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Genetic potential for methane metabolism in the Greenland subglacial ecosystem

Genetický potenciál pro metabolismus metanu v grónském subglaciálním ekosystému

Master thesis

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Prohlášení:

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Abstract

Subglacial environments, located at the interface of glacier ice and bedrock, represent one of the major ecosystems associated with glaciers and ice sheets. This environment contains liquid water and underlying sediment with large amounts of organic matter overridden during periods of ice advance. Large areas of subglacial sediment are exposed annually by glacier melting and retreat, which affects the subglacial carbon stores and may significantly impact regional carbon fluxes. Due to the widespread anoxia at glacier beds, subglacial environments are potential producers and reservoirs of methane that can be released into the atmosphere as a consequence of glacial retreat. While the presence of methanogens and methane oxidisers has been shown based on 16S rRNA gene data, no data on the functional genetic potential for methane metabolism currently exist. In this study, the first subglacial metagenomes obtained from subglacial sediment exported from beneath the Greenland ice sheet by a meltwater river were used to characterize the metabolic potential for methane metabolism by the identification and quantification of genes involved in methane production and consumption. Functional genes of potential syntrophic pathways were also investigated. Functional genes of methanogenesis/anaerobic oxidation of methane and methanotrophy were detected in all four metagenomes. The data provide strong evidence for the presence of methanotrophic organisms within the family *Methylococcaceae*, which indicates the presence of methane as a substrate. Hydrogenotrophic methanogens were also identified, albeit in lower quantities, while no taxa potentially capable of methane oxidation under anaerobic conditions were detected. The presented data broaden our understanding of the functioning of the subglacial ecosystem and bring a fresh look at the production and recycling potential of methane at glacier beds.

Key words: subglacial ecosystem, glaciers and ice sheets, microbial activity, organic matter, carbon cycling, methane

Abstrakt

Subglaciální prostředí se nachází na rozhraní ledovců a jejich podloží. Představuje jeden z hlavních ekosystémů spojených s ledovci a ledovcovými pokrývkami. Zahrnuje tekutou vodu i jemný materiál rozdrcený pohybem ledovců obsahující organický materiál. Velké oblasti subglaciálního sedimentu jsou každoročně obnaženy kvůli tání a ústupu ledovců, což ovlivňuje subglaciální zásoby uhlíku a může mít významný dopad na regionální toky uhlíku. Vzhledem k rozšířené anoxii v ledovcových podloží jsou subglaciální prostředí potenciálními producenty a zásobárnami metanu, který může být uvolněn do atmosféry v důsledku ústupu ledovců. Zatímco přítomnost metanogenů a organizmů oxidujících metan v subglaciálním prostředí byla ukázána na základě 16S rRNA genových dat, v současné době neexistují žádná data o funkčním genetickém potenciálu metabolismu metanu. V této studii byly poprvé využity metagenomy, získané ze subglaciálního sedimentu exportovaného řekou zpod Grónského ledovce, k charakterizaci metabolického potenciálu metabolismu metanu a identifikaci a kvantifikaci genů zapojených do produkce a spotřeby metanu. Byly také zkoumány funkční geny potenciálních syntrofních drah. Funkční geny metanogeneze/anaerobní oxidace metanu a metanotrofie byly zaznamenány ve všech čtyřech metagenomech. Výsledky analýz poskytují přesvědčivý důkaz o přítomnosti metanotrofních organismů z čeledi *Methylococcaceae*, což naznačuje přítomnost metanu jako substrátu. Přítomnost hydrogenotrofních metanogenů byla rovněž zaznamenána, byť v nižších množstvích, zatímco žádné taxony potenciálně schopné oxidace metanu za anaerobních podmínek nebyly nalezeny. Prezentovaná data rozšiřují naše chápání fungování subglaciálního ekosystému a přinášejí nový pohled na potenciál tvorby a koloběhu metanu v podledovcových ekosystémech.

Klíčová slova: subglaciální ekosystém, ledovce, mikrobiální aktivita, organická hmota, cyklus uhlíku, metan

Contents

1. Introduction

The subglacial environment comprises the interface of glaciers and ice sheets and their bedrock and represents one of the major ecosystems associated with glaciers (Wadham et al. 2004). Microbial communities play significant roles in carbon cycling in these systems and increasing attention has been paid to biogeochemical processes in the subglacial environment due to their possible global impacts (Wadham et al. 2008, 2019). The subglacial microorganisms are adapted to low temperature, eternal darkness, and low nutrient contents of glacier and ice sheet beds (Margesin & Miteva 2011), and the end product of their carbon metabolism is methane, a potent greenhouse gas (Stibal et al. 2012, Christiansen & Jørgensen 2018, Lamarche-Gagnon et al. 2019).

Despite the potential importance of glaciers and ice sheets in present and past carbon cycling, microbial communities and their functions in subglacial ecosystems have not been sufficiently described and understood. The fast development of molecular microbiology methods means new approaches may be used to gain insights into the functioning of the microbe-dominated subglacial ecosystems. In this thesis, a functional gene analysis of the first metagenome obtained from a Greenland ice sheet meltwater river is presented, focusing on methane cycling and the metabolic potential of the organisms associated with these processes.

2. Aims

The main aim of this thesis is to create a picture of the potential metabolism of methane in the subglacial environment of the Greenland ice sheet. To do this, we analyze a unique set of Greenland ice sheet subglacial metagenomes, focusing on identifying the functional genes involved in the production and consumption of methane as well as the genes for possible

syntrophic metabolisms. We quantify individual genes of methane metabolism pathways, identify their owners by comparing the gene sequences to available database records, and put the results into context of the available environmental data.

3. The subglacial ecosystem

Glaciers and ice sheets represent an integral part of our planet. At present, more than one tenth of the world's terrestrial surface is covered by ice, and its importance was even greater in the past. For example, during the last glacial maximum (33,000 years before present) the area of glaciers and ice sheet was three times larger than today (Clark et al. 2009).

Once thought to be abiotic, glaciers and ice sheets are now recognized to host distinct ecosystems, mostly dominated by microorganisms. One of the major ecosystems associated with glaciers and ice sheets is the subglacial ecosystem, situated at the interface between glacial ice and the bedrock. Subglacial habitats harbor phylogenetically and functionally diverse microbial communities that face severe environmental challenges, such as low temperature, the low availability of liquid water, eternal darkness, and low nutrient concentrations (Cavicchioli et al. 2002). As a response, the subglacial microorganisms have developed a number of adaptations for survival in this inhospitable ecosystem.

3.1. Low Temperature

The first problem that microorganisms are facing in subglacial environments is low temperature. Low temperatures below 5 °C are common in a significant portion of planet Earth, and permanently low-temperate environments can be found in deep oceans, snow, permafrost, sea ice, glaciers, and ice sheets. Some organisms - called psychrophilic or psychrotolerant - can live and even prosper in this kind of environment.

During their evolutionary path psychrophiles have evolved several adaptations that help them to be functional in cold environments. These adaptations can also complement each other to increase functionality (Margesin & Miteva 2011). One of the crucial factors that are

important for living in cold environment is the fluidity of cell membrane. Rigidity of cell membrane that reduce functionality in cold environments is a common problem that subglacial microorganisms face. This problem can be solved by the multiplication of unsaturated fatty acids which leads to decreasing the average fatty acid chain length and increasing membrane fluidity (Metz et al. 2001). Another adaptation for low temperature is the production of anti-freezing proteins. Some bacteria are able to reduce destructive effect of crystallization by producing this type of proteins. Antifreeze proteins or ice structuring proteins are polypeptides and they bind to small ice crystals to inhibit growth and recrystallization of ice that would otherwise be fatal. This creative way of living helps them to be functional even during extreme life conditions (Gilbert et al. 2005). Producing cold-adapted enzymes is another way to cope with a cold environment. The main goal of these enzymes is to increase flexibility of cell which helps them to be active in specific cold environment conditions (Siddiqui & Cavicchioli 2006, Brizzio et al. 2007).

3.2. Liquid water

The availability of liquid water is a key environmental factor for active life. There are several ways how liquid water can be generated in the subglacial environment and become available for microorganisms. First, surface meltwater may reach the basal zone of glaciers through moulins and crevasses, and subsequently accumulated at glacier beds (Fountain & Walder 1998, Hodson et al. 2008). Second, glacier ice is an excellent thermal insulator and the heat generated at the glacier bed is primarily used to warm the basal ice and so to generate liquid water. The two dominant sources of heat are viscous heat dissipation due to vertical shearing and friction and geothermal heat that comes form within the Earth (Veen et al. 2007).

Water does not only appear in the subglacial environment as a thin layer between the glacier and glacier base but can also create large lakes. For example, several hundred subglacial lakes have been discovered under the Antarctic ice sheet (Wright & Siegert 2011). Lake Vostok can be considered as one of the best known subglacial which is the largest of Antarctica's subglacial lakes and also one of the largest lakes in the world, being 250 km in length, up to 50 km wide and more than 500 m deep. Subglacial lakes are an established and important component of the basal hydrological system and can be also found under the Greenland ice sheet (Palmer et al. 2013).

3.3. Energy and nutrient sources

The availability of energy and nutrition is another crucial factor influencing life under the glaciers. Minerals are present in the form of fine debris created by the physical weathering of rock covered by glaciers or ice sheets (Rothschild and Mancinelli 2001).

The principal source of organic carbon (OC) for the subglacial ecosystem is organic matter overridden during periods of glacier advance (Fig. 1). Large areas of subglacial sediment are exposed annually by glacier melting and retreat, which affects the subglacial carbon stores (Bardgett et al 2007, Bradley et al 2014) and may significantly impact regional carbon fluxes (Wadham et al 2012). However, not all organic matter found in the subglacial environment is available for microbial communities, and it is likely that only a small portion of organic carbon is further used and processed. While simple soluble organic molecules and relatively labile polymers such as hemicellulose and non-lignified cellulose can be degraded even anaerobically, other polymers such as lignin and lignified cellulose are very difficult to degrade (Miyajima et al. 1997). The composition of subglacial organic substrates depends on the type of ecosystems (e.g. boreal forest and tundra) that were overridden during the last glaciations (Trumbore 2000).

Figure 1: The front of a glacier (terminus) containing basal layers of subglacial material (photo: Jakub Žárský)

The presence of microorganisms beneath glaciers and ice sheets is also dependent on other macronutrients such as nitrogen or phosphorus. While nitrogen is present mainly in the organic form due to the degradation of organic matter or supplied with surface meltwater as nitrate, phosphorus comes from the Earth's crust and is available for microbial communities due to glacial erosion (Wynn et al. 2007, Hodson et al. 2005). Overall, the composition of the bedrock and the material that contains is therefore considered to be the limiting factor for the presence of microorganisms.

A wide range of microbial metabolic processes have been identified in subglacial environments. Chemolithoautotrophs derive energy from reduced compounds of mineral origin and utilize CO_2 as the sole carbon source for growth, and thus may form the base of the subglacial food web. For example, samples from Robertson Glacier, Alberta, Canada show the presence of chemolithoautotrophs that include populations inferred to be involved in iron or

iron sulfide oxidation, including putatively autotrophic *Sideroxydans* spp. and *Thiobacillus* spp. (Boyd et al. 2014, Christner et al. 2014, Hamilton et al. 2013, Mitchell et al. 2013). Heterotrophy is widespread in subglacial environments and it can be found there in many forms. Heterotrophic microbes use organic compounds as their carbon source and obtain energy through the oxidation of these compounds. Organic carbon can be derived from chemolithoautotrophic production and ancient organic matter from the sediments overridden during glacier advance (Mikucki et al. 2009).

Metabolic activities of microbial life are influenced by bedrock composition (Skidmore et al. 2005). When all available oxygen is consumed by aerobic chemolithoautotrophs and heterotrophs, anaerobic respiration takes place and alternative electron acceptors become crucial in subglacial environments (Wadham et al. 2004, Tranter et al. 2005). Nitrate and sulphate reduction have been measured in sediments sampled from the debris-rich basal ice layers of a high Arctic John Evans Glacier (Skidmore et al. 2000). Another type of anaerobic respiration that has been measured in subglacial environment is Fe(III) reduction, identified beneath Taylor Glacier, an outlet glacier of the East Antarctic Ice Sheet (Mikucki et al. 2009). When there is an absence of higher-energy-yielding electron acceptors, fermentative metabolism may occur (Dieser et al. 2014). This heterotrophic type of metabolism may convert organic compounds, such as fatty acids, alcohols and others, to acetate, carbon dioxide, hydrogen, and formate. Afterwards, some microbes may use fermentation products (mostly acetate) as substrates for methane production in the process of acetoclastic methanogenesis (Schink 1997).

4. Carbon cycling at glacier beds

Carbon cycling in subglacial ecosystems is usually studied through the analysis of glacial meltwater exported from subglacial ecosystems (Fig. 2). The origin of dissolved organic carbon exported from subglacial systems varies over the course of the melting season. Early season subglacial discharge has a higher proportion of protein-like and lipid-like elemental formulae, whereas late season subglacial water dissolved organic carbon has a higher proportion of lignin type material. The development of a subglacial outflow is likely to have a significant impact on the origin and type of material flowing from the subglacial in different parts of the season. During the winter period, the dissolved organic material is rather similar to microbial origin, and in the summer period, the terrestrial origin prevails. This feature of the subglacial outflow may indicate the presence of microbial activity during the season (Bhatia et al. 2010).

Figure 2: Subglacial outflow (photo: Jakub Žárský)

Organic carbon is present in the subglacial environment as a complex mixture of compounds from different sources, with different availabilities for the microorganisms. Easily biodegradable substrates include e.g. proteins, n-alkanoic acids, steroids and other functionalized compounds typical of old microbial (cyanobacterial and algal) and plant material (Stibal et al. 2012b) while compounds such as cellulose or lignin are more recalcitrant, but may also potentially be degraded by microorganisms (Miyajima et al. 1997).

Organic matter degradation is a complex process. Often, products of one set of reactions are used as substrates in other reactions. Organic carbon is oxidized by aerobic or anaerobic respiration, depending on the available terminal electron acceptors, or it can be disproportionated during fermentative processes (Fig. 3). Final products of these reactions can subsequently be used by methanogens. However, very little information exists about these intermediate processes in the subglacial ecosystem.

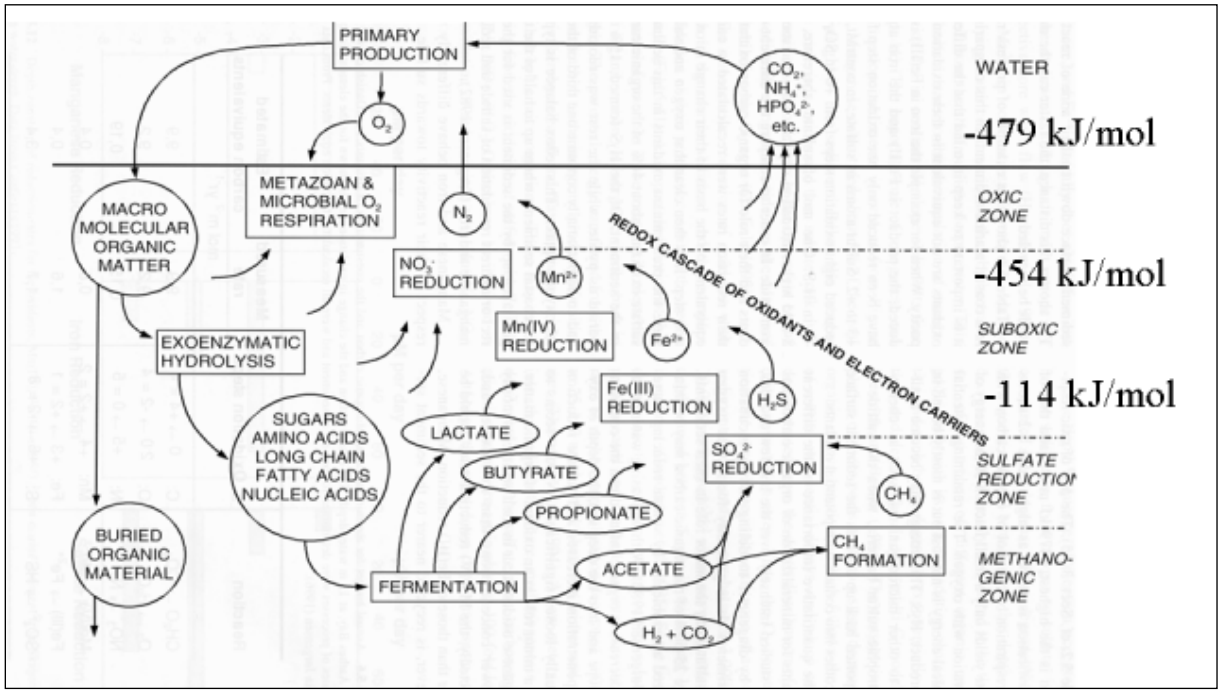

Figure 3: The sequence of redox processes involved in mineralization of organic substances (Jorgensen 2000)

4.1. Methanogenesis

The production of methane is the last step for organic matter degradation in anoxic environments including the subglacial ecosystem (Wadham et al. 2008). Methanogens are a very diverse group of microorganisms, yet they can consume only a limited number of substrates. There are three main types of substrates they can use: CO_2, methyl-group containing compounds, and acetate.

The life of methanogens is influenced by the type of substrates they utilize. The primary type substrate for methanogenesis is CO_2. Most methanogens are hydrogenotrophs that can reduce CO_2 to methane using H_2 as the primary electron donor:

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \qquad \text{(equation 1)}$$

The second choice of electron donor for hydrogenotrophic methanogens is formate. They may also use methyl-group containing compounds, such as methanol, methylated amines (monomethylamine, dimethylamine, trimethylamine, and tetramethylammonium), and methylated sulfides (methanethiol and dimethylsulfide). Acetoclastic methanogenesis is another major pathway of methane production. Acetate -- the most abundant intermediate compound in the anaerobic food chain -- is used as a substrate. The major part of biologically generated methane is likely derived from acetate (Liu & Whitman 2008):

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \text{(equation 2)}$$

Subglacial methanogens may play an important role in carbon cycling in subglacial environments. Moreover, they have been considered as a factor that can influence the Earth

atmospheric system and global carbon cycling (Wadham et al. 2008). While it is known that methanogens occur in subglacial environment by collecting samples from different locations (Boyd et al. 2010, Stibal et al. 2012b), and that microbially produced methane is released from underneath the Greenland ice sheet (Christiansen & Jørgensen 2018, Lamarche-Gagnon et al. 2019) and elsewhere (Burns et al. 2019), the amount of produced methane and its fate are still not fully understood. Further research on this type of ecosystems is, therefore, necessary to increase our knowledge about subglacial environment and processes occurring under glaciers.

Methane is a powerful greenhouse gas emitted by human activities such as leakage from natural gas systems and the raising of livestock, as well as by natural sources such as wetlands, permafrost, subglacial and others. It has a direct influence on climate where methane (CH_4) has 84 times more potent than carbon dioxide (CO_2) as a greenhouse gas over a 20-year timeframe and 25 times more potent over a century on a per unit mass basis (Ruppel & Kessler 2017). 10–20 % of organic carbon produced in soils and sediments is converted to methane, and ~85 % of the annual global methane production and ~60 % of its consumption are based on microbial processes (Reeburgh 2007, Knittel & Boetius 2009). The importance of microorganisms in regulating the amount of methane, as well as processes and main agents are still not fully understood, and a lot of research is still needed to understand them. The subglacial environment could play an important role in releasing methane stocks during the melting of glaciers (Wadham et al. 2008). Recent research has suggested that the Antarctic ice sheet can play a comparable role in releasing methane as an Arctic permafrost, and significantly contribute to the overall methane budget (Wadham et al. 2012).

4.2. Methanotrophy

Methane can be biologically oxidized to CO_2 by microorganisms called methanotrophs. They use methane as a carbon and energy source and they remain an essential component of many natural ecosystems, in particular in areas where methane is produced and subsequently consumed. Atmospheric methane may be consumed by aerobic methanotrophs, and so they influence the cycle of methane on Earth (Hanson & Hanson, 1996). A typical environment for these microorganisms is the boundary of aerobic and anaerobic conditions in wet areas (Sundh et al. 1994). Methanotrophic bacteria often act as a biofilter for diffusing methane from anaerobic zones and so serve as a controlling factor for the potential leakage of methane into the atmosphere. Methanotrophs have already been identified in samples of sediment freshly thawed or flushed from subglacial ecosystems (Achberger et al. 2016, Dieser et al. 2014, Cameron et al. 2017), but their impact on methane cycling in the system is still not fully understood.

Methane oxidation can be divided into two main types - aerobic and anaerobic. They are two completely different processes involving different groups of prokaryotes. Aerobic methane oxidation occurs in the presence of oxygen and aerobic methanotrophs, while anaerobic oxidation of methane (AOM) requires anaerobic methane oxidizers (ANME) and other electron acceptors such as sulphate, nitrate, iron or manganese (Boetius et al. 2000, Raghoebarsing et al. 2006).

4.2.1. Aerobic oxidation of methane

Aerobic methanotrophs belong to several groups of methylotrophic bacteria (Type I, Type II, *Verrucomicrobia*) and use methane as a source of energy and carbon (Hanson & Hanson, 1996).

Type I methanotrophs include the genera *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylosphaera*, *Methylothermus*, *Methylosarcina*, *Methylohalobius*, *Methylosoma*, and *Methylococcus*, which belong to the phylum *Gammaproteobacteria*. Type II methanotrophs (e.g. genera *Methylocystis*, *Methylosinus*, *Methylocella*, and *Methylocapsa*) belong to *Alphaproteobacteria* (Hanson & Hanson, 1996). In 2007–2008 three research groups independently described the isolation of thermoacidophilic methanotrophs that represented a distinct lineage within the bacterial phylum *Verrucomicrobia*. Isolates were obtained from geothermal areas in Italy, New Zealand and Russia (Dunfield et al. 2007, Pol et al. 2007, Islam et al. 2008).

In the process of oxidizing methane to carbon dioxide, the first step is to convert methane to methanol. This reaction is catalysed with the enzyme methane monooxygenase. This enzyme occurs in two forms: a particulate membrane bound form (pMMO) and a soluble cytoplasmic form (sMMO). While pMMO has been found in all aerobic methanotrophs except for the genus *Methylocella* (Theisen et al. 2005), the sMMO is found only in some methanotrophic groups (Murrell et al. 2000). To identify the possible presence of aerobic methanotrophs in a sample, genes encoding these specific enzymes (*pmoA* and *mmoX* for pMMO and sMMO, respectively) may be used as molecular markers. While *pmoA* genes have been sequenced from a considerable number of methanotrophs in different environmental studies, *mmoX* sequences are only available from a small number of studies, therefore only *pmoA* is used for metagenomics. Other functional genes have also been considered to participate on aerobic methanotrophy, but they are not unique to methanotrophs and therefore do not represent suitable genes to be investigated

4.2.2. Anaerobic oxidation of methane

Since the environment under the glaciers provides a large degree of anoxic conditions, anaerobic oxidation of methane (AOM) may occur in subglacial ecosystems. This might affect the amount of present methane, as is the casein subseafloor sediments, where more than 90 % of the produced methane is consumed by AOM. At a global scale, the oceans only make up about 2 % of the global methane emission (Reeburgh 2007) budget due to prokaryotic oxidation of methane in marine sediments and bedrocks before it reaches the water column (Reeburgh 1996). In that case, we might consider AOM in subglacial environments as one of the key processes that affect the amount of methane released into the atmosphere.

Overall, three metabolic groups of anaerobic methane-oxidizing archaea (ANME) were identified: ANME-1 (subclusters a and b), ANME-2 (subclusters a, b, and c), and ANME-3. ANME-1 are related to *Methanomicrobiales* and *Methanosarcinales* forming a separate cluster (Boetius et al. 2000), ANME-2 are related to cultivated *Methanosarcinales* (Hinrichs and Boetius 2002) and ANME-3 are related to *Methanococcoides* spp. (Knittel et al. 2005, Fig. 4). Different groups of ANME have large phylogenetic distance and they are not monophyletic. Also ANME-2a and 2b are distinguished from ANME-2c (Orphan et al. 2001). The wide phylogenetic distribution is reflected in the ecological niche adaptation of the different ANME clades.

ANME perform anaerobic oxidation of methane via reversal of the methanogenic pathway. ANME were first discovered in marine sediments. These microorganisms performed AOM coupled with sulfate reduction (Tab. 1), so they formed consortia with sulfate-reducing bacteria that belong to the Deltaproteobacteria (Boetius et al. 2000, Hinrichs et al. 1999, Orphan et al. 2001).

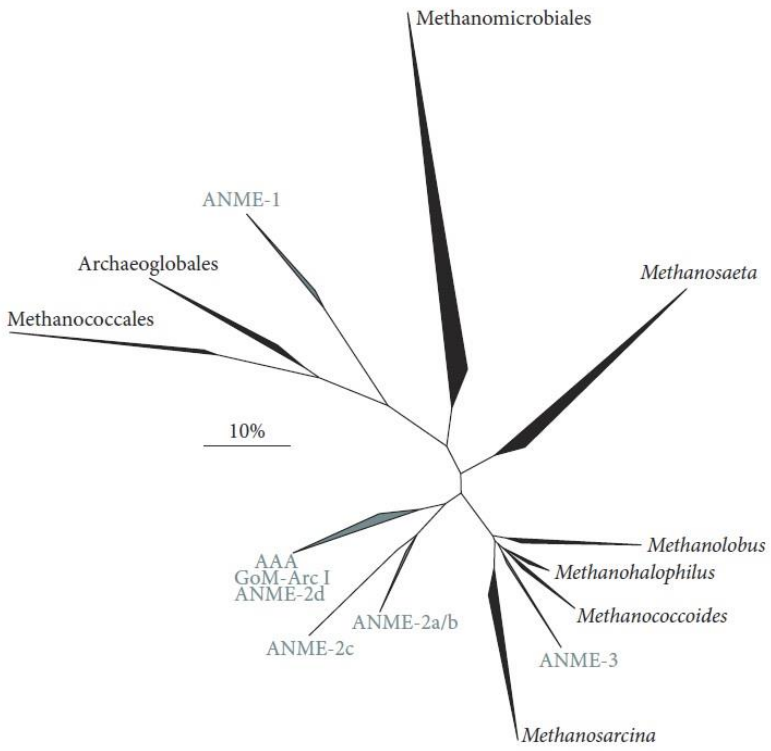

Figure 4: Phylogenetic tree of full length archaeal 16S rRNA sequences showing all methanotrophic clades so far described (grey) (Timmers et al. 2017)

process	stoichiometry	Gibs free energy (ΔG^0, kJml^{-1})
AOM	$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	−16.3
AOM	$CH_4 + 4NO_3 \rightarrow CO_2 + 4NO_2 + 2H_2O$	−517.2
AOM	$CH_4 + 8Fe(OH)_3 + 15H^+ \rightarrow HCO_3^- + 8Fe^{2+} + 21H_2O$	−571.2
AOM	$CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O$	−763.2
AOM	$CH_4 + 4/3Cr_2O_7^{2-} + 32/3H^+ \rightarrow 8/3Cr^{3+} + CO_2 + 22/3H_2O$	−841.4

Table 1: Gibbs free energy changes under standard conditions (ΔG^0) for anaerobic methane oxidation coupled to different electron acceptors (possibly) performed by ANME. (Kim et al. 2018)

The reversed methanogenesis model for AOM has gained support by a metagenomic study on ANME at Eel River (Hallam et al. 2004) and sequencing of an ANME-1 draft genome (Meyerdierks et al. 2010). ANME are described to perform “reverse methanogenesis” (Hallam et al. 2004) which implies the complete reversal of hydrogenotrophic methanogenesis from H_2 and CO_2 (Fig. 5). The enzyme methyl-coenzyme M reductase (encoded by the gene *mcrA*) is

assumed to catalyze the first step of AOM and the last step of methanogenesis and is therefore a marker gene for both processes.

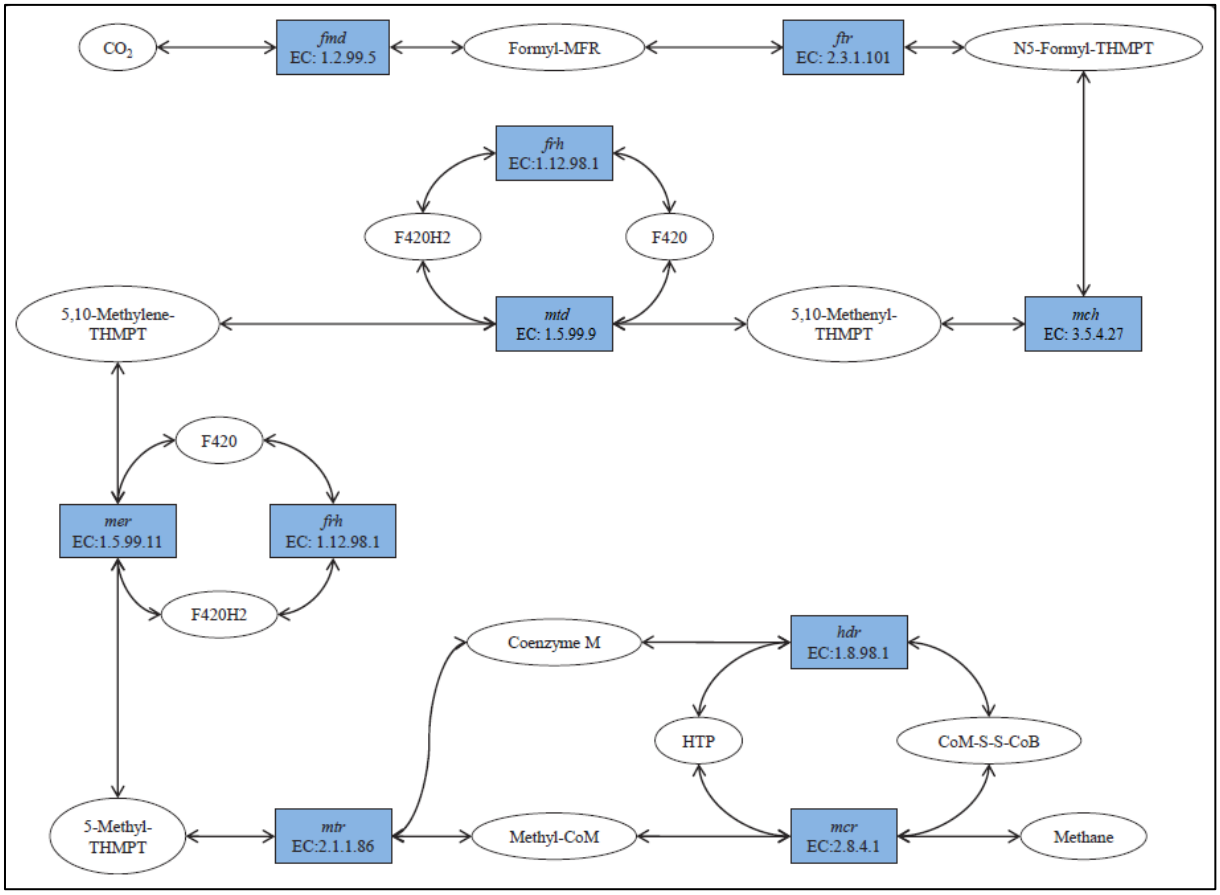

Figure 5: Anaerobic oxidation of methane/methanogenesis pathway. The figure is based on the KEGG-map for methane metabolism and includes the enzymes involved in methanogenesis and reverse methanogenesis (Håvelsrud et al. 2011).

Anaerobic oxidation of methane is critical for controlling the flux of methane from anoxic environments and has been studied mostly in ANME-1 and ANME-2 that work in concert with sulphate-reducing bacteria, a process by which methane is oxidized to carbon dioxide and sulphate is reduced to hydrogen sulphide (Knittel & Boetius 2009).

For sulfate-dependent AOM, marine sediments can be used as a useful analogue. It is not only useful for its permanently cold environment, but mainly due to the stratification of the sediments with respect to their redox conditions and microbial communities. This stratification

could be comparable not due to space stratification, but it is taught as a time scale stratification during ages processes in subglacial sediments.

Marine sediments are permanently cold environment with temperatures below 4 °C so this and it is also inhabited by psychrophilic microorganisms (Knoblauch et al. 1999). Composition of sediments is created by particles accumulating in seafloor from overlying water column. The thickness of this layer is variable (Ryan et al. 2009). The material is used by microorganisms and the most important substrate for them is organic matter which is deposited, degraded and remineralized. This process is influenced by the quantity and quality of organic matter (Hedges et al. 1988, Niggemann et al. 2007). Sediments obtain terminal electron acceptors that play main role in the rate of organic matter remineralization. As the seawater circulates through the oceanic crust, sediments are transferred from midocean ridges. This change could cause sediments containing oxygen, nitrate and sulfate to diffuse upwards from the crust. Furthermore, these sediments also include terminal electron acceptors which are available for organisms (Orcutt et al. 2011).

Since redox potential and availability of terminal electron acceptors controls microbial activity, it is crucial to fully understand them. The presence of terminal electron acceptors in marine sediments is stratified in well-defined zones. The oxygen penetration level in marine sediments is shallow, so most of the methane oxidation takes place at anaerobic conditions with different terminal electron acceptors. Nitrate, manganese and iron reduction occur but sulphate reduction is the quantitatively most significant organic matter remineralization process (Thamdrup & Canfield 1996, Berner 1978, Reeburgh 1983). Methane created by methanogenesis is deeper zones of sediments diffuses upwards to the sulfate zone and it is consumed by anaerobic oxidisers of methane by the process of sulfate reduction (Iversen & Blackburn 1981, Thomsen et al. 2001). This process is likely performed by the yet uncultured anaerobic methanotrophic archaea in syntrophy with sulphate reducing bacteria (SRB). Both

ANME-1 and ANME-2 are associated with sulphur reducing deltaproteobacteria of the *Desulfosarcina/Desulfococcus*-branch (Knittel et al. 2005, Orphan et al. 2001, Boetius et al. 2000). ANME-3 is mainly associated with SRB strains closely related to *Desulfobulbus* (Niemann et al. 2006). Dissimilatory sulphite reductase (*dsrAB*), where adenylylsulphate reductase first reduces sulphate to sulphite before dissimilatory sulphite reductase reduces sulphite to sulphide, is often used as a marker gene for SRB as well as in this study (Knittel et al. 2009).

Nitrate-dependent anaerobic oxidation of methane (AOM) is catalyzed by the anaerobic bacterium *Methylomirabilis oxyfera* that belongs to the NC10 phylum (Ettwig et al. 2010). Nitrate-dependent AOM seems to be catalyzed by an archaeal methanotroph alone that was named *Methanoperedens nitroreducens* and is affiliated to the ANME-2d clade (Raghoebarsing et al. 2006, Haroon et al. 2013). Nitrate reduction to nitrite seems to be located in the pseudoperiplasm and maybe catalyzed by an unusual Nar-like protein complex (Arshad et al. 2015). Bacterial Nar-type nitrate reductase has its active site directed toward the cytoplasm, whereas archaeal nitrate reduction by the Nar enzyme seems to take place at the extracellular side of the cytoplasmic membrane (Yoshimatsu et al. 2000, Martinez-Espinosa et al. 2007, de Vries et al. 2010). The membrane-bound nitrate reductase is comprised of a molybdopterin oxidoreductase domain and twin-arginine signal peptide for translocation across the membrane (*narG*) and iron-sulphur centres (*narH*). The *narGH* genes seem to have been acquired laterally from a bacterial donor (Haroon et al. 2013). These genes were used to identify nitrate-dependent AOM in this study.

Iron or manganese – dependent AOM Iron or manganese – dependent AOM might also occur in subglacial environments and play a role in methane cycling. But evidences for iron or manganese – dependent AOM have been found only in marine sediments (Egger et al. 2015, Treude et al. 2014, Riedinger et al. 2014). Organisms responsible for metal-dependent AOM

were not identified in these studies. It was speculated that methanogenic archaea, and *Methanohalobium*/ ANME-3 could be responsible for iron-dependent AOM (Oni et al. 2015). Other researchers speculated that either ANME- 1 or *Methanococcoides*/ANME-3 together with a bacterial partner were responsible for manganese-dependent AOM (Beal et al. 2009). However, processes of iron/manganese-dependent AOM have common enzymes with other metabolic pathways and therefore cannot be selected for them marker genes to identify these pathways in subglacial environment. More research is needed for a better understanding this problematic.

5. Materials and methods

5.1. Sampling

Sampling of proglacial rivers draining subglacial catchments offers an indirect access to the subglacial system of land terminating glaciers (Wilhelm et al. 2013). During the ablation season, surface meltwaters are routed to the glacier's bed, flushing subglacial flours (sediments), basal waters, and concomitantly, cells, to glacial margins and proglacial landscapes (Sharp et al. 1999, Skidmore et al. 2000).

5.1.1. Location description

Samples for metagenomic data analysis were taken from polythermal-based, land terminating outlet glacier from Greenland Ice Sheet (GrIS) which has subglacial drainage systems that develop seasonally (Bartholomew et al. 2011). Data were collected from the proglacial river exiting Leverett Glacier, (LG; 67.06N, 50.17W, Fig. 6) from May to July 2015 to capture the composition of melt- waters exiting the glaciers during the summer ablation season. LG is a

large glacier, with a hydrologically active catchment of around 600 km^2 (Cowton et al. 2012), which feeds into Watson River and then into the Davis Strait via the Søndre Strømfjord (Hawkings et al. 2016). The composition of bedrock beneath LG is dominated by Precambrian Shield crystalline gneiss and granite, which is representative of the majority of GrIS (Escher & Watt 1976) and much of the bedrock that was covered by the Eurasian and North American Ice Sheets (Bouysse 2014).

The LG acts as the main outlet to the Russell-Leverett catchment, one of the three large GrIS hydrological basins of the Kangerlussuaq area in South West Greenland (Lindbäck et al. 2015). Many studies have been written about ice-dynamics, hydrology and subglacial biogeochemistry in the last decade and thanks (Bartholomew et al. 2010, Bartholomew et al. 2011, Cowton et al. 2012, Chandler et al. 2013, Tedstone et al. 2013, Hawkings et al. 2016) to its geology it could be considered as a representative area common to much of Greenland (Dawes 2009). LG has polythermal conditions, common conditions for much of the Western margin of the ice sheet which is considered thawed at the bed (MacGregor et al. 2016).

5.1.2. Pre-melt season/ upwelling stage sampling

Earlier samples (May 04-13), used for metagenome sample number 1, were collected beneath the frozen proglacial river through boreholes and a chainsawed hole in front of the LG prior (~20 m downstream of the then-closed subglacial portal) to the onset of the melt-season and a set of samples were collected on June 7th from a subglacial upwelling through river ice ~ 50 m from the glacier's terminus.

5.1.3. Outburst period sampling

The remainder of the samples used for metagenome samples 2, 3, and 4 were collected approximately 500 m downstream from the LG portal. The evolution of the subglacial drainage system at LG, and (its) connectivity to the proglacial river, can be inferred from changes and evolution in discharge, turbidity and other features. The LG has seasonal hydrological features that show episodic and abrupt subglacial "outburst" events, which flush out high concentrations of subglacial sediments from hydrologically poorly connected parts of the ice sheet bed, sampling during the outburst period, therefore, allows us to better capture microbial information from more isolated (e.g. distributed) parts of the glacial bed.

The series of four pulses in suspended sediment concentrations (SSC) between June 19 to July 15 reflect the rapid drainage of supraglacial lake waters to the base of the glacier (Bartholomew et al. 2011). This massive penetration of supraglacial water into the subglacial environment mechanically disrupted the bed of glacier, which subsequently caused the flushing out of the isolated parts of the iceberg. In these isolated parts of subglacial system, contact between rock and water was longer and less disturbed during the time. The result of this process of flushing out the subglacial material was the series of subglacial outburst events (Nienow et al. 1998, Tranter et al. 2002). Outburst water events are characterized by stronger chemical weathering signal. The export of subglacial material from more remote sources can also be inferred by the export of suspended sediments bearing an older particulate organic carbon (POC) content during that period (Kohler et al. 2017). Lastly, the hydrological period following the last outburst at LG (after July 15) is reflective of a fully expanded, efficient and channelized drainage system (Hatton et al. 2019).

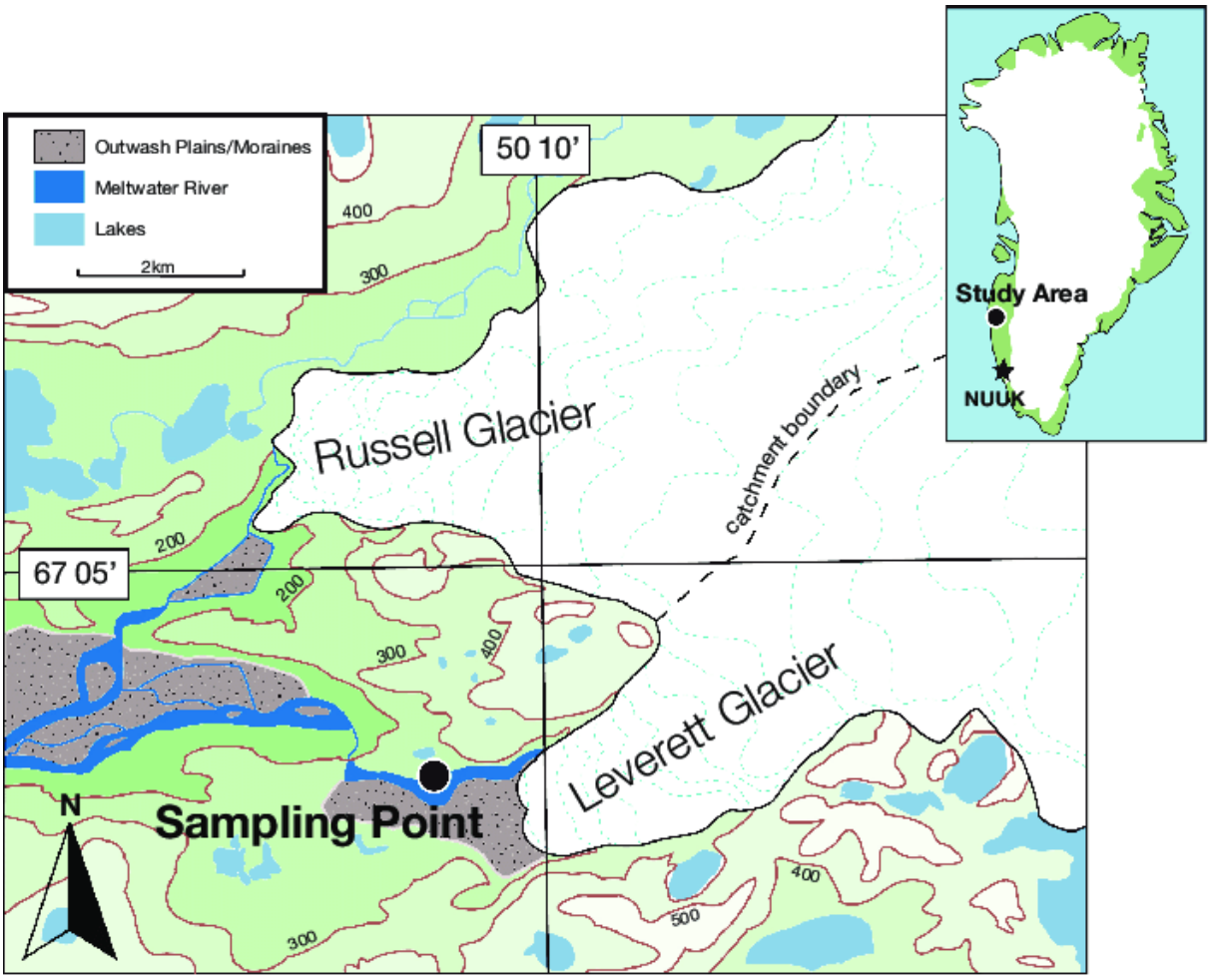

Figure 6: Map of the terminus of Leverett Glacier (sampling location is noted) and context within the wider Greenland Ice Sheet (Hawkings et al. 2016).

Overall, the sampling of the LG subglacial outflow can be divided into several different stages: "pre-melt" and "upwelling" stages (June 1-18), "outburst", which we divide into two: "early outburst" stage (June 19 – July 3) and "late outburst" and "post-outburst", or channelized, (July 15th onward, Fig. 7).

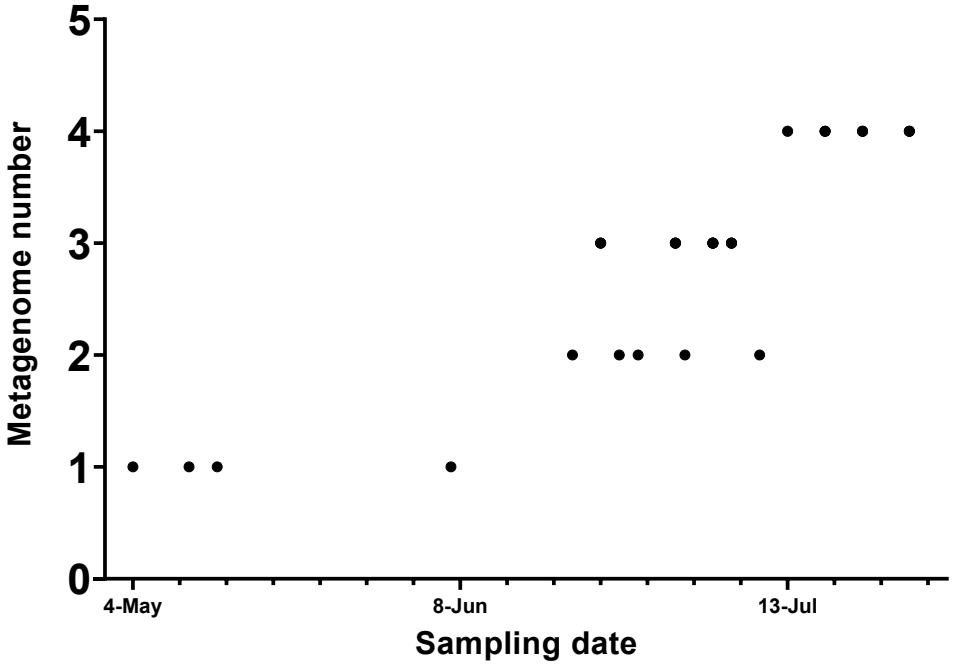

Figure 7: Schematic of samples collected from the LG outflow and their pooling into metagenome samples

5.2. Metagenome analysis

Metagenome studies provide a set of DNA sequences, reflecting genetic makeup of living organisms and help to identify genetic content of all microbiota members in a natural habitat. By offering direct access to the entire genetic make-up of microbial communities, metagenomics can provide valuable molecular insights into genomic linkages between community function and structure. In this study were used metagenomics to characterize the metabolic and taxonomic potential for methane metabolism of the subglacial environment of the LG catchment. Four metagenomes from different stages of pre-melt or during melting season were sequenced. To gain insight into the metabolic potential the metagenomes were searched for marker genes associated with methane metabolism. By avoiding PCR amplification and primer target specificity, the metagenomics approach offered further insight

into the taxonomy and metabolic potential of the prokaryotic communities of the methane seep sediments.

For the metagenome analysis, DNA was extracted from pooled samples (Fig. 6) using the DNeasy PowerWater Sterivex kit (MoBio Laboratories, USA) following the manufacturer's protocol. A total of at least 20 ng of treated DNA (Tab. 2) served as the input for the TruSeq Nano DNA library preparation kit (Illumina). Libraries were sequenced on an ILLUMINA HISEQ2000 at Bristol University's Genomics Centre, UK, to generate 150-base paired-end reads.

Sample number	**amount of DNA (ng)**
1	232
2	28
3	20
4	49

Table 2: Amounts of extracted DNA throughout samples 1-4.

The reads were quality trimmed by removing adapters with Trimmomatic (v 0.27) using Illumina TruSeq2-PE adapters with a seed mismatch threshold, palindrome clip threshold, and simple clip threshold set at 2, 30, and 10, respectively (Bolger et al. 2014). Furthermore, sequencing reads were filtered by base call quality using the FASTX-Toolkit (http://hannonlab.cshl.edu/ fastx_toolkit/index.html), specifically fastq_quality_filter, with the following parameters: -Q33 -q 30 -p 50. Resulting sequences were normalized using methods previously described in (Howe et al. 2014, Pell et al. 2012) and Khmer (v 0.7.1) and command normalize-by-median.py with the following parameters: -k 20 -C 20 -N 4 -x 50e9. Next, errors were trimmed by removing low abundance fragments of high coverage reads with Khmer and command filter-abund.py -V. The paired-end assembly of the remaining reads was performed with the Velvet assembler (v 1.2.10, -exp_cov auto -cov_cutoff auto -scaffolding on (Zerbino

& Birney 2008)) using odd k-mer lengths ranging from 33 to 63. Resulting assembled contigs were merged using CD-HIT v4.6 (Fu et al. 2012, Li 2015) and minimus2 Amos v3.1.0 (Sommer et al. 2007). Sequence data of all contig sequences have been deposited in the MG RAST data set number 4627252.3). Contig annotation was performed in MG RAST with an E value threshold of 10−5 while also considering the representative hit option (i.e., single best annotation for each feature) and taxonomic information was retrieved for each identified contig.

The resulting metagenomes were further analyzed for the quantities of relevant contigs in individual metagenomes. The retrieved counts were then normalized with *rpoB*, a housekeeping gene that has a constant amount in all cells. Its frequency was calculated in each sample (normalized to length of contigs and number of sequences), the average of those numbers was determined, and the calculated ratio between *rpoB* (avg.) / *rpoB* (sample). The calculated coefficients were multiplied with the frequencies of the other genes before comparing their frequencies in each sample.

5.3. Phylogenetic analysis

The newly determined sequences were aligned using MAFFT (Katoh et al. 2002) and positions consisting mainly of gaps or manifestly unidentical sections have been manually removed. All the datasets contained first BLAST hits of the newly determined sequences as well as representatives of the broad diversity of the studied genes. Sequences from each data set were aligned by MAFFT and coding sequences were in silico translated. Phylogenetic trees of functional genes were constructed by maximum likelihood of functional genes in Phyml 3.0 under LGGAMMA model (Guindon & Gascuel 2003). Node support was assessed by maximum likelihood analysis of 1000 bootstrap datasets.

6. Results and Discussion

To gain insights into the metabolic pathways for methane metabolism in the LG subglacial environment, this study analyzed contigs of metagenomes using the KEGG pathway maps (Kanehisa & Goto 2000).

6.1. Genes for methanogenesis/AOM

Table 3 lists the genes of the methanogenesis/AOM pathway. All genes of this pathway were detected in all four metagenomes. This is the first report of a full pathway of methanogenesis in a subglacial environment.

gene	full name
mcr	methyl-coenzyme M reductase
mtr	tetrahydromethanopterin S-methyltransferase
mer	5,10-methylenetetrahydromethanopterin reductase
mtd	methylenetetrahydromethanopterin dehydrogenase
mch	methenyltetrahydromethanopterin cyclohydrolase
ftr	formylmethanofuran--tetrahydromethanopterin N-formyltransferase
fmd	formylmethanofuran dehydrogenase
frh	coenzyme F420 hydrogenase
hdr	heterodisulfide reductase

Table 3: Enzymes involved in methanogenesis/AOM (see also Figure 4)

Table 4 summarises the normalized quantities of methanogenesis/AOM genes detected in the LG subglacial metagenomes. The total quantities of some genes, such as *mcr* (with all found subunites) or *frh*, was orders of magnitude smaller compared to the rest of the methanogenesis genes. In contrast, the gene *fmd* had a higher amount in the analyzed metagenomes compared to other genes. The differences in the quantities of the studied genes might be caused by their different expression of many seemingly “redundant” genes. For example, the genome of

Methanosarcina acetivorans contains seemingly redundant copies of many other genes with implied roles in carbon or energy metabolism (Galagan et al. 2002). *M. acetivorans* possesses four gene clusters annotated for formylmethanofuran dehydrogenase, five distinct clusters of genes encoding membrane-bound and/or soluble-type heterodisulfide reductase enzymes. Orthologs of many of these genes are present in other described *Methanosarcinaceae* species including *M. acetivorans, M. mazei*, and *M. barkeri* (Rohlin & Gunsalus 2010). In addition, some genes have multiple forms consisting of different subunits, which may contribute to their higher counts in the dataset.

	1	***2***	***3***	***4***	***SUM***
mcr	14	27	40	197	278
mtr	77	145	281	875	1378
mer	2463	3783	4389	1776	12411
mtd	117	142	181	1921	2361
mch	1962	1791	1455	7586	12795
ftr	2603	2354	1711	8216	14884
fmd	4384	4385	3676	30258	42704
frh	112	207	203	168	690
hdr	2848	4106	5011	2890	14855

Table 4: Total amounts of genes from subglacial samples of Leverett Glacier. Functional genes involved in methanogenesis/AOM throughout metagenomes 1-4

Figure 8 visualises the differences in relative amounts of individual genes between the different sampling periods. The most functional genes have the largest relative quantities in Metagenome 4, while the genes *mer*, *frh*, and *fdh* show similar concentrations in all the periods of sampling. The higher concentrations of the genes *mcr*, *mtr*, *mtd*, *mch*, *ftr*, and *fmd* in the last sample might be explained by the fact that sampling for Metagenome 4 was done in the post-outburst season, when new regions of the bed become accessible and connected to efficient drainage channels (Bartholomew et al. 2011). The export of long-residence-time subglacial waters from the glacier bed are common during melting season. Large pulses of meltwater alter

the basal water pressure and enable the expansion of efficient subglacial drainage pathways into previously inefficient areas of the ice sheet bed (Nienow et al. 1998, Stevens et al. 2015).

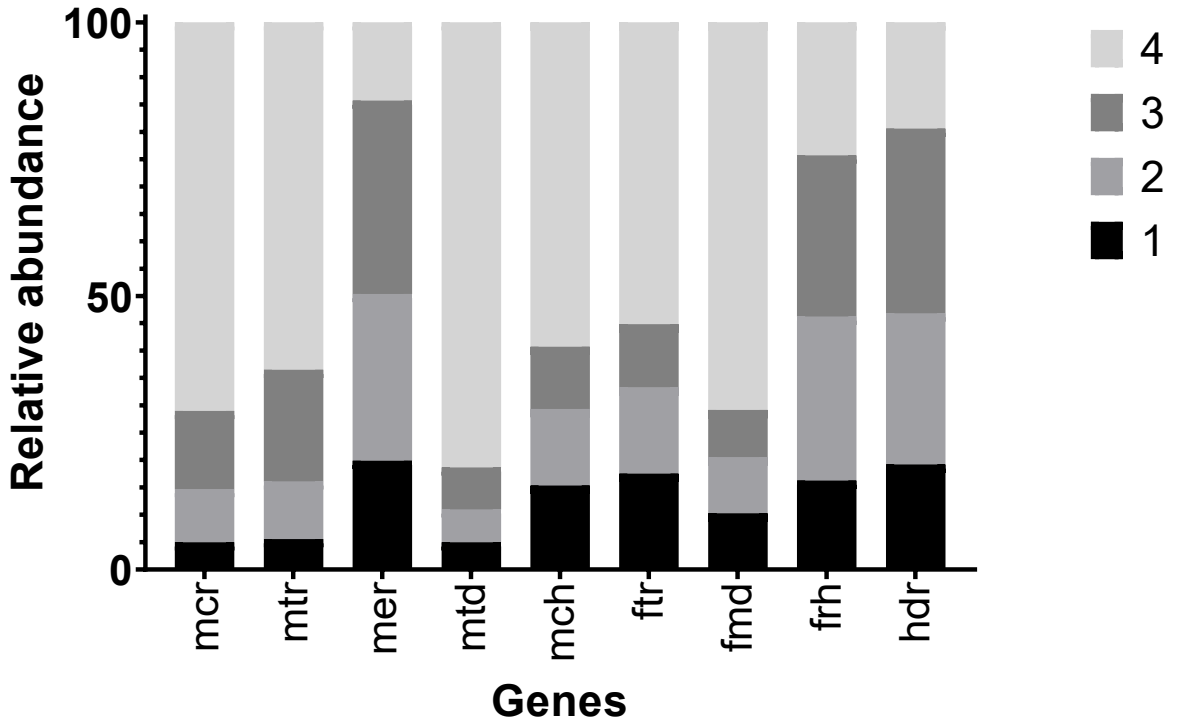

Figure 8: Relative abundances of genes from subglacial samples of Leverett Glacier. Functional genes involved in methanogenesis/AOM throughout metagenomes 1-4.

Periods of subglacial water release via outbursts are consistent with increased mobilisation of long residence time subglacial waters which have undergone substantial chemical weathering (Tranter et al. 2002). Therefore, in the post-outburst season more functional genes involved in anaerobic methane metabolism (methanogenesis and/or AOM) may be exported from the long-term isolated, possibly anoxic, areas. However, since the genes *mer*, *frh*, and *hdr* did not follow this trend, potentially due to differences of involvement in other metabolic pathways, this question remains open, and more research into functional genes needs to be done for a better understanding of the subglacial metabolic potential. It should also be emphasized that it is not possible to distinguish between methanogenesis and AOM by analyzing these marker genes,

and the identity of the organisms must be ascertained via phylogenetic analysis of the respective gene sequences.

6.2. Genes for potential AOM syntrophy

Anaerobic oxidation of methane may coupled to dissimilatory reduction of sulphate or reduction of nitrate, Fe, Mn (section 4.2.2.). All four metagenomes yielded contigs assigned to marker genes from both mentioned reactions (Fig. 9): the gene *dsrAB* is the marker genes for the dissimilatory reduction of sulphate and the *narGH* gene marks the reduction of nitrate. The presence of these marker genes may indirectly support the presence of active anaerobic methanotrophs in the subglacial environment, however, the relationship between the partners of the metabolic pathways is often unclear and it remains questionable how these partners are cooperating in this kind of environment.

Additionally, the quantities of *dsrAB* in the subglacial samples were significantly higher compared to *mcrA* genes (Tab. 4, Fig. 9). which may indicate the presence of microorganisms containing *dsrAB* genes, that are not partnered with ANME. The *dsrAB*-type dissimilatory sulfite reductase is a key microbial enzyme in both the reductive and the oxidative steps of the biogeochemical sulfur cycle. Utilized by microorganisms that catalyze redox reactions involving sulfur-containing compounds as components of energy metabolism, it catalyzes the reduction of sulfite to sulfide during anaerobic respiration with sulfate, sulfite or organosulfonates as terminal electron acceptor, and functions in reverse during sulfide oxidation (Dahl et al. 1993). *DsrAB* enzymes do not occur only in syntrophic partners of ANME microorganisms but are also in sulfate-reducing microorganisms that have no partnership with ANME and play important role in biogeochemical cycling of sulfur and carbon (Pester et al. 2012, Bowles et al. 2014). *DsrAB* catalyzes the last and main energy-conserving

step in the dissimilatory sulfate reduction pathway that is conserved in all cultivated sulfate-reducing microorganisms, which are distributed in four bacterial (*Deltaproteobacteria*, *Nitrospirae*, *Firmicutes*, *Thermodesulfobacteria*) and two archaeal phyla (*Euryarchaeota*, *Crenarchaeota*). So far it is known that anaerobic methanotrophic archaea are associated only with sulfate reducing microorganisms of phylum *Deltaproteobacteria* (Knittel et al. 2005, Orphan et al. 2001, Boetius et al. 2000, Niemann et al. 2006, section 4.2.2.), therefore it remains unclear to which representatives belong found *dsrAB* genes from the subglacial environment.

A similar situation to *dsrAB* genes occurs in *narGH* genes compared to the quantities of *mcrA* genes. Again, this might be due to the fact that these genes do not occur only in microorganisms that are in syntrophic partnerships with ANME but they could also be found in microorganisms that have no relationship with ANME and are present in a subglacial environment. Nitrate-dependent AOM pathway is even less explored than in the case of sulfate-dependent AOM and therefore further research is needed.

6.3. Methanotrophy genes

The presence of *pmoA* genes indicates aerobic oxidation of methane (methanotrophy). Due to limited oxygen penetration, active aerobic methane oxidation is probably limited in the subglacial environment to marginal areas and areas adjacent to the channelized drainage system which brings oxygenated water from the glacier surface. Nevertheless, *pmoA* genes were detected in all four metagenomes (Fig. 9). Microorganisms capable of methanotrophy may be responsible for the recycling of methane produced in the subglacial environment, however, the rates and the importance of this process remains unclear.

The quantities of *pmoA* in the subglacial samples (especially in metagenome 4, Fig 9) were higher compared to the very low quantities of *mcrA* (Tab. 4), which may indicate the

presence of methane as a carbon source, potentially delivered from deeper parts of subglacial system. Given the results, there seems to be a significant influence of hydrology and hydrochemistry on subglacial microbial assemblages flushed out during the melting season, affecting the presence of aerobic methanotrophs in the samples. It may also indicate higher concentrations of methane flushed out of the GrIS subglacial system during the post outburst period. The hydrological networks evolve over the course of a melt season and this dictates the proportion and origin of water and sediments that is exported from microbial habitats of subglacial systems. The subglacial drainage system of the GrIS can include permanent elements as well as elements that form, grow, and change structure over the course of a melt season (section 5.1.3.). The marginal parts can be washed out regularly every year, but during later period of melting season, the drainage system may also disrupt distant areas of the subglacial environment that have remained intact. These areas might contain accumulated methane, which could be consequently distributed to other parts of subglacial environment. It will be interesting to observe the development of glaciers in the near future, given that it is expected the glacial retreat and it will be exciting to see the evolution of newly accessible subglacial areas, which might subsequently affect the regional carbon cycle. However, the amount of methane accumulated in the subglacial environment as well as the possible factors affecting the amount of methane such as anaerobic oxidation of methane remain questionable (section 4.2.2.).

The very low values of the *mcrA* genes assigned to methanogens producing methane in the subglacial environment provide a question about the origin of methane in the subglacial environment that is consequently consumed by methanotrophic organisms. Methanogens might not be evenly distributed throughout the GrIS subglacial system and they may occur only in some certain places where appropriate conditions and resources for their functioning are met. These subglacial areas might vary in terms of physical conditions, type/quantity of sediment available, allochthonous input of nutrients and microbes, redox conditions, temperature,

seasonality, and meltwater residence times. Even within the subglacial environment, microbial assemblages consist of both locally distinct (Bhatia et al. 2006, Lanoil et al. 2009) and globally distributed species (Foght et al. 2004, Tranter et al. 2005, Bhatia et al. 2006, Lanoil et al. 2009). Areas containing methanogens might not be disrupted during the melting season when samples were taken by the drainage processes and they could be located in more distant parts of glacial bed. Additionally, methane as a gas can spread and from its origin location and penetrates to other parts of the subglacial system, providing a substrate for methanotrophic microorganisms.

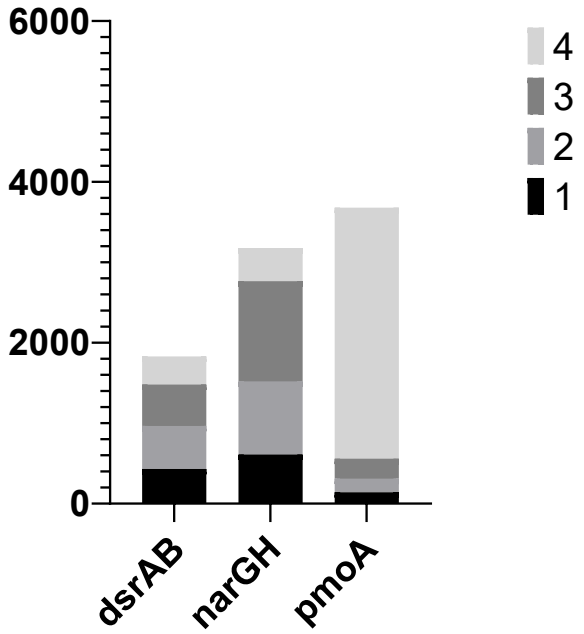

Figure 9: Total abundances of genes from subglacial samples of Leverett Glacier. Functional genes of sulphate-reducing bacteria, nitrate-reducing bacteria and aerobic methanotrophs in metagenomes 1-4.

6.4. Taxonomic analyses

To obtain a picture of specific taxa capable of methane cycling in sampled subglacial sediments from the LG catchment, phylogenetic analysis was conducted, focusing on *mcrA* and *pmoA*

genes. Both are referred to as the marker genes, where *mcrA* represents methanogenesis/anaerobic methanotrophy and *pmoA* aerobic methanotrophy.

The marker gene *mcrA* was found in all four metagenomes, but only two sequences corresponded to the structure of the *mcrA* gene coding the Alpha sub-unit of the methyl-coenzyme M reductase gene. The *mcrA* gene is highly conserved (Lehmacher & Klenk 1994, Springer et al. 1995, Nölling et al. 1996), which renders it suitable for use in molecular ecology studies. Although sequences of other mcr subunit genes were found in the metagenome analysis, only *mcrA* are usually used as marker genes. The other subunits are also used in methanogenesis/anaerobic methanotrophy, but their structure is similar to other genes used in other pathways and could therefore be confused. Thus, only the two sequences identified as *mcrA* were selected for taxonomic analysis. The found *mcrA* genes were assigned to the methanogenic orders *Methanobacteriales* and *Methanomicrobiales* (Fig. 10). One of the observed sequences was identified as being a representative of the genus *Methanobacterium*. One of the close relatives of this sequence is the species *Methanobacterium lacus*, an autotrophic, hydrogenotrophic methanogen. The second one was assigned to the family *Methanoregulaceae* that consists three genera: *Methanoregula* (2 species), *Methanolinea* (2 species), and *Methanosphaerula* (1 species). Both these taxa are assumed to be hydrogenotrophic methanogens, since acetoclastic methanogens only occur in the order *Methanosarcinales*.

The marker gene *pmoA* was also found in all four metagenomes. All sequences were assigned to Type I methanotrophs which belong to the phylum *Gammaproteobacteria* (Fig. 11) and placed in the order *Methylococcales*. The order includes members of the families *Methylococcaceae*, *Methylothermaceae*, and *Crenotrichaceae,* but only relatives of *Methylococcaceae* were represented in the metagenomes. Most of the detected *pmoA* sequences were assigned to 'unclassified M*ethylococcaceae*'. The family *Methylococcaceae* are

methanotrophs able to use methane and methanol as sole carbon and energy sources but are unable to use substrates containing carbon-carbon bonds. Phylogenetically the family is polyphyletic and includes three distinct clades. Members of the *Methylococcaceae* are found in any environment where methane and oxygen coexist including cold to thermal environments in both terrestrial and marine locations (Bowman 2006).

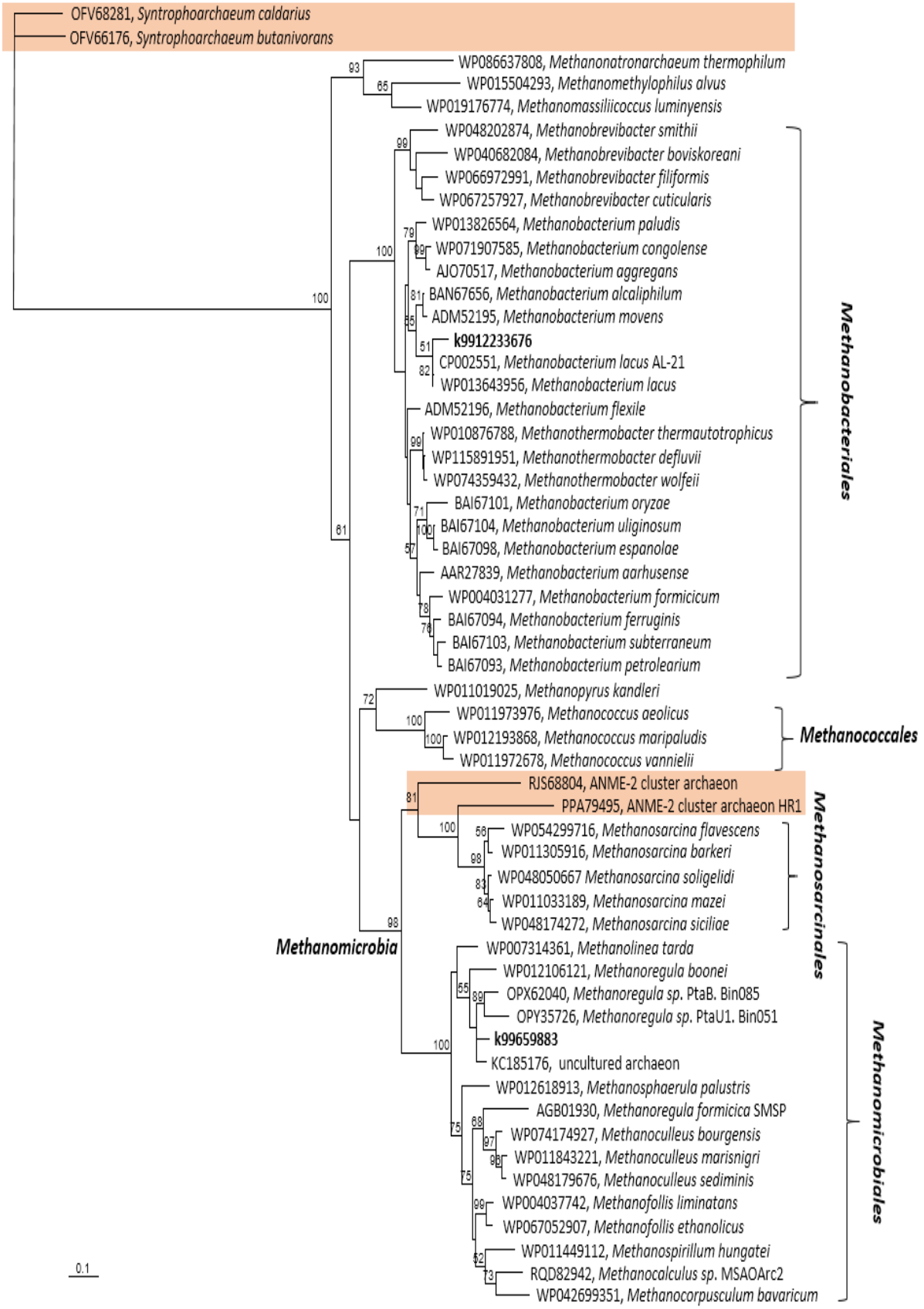

Figure 10: Phylogenetic tree of mcrA subglacial sequences (k99-numbers) with relative microorganisms of other methanogenic (and some methanotrophic) strains. Bootstrap values (percentages of 1000 data resamplings) ≥50% are shown at each node. ANME marked with an orange color.

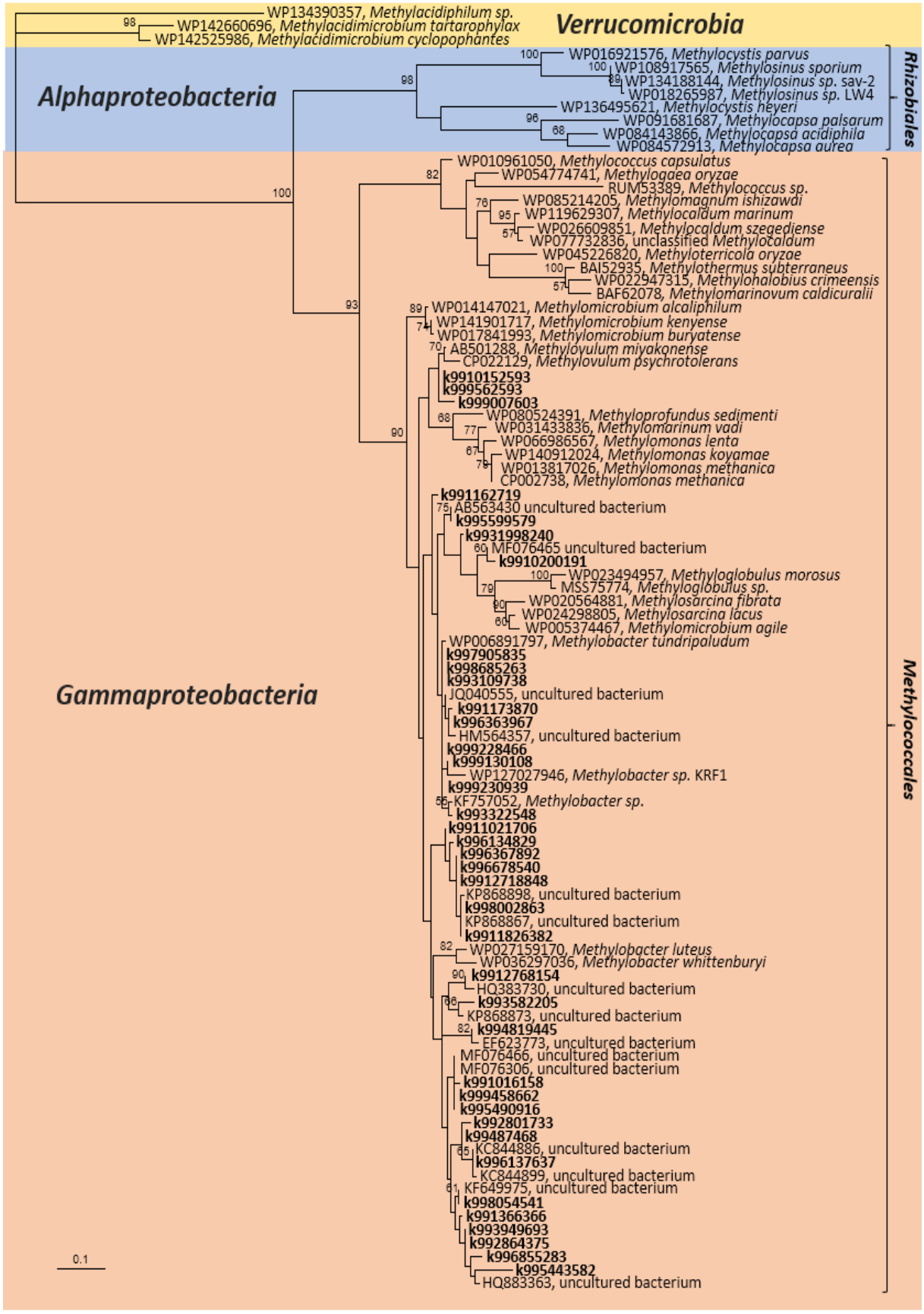

Figure 11: Phylogenetic tree of pmoA subglacial sequences (k99-numbers) with relative microorganisms of other methanotrophic strains. Bootstrap values (percentages of 1000 data resamplings) ≥50% are shown at each node.

7. Conclusion

This study used a metagenomic approach to gain an insight into processes that occur in the subglacial environment by analyzing sequences of functional genes associated with methane metabolism in four pooled samples of sediment exported from beneath the Greenland ice sheet in the summer 2015. All functional genes of methanogenesis/anaerobic oxidation of methane and methanotrophy were detected in all four metagenomes. Differences between the quantities of genes within the metagenomes might indicate the difference in origin of sediments that were exported from more or less distant and isolated regions of the GrIS subglacial system. The data provide strong evidence for the presence of taxa responsible for methane oxidation, which indicates the presence of methane as a substrate. The presence of methanogens is also shown, albeit in lower quantities, while no taxa potentially capable of methane oxidation under anaerobic conditions were detected.

The understanding of the subglacial ecosystem is far from complete despite the increasing interest and the amount of works about the subglacial environments. Therefore, future research could be focused subglacial sediments and studying the potential of releasing methane with regional or large-scale impacts.

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