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Atmospheric Measurement Techniques Discussions

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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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 gradient 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 (deviations <0.5 K), enabling measurements over a wide range of temperatures (~233-270 K) at variable cooling rates (up to 10 K min^{-1} and more).

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® samples to O_3 and NO_2, the concentration of IN active around 269 K (-4 °C, "class A") decreased by 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. Furthermore, TINA was used to study aqueous extracts of atmospheric aerosols, simultaneously investigating a multitude of samples that were treated in different ways to distinguish different kinds of IN. For example, heat treatment and filtration experiments indicated that highly efficient biological IN were smaller than 0.1 µm, and many IN active between 263 K (-10 °C) and 256 K (-14 °C) were heat-resistant and 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.

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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, 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.

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 infrared (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), 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 gradient and freezing of each droplet is tracked by an infrared detector. In the fully automated setup, a couple of independently cooled

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aluminum blocks carrying two 96-well and two 384-well plastic plates, respectively, are available to study ice nucleation and freezing events in nearly 1000 microliter range droplets simultaneously. 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). 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. 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.

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). 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 trough is at the bottom (Fig. 1c) and the cap is at the top (Fig. 1b), 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 due to the usage 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 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 NTC thermistor (10K3MRBD1, resistance: 10000 Ω/298 K, interchangeability: ±0.2 K, TE Connectivity Company, Galway, Ireland) in the elevation. 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, resolution: 0.01 K, maximum system error: ± 0.08 K at 223 K and ± 0.07 K at 273 K, Fluke Deutschland GmbH, Glottertal, Germany). 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 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

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temperature measured by the reference thermometer were monitored. For the conversion of the measured resistance of the thermistors into temperature, cubic spline interpolation was used.

2.3 Infrared video thermography

Droplet freezing is determined by a distinct detection system, where the temperature gradient 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. This 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. 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 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 gradient 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 concentration of IN (n_m) active at a certain temperature (T) per unit mass of material is given by Eq. (1) (Vali, 1971a).

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

where f_{ice} is the fraction of frozen droplets at a particular temperature, V_{drop} the droplet volume, m the mass of the particles in the initial suspension, and d the dilution factor of the droplets relative to m. To simplify data 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 96-well plates (Axon Labortechnik, Kaiserslautern, Germany) and 384-well plates (Eppendorf), respectively, 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).

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 have reported average freezing temperatures for microliter volume droplets of pure water at significantly warmer subfreezing temperatures due to 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 show that most pure water droplets froze around 247 K (-26 °C) in 96-well plates (Fig. 5a) and around 244 K (-29 °C) in 384-well plates (Fig. 5b), respectively. This

discrepancy can have different explanations. First, the 96-well plates are obtained from a different manufacturer than the 384-well plates. Second, the different well shape leads to an altered shape of the droplet, which could influence its freezing abilities at very low temperatures. 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® 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 with the results of Budke and Koop (2015). Below 259 K (-14 °C), a flat plateau arises where no IN are active.

The analysis of different IN active within a wide temperature range was only possible due to 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.

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Mortierella alpina 13A was grown on full-strength PDA (VWR International GmbH, Darmstadt, Germany) at 269 K 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. S2). The cumulative number of IN per gram mycelium only varies 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 (Fig. 6b). The cumulative number of IN and the initial freezing temperature of 267.7 K (-5.5 °C) are in good agreement with the literature (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015).

3.3 Ozonized and nitrated samples

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

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.

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

Filters were cut with a sterilized scissor into aliquots (~1/16), and the exact percentage was determined gravimetrically. For reproducibility, aerosol and blank 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. S3). For better clearness, data of different dilutions were averaged for each treatment (Fig. 8).

The untreated filter extract showed IN activity at relatively warm subfreezing 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 10^{-3} L^{-1}, but heat treatment led to a loss of IN activity above 263 K (-10 °C), which indicates the presence of biological IN. The concentration of IN between 263 K (-10 °C) and 255 K (-18 °C) increased continuously about two orders of magnitude and in a sudden increase another two orders between 255 K (-18 °C) and 254 K (-19 °C). A maximum IN concentration of 10^2 L^{-1} was reached around 250 K (-23 °C), but heat treatment reduced the maximum IN concentration to 10^1 L^{-1}at 250 K (-23 °C). Filtration experiments did not affect the initial freezing temperature, but the concentration of biological IN was decreased about half an order of magnitude, at which the 0.1 µm filtration showed a slightly bigger effect. The concentration of IN active between 263 K (-10 °C) and 256 K (-17 °C) decreased about two orders of magnitude upon 5 µm filtration, and 0.1 µm filtration reduced the IN concentration slightly more. The maximum IN concentration of 10^2 L^{-}

[1] around 250 K (-23 °C) was not affected 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 findings show that many IN active between 263 K (-10 °C) and 256 K (-17 °C) were larger than 5 µm, whereas IN active between 256 K (-17 °C) and 250 K (-23 °C) were smaller than 0.1 µm. 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.

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 mycelium, were in accordance 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, we found highly efficient biological IN smaller than 0.1 µm and heat-resistant IN 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.

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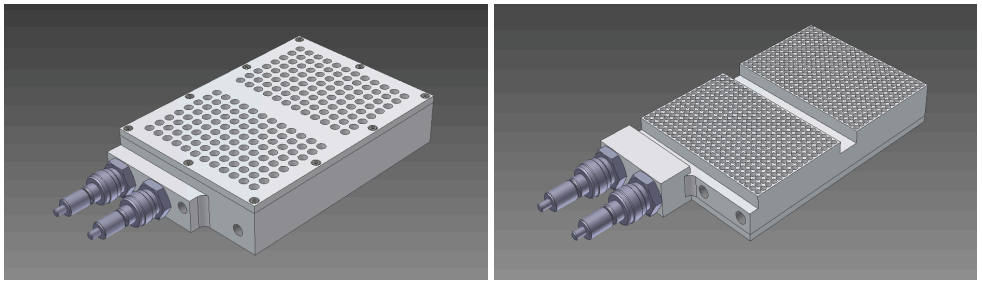
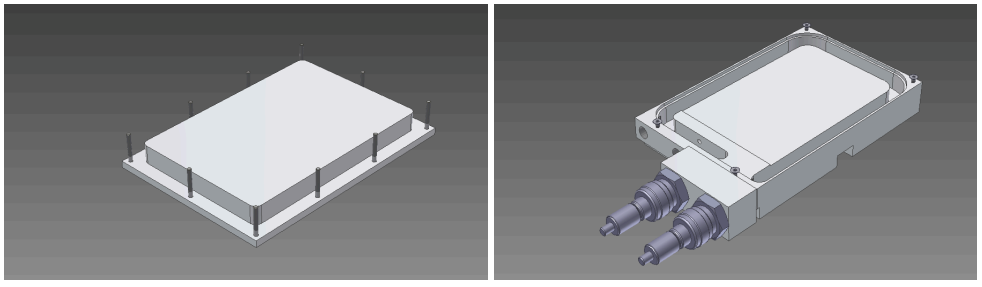
(a) 96-well sample holder block (d) 384-well sample holder block



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



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

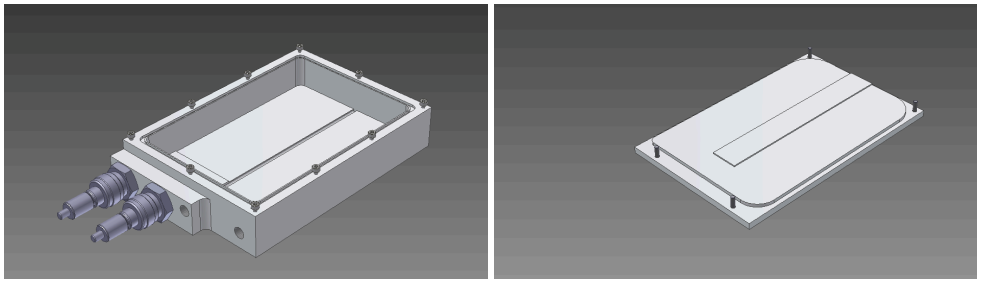


Figure 1. Sample holder and cooling blocks of the Twin-plate Ice Nucleation Assay (TINA) with **(a-c)** 96-well plates and **(d-f)** 384-well plates (CAD drawings).

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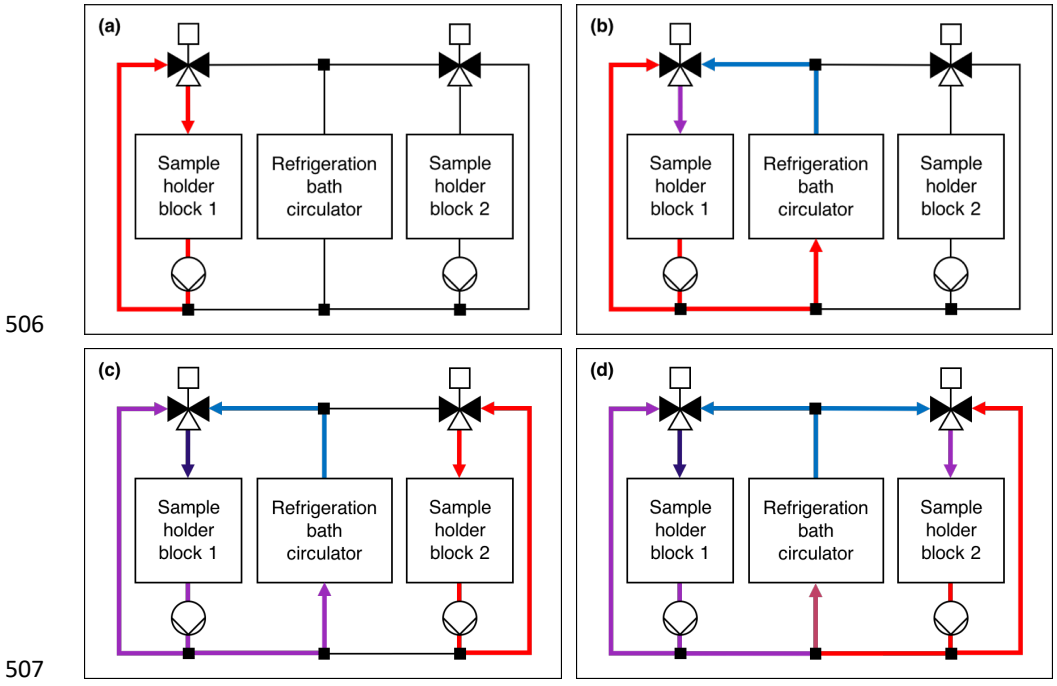

Figure 2. Cooling system layout and operating principle of the Twin-plate Ice Nucleation Assay (TINA). **(a)** Cooling liquid is pumped in warm cooling loop of sample holder block 1 without connection to colder cooling liquid provided by refrigeration bath circulator. **(b)** Mixing valve is opened for 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.

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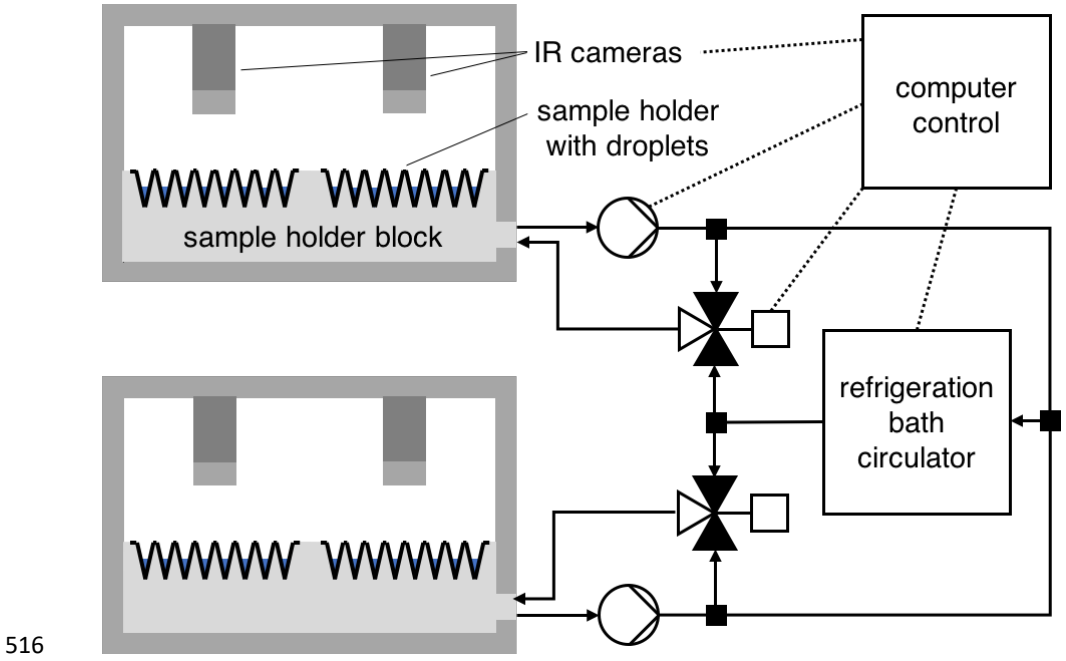

Figure 3. Schematic illustration of the overall setup: sample holder blocks, sample holders with droplets, IR cameras, cooling system with refrigeration bath circulator, pumps and mixing valves, computer control.

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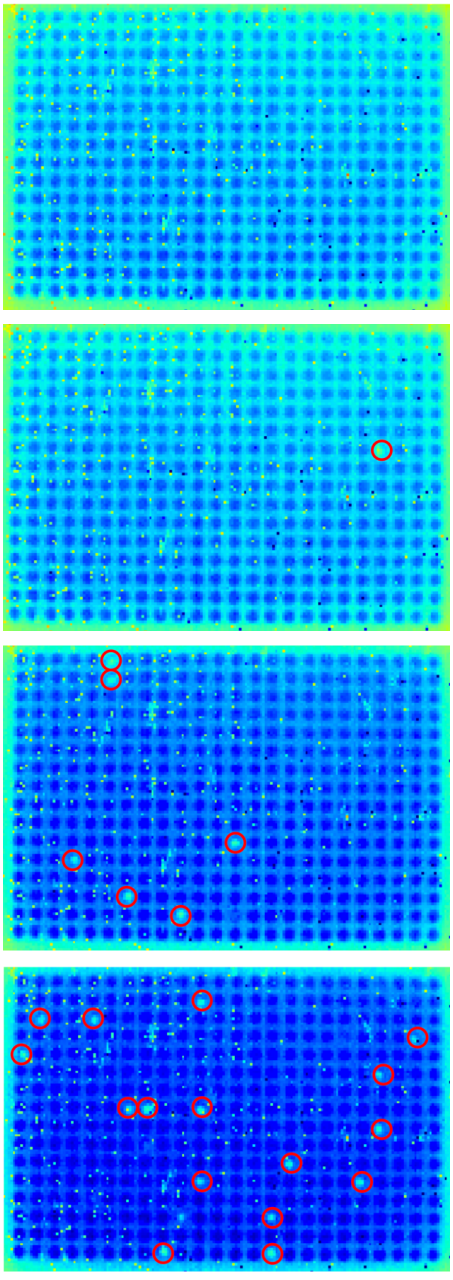

Figure 4. Sequence of infrared camera images showing 384 droplets during cooling. Red circles indicate freezing droplets.

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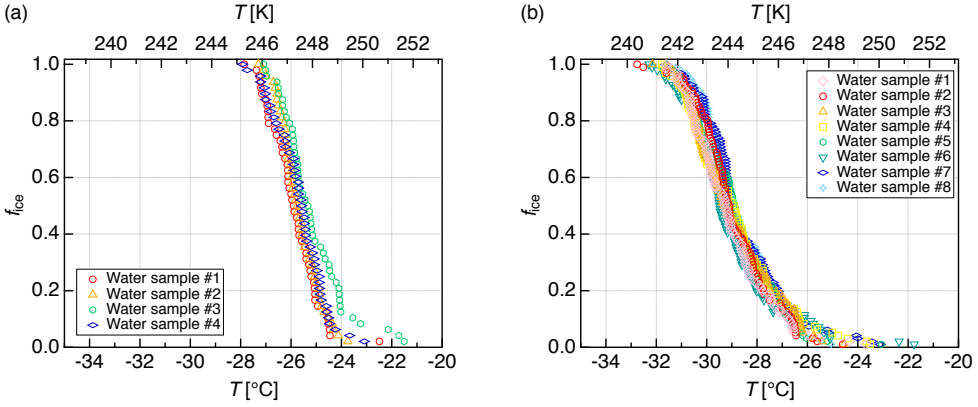

526

527 **Figure 5.** Freezing experiments with pure water droplets. Fraction of frozen droplets (f_{ice}) vs.
528 temperature T obtained with a continuous cooling rate of 1 K min^{-1} and a droplet volume of 3
529 µL. **(a)** Four different samples with 48 droplets each apportioned over two 96-well plates. **(b)**
530 Eight different samples with 96 droplets each apportioned over two 384-well plates.

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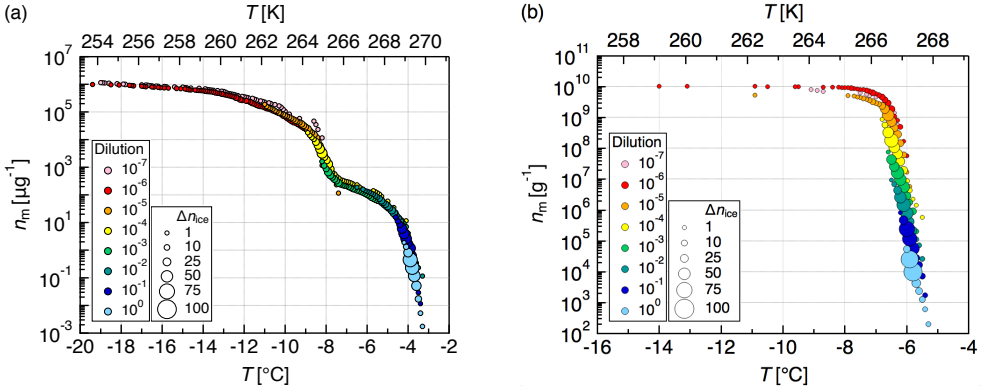

Figure 6. Measurements of dilution series of **(a)** bacterial IN (Snomax®) and **(b)** fungal IN (*Mortierella alpina* 13A). Cumulative number of IN (n_m) per unit mass of Snomax® and mycelium, respectively, vs. temperature T. Droplets of the same dilution of three independent measurements were added 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 temperature interval ($\Delta T = 0.1$ K).

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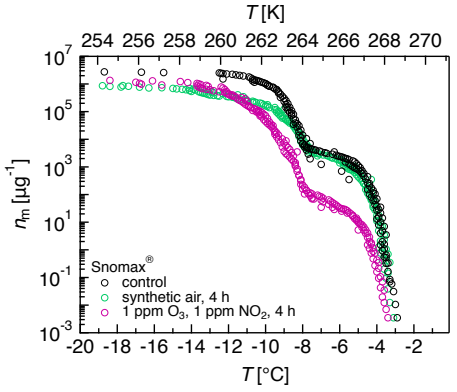

Figure 7: Freezing experiments with ozonized and nitrated bacterial IN. Cumulative number of IN (n_m) per unit mass of Snomax® vs. temperature T. Symbol colors indicate different exposure conditions.

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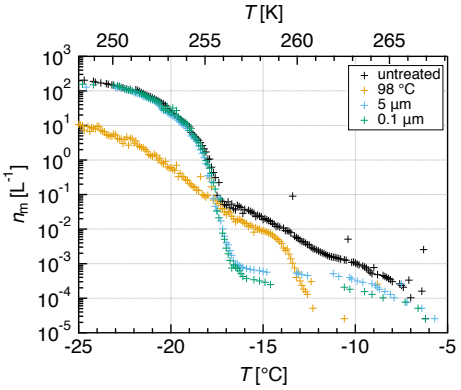

543

544 **Figure 8.** Freezing experiments with aqueous extracts of atmospheric aerosols. Number of IN
545 per liter air vs. temperature *T* for untreated (black), heated (orange), 5 µm filtered (blue), and
546 0.1 µm filtered (green) filter extracts. Data of different dilutions were averaged for each
547 treatment.