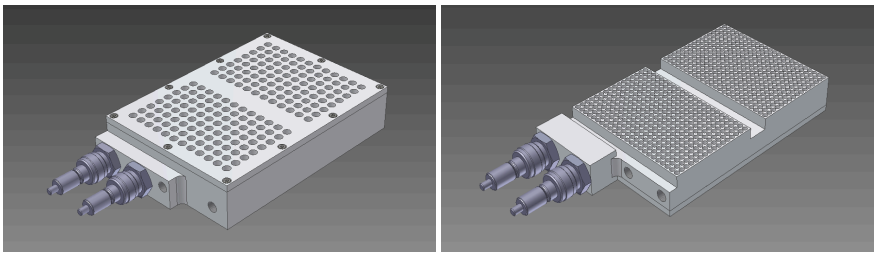
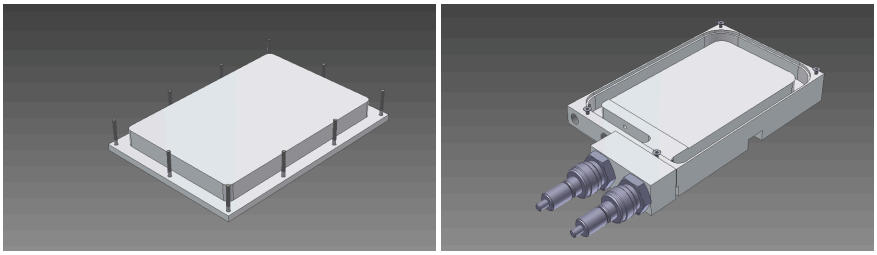
(a) 96-well sample holder block (d) 384-well sample holder block



(b) 96-well top (flipped) (e) 384-well top (flipped)



(c) 96-well bottom (f) 384-well bottom

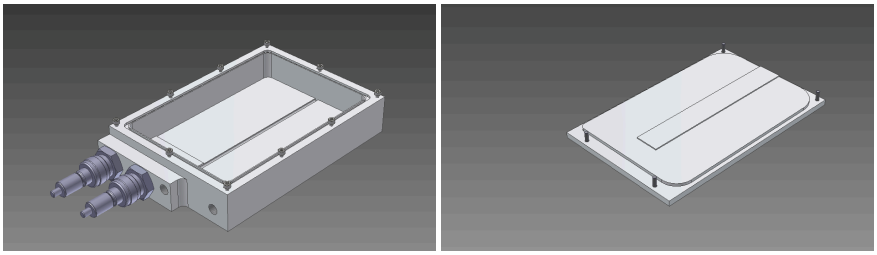


Figure 1. Sample holder and cooling blocks of the Twin-plate Ice Nucleation Assay (TINA) with **(a-c)** 96-well plates and **(d-f)** 384-well plates (CAD drawings).

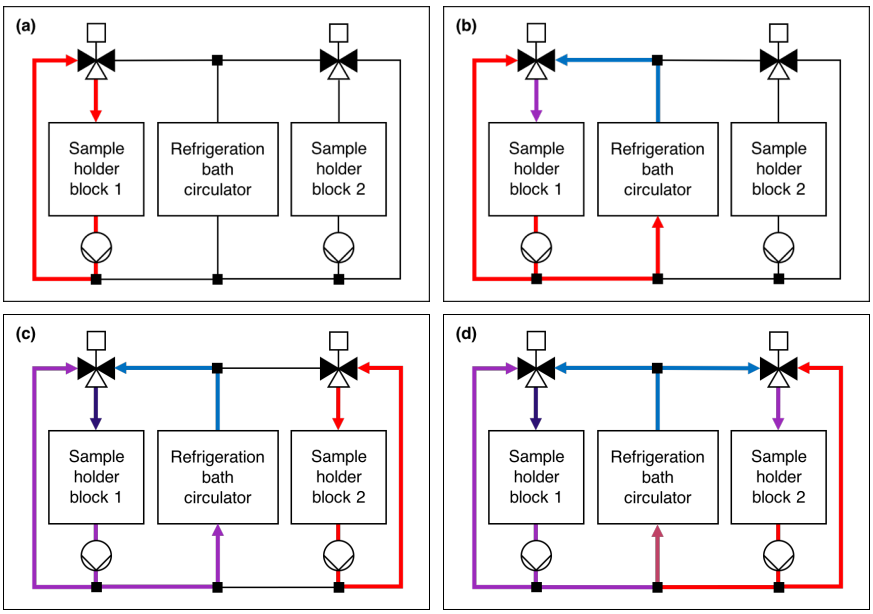


Figure 2. Cooling system layout and operating principle of the Twin-plate Ice Nucleation Assay (TINA). **(a)** Cooling liquid is pumped in warm cooling loop of sample holder block 1 without connection to colder cooling liquid provided by refrigeration bath circulator. **(b)** Mixing valve is opened for both, warm cooling liquid of warm cooling loop and cold cooling liquid of refrigeration bath circulator. Position of mixing valve defines temperature within sample holder block 1. **(c)** Sample holder block 1 is cooled further down, while cooling liquid is pumped in warm cooling loop of sample holder block 2. **(d)** Sample holder block 2 can be run in parallel independently from the temperature in sample holder block 1.

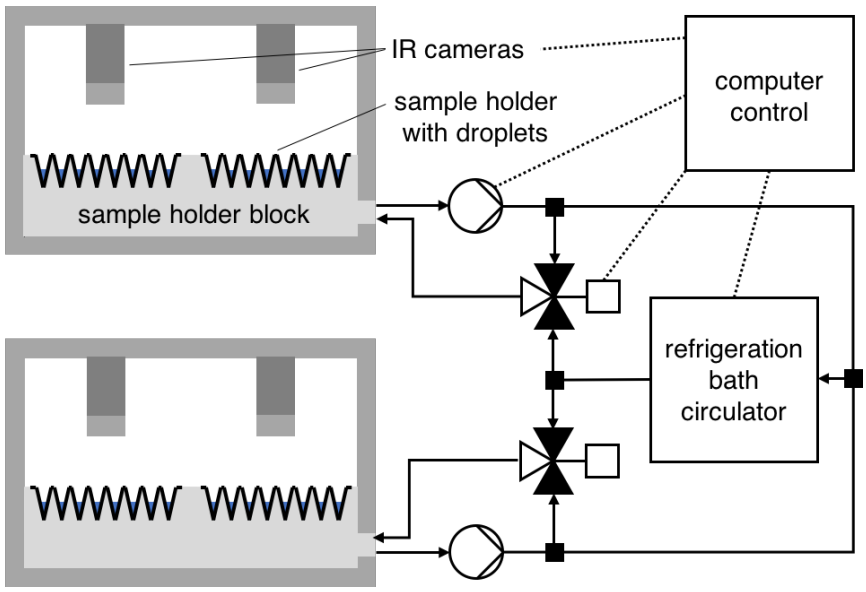


Figure 3. Schematic illustration of the overall setup: sample holder blocks, sample holders with droplets, IR cameras, cooling system with refrigeration bath circulator, pumps and mixing valves, computer control.

Gelöscht: ¶

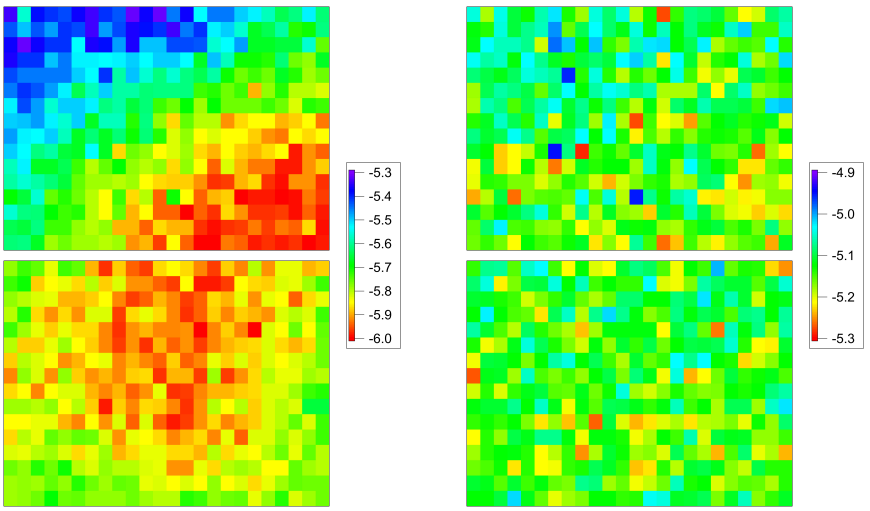


Figure 4: Measurement of temperature gradient of 384-well sample holder block using *Mortierella alpina* 13A as calibration substance. A correction matrix was calculated to compensate for temperature gradient and offset. (a) Data before correction. (b) Data after correction.

Gelöscht:

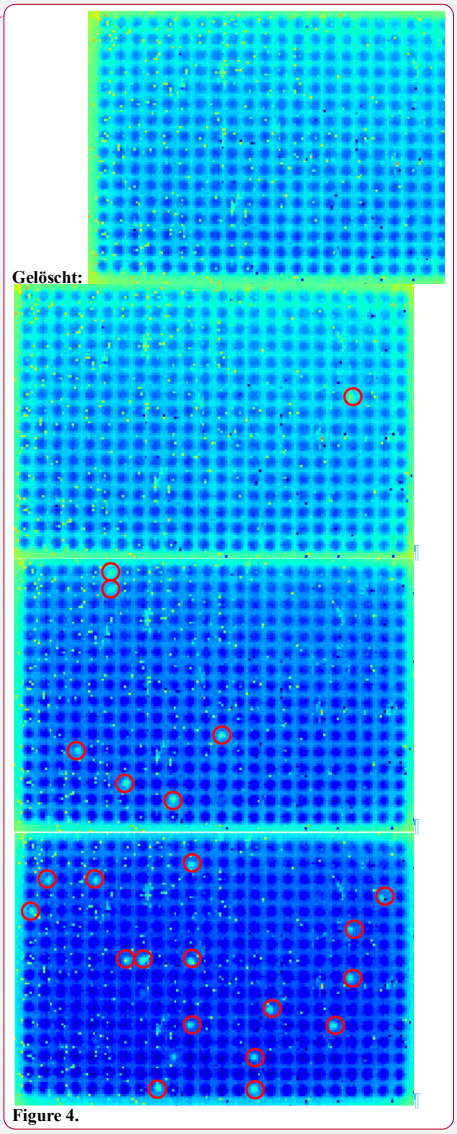


Figure 4.

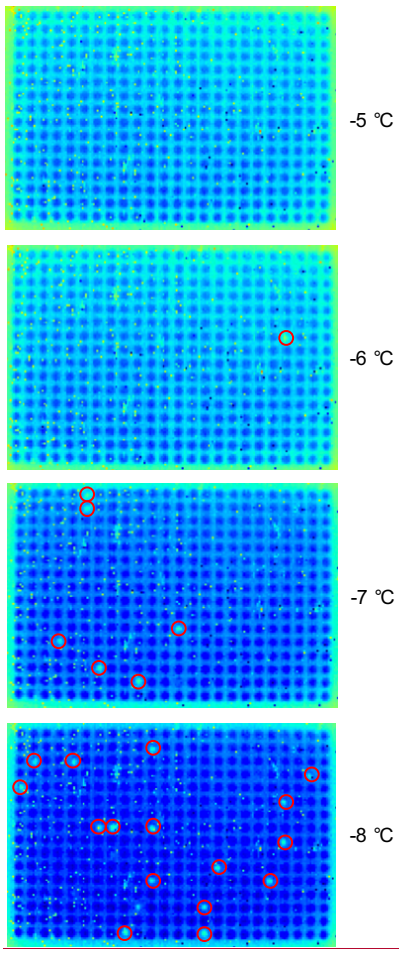


Figure 5. Sequence of infrared camera images showing 384 droplets during cooling. Red circles indicate freezing droplets.

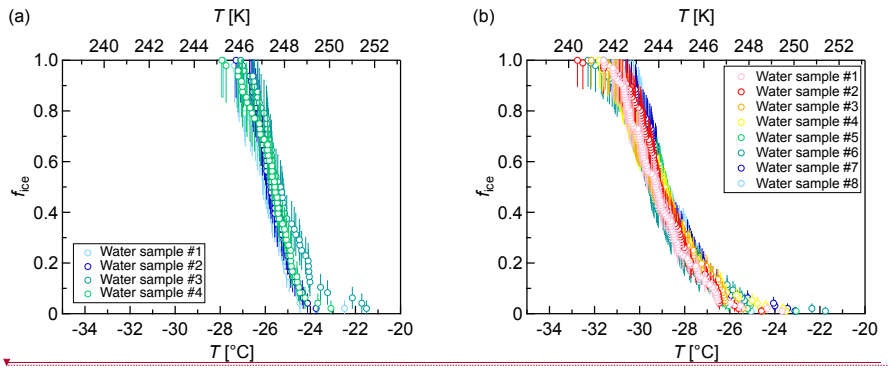
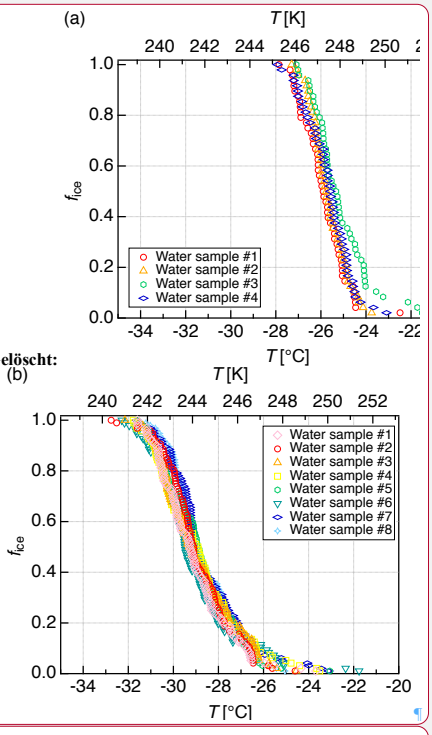


Figure 6. Freezing experiments with pure water droplets. Fraction of frozen droplets (f_{ice}) vs. temperature (T) obtained with a continuous cooling rate of 1 K min^{-1} and a droplet volume of 3 µL. **(a)** Four different samples with 48 droplets each apportioned over two 96-well plates. **(b)** Eight different samples with 96 droplets each apportioned over two 384-well plates. The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

Gelöscht:



Gelöscht: 5

Gelöscht: f_{ice})

Gelöscht: Seitenumbruch

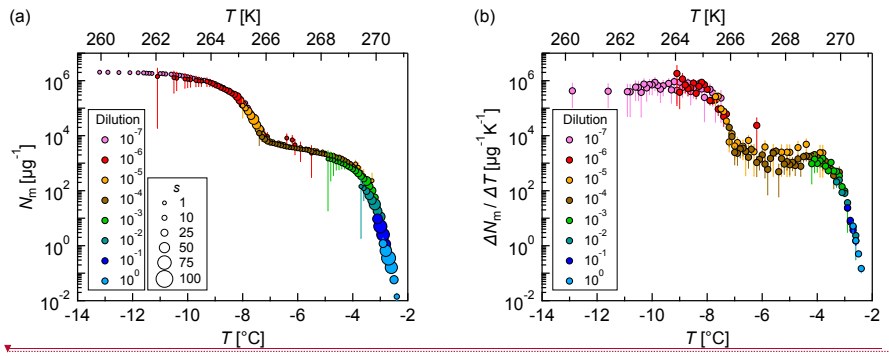
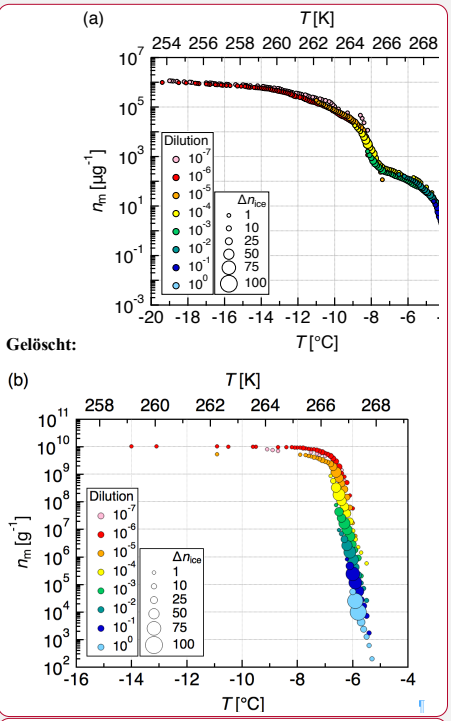


Figure 7. Measurements of dilution series of bacterial IN (Snomax®). **(a)** Cumulative number of IN (N_m) and **(b)** differential number of IN ($\Delta N_m/\Delta T$) per unit mass of Snomax® vs. temperature (T). Droplets of the same dilution of three independent measurements were added to a total droplet number of 288 (3 x 96 droplets). Symbol colors indicate different dilutions; symbol size indicates the number of frozen droplets per 0.1 K bin (s). The error bars were calculated using the counting error and the Gelöscht: Gaussian error propagation. The temperature error is 0.2 K.

Gelöscht:



Gelöscht: 6

Gelöscht: (a)

Gelöscht: ®) and **(b)** fungal IN (*Mortierella alpina* 13A).

Gelöscht: n_m

Gelöscht: and mycelium, respectively,

Gelöscht: .

Gelöscht: temperature interval (ΔT = 0.1 K).

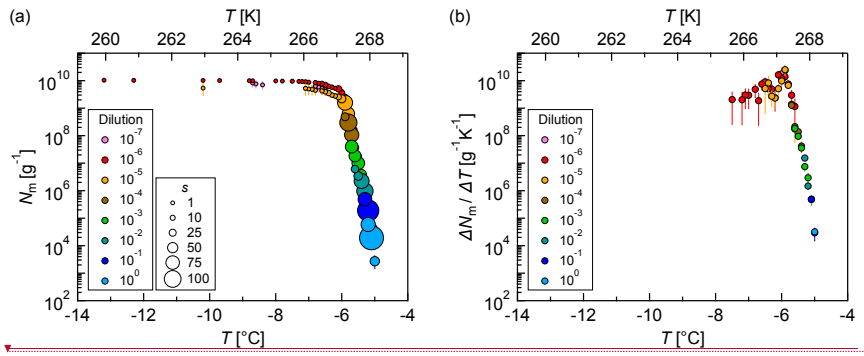


Figure 8. Measurements of dilution series of fungal IN (*Mortierella alpina* 13A). **(a)** Cumulative number of IN (N_m) and **(b)** differential number of IN ($\Delta N_m/\Delta T$) per unit mass of mycelium vs. temperature (T). Droplets of the same dilution of three independent measurements were added to a total droplet number of 288 (3 x 96 droplets). Symbol colors indicate different dilutions; symbol size indicates the number of frozen droplets per 0.1 K bin (s). The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

Gelöscht: Seitenumbruch

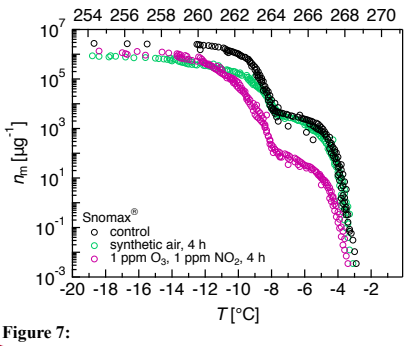


Figure 7:

[2] verschoben (Einfügung)

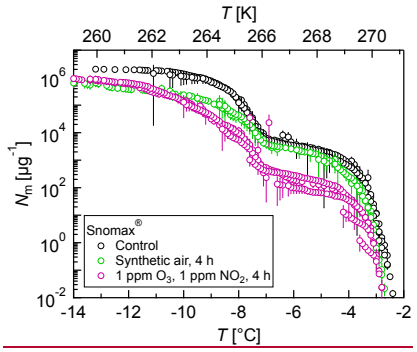


Figure 9. Freezing experiments with ozonized and nitrated bacterial IN. Cumulative number of IN (N_m) per unit mass of Snomax® vs. temperature (T). Droplets of the same dilution of three independent measurements were added to a total droplet number of 288 (3 x 96 droplets). Symbol colors indicate different exposure conditions. The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

Gelöscht: n_m

Gelöscht: temperature T.

Gelöscht: Seitenumbruch

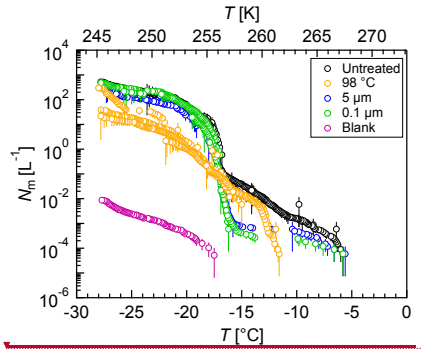
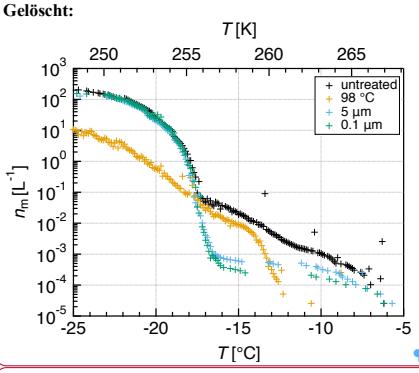


Figure 10. Freezing experiments with aqueous extracts of atmospheric aerosols. Cumulative number of IN per liter air (N_m) vs. temperature (T) for untreated (black), heated (orange), 5 µm filtered (blue), 0.1 µm filtered (green), and blank (purple) filter extracts. Droplets of the same dilution of two aliquots were added to a total droplet number of 192 (2 x 96 droplets). The error bars were calculated using the counting error and the Gelöscht: Data of different dilutions were averaged for each treatment Gaussian error propagation. The temperature error is 0.2 K.

Gelöscht:



[2] nach oben verschoben: Figure 8.

Gelöscht: Number

Gelöscht: and