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EXPERIMENTAL ARTICLES

Modeling of Dissemination of Microbial Cells and Phages from the Sites of Permafrost Thawing

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Abstract—A method is proposed for integral assessment of the propagation of microbial cells and phage particles during seasonal thawing of relic ice wedge layers. The results of field and laboratory investigation carried out in the upper part of permafrost exposure at Mamontova Gora (Yakutiya, Russia) are presented. Suspensions of yeast, bacteria, and two coliphages were introduced as biomarkers directly on the surface of thawing ice and in the meltwater flow. Microorganisms and phages were shown (a) to possess particular parameters of dissemination in the meltwater flow and (b) were able to move 132 m in 25–35 min with the stream water flow.

Keywords: Arctic, permafrost, relic ice wedge, thawing, dissemination of microorganisms and phages
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This investigation is devoted to the refinement of procedures for studying the propagation of microorganisms and viral particles in the zones of thawing of permafrost ice. Efficient monitoring of spatial and temporal dissemination of any microbial objects, including pathogens, during thawing of Arctic permafrost is unquestionably topical, especially under the potential global climate warming conditions.

Microorganisms of the ecological niches of the polar regions, including those revealed within permafrost ice, are of special interest, because under the conditions favoring long-term preservation of cells and viruses, very ancient forms may be retained in a viable state (Gilichinsky et al., 2007; Mel'nikov et al., 2011; Wilhelm et al., 2011; Meiring et al., 2012). Almost all microbiological investigations of polar ecological niches dealt with determining the taxonomic composition of the microbial communities (Junge et al., 2002; Steven et al., 2006; Segawa et al., 2011; Filippova et al., 2013), as well as with establishing the molecular mechanisms of biostructure preservation under freezing conditions (Mitova et al., 2005; Papa et al., 2009; Merlino et al., 2010; Piette et al., 2010; Satory et al., 2011; Kryazhevskikh et al., 2013). Abundance and diversity of culturable aerobic heterotrophic microorganisms in the samples are usually assessed using microbiological and molecular methods (Mulyukin et al., 2002; Williamson et al., 2007; Kryazhevskikh et al., 2012; Mulyukin et al., 2014). However, there is a possibility that microbial cells and phages disseminate with water and air flows on the surface and via capillaries and fissures during thawing of permafrost sediments and ice wedge layers.

The goal of the present work was to develop a method for assessing the dissemination dynamics of aboriginal microorganisms and phages thawing from permafrost ice using the biological markers introduced directly into the thawing zone. Presumably, their propagation pattern was similar to that of natural microbiota of thawing ice. Such investigations are important for insufficiently studied regions of the Russian Arctic in view of ambitious plans of intensive use of Russian polar zones in economic and defense activities.

MATERIALS AND METHODS

Biological markers. The yeast, actinobacteria, and two coliphages were used as four biological markers. The first biomarker was a specially constructed strain of nonpathogenic ascomycetous yeasts *Yarrowia lipolytica* Y-3603 (Scioli et al., 1997; Yuzbasheva et al., 2011; Zinjarde et al., 2014). The cells of *Y. lipolytica* Y-3603 were tagged with the structural gene encoding TurboFP635 red fluorescent protein covalently bound to the YlPir1 surface protein. Under laboratory conditions, the presence of *Y. lipolytica* Y-3603 cells in the meltwater samples was determined using two indepen-

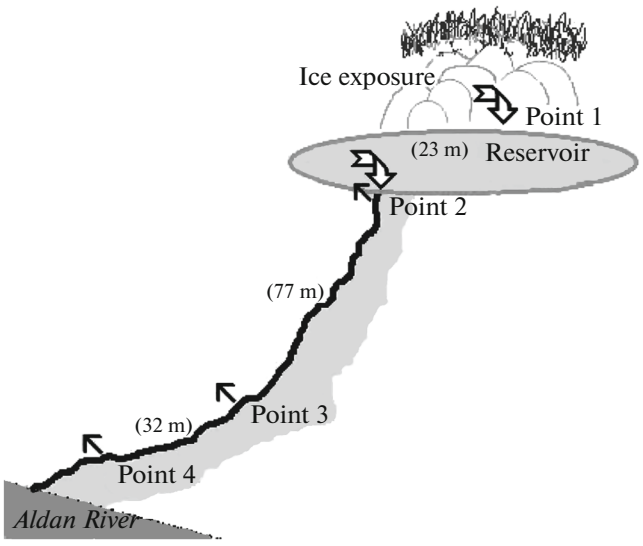

Fig. 1. Schematic view of the area of the model experiment. The points of introduction of the marker biological objects: the foot of the ice wedge (point 1) and the meltwater outflow from the reservoir (point 2). At point 1, *Y. lipolytica* and phage G7C suspensions were introduced. At point 2, *Dietzia* L18 and phage 9G suspensions were introduced. The meltwater for analyzing the presence of introduced marker biological objects was sampled at points 2–4. The distance between the neighboring sampling points is indicated in brackets.

dent methods: (1) by the number of colony-forming units (CFU) on LBagar with ampicillin, kanamycin, and tetracycline (*Y. lipolytica* Y-3603 cells contain the corresponding resistance genes) and (2) by the presence of red fluorescent cells using fluorescence microscopy (Axioplan, Carl Zeiss, Germany).

The actinobacterial strain L18, isolated earlier from the relic ice wedge sample from Mamontova Gora (the 2012 expedition), was used as the second marker. Isolate L18 grew well on LB medium and was resistant to unfavorable effects. Based on the 16S rRNA gene analysis, strain L18 was assigned to the genus *Dietzia* with high similarity (99%) to *D. natronolimnaea, D. dagingensis,* and *D. psychralcaliphila* (Koerner et al., 2009). CFU numbers of *Dietzia* sp. L18 cells in the samples were counted on LB agar based on pink pigmentation and specific colony morphology. The numbers of viable cells were determined in three to five replicates in two or three independent series of experiments.

The investigation used two different bacteriophages that have a narrow range of host enterobacteria. Phage G7C forms clearly defined negative colonies on the lawn of *Escherichia coli* 4s (Kulikov et al., 2012). The second phage 9G forms small negative colonies on the lawn of another strain *E. coli* C600. The abundance of phages in the supernatant of natural samples (after standing for 1 h) was determined by counting negative colonies (PFU). The sampled supernatants were titrated on the lawns of both host strains in five replicates.

Field experiments were carried out in the region of Mamontova Gora (Central Yakutia, Russia) (Mel'nikov et al., 2011). The marker microorganisms and bacteriophages were introduced at two points: the upper point was at the foot of the thawing ice exposure layer, while the second point was at the outflow from the storage reservoir formed by meltwater (Fig. 1, points 1 and 2).

The biomarkers were introduced directly into the permafrost ice thawing site after resuspending of the fresh, highly concentrated microbial and phage cultures in the meltwater. The sample for introduction at point 1 (a total volume of 2500 mL) contained the cells of the yeast *Y. lipolytica* (1150 g) and 200 mL of phage G7C suspension (10^{11} PFU/mL). The sample for introduction at point 2 (a total volume of 500 mL) contained 100 mL of *Dietzia* sp. L18 cell suspension (4.8×10^8 CFU/mL) and 200 mL of phage 9G suspension (10^{11} PFU/mL).

The dissemination rate of biological markers was assessed by their abundance in the flowing meltwater sampled at certain time intervals. Three sampling points were located along the main meltwater flow course at a distance of 23 to 77 m from one another (Fig. 1, points 2, 3, 4). A total of 28 meltwater flow samples were collected. The flowing meltwater was sampled into screw-capped sterile plastic test tubes (50 mL). Four control meltwater samples were taken at each point prior to introduction of the biological objects. All the samples were stored and transported at temperature not exceeding 12ºC and assayed for the biomarkers after 72 h in the laboratories of the Federal Research Center of Biotechnology, Russian Academy of Sciences.

RESULTS AND DISCUSSION

Description of the area of field experiment. The most expressed exposures of permafrost ice wedges are in the region of Mamontova Gora on the left bank of the Aldan River. The third terrace exposes alluvial sediments filling the Nizhnealdanskii trough developed along the suture zone between the Predverkhoyansk marginal trough and the Siberian platform. The terrace consists of mostly Miocene sands. The sands and sandy loam of the Middle Pleistocene age are exposed in the middle part of the section. They contain frequent horizontal layers of the floodplain sandy loams with high ice content. The Ice Complex with large ice wedges of the Late Pleistocene lies higher. The geological data show that climatic cooling and the related freezing of sediments began here at the end of the Pliocene Time (approximately 3–3.5 million years ago). The cryolithological indicators, i.e., the ice wedges overlying the section, point to the absence of perma-

frost thawing during the Holocene optimum at least (Mel'nikov et al., 2011).

Seasonal thawing of relic ice wedge layers is followed by the formation of a reservoir where the meltwater stagnates and then ends up in the Aldan River in the form of water flows (Fig. 1). Accordingly, the dissemination of microorganisms and phages includes two steps: (1) from the ice surface in the meltwater flow within the reservoir and (2) from it with flows to the Aldan River. In order to measure the rate of movement at these two steps, a scheme of introduction of two pairs of biological markers at distant points was chosen (Fig. 1, points 1 and 2). The biomarkers introduced at point 1 had to cover the maximal distance (132 m) from exposed ice to the Aldan River; when introduced at point 2, they were able to move only within the meltwater flow (109 m).

It is important to note that an obligatory requirement for the cultures of introduced biological markers was taken into account in this investigation: they must not alter substantially the natural biota composition of the region studied. Therefore, we chose four ecologically safe biological objects: the nonpathogenic yeast *Y. lipolytica*, the actinobacterial isolate of the genus *Dietzia* (isolated earlier from the cryozone investigated), and two strains of easily detectable coliphages.

Preliminary measurements of the water flow rate showed the following values: 4 L/min in the upper part of the flow (point 2) and 6 L/min in the lower part (point 3). The meltwater flow rate at point 3 was 1 m/s. Noteworthy, this flow was non-uniform because of temporary obstruction due to accumulated sand, clay, and plant fragments.

Since significant amounts of insoluble components in water were expected to influence the propagation of biomarkers flows, we assessed the volume proportion of sediment fraction in the studied samples (Fig. 2). The maximal volume ratio of sediments (60 vol %) was observed in the samples taken at the site of the meltwater outflow from the reservoir (point 2) and decreased as the Aldan River was approached (up to 37 vol %). The volume ratio of sediments in the water thawing directly from permafrost ice (point 1) was minimal (2 vol %).

Dissemination of biological markers introduced on the surface of ice exposure layer (at point 1). The initial suspension of *Y. lipolytica* cells before introduction on the ice surface at point 1 contained approximately 10^6 CFU/mL (Fig. 3a). The first water sample was taken 10 min after the beginning of the experiment at point 2 at the site of meltwater outflow from the reservoir at a distance of 23 m from the point of introduction. A marked decline in the yeast cell concentration was observed in the samples sequentially taken at point 2: 2×10^3 and 5×10^2 CFU/mL after 10 and 30 min, respectively, which indicated a rapid dissemination of the marker cells throughout the reservoir. When the yeast cells moved in the water flow, they also dispersed

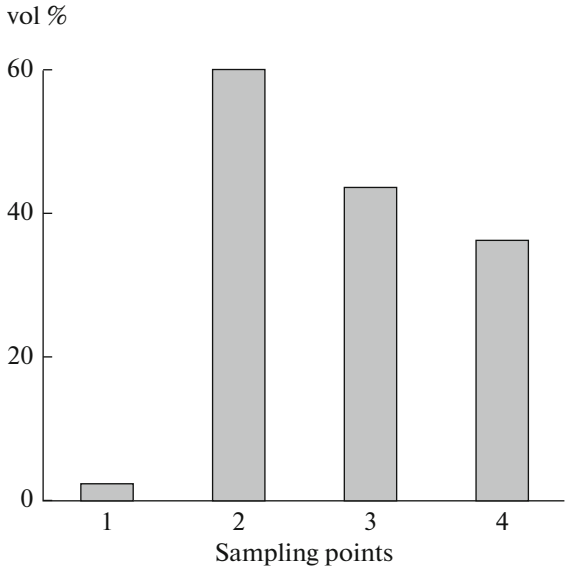

Fig. 2. Volume ratio of insoluble components in the meltwater sampled in the course of the experiment.

in it, which was indicated by cell number reduction in samples 3 and 4 taken in the middle of the flow (point 3: $(1–3) \times 10^1$ CFU/mL; point 4: $(1–3) \times 10^1$ CFU/mL). A weakly pronounced spot of the biomarker yeasts traveled a distance of 132 m from the introduction point (point 1) to the lower sampling point (point 4) in 30–35 min. Analysis of the meltwater samples taken 24 h after the introduction of *Y. lipolytica* showed the presence of low numbers of viable yeast cells in the sample obtained from point 4 (Fig. 3a).

The coliphage G7C suspension was introduced directly on the ice (together with *Y. lipolytica* cells) at a concentration of 2×10^6 PFU/mL. In the samples taken at point 2 of outflow within 60 min, the coliphage G7C titer was almost constant: 4×10^6 PFU/mL was found 10 min after introductions; 9×10^6 PFU/mL after 30 min; 6×10^6 PFU/mL after 60 min (Fig. 3b). Thus, the content of phages in the reservoir was very stable in contrast to the marker yeasts.

The main marker spot, containing the maximal number of phages, moved in the meltwater flow in proportion to the flow rate along the verge of the stream as the spot arrived from point 2 to point 3 (a distance of 77 m) within 7 min. At point 4 (a distance of 32 m), the spot was detected 8 min later. The entire way from the introduction point at the foot of ice exposure (point 1) to the lowest sampling point (point 4, the total distance 132 m) was covered by the spot of coliphages G7C in 25–30 min. The concentration of phages in the samples from the lower points 3 and 4 was found to decrease only after 12 h. In general, the marker biological objects with substantially differ-

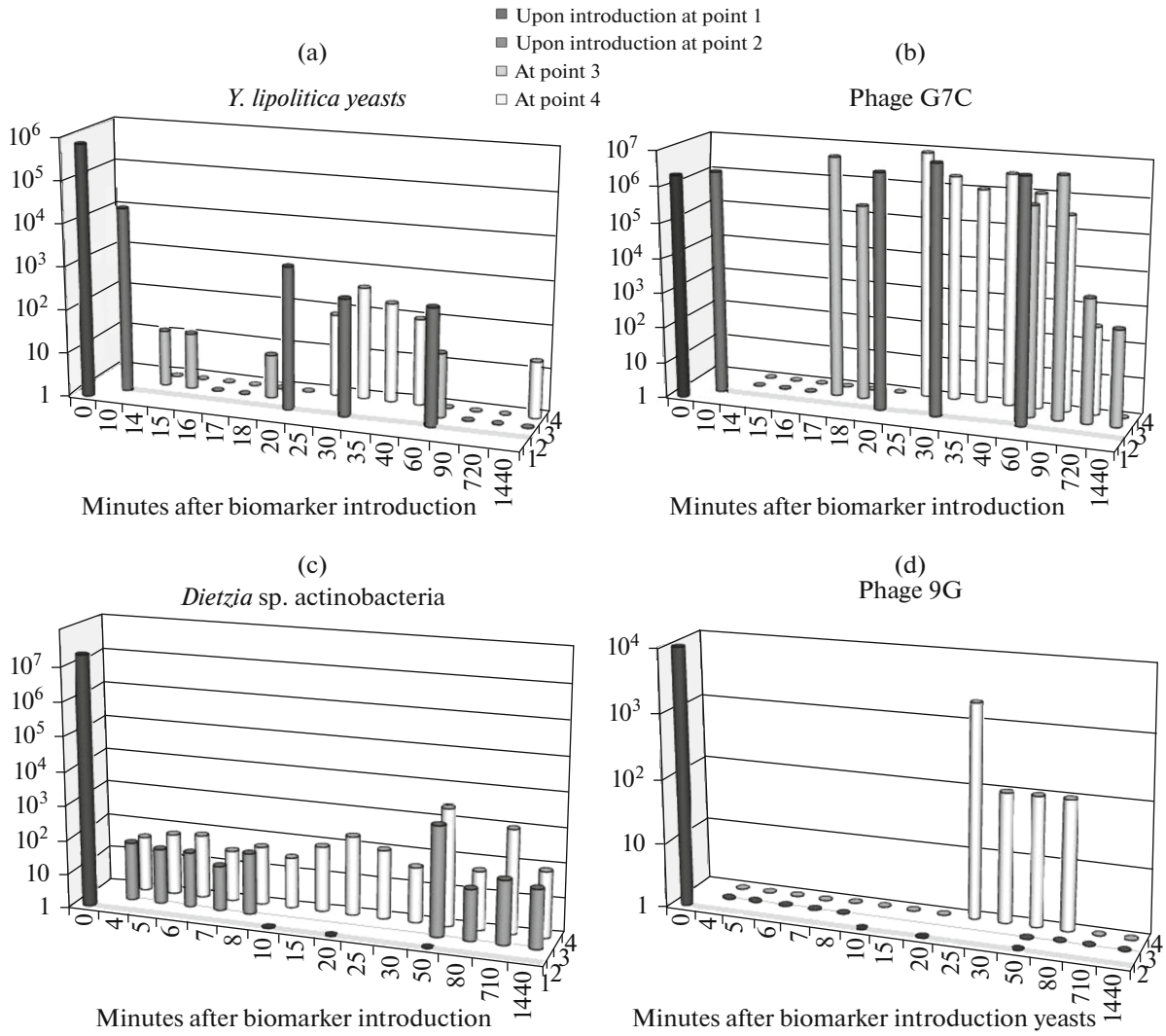

Fig. 3. Content of viable cells (a and c, CFU/mL) and active phages (b and d, PFU/mL) in the meltwater sampled at points 1–4. The intervals between the moments of biomarker introduction (zero time) and the moment of sampling are indicated.

ing masses and volumes (the yeast *Y. lipolytica* and coliphage G7C) moved at virtually the same rate.

Dissemination of the biological markers introduced at the site of meltwater outflow (at point 2). The abundance of the bacterial marker, the actinobacteria *Dietzia* sp. L18 introduced at point 2, was determined by the number of colonies with a characteristic shape and pigmentation on LB agar inoculated with sampled meltwater. In order to determine the background level of the presence of aboriginal taxonomically and phenotypically close bacteria, the control samples were analyzed. The count of characteristic pigmented colonies showed the presence of a natural background of actinobacteria in the lower flow course at a concentration of (1–7) × 10^1 CFU/mL. Since the *Dietzia* sp. L18 cell suspension prepared for introduction into the meltwater flow contained approximately 5 × 10^8 CFU/mL, the movement of the spot of the marker actinobacteria introduced was reliably observed against the background of aboriginal actinobacteria (Fig. 3c). It took the weakly pronounced bacterial spot 30 min to shift from the flow origin (point 2) to the lower sampling point (point 4, the total distance 109 m). After 30 min, the second, more noticeable peak of the presence of marked cells, (2–3) × 10^3 CFU/mL, was observed at point 4. This pattern of the *Dietzia* cell movement might indicate the possibility of the cells and certain mobile components association (adsorption) in the meltwater flow.

The use of coliphage 9G as a biological marker yielded minimum information. First of all, the phage particle suspension concentration sharply decreased to 2 × 10^4 PFU/mL before introduction into the flow (Fig. 3d). Nevertheless, the distance from the introduction point (point 2) to point 4 was covered by the weakly pronounced spot of phages in 25 min. A com-

plete absence of coliphages 9G in all the samples from point 3 should be noted, whereas they were present in the samples from point 4 located lower along the flow course. It may be suggested that the coliphage 9G was concentrated in the lower flow course by sorption on certain components of meltwater flow.

The proposed method is based on the use of complex biological markers introduced into the natural area where permafrost thawing is observed and made it possible to assess the propagation rates for microbial cells and phages in the meltwater flow. The data obtained showed, firstly, all the biomarkers to be able to travel a sufficiently long distance (132 m) in meltwater in a limited time (25–35 min) and, secondly, the biological markers possess unique parameters of movement in the meltwater flow. Thirdly, significant differences were revealed in the pattern of the introduced biomarkers' movement from thawing permafrost ice above the reservoir and at the point of meltwater outflow from it. Analysis of the data prompted a preliminary conclusion that movement of microscopic biological objects (and, possibly, their survivability) in permafrost meltwater flows under natural environment is considerably determined by the degree of their adsorption on the structural components in the flow. Accordingly, it is expedient to obtain information about the dynamics of movement in meltwater flows for such insoluble components as carriers of adsorbed microbial cells and phages particles.

The dissemination dynamics of G7C and 9G phages in the flow above the reservoir and at the site of outflow differed significantly. The concentration of 9G phage dropped to the nominally detected level immediately after their being introduced into the meltwater. On the contrary, the coliphage G7C introduced directly on the ice surface was detected at an invariably high concentration in all the samples from the reservoir, as well as in meltwater samples including those taken 24 h after introduction. On the whole, it may be concluded that the coliphage suspensions are easy to use as biomarkers in environmental monitoring studies.

A similar conclusion can be made for the nonpathogenic yeast strain *Y. lipolytica* Y-3603 marked with the red fluorescent protein gene, which have not been used previously in ecological research. An additional advantage of Y-3603 is the possibility of assessing not only the phenotypic characteristics of ~~its~~ colonies but also using a highly selective antibiotic-containing medium: the ability of yeast to grow on medium supplemented with ampicillin, kanamycin, and tetracycline provided for the accuracy of precise detection of this biological marker in the samples from the surface of thawing ice to as far as the Aldan River.

The interest in the use of the actinobacterium *Dietzia* sp. L18 in this investigation was explained by the fact that it represented a component of the microbiota of relic permafrost ice wedges (unpublished data) and was isolated from the thawing ice sampled in this region of Mamontova Gora. The use of high cell density actinobacterial suspensions, firstly, enabled evaluation of the dissemination dynamics in the thawing water even against the background of aboriginal actinobacteria and, secondly, supported the conclusion that the movement of microobjects in the meltwater flow may be influenced by their sorbed or planktonic state. For further use of pigmented actinobacteria, application of genetically marked strains is much more advisable.

Analysis of the obtained data allowed the following conclusions.

Each of the four marker biological objects had its own parameters of movement in the meltwater flow. The presence of the reservoir on the way of the meltwater flow determined the successfulness of adsorption of the biological objects on insoluble flow components. The information about the movements of such components may give additional material about the dynamics of movement of thawing microbial cells and phages.

Comparison of the dynamics of movement and distribution of coliphages G7C and 9G introduced before the entrance into the reservoir and at the site of outflow accordingly shows that, only in the first case, the coliphage was present in the flow for 24 h, whereas in the second case, the concentration decreased to the nominally detected level almost immediately after the introduction. Hence, careful enumeration and surveillance studies at all points on the way of meltwater flows as niches of accumulation of thawing biological objects are needed.

On the whole, we should state the expediency of the use of a new integrated method of biological marking for monitoring the dissemination of thawing microbiota in meltwater flows, as well as the necessity for further research in this direction. In particular, it is necessary to look into a relationship between the capacity of microbiological objects (cells and phage particles) for lateral surface displacements and the bioobjects sorption on the structural mineral components of the flows.

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