Feasibility of an In-Situ Microbial Decontamination of an Ice-Melting Probe

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Abstract

Autonomous robotic systems for penetrating thick ice shells with simultaneous collecting of scientific data are very promising devices in both terrestrial (glacier, climate research) and extra-terrestrial applications. Technical challenges in development of such systems are numerous and include 3D-navigation, an appropriate energy source, motion control, etc. Not less important is the problem of forward contamination of the pristine glacial environments with microorganisms and biomolecules from the surface of the probe.

This study was devoted to establishing a laboratory model for microbial contamination of a newly-constructed ice-melting probe called IceMole and to analyse the viability and amount of the contaminating microorganisms as a function of distance. The used bacterial strains were *Bacillus subtilis* (ATCC 6051) and *Escherichia coli* (ATCC 11775). The main objective was development of an efficient and reliable in-situ decontamination method of the melting probe. Therefore, several chemical substances were tested in respect of their efficacy to eliminate bacteria on the surface of the melting probe at low temperature (0 - 5°C) and at continuous dilution by melted water.

Our study has shown that at least 99.9% decontamination of the IceMole can be successfully achieved by the injection of 30% (v/v) hydrogen peroxide and 3% (v/v) sodium hypochlorite into the drilling site. We were able to reproduce this result in both time-dependent and depth-dependent experiments. The sufficient amount of 30% (v/v) H_2O_2 or 3% (v/v) NaClO has been found to be approximately 18 µL per cm² of the probe's surface.

Introduction

Recently, there are two main branches of special importance in respect of glacial/subglacial research. One of them is related to terrestrial applications (e.g. examining sub-glacial lakes and climatic studies) and the other is related to exploration of the solar system. For example, a question of great interest is origin and fate of the Lake Vostok, stayed buried under thick ice shield in Antarctic for more than 500 000 years [1;2]. In recent years, psychrophilic microflora from Vostok and other sub-glacial lakes in Antarctica have been the focus of increasing attention from both basic science and biotechnology perspectives, but the main obstacle for such lake's exploration is the danger of their contamination during sampling process [3-8].

The extraterrestrial direction of glacial research is based on the multiple evidences that many planets and moons of our solar system (e.g. Europa, Titan, Enceladus and etc.) contain great amounts of ice. This huge amount of frozen water raises the probability of finding extraterrestrial life. The Committee on Space Research (COSPAR) pays special attention to planetary protection policy for the reference of space-faring nations, both as an international standard on procedures to avoid organic-constituent and biological contamination in space exploration and to provide accepted guidelines in this area [9;10;16].

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Thus, for both terrestrial and extraterrestrial applications, an efficient and reliable biological decontamination protocol of an exploration probe is necessary to prevent biological contamination of the pristine environment. Usually, sampling equipment is sterilised before its transportation to the application site [11]. Unfortunately, this approach does not prevent contamination appearing shortly after the mission begins, for instance, if the ice-melting probe has to penetrate contaminated upper ice layers on its way to the subglacial locations of interest. In this situation, a thorough and reliable in-situ decontamination method is necessary.

In our opinion, the most suitable in-situ decontamination procedure would be local injection of some disinfecting chemical agent from the probe and bringing the total probe surface into contact with this substance. The main requirements for a substance appropriate for efficient in-situ chemical decontamination in glacial environments are: high killing rate and activity against broad range of microorganisms (spores, fungi, viruses, small insects and etc.), fast decomposing, environmentally friendly end products, easy handling, good solubility in water at any temperatures, good penetration ability, activity in cold environments, easy storage (not using too much space) and easy application.

In this study, we proposed the use of hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO, bleach). They are among the most studied chemicals regarding the removal of contaminants from drilled ice cores and have high success rate of eliminating of various contaminants [12-14].

The objectives of this study were a) to develop an efficient and reliable in-situ decontamination method of newly-constructed ice-melting probe, "Ice Mole", b) to establish a laboratory model for microbial contamination of the melting probe, and c) to analyze the viability, amount and distribution of the contaminating microorganisms as a function of time and distance.

Materials & Methods

Microorganisms and culture conditions

The used bacterial strains for our study were *Bacillus subtilis* (ATCC 6051) and *Escherichia coli* (ATCC 11775). Bacteria were cultured in 10mL of tryptone soy broth (International Diagnostics Group plc, Lab M, Germany) in sterile polypropylene centrifuge 50 mL-tubes. Temperature of incubation was 30°C for *Bacillus subtilis* and 37°C for *Escherichia coli*. The bacterial working suspension obtained after 24 hours incubation had in average 10^9 – 10^{10} colony forming units (CFUs) per mL. For CFU counting, the bacteria were cultured in Petri dishes containing tryptone soy agar (Carl Roth Co., Germany) for *B. subtilis* and MacConkey agar (Carl Roth Co, Germany) for *E. coli.*

Melting device

The melting probe, called IceMole (Fig. 1-A), has been developed at the Aerospace Technology Department at Aachen University of Applied Sciences, Germany. The probe possesses a melting copper head equipped with four resistive heaters with the net power of around 8 kW in order to melt through thick ice sheets. To ensure the advancement by mechanical drilling of the probe in the dirty ice, a screw is attached, driven by a special motor. As each heater can be independently turned on and off, the probe functions like a remote-controlled toy car, which can manoeuvre in different directions through the ice. The empty space in the rear part of the probe can be used to place measuring devices such as pH – meter, barometer, microscope, video-camera, etc.

For this study, to analyse the contamination/decontamination processes, we used only the screw itself (Fig. 1-B), further referred to in this study as "melting probe", which is situated at the leading side of the instrument. The dimensions of this stainless steel screw are: 21.5 cm long, 2.1 cm outer diameter and 1.5 cm inner diameter.

Ice & drill preparation

Sterile ice-cubes (length of 25 cm, width of 25 cm and height of 30 cm) were made of autoclaved distilled water poured into sterile cubic containers and incubated at -20°C overnight.

The melting probe was contaminated by submerging it into 10 mL of previously prepared bacterial suspension (10^8 – 10^9 CFUs per cm^3) of either *Bacillus subtilis* or *Escherichia coli.* The submerged drill was then covered with sterile aluminium foil and incubated at strain-specific optimal temperature for 24 hours to allow bacteria attaching to the surface.

Fig. 1. (A) The melting probe IceMole has been developed at Aachen University of Applied Sciences, Aerospace Technology Department. The probe consists of the copper melting head (1), containing four heaters, which it can melt through the thick ice sheet. To ensure the advancement of the probe in the dirty ice, an ice screw (2) driven by a small motor (3) is attached, which can facilitate drilling through layers of non-melting material. The measurement equipment can be loaded into the rear chamber of the device (4) made of aluminium. **(B)** The stainless steel screw used in this microbiological study is shown together with a 20 cm-long scale.

Drilling process

The contaminated melting probe was unwrapped inside the clean bench and gently rinsed with sterile distilled water for 2 minmin. The washed melting probe was placed on top of the sterile ice-cube (side-covered with a 2 cm thick Styrofoam after taken out of the freezer). When the ice began to melt under the drill's tip (~2 min), the "0 centimetre" point sample was taken.

In this study the drilling was carried out using solely mechanical force of hands, with applying downwards force of up to ~300 N and with the drill's rotation speed of up to ~15 rpm. To increase the applied torque, a stainless steel bar was inserted onto the hole made at the top of the drill (Fig. 1, B). During the drilling, the melting probe has been heated by a heat gun (GT-HAG-01, 2000 W, King Craft, Germany) placed at the level of the upper 5cm of the drill and 3 cm away horizontally.

The samples (1 mL of the melted water at the bottom of the drilled hole) were collected at every 2 centimetres of the drilling depth. When collecting samples, the melting probe was lifted up briefly. The collected samples (aliquoted as 100 µL) were dispensed into Petri dishes (∅ 99mm) containing tryptone soy agar (Carl Roth, Germany) for *B. subtilis* and MacConkey agar (Carl Roth, Germany) for *E. coli*, in 3 replications. The inoculated agar plates were then incubated at the strain-specific optimal temperature for 24 hours. The number of colonies formed per plate was then counted with the help of molecular imager gel doc XR system (Bio-Rad, Germany) and QuantityOne® software (Bio-Rad, Germany).

In the control trials, 1 ml of sterile distilled water was injected at the 4 centimetre depth before the sample was taken as the "control" one. In the experimental trials, 1ml of either 30% (v/v) H_2O_2 (Sigma-Aldrich, Germany) or 3% (v/v) NaClO (DanKlorix, Colgate-Palmolive, Germany) was introduced into the system instead of water.

In a special series of experiments, the temperature of the tip of the melting probe during ice melting has been recorded using Voltcraft K204 digital thermometer (Conrad Electronic SE**,** Germany) while drilling the ice cube situated in a 2 cm thick Styrofoam box. During drilling, the melting probe was continuously heated with the heat gun, as described above and the probe's temperature was measured every 2 min.

Statistical treatment and data processing

Data were collected into a Microsoft® Excel® 2007's spreadsheet for calculation of means, standard deviations and standard errors. For statistical accuracy, each drilling experiment was repeated at least 2 times and for each sampling depth point, 3 CFU counts (replicates) were made. To improve the comparison of the CFU data, obtained in independent experiments, the weighted mean values were transformed into relative values, considering the "0 cm" samples' averages as 100%.

Results and Discussion

Melting probe temperature during drilling

In order to check if the temperature of the melting probe itself was high enough to cause inactivation of bacteria, a special series of tests was done where the temperature of the melting probe was monitored under typical experimental conditions. Fig. 2 shows the melting probe's mean temperature over the drilling time period of 23 min for 3 independent drillings.

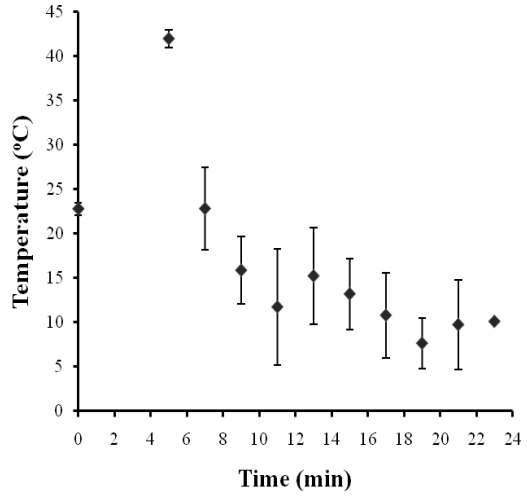

Fig. 2. Mean temperature of the tip of the melting probe during 23 min of drilling. The error bars represent the standard deviation for 3 independent experiments.

As visible from Fig. **2**, the temperature of the melting probe's tip never exceeded 45°C. This means that the supplied heat itself could not significantly contribute to inactivation of the bacteria on the surface of the melting probe. Apparently, the drill could not reach higher temperature because of efficient heat transduction in the metal-ice-water system.

"Natural" in-situ melting probe decontamination

During the ice-drilling process, large amounts of melted water are produced. This can provide a mechanism of the probe's self-cleaning because of induced water flow, shear stress and dilution of the detaching bacteria [15]. In order to examine to what extent the detectable amount of bacteria is affected by these factors, in the next series of tests the drilling was conducted without application of disinfecting agents with the samples taken as described in the materials and methods.

Fig. **3** shows relative number of colony-forming units in a Petri dish as a function of the drilling depth for such "disinfectant-free" drills done up to 16 cm.

As follows from Fig. 3, for both microorganisms, with the increased sample's depth, the number of viable cells was subjected to high fluctuations. *E.coli* has demonstrated generally higher susceptibility to mechanical friction, shear force and dilution. This is probably because it is a Gram-negative bacterium and therefore it has thinner cell wall compared to those in Gram-positive bacteria (such as *B. subtilis*). For Escherichia, the number of viable cells at the maximal drilling depth was only 1.04±0.9% from the initial (surface) value. In contrast to that, for, the number of CFUs in the deeper samples was even more than the initial value. A reason for this may be that the bacteria were initially distributed non-uniformly over the surface of the drill. Since *B. subtilis* is aerobic, during the incubation of the drill with bacterial suspension more colonies are found on the upper part of the tryptone soy broth compared to the bottom part of the tryptone soy broth. The obtained data imply that probe's self-cleaning process is not efficient, especially for *Bacillus*, where the number of viable cells at the maximal drilling depth is still 106.2±11.9% from the initial (surface) value.

Additional data obtained by us for other bacterial strains, *Micrococcus roseus* and *Pseudomonas syringae* (data not shown) have also instantiated that the natural factors are not efficient enough to eradicate bacterial contaminants during ice penetration.

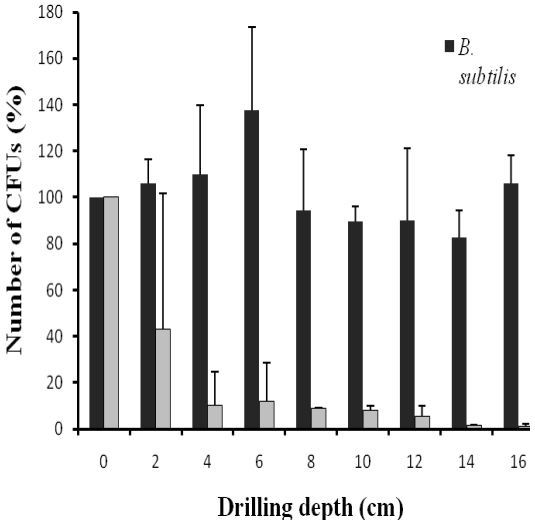

Fig. 3. Relative number of colony-forming units in the samples taken at different depths when no disinfecting agent was applied. The error bars represent standard deviation for B. subtilis (N=9) and for E. coli (N=6).

In-situ melting probe decontamination using H_2O_2 and NaClO

In order to check if 30% (v/v) H_2O_2 at low temperature can inactivate bacteria and also to check whether drilling depth will have an effect, a series of tests was done for each bacterial strain. In this case, 1 ml of 30% (v/v) H_2O_2 was added at the 4 cm drilling depth.

Fig. 4 shows how relative number of bacteria change during drilling up to the depth of 18 cm when hydrogen peroxide was injected into the drilling hole.

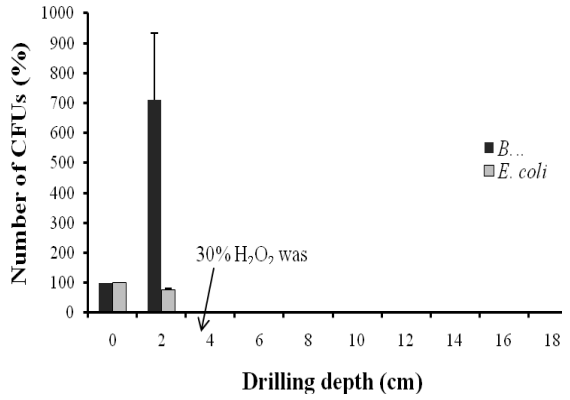

Fig. 4. Relative number of colony-forming units in the samples taken at different depths when hydrogen peroxide was injected as disinfecting agent. The error bars represent the standard error for 3 experiments.

As we can see from Fig. 4, there was an abrupt drop to 0% at 4 centimetre sample point, due to the introduction of H_2O_2. Fig. 5 shows the results of a similar series of tests where 1 ml of 3% (v/v) NaClO was injected at the 4 cm-deep point. The standard error is very small for most part of the experiment, except the 2 cm sampling point for *B. subtilis*. Again, probably for the reasons mentioned above, the number of CFUs (%) in the deeper samples was more than the initial value for *B. subtilis*.

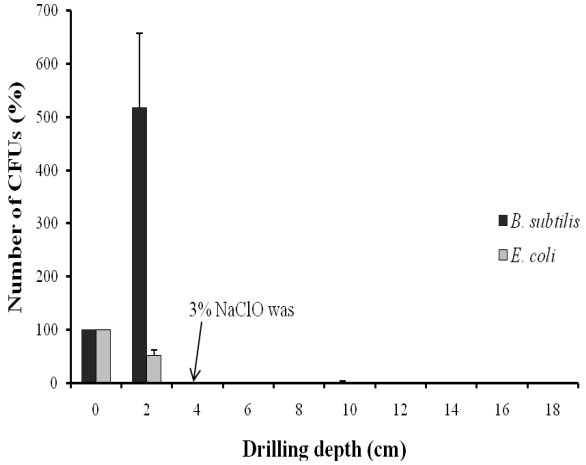

Fig. 5. Relative number of colony-forming units in the samples taken at different depths when 3% sodium hypochlorite was injected as disinfecting agent.
The error bars represent the standard error for 3 experiments.

Obviously, in both cases, there were no survived bacteria in the in the drilling hole after the 4 centimetre sample point of the experiment till the end of the drill. We have concluded that 1 mL of 30% (v/v) H_2O_2 as well as 1 mL of 3% NaClO was enough to eradicate both *B. subtilis* and *E. coli* contaminants at ice-drilling conditions over the whole range of depths checked.

Conclusions

Our study has demonstrated that the ice-melting probe's heating itself does not contribute significantly to inactivation of microbial contaminants on its surface, since the temperature dose not reach more than 50°C. During the IceMole propagation in ice, there are dilution and abrasion effects leading to decrease in both viability and concentration of bacterial cells but their role is not big enough to reach a sufficient decontamination level.

Successful decontamination of the surface of a melting probe can be successfully achieved by usage of 30% (v/v) H_2O_2 and 3% (v/v) NaClO. Our group was able to reach the threshold of 0 colony forming unit per mL for in-situ drilling for both time-course and depth-course experiments. According to our estimations and assuming that the total surface area of the drilling device is 57.39 cm^2, the amount of 30% (v/v) H_2O_2 and 3% (v/v) NaClO providing 99.9% decontamination is 17.42 $\mu l/cm^2$ in both cases. Moreover, biochemical analyses of the studied compounds (data not published yet) have shown their potency in the breakup of bacterial proteins and DNA. We also suggest that a combination of NaClO and H_2O_2 consequent treatments may be applied to increase the microbial safety even more.

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