AN EXPERIMENTAL TECHNIQUE FOR ACCURATE MEASUREMENT OF THE FREEZING POINT OF SOLUTIONS AND ICE MELTING IN THE PRESENCE OF BIOLOGICAL OBJECTS ON THE EXAMPLES OF P. SYRINGAE AND E. COLI

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Abstract. *Pseudomonas syringae* is a widely distributed bacterial epiphyte of plants. When the temperature of the environment drops below zero, *P. syringae* can serve as biological ice nuclei due to the presence of specialized ice-nucleating proteins. This property has found application in various fields, but despite its evident importance, the molecular mechanisms behind protein-induced freezing have remained largely elusive. One of the problems in the study of the ice-nucleating process is the difficulty of carrying out experiments with freezing/melting solutions at near-zero temperatures. The experimental design implies special requirements for the equipment used and measurement technique. In this work, we used an experimental setup assembled from a dry thermostat that maintains a constant temperature and an accurate digital thermometer. We experimentally tested the possible sources of errors of the setup. As a result, we have shown that the accuracy of determining the freezing temperature of liquids and the coexistence of ice and water is mainly determined by the accuracy of the thermometer. The accuracy of determining the melting point of ice depends on the volume of the sample and is systematically underestimated in our setup. Using the proposed experimental technique, we performed a comparative study of *P. syringae* and *E. coli* cells, which revealed that *P. syringae* cells affect not only the freezing point of the solution but also the temperature of the coexistence of ice and water. The observed effect can be explained by the binding of *P. syringae* cells to the ice surface.

Keywords: freezing point of the solution, the temperature of coexistence of ice and water, melting point of ice, pseudomonas syringae.

Introduction

Pseudomonas syringae is a widely distributed bacterial epiphyte of plants. It was discovered (Maki *et al.*, 1974) that *P. syringae* can reduce supercoiling of liquid and serve as biological ice nuclei at fairly high temperatures (about –2 °C). It is assumed that the ability to initiate ice crystallization results from the presence of specialized ice-nucleating proteins and is one of factors of pathogenicity of this bacterium. This property has found application in different fields (Wolber & Warren, 1989; Gurian-Sherman & Lindow, 1993) but despite their evident importance, the molecular mechanisms behind protein-induced freezing have remained largely elusive.

One of the reasons why the influence of cells and proteins on the freezing of water is poorly understood is technical difficulties in performing experimental study. It is difficult to work with freezing and melting liquids at near-zero temperatures. Commonly used devices with glass cells are not suitable for such experiments (the measuring cell cracks when the ice freezes), optical measurements are difficult due to the opacity of the ice, etc. So, the experimental design implies special requirements for the equipment used and measurement technique.

Two techniques are commonly used to study the freezing of solutions containing various biological objects (proteins or cells). The first one is the study of the freezing of small droplets of liquid. This approach predominantly applied in works analyzing the effect of *P. syringae* cells on the process of ice formation (Cochet & Widehem, 2000; Maki *et al.*, 1974; Kozloff *et al.*, 1983; Goodnow *et al.*, 1990; de Araujo *et al.*, 2019; Weng *et al.*, 2016; Pietsch *et al.*,

2017). Small droplets on the metal surface or in the oil layer is cooled and the freezing is visually monitored. The drops are, mostly, maintained at a certain temperature and the number of frozen drops is counted.

The second technique is based on the determination of the temperature of the coexistence of ice and water using a cryo-osmometer. This method has become a classic in the study of ice binding proteins (Graether *et al.*, 2000; Leinala *et al.*, 2002; Glukhova *et al.*, 2020).

The first technique is very time-consuming and not very accurate being based on statistics of frozen droplets counting, and allows to determine the freezing point of the solution. The second method allows to measure with a very high accuracy a single parameter - the temperature of the coexistence of ice and water. We must highlight the existing confusion in terminology. In different works, the temperature of the coexistence of ice and water is called differently; for example, it can be called the freezing point of water or the melting point of ice or thermal hysteresis (in the study of the solutions of ice-binding proteins). To be precise, the term thermal hysteresis refers to the difference between the temperature of coexistence of ice and water in the presence and absence of protein, but considering that for pure water this temperature is zero it turns out that thermal hysteresis and the temperature of coexistence of ice and water are the same thing. In our work, we will adhere to the following terminology: we will call the freezing point the temperature at which ice arises and water crystallization begins; the temperature of coexistence of ice and water is the temperature of a mixture of fine ice and water; the melting point of ice is the melting point of a completely frozen piece of ice. Below we will show that from an experimental point of view these are completely different tempera-tures and they are measured with different accuracy.

The aim of our work was to assemble and test an experimental setup in which it is possible to continuously monitor the process of freezing water and melting ice and determine parameters of this process with sufficient accuracy. In addition, one of the objectives of this work is to study the effect of the *P. syringae* on the freezing point of water and the temperature of coexistence of ice and water. These two temperatures are related to the peculiarities of the functioning of ice-nucleating proteins on the surface of the *P. syringae* cells.

We used a simple experimental setup that can be assembled in the laboratory if there are an accurate digital thermometer and a thermostat with minor modifications. The experimental setup we have assembled allowed us to obtain three parameters: the temperature of the beginning of ice crystallization (freezing) T_f, the temperature of coexistence of ice and water T_{iw} and the melting temperature of ice T_m. We tested the experimental setup and checked how the amount of liquid in the test tube and the position of the thermometer probe affect the accuracy of the data obtained. Also, the accuracy of the measurements was tested on salt solutions that should affect the temperature of T_{iw} and the temperature of T_m. Using the setup, we conducted a study of solutions containing *P. syringae* cells which have the ability to «trigger» the formation of ice at near-zero temperatures (Maki *et al.*, 1974; Wolber & Warren, 1989; Gurian-Sherman & Lindow, 1993).

Materials and Methods

Cell culture experiments

E. coli BL21 (DE3) cells were grown on LB growth medium (VWR Life Science AMRESCO) at a temperature of 37 °C. *P. syringae* cells (*Pseudomonas syringae pv. syringae*) were grown on medium L (yeast extract 5.0 g/l; peptone 15.0 g/l; NaCl 5.0 g/l) at a temperature of 26 °C. All the cells were grown in a liquid medium up to cell density of OD600 = 1.0OU, then precipitated on a centrifuge at 6000 g, and washed twice with a solution of 20 mM Tris-HCl, pH 7.5. The initial cell solution was diluted with a buffer solution of the same composition to the desired concentration. The concentration of cells was controlled by absorption at 600 nm.

Experimental setup for measuring the temperature characteristics of liquid freezing and ice melting

Fig. 1A shows an experimental setup assembled using solid-state thermostat Biosan

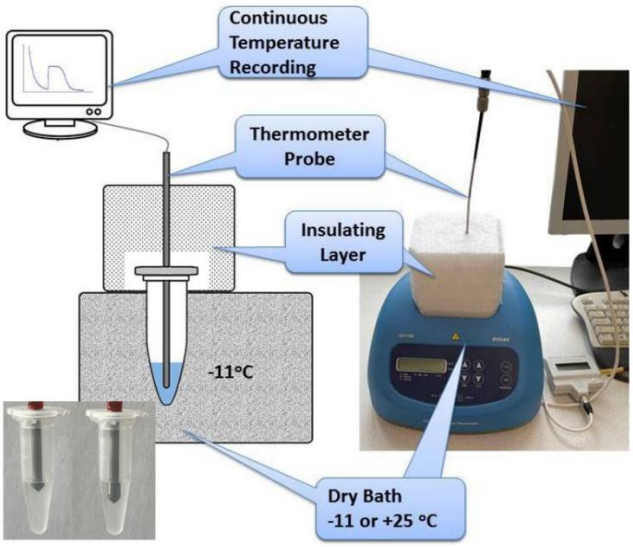

Fig. 1. Diagram and photo of an experimental setup for measuring the features of freezing solutions and melting ice. At the bottom left, photos of test tubes with 0.5 ml of solution and a thermometer probe immersed at different levels are shown

CH-100 (Latvia) and digital thermometer LT-300 (Russia). On the upper metal part of the thermostat in which the sample is cooled/heated, a protective shell (insulating layer) in the form of a cube of insulating material is put on. We used foamed polyethylene (Warmlex). This part serves as a thermal insulator for the sample and at the same time as a holder for the metal probe of the thermometer. The design is quite simple; however, careful manufacturing of the thermal insulator is required, ensuring the thermometer probe always fixed in the same position – vertically in the middle of the tube. The only thing that can be changed in this design is the depth of immersion of the probe into the liquid. Fig. 1 (at the bottom left in the picture) shows photos of a test tubes in which the thermometer probe is immersed in liquid to different depths.

The design and protocol of the experiment

All the experiments were performed following the same sequence of steps. The sample was poured into a test tube. A thermometer probe was inserted into the test tube (Fig. 1). The test tube was lowered into a metal cell of the thermostat, the temperature of which was –11 °C. A recording of temperature measurement in a test tube was started. After the sample was completely frozen, it was moved to the second same thermostat but with a set temperature of +25 °C. As a result, we recorded the time-dependence of the sample temperature. The example of a typical recorded curve is presented in Fig. 2. The different stages are indicated in the curve: the sample (water) first cools, then exists in a supercooled state for some time, then an ice embryo appears and the sample temperature increases rapidly since heat is released during the crystallization of water. The mixture of water and ice coexists for a while until all the water freezes, then the ice is cooled to the temperature of the thermostat. After that, we moved the test tube together with the thermometer probe into the second thermostat heated to +25 °C. The ice is heated and then melted.

Determining the temperatures T_f, $T_{i\text{-}w}$ and T_m

From the plot of the time-dependence of the sample temperature (an example is shown in Fig. 2) we determined three temperatures T_f, $T_{i\text{-}w}$ and T_m. The freezing point T_f was determined as the minimum value of the temperature at the time of the beginning of water crystallization in the sample. The temperature of coexistence of ice and water T_{iw} was defined as the

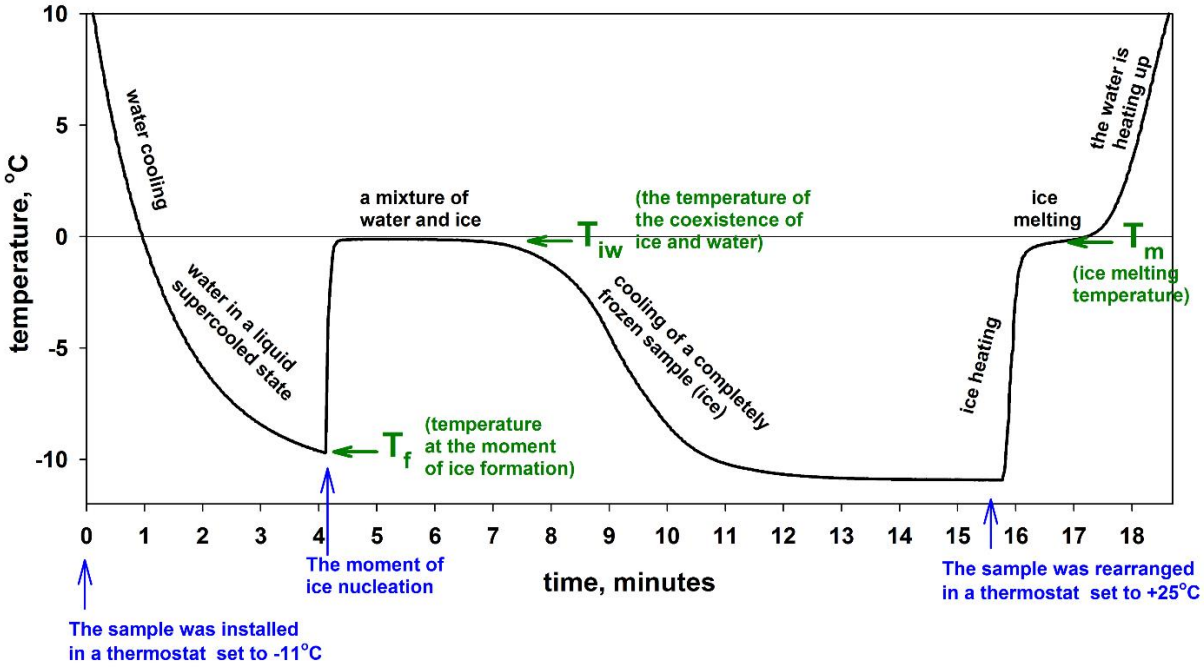

Fig. 2. The dependence of the sample temperature on time during its cooling and subsequent heating

temperature of the horizontal section of the curve after the moment of freezing of water. For example, in Fig. 2, such a section of the curve is between the 4th and 7th minute.

To determine the melting temperature of ice T_m, the first derivative of the temperature dependence curve was plotted. In Fig. 3, the blue solid line shows the section of the temperature dependence curve where ice melting occurs. The black dotted line shows the e first derivative of this section of the curve. The arrows in Fig. 3 show how the temperature T_m is determined. Obviously, with such a definition of Tm, this parameter will always differ from the actual melting temperature of the sample T_{mReal}. If the sample had a sufficiently large volume and the thermometer could correctly measure its average temperature, then the curve would asymptotically tend to the T_{mReal} temperature. In Fig. 3, the red dash line shows the possible shape of the curve with a large sample volume. Moreover, a very accurate determination of the sample temperature is not possible due to the device. The temperature measurement takes place in the middle of the sample (ice), and its melting begins on the walls of the tube (which are in contact with the thermostat). Therefore, the temperature readings are always underestimated (in the case of melting) compared to the average temperature of the sample. Nevertheless, the temperature determination method shown in Fig. 3 allows the T_m temperature to be determined in the same way for different samples and to compare the effect of different substances on the melting temperature of ice.

Results

Verification of the stability of measurements using the assembled setup

Fig. 4 shows the freezing and melting curves of distilled water samples (Fig. 4 A and B) and deionized water (miliQ purification system) (Fig. 4 C and B) recorded using the assembled experimental setup. From the figures it can be seen that the appearance of an ice nucleus is a random process and depends both on the temperature and on the dust particles or irregularities present in the water or on the walls of the test tube. Fig. 4 illustrates that the freezing point of the solution is very different for different samples. At the same time, the temperatures T_{iw} and T_m are measured very stably. The inserts demonstrate sections of curves on an enlarged scale. It can be seen that the plateau

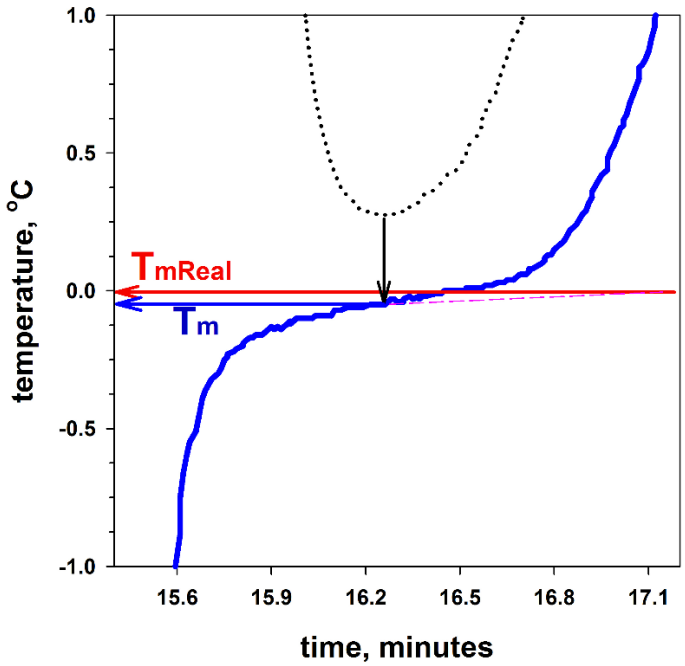

Fig. 3. Determination of the melting temperature of ice T_m. The blue solid line is an example of the dependence of the sample temperature on time when it is heated. The black dotted line is the graph of the first derivative of the blue curve. The minimum on the graph of the first derivative allows to determine the inflection point of the blue solid curve (shown by the black arrow), by the position of which T_m is determined (shown by the blue arrow). The red dash line shows a possible change in the experimental curve (blue) if the sample volume was large and the thermometer could accurately detect its average temperature. The red dash line asymptotically tends to the real melting temperature of the T_{mReal} ice. The red arrow shows the actual melting point of the ice

(from the position of which the temperature T_{iw} is calculated) is at the same level for different curves. In Fig. 4B and 4D, the curves are shifted along the X-axis for ease of comparison. The calculated values of T_f, T_{iw} and T_m are summarized in Table 1. From the data obtained, it can be concluded that none of the calculated parameters depends on the degree of water purification. It can be assumed that the number of inhomogeneities or surfaces that can serve as ice nuclei in supercooled water (Melnik *et al.*, 2021) weakly depends on the degree of water purification, so the T_f values do not differ. The temperature value T_{iw} is related to the amount of substance dissolved in water (Suzuki *et al.*, 1993; Sweeney & Beuchat, 1993), so it is obvious that the Tiw values should be close to zero. The T_m temperature value should also be close to the zero but may differ due to the technical pecularities of the experimental setup and the calculation method (see explanations above). It is also seen that the average deviation during measurements is comparable to the relative error of the thermometer, which is 0.01 degrees. The absolute error of the thermometer is 0.05 degrees.

The influence of different sample volume on the temperature determination error

Fig. 5A shows the time-dependence of the samples' temperature during cooling of deionized water of different volumes. The curves in Fig. 5A are shifted along the time axis to combine the moment of freezing (crystallization) of water. It can be seen that the freezing of solutions occurs at a temperature of about –10 °C with a small variation. The shape of the peak that occurs after freezing of the solution noticeably varies depending on the volume of the sample. At the same time, the plateau after the appearance of ice in the test tube is the same for all the samples. Temperature T_{iw} differs by no

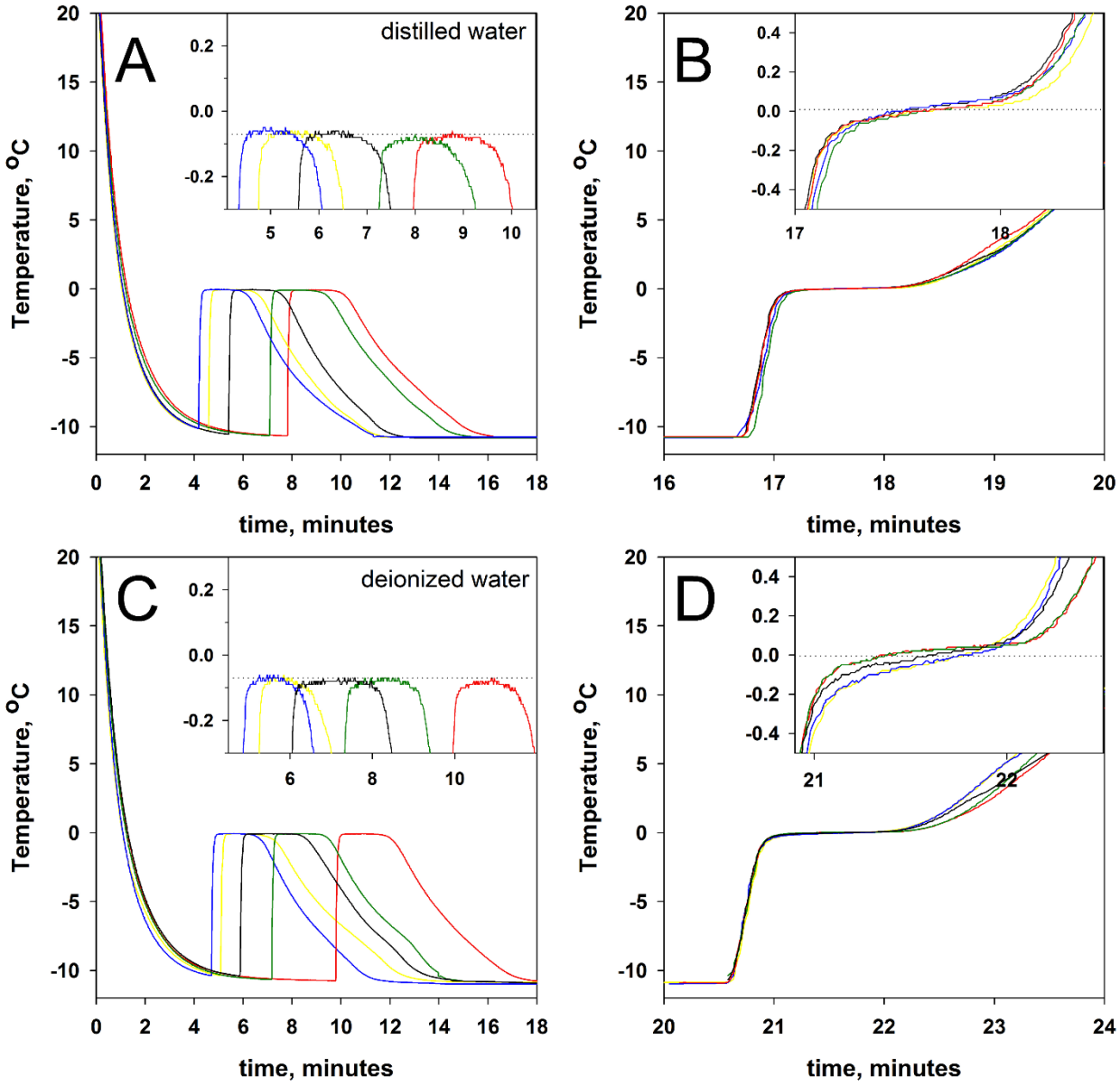

Fig. 4. Dependences of sample temperature on cooling time (A and C) and heating time (B and D) for distilled water (A and B) and for deionized water (C and D). The insert in each figure shows a part of the graph by which the temperatures T_{iw} (on panel A and C) and T_m (on panel B and D) are determined. The melting curves of the samples are shifted along the time axis for ease of comparison. The volume of samples is 0.5 ml

more than 0.025 °C. Fig. 5B shows an example of ice melting curves recorded for samples of different volumes. The sample with a volume of 250 µl differs from the rest by inflated temperature values. It can be concluded that the melting point of ice is determined incorrectly (overestimated) when measuring samples of small volume.

The influence of the position of the thermometer probe on the accuracy of temperature determination

Fig. 6A and 6B show temperature curves of samples (deionized water) with a volume of 0.5 ml and different positions of the thermometer probe. Fig. 1 shows photos of a test tube with a thermometer probe lowered to different depths

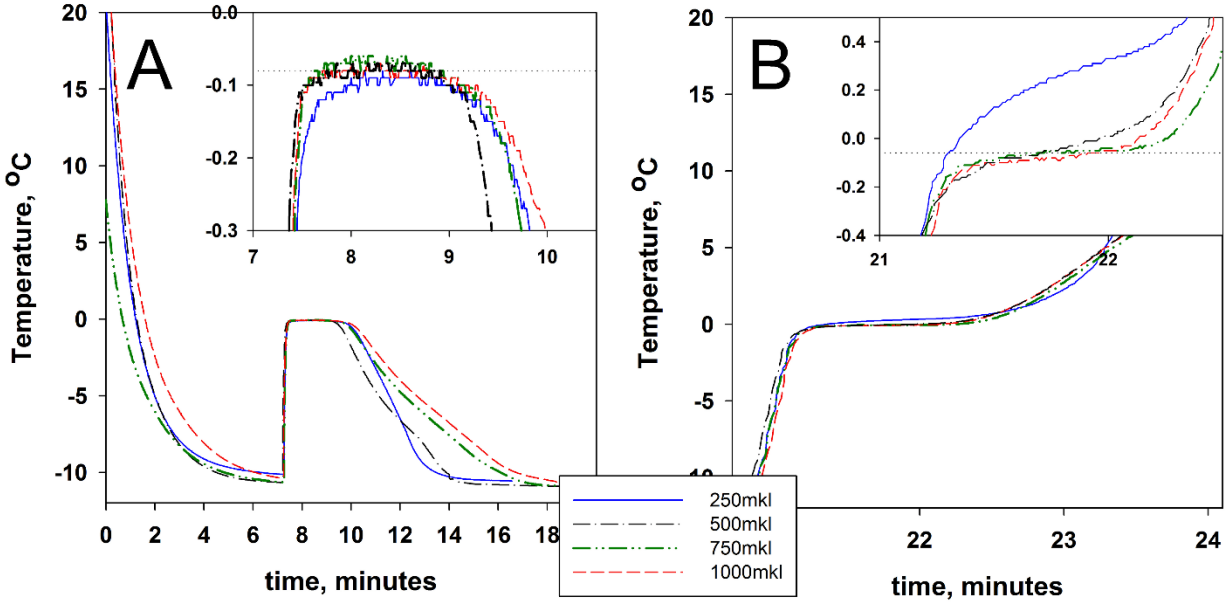

Fig. 5. Temperature dependences of deionized water samples of different volumes on cooling time (A) and heating time (B). Average temperature value: T_f = –10.4 ± 0.02 °C, T_{iw} = –0.08 ± 0.01 °C. For samples with a volume of 500 to 100 mkl T_m = –0.06 ± 0.02 °C, for a sample with a volume of 250 mkl T_m = –0.29 °C

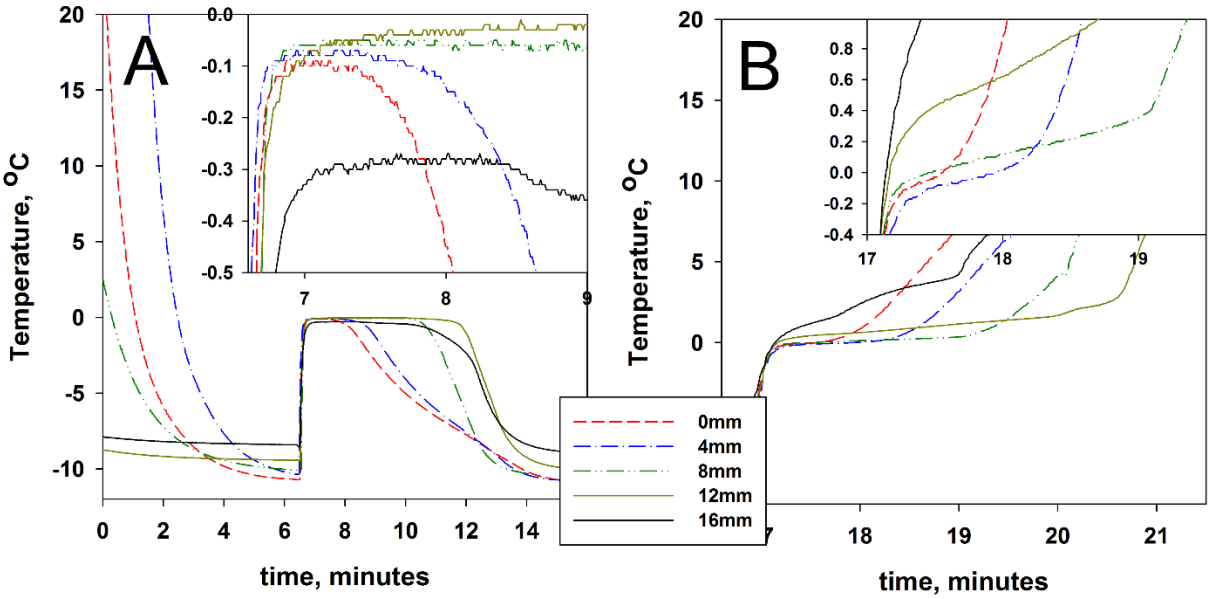

Fig. 6. Temperature dependences of samples (deionized water) on cooling time (A) and heating time (B) at different positions of the thermometer probe. The positions changed from 0 to 16 mm in increments of 4 mm

(16 mm and 12 mm). The position of the thermometer varied from the maximum lowered (0 mm from the bottom of the tube) to the position at which the thermometer slightly touches the surface of the water (16 mm from the bottom of the tube). It can be noticed that at the 0 mm position, the curves in Fig. 6A and 6B have a short section by which T_{iw} and T_m are determined. For curves recorded at the positions of the thermometer probe at 12 mm and 16 mm, the temperature values are overestimated when measuring ice melting (Fig. 6B).

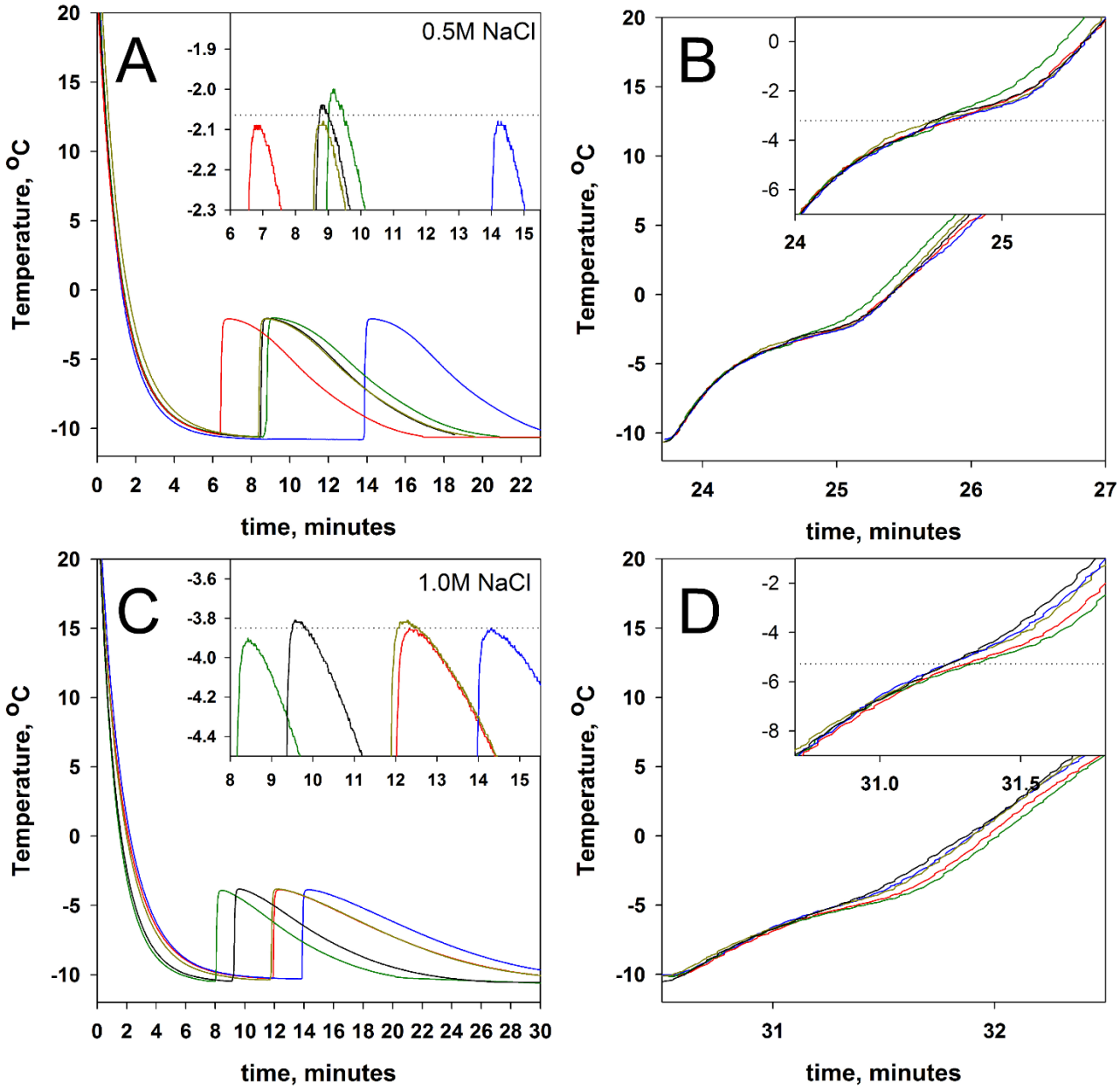

Fig. 7. Temperature dependences of NaCl solutions (0.5 ml) on cooling time (A, C) and heating time (B, D). On panels A and B curves for solutions of 0.5 M NaCl, on C and D – 1.0 M NaCl

Thus, the optimal position of the thermometer probe for accurate measurement is 4 to 8 mm from the bottom of the tube.

Verification of the accuracy of the T_{iw} determination using salt solutions

Fig. 7 shows the temperature dependence curves for NaCl solutions at concentrations of 0.5 M and 1.0 M. The temperature of ice and water coexistence T_{iw} is related to the amount of substances dissolved in water, namely, 1 mol of any dissolved substance lowers the temperature of ice and water coexistence by $\Delta = 1.86$ °C (Suzuki *et al.*, 1993; Sweeney & Beuchat, 1993). It should be noted that for salts that dissociate into two ions the value of Δ is doubled. The study of salt solutions allows determining the correctness of the thermometer readings. Fig. 7 shows such studies. From the curves in Fig. 7A, for a 0.5M NaCl solution, we deter-

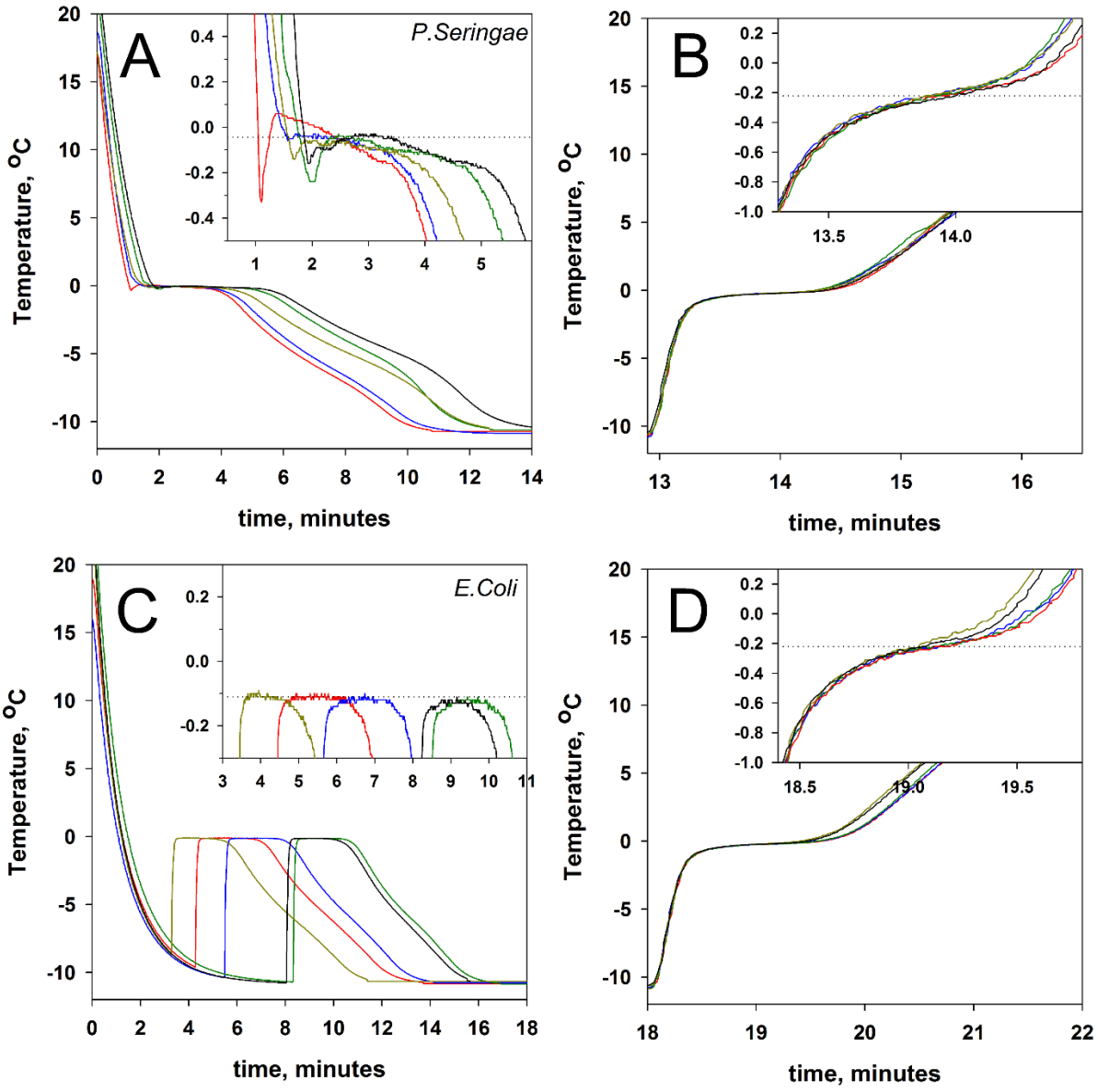

Fig. 8. Temperature dependences of *P. syringae* and *E. coli* solutions (0.5 ml) on cooling time (A, C) and heating time (B, D). On panels A and B curves for solutions of *P. syringae*, on C and D – *E. coli*

mined T_{iw} = –2.06 °C. Hence, given that for pure water T_{iw} = –0.08 °C (Table 1), a 0.5M NaCl solution shifts the value of T_{iw} by $\Delta 0.5_{experiment}$ = (2.06 – 0.08) = 1.98 °C. Similarly, from the curves in Fig. 7C, it can be determined that for 1.0M NaCl solution $\Delta 1.0_{experiment}$ = 3.77 °C. The values of Δ calculated theoretically from the molarity of solutions $\Delta 0.5_{theory}$ = 1.86 °C and $\Delta 1.0_{theory}$ = = 3.72 °C. Thus, it can be concluded that the absolute error in determining the T_{iw} tempera-ture using our experimental setup does not exceed 0.1 °C. The temperatures T_f, T_{iw} and T_m calculated from the curves are summarized in Table 2.

Investigation of solutions of P. syringae and E. coli cells

P. syringae cells were chosen as an example of cells that can reduce supercoiling of liquid (Maki *et al.*, 1974); *E. coli* cells do not affect the freezing process of water. Fig. 8 shows

Table 1

Average temperatures T_f, T_{iw} and T_m calculated from the freezing/melting curves

Sample 0.5 ml	T_f, °C	T_{iw}, °C	T_m, °C
Distilled water	-10.4 ± 0.2	-0.07 ± 0.01	0.01 ± 0.02
Deionized water	-10.5 ± 0.2	-0.08 ± 0.01	0.0 ± 0.03

Note: the error indicated in the table is the average of the absolute values of data deviations from the average value. The absolute error of the thermometer is ±0.05 °C

Table 2

Average temperature values T_f, T_{iw} and T_m calculated from the freezing/melting curves of solutions (0.5 ml) of NaCl salt with concentrations of 0.5M and 1.0M

Solutions 0.5 ml	T_f, °C	T_{iw}, °C	T_m, °C
0.5M NaCl	-10.6 ± 0.1	-2.06 ± 0.03	-3.2 ± 0.2
1.0M NaCl	-10.3 ± 0.1	-3.85 ± 0.1	-5.3 ± 0.1

Table 3

Average temperature values T_f, T_{iw} and T_m calculated from the freezing/melting curves of solutions (0.5 ml) of *P. syringae* and *E. coli*

Solution	T_f, °C	T_{iw}, °C	T_m, °C
P. syringae	-0.19 ± 0.08	-0.04 ± 0.04	-0.22 ± 0.01
E. coli	-10.01 ± 0.7	-0.12 ± 0.01	-0.22 ± 0.01

Note: the cell concentration is 0.1OU

temperature curves reflecting the process of freezing and melting of solutions of *P. syringae* and *E. coli* cells.

It can be seen that *P. syringae* cells affect the freezing temperature of T_f solutions. In Fig. 8A, the temperature spike associated with the appearance of ice in the solution occurs at near-zero temperatures. For comparison, Fig. 8C illustrates the freezing of *E. coli* cell solutions which occurs at low temperatures of about –10 °C. The freezing temperature of *E. coli* cell solutions does not differ much from T_f for pure water (Fig. 4). In addition, *P. syringae* cells affect the temperature of the coexistence of ice and water T_{iw} by increasing it. Comparing with a solution of *E. coli* cells, it can be seen that *P. syringae* increase T_{iw} by almost 0.1 °C (see Table 3). At first glance, it may seem to be a small difference. However, we must underline that usually dissolved substances reduce the temperature of T_{iw}, but do not increase it. In addition, it can be calculated that the increase in T_{iw} which occurs due to the presence of *P. syringae* cells is comparable, for example, to the effect of 20 mM NaCl. That is, the decrease in T_{iw} temperature caused by the presence of 20 mM NaCl in the solution can be compensated by the presence of *P. syringae cells*. Table 3 summarized the temperatures T_f, T_{iw} and T_m calculated from the graphs.

Discussion

As a result of the measurements, several conclusions can be made. The influence of various substances on the freezing point of ice (T_f), the temperature of coexistence of ice and water (T_{iw}), and the melting point of ice (T_m) can be studied on the experimental setup we have assembled. The most stable and accurate data is obtained when measuring T_{iw}. They virtually do

not depend on either the volume of the sample or the position of the thermometer probe. The temperature T_f depends on the occurrence of the ice nucleus and therefore can be measured with some variation of data. The T_m temperature is more difficult to measure task. This parameter clearly depends on the volume of the sample and the position of the thermometer probe. The error of T_m measurement is also related to the features of the experiment design. In our experimental setup, a small piece of ice in the test tube does not melt equally on the walls of the test tube, so the thermometer probe cannot measure the average temperature of the sample.

The performed studies demonstrate that using our experimental setup we have made it is possible to measure the freezing temperatures of solutions T_f, the temperature of the coexistence of ice and water T_{iw}, and the melting of ice T_m with high accuracy. For T_f and T_w, the error does not exceed 0.05 °C. Measurement of T_m temperature is more difficult, however, in comparative experiments, the result is reliable and the error does not exceed tenths of a degree.

A comparative study of *P. syringae* and *E. coli* cells made it possible to determine that *P. syringae* cells affect not only the freezing temperature T_f of the solution but also the temperature of the coexistence of ice and water T_{iw}. This interesting fact has been shown for the first time. The increase in the temperature of the coexistence of ice and water in the presence of *P. syringae* cells can be explained if we assume that *P. syringae* cells bind to the ice surface. In a test tube, after the ice nucleus has appeared, a mixture of ice and water is formed. The cells stabilize ice surface by binding to it. Thus, the thermodynamic equilibrium in the ice-water system is shifted. At the same time, it is surprising that we did not find the influence of *P. syringae* on the melting temperature of ice. Perhaps this is due to the fact that the T_m temperature is measured with less accuracy using the assembled experimental setup.

Acknowledgements

We are grateful to Alexei V. Finkelstein for fruitful discussions and Irina V. Balalaeva for assistance.

This research was funded by Russian Science Foundation, grant number 21-14-00268.

Authors declare no conflicts of interest.

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