Metagenomic analyses of the late Pleistocene permafrost – additional tools for reconstruction of environmental conditions

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Abstract

A comparative analysis of the metagenomes from two 30,000 year-old permafrost samples, one of lake-alluvial origin and the other from late Pleistocene Ice Complex sediments, revealed significant differences within microbial communities. The late Pleistocene Ice Complex sediments (which have been characterized by the absence of methane with lower values of redox-potential and Fe^{2+} content) showed both a low abundance of methanogenic archaea and enzymes from the carbon, nitrogen and sulfur cycles. The metagenomic and geochemical analyses described in the paper provide evidence that the formation of the sampled late Pleistocene Ice Complex sediments likely took place under much more aerobic conditions than lake-alluvial sediments.

1. Introduction

Permafrost, including constantly frozen sediments of the Arctic, is a unique subsurface complex environment where microorganisms retain viability over a long period of time, from thousands to millions of years (Gilichinsky and Rivkina 2011). The impact of climate change on permafrost stability has recently been discussed widely by the scientific community (Zimov et al 2006, Walter et al 2007, Anthony et al 2014). The permafrost deposits of the North-East Siberia, which did not thaw during the Holocene climatic optimum, have attracted particular interest, especially the late Pleistocene Ice Complex deposits (Yedoma Suite) that are widespread on the Kolyma-Indigirka lowland (152-162°E, 68-72°N) (Schirrmeister et al 2011). Earlier we found that the epigenetically (consecutively) frozen sediments of both lake and marine origin (independent of age) contain biogenic methane, whereas methane was either absent or present at trace concentrations in samples from the sincryogenic (simultaneously frozen) late Pleistocene Ice Complex (Rivkina et al 2007, Rivkina and Kraev 2008). However, low concentrations of methane have been detected in the Yedoma deposits located both far south in boreal forest (Brouchkov and Fukuda 2002) and near the Arctic coast in Lena Delta (Brouchkov and Fukuda 2002, Bischoff et al 2013). The presence of biogenic methane in the Ice Complex deposits located near the coast of the Arctic Ocean was also confirmed by our research conducted in 2013 on the Gydan Peninsula (unpublished data).

Anaerobic microcosm incubation of thawed permafrost samples in a carbon dioxide- and hydrogen- enriched atmosphere showed methanogenic activity in epicryogenic sediments only, while this process was not observed in samples from the sincryogenic late Pleistocene Ice Complex (Rivkina and Kraev 2008). Similar results were obtained during experiments with radioactively labeled substrates (Rivkina et al 2002, Rivkina et al 2004, Rivkina et al

2007), showing an absence of methanogenic activity in the sincryogenic late Pleistocene Ice Complex samples with this process evidently taking place in epicryogenic sediments of lake or lake-alluvial origin. Moreover, only from the latter sediments methanogenic archaea were isolated in pure culture (Rivkina et al 2007, Krivushin et al 2010, Shcherbakova et al 2011). Viable methanogens were also isolated from the permafrost-affected soils located in the Lena River Delta region (Morozova et al 2007, Wagner et al 2013).

The specific distribution of methane and methane-producing microorganisms in permafrost raises number of questions; for example, why the sediments of the late Pleistocene Ice Complex in Kolyma–Indigirka Lowland do not contain methane or methanogenic activity. To answer this question, it is thought that the application of new methodologies such as metagenomic analyses is required (Graham et al 2012, Jansson and Tas 2014). Until recently, the determination of microbial diversity in low biomass environments, including permafrost, was problematic. The microbial cell abundances in the ancient permafrost is 10–100 times lower than that in the active layer samples, thereby resulting in low yields of the total community genomic DNA (gDNA) (Yergeau et al 2010). However, using appropriate DNA extraction kits (Vishnivetskaya et al 2014) and the whole-community genome amplification technique (Yergeau et al 2010), a sufficient amount of gDNA can be obtained for next-generation sequencing technologies, producing sequences on an unprecedented scale. Indeed, the first metagenomic analyses of permafrost samples became available recently. Specifically, the analyses of the metagenomes from active layer soil and two-meter deep permafrost samples collected in the Canadian High Arctic and Alaska identified signature genes responsible for hydrogenotrophic and acetoclastic methanogenesis, methylotrophic methane oxidation, nitrification, and carbohydrate degradation (Yergeau et al 2010, Mackelprang et al 2011).

Here we report results of the comparative metagenomic analyses of the two ancient permafrost samples similar in age (*ca.* 30,000 years old), however of different origins (epigenetically frozen lake sediments versus sincryogenic sediments from the late Pleistocene Ice Complex). The results based on comparative analyses of microbial communities from sediments of different origin may be used for evaluation of responses associated with permafrost thawing due to global warming.

2. Materials and Methods

Sample collection and description

Samples were collected within the Kolyma-Indigirka Lowland in northeast Siberia (69°299N, 156°599E) during the summer field season of 2007 (Fig. 1A). Permafrost sediments were sampled using drilling equipment that operates without fluids and prevents down-hole contamination. The sampling technique was tested and described previously (Shi et al 1997). Briefly, the surfaces of the 20- to 30-cm-long cores were cleaned immediately by shaving melted layers out with an ethyl alcohol-sterilized knife and then the frozen internal part of the core was split into 5-cm-long segments; these were placed into sterile aluminum containers and kept frozen during storage in field and transportation to the Institute of Physicochemical and Biological Problems in Soil Science, Pushchino.

The physicochemical characteristics of the samples are presented in Table 1. Methane content was measured as described previously (Rivkina et al 2007, Rivkina and Kraev 2008).

Sample IC4 corresponded to the permafrost sediment of lake origin from the floodplain of the Ambolikha River, borehole DH-4/07, depth of 22.5 m (Fig. 1B). Total carbon concentration was ~1.1% (w/w). Methane content of this sample was 1.2 mmol/kg, $\delta^{13}C$=-

85‰ indicative of biogenic origin. The radiocarbon age of this sample was 30,696 ± 394 years (J–5829) (Kraev et al 2013).

Sample IC8 represented a permafrost soil from the late Pleistocene Ice Complex (Omolon River), borehole DH-2/07, depth of 16 m. Total carbon concentration was ~1.1% (w/w). Methane levels in all samples tested from this borehole were non- detectable (Fig. 1B). The age of this sample was estimated to be ~32,000 years, based on the age determination for the same outcrop which was described recently (Legendre et al 2014).

2.1 DNA extraction and sequencing

In the laboratory, material from the inner part of the permafrost cores was subsampled aseptically for DNA isolation. The gDNA was extracted from eight replicates of ~0.5 g each randomly taken from ~50 g of permafrost core collected at coresponding depth (Figure 1) using the PowerSoil® DNA Extraction Kit (MO BIO Laboratories, Inc., USA). Due to low yield (3-6 ng μl^{-1}), gDNAs from eight replicates were combined, then purified and concentrated using Genomic DNA Clean & Concentrator® Kit (Zymo Research Corporation, USA).

The gDNA sequencing libraries were prepared using NEBNext® reagents (New England BioLabs Inc., USA), according to protocol recommended by the manufacturer, having an estimated peak insert size of 150 nt. Metagenome sequencing was performed at the CRG Genomics Core Facility (Centre for Genomic Regulation, Barcelona, Spain) on an Illumina HiSeq 2000™ machine using Flow Cell v3 with TruSeq SBS v3 reagents and a 2 x 100 cycle sequencing protocol.

2.2 MG-RAST Analysis

Raw sequencing data, i.e., 19.8 Gb representing 143.7 M sequences with an average length of 138 bp for IC4 and 19.7 Gb representing 131.7 M sequences with an average length of 150 bp for IC8, were uploaded to the MG-RAST server (Meyer et al 2008) for gene calling and annotation under ID 4606864.3 for IC4 and 4606865.3 for IC8. A total of 6.6% (IC4) and 3.4% (IC8) sequences failed to pass the quality control (QC) pipeline, whereas 0.3% of total sequences in both data sets were assigned to ribosomal RNA genes.

For functional assignment, protein sequences of putative ORF were searched against the M5NR non-redundant protein database (Wilke et al 2012) with an e-value threshold of $1e^{-5}$, minimum percentage identity of 60%, and minimum alignment length of 15 aa. The taxonomic assignments of Illumina reads were performed against M5NR and M5RNA databases at default parameters. The best-hit classification method was used in both cases for match assessment.

2.3 Comparative metagenomic data analyses

Statistical analyses of the metagenomes were performed in order to compare community composition and functional profiles of the permafrost samples. Statistical significance was calculated using two-sided Fisher's exact test. The differences between proportions were analyzed in STAMP (Parks and Beiko 2010) using MG-RAST taxonomic profile and the Newcombe-Wilson method (Newcombe 1998) at a 95% confidence interval and with Storey's FDR correction (Storey and Tibshirani 2003, Storey et al 2004). Original data sets were deposited at the NCBI Sequence Read Archive (SRA) under the accession numbers SRX763249, SRX751044 (Krivushin et al 2015).

151 **3. Results and Discussion**

152 **3.1 Community description**

153 The gDNA yield was higher in IC8 sample, with an average of 0.5 µg g^{-1} of wet sediment, in
154 comparison to 0.37 µg g^{-1} in the IC4 sample. Efficiency of gDNA extraction from
155 permafrost for the PowerSoil® DNA Extraction Kit (MO BIO Laboratories, Inc., USA) was
156 estimated at 16 ± 8% (Vishnivetskaya et al 2014). Based on the metagenomics data (Angly
157 et al 2009, Raes et al 2007) giving an average genome length of 4.7 Mb for the soil
158 bacterial/archaeal population and an estimated weight of 4.05 fg (Ellenbroek and
159 Cappenberg 1991) for a genome of this size, the theoretical level of the prokaryotic cell
160 populations calculated from the total gDNA recovered accounting average extraction
161 efficiency were 4.3 x 10^8 for IC4 and 5.9 x 10^8 for IC8, including a reduction of the total cell
162 population by the eukaryotic component equal to 25% (Raes et al 2007).

163 The PowerSoil® DNA Extraction Kit (MO BIO Laboratories, Inc., USA) was selected based
164 on its performance and cost per sample. For extractions of gDNA from permafrost, this kit
165 provided similar microbial community structure results with minimal variation among
166 triplicates ($P > 0.05$) (Vishnivetskaya et al 2014). Analyses of metagenomes of the two
167 permafrost samples showed that bacterial genes were dominant and 96.4% and 97.7% of
168 sequences were assigned to the domain Bacteria in the IC4 and IC8 samples, respectively.
169 Archaea were the second dominant domain followed by Eukaryotes, while viruses
170 comprised only 0.06% in IC4 and 0.03% in IC8 samples. Archaea and Eukaryotes were
171 more abundant in the IC4 sample compared to the IC8 sample, i.e., 2.4% versus 1.3% and
172 1.0 versus 0.9%, respectively. While Bacteria were significantly more abundant than
173 Eukaryotes, the functional diversity was higher among the Eukaryotic organisms, which
174 play essential roles in the biology and fertility of soils. A number of detected unparalleled

genes of Eukaryotic organisms including plants, algae, fungi, and protists was 3.4 and 2.6 times higher in comparison to a number of Bacterial unparalleled genes detected in the IC4 and IC8 samples, respectively. The most abundant microorganisms were comprised of nine bacterial and one Archaeal phyla, with Proteobacteria being the most plentiful, followed by Actinobacteria, Firmicutes, Bacteroidetes, Chloroflexi, Planctomycetes, Euryarchaeota, Acidobacteria, Cyanobacteria, and Verrucomicrobia in that order (Fig. 2). The quantities of all other phyla were less than 1%. Sequences affiliated with Actinobacteria ($P < 1e^{-15}$), Firmicutes ($P < 1e^{-15}$), Chloroflexi ($P < 1e^{-15}$), Cyanobacteria ($P < 1e^{-15}$), Acidobacteria ($P < 1e^{-15}$), Verrucomicrobia ($P < 1e^{-15}$) were dominant within IC8, whereas α-, β-, γ-, and δ-Proteobacteria ($P < 1e^{-15}$), Bacteroidetes ($P < 1e^{-15}$), Planctomycetes ($P < 1e^{-15}$), and Euryarchaeota ($P < 1e^{-15}$) dominated within IC4. The microorganisms that are involved into methane formation and methane metabolism, for example methanogenic archaea (phylum Euryarchaeota) or methanotrophic and methylotrophic bacteria (phylum Proteobacteria), were more abundant in more reduced IC4 sample (Table 1). Microorganisms without strong association to methane metabolism (e.g. phyla Actinobacteria and Chloroflexi) dominated in IC8.

3.2 Dependence of the microbial community structure on permafrost characteristics as detected from genus taxonomical level

3.2.1 Methanogenic community. As was shown in previous research (Kraev et al 2013, Legendre et al 2014), methane was not detected in the IC8 sample, but its level in the IC4 sample reached 1-2 mmol/kg; hence, one would expect to find a greater abundance of methanogenic species in the IC4 sample. Indeed, the phylum Euryarchaeota, which contains methanogens, was twice as prevalent in IC4 sample, and methanogenic Archaea constituted as much as 1.5% of the microbial communities in IC4 (compared to 0.5% in IC8). The most

199 abundant methane-producing species was *Methanosarcina* (0.32% in IC4 versus 0.14% in
200 IC8), followed by *Methanoregula* (0.28% versus 0.03%), *Methanoculleus* (0.1% versus
201 0.05%), *Methanosphaerula* (0.1% versus 0.03%), *Methanospirillum* (0.1% versus 0.03%),
202 *Methanosaeta* (0.1% versus 0.03%). Twelve other methanogenic species were detected at
203 <0.1% in IC4 and at an even lower percentage in IC8.

204 **3.2.2 Methanotrophic bacteria**. In the vicinity of the environments where methane is
205 produced, methane-oxidizing (methanotrophic) bacteria can be found (Khmelenina et al
206 2002). Thus, we analyzed the presence of methanotrophic DNA in our data. To date two
207 types of methane-oxidizing bacteria are recognized, Type I methanotrophs belonging to γ-
208 Proteobacteria and type II methanotrophs from α-Proteobacteria. Indeed, the phylum
209 Proteobacteria clearly dominated in the IC4 sample (50.0% versus 26.5% in IC8) with the α-
210 Proteobacteria being more abundant in IC4 (32.7%) in comparison to IC8 (12.3%). On a
211 more refined taxonomical scale the most abundant order of α-Proteobacteria was
212 Rhizobiales (24.2% in IC4 versus 7.7% in IC8), which contains both methanotrophic and
213 nitrogen-fixing bacteria. The α-Proteobacteria Type II methanotrophs (3.32% versus 1.06%)
214 were represented by the genera *Methylocella* (0.53% versus 0.14%), *Methylosinus* (0.24%
215 versus 0.07%), *Methylocystis* (0.22% versus 0.06%), and *Methylobacterium* (2.32% versus
216 0.8%) in IC4 and IC8. The last genus (*Methylobacterium*) is a facultative methylotroph,
217 however, some species are capable of growth on methane. The class γ-Proteobacteria was
218 the most diverse Proteobacteria class (184 species); nonetheless, it was six-fold less plentiful
219 in comparison to α-Proteobacteria. The γ-Proteobacteria Type I methanotrophs, such as
220 *Methylococcus* (0.13% versus 0.11%), *Methylobacter* (0.12% versus 0.05%), and
221 *Methylophaga* (0.02% versus 0.01%) were again more abundant in the IC4 than in the IC8
222 sample. In this Siberian permafrost, Type II methanotrophs dominated over Type I
223 methanotrophs, which is similar to methanotrophic bacteria abundance and diversity in

224 Canadian high Arctic permafrost (Lau et al 2015). Methylotrophs, as well as the subset
225 methanotrophs, play an essential role in the carbon cycle. Interestingly, obligate
226 methylotrophic bacteria belonging to the β-Proteobacteria, such as *Methylibium* (0.17%
227 versus 0.16%), *Methylobacillus* (0.07% versus 0.08%), *Methylovorus* (0.04% versus 0.04%),
228 and *Methylotenera* (0.06% versus 0.05%), were detected in both the IC4 and IC8 samples at
229 equally low levels.

230 **3.2.3 Bacteria of nitrogen cycle**. Another important metabolic process in an environment is
231 the nitrogen cycle. Nitrogen-fixing bacteria were more abundant in the IC4 of the following
232 genera: *Bradyrhizobium* (1.85% versus 0.5%), *Sinorhizobium* (1.3% versus 0.6%),
233 *Rhizobium* (0.82% versus 0.4%), *Rhodospirillum* (0.63% versus 0.3%), *Afipia* (0.4% versus
234 0.12%), the γ-Proteobacteria nitrogen-fixing species. Other species involved in the nitrogen
235 cycle from the *Hyphomicrobium* (2.5% versus 0.23%, capable of denitrification with
236 methanol), *Nitrobacter* (1.9% versus 0.6%, capable of oxidizing nitrite into nitrate) and
237 *Rhodopseudomonas* (3.8% versus 1.2%, capable of carbon dioxide and nitrogen fixation)
238 genera were also more abundant in the IC4 sample. However, ammonia-oxidizing and
239 nitrifying bacteria of the class β-Proteobacteria, such as *Nitrosomonas* (~0.1%), *Nitrosospira*
240 (~0.17%), were detected in both samples at the similar level. Other bacteria involved in the
241 nitrogen cycle are members of the phylum Planctomycetes, many of which conduct
242 anaerobic ammonium oxidation or so-called "anammox" metabolism, a process of ammonia
243 oxidation by nitrite involvement to yield nitrogen gas. Four planctomycetes genera were
244 more abundant in IC4 compared to the IC8 sample with *Plantomyces* (0.63% versus 0.34%)
245 being the most abundant, followed by *Pirellula* (0.61% versus 0.28%), *Blastopirellula*
246 (0.57% versus 0.24%), and *Isosphaera* (0.16% versus 0.13%). Some planctomycetes, e.g.,
247 *Pirellula*, are able to live in environments with high inorganic sulfate concentrations
248 (Glockner et al 2003). Nitrogen-fixing cyanobacteria slightly dominated in IC8 (0.82%) in

249 comparison to IC4 (0.71%); however, the proportion of nitrogen-fixing to total amount of
250 cyanobacteria was higher in the IC4 (55.5%) than in the IC8 (48.6%) sample. Another
251 nitrogen-fixing bacterium dominating in IC8 (2.6%) in comparison to IC4 (0.8%) was the
252 actinobacterium *Frankia*, which is characterized by the ability to engage in a symbiotic
253 relationship with plants, producing nitrogen-fixing root nodules.

254 **3.2.4 Sulfate-reducing bacteria**. The δ-Proteobacteria were also more abundant in IC4
255 (5.8%) than in IC8 (4.5%). Sulfate-reducing bacteria, namely, *Desulfovibrio* (0.49% versus
256 0.41%), *Desulfatibacillum* (0.22% versus 0.08%), *Desulfococcus* (0.18% versus 0.07%),
257 *Desulfobacterium* (0.14% versus 0.04%), *Desulfomicrobium* (0.11% versus 0.06%), and
258 metal-reducing bacteria of the genus *Geobacter* (1.13% versus 0.82%) were more plentiful
259 in the IC4 sample. Two species from the order Syntrophobacterales were more abundant in
260 IC4; these included the strictly anaerobic, sulfate-reducing, propionate-degrading bacterium
261 *Syntrophobacter* (0.33% versus 0.18% in IC8) and the benzoate-degrading bacterium
262 *Syntrophus* (0.62% versus 0.13%). During growth on certain compounds, both of these
263 organisms are known to form syntrophic associations with methanogens, e.g.,
264 *Methanospirillum hungateii*, facilitating methane production (Harmsen et al 1998, Jackson
265 et al 1999). The sulfate-reducing bacterium *Desulfotomaculum* from the phylum Firmicutes
266 was found at similar concentration in both samples (i.e., 0.32-0.33%). However, another
267 stricly anaerobic bacterium *Desulfitobacterium*, which is capable of using a wide variety of
268 electron acceptors, such as nitrate, sulfite, metals, humic acids, and halogenated organic
269 compounds can use hydrogen gas as an electron donor at extremely low concentrations to
270 facilitate sulfate reduction and methanogenesis (Villemur et al 2006) was twice as abundant
271 in the IC4 (0.2%) than in the IC8 sample (0.1%). Sulfate-reducing *Thermodesulfovibrio*
272 species from the Nitrospirae division were found at 0.09% and 0.06% in the IC4 and IC8
273 samples, respectively.

3.3 Similarities and dissimilarities in the microbial communities based on functional annotation

In contrast to the taxonomical assignment, the functional annotation of the metagenomes exhibited a similar structure for the microbial communities. Essential portions of sequencing data ranging from 51.9% in IC4 to 49.1% in IC8 were issigned to unknown proteins, while 37.6% (IC4) and 44.1% (IC8) were annotated proteins. A comparison of the metagenomes at the function level using the SEED (Overbeek et al 2005) genome annotations and profile scatter plot showed that the IC4 and IC8 metagenomes possess >83% similarity at the function level (Fig. 1S). Functional analysis of the IC4 and IC8 metagenomes demonstrated that among the annotated protein sequences, the most abundant groups represented housekeeping functions, including carbohydrate metabolism (16.6% and 17.7%), amino acid biosynthesis (13.8% and 14.8%), and vitamin and protein metabolism (12.1% and 13.2%). Approximately 4.1% and 3.7% of annotated reads were related to stress response including a high proportion of oxidative stress- (52% and 48%) and osmotic stress- (13% and 12%) related sequences (Fig. 3). The functional potential documented through the metagenome provides the information on how many different genes may exist in a sample, but it does not give any indication if these genes are expressed in permafrost. If slow metabolic activity at subfreezing temperatures in permafrost exists as shown in the laboratory studies (Rivkina et al 2000) then such a community may develope adaptation directed by sediments' physicochemical properties.

3.3.1 Methane metabolism. Analyses using KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Kanehisa and Goto 2000) demonstrated that several functional genes directly related to methanogenesis are abundant in the IC4 metagenome (Fig. 4), including genes coding for F420-dependent methylene-H4 MPT reductase (Enzyme Commission

298 number, EC 1.5.99.11), formylmethanofuran dehydrogenases (*fmd*) (EC 1.2.99.5), CoB-
299 CoM heterodisulfide reductases (EC 1.8.98.1), F420-reducing hydrogenases (EC 1.12.98.1),
300 and methylenetetrahydromethanopterin dehydrogenases (*mer*) (EC 1.5.99.9). All of these
301 genes, with exception of F420-dependent methylene-H4 MPT reductase, were also present
302 in the IC8 metagenome though in lower amounts. For example, the gene coding for the
303 enzyme catalyzing the last step of methanogenesis, methyl-coenzyme M reductase (MCR)
304 (EC 2.8.4.1), was represented by 1333 hits in IC4 but only by nine hits in the IC8
305 metagenome.

306 **3.3.2 Nitrogen metabolism.** The abundance of genes associated with nitrogen metabolism
307 was higher in the IC4 metagenome than in that of IC8 (2.2% versus 1.7%). The percentage
308 of genes related to nitrogen metabolism detected in permafrost was slightly higher than the
309 relative abundance (1.3%) of genes responsible for nitrogen metabolism reported in
310 metagenomes examined from glacier ice of the Northern Schneeferner (Simon et al 2009).
311 Notably, in the IC8 metagenome the gene *nifH* coding for a nitrogenase (EC 1.18.6.1),
312 which performs nitrogen fixation, was represented only by seven sequences. These genes
313 were associated mainly with Proteobacteria (43.7%) represented by *Bradyrhizobium* sp.,
314 *Azoarcus* sp., *Mesorhizobium loti*, and Actinobacteria (*Frankia* sp., 41%). In the IC4
315 metagenome 90 features corresponding to *nifH* gene were detected, and these nitrogenase
316 sequences were linked predominantly with Proteobacteria (33.5%). Nitrogenase genes
317 related to Firmicutes (primarily *Desulfitobacterium hafniense* and *'Alkaliphilus*
318 *metalliredigens'*) and Actinobacteria (*Frankia* species) constituted 17.6% and 15%,
319 respectively. Interestingly, *nifH* genes of cyanobacterial origin (primarily linked to *Nostoc*
320 species) were detected exclusively within the IC4 metagenome (12.9%).

Genes connected with the denitrification processes, such as nitrate reductase (EC 1.7.99.4) and nitrite reductase (EC 1.7.2.1) were found in both metagenomes (Fig. 4). The nitrate reductase (*narG*, EC 1.7.99.4) sequences predominating within the IC4 metagenome came from more diverse phylogenetic groups in contrast to *narG* gene from IC8. By contrast, even though nitrite reductase (*nirS*) genes were significantly overrepresented in IC8, their presence was detected in similar phylogenetic groups in both metagenomes (Fig. 5). The sequences related to both nitrite reductase (EC 1.7.1.4) and nitric oxide reductase (EC 1.7.2.5) were found in similar phylogenetic groups in both metagenomes with prevalence in IC4 (Fig. 5).

The ammonium oxidation pathway was represented by a few sequences related to hydroxylamine oxidase (EC 1.7.3.6) genes in known nitrifying bacteria such as *Nitrosomonas eutropha*, *Nitrosococcus oceani*, *Nitrosospira multiformis* and some others. Genes coding for ammonia monooxygenase (EC 1.14.99.39) were not detected in either metagenome by a search with its EC number. However, a search for ammonia monooxygenase using functional hierarchies such as KEGG orthologs yielded ten hits in IC4 and four hits in IC8, while SubSystems showed presence of 509 hits in IC4 and 324 hits in IC8. The ammonia monooxygenase sequences were annotated as methane monooxygenase (EC1.14.13.25). It should be noted that the particulate methane monooxygenase and ammonia monooxygenase are related and occur in both methanotrophs and ammonia oxidizers (Holmes et al 1995). These enzymes have wide substrate specificity catalyzing the oxidation of various substrates including ammonia, methane, halogenated hydrocarbons, and aromatic molecules (Arp et al 2002). Overall, the low abundance of ammonia-oxidizers presumably represents the rare utilization of this pathway by permafrost bacteria in studied environments. Similar results were also reported for metagenomes from other cold

345 environments, e.g., high Arctic hypersaline subzero spring (Lay et al 2013) and Arctic snow
346 packs (Larose et al 2013).

347 **3.3.3 Sulfur metabolism.** Sequences associated with sulfur metabolism were present in both
348 IC4 and IC8 metagenomes and related to both reduction and oxidation (Fig. 6). Genes
349 coding for sulfate reduction were more abundant in the IC8 metagenome including genes for
350 sulfate adenylyltransferase (EC 2.7.7.4), phosphoadenylyl-sulfate reductase (EC 1.8.4.8),
351 and ferredoxin-sulfite reductase (EC 1.8.7.1). Taxonomic distribution of associated species
352 was similar in both metagenomes with the exception of sulfate adenylyltransferase, which
353 was represented in IC4 by sequences related mainly to Proteobacteria and Actinobacteria. In
354 the IC8 sample this gene was of more diverse phylogenetic origin (Fig. 6). A few sulfur
355 oxidation genes detected were associated with *Renibacterium salmoninarum* and *Gordonia*
356 *bronchialis* in IC8 and *Mycobacterium* species and *Sinorhizobium meliloti* in IC4.

357 **3.3.4 Stress response.** Genes associated with stress response were detected in both of the
358 metagenomes (33683 hits in IC4 metagenome and 28557 in IC8). The three most abundant
359 groups present corresponded to oxidative stress, heat shock, and osmotic stress response
360 genes. Sequences related to oxidative stress originated principally from Proteobacteria,
361 Actinobacteria and Firmicutes (5148 features in IC4 and 3832 features in IC8) and included
362 genes for catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), and different superoxide
363 dismutases (EC 1.15.1.1.). Their occurrence is presumably explained by increased oxygen
364 solubility at low temperatures and associated increase of reactive oxygen species
365 concentration (Chattopadhyay 2006). Sequences related to osmotic stress were represented
366 by the genes involved in the synthesis and uptake of compatible solutes including choline,
367 betaine, periplasmic glucan, and ectoine. Genes for osmoprotectant ABC transporters were
368 also detected. Choline dehydrogenase (EC 1.1.99.1) (222 features in IC4 and 213 features in

IC8 mainly from Proteobacteria and Actinobacteria) and betaine-aldehyde dehydrogenase (EC 1.2.1.8) (166 features in IC4 and 186 features in IC8 from Proteobacteria, Actinobacteria and Firmicutes) were the most abundant enzymes of this class. This emphasizes the importance of betaine osmolyte for the osmoprotection of members in microbial communities from subfreezing environments. The genes encoded the heat shock proteins were mainly represented by the chaperone protein DnaK (816 hits in IC4 and 54 in IC8) and its interacting protein DnaJ (759 hits in IC4 and 67 in IC8). These proteins are among the most plentiful chaperons in the bacterial cell and often prevalent in microorganisms from cold environments (D'Amico et al 2006).

3.4. Implication of metagenomic sequencing data for the analysis of the samples origin and evolution.

The application of biological markers for paleo-reconstructions in various environmental sites has been used occasionally. For example, utilization of lipid analyses for petroleum reservoirs formation and maturation (Seifert and Moldowan 1981); analysis of fossil chironomid assemblages in the Holocene lake-sediment cores (south-central Alaska) for evaluating anthropogenic climatic changes and quantitative paleo-temperature reconstructions (Clegg et al 2010); and analyses of fossil ostracodal assemblages from the Arctic seas for reconstruction of coastline and interpretation of environmental differences in Arctic areas (Stepanova et al 2010). To track the occurrence and distribution of microorganisms in the environment, the gDNA and DNA fragments amplified with PCR can be employed as biological markers. Therefore, we anticipated that integration of the next generation sequencing capabilities and approaches of microbial ecology (such as linking microbial community composition and environmental processes involved in carbon, nitrogen and sulfur cycling) could be used for paleo-reconstructions.

393 The late Pleistocene Ice Complex or Yedoma deposits are widely distributed in northeast
394 Siberia and because of their wide occurrence on the Kolyma lowland, these deposits may
395 play a significant role in climate warming, permafrost degradation and greenhouse gases
396 emission. A question, which processes formed Yedoma has been under dispute in the last
397 several decades. Several hypotheses have been proposed about the origin of the late
398 Pleistocene Ice Complex, including eolian (Tomirdiaro et al 1984, Tomirdiaro and
399 Chernen'k'ii 1987), alluvial (Rozenbaum 1981), and polygenetic (Konishchev and
400 Kolesnikov 1981, Sher et al 1987) formation. Different opinions on the origin of these
401 deposits have been summarized in the recent publications of Lutz Schirrmeister and co-
402 authors (Schirrmeister et al 2011, Schirrmeister et al 2013). The researchers suggested that
403 the ice rich syngenetic permafrost of the late Pleistocene Ice Complex was developed under
404 a cold-arid climate at less hydromorphic conditions than the lake and lake-alluvial
405 sediments. In general, Yedoma sediments have been characterized by the absence of
406 methane (Rivkina et al 2007, Rivkina and Kraev 2008) and much lower redox-potential and
407 iron (Fe^{2+}) content in comparison to permafrost layers of lake and lake-alluvial origin
408 (Rivkina et al 2006).

409 A comparison of the two late Pleistocene permafrost metagenomes of different genesis, IC4
410 and IC8, revealed differences in the composition of the microbial community that reflects
411 the conditions under which these deposits were formed. These data uncovered significant
412 distinctions in microbial community compositions between Yedoma and lake-alluvial
413 sediments. The relatively low abundance of methanogenic archaea, limited presence of
414 enzymes from the carbon, nitrogen, and sulfur cycles, as well as the presence of
415 methanotrophic bacteria could explain the absence of methane in Yedoma deposits and
416 provide evidence that the formation of these sediments took place under much more aerobic
417 conditions. In other words, we may assume that during the late Pleistocene period, nearly

418 30,000 years ago, different environmental conditions predetermined biogeochemical regimes
419 and composition of microbial communities of the studied samples. Similarly to our study the
420 recent survey of soil taxonomic, phylogenetic, and functional diversity have demonstrated
421 that metagenomic approaches can be used to build a predictive understanding of variations
422 in microbial diversity and functions across terrestrial biomes (Fierer et al 2012). Thus, the
423 involvement of metagenomic analyses, along with geological and biogeochemical methods,
424 may be used for characterization of the permafrost microbial community, its contribution
425 during climate warming and permafrost thawing, as well as it may become an additional
426 instrument in paleo-reconstructions.

427 **4. Conclusions**

428 In the current study, we performed a comparative analysis of the two permafrost samples
429 isolated from lake sediments and ice complex with utilization of metagenomic sequencing
430 approach. We have shown that the presence of methane in one sample and its absence in the
431 second are associated with the prevalence of the certain groups of microorganisms and their
432 corresponding genes. The possible scenario of the development of both Yedoma and lake
433 sediments can be proposed on the basis of these findings. Analysis of metagenome from
434 IC4, a sample of the lake sediments, demonstrated occurrence of many physicochemical
435 reactions such as denitrification, iron reduction and sulfate reduction, which could reduce
436 environmental redox potential and ultimately create favorable conditions for development of
437 methanogenic community and methanogenesis. As reflected from the composition of IC8
438 metagenome, the sporadic occurrence of such physicochemical reactions brought to
439 deficiency of methanogenic activity and lack of biogenic methane in the late Pleistocene ice
440 complex on Kolyma-Indigirka lowland.

The obtained results demonstrate that the metagenomic analysis of permafrost microbial communities can represent a valuable instrument for paleo-reconstruction of conditions under which the permafrost sediments were formed in geological perspective. However more studies would be needed before a predictive capability can be achieved with respect to microbial community function after permafrost thaw.

Author contribution:

E.R., L.P. and F.K. designed research; L.S., M.T., L.P., E.R. performed research; L.S. and M.T. extracted DNA; F.K. performed DNA sequencing; E.R., L.P., K.K., T.V., A.M. analyzed data; E.R. and F.K. contributed new reagents and analytical tools; E.R., L.P., T.V. wrote the paper.

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