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Detection of Microorganisms in Low-Temperature Water Environments by *in situ* Generation of Biogenic Nanoparticles

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A new nanobiotechnological approach for the detection of extraterrestrial Earth-like biological forms is proposed. The approach is based on the ability of microbial cells to reduce artificially added cations with the generation of crystalline nanoparticles (NPs) from zero-valent atoms. The method is named DBNG (Detection of Biogenic Nanoparticles Generation). The subglacial low-temperature oligotrophic Lake Untersee in Antarctica was used as a model of putative extraterrestrial water environments inhabited by Earth-like type microorganisms. The DBNG protocol for the comparative study of microbial communities of low-temperature oligotrophic environments was optimized on the base of experiments with the pure culture of psychroactive bacterium *Cryobacterium* sp. 1639 isolated earlier from Lake Untersee. The formation of silver nanoparticles (Ag°NPs) has been conducted in natural water samples of three horizons at low temperature (+5°C), which was in the temperature range registered in the Lake Untersee. The generation of biogenic Ag°NPs was detected only at the presence of indigenous microorganisms in all studied samples. No Ag°NPs generation was observed in the lake water samples artificially free of cells or exposed to pasteurization (two types of controls). The miniature microfluidic chip for an automated version of the device, based on using different analytical methods for recording *in situ*-formed biogenic nanoparticles, is proposed. The device allows the detection of the biological objects directly at the sampling site.

Keywords: astrobiology, extraterrestrial life, nanoparticles, psychrophiles, DBNG method

INTRODUCTION

Nowadays, the topical challenge of astrobiology is the development and testing of new methods for searching for Earth-like biological forms in Space. It is assumed that metabolically active microorganisms are present mainly in a state of aqueous suspensions in terrestrial environments. Many cells of such suspensions can remain viable for a very long time, especially in oligotrophic environments (Gordon et al., 2000; Vishnivetskaya et al., 2000; Ponder et al., 2008). Accordingly, searching for extraterrestrial biosignatures or biological Earth-like objects in most studies is focused

on cosmic bodies containing water and ice (Andersen et al., 1995; Priscu et al., 1998; Storrie-Lombardi and Sattler, 2009; Haendel et al., 2011; Filippova et al., 2013; Westall et al., 2013; Koo et al., 2017, 2018). Various approaches to detect biosignatures have been suggested (Rivkina et al., 2000; Gilichinsky et al., 2007; Rogers et al., 2010; Andersen et al., 2011; Zhang et al., 2014; Bedrossian et al., 2017; Managadze et al., 2017). Nevertheless, the development of new effective techniques for the detection of putative life beyond Earth is still an important problem to be solved (Nadeau et al., 2008; Garcia-Descalzo et al., 2012; Judge, 2017; Nelis et al., 2018). The basic astrobiological methods are mostly related to search for biosignatures of a geological, chemical, and biomorphological nature (Cockell, 2010; Westall et al., 2015; Hays et al., 2017; Mickol et al., 2018). Here, we propose a novel nanobiotechnological approach for the detection of microbiological objects in water or melted ice samples from extraterrestrial low-temperature environments. The approach is named the Detection of the Biogenic Nanoparticles Generation (DBNG), and it based on the ability of microorganisms, in particular bacteria, to produce biogenic crystalline nanoparticles of reduced Ag° atoms of the artificially added silver salt solution.

It has already been shown that microorganisms are capable of reducing cations and generating nanoparticles from reduced zero-valent atoms of metals (Wang et al., 2012; Wu et al., 2013). This is one of the natural non-specific mechanisms that are probably involved in the detoxification of metals, including the protection from cation entry into the cell (Merroun, 2007; Gadd, 2010). All tested microorganisms had this ability to reduce cations with different valences (Velusamy et al., 2016; Patil and Kim, 2017; Sorokin et al., 2019; Singh et al., 2020). It was also demonstrated that a much greater variety of nanoparticle assemblies could be produced by cells along the "bottom-up" approach (Zhang et al., 2011; Woehl et al., 2014; Sánchez-López et al., 2020) (**Figure 1**). Observations clearly show that nanocrystals can nucleate from an aqueous solution *via* a three-step mechanism: spinodal decomposition, clusterization, and nanocrystallization (Luo et al., 2017; Tan et al., 2017; Xie et al., 2017; Liao et al., 2019; Mikhailov and Mikhailova, 2019; Wang et al., 2019). The amorphous clusters of zero-valent atoms (up to 1–2 nm) are direct precursors of the bigger crystalline nanoparticles (Malis et al., 2011; Wan et al., 2013; Loh et al., 2017; Singh et al., 2020). Several studies have reported natural polymers (such as chitosan, proteins and amino acids, sugars, starch, and tannic acid) as reducing agents for the synthesis of metallic nanoparticles (Prakash and Sharma, 2010; Kharissova et al., 2013; Velusamy et al., 2016; Masse et al., 2019; Navarro Gallón et al., 2019). Differences in generated biogenic nanoparticles depend on the physiology of microorganisms as well as on the medium composition and conditions at their formation (Narayanan and Sakthivel, 2010; Zhang et al., 2011; Wang et al., 2012; Sorokin et al., 2013; Wu et al., 2013; Tyupa et al., 2016; Muller, 2018; Siddiqi et al., 2018). However, only metabolically active cells can rapidly (in minutes) generate metal nanoparticles from metal ions of the added salt due to their ability to constantly "efflux" electrons and electron donors into the medium (Zhou et al., 2013; Skladnev et al., 2017a; Wang et al., 2019). Inactive cells or cells with destroyed and non-active biostructures have a much weaker ability to reduce cations and form only the amorphous nanoclusters (up to 1–2 nm) as precursors of nanoparticles (Woehl et al., 2014; Liao et al., 2019). In solution, the organic components of the cytoplasms of destroyed cells can only act as low concentrated and "slow" chemical reducing agents. The generation of nanoparticles, when a source of metal cations is added into a sample, can be considered as an indicator of the presence of biological objects.

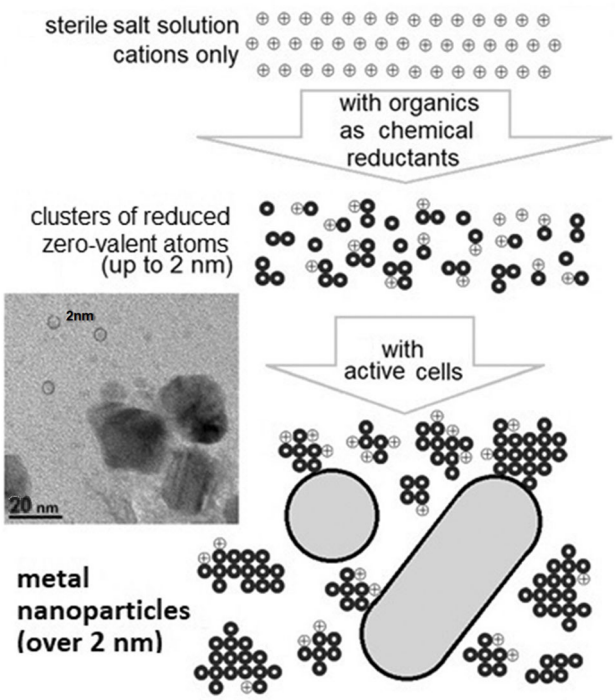

FIGURE 1 | The scheme of stages of biogenic nanoparticles generation.

To confirm the efficiency of the proposed DBNG method, we applied it to natural water samples taken from the Antarctic Lake Untersee—an analog terrestrial system for putative cold extraterrestrial ecosystems (Andersen et al., 1995, 2011; Filippova et al., 2013; Heinz et al., 2018). The lake is an ice-covered reservoir, oligotrophic, and isolated from the Earth's atmosphere for millions of years. In previous studies, microbial communities present in the Lake Untersee have been shown to survive in extreme conditions, such as near-zero temperatures and low content of organic compounds (Fomenkov et al., 2017; Pikuta et al., 2017). The water reservoir with likely similar characteristics, was found under a layer of Martian ice in the southern polar region (Jones et al., 2018; Orosei et al., 2018; Post et al., 2019). The goal of the present work was to demonstrate the efficiency of the proposed nanobiotechnological DBNG method for astrobiology applications. The aim of the investigation was to study the biogenic Ag°NPs formation *in situ* in water samples from different horizons of subglacial Antarctic Lake Untersee containing indigenous microorganisms under low-temperature conditions.

TABLE 1 | Some characteristics of water sampling horizons from the Lake Untersee (Andersen et al., 2011).

Horizon depth, m	T, °C	pH	Dissolved oxygen, mg L^{-1}	Features of lake water horizons
20	0.3	10.1	20	Aerobic layers with
40		10.3	18	very low organic content
72	4.9	7.9	0.2	Microaerobic conditions in chemocline

MATERIALS AND METHODS

Sampling of Different Horizons of the Lake Untersee, East Antarctica

Three water samples were taken aseptically from the subglacial low-temperature Lake Untersee, in East Antarctica (71°20′ S, 13°45′ E) located in the interior of the Gruber Mountains of central Queen Maud Land. This extremely oligotrophic lake is situated 563 m above the sea level and is the largest by surface area (11.4 km^2) lake in East Antarctica. Samples from the lake were taken using 1-liter bathometers at the station of the ice camp, as described in the NASA 2015 Annual Science Report[1] The water samples were collected by the depth profile of the column at 20, 40, and 72 m (starting from the smallest depth). Some environmental parameters are shown in **Table 1**. Water temperature in the horizons was from +0.3 to +4.9°C. The samples were aseptically transferred to sterile laboratory flasks and transported to the laboratory in heat-insulating containers and subsequently stored at +5°C. For the current study, about 4 ml of those water samples were kindly provided by Acad. Gal'chenko. The experiments for the generation of nanoparticles were started in 4 months of sample collection. The aliquots for analysis were also taken aseptically.

The Pure Culture of Psychroactive Bacterium

The psychroactive bacterium *Cryobacterium* sp. 1639 was isolated in pure culture from the Lake Untersee water sample taken from a depth 72 m with registered *in situ* temperature of +4.9°C. The 16S rRNA sequence of the isolate was deposited in the NCBI GenBank database (MT364260).

Formation of Biogenic Silver Nanoparticles at DBNG Method

The principal scheme of the DBNG method is presented in **Figure 2**. The formation of biogenic Ag°NPs was carried out in 50-μL aliquots. The source of silver cations for generation of Ag°NPs was an aqueous solution of $Ag(NH_3)_2NO_3$ synthesized from $AgNO_3$ and ammonia according to a modified Tollens protocol (Anh-Tuan et al., 2010). Sterile 2 mM $Ag(NH_3)_2NO_3$ solution was added directly to samples to achieve a final concentration of 0.1 mM. The generation of silver nanoparticles in native water samples was carried out at +5°C. The generation of silver nanoparticles by cells of strain *Cryobacterium* sp. 1639 was carried out at the exponential growth phase at two cultivation temperatures: +5°C (close to the Lake Untersee horizon temperature) and at +24°C (room temperature). The duration of exposure to silver salt in all experiments was 20 min (Sorokin et al., 2013). The experiments were conducted in triplicate. Sterility of $Ag(NH_3)_2NO_3$ solution was proved by the absence of microbial growth in the LB medium. The absence of NPs in native water samples and $Ag(NH_3)_2NO_3$ solution was confirmed by transmission electron microscopy. As cell-free control aliquots, 1.0 mL of the native water samples and the cultural fluid of *Cryobacterium* sp. 1639 free of microbial cells previously removed by centrifugation (10,000 g, 15 min) were used. In all cell-free controls formation of silver nanoparticles was carried out at two temperatures +5 and +24°C.

Electron Microscopy and X-Ray Microanalysis

Specimens were studied using a JEM-1400 microscope (JEOL, Japan) equipped with an X-ray microanalyzer (Oxford Instruments, United Kingdom) at an accelerating voltage of 80 keV. The samples were prepared using standard copper grids with Formvar film reinforced with carbon. Five microliters aliquots of each variant were applied to the copper grids and dried for 15 h.

A direct cell counting was conducted for the samples taken from different water horizons by the TEM examination of grids with native variants.

Analysis of the linear dimensions of Ag°NPs and their classification was carried out using the "Compass 3D-V14" software and a specially developed algorithm for calculating linear dimensions based on electron microscope images of at least 300 nanoparticles.

Inactivation of Cells of *Cryobacterium* sp. 1639 Culture and Water Samples From Horizons of the Lake Untersee

Four samples were pasteurized at 70°C for 30 min and used as negative controls (Cebrián et al., 2017).

RESULTS

Formation of Silver Nanoparticles by Cells of Psychroactive Bacterium *Cryobacterium* sp. 1639

The psychroactive bacterium *Cryobacterium* sp. 1639 was isolated from horizon depth 72 m of the Lake Untersee. The culture *Cryobacterium* sp. 1639 grew well at +5°C and was able to reduce silver cations and forming Ag°NPs at +5°C during 20 min (**Figure 3A**). The size of 50% of these particles did not exceed 20 nm, while larger size appeared rare (**Figure 3B**). When the temperature was higher than 20°C, the growth of psychroactive bacterium *Cryobacterium* sp. 1639 was very slow, and the formation of Ag°NPs was not detected (**Figure 3C**). Cells of *Cryobacterium* sp. 1639 grown at +5°C lost their ability

[1]https://astrobiology.nasa.gov/nai/annual-reports/2015/seti/lake-sediment-habitats-lake-habitability-and-sediment-biosignatures/

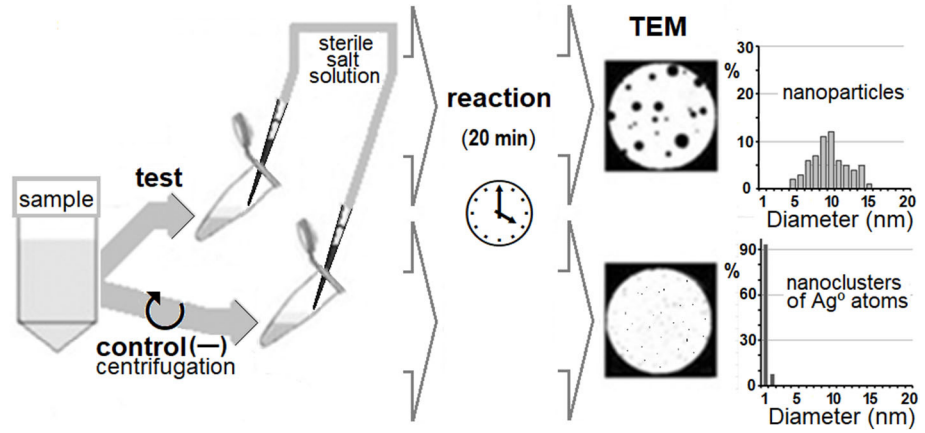

FIGURE 2 | The principal scheme of the DBNG method.

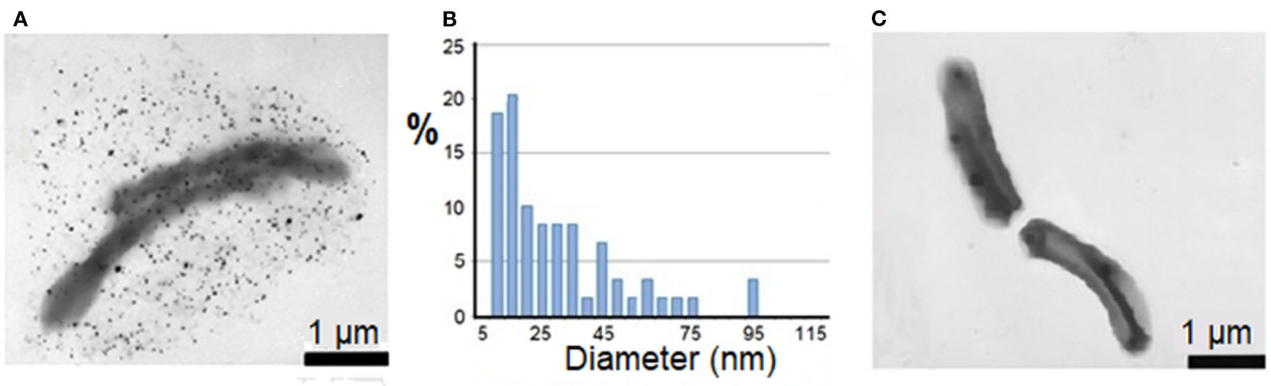

FIGURE 3 | Distribution in sizes **(B)** of biogenic Ag°NPs generated by psychroactive *Cryobacterium* sp. 1639 at +5°C in 20 min **(A)**. No Ag°NPs generated by cells *Cryobacterium* sp. 1639 at +24°C **(C)**.

to reduce Ag^+ cations fast and generate nanoparticles under non-optimal temperature conditions (at +24°C).

Formation of Silver Nanoparticles in Native Water Samples From the Lake Untersee

The direct cell counting in water horizons confirmed the presence of numerous indigenous microorganisms (from $3{\cdot}10^4$ to $2{\cdot}10^6$ mL^{-1}). In all three studied water samples, the formation of biogenic Ag°NPs of 15–150 nm in diameter at +5°C in 20 min has been observed (**Figure 4**). In cell-free controls no Ag°NPs have been detected.

The difference in the size distribution of Ag°NPs formed in water samples taken from three different horizons of the Lake Untersee was revealed. For these samples, distribution in size could be characterized by the normal law. Biogenic nanoparticles detected in the sample taken from 20 m depth had a maximum size of 40 nm. In the samples from depth 40 m and 72 m, the formed nanoparticles were twice as large. Nanoparticles from deeper samples had a larger size (35–120 nm for depth 40 m and 65–155 nm for depth 72 m), the predominant size was 80 nm.

Pools of biogenic nanoparticles formed in samples were different in both linear sizes and quantity. The same conditions for silver reduction in lake water samples made it possible to compare concentrations of biogenic Ag°NPs formed in these reactions. Amounts of biogenic Ag°NPs detected in water samples were normalized to those detected in the sample from the depth of 40 m (**Figure 5**).

Formation of Nanoparticles by Inactivated Cells of *Cryobacterium* sp. 1639 and Native Microflora of Water Horizons of the Lake Untersee

The standard protocol of the DBNG method was used for experiments with non-specific inactivation of cell suspensions of psychoactive bacterium *Cryobacterium* sp. 1639 and three studied water samples with indigenous microorganisms of the Lake Untersee. No silver nanoparticles were observed in these four samples with their previous pasteurization.

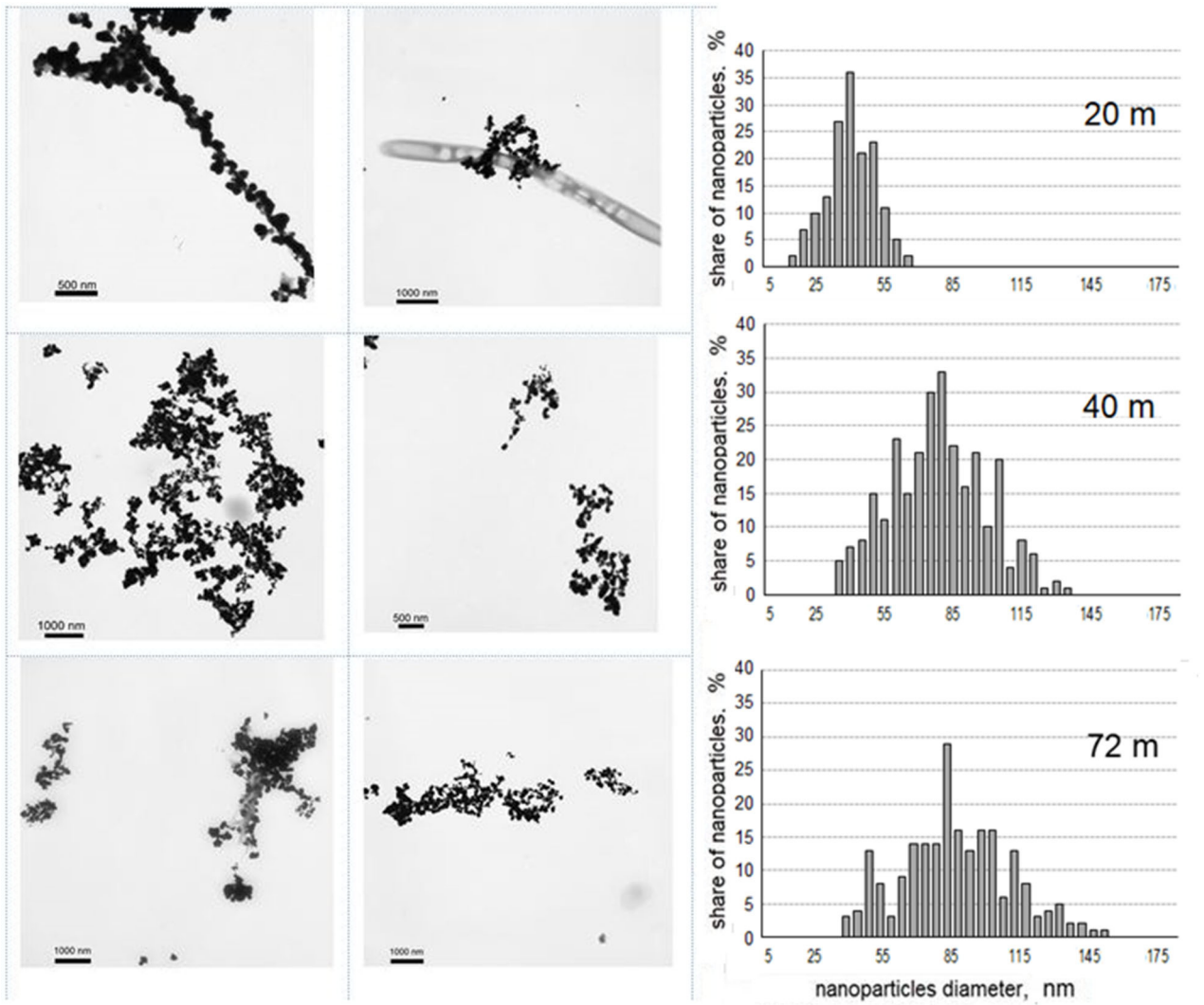

FIGURE 4 | Biogenic Ag°NPs, formed at +5°C in 20 min in water samples of the Lake Untersee and their distribution in size (in percentage to the total amount).

DISCUSSION

The Lake Untersee is highly oligotrophic, ice-covered, and has no anthropogenic impact. All these features make it a good terrestrial analog to putative cold extraterrestrial ecosystems and therefore an attractive subject for astrobiologists (Andersen et al., 1995, 2011; Cockell, 2010; Garcia-Lopez and Cid, 2017; Nelis et al., 2018; Post et al., 2019). All these characteristics make water samples from this lake acceptable for the approbation of the new method based on the ability of microbial forms to reduce Ag^+ cations followed by the formation of biogenic Ag°NPs from zero-valent silver atoms.

Adaptation of the DBNG Method for the Detection of Microorganisms in Low-Temperature Water Environments

To adapt the DBNG method for the detection of microorganisms in low-temperature water environments (by *in situ* formation of the biogenic nanoparticles), we used the results of experiments with a pure culture of psychroactive bacteria isolated from the 72 m horizon of the Lake Untersee. It has been demonstrated that the psychroactive bacterium *Cryobacterium* sp. 1639 has good growth at +5°C (Bajerski et al., 2011), within the temperature range characteristic of its native habitat in the horizon of the Lake Untersee (while at +25°C bacterium *Cryobacterium* sp. 1639 grew three times slower). For the first time, it has been shown that psychroactive bacteria are capable of biogenic Ag°NPs formation at +5°C during 20 min. It is important to note that silver nanoparticles generated at these conditions were relatively small in size (20 nm or smaller in average) in comparison to those formed by indigenous microorganisms in water samples of the Lake Untersee. The possible explanation of the difference in size is the biosynthesis of low-molecular extracellular bioactive compounds of *Cryobacterium* sp. 1639. These compounds can be absorbed by the "young" nanoparticles (nanoclusters) and prevent their further growth (Zhang et al., 2011; Zhou et al.,

2013). There were no biogenic Ag°NPs generated at +24°C for 20 min. Thus, the temperature +5°C is optimal for the DBNG method for detecting microorganisms in a low-temperature water system.

The formation *in situ* biogenic Ag°NPs in two horizons of the Lake Untersee at +24°C were described earlier (Skladnev et al., 2017a). It was shown that: (i) for the depth of 40 m—the size distribution of nanoparticles had the normal distribution with a distinct peak at 12–13 nm; (ii) for the depth of 72 m—half of Ag°NPs had a very small diameter (about 5 nm), whereas other nanoparticles were larger (from 12 to 50 nm).

In our study, at +5°C Ag°NPs showed different size distribution (**Figure 4**). In this case, Ag°NPs size distributions for horizons 40 and 72 m had the pick at 80 nm. We emphasize that large nanoparticles were formed over 20 min, indicating the presence of metabolically active microorganisms in these horizons.

The comparison of average amounts of formed Ag°NPs demonstrate that in the water sample from 72 m, the number of biogenic Ag°NPs is larger than in the water sample of the 40 m depth. It was true for both temperature conditions: at +5°C (60: 1), and +24°C (60: 2.5) (**Figure 5**). The value of relative amount Ag°NPs at +5°C is higher, which means that cells at low temperature demonstrate higher reducing activity. The high sensitivity of the DBNG method directly for native water samples was shown even at low-concentrated cell suspensions (20 m—$3{\cdot}10^4$, 40 m—$4.1{\cdot}10^4$ cells per mL).

In general, based on the data obtained in our experiments, we can conclude that the reduction efficiency for artificially added silver cations to samples is higher at the temperature close to the native environment (+5°C) than at the room temperature (+24°C), and this indicates to the true psychrophilic physiology of inhabitants. Biogenic Ag°NPs with a diameter larger than 10 nm can be detected by a wide range of analytical methods (visible and fluorescent spectrometry, spectroscopy of surface-enhanced Raman scattering, etc.).

Technical Principles of Analytical Methodology of the Proposed DBNG Method for Perspective Astrobiology Missions

For reliable detection of life-forms using the DBNG method, two controls have to be used: (i) an aliquot of the test sample mechanically purified from microbial cells (for example, by centrifugation) and (ii) an aliquot with inactivated cells (for example by pasteurization). The formation of nanoparticles (from artificially added cations) in the native sample would prove the presence of Earth-type active living forms, if there are no nanoparticles (from artificially added cations) in the two controls.

UV-visible and fluorescence spectrometry are the most frequently used tools for the detection of metal nanoparticles (Gomez et al., 2014; Loh et al., 2017; Liao et al., 2019; Sánchez-López et al., 2020). In earlier work (Sorokin et al., 2013), it was shown that biogenic Ag°NPs generated under DBNG protocol were confidently registered by visible spectrometry

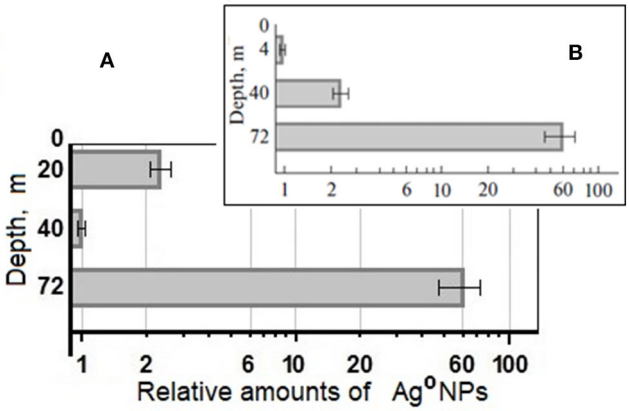

FIGURE 5 | Relative number of Ag°NPs, formed at +5°C in water samples of the Lake Untersee from different depths, normalized to the amount of biogenic nanoparticles in the sample from the depth 40 m **(A)**, **(B)** relative number of Ag°NPs, formed in the same water samples at +24°C (Skladnev et al., 2017a).

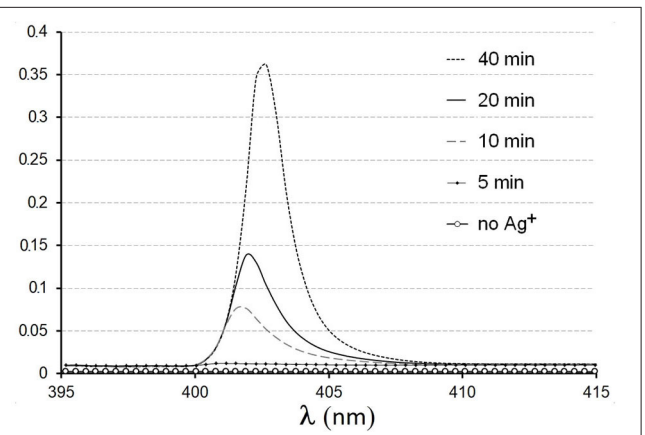

FIGURE 6 | Visible absorption spectra for different duration of biogenic Ag°NPs generation.

(**Figure 6**). Several models of miniature spectrometers with high sensitivity are available now. The protocol of the DBNG method permits the use of microliter volumes of examined samples and salt solutions. Devices for the production of nanoparticles by chemical methods based on microfluidic lab-on-a-chip (LOC) technologies have already been demonstrated (Wu et al., 2012; McLeod et al., 2013; Gomez et al., 2014; Buja et al., 2017). So, similar chips for detecting biogenic nanoparticles formed by microorganisms in aqueous samples can be developed (Skladnev et al., 2017b). Such devices can certainly be useful for searching for Earth-like living forms directly on extraterrestrial objects.

CONCLUSION

The fast *in situ* formation of the biogenic Ag°NPs in water samples of the Lake Untersee at low temperature was

demonstrated. The efficiency of the DBNG method to detect living biological forms directly in native water samples (from 10^4 cells per mL) was confirmed. For evidence of the biogenic character of formed nanoparticles, we used two controls: water samples after the indigenous cells were artificially removed by centrifugation and water samples exposed to pasteurization. Biogenic nanoparticles formed due to application of the DBNG protocol can be detected by spectrometry and/or other physical methods. A compact chip, based on principles of the DBNG method, can be constructed. In summary, the proposed and tested in this work method can be used for the detection of active microbial Life in native samples and can be considered as a new nanobiotechnological tool for searching for Earthlike living forms elsewhere in Space.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

DS: general concept, nanoparticles forming experiments, literature review, and preparing the manuscript. LV and YB: isolation of microbial culture and incubation experiments. OK: general concept, correction of the manuscript, and organizational support. SK: literature review and correction of the manuscript. VS: general concept, electron microscopy investigations, and preparing the manuscript. All authors contributed to the article and approved the submitted version.

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