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Ciclo del metano en lagos árticos, templados y tropicales

Tesis que presenta

Karla Catalina Martínez Cruz

Para obtener el grado de

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Director de Tesis: **Frédéric Thalasso**

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Methane cycling in arctic, temperate and tropical lakes

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Abstract

Methane (CH_4) is an important greenhouse gas biologically produced in anoxic environments, such as lake sediments. Lakes are responsible for about 13% of total CH_4 emission, although they cover only 0.8% of the earth's total surface. The CH_4 emission from lakes are governed by CH_4 production and oxidation processes. This study covers the three main processes involved in CH_4 cycling: CH_4 production in sediments by methanogenesis, aerobic oxidation by methanotrophy, and anaerobic oxidation of methane (AOM). These processes are carried out in sediments and in water column, with remarkable variations subject to the season, landscape, nutrient state and limnological characteristics.

In order to get more and precise determinations of both, CH_4 production and CH_4 oxidation rates in lakes, the development of techniques that allow a precise measurement of a larger number of samples in shorter time and in the field were required. First, an alternative method for non-invasive measurement of CH_4 produced during activity tests in closed vials is presented. This new method is based on Infrared Tunable Diode Laser Absorption Spectroscopy (TDLAS), and was evaluated during methanogenic activity tests and compared to a more traditional method based on gas chromatography. From the results obtained, the minimum measurable methane production rate was estimated to 1.09×10^{-3} mg l^{-1} h^{-1}, which is below CH_4 production rate usually reported in natural ecosystems. Additionally, to sensitivity, the method has several potential interests compared to more traditional methods among which; (i) short measurements time allowing the measurement of a large number of samples, (ii) non-invasive measurements avoiding leakage or external interferences and, (iii) similar low costs as GC based methods can be mentioned. Therefore, this TDLAS based method was also used in aerobic methanotrophic rates determinations in several sections of this thesis.

This thesis first assessed the aerobic methanotrophy in lake water columns. In lakes, it has been demonstrated that up to 99% of the dissolved CH_4 can be oxidized by aerobic methanotrophy. This oxidation process mainly depends on the lake CH_4 and oxygen (O_2) concentrations. However, other environmental factors also influence the aerobic CH_4

oxidation rates, generating seasonal, nutrient state and geographic variations. In this study, the aerobic methanotrophic rates in 30 Alaskan lakes along a north-south latitudinal transect was determined during winter and summer. The results showed that in winter, aerobic CH_4 oxidation was mainly controlled by the dissolved O_2 concentration, while during summer it was controlled primarily by the CH_4 concentration. The permafrost type in which lakes are located was identified as another key factor. The aerobic CH_4 oxidation rates in thermokarst (thaw) lakes formed in yedoma-type permafrost was significantly higher compared to other lakes formed in non-yedoma permafrost environments. These results confirm the significant impact of thawing permafrost on CH_4 cycling in northern lakes, which directly impact on the CH_4 emission to the atmosphere.

This research also evaluated methanogenesis and AOM in surface sediments of 29 lakes that varied in latitude, biome, soil type and limnology. That study aimed at three main goals: (i) the determination of the effect of sulfates and nitrates as electron acceptors on AOM rates, (ii) the quantification of the fraction of CH_4 being oxidized by AOM in actual conditions, and (iii) the identification of the microbes involved in AOM. The first aim was assessed by incubation tests performed with surficial sediments from fifteen Alaskan lakes with different permafrost type and limnological characteristics. AOM, together with methanogenesis in all fifteen lakes was observed. However, the role of sulfate and nitrates, as final electron acceptors remained uncertain since incubations with and without electron acceptors interchangeably showed similar AOM activity in the Alaskan lake sediments. No correlation among the geographic and limnological properties of the lakes and AOM rates was found. The second aim, included the demonstration of AOM occurrence in fourteen lakes distributed in tropical (Mexico), temperate (Germany and Mexico) and arctic (Alaska) regions, with the subsequent detailed analysis of three lakes with contrasting trophic states and water physicochemical regimes. By combining a span of incubation tests together with isotopic measurements, AOM was quantified and a CH_4 cycling model was developed and validated with field measurements. From the model, its field validation and the AOM incubations of the three lake sediments, it was concluded that AOM is a widespread process that mitigates 38 $\pm$ 23 % of the total CH_4 produced in sediments. The latter is similar to those mitigation percent determined from the fifteen Alaskan lakes and slightly higher than the percent calculated from previous reports. Altogether, these findings

indicated AOM is a widespread process in tropical, temperate and arctic lakes that should be incorporated in future estimations and models of the global CH_4 budget. The third aim was evaluated by a study in surficial sediments of one sub-arctic lake. By using DNA- and phospholipid fatty acid- (PLFA) based stable isotope probing, quantitative (q)-PCR, and gene-targeted metagenomics, carbon derived from $^{13}CH_4$ was tracked through the active anaerobic microbial community. Results indicated that known aerobic methanotrophs belonging to the genera *Methylobacter* might be responsible for oxidizing CH_4 in anaerobic conditions in lake sediments.

Resumen

El metano (CH_4) es un importante gas con efecto invernadero, biológicamente producido en condiciones anaeróbias, como los sedimentos de lagos. Los lagos son responsables de hasta 13% de las emisiones totales de CH_4 a la atmósfera, a pesar de sólo cubrir el 0.8% de la superficie terrestre total. Las emisiones de CH_4 provenientes de los lagos dependen de procesos de producción y oxidación de CH_4. Este trabajo abarca los tres procesos principales involucrados en el ciclo del CH_4: la producción de CH_4 en sedimentos por medio de metanogénesis, la oxidación aerobia de CH_4 por medio de metanotrofía y el enigmático proceso de oxidación anaerobia de metano (AOM, por sus siglas en inglés). Dichos procesos se llevan a cabo a lo largo de los sedimentos y la columna de agua con variaciones importantes, sujetas a cambios estacionales, geográficos, tipo de nutrientes y características limnológicas.

Con el objetivo obtener más y mejores mediciones, tanto de producción como de oxidación de CH_4, se requiere desarrollar técnicas que permitan mediciones precisas de un mayor número de muestras en poco tiempo e in situ. Por lo tanto, en primer lugar, se presenta un método alternativo no invasivo para mediciones de CH_4 producido durante incubaciones en viales cerrados. Dicho método se basa en espectroscopia de absorción infrarroja de diodo láser sintonizable (TDLAS, por sus siglas en inglés) y fue evaluado durante incubaciones de actividad metanogénica y comparado con el método tradicionalmente utilizado, basado en cromatografía de gases. Los resultados obtenidos indican que el mínimo valor medible de velocidad de producción de CH_4 con este método corresponde a 1.09×10^{-3} mg l^{-1} h^{-1}, que está por debajo de las velocidades de producción de CH_4 usualmente reportadas en ecosistemas naturales. Además de la sensibilidad, este método tiene diversos atributos en comparación con el método tradicional de cromatografía de gases, por ejemplo: corto tiempo de medición que permite la determinación de un gran número de muestras, mediciones no invasivas que evitan fuga o interferencias externas, y costo similar a los métodos basados en cromatografía. Por en todo lo anterior, el método basado en TDLAS se utilizó también para la determinación de velocidades de oxidación aerobia en diferentes secciones de esta tesis.

El presente estudio evaluó, en primer lugar, la metanotrofía aerobia en la columna de agua de diversos lagos. Se ha demostrado que la metanotrofía aerobia oxida hasta el 99% del metano disuelto en la columna de agua. La metanotrofía depende, principalmente, de la concentración de CH_4 y O_2. Sin embargo, otros factores ambientales, tales como cambios estacionales, geográficos, tipo de nutrientes y características limnológicas, también influyen en las velocidades de oxidación de CH_4 por metanotrofía. En este estudio, se determinó la velocidad de oxidación aerobia de metano en 30 lagos Alaskeños a lo largo de un transecto latitudinal de norte a sur, durante invierno y verano. Los resultados muestran que, en invierno, la metanotrofía aerobia estaba principalmente controlada por la concentración de O_2 disuelto, mientras que, en verano, la metanotrofía dependía, principalmente, de la concentración de CH_4 en la columna de agua. El tipo de permafrost sobre el cuál se localizan los lagos, también fue identificado como un factor clave en la metanotrofía, mostrando velocidades metanotróficas significativamente mayor en lagos termokársticos (con derretimiento de permafrost) localizados en permafrost tipo yedoma, comparados con lagos formados en permafrost tipo no-yedoma. Estos resultados confirman el gran impacto del derretimiento del permafrost en el ciclo del CH_4 en lagos nórdicos.

Esta tesis, también evaluó la metanogénesis y la AOM en sedimentos superficiales de 29 lagos que varían con respecto a su latitud, bioma, tipo de permafrost y características limnológicas. Dicho estudio, cubre tres metas principales: (i) la determinación del efecto de sulfatos y nitratos como aceptores de electrones en velocidades de AOM, (ii) la cuantificación de la fracción de CH_4 que es oxidada por AOM en condiciones reales y (iii) la determinación de los microorganismos involucrados en AOM. El primer objetico fue evaluado a partir de incubaciones llevadas a cabo en sedimentos superficiales de quince lagos Alaskeños con diferentes características limnológicas y tipo de permafrost. Se observó AOM a la par de metanogénesis in los quince lagos. Sin embargo, el rol de los sulfatos y nitratos, como aceptores finales de electrones es incierto, puesto que las incubaciones, con y sin aceptores finales de electrones mostraron, actividad similar de AOM en sedimentos de los lagos estudiados. Adicionalmente, no se encontró ninguna correlación entre las características limnológicas y geográficas de los lagos, y las velocidades de AOM. El segundo objetivo, incluyó, en primer lugar, la demostración de la ocurrencia de AOM en catorce lagos distribuidos en regiones tropicales (México),

templados (Alemania y México) y árticos (Alaska), con el subsecuente análisis detallado en sedimentos de tres de estos lagos, con condiciones contrastantes de estado trófico y regímenes fisicoquímicos en la columna de agua. La combinación de incubaciones de sedimentos de dichos lagos y mediciones de isótopos estables, permitió la cuantificación de AOM y el desarrollo de un modelo del ciclo de CH_4, mismo que fue validado con mediciones en campo. A partir del modelo, su validación en campo y la determinación de AOM en los tres sedimentos de lagos, se concluyó que la AOM es un proceso ampliamente distribuido que mitiga 38 ± 23 % del total del CH_4 producido en sedimentos de lagos árticos, que es similar al porcentaje estimado en los quince lagos Alaskeños y ligeramente mayor que el estimado a partir de reportes previos. En general, dichos hallazgos indican que AOM es un proceso ampliamente distribuido en sedimentos de lagos tropicales, templados y árticos, por lo cual debería ser incorporado en estimaciones futuras y modelos de balance global de CH_4. El tercer objetivo se evaluó en sedimentos superficiales de un lago subártico, para ello se usaron técnicas de sondeo de isótopos estables en DNA y ácidos grasos (PLFA por sus siglas en inglés), PCR cuantitativa y análisis metagenómico para rastrear el carbón derivado de $^{13}CH_4$ en la comunidad microbiana activa en condiciones anaerobias. Los resultados indicaron que los metanótrofos aerobios, pertenecientes al género *Methylobacter*, podrían ser responsables de la oxidación de CH_4 en condiciones anaerobias en sedimentos de lagos.

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Chapter 1

Introduction

Methane (CH_4) is a trace gas involved in the Earth's radiative budget and atmospheric chemistry. It is the second most important greenhouse gas, after carbon dioxide (CO_2), with a global warming potential 34 times greater than CO_2 on a 100-year time horizon (Myhre et al., 2013). Atmospheric CH_4 concentration has risen from 722 ± 25 ppb in 1750 to 1803 ± 2 ppb in 2011; over that time scale the increment has been attributed mostly to changes in anthropogenic CH_4 emissions. The global CH_4 emission to the atmosphere is estimated to 556 ± 56 Tg $year^{-1}$ (Myhre et al., 2013).

Methane emission sources are classified as biogenic and abiogenic, and they are the result of both anthropogenic and natural processes. Biogenic CH_4 is produced either by microbial degradation of organic matter in anaerobic conditions, such as ruminants and termites' digestion, landfills, rice paddies and freshwater ecosystems, or by thermogenic degradation of organic matter in sedimentary rocks (Ferreti et al., 2005; Neef et al., 2010; Etiope and Sherwood Lollar, 2013). Abiogenic or abiotic CH_4 is formed by chemical reactions such as pyrogenic CH_4, which is produced by the incomplete combustion of organic matter (biomass and biofuel burning, Myhre et al., 2013). Abiotic CH_4 do not directly proceed from organic matter, for example, CH_4 is also formed by magmatic processes, generally in volcanic and high temperature hydrothermal settings, and gas-waterrock interactions (or postmagmatic processes), at lower temperatures (Etiope and Sherwood Lollar, 2013). Each type of emissions is characterized by specific ranges in CH_4 isotopic composition, with $\delta^{13}C$-CH_4; being typically from –55 to –70‰ for microbial degradation processes, –from 25 to –45‰ for thermogenic, and from –13 to –25‰ for abiogenic sources (Myhre et al., 2013). Nevertheless, in most geologic environments biogenic and abiogenic gases are mixed, and notwithstanding the modern molecular and isotopic analytical and interpretative tools, in many cases it is not easy (or even possible) to distinguish them (Etiope and Sherwood Lollar, 2013).

Microbial degradation processes are the major source of atmospheric CH_4, contributing 91% of the total sources. Approximately half of the total emissions; i.e., 51%, are attributed to anthropogenic processes, while 49% of all CH_4 emissions correspond to

natural processes (Fig. 1), being natural wetlands the main contributors, followed by geological sources and other freshwater ecosystems, such as lakes. Hydrates, consisting of solid state CH_4 at high pressure habitats, are estimated to represent only a small fraction of total CH_4 emission, between 2 and 9 Tg yr^{-1} under the current time period. However, emissions from thawing permafrost and CH_4 hydrates in the northern circumpolar region could increase dramatically, owing to the rapid climate warming of the Arctic and the large carbon pools stored there (Tarnocai et al., 2009; Walter Anthony et al., 2014). The emission of CH_4 from decomposing, thawing lake sediments in north Siberia with an estimated flux of ~4 Tg yr^{-1} shows its significance for the future (Walter et al., 2006; van Huissteden et al., 2011) and the need of further studies on thawing lake sediments.

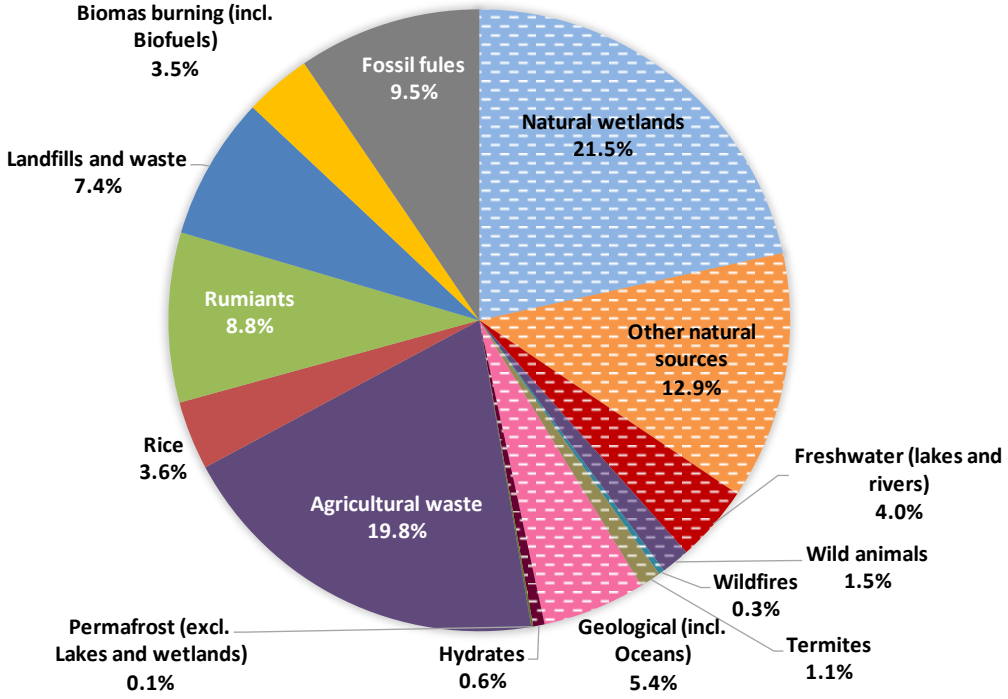

Figure 1. Sources of CH_4 during 2000-2009. Full color fractions represent CH_4 anthropogenic sources whereas dotted fractions represent natural CH_4 sources (From table 6.8, Myhre et al., 2013).

Only part of the CH_4 microbiologically produced reaches the atmosphere, as several processes reduce the total CH_4 emitted. Among them, the most important is certainly

microbial oxidation (Conrad, 2009), which is estimated to uptake up to 60% of the CH_4 microbiologically produced in the environment (Reeburgh, 2007). The CH_4 microbial oxidation processes are not fully understood and still require further investigation in order to be included in the global CH_4 budget, as it will be discussed in sections 6.2 to 6.4. Once in the atmosphere, CH_4 is predominantly removed by photochemistry, through atmospheric chemical reactions with OH radicals (Cicerone and Oremland, 1988)

1.1 Lakes

A lake is defined as a body of permanent still water (lentic water body), completely isolated from the sea and having an area of open and deep water, sufficiently large to produce a clearly defined shore (Welch, 1952). Lake basins originate in many different ways, the most important are: 1) glaciation; 2) landslides that obstruct valleys; 3) as result of sinking of underlying rock or soil surface, i.e. limestone sinks and thawing permafrost; 4) crustal movements of the earth, such as up-warping which forms a dam or down-warping which forms a basin; 5) craters of extinct volcanoes; and, 6) river channel changes (Welch, 1952).

Because lakes are minor part of the earth surface, the activity of these ecosystems was commonly ignored in global estimates of ecosystem processes such as carbon budgets. Nevertheless, lakes play a significant role in many key processes, such as, methane (CH_4) efflux, carbon dioxide (CO_2) source/sink and large organic carbon (OC) storage in sediments (Dean and Gorham, 1998; Cole and Caraco, 2001). In lakes, OC burial and CH_4 production are mostly restricted to the sediments. From the OC being deposited onto the sediment surface, a portion is mineralized to CO_2 and/or CH_4 while the remainder is buried in the sediment over geologic timescales (Sobek et al., 2003). Knowledge about factors regulating the OC fate in lakes sediments is relevant, since they have been estimated to bury more OC in their sediments than the entire ocean (Tranvik et al., 2009). Due to the high amount of OC in sediments, CH_4 could be produced in lakes, leading to a potentially high CH_4 emission rates and an offset the continental C-sink. Indeed, lakes are considered to account for up to 13% of the total atmospheric CH_4 emission (Bastviken et al., 2011).

Dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) are the major carbon inputs to most lakes, followed by particulate organic carbon (POC) and particulate

inorganic carbon. The importance of these inputs varies with lake location and hydrology (Tranvik et al., 2009). The proportion of POC, DOC, and total inorganic carbon that enter lakes (Meybeck, 1993), is highly variable and depends on differences in climate, soil texture and geochemistry, and land use. In temperate regions and boreal forests with carbonate terrain, DIC is the dominant form of aquatic C received by lakes (Finlay et al., 2009; Stets et al., 2009), due to high soil respiration, carbonate weathering, and groundwater flow. By contrast, DOC dominates in the humid tropics and in non-carbonate boreal forest (Tranvik et al., 2009).

The latitudinal distribution of lakes is presented in Fig. 2A, showing a larger lake area between 40 and 70° North, peaking at 45° North. Similarly, the latitudinal distribution of lakes belonging to different size classes is presented in Fig. 2B, showing higher number of small lakes between 50 and 80° North, but peaking at 70° North. Thus, approximately one-quarter of lakes on Earth occur in the northern high latitudes (Lehner and Döll, 2004). The distribution of lakes in the Arctic is largely controlled by the presence of permafrost as well as glacial history (Mostakhov, 1973; Smith et al., 2007). The lake distribution skewed to north, is of major relevance for the global CH_4 cycling because it has been reported that northern lakes are responsible for 6% of the total CH_4 emission to the atmosphere (Walter et al., 2007). Moreover, permafrost-melting scenarios caused by global warming suggest that a significant fraction of the C pool will become available for increased microbial OC transformations (Tranvik et al., 2009). Thus, larger emissions of CO_2 and CH_4 from lakes are expected, especially where thermokarst erosion and ponding is occurring (Walter et al., 2006).

Tropical lakes are less numerous than temperate and arctic lakes, because lakes of glacial origin are rare in the tropics (Lewis, 1996). By comparison to northern lakes, tropical lakes present a primary production that is about twice as high, on a given nutrient base. The latter is primarily because of the efficient nutrient cycling combined with higher temperatures and greater stability in solar irradiance, although nitrogen is more often a limiting element in the tropics than in temperate or northern latitudes where phosphorus is more often limiting (Lewis, 1996). Thus, the OC input to tropical lakes is largely caused by autochthonous origin while, in northern lakes, most of the OC is coming from landscape processes.

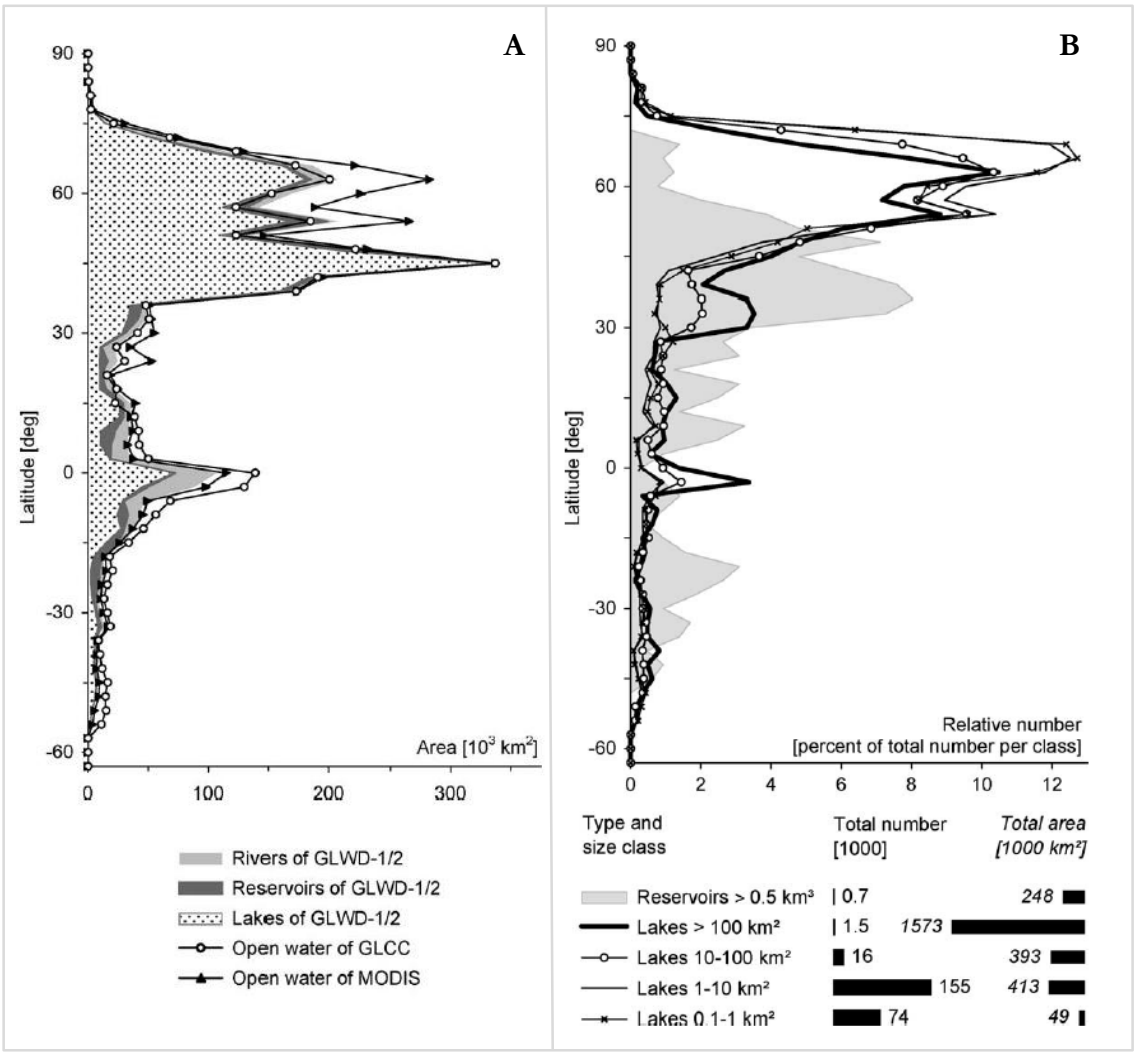

Figure 2. A) Latitudinal distribution of global open water areas. Area values are aggregated in steps of 38 latitudes; B) latitudinal distribution of global lake and reservoir numbers for different size classes according to Global Lakes and Wetlands Database. (Lehner and Döll, 2004).

Independently of their latitudinal locations, lakes can be sites of intense OC mineralization with subsequent CO_2 and CH_4 emissions to the atmosphere. Allochthonous OC inputs promotes respiration to dominate over primary production in most lakes (Del Giorgio and Peters, 1993; Jansson et al., 2000; Duarte and Prairie, 2005), resulting in CO_2 supersaturation (Sobek et al., 2005), and favoring anaerobic conditions, which in turn, promote CH_4 production. With decreasing allochthony, the efflux of CH_4 and CO_2 is

expected to decrease (Jansson et al., 2008; Gonzalez-Valencia et al., 2014); moreover, lakes can even become a sink of CO_2.

Thus, lakes can act mainly as a source of CH_4 and as sink or source of CO_2. The amount of CO_2 and CH_4 emitted to the atmosphere rest on a fragile equilibrium involving several lake physical and chemical parameters. Some studies pointed out the lakes as net sources of both, CH_4 and CO_2, but propose that climate warming impact of lake CH_4 emissions is two times higher than that of CO_2 (Sepulveda-Jauregui et al., 2015). In addition, lakes account for up to 70% of the total inland freshwater CH_4 emission (Bastviken et al., 2011). Therefore, studies focused on CH_4 cycling in lakes are required to better constrain the total CH_4 emitted by lakes and the transport mechanisms of CH_4 in the water column before being released to the atmosphere.

1.2 The methane cycle in lakes

Lakes cover about 0.8% of the Earth's surface (Downing et al., 2006), however they are responsible for up to 13% of total CH_4 emission (Bastviken et al., 2011), compared to less than 1% for oceans (Rhee et al., 2009).

The CH_4 cycle is part of the global carbon cycle, thus it depends on the degradation chain of organic matter. The CH_4 budget in lakes mainly depends on two biological antagonistic processes: CH_4 production and oxidation. Whereas CH_4 production is a strictly anaerobic process, CH_4 oxidation occurs in both, aerobic and anaerobic conditions (Fig. 3).

In general, aqueous CH_4 produced by methanogens accumulates in sediments until it reaches saturation and comes out of solution as bubbles. CH_4 also diffuses through the water column, where it is susceptible to other processes and serves as carbon source and an electron donor for methane oxidation. The balance between CH_4 production and oxidation has been previously studied. It has been estimated that globally, 30–99% of total CH_4 produced in lakes is microbiologically oxidized under aerobic conditions in the water column rather than being released to the atmosphere (Bastviken et al., 2002; Thauer et al., 2008).

In addition to aerobic methane oxidation, anaerobic oxidation of methane (AOM) is also a process that may reduce substantially the amount of CH_4 released to the atmosphere. AOM has been first acknowledged in marine sediments, where it has been suggested that between 70-85% of CH_4 produced by methanogens is oxidized anaerobically (Reeburgh, 2007; Hinrich and Boetius, 2002). More recently, the existence of AOM was explored in other ecosystems and has been acknowledged in peatlands, wetlands, soils and lakes. In lakes, the potential of AOM generates a large interest as being possibly a major piece of the global biogeochemical carbon puzzle, still partially constrained.

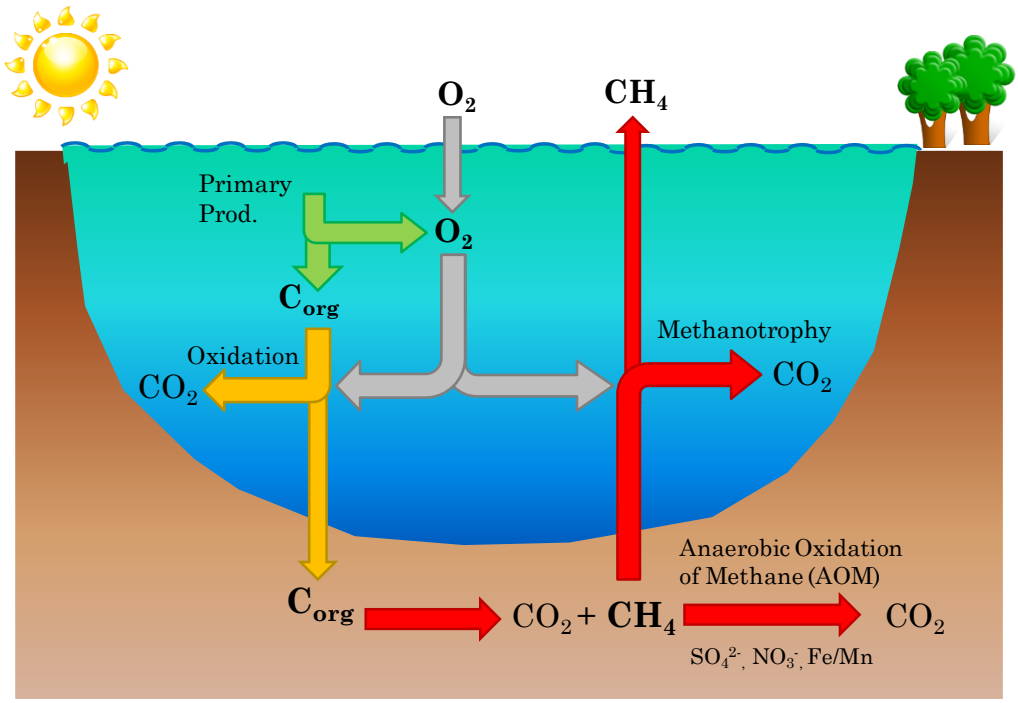

Figure 3. Methane cycling in lakes. Red arrows indicate flow of the direct process involved in the CH_4 cycle. Green arrows indicate the fate of photosynthesis products, yellow arrows indicate the organic matter degradation processes, gray arrows indicate the fate of dissolved O_2, and red arrows indicate CH_4 cycling.

1.2.1 Methanogenesis

CH_4 is produced during the biological degradation of organic matter under low redox potential (Madigan et al. 2003), conditions that are often found in the sediments and/or hypolimnion of aquatic ecosystems. Anaerobic degradation of organic matter proceeds via

several microbial processes, including hydrolysis, acidogenesis and acetogenesis; which produce hydrogen, CO_2, formate, acetate and others simple compounds (Muyzer and Stams, 2008), that are finally used by methanogens. Methanogens produce CH_4 from H_2 and CO_2 (hydrogenotrophic pathway), acetate (acetoclastic pathway) or methylated compounds (methylotrophic pathway) as substrate (Canfield et al., 2005).

The ability to utilize H_2 as an electron donor for CO_2 reduction is distributed among all five methanogen order (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales). In sediments of freshwater lakes, only about 33% of the total methane is formed from CO_2 reduction (Conrad et al., 1999). Acetate is the main precursor of CH_4 in many environments, but the ability to catabolize this substrate is limited to *Methanosarcina* and *Methanosaeta* species, belonging to Methanosarcinales order. The CH_4 synthesis from acetate proceeds by an acetoclastic reaction, in which the methyl carbon of acetate is reduced to CH_4 and the carboxyl carbon is oxidized to CO_2 (Whitman et al., 2006). Acetoclastic pathway is the most common in anoxic freshwater sediments (~67 %) where the catabolism of acetate by other anaerobes is limited by the availability of alternate electron acceptors such as sulfate or nitrate. Methyl-containing C-1 compounds (e.g., methanol, methylamine, formate etc.), can serve as substrates only for one taxon of methanogens (Methanosarcinales). Methylotrophic methanogenesis pathway is common only where methyl-containing C-1 compounds are abundant i.e. in marine sediments or the large intestine of mammals, hence it is not expected to occur in lake sediments (Whitman et al., 2006)

1.2.2 Aerobic methanotrophy

Methanotrophs are a widely spread group of gram-negative bacteria that use methane as carbon and energy source in presence of O_2 as electron acceptor (Trotsenko and Murrell, 2008). Methanotrophs oxidize CH_4 to carbon dioxide and water via several intermediates; i.e., methanol, formaldehyde, and formate. The first oxidative stage of CH_4 is catalyzed by methane monooxygenase (MMO). Two distinct types of MMO are known, a cytoplasmic soluble form (sMMO) and a membrane-bound particulate form (pMMO). The latter has been found in most methanotrophs. The occurrence of sMMO is not a taxonomic property of methanotrophs; instead, it is induced by low Cu to biomass ratios (Trostenko

and Murrell, 2008; Semrau et al., 2010). The pMMO generally has a relatively high affinity for CH_4 (Km= 1–2 mM) and O_2 (Km= 0.1 mM), wheares the sMMO has a lower affinity for CH_4 (Km= 3 mM) and O_2 (Km= 16.8 mM; Chan et al., 2004; Trostenko and Murrell, 2008; Semrau et al., 2010)

Methanotrophs use two main pathways for C-1 assimilation: the ribulose monophosphate (RuMP) and the serine cycle (Quayle, 1969, 1972, 1980). In both cycles, formaldehyde is the key intermediate in carbon metabolism.

Methanotrophs are classified into two types. Type I strains are characterized by: (i) having intracytoplasmic membranes all over the cell as bundles of vesicular disks, (ii) using the RuMP pathway for carbon assimilation, and (iii) having a specific signature of phospholipid fatty acids, of 14 and 16 carbons in length. Type II strains are characterized by: (i) intracytoplasmic membranes aligned along the periphery of the cell, (ii) using the serine pathway for carbon assimilation, and (iii) having a distinctive signature of phospholipid fatty acids of 18 carbons in length. Previously, a third methanotrophic group was described; i.e., Type X strains, which were reclassified as a subset of Type I (Bowman et al., 1993).

There are three phylum of aerobic methanotrophs: 1) α-Proteobacteria, which includes 12 genus and belongs to type I methanotrophs; 2) γ-Proteobacteria, which comprises two different families and four genus and correspond to type II methanotrophs; 3) Verrucomicrobia, which comprises only one family and one genera and is not classified as type I or II but carry out the Rubisco cycle for carbon assimilation (Op den Camp et al., 2009; Semrau et al, 2010). Type I methanotrophs have been demonstrated to dominate CH_4 oxidation in temperate and arctic lakes sediment (Sundh et al., 2005; Rahalkar et al., 2009; He et al., 2012a, 2012b). Very recently, (e.g. Knief 2015) it was recommended to replace the concept of type-classification by the phylogenetic groups (type I and X as γ-Proteobacteria; type II as α-Proteobacteria). Within this thesis the type concept will be used.

1.2.3 Anaerobic oxidation of methane

The CH_4 flux throughout the sediment is also potentially reduced by anaerobic oxidation of methane (AOM), probably playing a significant role in constraining CH_4 emissions to the atmosphere (Reeburgh, 2007; Smemo and Yavitt, 2007; Sivan et al., 2011). AOM is a controversial process which occurrence has been discussed for several decades (e.g., Reeburgh, 1976; Zehnder and Brock, 1980), but has only been described relatively recently (Boetius et al., 2000; Schubert et al., 2011; Blazewicz et al., 2012). AOM is thermodynamically feasible by exchanging electron acceptors with a reductive process (Fig. 4).

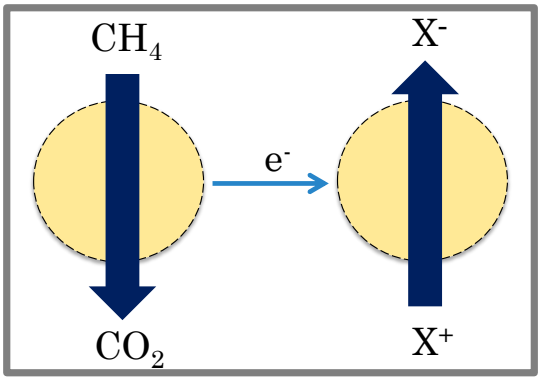

Figure 4. Electron exchange among an anaerobic methane oxidizer and another reductive process.

Table 1 presents several possible electron acceptors that can be coupled to CH_4 oxidation. The most thermodynamically favorable is the known aerobic methanotrophy and the less thermodynamically favorable is the AOM coupled to sulfate reduction (AOM-S). The later reaction is the most important AOM in ocean sediments, because of the relatively high sulfate (SO_4^{-2}) concentration in seawater. In general, AOM has been found in marine sediments to be also coupled to the reduction of mineral electron acceptors, such iron (Fe^{+3}) and manganese (Mn^{+4}) (Hallam et al., 2004; Moran et al., 2008; Beal et al., 2009). In freshwater sediments, the AOM has been found mainly coupled to denitrification, namely AOM-D (Deutzmann and Schink, 2011) and Fe^{+3} reduction (Sivan et al., 2011).

Table 1. Possible reactions for anaerobic oxidation of methane with its ΔG° values.

Reaction	Pathway name	ΔG^0 (KJ/molCH_4)
$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$	Aerobic Methanotrophy	-822
$5CH_4 + 8NO_3^- \rightarrow 5CO_2 + 4N_2 + 8H_2O + 6OH^-$	AOM-D	-766
$CH_4 + 8Fe^{+3} + 3H_2O \rightarrow HCO_3^- + 8Fe^{+2} + 9H^+$	AOM-Fe	-434
$CH_4 + 4HAsO_4^{-2} + 4H^+ \rightarrow CO_2 + 4H_2AsO_3^- + 2H_2O$	AOM-As	-278
$CH_4 + SO_4^{-2} + 2H^+ \rightarrow HCO_3^- + HS^- + H_2O$	AOM-S	-33

Methanogen-like archaea called anaerobic methanotrophic (ANME) archaea have been identified to be involved in AOM-S, in syntrophic association with sulfate-reducing bacteria (Hoehler et al., 1994; Boetius et al., 2000; Valentine, 2002). The proposed AOM-S mechanism is described as a reverse methanogenesis (Hallam et al., 2004). More recently, it has been shown that ANME-1 archaea anaerobically oxidize CH_4 but assimilates inorganic carbon (Kellermann et al., 2012). In freshwater ecosystems, with the exception of areas prone to acid rain or in watersheds containing acid SO_4^{-2} soils and/or rocks, SO_4^{-2} concentration is probably too low for AOM-S to be thermodynamically favorable (Alperin and Reeburgh, 1984; Smemo and Yavitt, 2007). AOM coupled to denitrification (AOM-D) is thermodynamically more favorable than AOM-S, and was first demonstrated in enriched-cultures (Islas-Lima et al., 2004; Raghoebarsing et al., 2006) but also reported in lake sediments with high NO_3^- input (Deutzmann and Schink, 2011). *Methylomirabilis oxyfera*, a member of NC10 phylum, has been described to carry out AOM by an intra-aerobic pathway of nitrite (NO_2^-) reduction (Ettwig et al., 2010). More recently, it was revealed that *Candidatus Methanoperedens nitroreducens*, an ANME-type archeae, is capable of independent AOM through reverse methanogenesis using nitrate (NO_3^-) as the terminal electron acceptor. The latter is possible because *Methanoperedens nitroreducens* contains genes encoding for NO_3^- reductase laterally transferred from a bacterial donor (Haroon et al., 2013). Despite the fact that NO_3^- and NO_2^- are lacking in most anoxic sediments (Smemo and Yavitt, 2007), populations of NC10 bacteria have been detected, i.e. NC10 type were found, in low abundance, in oxic and semi-oxic sediment layers of Lake Washington (Beck et al., 2013) and in anoxic sediments of Lake Constance (Deutzmann et al., 2014). Anaerobic iron (Fe^{+3}) and manganese (Mn^{+4}) reduction coupled to AOM is a more favorable reaction and they have been demonstrated to significantly occur in freshwater ecosystems (Sivan et al., 2011; Nordi et al., 2013). Little is known about the

microorganisms involved in AOM coupled to Fe^{+3} and Mn^{+4}, however one study revealed that the most abundant microorganisms in the sediments after incubations of AOM linked to Fe^{+3} and Mn^{+4} were affiliated with the marine benthic group D and ANME (Beal et al., 2009).

Other electron acceptor candidates have been suggested, in addition to sulfate, nitrite/nitrate, Fe^{+3} and Mn^{+4}. These candidates include: (i) humic substances as being highly abundant in freshwater ecosystems such as lakes (Smemo and Yavitt, 2011), (ii) fumarate which can be theoretically regenerated after reduction, via at least two well-known metabolic cycles (Thauer and Shima, 2008; Beasley and Nanny, 2012); and (iii) micro-aerobic CH_4 oxidation in anaerobic environments by using O_2 derived from chlorite dismutation (Miller et al., 2014). These hypotheses suggest a wider option of AOM metabolic pathways to be explored.

1.2.4 Mechanisms of CH_4 emission to the atmosphere

In general, aqueous CH_4 produced by methanogens accumulates in sediments. Since CH_4 is relatively insoluble in water, (saturation in freshwater is about 1.6 mol m^{-3} at 20 °C, Casper, 2000) high CH_4 concentrations lead to bubble formation. When the CH_4 bubble buoyancy overpasses the entrapment forces, bubbles are released from the sediments and migrate to the atmosphere. This emission mode is commonly called ebullition. Ebullition results in direct flux of CH_4 from the sediment to the atmosphere. Despite its low solubility in water, CH_4 also diffuses through the water column to the atmosphere, where it is released. This emission mode is called diffusive flux. During its migration through the water column CH_4 is susceptible to other processes and for which it serves as carbon source and as electron donor.

Although, ebullition and diffusion are the main pathways of CH_4 emission, emission may also involve three additional modes:

1) Storage flux, which occurs in stratified lakes that accumulate CH_4 in the anoxic hypolimnion of the water column or under the ice cover during winter periods. CH_4 storage is emitted rapidly by diffusion during periods of lake overturn or ice break (Michmerhuizen et al., 1996; Bastviken et al., 2004a);

2) Ice-bubble storage (IBS) flux, which refers to the released of the ebullition bubbles sealed in under the ice formed during winter periods in northen lakes subject to winter-freezing (Greene et al., 2014; Sepulveda-Jauregui et al., 2015); and

3) Plant mediated transport that has been extensively studied in wetlands, more than in lakes (Boon and Sorrell, 1995).

The mechanisms for CH_4 emissions depends on the chemical and physical characteristics of the lakes as well as the biological activities. Each lake can present several CH_4 emission modes changing over the year, and it is important to know the amount of CH_4 emitted by each pathway in order to understand its contribution to the total CH_4 emission of the ecosystem.

1.2.5 Physical and chemical parameters affecting CH_4 cycle.

Several physical and chemical factors can directly or indirectly alter the CH_4 budget by favoring or impeding the production or oxidation of CH_4. Some of them are inherent to the geographical location or seasonal cycles in lakes. The major factors affecting CH_4 cycling are listed hereafter.

Oxygen. Dissolved O_2 concentrations above 10 mg L^{-1} completely inhibit methanogens because several cofactors and enzymes; e.g., dehydrogenaseeacetyl-CoA synthase and F_{420}, are O_2-sensitive (Thauer et al., 2008). In freshwater lakes, anoxic conditions are typically found in the sediment where O_2 is generally depleted few millimeters below the water/sediment interface. Anoxic conditions can also be found in the hypolimnion of stratified lake water columns. By the contrary, the activity of aerobic methanotrophic bacteria depends on the availability and concentrations of both CH_4 and O_2. The highest methane consumption rates are often located at the oxic/anoxic interface where both CH_4 and O_2 are present (Utsumi et al., 1998; Bastviken et al., 2002; Liikanen et al., 2002; Carini et al., 2005; Schubert et al., 2010).

Organic matter content and quality. Labile organic carbon fuels carbon mineralization and methanogenesis. Seasonal inputs of fresh organic carbon stimulate methanogenesis in surface sediment, but also in the deeper sediment layer by diffusion of dissolved labile

organic matter such as acetate, propionate and isopropanol (Chan et al., 2005; Schwarz et al., 2008; Borrel et al., 2011). The methanogenesis rate is positively linked to the lake trophic state, probably due to higher quantity and better quality of organic matter available under higher trophic states (Bastviken et al., 2004b). Indeed, methanogenic rates are higher in lake sediments with a low C:N ratio (<10) than in lake sediments with a high C:N ratio (>10) (Duc et al., 2010; Borrel et al., 2011).

Temperature. Despite the existence of thermophilic and psychrophilic CH_4 producers and oxidizers, both processes are mesophilic and sensitive to temperature changes processes (Schulz et al., 1997; Semrau et al., 2010). The influence of temperature seems to play a major role in the regulation of CH_4 production rather than for CH_4 oxidation (Schulz et al., 1997; Duc et al., 2010; Lofton et al., 2014). In CH_4 oxidation, the temperature effect is more pronounced at high CH_4 concentration than at low CH_4 concentration (Lofton et al., 2014). Moreover, the emission of CH_4 has been proved to increase markedly with seasonal increases in temperature, suggesting that global warming may have a large impact on the relative contributions of CH_4 to total greenhouse gas emissions from aquatic ecosystems (Yvon-Durocher et al., 2014).

Availability of alternative electron acceptors. The availability of alternative electron acceptors, such as SO_4^{-2}, Fe^{+3}, NO_3^- favors anaerobic respiration by sulfate-reducing bacteria, iron-reducing bacteria and denitrifying bacteria, respectively, that outcompete methanogens for the uptake of H_2 and acetate (Winfrey and Zeikus, 1977). However, sulfate concentrations are relatively low in most freshwater lakes; i.e. 100-200 μM, compared to a concentration of 20-30 mM in seawater (Capone and Kiene, 1988). With these low concentrations, sulfate is rapidly depleted within sediment, allowing significant methanogenesis a few centimeters below the sediment surface (Borrel et al., 2011). Similarly, NO_3^- and NO_2^- are depleted in most lakes and even when they are present, NO_3^- and NO_2^- are rapidly depleted in the superficial sediment layers. Although the lack of alternative electron acceptors favors the methanogenesis, it reduces the possibilities of AOM coupled to sulfate-reduction or denitrification.

Chapter 2

State of the art

Despite the importance of freshwater ecosystem in global CH_4 cycling and its meaning for the greenhouse gas effect on earth, there is still a lack of knowledge and a clear requirement to better understanding the biological processes involved: methanogenesis, aerobic methanotrophy and anaerobic methane oxidation. In the framework of this thesis, five aspects related to CH_4 cycling have been addressed.

2.1. Field measurement of dissolved CH_4 concentration and CH_4 production/oxidation rates

An important step for understanding the complexity of methane cycling in freshwater ecosystems is the quantification of dissolved CH_4 concentration (C_s). Quantification of C_s allows a better understanding of biological processes related to methane production and oxidation. It also allows the quantification of total diffusive CH_4 emissions to the atmosphere (Kling et al., 1992; Reeburgh, 2007).

Headspace equilibration (HE) methods are the most widely used technique for measurement of dissolved gas concentrations in environmental water (Kling et al., 1992, Jacinthe and Groffman, 2001). Quantification of C_s by HE is based on Henry's law, which establishes the equilibrium between gas solubility and partial pressure for a given temperature. Most gases are poorly soluble in water and therefore the water/gas equilibrium often favors the latter, making them more easily detectable in the gas phase than in the liquid phase. Headspace equilibration methods typically involve measurement of the concentration of a gas extracted from water by equilibration (via agitation) with an air- or nitrogen-filled headspace (Kling et al., 1992). Although HE methods are simple, reliable, and extensively used (Mengis et al., 1997; Cole and Caraco, 1998; Jacinthe and Groffman, 2001; Gardner and Solomon, 2009), standard analysis is also time consuming and has some logistical drawbacks.

HE methods depend on precise quantification of the gas concentration in the headspace of the equilibration cell. Gas chromatography is the most frequently used technique

because of its accuracy and versatility. However, in most cases gas chromatography cannot be performed on-site, resulting in significant delays between sampling and measurement. These delays (i) impede feedback of results to researchers that would otherwise inform field sampling strategies; (ii) may affect the precision of the results due to biological activity or leakage in sample vials; and (iii) often requires logistically complex procedures of transport and storage of samples between the field and laboratory.

In this context, Sepulveda-Jauregui (2012) developed a new method, based on HE but using Infrared Tunable Diode Laser Absorption Spectroscopy (HE-TDLAS), that allows immediate and non-invasive determination of dissolved CH_4 concentrations in environmental water samples in a closed glass vial. That method was based on a commercially available TDLAS, modified in such manner that the laser beam crosses the headspace section of an equilibration cell before being reflected back to the detector, crossing again through the headspace of the equilibration cell on the return. After proper calibration, this method allows the determination of the CH_4 concentration in the headspace, which is proportional to the dissolved gas concentration in the water sample. That non-invasive method offers also a unique opportunity to determine CH_4 production/oxidation rates in water or sediment samples incubated in headspace equilibration cells, which has not been reported before.

2.2. Variability of methanotrophy in northern lakes

Aerobic CH_4 oxidation; i.e., methanotrophy, is a process that reduces substantially the CH_4 emissions from freshwater ecosystems to the atmosphere. Several environmental factors directly affect aerobic methanotrophy. First, methanotrophy depends on the availability of both CH_4 and O_2. Higher MO rates are usually found at the oxic/anoxic interface, where both CH_4 and O_2 are present (Utsumi et al., 1998a, 1998b; Bastviken et al., 2002; Liikanen et al., 2002; Carini et al., 2005; Schubert et al., 2010). In turn, CH_4 and O_2 concentrations depend on numerous other processes involved in biogeochemical carbon cycling. Among these, the most important are methanogenesis producing CH_4, primary production and atmospheric diffusion supplying O_2, and several aerobic metabolic processes that compete with MO for available O_2 (Dzyuban, 2010). In addition, methanotrophy occurs more efficiently at mesophilic temperatures, from 20 to 35 °C

(Semrau et al., 2010), thus methanotrophy in lakes are expected to be less active in cold lakes. Given the number of parameters having a potential effect on MO, as well as the patchwork of seasonal and geographic conditions found among lakes, MO is expected to exhibit large geographic and seasonal variations that still remain to be characterized. This is particularly clear in northern lakes, where the variability of methanotrophy has not been well established.

2.3. Distribution of anaerobic CH_4 oxidation in the sediments of northern lakes and possible electron acceptors

In addition to aerobic CH_4 oxidation, anaerobic oxidation (AOM) also contributes to mitigate CH_4 emission to the atmosphere. In marine sediments, were AOM was first and best described, AOM is responsible for the reduction of >80% of the annual CH_4 produced in marine sediments (Reeburgh, 2007). More recently, several studies have demonstrated the occurrence of AOM in a reduced number of freshwater ecosystems (Eller et al., 2005; Smemo et al., 2007; Schubert et al. 2010; Sivan et al., 2011; Deutzmann et al. 2011; Segarra et al., 2013, 2015).

AOM might be therefore a significant operator of the global biogeochemical carbon cycling in different freshwater and terrestrial ecosystems, as it is in marine environment. However, our knowledge of AOM does not go beyond evidences of its ecological importance. Few studies have reported AOM from northern highland latitudes ecosystems: Blazewicz et al. (2012) studied the ecological significance of AOM in boreal soils, while Gupta et al. (2013) and Smemo and Yavitt (2007) showed that AOM is widespread in Canadian and USA freshwater peatlands from subarctic to cool-temperate regions. No studies have reported on AOM in boreal and arctic lakes.

Furthermore, AOM linked to sulfate-reduction has been extensively demonstrated in marine sediments (Zehnder and Brock, 1980; Thomsen et al., 2001) and later suggested in lake sediments (Schubert et al., 2011). AOM coupled to denitrification has also been demonstrated to occur in lake sediments (Deutzmann et al., 2014). However, under natural conditions, both, SO_4^{-2} and NO_3^- are usually lacking in lake sediments (Smemo and Yavitt, 2007). AOM linked to iron and manganese-reduction was also proved (Sivan et al., 2011)

and other hypothesis regarding another possible electron acceptor have been shaped (Miller et al., 2014; Beasly and Nanny, 2012). Thus, the role of AOM in methane cycling in northern highland lake ecosystems and how widespread it is and what are the appropriate electron acceptors still remain to be determined.

2.4. Profiles of anaerobic CH_4 oxidation in the sediments of lakes

In complement to AOM distribution, a quantitative approach to further constrain the relative importance of this process in the global CH_4 cycling is still required. Previous quantitative determinations of AOM based on diffusive models of CH_4 concentration profiles in sediments (Alperin and Reeburgh, 1984; Thomsen et al., 2001; Schubert et al., 2011) or based on incubations (Smemo et al., 2007; Deutzmann et al., 2011), reported AOM rates ranging from 0.1 to 100 nmol mL^{-1} d^{-1}. These previous reports; (i) focused on identifying the electron acceptor of AOM; i.e., in most of the cases by adding an external compound, (ii) were based on sediment samples obtained from a single lake and (iii) were mainly based on CO_2 production rate. Additional data on AOM are still required, preferably by quantifying AOM without addition of external electron acceptors, in several lakes, by quantifying CH_4 uptake rate and at several depths of lake sediments.

2.5. Microorganisms involved in anaerobic CH_4 oxidation

Several important breakthroughs have been made in the identification of the microbes involved in AOM, but so far, the evidences are still indirect and not definitive. Methanogen-like archaea called anaerobic methanotrophic (ANME) archaea have been found in syntrophic association with sulfate-reducing bacteria to perform AOM in oceans (Hoehler et al., 1994; Boetius et al., 2000; Valentine, 2002). In marine sediments, AOM linked to Fe^{+3} and Mn^{+4} has been attributed to the marine benthic group D and ANME (Beal et al., 2009). Microbes involved in AOM coupled to nitrite or nitrate reduction has been also investigated. *Methylomirabilis oxyfera*, a member of NC10 phylum, has been described to carry out AOM by an intra-aerobic pathway of nitrite (NO_2^-) reduction (Ettwig et al., 2010). *Candidatus Methanoperedens nitroreducens*, an ANME-type archeae, is thought to be capable of independent AOM through reverse methanogenesis using nitrate (NO_3^-) as the terminal electron acceptor (Haroon et al., 2013). Even though NO_3^- and

NO_2^- are lacking in most anoxic sediments (Smemo and Yavitt, 2007), populations of NC10 bacteria have been detected in anoxic sediments of lakes (Deutzmann et al., 2014).

Despite the effort to determine the biochemistry and microbiology of AOM in laboratory tests, no study has directly identified and isolated the microbes involved in AOM in the natural environment. A commonly used strategy for microbes' identification within a community that use a particular growth substrate is stable isotope probing (SIP), which consists of providing a stable isotope-labeled substrate and analyzing the isotope-labeled biomarkers that are produced in the target organisms. The approach has been applied to lipids (Evershed et al., 2006), nucleic acids (Neufeld et al., 2007) and proteins (Jehmlich et al., 2008). The identification of active aerobic methanotrophs in lakes has been done by using DNA, RNA and PLFA-SIP (He et al., 2012a, 2014, Dumont et al 2011). Hence, SIP techniques may be a good approach to identified microorganisms involved in AOM as well.

Chapter 3

General and specific objectives

General Objective

To reach a better understanding of aerobic and anaerobic methane oxidation processes involved in CH_4 cycling in lakes.

Specific objectives

For each aspect related to CH_4 cycling that has been addressed in the present work, at least one specific objective is established.

1. Field measurement of dissolved CH_4 concentration and CH_4 production/oxidation rates.

   Obj: To develop a non-invasive field technique that can be used *in-situ* for a precise determination of CH_4 concentrations in water samples, also for the estimation of production and oxidation rates.
2. Variability of methanotrophy in northern lakes

   Obj: To determine the seasonal and geographic variations of aerobic methanotrophy in 30 lakes along a south-north transect in Alaska.
3. Distribution of AOM in the sediments of northern lakes and possible electron acceptors

   Obj 1: To establish the existence of AOM in 15 arctic lakes with contrasting trophic states and water column physicochemical regimes.

   Obj 2: To evaluate the potential participation of SO_4^{-2} and NO_3^- as electron acceptors.
4. Profiles of anaerobic CH_4 oxidation in the sediments of lakes

   Obj: To determine CH_4 production and actual AOM (without addition of external electron acceptors) rates along sediment cores of three lakes with contrasting trophic states and water column physicochemical regimes.
5. Microbes involved in anaerobic CH_4 oxidation.

   Obj: To identify the microorganisms involved in AOM in lake sediments by using stable isotopic probing methods.

Chapter 4

Hypotheses

To address the objectives previously mentioned, the following hypotheses were formulated:

1. A method based on infrared Tunable Diode Laser Absorption Spectroscopy (TDLAS) combined with incubations in equilibration cells enables determination, in the field, of the CH_4 production/oxidation rates with several benefits compared to standard methods.

   This hypothesis is addressed in section 6.1 (method for determination of CH_4 production by TDLAS) and in section 6.2 (method for determination of aerobic CH_4 oxidation rates)

2. Aerobic methanotrophy in northern lakes is highly variable and dependent on dissolved CH_4 concentrations, which in turn is a function of the permafrost soil type in which these lakes are located.

   This hypothesis is addressed in section 6.2 which describes spatial and temporal variation of methanotrophy in Alaskan lakes.

3. AOM is a widespread process among northern lakes, independent of lake trophic state and water physicochemical regime.

   This hypothesis is addressed in section 6.3, which describes the assessment of AOM in 15 Alaskan lakes.

4. The addition of SO_4^{-2} or NO_3^- stimulates AOM, suggesting they are effective electron acceptor in lakes.

   This hypothesis is also addressed in section 6.3, which describes the assessment of AOM in 15 Alaskan lakes.

5. A significant fraction of the CH_4 produced in the anaerobic sediments of lakes is anaerobically oxidized with no added electron acceptor.

   This hypothesis is addressed in section 6.4, which describes the AOM profiles in sediment cores of three Alaskan lakes.

6. The identification of microorganisms involved in AOM in lake sediments is possible by using stable isotopic probing techniques.

   This hypothesis is addressed in section 6.5, which describes the possible participation of aerobic methanotrophs in AOM, as evidenced by DNA-SIP.

Chapter 5

Materials and Methods

In this chapter, we describe the general material and methods used in the present work. Specific methodologies are presented in each subsection of the chapter 6.

The experimental work was divided into four main methodologies:

1. Development of a non-invasive technique based on TDLAS to determine CH_4 production and oxidation.
2. Sample collection and measurement of physicochemical parameters in water and sediment cores, including, temperature, pH, total organic carbon (TOC), nitrates (NO_3^-), nitrites (NO_2^-), sulfates (SO_4^{-2}), total iron/manganese and dissolved CH_4/$\delta^{13}C$-CH_4 and CO_2/ $\delta^{13}C$-CO_2.
3. Determination of aerobic methanotrophic rates in lake water and methanogenic, methanotrophic and AOM in lake sediment.
4. Identification of microorganisms involved in AOM by stable isotope probing lipids and DNA analyses.

5.1 Study sites

The study sites selected were lakes located at different latitudes, in table 2 the general information about the study lakes were compiled. The study sites are located in:

a) Alaska, USA. To evaluate the effects of latitudinal variation and permafrost type on aerobic methane oxidation (MO), lakes were selected along a transect from the southcentral Alaskan coast on the Kenai Peninsula to the Arctic Ocean near Prudhoe Bay. The transect crossed through glaciated mountain ranges and discontinuous, sporadic, or no permafrost in south-central Alaska; discontinuous to isolated yedoma permafrost in the interior of Alaska; and continuous permafrost in northern Alaska. In this work, lakes located in yedoma-type permafrost soil areas will be referred to as "yedoma lakes" and all others as "non-yedoma lakes". Fig. 5 shows the distribution of studied lakes.

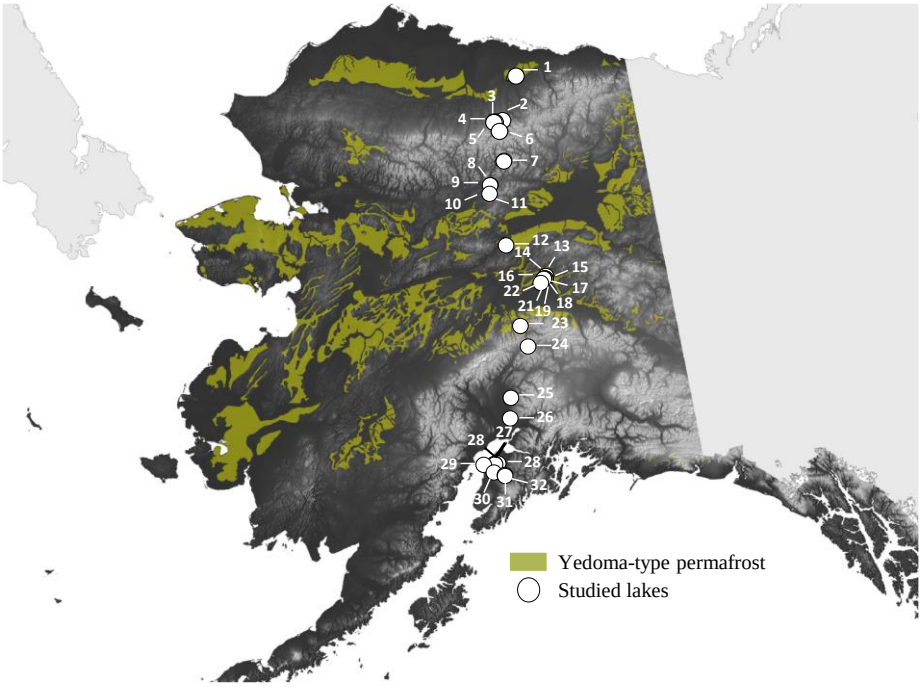

Figure 5. Location of arctic studied lakes. The identification numbers correspond to the given number presented in table 2. From: Martinez-Cruz et al., 2015.

b) Lake Stechlin area, Germany. Dagow Lake is a eutrophic lake located in northern Brandenburg, Germany. The main morphological and limnological characteristics of the lake were described by Casper (1996).

Figure 6. Location of Dagow Lake in Germany. "Relief Map of Germany" from: www.ginkgomaps.com/en/rl3c_de_germany_map_illdtmcolgw30scut_ja_mres.jpg.

c) Mexico: two tropical (low elevation) and three temperate (high elevation) lakes as well as one tropical and two temperate reservoirs with different physicochemical regimes, morphologies and trophic states were analyzed in Mexico (Fig. 7). The analyses were done in superficial layers of the sediments in order to determine the AOM occurrence.

Figure 7. Location of tropical and temperate lakes and reservoirs in Mexico. The identification numbers correspond to the given number presented in table 2. Map from: SRTM-30 relief data, by Carport.

Table 2. Identification, location, and permafrost soil type for lakes included in the study. *Indicates informal lake name, TSI = Trophic state index: UO – ultraoligotrophic; O – oligotrophic; M – mesotrophic; E – eutrophic; Mx – mixotrophic; HE– hypereutrophic

#	Name	Lat.	Long.	Country	TSI	Permafrost type
1	Big Sky* A31	69.581	-148.639	USA	O	Non-Yedoma
2	GTH 112	68.672	-149.249	USA	Mx	Non-yedoma
3	NE2	68.647	-149.582	USA	O	Non-yedoma
4	Toolik A28	68.632	-149.605	USA	UO	Non-yedoma
5	E1	68.626	-149.555	USA	UO	Non-yedoma
6	Julieta* A27	68.447	-149.369	USA	UO	Non-yedoma
7	El Fuego* A36	67.666	-149.716	USA	UO	Non-yedoma
8	Jonas* A26	67.647	-149.722	USA	UO	Non-yedoma
9	Augustine Zoli* A25	67.138	-150.349	USA	O	Non-yedoma
10	Ping*	67.136	-150.370	USA	UO	Non-yedoma
11	Grayling* A24	66.954	-150.393	USA	O	Non-yedoma
12	Eugenia*	65.834	-149.631	USA	Mx	Yedoma
13	Vault*	65.029	-147.699	USA	Mx	Yedoma
14	Goldstream*	64.916	-147.847	USA	Mx	Yedoma
15	Doughnut*	64.899	-147.908	USA	O	Non-yedoma
16	Killarney*	64.870	-147.901	USA	Mx	Yedoma
17	Smith A13	64.865	-147.868	USA	Mx	Non-yedoma
18	Stevens Pond*	64.863	-147.871	USA	Mx	Yedoma
19	Duece A2	64.863	-147.942	USA	Mx	Yedoma
20	Ace A1	64.862	-147.937	USA	Mx	Yedoma
21	Rosie Creek*	64.770	-148.079	USA	Mx	Yedoma
22	Otto	63.842	-149.037	USA	O	Non-yedoma
23	Floatplane* A16	63.394	-148.670	USA	O	Non-yedoma
24	Montana A40	62.143	-150.048	USA	O	Non-yedoma
25	Rainbow Shore* A41	61.694	-150.089	USA	M	Non-yedoma
26	Big Merganser A49	60.726	-150.644	USA	O	Non-yedoma
27	Rainbow A48	60.719	-150.808	USA	UO	Non-yedoma
28	Dolly Varden A47	60.704	-150.787	USA	UO	Non-yedoma
29	Abandoned Cabin* A50	60.696	-151.315	USA	O	Non-yedoma
30	Scout A46	60.533	-150.843	USA	O	Non-yedoma
31	Engineer A45	60.478	-150.323	USA	O	Non-yedoma
32	Lower Ohmer A44	60.456	-150.317	USA	O	Non-yedoma
33	Dagow	53.151	13.0513	Germany	E	NA
34	Cuitzeo	19.940	-101.200	Mexico	HE	NA
35	Umecuaro	19.523	-101.254	Mexico	O	NA
36	Zirahuen	19.435	-101.738	Mexico	O	NA
37	El Llano	19.658	-99.507	Mexico	M	NA
38	Guadalupe	19.633	-99.256	Mexico	HE	NA
39	San Julian	19.256	-96.261	Mexico	HE	NA
40	Olmeca	19.147	-96.151	Mexico	HE	NA
41	Martintela	18.570	-95.680	Mexico	HE	NA

5.2 Methane measurement by TDLAS prototype

We used a commercial infrared TDLAS (GasFinder 2.0, Boreal Laser Inc., Canada) to detect and quantify gaseous CH_4 produced during CH_4 production (MA) and aerobic oxidation (MO) tests as well as dissolved CH_4 concentration in sediments. The GasFinder 2.0 is a portable instrument with 1 ppmm sensitivity and a measurement frequency of 1 s^{-1}. We modified the GasFinder 2.0 to support a frame for a closed glass vial, used as MA/MO test vial and a laser reflector, perfectly aligned with the laser beam source (Figure 1). This design allowed the laser beam to cross the superior section (headspace) of the test vial containing the sample before being reflected back to the detector, crossing again through the test vial on the return path. Standard test vials of 100 ± 1.0 mL volume were custom made for MA/MO incubations and 412 ± 10 mL test vials were custom made for dissolved CH_4 concentration in sediment core slices, all test vials had 1.473 refraction index. GasFinder 2.0 was not initially designed for measurement of gas concentration within a glass enclosure or with such a short path length; therefore, all measurements were made with reference to a calibration curve.

5.3 Sample collection and processing

5.3.1 Water

Lake water was sampled offshore and usually near the center of each lake. In the winter, the ice cover was drilled through with a motorized auger (0.3m in diameter). Using a Hydrolab DataSonde (Hach Hydromet, Loveland, CO, USA), temperature, pH, chlorophyll *a*, and dissolved oxygen (DO) were determined at 1m depth intervals throughout the water column. Water samples for aerobic methanotrophic rates and dissolved CH_4 concentration were taken at a depth of within 1m of the ice–water interface in winter and usually at 0.75–1m water depth in 25 summer. Samples were taken with a horizontal Van Dorn bottle (Wildco, Yulee, FL, USA) and kept at 4°C before incubation tests or frozen before dissolved CH_4 concentration measurements.

5.3.2 Sediments

Superficial sediments were collected with an Ekman dredge (Wildco, USA) and sediment cores were collected in polycarbonate tubes (7.5 cm diameter) with overlaying water, with a gravity corer (Universal Percussion Corer, Aquatic Instruments, USA and Uwitec®, Austria) and sealed

without headspace. The superficial sediments and sediment cores were transported to the laboratory and stored in the dark at 4°C until their analyses within 24 h after collection.

Each core was sliced in six sections (0-2.5 cm, 5-10 cm, 10-15 cm, 15-20 cm and 20-25cm) along the core. For each subsample, sediment plugs were collected per triplicate by 5 mL polyethylene syringes and processed for determining CH_4 and CO_2 concentrations by gas chromatography. After collecting the samples for dissolved gases, the subsamples from the same section of each core were pooled for physicochemical analysis and incubation tests. Aliquots of ~30 g from each pooled section of the core were centrifuged at 415 G for 15 min, the supernatant was the pore water, which was filtered through 0.45 μm pore size (Whatman OE67) and used for physicochemical analysis. We also collected ~5 g aliquots of each interval to determine dry weight and amount of organic matter as loss on ignition; the total organic carbon (TOC) was calculated from the loss on ignition.

5.4 Sample analyses

5.4.1 Physical and chemical analysis

Dissolved nitrate (NO_3^-) and nitrite (NO_2^-) in pore water were determined according to standard methods (APHA, 1999). For all lakes, sulfate (SO_4^{2-}) concentration was measured with an ion chromatograph (eluent 3.2 mM Na_2CO_3, 1 mM $NaHCO_3$, detection limit 5 mmol L^{-1}; electrical conductivity determination, DIONEX ICS1000, USA and Germany). Total iron (Fe^+) and manganese (Mn^+) were determined according EPA (Method 3050 B, 2000) for all lakes. The DOC and DIC were measured with an Aurora 1030W TOC Analyzer (O.I. Analytical, Canada) in Alaska, and with Shimadzu-TOC analyzer (Shimadzu, Japan) for Dagow Lake. Dry weight and loss on ignition were determined following APHA (1999) methods, and TOC and TIC were determined with a TOC Shimadzu Analyzer coupled to a Solid Sample Combustion Unit SSM-5000A (Shimadzu, USA and Germany) for all lakes.

5.4.2 Dissolved CH_4 concentration and stable isotopic ratios determination

Except for the lake sediments used to test the TDLAS prototype, the slurry and water samples for gas testing were unfrozen and vigorously shaken for several minutes for promoting the gas-liquid equilibration. In both, Alaska and Germany, the headspace was analyzed for CH_4 and CO_2 concentration with a GC-2014 gas chromatograph (Shimadzu, Japan), equipped with a Flame

Ionization Detector (FID) and a PLOT alumina column; however, in Germany it was equipped with a Shimadzu HS-20 auto-sampler. In Alaska, the gas chromatograph was calibrated with pure CH_4 and CO_2 standards (Scott Specialty Gases, USA) of three different concentrations 1, 1,000 and 10,000 ppm; while in Germany, the gas chromatograph calibration was done by diluting a CH_4/CO_2 standard mix (Scott Specialty Gases, Germany) of 1% concentration for both gases. After gas analysis, the slurry of each bottle was tested for dry weight for further calculations. We determined the concentration of dissolved gases as follows.

$$C_s = \frac{(C_g^* \cdot V_g) + \left(\frac{C_g^*}{H'} V_l\right)}{dw} \quad (1)$$

Where C_s is the dissolved methane concentration in the slurry sample (g L^{-1}); C* g is the gas concentration measured in the headspace of the vial at equilibrium (g L^{-1}); V_l and V_g are the water and gas volumes in the vial respectively (L); dw is the slurry dry weight of; and H' is the CH_4 air/water partition coefficient (dimensionless), defined as follows.

$$H' = \frac{1}{R \cdot T \cdot K_H \cdot e^{\left[\alpha \cdot \left(\frac{1}{T} - \frac{1}{298.15}\right)\right]}} \quad (2)$$

Where R is the universal gas constant (0.082 L atm K^{-1} mol^{-1}); T is the equilibration temperature (K) at the time of measurement; K_H is the CH_4 Henry's law constant at 298.15 K (1.39 10^{-3} mol L^{-1} Bar^{-1}, NIST 2013), and α is the temperature dependence coefficient of the Henry's law constant (1750 K, NIST 2013).

The $^{13}CH_4$ and $^{13}CO_2$ isotopic ratios at every sediment section from Alaskan lakes were determined with Picarro G2201-i Analyzer (Picarro Inc., California, USA), whereas for Dagow Lake, they were obtained from Conrad et al. (2009).

5.5 Incubations tests

All incubation experiments were carried out as triplicate for each water and sediment sample from each lake. Methanogenic, methanotrophic and anaerobic oxidation of methane (AOM) tests, were developed according to the hereafter explained procedure.

For aerobic methanotrophic incubation tests in water, we homogenized the water sample by strong agitation in aerobic conditions. Then, we transferred 30 mL of the homogeneous water to each sterile serum 50 mL bottles. For sediment, we homogenized each sediment sample by transferring them into anaerobic water while flushed with ultrahigh purity N_2 (99.999%, AirLiquid, USA or Germany) to keep anaerobic conditions and get a homogeneous slurry with 60% of wet sediment. Then, we transferred 30 mL of the homogeneous slurry to each sterile serum 50 mL bottles.

The serum bottles containing water or slurry, designed for aerobic methanotrophic tests, were flushed with zero air (AirLiquid, USA) to ensure aerobic conditions. All methanotrophic test were spiked with 2 mL of CH_4 (99%, AirLiquid, USA or Germany) to get a final concentration of 5% of CH_4 in the headspace and 1.8 mg L^{-1} in the liquid phase.

Bottles, designated for AOM and methanogenic tests, were kept in anaerobic conditions by flushing ultrahigh purity N_2 (99.999%) to each bottle for several minutes, we immediately closed the bottles with blue butyl rubber stoppers (Bellco, USA or Germany) and aluminum crimp caps. Finally, we added a solution of Na_2S to get a final concentration of 0.001% in the slurry and reduce the media. To ensure that CH_4 was not a limiting factor, all AOM tests were spiked with 2 mL of $^{13}CH_4$ (99%, Sigma Aldrich, USA or Germany) to get a final concentration of 5% of $^{13}CH_4$ in the headspace and 1.8 mg L^{-1} in the liquid phase.

The anaerobic conditions of AOM and methanogenic incubations, and the aerobic conditions of methanotrophic incubations along the whole incubation were tested by determining O_2 concentration in the headspace with a GC-2014 gas chromatograph (Shimadzu, Japan), equipped with a Thermal Conductivity Detector (TCD) and a packed column. Incubation tests in Alaska were maintained in darkness at 4 ± 2 °C, which is the annual mean temperature in Boreal lakes (Sepulveda-Jauregui et al., 2015); whereas incubation tests in Germany were kept in darkness at 8 ± 3 °C, which is the annual mean temperature in Dagow Lake (Casper, 1996).

Incubations from all lake water and sediments were monitored for CH_4, CO_2 and O_2 concentration with a GC-2014 gas chromatograph along the incubation time. AOM tests were monitored for isotopic ^{13}C-CH_4 ratios every 30 days with Picarro G2201-i Analyzer (Picarro Inc., USA) and for isotopic ^{13}C-CO_2 ratios only at initial and final times using a gas chromatography

combustion isotope ratio mass spectrometer (GC-C-IRMS) system (ThermoQuest, USA and Germany). Both equipment reported in the delta notation vs. V-PDB (see below). Methanotrophic tests were analyzed until most of the CH_4 was oxidized; methanogenic tests were analyzed until CH_4 production reached a plateau; and AOM tests were analyzed for ~200 days. Five killed controls for each treatment were prepared by sterilizing 50 mL bottles containing 30 mL of a homogenous slurry from each section of the core at 121 °C for 20 min, followed by the addition of the same headspace according to the incubation test designated for.

5.6 Stable isotope probing (SIP)

Sediment samples were collected along AOM incubation tests and frozen to -80 °C. After the incubation, DNA and phospholipid fatty acids (PLFAS) were extracted from the sediment samples. The DNA and PLFAs were first tested for the incorporation of the ^{13}C into the DNA and PLFAs, by determining the $\delta^{13}C$ in an elemental analyzer (Finnigan DeltaPlus XP, Thermo Scientific) coupled to a Costech ECS4010 Elemental Analyzer (Costech Scientific, Valencia, California, USA) at the University of Alaska Stable Isotope Facility, Fairbanks, Alaska, USA. When the presence of ^{13}C-DNA and ^{13}C-PFLAs was proved, DNA-SIP and PLFA-SIP were done following the methods described by He et al. (2012). The sequencing of heavy fractions of DNA was done at Michigan State University (MSU) by Illumina sequencing and the analysis of ^{13}C-PFLAs was done at Stable Isotope Facility in the University of California Davis.

5.6.1 Illumina sequencing and analyses of 16S rRNA genes

The V4 region of the 16S rRNA gene was amplified using Illumina fusion primers as described by Caporaso et al. (2012). PCR output for all samples was normalized using a Life Technologies SequalPrep Normalization plate. The normalized products were pooled. After Ampure clean up, QC and quantitation the pool was loaded on a standard v2 MiSeq flow cell and sequenced in a 2x250bp format using custom V4 sequencing and index primers (see Caporaso et al.) and a MiSeq 500 cycle reagent cartridge (v2). Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ with Illumina Bcl2fastq v1.8.4.

Sequences were processed using the mothur software package, version 1.33.3 as previously described (Kozich et al., 2013; Uhlik et al., 2012). Briefly, the read was processed by removing

primers and tags, and sequences that had an average quality score of above 25 and read length of no longer than 275 nt. Identical sequences were grouped and representatives aligned against the SILVA database. A further pre-clustering screening step was applied to reduce sequencing noise by clustering reads differing by only 1 bp per 100 nt of each read. Chimeric sequences were detected and removed using the mothur-implementation of Uchime. The remaining high-quality reads were used to generate a distance matrix and clustered using a furthes-neighbor algorithm. Operational taxonomic units were defined by 97% similarity, and identified using the RDP database.

Sequences were first trimmed of the primer region and low-quality sequences were removed by illumina sequencing pipeline. The sequences that passed this filter (average length of 331 bp) were assigned by the RDP Naïve Bayesian Classifier (80% confidence threshold). The nucleotide sequences have been deposited to the NCBI Short Read Archive under the accession number of SRP005485.

5.7 Data analysis and rate calculations

The methanogenic production rates were determined from the linear increase of CH_4 concentration against time. Methanotrophic potential rates were determined from the linear decrease in CH_4 in methanotrophic bottles and AOM rates were calculated from the linear decrease of $^{13}CH_4$ concentration against time, for that purpose we measured the isotopic fraction of ($\delta^{13}CH_4$) and the total CH_4 concentration as indicated above. We determined $^{13}CH_4$ concentration, based on equation (3).

$$^{13}CH_4 = \frac{TCH_4}{\frac{1}{\left(\frac{\delta^{13}C.C_4}{1000\ ‰}+1\right)\times VPDB}+1} \qquad (3)$$

Where, $\delta^{13}C.CH_4$ is the isotopic fraction measured by the equipment, TCH_4 is the total CH_4 concentrations of the sample measured by gas chromatography (mol g_{dw}^{-1}), and VPDB is the Vienne Pee Dee Belemnite standard = 0.0112372.

The CH_4 production rates determined from methanogenic incubations comprises not only production but also oxidation of CH_4 being produced, therefore it will be called net methane production rates (NMPR). The total methane production rates (TMPR) were then determined

as the sum of AOM rates (AOMR) and NMPR. From those three values it is possible to determine the percent of CH_4 being oxidized in anaerobic conditions. This assumption neglects the ^{12}C-CH_4 being oxidized by AOM as well as the ^{13}C-CH_4 produced by methanogenesis which may underestimate the actual percent of total CH_4 being oxidized. We also detected methanogenic activity in AOM incubations, nevertheless we only accounted for the ^{12}C-CH_4 assuming that all the ^{13}C-CH_4 was due to the initial addition, this methane produced calculated only from the ^{12}C-CH_4 was called ^{12}C methane production rate (^{12}MPR) and it also corresponds to a net methane production.

5.7.1 Statistical Analysis

Normal distribution for all parameters was tested with Shaphiro-Wilk test. Most of the analyzed parameters were non-normal distributed. Therefore, significant differences were determined using the Kruskal–Wallis (KW) test for nonparametric data ($p < 0.05$). Statistical analyses were conducted using the NCSS 2000 Statistical Analysis System software (Number Cruncher Statistical Systems, Kaysville, 15 UT, USA). All linear regressions were constructed using Wolfram Mathematica 7.0 (Wolfram, Minneapolis, MN, USA).

Chapter 6

Results and discussion

6.1 Methanogenic activity tests by Infrared Tunable Diode Laser Absorption Spectroscopy

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Abstract

Methanogenic activity (MA) tests are commonly carried out to estimate the capability of anaerobic biomass to treat effluents, to evaluate anaerobic activity in bioreactors or natural ecosystems, or to quantify inhibitory effects on methanogenic activity. These activity tests are usually based on the measurement of the volume of biogas produced by volumetric, pressure increase or gas chromatography (GC) methods. In this study, we present an alternative method for non-invasive measurement of methane produced during activity tests in closed vials, based on Infrared Tunable Diode Laser Absorption Spectroscopy (MA-TDLAS). This new method was tested during model acetoclastic and hydrogenotrophic methanogenic activity tests and was compared to a more traditional method based on gas chromatography. From the results obtained, the CH_4 detection limit of the method was estimated to 60 ppm and the minimum measurable methane production rate was estimated to $1.09 \cdot 10^{-3}$ $mg \cdot l^{-1} \cdot h^{-1}$, which is below CH_4 production rate usually reported in both anaerobic reactors and natural ecosystems. Additionally to sensitivity, the method has several potential interests compared to more traditional methods among which short measurements time allowing the measurement of a large number of MA test vials, non-invasive measurements avoiding leakage or external interferences and similar cost to GC based methods. It is concluded that MA-TDLAS is a promising method that could be of interest not only in the field of anaerobic digestion but also, in the field of environmental ecology where CH_4 production rates are usually very low.

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Methanogenic activity tests by Infrared Tunable Diode Laser Absorption Spectroscopy

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ABSTRACT

Methanogenic activity (MA) tests are commonly carried out to estimate the capability of anaerobic biomass to treat effluents, to evaluate anaerobic activity in bioreactors or natural ecosystems, or to quantify inhibitory effects on methanogenic activity. These activity tests are usually based on the measurement of the volume of biogas produced by volumetric, pressure increase or gas chromatography (GC) methods. In this study, we present an alternative method for non-invasive measurement of methane produced during activity tests in closed vials, based on Infrared Tunable Diode Laser Absorption Spectroscopy (MA-TDLAS). This new method was tested during model acetoclastic and hydrogenotrophic methanogenic activity tests and was compared to a more traditional method based on gas chromatography. From the results obtained, the CH_4 detection limit of the method was estimated to 60 ppm and the minimum measurable methane production rate was estimated to $1.09{\cdot}10^{-3}$ mg l^{-1} h^{-1}, which is below CH_4 production rate usually reported in both anaerobic reactors and natural ecosystems. Additionally to sensitivity, the method has several potential interests compared to more traditional methods among which short measurements time allowing the measurement of a large number of MA test vials, non-invasive measurements avoiding leakage or external interferences and similar cost to GC based methods. It is concluded that MA-TDLAS is a promising method that could be of interest not only in the field of anaerobic digestion but also, in the field of environmental ecology where CH_4 production rates are usually very low.

1. Introduction

Anaerobic degradation of organic matter proceeds via a number of microbial processes, including hydrolysis, acidogenesis and acetogenesis; which produce hydrogen, CO_2, formate, acetate and ammonium (Muyzer and Stams, 2008). As the last step of anaerobic digestion, methanogens produce CH_4 from H_2 and CO_2 (hydrogenotrophic pathway), acetate (acetoclastic pathway) or methylated compounds (methylotrophic pathway) (Liu and Whitman, 2008). In sewage sludge digesters, about 70% of CH_4 is produced via acetoclastic pathway and the other 30% is produced by the hydrogenotrophic pathway (Kruger et al., 2005; Pavlostathis and Giraldogomez, 1991). With soluble substrates, methanogenesis is generally considered as the rate limiting step and methanogens are also regarded as the microbial community most sensitive to the environmental or operational conditions. Thus, CH_4 production rate is an important parameter which informs about the all anaerobic process and is often used (i) to quantify or characterize anaerobic digestion processes such as wastewater treatment or soil remediation or (ii) to evaluate anaerobic processes in natural environments such as soils, peatlands or aquatic ecosystems.

CH_4 production rate is usually measured during methanogenic activity (MA) tests, using several procedures that have been exhaustively listed by Souto et al. (2010) and earlier by Soto et al. (1993). Despite a large diversity, MA tests are usually based on one of the three following methods; (i) the recovery and measurement of the volume of biogas produced, combined with the determination of CH_4 content of the biogas, (ii) the measurement of CH_4 produced in closed vials by gas chromatography (GC) or (iii) the measurement of pressure increase in closed vials, combined with the determination of CH_4 content of the biogas.

These methods are largely used on a daily basis in countless applications and give confident results but they also have some drawbacks. Methods based on recovery and measurement of the volume of biogas produced require a measurement device for each MA test and are limited to the measurement of relatively high methanogenic activities; i.e. producing a measurable volume of biogas. On the contrary, methods based on gas chromatography are very sensitive but are time demanding which limit the number of test vials that can be processed together. Methods based on pressure can be applied to very large number of samples but require the quantification of CH_4 content of the biogas produced and are limited to relatively high methanogenic activities, although high sensitivity pressure sensors have been developed. Pressure based methods are also of limited interest for hydrogenotrophic MA tests.

A relatively new technology with the potential for improving determinations of dissolved gas measurement is Infrared (IR) Tunable Diode Laser Absorption Spectroscopy (TDLAS). TDLA spectrometers are based on the emission and reflection, back to a detector, of a laser beam. Along

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the laser beam path, the presence of the target gas modifies the laser spectrum through the absorption of light in a specific wavelength range, which is detected by the instrument upon return of the laser beam. This modification of the laser spectrum can be used to quantify the concentration of the target gas with a sensitivity as low as 1 ppm meter (ppmm), defined as the minimum concentration detectable multiplied by the path length. A modification to the commercially available open path technique allows the measurement of the gas concentration within the headspace of a closed cell, providing a convenient technique for non-invasive determinations of methane concentration. A method, based on the same principle has been previously described for the determination of dissolved methane concentration in aquatic ecosystems (Sepulveda-Jauregui et al., in press).

In this paper, we developed and tested a method based on TDLAS, called MA-TDLAS, for the determination of methane production rate during MA tests. We put emphasis on quantifying the precision and accuracy of the method by comparing the results obtained by MA-TDLAS to classical gas chromatography method.

2. Methods

2.1. MA-TDLAS prototype

We used a commercial infrared TDLAS (GasFinder 2.0, Boreal Laser Inc., Edmonton, Canada) to detect and quantify gaseous CH_4 produced during MA tests. The GasFinder 2.0 is a portable instrument with 1 ppmm sensitivity and a measurement frequency of 1 s^{-1}. We modified the GasFinder 2.0 to support a frame for a closed glass vial, used as MA test vial and a laser reflector, perfectly aligned with the laser beam source (Fig. 1). This design allowed the laser beam to cross the superior section (headspace) of the MA test vial before being reflected back to the detector, crossing again through the MA test vial on the return path. Standard MA test vials of 100 ± 1.0 mL volume were custom made from Schott Duran 3.3 borosilicate glass having a 38 mm external diameter, 34 mm internal diameter, 104 mm length and 1.473 refraction index. Additionally, each MA test vial was fabricated with a serum vial type bottle neck at the bottom to allow sealing with a 20 mm inner diameter rubber stopper and aluminum crimp cap. GasFinder 2.0 was not initially designed for measurement of gas concentration within a glass enclosure or with such a short path length; therefore all measurements were made with reference to a calibration curve.

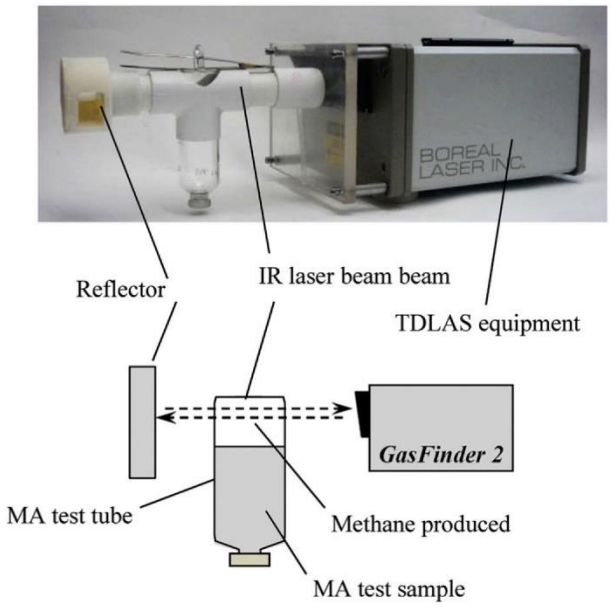

Fig. 1. Schematic of MA-TDLAS measurement method and prototype.

2.2. Methanogenic activity tests

MA-TDLAS method was assessed during MA tests done with anaerobic sludge obtained from a full scale upflow anaerobic sludge blanket (UASB) plant treating urban wastewater (Metropolitan Autonomous University, Mexico). Experiments were conducted in MA test vials containing 60 ml of sludge and 40 ml headspace. In order to test MA-TDLAS method over a wide range of methanogenic activity, three sludge concentrations (X) were used; namely 1.021, 0.102 and 0.010 $g_{VSS}\ l^{-1}$ obtained by diluting the original sludge sample with mineral medium (Park et al., 2010). It should be noted that due to granular nature of the original sludge, dilutions as well as concentrations were approximate. Medium preparation and inoculation were done under strict anaerobic conditions by continuous flushing with He (99.998%, Infra, Mexico) upon vials closure.

Two distinct MA tests were performed; (1) Hydrogenotrophic MA tests, where headspace of test vials was replaced by H_2/CO_2 (80/20, Infra, México) as carbon and energy source (Sorensen and Ahring, 1993) and (2) Acetoclastic MA tests by addition of 3.0 g l^{-1} of acetate as carbon and energy source (Park et al., 2010) and with He (99.998%, Infra, México) as headspace. Each experimental condition (3 sludge concentrations, each with 2 different substrates) was tested in triplicate, each MA test vial being treated independently from the others (total of 18 MA test vials). All MA test vials were incubated at 37 °C without shaking. CH_4 produced by methanogenesis was measured each hour by MA-TDLAS, according to the following procedure; (1) control MA test vials containing standard CH_4 concentrations were read for calibration, (2) each MA test vial containing sample was vigorously shaken for 10 s to allow for phase equilibrium and then immediately placed in the laser beam path, (3) a stable MA-TDLAS reading was typically observed within 5 seconds, (4) five readings for each MA test vial were done and (5) a new calibration was performed after measurement of all test vials, to ensure reading stability.

In order to compare results obtained by MA-TDLAS with a more conventional method, CH_4 concentration in MA test vials were also measured in triplicate by gas chromatography (GC) using a Clarus-500 (Perkin Elmer, Mexico) chromatograph equipped with a FID detector and an Elite-QPlot column (Perkin Elmer, Mexico). Significant difference between results was determined using the Tukey–Kramer's multiple comparison (TK) tests performed after analyses of variance ($\alpha < 0.05$) using the NCSS 2000 Statistical Analysis System software (Number Cruncher Statistical Systems, USA).

3. Results and discussion

We first tested the MA-TDLAS method against GC, by measuring seven MA test vials containing 60 ml of distilled water and different CH_4 concentrations, ranging from 2500 to 25000 ppm. CH_4 concentration in the headspace of each test vial was measured, first in triplicate by GC and then, in quintuplicate by TDLAS. Fig. 2 shows the results observed. A linear correlation was observed between both techniques ($R^2 > 0.99$), with a slope of 1.00. These results confirm that CH_4 concentration in the headspace of MA test vials can be measured by TDLAS.

MA-TDLAS method was then tested during actual MA tests, under both acetoclastic and hydrogenotrophic conditions, with three sludge concentrations. Fig. 3 shows the average CH_4 concentration increased observed for each sludge concentration, during acetoclastic (Fig. 3A) and hydrogenotrophic (Fig. 3B) tests. As observed, TDLAS methods allowed the detection of a clear CH_4 production during 10 hours of experimental time.

Table 1 shows methanogenic activities, measured by TDLAS and by GC during acetoclastic and hydrogenotrophic MA tests. Except for the higher sludge concentration measured, no significant difference was

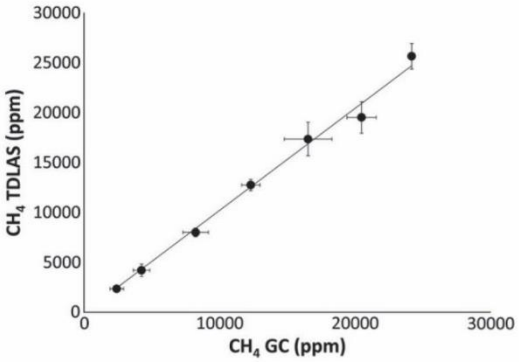

Fig. 2. CH_4 concentrations in MA test vials measured by GC and TDLAS.

observed between both measurements techniques. It is worthwhile to underline that standard deviations of both techniques were similar.

In order to further explore the sensitivity and accuracy of the MA-TDLAS method, a total of 100 independent measurements done in quintuplicate during MA tests were used to determine the standard deviation (σ) observed at several CH_4 concentrations. Fig. 4 shows the results obtained, for CH_4 concentrations ranging from 30 to 20,000 ppm. Fig. 4 also shows the theoretical curve and equation that best fitted experimental data. As expected, standard deviation increased exponentially as CH_4 concentration decreased. According to Fig. 4 an arbitrary cut-off index for standard deviations of 5, 10 and 30% gave CH_4 concentrations of 720, 340 and 86 ppm, respectively. The detection limit, defined as the lowest CH_4 concentration that can be distinguished from a blank concentration ($\alpha < 0.05$) was 60 ppm. At 60 ppm, the standard deviation was

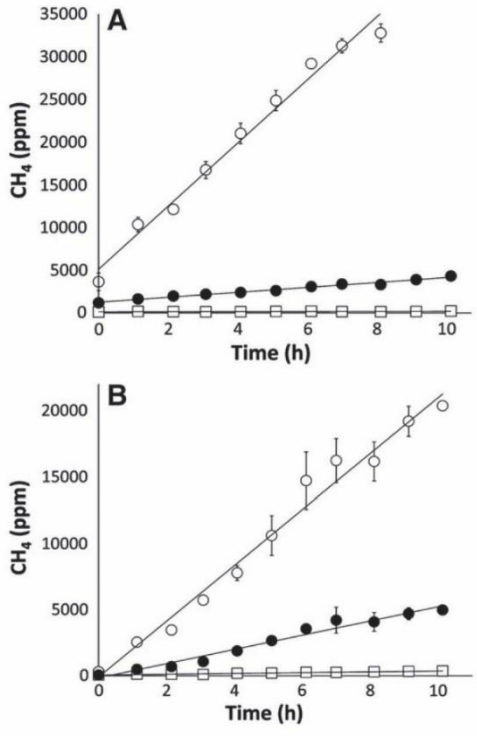

Fig. 3. Average methane production observed during (A) acetoclastic and (B) hydrogenotrophic MA tests, measured by MA-TDLAS and performed with 1.021 (○), 0.102 (●) and 0.010 (□) $g_{VSS} \cdot l^{-1}$ of sludge. Error bars indicate standard deviation; 3 test vials for each condition, quintuplicate methane measurements.

Table 1
Acetoclastic and hidrogenotrophic activities measured by GC and by TDLAS. Tukey–Kramer test results for each treatment are presented in parenthesis ($\alpha<0.05$; $n=3$ for each substrate and biomass concentration); values with the same capital letter are not significantly different.

	X ($g_{VSS} \cdot l^{-1}$)	CH_4 production rate ($mg \cdot l^{-1} \cdot h^{-1}$)	
		TDLAS	GC
Acetate	1.021	1.144 ± 0.033 (A)	0.997 ± 0.038 (B)
	0.102	0.082 ± 0.015 (C)	0.093 ± 0.007 (C)
	0.010	0.001 ± 0.001 (D)	0.009 ± 0.008 (D)
H_2/CO_2	1.021	0.644 ± 0.044 (E)	0.509 ± 0.059 (F)
	0.102	0.162 ± 0.024 (G)	0.185 ± 0.036 (G)
	0.010	0.010 ± 0.001 (H)	0.020 ± 0.006 (H)

38% (Fig. 4). Compared to TDLAS, standard deviation of GC was estimated, under experimental conditions of this work, to 15% for 300 ppm and below and 7.3% for CH_4 concentrations over 1000 ppm. Thus GC method was more precise than TDLAS at low CH_4 concentrations but less precise at relatively high concentrations. It should be emphasized that GC error was mainly due to sampling and injection errors, inexistent in MA-TDLAS. However, GC errors can be significantly reduced by using auto-sampler and auto-injection devices.

At standard temperature for methanogenesis tests (37 °C), CH_4 Henry constant is $1.11 \cdot 10^{-3}$ $mol \cdot l^{-1} \cdot bar^{-1}$ (NIST, 2012). Under these conditions, a headspace CH_4 concentration of 60 ppm, which is the detection limit of the TDLAS method, corresponds to a CH_4 concentration in the headspace of test tubes of 0.038 $mg \cdot l^{-1}$. If observed in MA test tubes of 100 ml with 60 ml sample, this concentration would be found after a CH_4 production of 0.026 $mg \cdot l^{-1}$, in the liquid sample. Although no time limit between measurements has to be set, an arbitrary CH_4 production rate of one detection limit per day would correspond to a minimum measurable CH_4 production rate of $1.09 \cdot 10^{-3}$ $mg \cdot l^{-1} \cdot h^{-1}$. CH_4 production rate in anaerobic reactors is usually within a range from 0.14 to 133 $mg \cdot l^{-1} \cdot h^{-1}$ (Gupta et al., 1994; Park et al., 2010), largely above the minimum measurable CH_4 production rate by MA-TDLAS. Similarly, in natural ecosystems, such as bogs or rice fields, methanogenic activities usually range from $2.40 \cdot 10^{-2}$ to 0.61 $mg \cdot l^{-1} \cdot h^{-1}$ (Kotsyurbenko et al., 2004; Roy and Conrad, 1999). Thus MA-TDLAS method is adequate not only for the field of anaerobic digestion treatment and environmental engineering but also for ecological studies.

It is well known that TDLAS is pressure-sensitive and the scientific background of such sensitivity has been well established (Hoffman and Davies, 2008). In order to determine the impact of pressure on MA-TDLAS method proposed here, different methane/air mixture were measured at several total pressures and with different CH_4 partial pressures. The results showed that MA-TDLAS reading were indeed dependent of the total and partial pressure of methane, showing a perfect linearity ($R^2 > 0.99$) of MA-TDLAS readings with CH_4 volume percentages

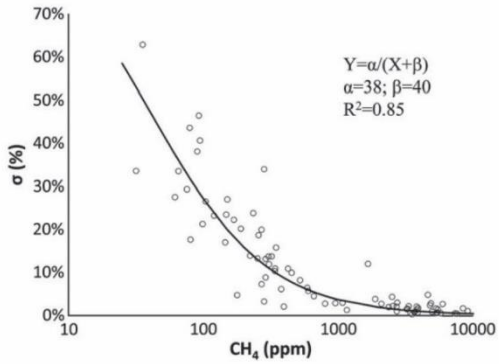

Fig. 4. Standard deviation observed during independent samples measurements (○) and best fitting correlation (——).

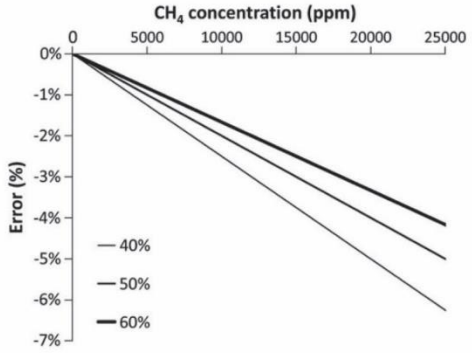

Fig. 5. Error on CH_4 determination due to pressure increase in MA-TDLAS tests, if not taken into account, for 100 ml test tube, 60 ml sample, 40 ml headspace, as a function of CH_4 concentration in headspace, assuming several percentage of CH_4 in biogas produced.

(results not shown). Thus, a significant pressure increase in MA test tubes due to biogas production, may generate a significant error if not taken into account. Fig. 5 shows the magnitude of such error. As shown, in the range of MA-TDLAS measurements (0–20,000 ppm) the error range in CH_4 determination ranges from 0 to −5%, which is probably within acceptable margins.

However, error on estimation can be easily eliminated by pressure measurements or, at least, significantly reduced, assuming a certain CH_4 percentage in biogas produced. Eq. (1) shows how to correct TDLAS reading; where C_g is the corrected CH_4 concentration ($g \cdot l^{-1}$), C'_g is CH_4 concentration read by TDLAS equipment (ppm), R is the ideal gas constant (0.0821 $l \cdot atm \cdot K^{-1} \cdot mol^{-1}$), T is temperature (K), P_{atm} is atmospheric pressure at which the MA test tube was closed and % CH_4 is the percentage of CH_4 in biogas produced.

$$C_g = \frac{16 \cdot C'_g \cdot P_{atm}}{1 \cdot 10^6 \cdot R \cdot T \cdot \left(1 - \frac{100 \cdot C'_g}{1 \cdot 10^6 \cdot P_{atm} \cdot \%CH_4}\right)} \quad (1)$$

The MA-TDLAS proposed here, requires relatively low analytical effort. Quintuplicate measurement of CH_4 concentration took approximately 1 min, while triplicate measurement of CH_4 concentrations by GC took approximately 60 min. MA-TDLAS allows therefore the analysis of a large samples number, with an effort probably similar to method based on pressure measurements, but with a similar sensitivity and accuracy than methods based on GC.

4. Conclusion

From the results obtained, the interest of MA-TDLAS for the determination of acetoclastic and hydrogenotrophic methanogenic activity is confirmed. This method has several potential interests compared to more traditional methods; (i) MA-TDLAS is fast; each quintuplicate measurement can be done in approximately 1 minute, allowing the measurement of a large number of MA test vials, (ii) MA-TDLAS is non-invasive as it does not require sampling of any kind, (iii) MA-TDLAS is similar to GC in sensitivity, allowing measurement of low methanogenic activities and (iv) MA-TDLAS cost is estimated to be similar to GC, as the GasFinder 2.0 used in this work costs 35.000 US$ and does not require any consumable. MA-TDLAS is a promising method that could be of interest not only in the field of anaerobic digestion treatment but also in the field of environmental ecology where CH_4 production rates are usually very low.

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6.2 Geographic and seasonal variation of dissolved methane and aerobic methane oxidation in Alaskan lakes

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Abstract

Methanotrophic bacteria play an important role oxidizing a significant fraction of methane (CH_4) produced in lakes. Aerobic CH_4 oxidation depends mainly on lake CH_4 and oxygen (O_2) concentrations, in such manner that higher MO rates are usually found at the oxic/anoxic interface, where both molecules are present. MO also depends on temperature, and via methanogenesis, on organic carbon input to lakes, including from thawing permafrost in thermokarst (thaw)-affected lakes.

Given the large variability in these environmental factors, CH_4 oxidation is expected to be subject to large seasonal and geographic variations, which have been scarcely reported in the literature. In the present study, we measured CH_4 oxidation rates in 30 Alaskan lakes along a north-south latitudinal transect during winter and summer with a new field laser spectroscopy method. Additionally, we measured dissolved CH_4 and O_2 concentrations. We found that in the winter, aerobic CH_4 oxidation was mainly controlled by the dissolved O_2 concentration, while in the summer it was controlled primarily by the CH_4 concentration, which was scarce compared to dissolved O_2. The permafrost environment of the lakes was identified as another key factor. Thermokarst (thaw) lakes formed in yedoma-type permafrost had significantly higher CH_4 oxidation rates compared to other thermokarst and non-thermokarst lakes formed in non-yedoma permafrost environments. As thermokarst lakes formed in yedoma-type permafrost have been identified to receive large quantities of terrestrial organic carbon from thaw and subsidence of the surrounding landscape into the lake, these results confirm that coupling of terrestrial and aquatic habitats.

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Geographic and seasonal variation of dissolved methane and aerobic methane oxidation in Alaskan lakes

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Abstract. Methanotrophic bacteria play an important role oxidizing a significant fraction of methane (CH_4) produced in lakes. Aerobic CH_4 oxidation depends mainly on lake CH_4 and oxygen (O_2) concentrations, in such a manner that higher MO rates are usually found at the oxic/anoxic interface, where both molecules are present. MO also depends on temperature, and via methanogenesis, on organic carbon input to lakes, including from thawing permafrost in thermokarst (thaw)-affected lakes. Given the large variability in these environmental factors, CH_4 oxidation is expected to be subject to large seasonal and geographic variations, which have been scarcely reported in the literature. In the present study, we measured CH_4 oxidation rates in 30 Alaskan lakes along a north-south latitudinal transect during winter and summer with a new field laser spectroscopy method. Additionally, we measured dissolved CH_4 and O_2 concentrations. We found that in the winter, aerobic CH_4 oxidation was mainly controlled by the dissolved O_2 concentration, while in the summer it was controlled primarily by the CH_4 concentration, which was scarce compared to dissolved O_2. The permafrost environment of the lakes was identified as another key factor. Thermokarst (thaw) lakes formed in yedoma-type permafrost had significantly higher CH_4 oxidation rates compared to other thermokarst and non-thermokarst lakes formed in non-yedoma permafrost environments. As thermokarst lakes formed in yedoma-type permafrost have been identified to receive large quantities of terrestrial organic carbon from thaw and subsidence of the surrounding landscape into the lake, confirming the strong coupling between terrestrial and aquatic habitats and its influence on CH_4 cycling.

1 Introduction

Northern lakes are an important source of atmospheric CH_4 (Bastviken et al., 2011), and it has been estimated that they are responsible for as much as 6 % of global emission to the atmosphere (Walter et al., 2007). Methane emission from aquatic ecosystems is significantly mitigated by CH_4 oxidation (MO) by aerobic methanotrophs, a group of gram-negative bacteria that use CH_4 as a carbon and energy source (Murrell et al., 1993; Trotsenko and Murrell, 2008). It has been estimated that globally, 30 to 99 % of total CH_4 produced in freshwater ecosystems is microbiologically oxidized in the water column rather than being released to the atmosphere (Bastviken et al., 2002; Thauer et al., 2008). Likewise, MO plays an important role in northern lakes specifically by oxidizing up to 88 % of the CH_4 diffusing through the water column (Kankaala et al., 2006, 2007; Bellido et al., 2011). As recently demonstrated using stable isotopes, after assimilating CH_4, methanotrophs are incorporated into the lake food web by zooplankton (Kankaala et al., 2006; Jones and Grey, 2011), Daphnia magna (Taipale et al., 2012), Odonata spp. (Seifert and Scheu, 2012), and Chironomus larvae (Gentzel et al., 2012; Wooller et al., 2012), among others. In addition to CH_4 respiration and conversion to CO_2, MO is therefore a pathway that reincorporates a fraction of the CH_4-C produced into the biogeochemical carbon cycle within lakes.

Several environmental factors directly affect aerobic MO in freshwater ecosystems. First, methanotrophy depends on the availability of both CH_4 and O_2. Higher MO rates are usually found at the oxic/anoxic interface, where both CH_4 and O_2 are present (Utsumi et al., 1998a, b; Bastviken et al., 2002; Liikanen et al., 2002; Carini et al., 2005; Schu-

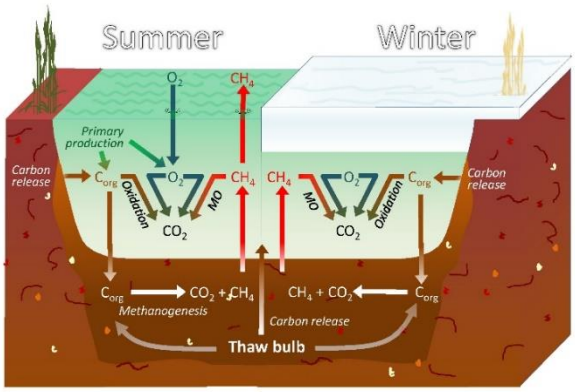

Figure 1. Carbon cycling in northern high-latitude lakes during the summer and winter. Carbon (C_{org}) release from primary production and landscape processes promotes CH_4 production and competes with MO for O_2.

bert et al., 2010). In turn, CH_4 and O_2 concentrations depend on numerous other processes involved in biogeochemical carbon cycling (Fig. 1). Among these, the most important are methanogenesis producing CH_4, primary production and atmospheric diffusion supplying O_2, and several aerobic metabolic processes that compete with MO for available O_2 (Dzyuban, 2010).

In addition to autochthonous and allochthonous carbon inputs to lakes, permafrost thaw can provide an additional source of labile organic carbon to fuel methanogenesis and carbon mineralization in thermokarst (thaw) lakes (Zimov et al., 1997; Walter et al., 2006). MO in northern regions is therefore directly and indirectly linked to permafrost type and landscape processes that are highly variable. Permafrost ranges from sporadic to continuous and is also variable in composition (Jorgenson et al., 2008). Yedoma-type permafrost is an organic-rich (about 2 % carbon by mass) Pleistocene-age permafrost with ice content of 50–90 % by volume (Zimov et al., 2006), which occurs mainly in the previously unglaciated regions of Siberia, Alaska, and NW Canada (Czudek and Demek, 1970; Walter et al., 2007; Kanevskiy et al., 2011; Grosse et al., 2013). Non-yedoma permafrost has a more widespread distribution than yedoma permafrost and is also characterized by a much thinner carbon-rich horizon, variable in composition (Ping et al., 2008; Tarnocai et al., 2009; Hugelius et al., 2014).

Many northern lakes are located in continental climate zones, subject to contrasting seasonal conditions with long, cold winters followed by relatively short, but warm summers. Although psychrotolerant and psychrophilic methanotrophs have been reported (Omelchenko et al., 1993, 1996; Bowman et al., 1997; Trotsenko and Khmelenina, 2002), MO was reported to occur more efficiently at mesophilic temperature (Semrau et al., 2010). During winter, northern lakes are covered by a thick ice layer for 7 to 9 months. Surface lake ice impedes oxygen transfer from the atmosphere to the lake and, when snow-covered, substantially reduces light penetration and oxygen production by photosynthesis (White et al., 2008; Clilverd et al., 2009). Thus, the combination of low temperature and limited oxygen availability suggests lower MO rates in northern lakes in winter than in summer.

Given the number of parameters having a potential effect on MO, as well as the patchwork of seasonal and geographic conditions found among northern lakes, MO is expected to exhibit large geographic and seasonal variations that still remain to be characterized. The goal of our study was to determine these variations through measurement of dissolved CH_4 and O_2 as well as MO rates in the winter and summer in 30 lakes along a south-north transect in Alaska.

2 Materials and methods

2.1 Site description

We sampled 30 Alaskan lakes during two field campaigns, one in late winter (March–April 2011) and one in summer (June–July 2011). To evaluate the effects of latitudinal variation and permafrost type on MO, lakes were selected along a transect from the south-central Alaskan coast on the Kenai Peninsula to the Arctic Ocean near Prudhoe Bay (Fig. 2). The transect crossed through glaciated mountain ranges and discontinuous, sporadic, or no permafrost in south-central Alaska; discontinuous to isolated yedoma permafrost in the interior of Alaska; and continuous permafrost in northern Alaska. In this work, for simplicity, lakes located in yedoma-type permafrost areas will be referred to as yedoma lakes and all others as non-yedoma lakes. Geographic variability along the north-south Alaska transect has been previously described for ecosystems, climate, geology, and permafrost type (Gregory-Eaves et al., 2000; Jorgenson et al., 2008; Smith et al., 2010). Additionally, Sepulveda-Jauregui et al. (2015) quantified the surface area of the selected lakes (0.002–1.45 km^2), their trophic states (ultraoligotrophic to eutrophic), and their annual CH_4 fluxes (0.5–317 g CH_4 m^{-2} yr^{-1}). Table 1 shows the location and permafrost type of the selected lakes.

2.2 Sampling and field measurements

We sampled lake water usually near the center of each lake. In the winter, the ice cover was drilled through with a motorized auger (0.3 m in diameter). Using a Hydrolab DataSonde (Hach Hydromet, Loveland, CO, USA), we measured temperature, pH, chlorophyll *a*, and dissolved oxygen (DO). The pH and DO sensors of the Hydrolab were calibrated regularly, before and after each section of the latitudinal lake transect (four sections per transect, approximately one calibration per week). Temperature and chlorophyll *a* sensors were not regularly calibrated. All parameters were measured at 0.5 or 1 m depth intervals throughout the water column, ex-

Table 1. Identification, location, and permafrost soil type for lakes included in the study.

#	Name	Lat.	Long.	Permafrost type
1	Big Sky* A31	69.581	−148.639	Non-yedoma
2	GTH 112	68.672	−149.249	Non-yedoma
3	NE2	68.647	−149.582	Non-yedoma
4	Toolik A28	68.632	−149.605	Non-yedoma
5	E1	68.626	−149.555	Non-yedoma
6	Julieta* A27	68.447	−149.369	Non-yedoma
7	El Fuego* A36	67.666	−149.716	Non-yedoma
8	Jonas* A26	67.647	−149.722	Non-yedoma
9	Augustine Zoli* A25	67.138	−150.349	Non-yedoma
10	Ping*	67.136	−150.370	Non-yedoma
11	Grayling* A24	66.954	−150.393	Non-yedoma
12	Eugenia*	65.834	−149.631	Yedoma
13	Goldstream*	64.916	−147.847	Yedoma
14	Killarney*	64.870	−147.901	Yedoma
15	Smith A13	64.865	−147.868	Non-yedoma
16	Stevens Pond*	64.863	−147.871	Yedoma
17	Duece A2	64.863	−147.942	Yedoma
18	Ace A1	64.862	−147.937	Yedoma
19	Rosie Creek*	64.770	−148.079	Yedoma
20	Otto	63.842	−149.037	Non-yedoma
21	Floatplane* A16	63.394	−148.670	Non-yedoma
22	Montana A40	62.143	−150.048	Non-yedoma
23	Rainbow Shore* A41	61.694	−150.089	Non-yedoma
24	Big Merganser A49	60.726	−150.644	Non-yedoma
25	Rainbow A48	60.719	−150.808	Non-yedoma
26	Dolly Varden A47	60.704	−150.787	Non-yedoma
27	Abandoned Cabin* A50	60.696	−151.315	Non-yedoma
28	Scout A46	60.533	−150.843	Non-yedoma
29	Engineer A45	60.478	−150.323	Non-yedoma
30	Lower Ohmer A44	60.456	−150.317	Non-yedoma

* Indicates informal lake name.

cept in Dolly Varden L. where measurement intervals were increased to every five meters from 15 to 25 m depth. In lakes shallower than 1 m, we measured Hydrolab parameters at three distributed depths throughout the lake water column.

Water samples for MO rates and dissolved CH_4 concentration were taken at a depth of within 1 m of the ice-water interface in winter and usually at 0.75 to 1 m water depth in summer. Due to differences in lake depth and thickness of the ice sheets, samples reflected surface water in deep lakes, but mid water column or even lake bottom water environment, in shallow lakes. Samples were taken with a horizontal Van Dorn bottle (Wildco, Yulee, FL, USA).

Water density derived from surface and bottom water temperatures were used to determine the relative water column stability (RWCS; Padisak et al., 2003). Lakes with RWCS > 56.5 were considered fully stratified, lakes with RWCS < 16.3 were considered fully mixed, and lakes with intermediate RWCS were considered partially stratified (Branco et al., 2009). Similarly, we determined whether an oxycline was present in each lake based on a sharp DO gradient or presence of an oxic/anoxic interface. Detailed temperature and DO profiles are available as a Supplement in Sepulveda-Jauregui et al. (2015). We report all results in mean ±standard deviation (SD).

2.3 Dissolved CH_4 concentration and MO rate

To avoid long delays in sample transfer from remote locations to the laboratory, we determined dissolved CH_4 concentrations with a previously described method based on Headspace Equilibration using Infrared Tunable Diode Laser Absorption Spectroscopy (HE-TDLAS; Sepulveda-Jauregui et al., 2012). This method consisted of creating a gas/liquid equilibrium in a 100 mL equilibration vial containing 60 mL of lake water sample and 40 mL of headspace (air) by vigorous shaking for 10 s. As previously reported (Sepulveda-Jauregui et al., 2012), this shaking time was enough to reach equilibrium and allowed the non-invasive determination of CH_4 concentration in the water sample by measuring headspace concentration with a laser beam crossing the headspace of the equilibration vial, through the glass walls

of the vial. This measurement was conducted with a modified open-field CH_4 analyzer (GasFinder 2; Boreal Laser, Edmonton, Canada). The CH_4 concentration in the water sample was calculated from the measured headspace concentration according to Henry's law. In the present work, all CH_4 concentrations are expressed in mg CH_4 L^{-1}.

We determined duplicate MO rates in one water sample from each lake taken as described above, using a modified HE-TDLAS method to allow for measurement of MO in the field. This new method was based on a previous development using the HE-TDLAS method for the determination of methanogenic activity (Martinez-Cruz et al., 2012). Two 60 mL lake water subsamples from a single Van Dorn bottle sample were gently transferred to two 100 mL equilibration vials (duplicates). Equilibration vials were immediately closed with rubber stoppers and vigorously shaken for 10 s to transfer most of the dissolved CH_4 contained in the water sample to the headspace. Next, the headspace was vented, the vial was closed, and the sample was shaken again to evacuate the residual CH_4 content of the water sample. Using this procedure, more than 99.5 % of the original CH_4 content of the sample was evacuated. The equilibration vials were then closed with rubber stoppers and aluminum crimp caps, spiked with 0.6 mL CH_4 (99.0 % purity; Air Liquide, Houston, TX, USA) injected with a disposable syringe, and vigorously shaken for 10 s. This approach allowed MO tests to be conducted with an initial standard CH_4 concentration in the liquid phase ($\sim 0.6\,mg\,L^{-1}$). It also provided an initial CH_4 to O_2 molar ratio of 0.062, significantly below the stoichiometric ratio (0.5), ensuring no O_2 limitation.

Equilibration vials were incubated for 10–12 days in a water bath inside insulated boxes placed in our vehicle. In the winter, the vials were maintained at $2 \pm 2\,°C$ in a water bath with ice supplements; in the summer, the vials were maintained at $15 \pm 2\,°C$. The temperature of the water bath was measured daily. We measured the CH_4 concentration in the equilibration vials daily using the HE-TDLAS method described in detail by Sepulveda-Jauregui et al. (2012). Briefly, dry control MO test vials containing only CH_4 standards were read by the TDLAS for calibration. Each experimental equilibration vial was vigorously shaken for 10 s to reach phase equilibrium and then immediately placed in the laser beam path, after which a stable HE-TDLAS reading was typically observed within 5 s. Five readings were taken for each MO test vial and recalibration was conducted after measuring each set of test vials to ensure instrument stability. The field HE-TDLAS method allowed measurement of dissolved CH_4 and MO rates. This technique was simple, rapid (about 60 s per sample measurement), non-invasive, and avoided complications and long delays in sample transfer from remote locations to the laboratory.

We calculated the total CH_4 concentration (C_{CH_4} = total CH_4 mass present in the gas and liquid phases divided by the sample liquid volume) in each vial during the MO tests. MO rates were determined from the decrease in C_{CH_4} in the equilibration vials with time. MO rates determined by this method represent the MO rate after aeration and CH_4 addition (vials spiked with CH_4 and vigorously shaken). Thus, these MO rates do not correspond to actual observations of in situ DO and dissolved CH_4 concentrations in the lakes. The measured CH_4 oxidation rate was considered the potential MO (r_{max}; $mg\,CH_4\,L^{-1}\,d^{-1}$) under non-limiting CH_4 and DO concentrations. To estimate the actual rate (r; $mg\,CH_4\,L^{-1}\,d^{-1}$) from r_{max}, a double Monod model was used (Bae and Rittmann, 1996; Segers, 1998) in which C_{CH_4} and C_{O_2} represent the actual dissolved CH_4 and DO concentrations measured in the lake, respectively, and K_{S-CH_4} and K_{S-O_2} are the apparent affinity constants of the methanotrophic community, for CH_4 and DO, respectively:

$$r = r_{max} \cdot \frac{C_{CH_4}}{K_{S\text{-}CH_4} + C_{CH_4}} \cdot \frac{C_{O_2}}{K_{S\text{-}O_2} + C_{O_2}}. \quad (1)$$

Average $K_{S\text{-}CH_4}$ and $K_{S\text{-}O_2}$ values for lakes have been determined by previous studies: $K_{S\text{-}CH_4} = 0.110 \pm 0.053\,mg\,L^{-1}$ (mean ±SD; Liikanen et al., 2002; Lofton et al., 2014) and $K_{S\text{-}O_2} = 0.624 \pm 0.064\,mg\,L^{-1}$ (mean ±SD; Lidstrom and Somers, 1984; Frenzel et al., 1990). To the best of our knowledge, the highest $K_{S\text{-}CH_4}$ reported in lakes is $0.704\,mg\,L^{-1}$ (Liikanen et al., 2002). It should be noted that these reported K_S values refer to the apparent affinity constants for the methanotrophic community, rather than the half-saturation constant for the CH_4 monooxygenase enzyme that catalyzes CH_4 oxidation. The potential error caused by using previously reported K_S, instead of experimentally determined values will be considered in the discussion section.

To establish the extent of potential MO limitation by CH_4 or DO, two limitation factors were defined, where β is the limitation factor for CH_4 (%) and γ is the limitation factor for DO (%):

$$0\,\% \le \beta = \left(1 - \frac{C_{CH_4}}{K_{S\text{-}CH_4} + C_{CH_4}}\right) \cdot 100 \le 100\,\% \quad (2)$$

$$0\,\% \le \gamma = \left(1 - \frac{C_{O_2}}{K_{S\text{-}O_2} + C_{O_2}}\right) \cdot 100 \le 100\,\%. \quad (3)$$

A limitation factor of 100 % means that 100 % of a process ceases to occur due to the absence of the limiting substrate, while a limitation factor of 0 % indicates a process occurring at maximum rate ($r = r_{max}$). When $\beta > \gamma$, CH_4 was considered to be the limiting factor; conversely, when $\gamma > \beta$, DO was considered to be the limiting factor.

2.4 Statistical analyses

Normality was assessed by the Shapiro-Wilk test. Since most of the data was non-normally distributed and with unequal samples number, significant differences among all parameters were determined using Kruskal-Wallis multiple comparison test (differences were considered significant at $p < 0.05$, $Z > 1.96$). To assess whether CH_4 was oxidized

during the MO incubation tests, significant differences between C_{CH_4} were determined by an analysis of variance (ANOVA; $p < 0.05$), after normality was assessed by the Shapiro-Wilk test. Statistical analyses were conducted using the NCSS 2000 Statistical Analysis System software (Number Cruncher Statistical Systems, Kaysville, UT, USA). Linear regressions were also conducted to determinate the MO rates using Wolfram Mathematica 7.0 (Wolfram, Minneapolis, MN, USA).

3 Results

3.1 Physicochemical parameters

The sampled lakes were shallow; other than four atypical lakes with a maximum known depth of > 20 m (lakes #4, #24, #26 and #30), the average lake depth in summer was 4.5 ± 2.6 m (mean ±SD). During winter, none of the lakes was completely frozen at the sampling stations. Liquid water was always present underneath the ice cover, which ranged in thickness from 0.60 to 1.25 m (mean ±SD, 0.81 ± 0.14 m). The mean temperature, measured at all depth throughout the lake water columns with the Hydrolab probe was $2.4 \pm 0.6\,^{\circ}C$ (mean ±SD, $n = 103$) in the winter and $13.9 \pm 2.4\,^{\circ}C$ (mean ±SD, $n = 235$) in the summer. According to RWCS, during the summer, 15 lakes of the 28 for which a complete temperature profile was determined were fully thermally stratified. Six lakes were partially stratified and seven lakes were mixed. During the winter, 16 of 18 lakes for which a complete temperature profile was determined were fully mixed, while two lakes were partially stratified and none was fully stratified. We observed temperature inversion in 15 of the 18 lakes, with temperature gradients ranging from -0.1 to $-3.4\,^{\circ}C$, with an average of $-1.4\,^{\circ}C$ (top minus bottom temperature). Overall, only one third of the temperature profiles indicated clear stratification. In both seasons, no correlation between RWCS and lake depth was found, probably due to the fact that lakes were shallow and with an uneven depth distribution.

Lake water pH ranged from 5.9 to 8.2 in winter and 6.3 to 9.2 in summer among the study lakes. Chlorophyll *a* was only detected during the summer, ranging from 1.0 to 45.9 $\mu g\,L^{-1}$ (manufacturer detection limit, 0.03 $\mu g\,L^{-1}$). The concentration of dissolved CH_4 in the 30 lakes ranged from 0.01 to 14.77 $mg\,L^{-1}$ during the winter and from 0.02 to 1.51 $mg\,L^{-1}$ during the summer (Table 2). The DO concentration at the same depths ranged from 0.10 to 13.63 $mg\,L^{-1}$ during the winter and from 0.22 to 11.07 $mg\,L^{-1}$ during the summer (Table 2). During summer, a clear oxycline was observed in all yedoma lakes, but only in six of 20 non-yedoma lakes. In contrast, during winter, an oxycline was not observed in any of the yedoma lakes, which were largely anaerobic throughout the whole water column. We observed an oxycline in winter in four of 13 non-yedoma lakes. Overall, an oxycline was observed in 30 % of the DO profiles. Temperature-oxygen profiles for all 30 studied lakes are shown in Sepulveda-Jauregui et al. (2015).

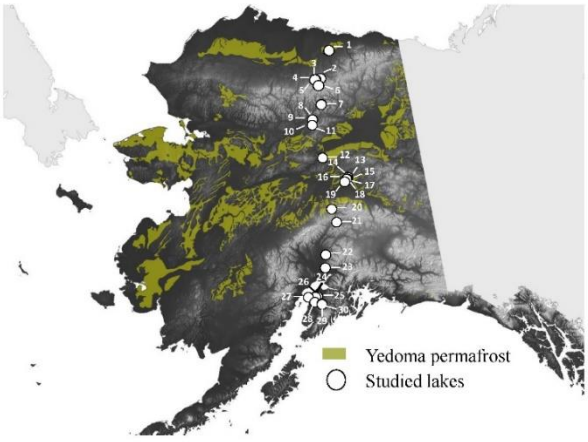

Figure 2. Locations of studied Alaskan lakes (white circles) plotted on the Alaska DEM hillshade raster. Information about the distribution of yedoma-type deposits (Pleistocene-aged, ice-rich silt containing deep thermokarst lakes) was from Jorgenson et al. (2008) and Kanevskiy et al. (2011). The Alaska map is the National Elevation Data Set 30 m hillshade raster.

Fig. 3 shows the statistical distributions of the dissolved CH_4 and DO concentrations, as well as the Kruskal-Wallis comparisons. Significant differences were observed between yedoma and non-yedoma lakes ($p < 0.05$). In yedoma lakes, the CH_4 and DO concentrations were significantly higher and lower, respectively, than in non-yedoma lakes during both seasons (Kruskal-Wallis test, $p < 0.05$). In addition to differences related to permafrost type, higher CH_4 concentrations and lower DO concentrations were observed during the winter than in the summer (Fig. 3) and an apparent geographic trend was observed. Higher dissolved CH_4 and lower DO concentrations were found in lakes from central Alaska than in those from southern and northern Alaska (Sepulveda-Jauregui et al., 2015).

3.2 Methane oxidation rates

The HE-TDLAS method allowed us to determine the MO potential in the field in all studied lakes. Fig. 4 shows three representative C_{CH_4} trends observed in the MO vials. In some cases, MO began on the first day of incubation (Fig. 4a) and the initial slope of the change in C_{CH_4} was taken into account in determining the MO rate. In about 60 % of the cases during the summer and 80 % during the winter, a lag phase was observed; i.e. period of time with no apparent MO (Fig. 4b). This behavior, termed induction of MO, has previously been reported for various soils (Bender and Conrad, 1995; Dunfield et al., 1999) and can be interpreted as an adaptation period of the CH_4 oxidizers to the culture conditions. In lakes in

Table 2. Methane oxidation parameters for 30 Alaskan lakes.

	CH_4 (mg L^{-1})		O_2 (mg L^{-1})		Potential MO (mg L^{-1} d^{-1})		Actual MO (mg L^{-1} d^{-1})		Limiting factor	
#	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
1	6.43	0.05	0.13	10.53	0.23	0.11	0.08	0.03	O_2	CH_4
2	0.58	0.09	0.43	8.28	0.14	0.36	0.05	0.15	O_2	CH_4
3	0.02	0.05	1.73	9.80	0.12	0.07	0.02	0.02	CH_4	CH_4
4	0.11	0.03	10.09	9.46	BDL	0.28	0.00	0.06	CH_4	CH_4
5	0.05	0.04	9.59	9.52	0.10	0.19	0.03	0.05	CH_4	CH_4
6	0.08	0.07	ND	9.81	0.12	0.20	ND	0.09	ND	CH_4
7	ND	0.06	ND	9.65	ND	0.18	ND	0.04	ND	CH_4
8	3.68	0.03	13.63	10.30	0.03	0.18	0.03	0.04	O_2	CH_4
9	8.83	0.11	3.64	9.87	0.05	0.39	0.04	0.19	O_2	CH_4
10	3.00	0.06	0.25	6.94	0.11	0.11	0.03	0.03	O_2	CH_4
11	8.43	0.88	0.19	9.31	BDL	0.28	0.00	0.23	O_2	CH_4
12	0.79	0.07	0.15	6.90	0.09	0.29	0.02	0.11	O_2	CH_4
13	8.43	0.19	0.11	6.23	0.49	0.54	0.07	0.48	O_2	O_2
14	12.59	0.31	0.09	0.31	0.20	0.92	0.03	0.30	O_2	O_2
15	1.30	0.02	0.23	3.93	0.05	0.31	0.01	0.05	O_2	CH_4
16	ND	0.72	0.11	1.36	ND	0.34	ND	0.20	ND	O_2
17	6.60	0.59	0.19	0.57	0.06	1.34	0.01	0.54	O_2	O_2
18	0.70	0.03	0.14	6.74	0.02	0.77	0.00	0.15	O_2	CH_4
19	14.77	1.51	0.13	0.22	0.20	0.74	0.04	0.19	O_2	O_2
20	1.24	0.03	0.31	9.47	0.05	0.67	0.01	0.15	O_2	CH_4
21	ND	0.04	ND	9.52	ND	0.33	ND	0.08	ND	CH_4
22	0.08	0.05	2.79	11.07	0.05	0.20	0.01	0.06	CH_4	CH_4
23	0.30	0.08	5.84	9.59	0.02	0.68	0.01	0.33	CH_4	CH_4
24	0.04	0.02	12.40	9.66	0.06	0.34	0.02	0.08	CH_4	CH_4
25	0.08	0.03	11.91	10.20	0.32	0.25	0.12	0.04	CH_4	CH_4
26	0.01	0.03	10.00	10.24	0.04	0.08	0.00	0.02	CH_4	CH_4
27	0.03	0.02	7.90	9.67	0.15	0.41	0.03	0.07	CH_4	CH_4
28	0.07	0.04	0.20	9.01	BDL	0.38	0.00	0.09	O_2	CH_4
29	0.04	0.04	9.13	10.19	0.02	0.28	0.00	0.05	CH_4	CH_4
30	ND	0.03	ND	10.25	ND	0.38	ND	0.11	ND	CH_4
					Mean					
Global	3.29	0.47	3.91	7.95	0.10	0.39	0.03	0.13	O_2^*	CH_4^*
Yedoma	7.53	1.73	0.14	3.19	0.18	0.71	0.03	0.28	O_2^*	CH_4^*
Non-yedoma	2.02	0.09	5.30	9.40	0.08	0.29	0.03	0.09	CH_4^*	CH_4^*

* Indicates median; ND – Not determined; BDL – Bellow detection limit. Yedoma lakes are marked in bold font.

which this pattern was observed, the lag phase was not taken into account and the MO rate was instead determined from the slope of C_{CH_4} after the lag phase. When no significant decrease in C_{CH_4} was observed during the first 7 days (Fig. 4c; ANOVA , $p < 0.05$), we assumed an MO rate of zero, consistent with previous reports for various soils (Whalen et al., 1990; Bender and Conrad, 1995; Dunfield et al., 1999). We observed MO rates of zero in only three non-yedoma lakes during winter. Otherwise, no correlation with lake morphology, season, or permafrost type was observed in regard to the existence of a lag phase or its duration.

The potential MO rate r_{max} ranged from 0.000 to 0.488 mg L^{-1} d^{-1} during the winter and from 0.073 to 1.339 mg L^{-1} d^{-1} during the summer (Fig. 5a). Seasonal variation of r_{max} was significant, with summer r_{max} up to 47 times higher than winter rates. Permafrost type was also an important determining factor, because during the summer, yedoma lakes had higher r_{max} than non-yedoma lakes (Kruskal-Wallis test, $p < 0.05$); specifically, r_{max} was 0.71 ± 0.36 and 0.29 ± 0.16 mg L^{-1} d^{-1} (mean $\pm$SD) for yedoma and non-yedoma lakes, respectively. However, during the winter, no significant differences were observed between yedoma and non-yedoma lakes. In addition to differences related to permafrost type, an apparent latitudinal pattern was also observed, with higher r_{max} for lakes from cen-

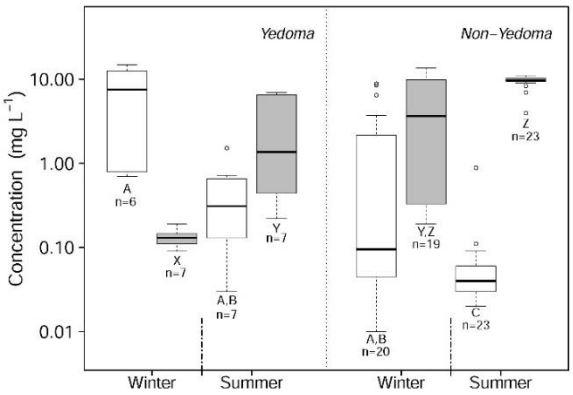

Figure 3. Statistical distributions of CH_4 (white boxes) and DO (grey boxes) water concentrations in yedoma and non-yedoma lakes during the winter and summer. The boxes include the median (Q2) and the quartile range (Q1 and Q3). The whiskers show minimum and maximum data. The open circles show outlier data. Capital letters are Kruskal-Wallis multiple comparison test; values with the same capital letter are not significantly different ($p < 0.05$, $Z > 1.96$). n represents the number of lakes measured.

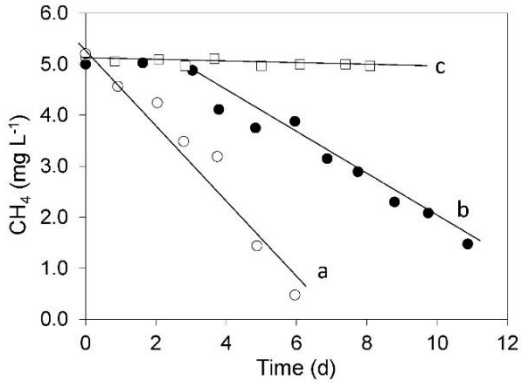

Figure 4. Examples of CH_4 oxidation patterns observed during the MO assays: (a) assay with no lag-phase, (b) assay with a 3-day lag-phase, and (c) assay with no detected activity. Straight lines are linear correlations.

tral Alaska compared to those from southern and northern Alaska (Fig. 5a).

4 Discussion

4.1 Geographic and seasonal variations in physicochemical parameters

In yedoma lakes, the CH_4 and DO concentrations were significantly higher and lower, respectively, than in non-yedoma lakes during both seasons. This observation is most likely due to higher organic carbon and nutrient inputs associated with thawing permafrost in yedoma-type lakes. Walter Anthony et al. (2014) and Sepulveda-Jauregui et al. (2015) showed that thawing yedoma permafrost not only provides ancient (Pleistocene-aged) organic carbon stimulating CH_4 production but also phosphate and nitrogen (ammonium), which promotes bacterial, algal and contemporary plant growth in and around lakes. Since terrestrial plant matter surrounding lakes gets deposited in thermokarst-lake sediments as lakes laterally expand, both enhanced allochthonous and autochthonous productivity of yedoma-type lake ecosystems results in higher rates of contemporary organic matter loading to sediments of yedoma-type lakes compared to non-yedoma lakes (Walter Anthony et al., 2014). Contemporary organic matter decomposes in part to form CH_4 in surface lake sediments. In contrast, ancient yedoma carbon is decomposed throughout the sediment profile (Fig. 1), with particularly high rates of methanogenesis occurring along the permafrost thaw front, located deep in the thaw bulb beneath the lake (Heslop et al., 2015). Methane produced at depth in the thaw bulb subsequently migrates, primarily as free-phase bubbles through bubble tubes in sediments, to the surface sediments where it escapes the lake via ebullition (Walter Anthony and Anthony, 2013; Tan et al. 2015). Thus, higher organic carbon and nutrient inputs in yedoma-type lakes promote higher anaerobic metabolism in the sediments together with aerobic metabolism in the water column, leading to higher CH_4 and lower DO concentrations in the water column.

In both yedoma and non-yedoma lakes, higher CH_4 concentrations and lower DO concentrations were observed during the winter than in the summer (Fig. 3). This seasonal variation can be attributed to thick ice covering the lakes in winter. Ice cover impedes gas exchange between the water and the atmosphere, promoting CH_4 build-up in the water column (Phelps et al., 1998; Bastviken et al., 2004; Juutinen et al., 2009) and hindering O_2 transfer from the atmosphere, except in some locations where high-flux ebullition seeps allow gas exchange through local holes in lake ice (Greene et al., 2014). Ice and snow also reduce light penetration and oxygen production by photosynthesis beneath the ice (White et al., 2008; Clilverd et al., 2009). The absence of detectable levels of chlorophyll *a* in ice-covered lakes during March and April (see results section) despite the longer springtime photoperiod was supportive evidence of reduced photosynthesis under the ice. In summer, although CH_4 production was probably higher due to warmer sediments, it did not cumulate in the water column and was released to the atmosphere (Fig. 1).

Geographic variations were also observed with higher dissolved CH_4 and lower DO concentrations being found in lakes from central Alaska than in those from southern and northern Alaska. However, this apparent latitudinal pattern was related to the higher proportion of yedoma lakes in central Alaska. No significant latitudinal trend was observed

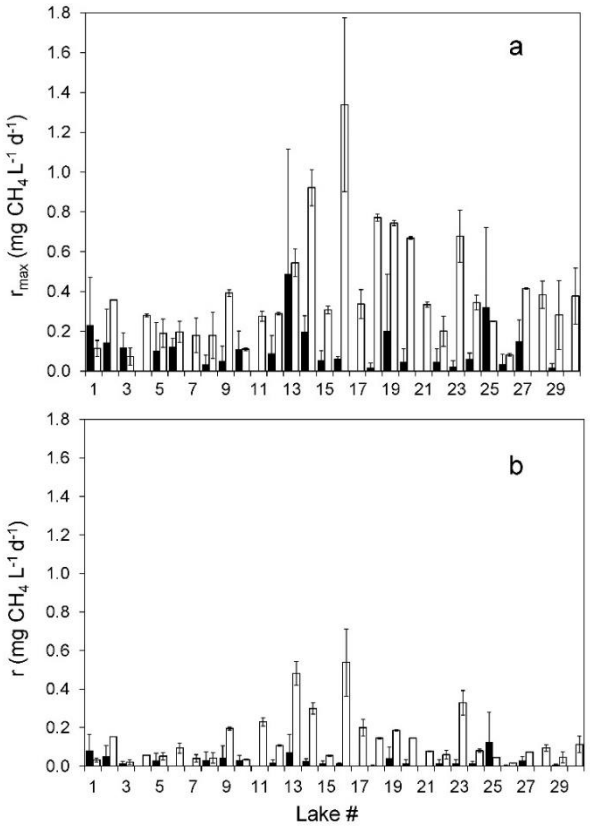

Figure 5. (a) CH_4 oxidation potential (r_{max}) and (b) CH_4 oxidation rates observed in 30 lakes along a north-south transect (left-right) in Alaska during the summer (white bars) and the winter (black bars).

when yedoma and non-yedoma lakes were analyzed separately (Kruskal-Wallis test, $p < 0.05$).

Figure 3 shows that when relatively high CH_4 concentrations were found, relatively low DO concentrations were observed and vice versa. This pattern was particularly clear in yedoma lakes: in winter, a CH_4 concentration of $7.32 \pm 5.86\,mg\,L^{-1}$ (mean ±SD) was found, while the DO concentration was $0.13 \pm 0.03\,mg\,L^{-1}$ (mean ±SD). In the same yedoma lakes, the summer CH_4 concentration was $0.49 \pm 0.52\,mg\,L^{-1}$ (mean ±SD), while the DO concentration was $3.19 \pm 3.24\,mg\,L^{-1}$ (mean ±SD). This observation suggests that MO was a significant contributor of the biogeochemical processes, actively controlling O_2 and CH_4 concentrations by oxidizing CH_4 when O_2 was present. To confirm the latter, it would be necessary to measure experimentally the O_2 uptake rate by methanotrophs and by other aerobic processes that compete with MO (Dzyuban, 2010).

The trend toward higher CH_4 concentrations and lower DO concentrations in winter than in summer was not as strong in non-yedoma lakes as in yedoma lakes (Fig. 4). These results provide additional evidence that in non-yedoma lakes, the lower organic carbon inputs fuels more weakly methanogenesis and aerobic processes including MO than in yedoma lakes, resulting in a lower seasonal variation of CH_4 and DO concentration. Another reason is that yedoma lakes have a significantly higher ebullition year round (Walter et al., 2007; Sepulveda-Jauregui et al., 2015). Even during winter, Greene et al. (2014) found that 80 % of CH_4 in ebullition bubbles trapped under the ice cover dissolves into the lake water column before being confined within the growing ice sheet, leading to elevated dissolved CH_4 beneath the ice.

4.2 Limiting factors of MO rates

The actual MO rates r ranged from 0.000 to $0.124\,mg\,L^{-1}\,d^{-1}$ during the winter and from 0.017 to $0.538\,mg\,L^{-1}\,d^{-1}$ during the summer (Fig. 6b), which as expected were significantly lower than the potential MO rates. These r values are within the range reported for arctic lakes, which ranges over 3 magnitude order, from 0.001 to $1\,mg\,L^{-1}\,d^{-1}$ (Liikanen et al., 2002; Kankaala et al., 2006; Lofton et al., 2014). Similarly, r values were 1 to 50-fold higher in the summer than in the winter. We attribute this finding to the temperature dependence of methanotrophy (Semrau et al., 2008; Borrel et al., 2011), but also to the limited DO concentration under the ice cover during the winter.

In addition to seasonal variations, permafrost type was also a determining factor of r and r_{max}. As mentioned before, although no difference in r_{max} was observed during winter between yedoma and non-yedoma lakes, r_{max} in yedoma lakes was about twice as high than in non-yedoma lakes during summer. We attribute that difference to a more active MO methanotrophic community in yedoma lakes, as all r_{max} tests were conducted in aerated vials with an initial standard CH_4 concentration in the liquid phase ($\sim 0.6\,mg\,L^{-1}$), thus ensuring similar and non-limiting conditions. As observed with r_{max}, during summer yedoma lakes showed 2–3 times higher r than non-yedoma lakes (Kruskal-Wallis test, $p < 0.05$; $r = 0.28 \pm 0.17$, mean ±SD, yedoma lakes; $r = 0.09 \pm 0.08\,mg\,L^{-1}\,d^{-1}$, mean ±SD, non-yedoma lakes). Higher r values for yedoma lakes in summer is explained by the higher dissolved CH_4 concentration in presence of a relatively high DO concentration above the oxycline (Fig. 3). As observed with CH_4 and DO concentrations, no significant latitudinal trend in MO was observed, when yedoma and non-yedoma lakes were analyzed separately.

The actual MO rates; r, were determined from r_{max} and CH_4 and DO concentrations using two affinity constants, K_{S-CH_4} and K_{S-O_2}. These affinity constants are highly variable, because their determination is challenging and subject to relatively high determination error (Segers et al., 1998) and because the methanotrophic community is sensitive to numerous factors and changes over time and space (Carini et al., 2005; He et al., 2012). For instance, Lofton et al. (2014) reported a variation of 150 % in K_{S-CH_4} within the hypolim-

netic water column of two lakes with similar characteristics. The determination of MO rates may, therefore, be subject to large error if reported values are used instead of experimental parameters or because of standard error associated to experimental K_S determination (Kovarova-Kovar and Egli, 1998). To quantify these potential errors, a sensitivity analysis was conducted. We arbitrarily modified K_{S-CH_4} and K_{S-O_2} and calculated the resulting r (Eq. 1) using the experimental r_{max}, C_{CH_4}, and C_{O_2} measured in the 30 lakes. Figure 6 shows the error on r caused by a given error on K_{S-O_2} (Fig. 6a) and K_{S-CH_4} (Fig. 6b), for yedoma and non-yedoma lakes, in winter and in summer. According to this analysis, an underestimation of K_{S-O_2} or K_{S-CH_4} would lead to an overestimation of the actual MO rate (positive error), while an overestimation of these affinity constants would produce an underestimation of r (negative error). Fig. 6a shows that, an error on K_{S-O_2} ranging from -50 to 200 %, would cause from 10 to -6 % error on r, for all lakes and all seasons, except in yedoma lakes during winter, where an error from 75 to -50 % would be generated. This relatively high sensitivity of r to error in K_{S-O_2} in yedoma lakes during winter is due to DO concentrations close to K_{S-O_2}. Likewise, Fig. 6b shows that, from an error on K_{S-CH_4} ranging from -50 to 200 %, a resulting error on r from 6 to -4 % would be done, for all lakes and all seasons, except in non-yedoma lakes during summer, where an error from 50 to -34 % would be generated. As above, the latter is due to CH_4 concentrations close to K_{S-CH_4} in non-yedoma lakes during summer. This sensitivity analysis shows that, other than for K_{S-O_2} in yedoma lakes during winter and K_{S-CH_4} in non-yedoma lakes during summer, errors on K_S would have relatively little impact on determination of methanotrophic rates. The potentially significant error on methanotrophic rates in yedoma lakes during winter and in non-yedoma lakes during summer encourages further studies including experimental K_S determination.

From Eqs. (2) and (3), we estimated that, during the summer CH_4 was the main limiting factor in 25 out of 30 lakes. In contrast, during winter, CH_4 was the main limiting factor in 10 of 26 lakes (Table 2). Notably, during the winter, DO was the limiting factor of MO for all seven yedoma lakes, while during the summer, CH_4 was the limiting factor for all non-yedoma lakes. A similar error analysis was done on β and γ, as done with r, to estimate if the estimated limiting factor would change as a result of error on K_S ranging from -50 to 200 %. The results showed no impact on the limiting factor in the 30 lakes and for both seasons. These results confirm that MO was mainly controlled by DO and CH_4 availability, which in turn depended on the season and landscape processes.

A potential bias in our r estimates may have arisen from taking water samples at a single depth in each lake. The literature has clearly shown that a higher MO rate is often found at the oxic/anoxic interface in stratified lakes (Utsumi et al., 1998a, b; Bastviken et al. 2002; Carini et al., 2005; Pimenov et al., 2010; Schubert et al., 2010). Estimation of MO rates consistently measured at a single depth that was not necessarily located at the oxic/anoxic interface may have neglected potentially higher rates occurring at the oxic/anoxic interface in stratified lakes. However, in the present study, the sampled lakes were in many cases shallow, relatively well mixed, and without a clear oxycline (see Results section), suggesting a relatively homogeneous water column. Utsumi et al. (1998b) observed homogeneous MO rates at all depths of a shallow and mixed temperate lake, while Rudd and Hamilton (1978) also reported homogeneous MO rates during overturn of a dimictic lake. Determination of MO rates at the oxic/anoxic interface, in the few cases in which such an interface was observed, would likely have indicated higher MO rates. Thus, the results of r presented here may be underestimated to an unknown extent.

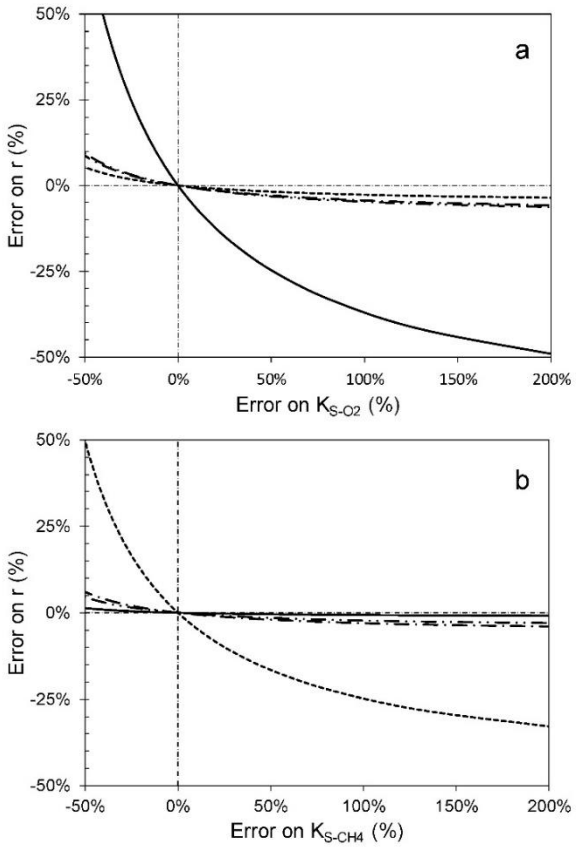

Figure 6. Sensitivity analysis of the impact of an error or variation in K_{S-O_2} (a) and K_{S-CH_4} (b) on r; yedoma lakes in winter (–), yedoma lakes in summer (– · – ·), non-yedoma lakes in winter (– ·· – ··) and non-yedoma lakes in summer (- - - -).

5 Conclusions

We developed a new method based on a TDLAS for the determination of MO rates together with dissolved CH_4 concentration in lakes in the field. This method was successfully applied to 30 lakes along a north-south transect and allowed for the determination of MO potentials ranging from 0.000 to 1.339 $mg\,L^{-1}\,d^{-1}$ in winter and summer. MO rates in water of Alaskan lakes showed high seasonal and geographic variability. In addition to temperature effects, the main factors controlling MO were: (1) CH_4 availability during the summer, limited both by exchange with the atmosphere and by MO itself; (2) DO availability during the winter, mainly due to ice cover impeding gas exchange with the atmosphere and primary production; and (3) inputs of organic substrates to lakes, mainly related to the presence or absence of yedoma permafrost as an additional source of carbon and nutrients. These results indicate that MO may substantially mitigate the increase in CH_4 emission predicted by permafrost thawing (Khvorostyanov, et al. 2008; Walter Anthony et al., 2014).

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6.3 Anaerobic Oxidation of Methane in Sediments of 15 Alaskan lakes: Response to Different Electron Acceptors

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Abstract

It has been suggested that methane (CH_4) emission from freshwater ecosystems is regulated by the balance between CH_4 production and CH_4 oxidation, involving several processes. Among those the most enigmatic process that consumes the CH_4 produced by methanogens in anoxic environments is the anaerobic oxidation of methane (AOM). This research evaluated the potential AOM in surface sediments of fifteen Alaskan lakes that varied by latitude, biome, permafrost regime and limnology. Incubation tests performed with surficial lake sediments spiked with $^{13}CH_4$ and amended with nitrate or sulfate, or left unamended, were used to determine the effect of electron acceptors on potential AOM rates. We found a significant depletion of $^{13}CH_4$ in all of the studied lakes compared to sterile controls. The role of sulfate and nitrate as the final electron acceptor remained uncertain since incubations with and without electron acceptors interchangeably showed AOM activity in several Alaskan lake sediments. We did not find any correlation among the geographic and limnological properties of the lakes and AOM rates. Altogether, our findings indicated that AOM can potentially mitigate around of 43% of the total CH_4 produced by methanogens in surface sediments of Alaskan lakes. We conclude that AOM is a widespread process in Alaskan lakes that should be incorporated in future estimations and models of the global CH_4 budget.

Keywords

Oxidation of methane · Greenhouse gas · Northern lakes · Rates · Surficial sediments

Introduction

Lakes are an important source of atmospheric methane (CH_4), contributing about around of 16% of global sources to the atmosphere (Bastviken et al. 2011). In lakes, the CH_4 molecule acts as a carbon source and an electron donor for several important aerobic and anaerobic biogeochemical processes, which significantly reduce the quantity of CH_4 emitted to the atmosphere. The most commonly reported process is the aerobic oxidation of CH_4 by methanotrophs (O_2 as final electron acceptor) that can reduce up to 99% of the total CH_4 produced in lakes (Bastviken et al. 2002; Kankaala et al. 2006; Trotsenko & Murrell 2008). Besides this well described aerobic process, CH_4 can also be oxidized by microbes in the absence of oxygen. In Anaerobic Oxidation of Methane (AOM), methane is oxidized by the mean of electron transfer from CH_4 to acceptors such as sulfate, nitrate, nitrite, magnesium, iron, chlorite or humic substances (Blodau & Deppe 2012; Caldwell et al. 2008; Cui et al. 2015, Miller et al. 2014). The sulfate-dependent oxidation of methane (S-AOM) was the first process to be clearly demonstrated in marine sediments and freshwater sediments (Barnes & Goldberg 1976; Marterns & Berner 1974; Reeburgh 1976; Zehnder & Brock 1980). S-AOM is thought to consume 5 to 20% of the CH_4 produced from marine environment (Thauer 2010; Valentine & Reeburgh 2000). The anaerobic oxidation of CH4 coupled to denitrification (D-AOM) in sludge from bioreactors (Islas-Lima et al. 2004) and from temperate freshwater sediments (Raghoebarsing et al. 2006) has also been suggested, although conclusive experimental evidence was only recently obtained (Deutzmann & Schink 2011; Deutzmann et al. 2014; Ettwig et al. 2010; Nordi et al. 2013; Norei & Thamdrup 2014). In addition to S-AOM and D-AOM, AOM coupled to humic substances, chlorite, iron and manganese, as electron acceptor has also been proposed to occur in soil and marine and freshwater sediments (Beal et al. 2009; Blodau & Deppe 2012; Miller et al. 2014; Sivan et al. 2011; Sivan et al. 2014; Smemo & Yavitt 2007; Smemo & Yavitt 2011).

Despite recent advances in the field, the role of AOM in methane cycling in northern high latitude lake ecosystems remains unclear and the extent of AOM as well as its potential contribution to global CH_4 cycling are still poorly constrained. Few studies have reported AOM from northern high latitude ecosystems: Blazewicz et al. (2012) studied the ecological significance of AOM in boreal soils, while Gupta et al. (2013) and Smemo and Yavitt (2007) showed that AOM is widespread in Canadian and USA freshwater peatlands from subarctic to

cool-temperate regions. AOM in northern high latitude lakes has not been reported and it is still required to improve approximations of the magnitude and occurrence of AOM in a global scale. Alaskan lakes are found in diverse landscapes characterized by different biomes, geology and permafrost types (Arp 2009; Gregory-Eaves et al. 2000), which result in a large variability of lake types. Thermokarst lakes, formed by thawing of permafrost and ground subsidence, are common in the Arctic (Grosse et al. 2013). In turn, thermokarst lakes have been categorized as "yedoma" and "non-yedoma" type lakes (Walter Anthony et al. 2012; Jorgenson et al. 2008; Sepulveda-Jauregui et al. et al. 2015). Yedoma lakes refers to lakes formed in syngenetic silt-dominated permafrost with high ice and organic carbon contents that were deposited in unglaciated regions of Siberia, Alaska and NW Canada during the Late Pleistocene (Czudek & Demek 1970; Ping et al. 2015; Zimov et al. 1997). Ice-rich yedoma deposits are often tens of meters thick, in such manner that when yedoma permafrost soils thaw, deep thermokarst "thaw" lakes are formed. In contrast, the icy, organic-rich horizons of non-yedoma permafrost soils is typically thinner, resulting in shallower non-yedoma thermokarst lakes, which are common on the Arctic Coastal Plain of Alaska. Other lakes formed in non-yedoma soils include glacial lakes, such as kettle and cirque lakes, found in the mountainous regions of Alaska. Previous work showed that CH_4 cycling in northern regions (i.e. circumpolar regions) is highly dependent of the lake type and, for instance, it has been established that CH_4 emission and aerobic CH_4 oxidation are higher in yedoma-type lakes compared to non-yedoma lakes in Alaska (Martinez-Cruz et al. 2015, Sepulveda-Jauregui et al. et al. 2015). AOM is therefore also potentially dependent on lake type. In this study, we hypothesized that AOM is a widespread process that plays a significant role in CH_4 cycling in Alaskan lakes. Given the higher concentration of dissolved CH_4 in yedoma lakes (Martinez-Cruz et al. 2015; Sepulveda-Jauregui et al. 2015), we also hypothesized that AOM is higher in yedoma lakes compared to non-yedoma lakes. To confirm these hypotheses, we conducted AOM incubations experiments using surficial sediments from a variety of Alaskan lakes. Furthermore, we assessed the impact of different electron acceptors on AOM by conducting incubations with and without added nitrate and sulfate.

Material and Methods

Study lakes and sampling

We collected surficial lake sediments from fifteen Alaskan lakes during the ice-free season in June-July 2012. Two surface lake sediment cores (5.5 cm diameter x 25 cm length) were collected with polycarbonate tubes, next to each other from a central lake location using a gravity corer (Universal Percussion Corer, Aquatic Instruments, ID). One core was used for physicochemical characterization, including determination of CH_4 concentration and the second core was used for AOM incubations. Geographical and sediment properties of the lakes and cores are summarized in Table 1. We distinguished yedoma-type thermokarst lakes as those formed in yedoma permafrost with active ongoing thermokarst activity, from non-yedoma type lakes, which are those occurring in all other non-yedoma deposits in permafrost and non-permafrost regions (Anthony et al. 2012; Gregory-Eaves et al. 2000; Sepulveda-Jauregui et al. 2015). Doughnut L. may have formed originally as a yedoma thermokarst lake and was therefore classified as yedoma in the present work. However, it must be kept in mind that this lake does not exhibit active thermokarst of undisturbed yedoma permafrost deposits today. Killarney L., classified here as a yedoma lake, receives input Pleistocene-aged mineral sediments via a large stream draining a yedoma-dominated watershed; however, thermokarst expansion of the lake itself may not be occurring in undisturbed yedoma sediments. Our study lakes were located near to the road system along a North-South transect in Alaska that spans a variety of geographic and limnological settings, described previously by Gregory-Eaves et al. (2000) and Sepulveda-Jauregui et al. (2015).

CH_4 concentration in sediments

In 10 lakes we measured on-site CH_4 concentration in sediment at 5 cm depth intervals throughout the sediment cores of up to 25 cm, using a headspace equilibration method and Tunable Diode Laser spectrometer (GasFinder 2.0, Boreal Laser Inc., Edmonton, Canada). This is a modified method that was adapted from Sepulveda-Jauregui et al. (2012). In the present work, we used a modified design of the headspace equilibrium (HE) cells (Fig. S1). Briefly, the sediment cores were sliced off from the top, typically with 5-cm intervals, and each 5-cm sediment layer was gently transferred into a borosilicate cell (412 $\pm$ 10 mL volume) for CH_4 concentration measurement. Cells were immediately sealed with rubber flanges and pressure clamps. We added a measured volume of CH_4-free, distilled water to reach a final volume of 150

mL. The difference between water volume added and final slurry volume was the volume of wet sediments measured. The cells were shaken vigorously for 60 s to reach equilibration between the liquid and the gas headspace. HE cells were then placed in the laser beam path of the spectrometer for CH_4 concentration measurement with replicates of five. To validate signal stability of the measurement device, cells containing gas standard CH_4 concentrations were read before and after sample measurements, as described by Martinez-Cruz et al. (2012). We determined CH_4 concentration in wet sediments using Henry's Law following Sepulveda-Jauregui et al. (2012). In the present work all CH_4 concentrations are expressed in milligrams of CH4 per liter of wet sediment (mg L_{ws}^{-1}).

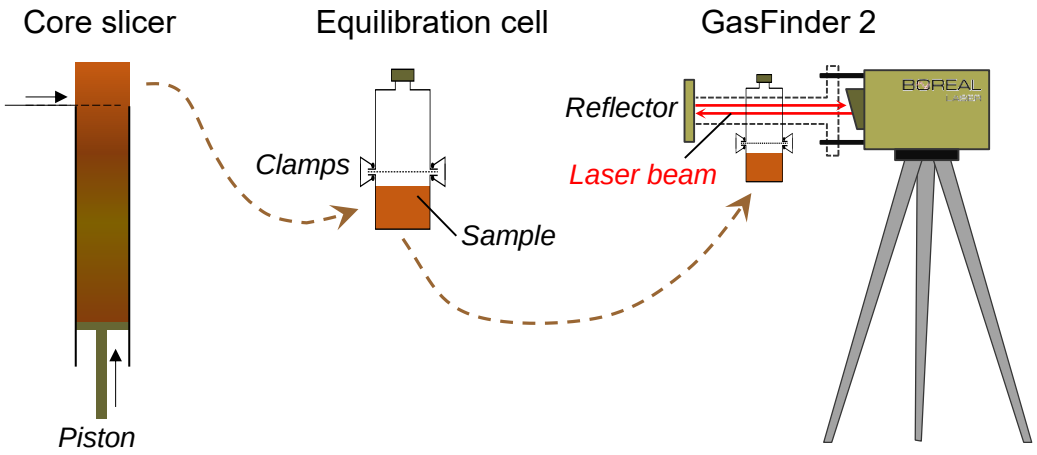

Fig. S1 Experimental on site HE-TDLAS method using GasFinder 2.0 for determining dissolved CH_4 in sediments from sediment core samples. A sediment slice is collected in the section A of the equilibration cell and immediately closed with the section B cell, a known volume of free CH_4 water is added through a septum on section B. The equilibration cell is vigorously mixed for 30 s and placed in the support frame of the TDLAS for determining the CH_4 concentration in the headspace. Method modified from Sepulveda-Jauregui et al. (2012).

Table 1 Lake sediment properties from fifteen Alaskan lakes. * indicates informal lake names. The "A-number" in the name, refers to lake identification numbers used by Gregory-Eaves et al. (2001). "N"- Lake number, "Lat"-Latitude, "Long"-Longitude, "Y/NY"- Permafrost type (yedoma or non-yedoma), "Eco"-Ecozonal according to Gregory-Eaves (2001), "A"-Area, "TOC"-Total organic carbon, "COD"-Chemical oxygen of demand, NO_2^--Nitrite, NO_3^--Nitrate, PO_4^{-3}-Phosphate, SO_4^{-2}-Sulfate. "ND" not determined, "BDL" indicates below detection limit.

N	Name	Lat	Long	Y/NY	Eco	A (km^2)	TOC (mg g_{dsw}^{-1})	COD (mg g_{dsw}^{-1})	NO_2^- (mg g_{dsw}^{-1})	NO_3^- (mg g_{dsw}^{-1})	PO_4^{-3} (mg g_{dsw}^{-1})	SO_4^{-2} (□ g g_{dsw}^{-1})
1	Big Sky A31	69.581	148.639	NY	ArT	0.349	191	ND	BLD	BDL	0.11	BDL
2	E5 Oil Spill A30	68.642	149.458	NY	ArT	0.116	66	4.25	BDL	BDL	0.51	BDL
3	Toolik	68.632	149.605	NY	ArT	1.449	61	16.68	BDL	BDL	0.64	BDL
4	Grayling A24	66.954	150.393	NY	FoT	0.401	206	6.58	BDL	BDL	0.11	BDL
5	Eugenia*	65.834	149.631	Y	FoT	0.027	235	ND	BDL	BDL	0.12	BDL
6	Vault	65.029	147.699	Y	NBF	0.003	102	ND	BDL	BDL	0.08	24.049
7	Goldstream	64.916	147.847	Y	NBF	0.010	46	6.02	BDL	BDL	2.24	BDL
8	Doughnut	64.899	147.908	NY	NBF	0.035	188	0.34	BDL	BDL	0.84	BDL
9	Killarney	64.870	147.901	Y	NBF	0.008	21	2.64	BDL	BDL	0.23	BDL
10	Otto	63.842	149.037	NY	FoT	0.515	97	2.05	BDL	BDL	25.78	0.53
11	Floatplane A16	63.394	148.670	NY	FoT	0.103	196	ND	ND	ND	ND	ND
12	Montana A40	62.143	150.048	NY	SBF	0.300	219	ND	ND	ND	ND	ND
13	Abandoned Cabin A50	60.696	151.315	NY	SBF	0.031	136	ND	ND	ND	ND	ND
14	Scout A46	60.533	150.843	NY	SBF	0.384	233	3.97	BDL	BDL	0.14	BDL
15	Engineer A45	60.478	150.323	NY	SBF	0.909	149	35.77	BDL	BDL	1.07	BDL
	Yedoma[1]	-	-	-	-	0.012 ± 0.010	101 ± 96	4.33 ± 2.39	-	-	0.67 ± 1.05	-
	Non-Yedoma[2]	-	-	-	-	0.417 ± 0.427	158 ± 61	2.39 ± 9.95	-	-	3.65 ± 8.95	-

[1]Mean from yedoma lakes and standard deviation. [2]Average from non-yedoma lakes and standard deviation.

CH_4 concentration in sediments

In 10 lakes we measured on-site CH_4 concentration in sediment at 5 cm depth intervals throughout the sediment cores of up to 25 cm, using a headspace equilibration method and Tunable Diode Laser spectrometer (GasFinder 2.0, Boreal Laser Inc., Edmonton, Canada). This is a modified method that was adapted from Sepulveda-Jauregui et al. (2012). In the present work, we used a modified design of the headspace equilibrium (HE) cells (Fig. S1). Briefly, the sediment cores were sliced off from the top, typically with 5-cm intervals, and each 5-cm sediment layer was gently transferred into a borosilicate cell (412 ± 10 mL volume) for CH_4 concentration measurement. Cells were immediately sealed with rubber flanges and pressure clamps. We added a measured volume of CH_4-free, distilled water to reach a final volume of 150 mL. The difference between water volume added and final slurry volume was the volume of wet sediments measured. The cells were shaken vigorously for 60 s to reach equilibration between the liquid and the gas headspace. HE cells were then placed in the laser beam path of the spectrometer for CH_4 concentration measurement with replicates of five. To validate signal stability of the measurement device, cells containing gas standard CH_4 concentrations were read before and after sample measurements, as described by Martinez-Cruz et al. (2012). We determined CH_4 concentration in wet sediments using Henry's Law following Sepulveda-Jauregui et al. (2012). In the present work all CH_4 concentrations are expressed in milligrams of CH_4 per liter of wet sediment (mg L_{ws}^{-1}).

Physicochemical characterization of sediments

To complement CH_4 concentration in sediments we also quantified other sediment parameters: Total Organic Carbon (TOC), Chemical Oxygen Demand (COD), nitrate (NO_3^-), nitrite (NO_2^-), soluble reactive phosphorus (SRP) and sulfate (SO_4^{-2}). TOC was determined on a dry weight (105 °C, 24 h) basis via loss on ignition at 550 °C according to Dean et al. (1974). COD was determined by dichromate oxidation using a colorimetric method (Skougstad et al. 1979). Soluble ions (NO_3^-, NO_2^-, SRP, SO_4^{-2}) were measured with a high-performance liquid chromatograph equipped with an electrochemical detector (ED40, Dionex, Dionex, USA).

Incubation setup

Lake sediments from the first 10 cm of each core were diluted (1:1 v/v) with sterile, CH_4 free and anaerobic distilled water. We transferred 80 mL of the slurry to 120 mL serological bottles

under continuous flushing with ultra-high purity nitrogen (Airgas, USA). Flushing was maintained for an additional five minutes until the serological vials were air-tight sealed with blue butyl rubber stoppers (Bellco, USA) and aluminum crimp caps. Into each vial, we injected 0.01% H_2S to reduce anoxic media, as commonly reported in previous works (Moran et al. 2008). Each vial was pre-incubated for five days to ensure the absence of oxygen, which we also confirmed by headspace measurement using a gas chromatograph equipped with a thermal conductivity detector (Shimadzu GC-2014, Japan). After pre-incubations we added 5 mL of $^{13}CH_4$ (99 atom % ^{13}C, Sigma Aldrich, USA) to the headspace of each incubation vial. In order to investigate the potential for AOM coupled to different electron acceptors, three treatments were applied; i) unamended, to determine AOM rates with the natural electron acceptors present in the sediments (N-AOM), ii) amended with SO_4^{-2} (10 mM), to determine the S-AOM potential rate, or iii) amended with NO_3^- (10 mM), to determine the D-AOM potential rate. Additionally, sterilized sediment samples (121 °C, 20 min) served as a control. Each incubation test was performed in duplicate, at 20 °C for 245 days.

CH_4 and CO_2 concentration and $\delta^{13}CH_4$ and $\delta^{13}CO_2$ isotopic measurements

At the beginning and end of each incubation test, headspace total CH_4 concentration was measured by gas chromatography with a flame ionization detector (FID, Shimadzu GC-2014). Simultaneously, measurements of $\delta^{13}C$ from both CH_4 and CO_2 were measured by Cavity Ring-Down Spectroscopy (CRDS) (G2201-i, Picarro, Ca. USA; precision ± 0.55 ‰ $^{\delta 13}$VPDB for CH_4, and ± 0.16 ‰ $^{\delta 13}$VPDB for CO_2) coupled to a Small Sample Isotope Module (SSIM2, Picarro, Ca., USA). A pre-dilution module (SSIM2, Picarro, Ca., USA) was also used to dilute the headspace samples with Zero Air (1:200 to 1:5000).

Data Analysis and Rate Calculations

High concentrations of ^{13}C-labeled CH_4 ($^{13}CH_4$) were added to allow for distinguishing $^{13}CH_4$ oxidized and $^{13}CH_4$ potentially produced at low concentration by methanogens, as previously suggested by Blazewicz et al. (2012) and Beal et al. (2009). AOM rates and $^{12}CH_4$ production rates were calculated from the difference between total $^{13}CH_4$ and $^{12}CH_4$ concentration in the headspace, respectively, at the initial and final time of each incubation test. The individual concentrations of $^{13}CH_4$ and $^{12}CH_4$ were determined from the isotopic fractions and the total

CH_4 concentration determined by gas chromatography. For our calculations, AOM was conservatively determined from $^{13}CH_4$ oxidation only, while CH_4 production by methanogenesis was conservatively determined from $^{12}CH_4$ production only.

Statistical Analysis

Lake sediments characteristics were not normally distributed and did not meet the assumption of homoscedasticity (based on Saphiro-Wilk test). We therefore tested statistical differences between results using the non-parametric Two-tailed Mann-Whitney U-test (M-W test) for comparison of two groups or Kruskal-Wallis one-way analysis of variance followed by Kruskal-Wallis Multiple-Comparison Z-value test (K-W test) for comparison of multiple groups. Difference was significant with a Z value > 1.96 ($\alpha < 0.05$).

Methane oxidation and production rates were also tested for normal distribution using Shapiro-Wilk test. When data were not normally distributed, they were converted to logarithmic data prior to student *t* testing for significant differences ($\alpha < 0.05$) between each AOM test with its respective sterile control. We used Tukey-Kramer test (T-K test) for multiple comparison among whole D-AOM, S-AOM and N-AOM ($\alpha < 0.05$). Statistical analyses were performed with NCSS 2000 Statistical Analysis 193 System software (Number Cruncher Statistical Systems, USA).

Results and discussion

CH_4 concentration and physicochemical characterization in sediments

Dissolved CH_4 concentration in surface sediments was highly variable among lakes and depth, ranging from 0.77 (E5) to 35.56 (Doughnut) mg L_{ws}^{-1} (Fig. 1); we did not find any pattern between CH_4 concentration profiles in yedoma and non-yedoma lakes type (M-W test). Dissolved CH_4 concentration was significantly lower in E5 than in any other lakes (K-W test). E5 is an oligotrophic lake with low CH_4 and CO_2 emissions (Sepulveda-Jauregui et al. 2015) that was previously used to study multi-year fertilization treatment (Hobbie & Kling 2014) using ammonium nitrate (NH_4NO_3). The addition of nitrate could have reduced the CH_4 production and/or enhanced the AOM (Cervantes et al. 2008; Deutzmann & Schink 2011; Liikanen et al. 2002).

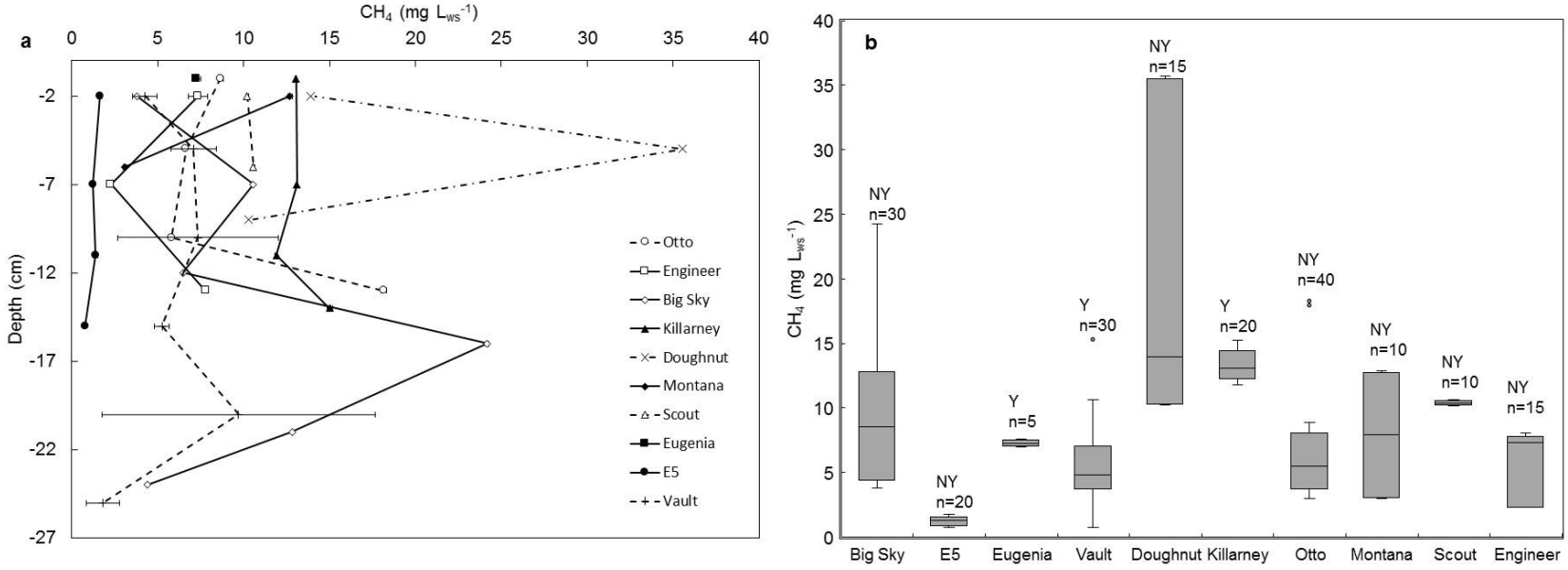

Fig. 1 (a) Depth profiles of in situ CH_4 concentration in lake sediments, standard deviation showed a variability for five measurements. (b) Statistical distributions of dissolved CH_4 concentration from all depth measured (ca. each 5 cm) in sediments from North-South transect lakes. Lake names are presented latitudinal from North (on the left) to South (on the right). The boxes include the median (Q2) and the quartile range (Q1 and Q3). The whiskers show minimum and maximum data. The open circles show outlier data. "n" represents the number of samples measured, "Y" yedoma lakes and "NY" non-yedoma lakes.

Our results indicate that CH_4 concentration in surface sediments is widely variable within the top 25 cm and among lakes. However, no significant differences were observed between latitudes and permafrost type. These results appear contradictory to previous observations in the same lakes by Sepulveda-Jauregui et al. (2015) and Martinez-Cruz et al. (2015), who found that the dissolved CH_4 concentration in the water column and the total emissions from yedoma lakes were higher than from non-yedoma lakes. This apparent antagonistic finding between CH_4 concentration in sediments and observed CH_4 emission suggests a competing processes of CH_4 production, oxidation and physical transport that may mask any underlying relationships between sediment CH_4 concentration and limnological or landscape properties. For instance, relatively high CH_4 concentration in the sediments of non-yedoma lakes might be compensated by higher aerobic CH_4 oxidation compared to yedoma lakes, which is consistent with observations that non-yedoma lakes tend to have well-oxygenated water columns in comparison to the more anoxic water columns of yedoma lakes (Sepulveda-Jauregui et al. 2015). The absence of apparent linkage between CH_4 concentration in sediments and observed CH_4 emission, may also be explained by CH_4 emission from deeper sediment layers than those sampled here, especially via ebullition. Deep sediment CH_4 production, particularly in yedoma lakes, is supported by previous observations of abundant ebullition seeps, which transmit CH_4 from deep sediments to the water column through well-established bubble transport tubes in sediments (Anthony & Anthony 2013; Anthony et al. 2012; Walter et al. 2007b).

Our observations of high spatiotemporal variability in sediment CH_4 concentration are in agreement with previous observations made Sobek et al. (2012) in reservoir sediments. In complement to these depth profiles, Coffin et al. (2013) found significant differences among cores collected from different locations in the Alaskan Beaufort Sea and Nordi et al. (2013) found significant differences between cores collected on different dates within the same Danish lake.

TOC and COD values in sediments were highly variable among all lakes (143 ± 73 mg g_{dsw}^{-1}, $n = 15$, 9.00 ± 11.19 mg g_{dsw}^{-1}, $n = 9$, respectively). The highest and lowest TOC values were found in yedoma lakes (Eugenia and Killarney respectively), meanwhile highest and lowest COD values were found in non-yedoma lakes (Engineer and Doughnut, respectively). We did not find significant differences in TOC and COD values between yedoma and non-yedoma sediment

lakes. Hence, in sediment microbial processes (e.g. methanogenesis, AOM) the organic matter "quality" are more important than TOC and COD values determined from sediments (Gonzalez-Perez et al. 2012; Reiche et al. 2010). Nitrogen oxides were not detectable in sediments (detection limit: 0.01 μg g_{dsw}^{-1}) and sulfates were detectable in low proportion in Vault and Otto sediments lakes only (detection limit: 0.01 μg g_{dsw}^{-1}), while SRP values in sediments were highly variable among all lakes (2.66 ± 7.31 mg g_{dsw}^{-1}, n = 12). We did not find significant differences in SRP values between yedoma and non-yedoma sediment lakes, therefore we could not attribute sediment CH_4 concentrations to nutrient availability.

Incubations of AOM

Results of anaerobic incubations of lake sediments left unamended or spiked with different electron-acceptors are shown in Figure 2. Tests without addition of any external electron acceptors showed a significant depletion of $^{13}CH_4$ in 11 lakes compared with the sterile control (Fig. 2A). Tests spiked with NO_3^- showed a depletion of $^{13}CH_4$ in 13 lakes compared to sterile control (Fig. 3B). Tests spiked with SO_4^{-2} showed a depletion of $^{13}CH_4$ compared to sterile control in all sediment lakes (Fig. 2C). In Big Sky, E5, and Otto, the addition of NO_3^- and SO_4^{-2} enhanced AOM. In Doughnut, the addition of SO_4^{-2} enhanced AOM while the addition of NO_3^- did not have any significant effect. In 10 lakes (Toolik, Grayling, Eugenia, Vault, Goldstream, Killarney, Floatplane, Montana, Abandoned Cabin and Scout) the addition of electron acceptors did not enhance AOM compared to unamended vials (Fig. 2). Overall, the percentage of $^{13}CH_4$ that was oxidized during incubation was 56 ± 13%, 53 ± 10 and 67 ± 4% for N-AOM, D-AOM and S-AOM, respectively, with no significant differences between them (T-K test). We did not find any correlation among anaerobic $^{13}CH_4$ oxidation and permafrost type (yedoma vs. non-yedoma, M-W test).

The isotopic CH_4 concentrations indicate that surface sediments from Alaskan lakes spanning different landscape and limnological properties (Table 1, Sepulveda-Jauregui et al. 2015) have the capacity to oxidize CH_4 under anaerobic conditions. These results suggest that AOM is a process independent of the landscape and limnological properties of surficial sediments in the 15 studied Alaskan lakes (K-W test). Our findings, together with previous reports of AOM in other terrestrial/freshwater ecosystems from different regions of the world (Antler et al. 2014; Avrahamov et al. 2014; Beal et al. 2009; Blazewicz et al. 2012; Deutzmann & Schink 2011; Deutzmann et al. 2014; Gupta et al. 2013; Nordi et al. 2013; Reeburgh 1976; Schubert et al. 2011;

Segarra et al. 2013; Segarra et al. 2015; Smemo & Yavitt 2007; Zehnder & Brock 1980), confirm that AOM is a widespread process.

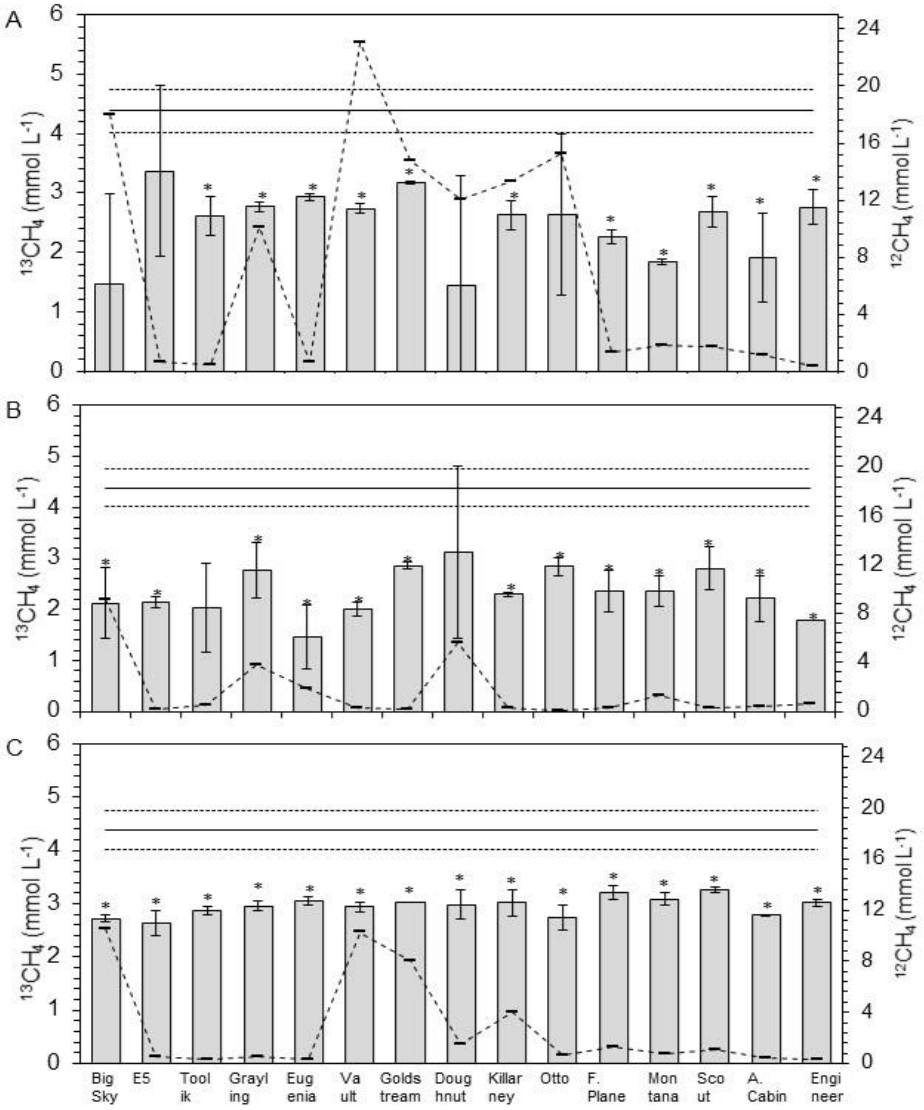

Fig. 2 Isotope $^{13}CH_4$ (gray bars) and $^{12}CH_4$ (-, separated by dashed line to improve visualization) concentrations in headspace of AOM incubation tests after 245 days, without electron acceptor addition (A), with NO_3^- (B) and with SO_4^{-2} (C). Lower $^{13}CH_4$values indicate higher AOM; higher $^{12}CH_4$ indicate higher CH_4 production in vials. * indicates significant difference ($\alpha < 0.05$, M-W test) in $^{13}CH_4$ concentration between samples and sterile controls. $^{13}CH_4$ final concentration in sterile controls is represented by continuous line at (4.4 mmol L^{-1}), standard deviation ($\pm$ 0.4 mmol L^{-1}) by dashed lines. Error bars indicate absolute error for duplicate assays. Lake name are ordered latitudinal from North (left) to South (right).

$^{12}CH_4$ concentration in Fig. 2 showed that, in most of the cases, CH_4 production occurred simultaneously with AOM. It is noteworthy that during incubations tests with added NO_3^- and SO_4^{-2}, $^{12}CH_4$ production was significantly lower compared to unamended incubations; with an overall decrease in $^{12}CH_4$ production by 78% and 65%, respectively (K-W test). This suggests that the addition of electron acceptor favored CH_4 oxidation and/or that NO_3^- and SO_4^{-2} promoted denitrification and sulfate reduction over methanogenesis, as previously reported (Canfield et al. 2005; Conrad 1996). Further research is required to clarify this issue. The simultaneous production and oxidation, suggest that AOM rates determined from labelled CH_4 oxidation most certainly underestimate the actual AOM rate, as discarding the oxidation of $^{12}CH_4$ produced by methanogens Gupta et al (2013), Segarra et al. (2013), Deutzmann and Schink (2011), Smemo and Yavitt (2007), Iversen and Jorgensen (1985) and Zendher and Brock (1980).

Potential AOM rates and CH_4 production

The potential AOM rates ranged from (mean ± SD) 0.33 ± 0.21 to 64.39 ± 9.35 ng $^{13}CH_4$ kg dry sediment^{-1} s^{-1} (Table 2). These AOM rates are in the range reported by Hinrichs and Boetius (2002), Gupta et al. (2013) and Blazewicz et al. (2013) for marine sediments, subarctic peatlands, and subarctic and tropical peatlands, respectively. However, our AOM rates were one order of magnitude lower than reported by Smemo and Yavitt (2007) for wetlands and peatlands using different electron acceptors. The wide range of AOM rates from our results and the literature in different habitats could be that CH_4 is oxidized under several mechanisms at the same time and they are regulated according to electron acceptors availability, enzymatic/redox changes in the sediments during the incubations (Holler et al. 2011a; Zehnder & Brock 1980). This variability may also be explained by the slow increase in active AOM microbial biomass during the incubation period in long-term experiments compared with the short-term experiments (Girguis et al. 2005; Holler et al. 2011b; Segarra et al. 2013). We also acknowledge that we could not compare our results with several AOM rates from other studies due to how rates were estimated and/or reported.

We observed simultaneous processes of AOM and methane production. CH_4 production rates ranged from 0.35 ± 0.18 to 111.77 ± 22.22 ng $^{12}CH_4$ kg dry sediment^{-1} s^{-1} (Table 2). On average, AOM can potentially consume around 43 ± 72% of the methane production rates. These results

must be considered with cautions for several reasons. First, as mentioned above our CH_4 oxidation rates are conservative, because AOM rates determinations were based on $^{13}CH_4$ only, excluding potential oxidation of the $^{12}CH_4$ produced by methanogens during incubations. Second, yedoma lakes (Vault, Goldstream, and Killarney) emit important amounts of CH_4 from the permafrost thaw front, which is several to tens of meters deeper than our surface sediment cores; thus our extrapolation based on surficial sediment. It does not reflect CH_4 production/oxidation budget in deeper layer (Anthony et al. 2012; Anthony et al. 2010; Brosius et al. 2012; Heslop et al. 2015; Walter et al. 2007a). Third, incubations were done with mixed sediments samples and therefore without mass transfer limitations that are a structural characteristic of sediments. Fourth, our assays were isolated from the interactions between the water column and sediments that are naturally occurring; i.e. gases and nutrients exchanges. Despite these experimental flaws, AOM was on the same order of magnitude as CH_4 production in surface sediments, which is a clear indication that AOM plays an important role in global CH_4 budget of Alaskan lakes. The absence of detectable levels of nitrite and nitrate in all lakes and of sulfate in 13 lakes, together with no overall significant differences between D-AOM, S-AOM and N-AOM (T-K test), suggests that nitrite, nitrate and sulfate were not involved in AOM in Alaskan lakes, which is contradictory to previous findings in long-term nitrate enriched cores (Deutzmann et al. 2014; Norei & Thamdrup 2014), and turns the attention to other candidates of electron acceptors, such as potentially; humic substances, iron or manganese, among others as postulates by other authors (Sivan et al. 2011; Smemo & Yavitt 2007; Smemo & Yavitt 2011). The isotopic CH_4 concentrations indicate that surface sediments from Alaskan lakes spanning different landscape and limnological properties (Table 1, Sepulveda-Jauregui et al. 2015) have the capacity to oxidize CH_4 under anaerobic conditions. These results suggest that AOM is a process independent of the landscape and limnological properties of surficial sediments in the 15 studied Alaskan lakes (K-W test). Our findings, together with previous reports of AOM in other terrestrial/freshwater ecosystems from different regions of the world (Antler et al. 2014; Avrahamov et al. 2014; Beal et al. 2009; Blazewicz et al. 2012; Deutzmann & Schink 2011; Deutzmann et al. 2014; Gupta et al. 2013; Nordi et al. 2013; Reeburgh 1976; Schubert et al. 2011; Segarra et al. 2013; Segarra et al. 2015; Smemo & Yavitt 2007; Zehnder & Brock 1980), confirm that AOM is a widespread process.

$^{12}CH_4$ concentration in Fig. 2 showed that, in most of the cases, CH_4 production occurred simultaneously with AOM. It is noteworthy that during incubations tests with added NO_3^- and SO_4^{-2}, $^{12}CH_4$ production was significantly lower compared to unamended incubations; with an overall decrease in $^{12}CH_4$ production by 78% and 65%, respectively (K-W test). This suggests that the addition of electron acceptor favored CH_4 oxidation and/or that NO_3^- and SO_4^{-2} promoted denitrification and sulfate reduction over methanogenesis, as previously reported (Canfield et al. 2005; Conrad 1996). Further research is required to clarify this issue. The simultaneous production and oxidation, suggest that AOM rates determined from labelled CH_4 oxidation most certainly underestimate the actual AOM rate, as discarding the oxidation of $^{12}CH_4$ produced by methanogens Gupta et al (2013), Segarra et al. (2013), Deutzmann and Schink (2011), Smemo and Yavitt (2007), Iversen and Jorgensen (1985) and Zendher and Brock (1980).

Potential AOM rates and CH_4 production

The potential AOM rates ranged from (mean ± SD) 0.33 ± 0.21 to 64.39 ± 9.35 ng $^{13}CH_4$ kg dry sediment^{-1} s^{-1} (Table 2). These AOM rates are in the range reported by Hinrichs and Boetius (2002), Gupta et al. (2013) and Blazewicz et al. (2013) for marine sediments subarctic peatlands and subarctic and tropical peatlands, respectively. However, our AOM rates were one order of magnitude lower than reported by Smemo and Yavitt (2007) for wetlands and peatlands using different electron acceptors. The wide range of AOM rates from our results and the literature in different habitats could be that CH_4 is oxidized under several mechanisms at the same time and they are regulated according to electron acceptors availability, enzymatic/redox changes in the sediments during the incubations (Holler et al. 2011a; Zehnder & Brock 1980). This variability also can be explained by the increase of active AOM microbial biomass during the incubation period in long-time experiments compared with the short-time experiments, which has been suggested that AOM growth rates are low (Girguis et al. 2005; Holler et al. 2011b; Segarra et al. 2013), but also we acknowledge that we could not compare with several AOM rates from other studies due to how rates were estimated and/or reported.

Together with AOM rates determination, we also observed CH_4 production rates ranging from 0.35 ± 0.18 to 111.77 ± 22.22 ng $^{12}CH_4$ kg dry sediment^{-1} s^{-1} (Table 2). On average, the AOM can potentially consume around 43 ± 72% of the methane production rates. These results must be considered with cautious, for several reasons. First, as mentioned above our CH_4 oxidation

rates are conservative, because AOM rates determinations were based on $^{13}CH_4$ only, excluding potential oxidation of the $^{12}CH_4$ produced by methanogens during incubations. Second, yedoma lakes (Vault, Goldstream, and Killarney) emit important amounts of CH_4 from the permafrost thaw front, which is several to tens of meters deeper than our surface sediment cores; thus our extrapolation based on surficial sediment, it may not reflect CH_4 production/oxidation budget in deeper layer (Anthony et al. 2012; Anthony et al. 2010; Brosius et al. 2012; Heslop et al. 2015; Walter et al. 2007a). Third, incubations were done with mixed sediments samples and therefore without mass transfer limitations that are a structural characteristic of sediments. Fourth, our assays were isolated from the interactions between the water column and sediments that are naturally occurring; i.e. gases and nutrients exchanges. Despite these experimental flaws, AOM was on the same order of magnitude than CH_4 production, which is a clear indication that AOM plays an important role in global CH_4 budget of Alaskan lakes. The absence of detectable levels of nitrite and nitrate in all lakes and of sulfate in 13 lakes, together with no overall significant differences between D-AOM, S-AOM and N-AOM (T-K test), suggests that nitrite, nitrate and sulfate were not involved in AOM in Alaskan lakes, which is contradictory to previous findings in long-term nitrate enriched cores (Deutzmann et al. 2014; Norei & Thamdrup 2014), and turns the attention to other candidates of electron acceptors, such as potentially; humic substances, iron or manganese, among others as postulates by other authors (Sivan et al. 2011; Smemo & Yavitt 2007; Smemo & Yavitt 2011).

Conclusion

Our study showed that AOM is a naturally occurring process; i.e., with no added electron acceptor, in surficial northern lake sediments, regardless of latitude, biome, permafrost regime and limnology. These results complement those previously obtained in tropical and boreal soils (Blazewicz et al. 2012; Gupta et al. 2013; Smemo & Yavitt 2007), freshwater and wetland sediments (Segarra et al. 2013; Segarra et al. 2015), confirming that AOM is a widespread process. The role of sulfate and nitrates, as potential final electron acceptor remained uncertain since incubations with and without added electron acceptors interchangeably showed AOM activity, with no significant difference, which is similar to previous findings from (Blazewicz et al. 2012; Gupta et al. 2013). Our findings indicated that AOM may potentially mitigate around of 43% of the total CH_4 produced by methanogens in surface sediments of Alaskan lakes and must be taken into account in future estimations and models of the global CH_4 budget.

Table 2 Mean rates of potential AOM (ng $^{13}CH_4$ kg dry sediment^{-1} s^{-1}) and CH_4 production (ng $^{12}CH_4$ kg dry sediment^{-1} s^{-1}) over 8 months. "BDL" below detection limit.

	Potential anaerobic oxidation of CH_4			CH_4 production		
Lake Name (#)	DAOM	SAOM	NAOM	DAOM	SAOM	NAOM
Big Sky A31(1)	15.63 ± 2.90	14.09 ± 1.63	-[1]	36.60 ± 14.82	42.17 ± 5.34	72.22 ± 17.48
E5 Oil Spill A30 (2)	34.49 ± 3.58	30.77 ± 4.69	-	2.20 ± 1.26	6.45 ± 4.78	7.43 ± 5.95
Toolik A28 (3)	-	3.10 ± 0.73	5.25 ± 3.26	2.86 ± 3.22	1.74 ± 0.41	2.33 ± 0.70
Grayling A24 (4)	2.94 ± 3.94	0.69 ± 0.50	4.03 ± 0.70	10.06 ± 0.57	1.27 ± 0.10	26.71 ± 2.56
Eugenia (5)	26.37 ± 9.97	5.12 ± 1.48	4.79 ± 1.02	14.72 ± 24.37	2.41 ± 0.21	4.87 ± 4.43
Vault (6)	9.13 ± 1.17	40.93 ± 2.11	17.05 ± 2.09	1.10 ± 0.55	40.93 ± 2.11	92.19 ± 4.92
Goldstream (7)	3.83 ± 0.51	6.70 ± 1.06	7.20 ± 0.80	0.35 ± 0.18	21.28[2]	39.53 ± 1.32
Doughnut (8)	-	7.29 ± 1.78	-	21.79 ± 1.41	5.80 ± 2.84	46.58 ± 0.60
Killarney (9)	8.95 ± 0.88	9.48 ± 1.87	14.21 ± 2.27	0.91 ± 0.54	16.34 ± 1.87	54.98 ± 0.74
Otto (10)	14.81 ± 2.24	15.29 ± 2.76	-	BDL	4.15 ± 0.74	111.77 ± 22.22
Float Plane (11)	58.35 ± 24.48	36.73 ± 9.29	64.39 ± 9.35	7.08 ± 2.38	42.49 ± 4.08	44.57 ± 0.41
Montana A40 (12)	61.22 ± 13.97	28.57 ± 5.50	63.43 ± 6.34	29.31 ± 43.38	16.67 ± 1.99	41.16 ± 33.40
Abandoned Cabin A50 (13)	0.55 ± 2.04	0.33 ± 0.21	1.93 ± 1.32	8.99 ± 9.26	11.84 ± 10.96	30.51 ± 23.33
Scout A46 (14)	45.52 ± 22.34	21.03 ± 2.07	58.75 ± 37.73	0.66 ± 0.07	2.68 ± 1.01	4.29 ± 0.45
Engineer A45 (15)	29.80 ± 13.59	13.49 ± 1.83	11.43 ± 5.93	7.47 ± 0.01	3.4 ± 0.29	3.63 ± 0.29

[1] - Indicates no significant difference between Potential of AOM rates and sterile control (based on statistical analysis from Figure 2). [2]duplicate lost.

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6.4 Anaerobic oxidation of methane in tropical, temperate and arctic lake sediments

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Abstract

Anaerobic oxidation of methane (AOM) is a biological process that plays a partially undetermined role in reducing global CH_4 emissions from a span of ecosystems. In marine sediments, where AOM was first and best described, AOM is responsible for the reduction of >90% of the annual CH_4 produced in marine sediments. More recently, the existence of AOM was explored in other ecosystems and has been observed in laboratory tests of peatlands, soils and aquatic freshwater aquatic ecosystems. In lakes, the potential of AOM generates a large interest as being possibly a major piece of the global biogeochemical carbon puzzle, still only partially constrained. Here we present evidences of AOM in the sediment of 14 lakes from tropical, temperate and arctic latitudes with contrasting climates, trophic states and water physico-chemical regimes. We also present a quantitative approach of AOM profiling in the surface sediments of three of these lakes. By combining a span of incubation tests together with isotopic measurements, we quantified AOM and we developed a CH_4 cycling model confirmed by field measurements. From our model and field validation, we concluded that AOM is a widespread process which account for 38 ± 23% of the total methane produced in lake sediments, superior to previous estimates. We also pointed out that aerobic methanotrophs might be involved.

Introduction

Several studies have demonstrated the occurrence of AOM in a number of freshwater ecosystems[1-7]. Although freshwater ecosystems are relatively minor in the earth continental landscape, i.e. only 3% of the continental earth surface, they are important contributors to global CH_4 emission to the atmosphere (16% for lakes and reservoirs[8]). Potentially, AOM has therefore important outcomes for global carbon cycling and quantitative assessment of its contribution in global methane cycling is crucial. Previous quantitative determinations of AOM based on lake sediment incubations[4,5,9-12] reported AOM rates ranging from 0.01 to 100 nmol mL^{-1} d^{-1} (Fig. 1), while methane production in the same lake sediments studied for AOM[5,9,10,12-14] has been reported in the range from 1 to 800 nmol mL^{-1} d^{-1}.

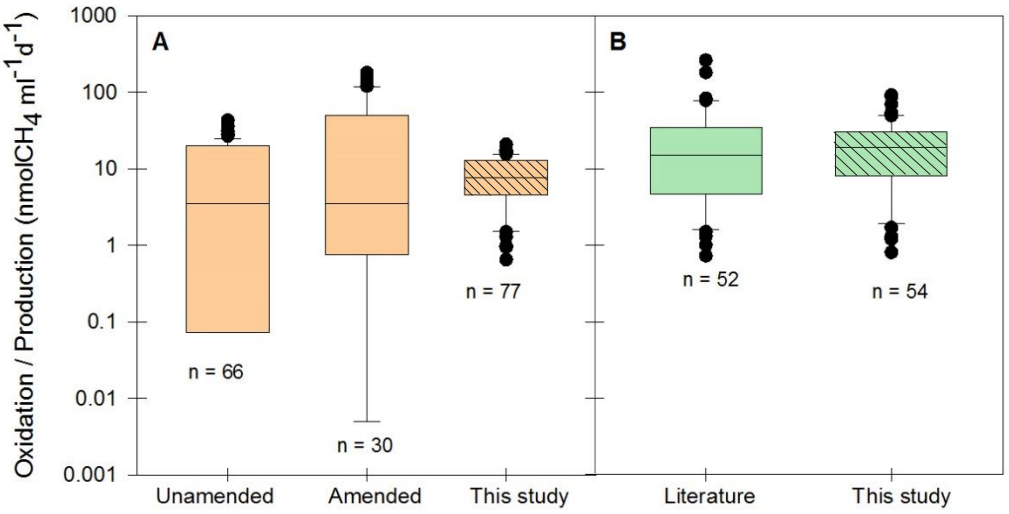

Fig. 1: AOM (A) and methanogenic (B) rates in lakes sediments, reported in the literature in assays without and with addition of exogenous electron acceptor and observed in the present study; n = number of data reported. Striped boxes represent value from this study whereas full color boxes represent values from the literature.

To understand the mechanisms involved in AOM, several researchers have strived to identify the electron acceptor involved, either by amended incubation[5,6,9,11,15] or by *in-situ* determination of potential electron acceptor profiles[2,7,9,10]. Despite significant efforts, the terminal electron acceptors associated with AOM in freshwater ecosystems is still unclear. Similarly, to marine environment, AOM coupled to sulfate reduction (SO_4^{-2}) has been first suggested [9], although SO_4^{-2} concentration in most freshwater systems are probably too low for the process to be thermodynamically favorable[2,16]. More recently, AOM coupled to nitrite (NO_2^-) or nitrate (NO_3^-) reduction has been demonstrated[11] but might be ancillary because NO_3^- and NO_2^- are lacking in most anoxic sediments[2]. In addition to the latter, AOM coupled to Fe (III) and Mn (IV)

respiration has been demonstrated[4,10], while the role of humic substances in AOM has only been suggested[2,17]. However, metadata analysis on previous reports shows no significant difference between AOM rates in presence or absence of a range of exogenous electron acceptor (KW-test, $p < 0.05$, Fig. 1). This suggests either that globally AOM is probably not supported by a specific electron acceptor common to all freshwater ecosystems, or that AOM is supported by a still unidentified oxidative agent. This study sought to determine the fraction of CH_4 being oxidized by AOM in lake sediments without addition of any external electron acceptors. The aim was assessed by performing AOM activity tests and designing a diffusion/reaction model to correlated laboratory results with field measurements.

Methods

We first investigated the existence of AOM in the sediment of 14 tropical, temperate and northern lakes with contrasting climates, trophic states and water physicochemical regimes (Table 1). In each lake we collected superficial lake sediment samples with an Ekman dredge. A slurry containing 60% of fresh sediment was prepared with anoxic distillated water. The slurry was flushed for 5 min with ultrahigh purity N_2 to ensure anaerobic conditions. Eighty mL of the slurry were transferred to 120 mL serum bottles maintaining continuous flushing with high purity helium and flushing was maintained for an additional 5 min before the serological vials were sealed with blue butyl rubber stoppers (Bellco, Vineland, NJ, USA) and aluminum crimp caps. Into each vial, Na_2S was added to a concentration of 0.5 g L^{-1} (ref. 18) to reduce anoxic media. We added 1 mL $^{13}CH_4$ (99 atom % ^{13}C) as a stable isotope tracer for methane oxidation. Additionally, sterilized sediment samples (121 °C, 20 min) from five lakes (#7-10) served as control to investigate abiotic factors that might affect the process studied. Abiotic water control tests were also done without sediments. The absence of O_2 in the vials was confirmed by gas chromatography equipped with a thermal conductivity detector (Agilent 4890 D in Mexico and Shimadzu GC-2014 in USA) and, in water control tests that received the same treatment as the experimental tests, including sampling, by a resazurin indicator. After 6 months of incubation at 25 °C, a 1 mL aliquot from each vial was transferred to a 20 mL analytical tube containing 0.5 mL 0.8 M phosphoric acid. Gaseous $^{13}CO_2$ was then measured by GC-MS (Thermo Finnigan GasBench II carbonate analyzer with a Delta^{Plus}XP Mass Spectrometer, precision ± 0.2 ‰ $\delta^{13}C_{PDB}$) at the Alaska Stable Isotope Facility of the University of Alaska, Fairbanks. Methane concentration in vial headspace was also determined at the beginning and the end of the

incubations with gas chromatograph equipped with a flame ionization detector (Agilent 4890 D in Mexico and Shimadzu GC-2014 in USA). The AOM rates were calculated from the total decrease of CH_4 concentration against time after the plateau of CH_4 production was reached.

Three lakes were further investigated to determine AOM activity in vertical sediment profiles; Vault Lake, an Alaskan thermokarst lake formed in yedoma permafrost with an actively expanding thaw bulb; Doughnut Lake, an Alaskan oligotrophic, partially-drained lake; and Dagow Lake, a eutrophic lake located in northern Brandenburg, Germany. Vault Lake and Doughnut Lake were described by Sepulveda-Jauregui et al.[19] while further details of Dagow Lake can be found in Casper[20]. Three sediment cores were collected with a gravity corer from the approximate center of each lake in March and April 2013 for Alaskan lakes and in June 2014 for Dagow Lake. Pore water from the section (0-2.5 cm, 5-10 cm, 10-15 cm, 15-20 cm and 20-25cm) of the cores were immediately subsampled for determination of dissolved CH_4 and CO_2 concentrations as well as physicochemical characterization (nitrate, NO_3^-; nitrite, NO_2^-; sulfate, SO_4^{2-}; total iron, Fe; total manganese, Mn; Dissolved Organic Carbon, DOC; Dissolved Inorganic Carbon, DIC, dry weight and loss on ignition). The $^{13}CH_4$ and $^{13}CO_2$ isotopic ratios in each sediment section from Alaskan lakes were determined with a Picarro G2201-i Analyzer, whereas for Dagow Lake, they were obtained from Conrad et al.[21].

Three sets of incubation experiments were done, each with sample tests performed in triplicate for each section of the sediment cores; methanogenic (MTG), aerobic methanotrophic (MTT) and anaerobic oxidation of methane (AOM) tests. From each core section, a slurry containing 60% of fresh sediment was made. The slurry was flushed for 5 min with ultrahigh purity N_2 to ensure anaerobic conditions. Then, we transferred 30 mL of the homogeneous slurry to nine sterile 50 mL serum bottles. Six of the serum bottles, designated to AOM and MTG tests, were kept under anaerobic conditions by flushing ultrahigh purity N_2 for five minutes while three bottles, designated to MTT tests, were flushed with zero air for the same time. All vials were closed with blue butyl rubber stoppers and aluminum crimp caps. In AOM and MTG bottles, Na_2S was added to a concentration of 0.5 g L^{-1} (ref. 18) to reduce anoxic media. All AOM vials were spiked with 2 mL of $^{13}CH_4$ (99 atom % ^{13}C, Sigma-Aldrich, USA) while all MTT vials were spiked with 2 mL of CH_4 99%. All vials were put to incubation at 4 ± 2 and 8 ± 3 °C, for Alaskan sediments and Dagow Lake sediments, respectively, which is the annual mean sediment

temperature of the lakes. Five killed controls for each treatment and each core sample were prepared by sterilizing 50 mL bottles with 30 mL of homogenous slurry from each section of the core at 121 °C for 20 min. These controls incubation received the same treatment as their correspondents.

We monitored incubations for CH_4, CO_2 and O_2 headspace concentration with gas chromatography, in 3, 15 and 30 day intervals for MTT, MTG and AOM, respectively. Additionally, in Alaskan lakes incubation, AOM vials were monitored for isotopic CH_4 and CO_2 ratios every 30 days with Picarro G2201-i Analyzer. In Dagow Lake AOM incubations, isotopic CH_4 and CO_2 ratios were determined with a gas chromatograph coupled to a combustion isotope ratio mass spectrometer (GC-C-IRMS) system (Thermoquest, Germany). The methanogenic production rates were determined from the linear increase of CH_4 concentration against time. Methanotrophic potential rates were determined from the linear decrease in CH_4, in methanotrophic bottles and AOM rates were calculated from the linear decrease of $^{13}CH_4$ concentration against time. The CH_4 production rates determined from methanogenic incubations comprises not only production but also oxidation of CH_4 being produced, therefore they corresponded to the net methane production rate (NMPR). The total methane production rates (TMPR) were then determined as the sum of AOM rates (AOMR) and NMPR. From these, the percentage of total CH_4 produced that was oxidized was determined from the ratio between AOM and TMPR. As during AOM tests the ^{12}C-CH_4 being produced by methanogenesis and then oxidized was neglected, therefore AOMR was considered conservative.

The results obtained from incubations were used to feed a diffusion-reaction mathematical model, which predicted dissolved CH_4 concentration in the cores (Fig 2). This model considered each volume element of the sediment cores as independent, with a homogeneous CH_4 concentration (C), exchanging dissolved gas with contiguous elements, according to the Fick's second law[22]. Each element was characterized by a CH_4 reaction rate equal to TMPR minus AOMR, measured during incubation tests. This model considered dissolved CH_4 concentration at the water/sediment interface and at the deepest segment of the core as boundary conditions. The model was fitted to CH_4 concentration profiles determined in the sediment of the three lakes, using the CH_4 diffusion coefficient as sole adjustment parameter and assuming steady-

state, i.e., concentration not changing over time. The model was fitted to the experimental data with a least square error minimization performed with R software.

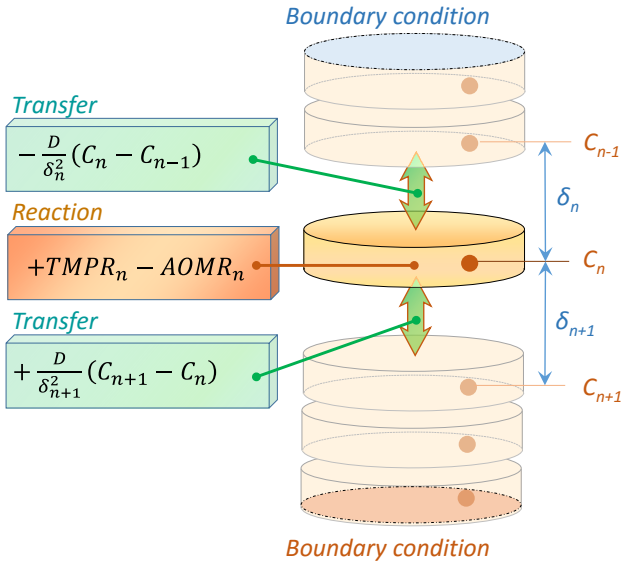

Fig. 2: Diffusion/reaction model of mass balance used to fit dissolved CH_4 concentration (C) in "n" sediment core sections.

Statistical analyses were carried out on AOMR and NMPR. First, normality was assessed by the Shapiro-Wilk test. As most of the data were non-normally distributed. Significant differences among AOMR and NMPR measured in this study and those reported in literature were determined using Kruskal-Wallis multiple comparison test (KW-test) for AOMR and U Mann-Whitney comparison test (UM-test) for NMPR.

Results and discussion

In order to complement previous studies and to better constrain AOM in lakes, we tracked AOM in the sediment of 14 tropical, temperate and northern lakes with contrasting trophic states and water physicochemical regimes (Table 1). Sediment samples spiked with labeled methane ($^{13}CH_4$) were incubated for six months, without addition of any electron acceptors, i.e. endogenous electron acceptor only. Methane oxidation, identified by measuring labeled carbon dioxide ($^{13}CO_2$) production, was significantly different than the sterile controls in all but two temperate lakes (#7 and #8; Table 1). The average AOM rate (AOMR), estimated from the total CH_4 decreasing in the headspace, was 4.61 ($\sigma \pm 5.15$) nmol mL^{-1} d^{-1}. These results, which are in the same range as those previously reported[5,9,10,12-14] (Fig. 1), indicate that AOM is a widespread

process that does not require exogenous electron acceptors, and that AOM takes place independently of the lake trophic state and climate.

Table 1. Relative abundance of ^{13}C in carbon dioxide produced from the anaerobic oxidation of ^{13}C-labeled methane in lakes from different climate. Values in italics indicate significant difference to sterile control according to KW-test (p = 0.05). TSI = Trophic state index: UO – ultraoligotrophic; O – oligotrophic; M – mesotrophic; E – eutrophic; Mx – mixotrophic; HE– hypereutrophic

#	Name	Climate	TSI	Latitude	Longitude	$\delta^{13}CO_2$[1]
1	Goldstream	Boreal	Mx	64.9155	-147.8488	*212 ± 2*
2	Killarney	Boreal	Mx	64.8698	-147.9020	*164 ± 15*
3	Otto	Boreal	O	63.8405	-149.0383	*406 ± 50*
4	Doughnut	Boreal	O	64.8981	-147.9086	*348 ± 100*
5	Vault	Boreal	Mx	65.0292	-147.6985	*2288 ± 877*
6	Dagow	Temperate	E	53.1512	13.0533	*1520 ± 514*
7	Guadalupe	Temperate	HE	19.6325	-99.2565	-120 ± 54
8	El Llano	Temperate	M	19.6577	-99.5073	96 ± 20
9	Cuitzeo	Temperate	HE	19.9400	-101.2000	*537 ± 51*
10	Umecuaro	Temperate	O	19.5228	-101.2454	*637 ± 94*
11	Zirahuen	Temperate	O	19.4355	-101.7379	*503 ± 106*
12	Olmeca	Tropical	HE	19.1470	-96.1506	*430 ± 69*
13	San Julian	Tropical	HE	19.2558	-96.2608	*479 ± 137*
14	Martintela	Tropical	HE	18.5700	-95.6800	*170 ± 18*

[1] $\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard}]$-1; VDPB, Vienna Pee Dee Belemnite,

After evidencing AOM in sediments of a span of lake ecosystems, we further investigated AOM in sediment cores of the two lakes which exhibited the higher AOM rates (Vault and Dagow; Table 1) and one lake with AOM rate similar to the average (Doughnut). This selection allowed the characterization of AOM in the sediments of a thermokarstic, an oligotrophic, and a eutrophic lake, respectively. We observed significant AOM and CH_4 production at all sediment depths of the three lakes (Fig. 3). The AOMR, determined from $^{13}CH_4$ uptake, ranged from 3.32 ± 0.36 to 14.90 ± 1.17 nmol mL^{-1} d^{-1}, observed at the bottom sediment section of Doughnut Lake and mid-section of Dagow Lake, respectively. The mean AOMR for all lakes and at all sediment depths was 9.28 ± 4.75 nmol mL^{-1} d^{-1}, which is not significantly different than the mean AOMR previously reported in the literature, in presence or absence of exogenous electron

acceptors; i.e., 34.07 ± 47.60 and 9.44 ± 11.30 nmol mL^{-1} d^{-1}, respectively (KW-test, $p < 0.05$, Fig. 1). Net CH_4 production rate (NMPR) ranged from 1.10 ± 0.26 to 83.18 ± 11.85 nmol mL^{-1} d^{-1} observed at the bottom of Vault Lake core, and top section of Dagow Lake, respectively. The percentage of CH_4 produced that was oxidized anaerobically ranged from 12 % in the bottom section of Doughnut Lake sediment core, to 87% in the bottom sediment section of Vault Lake. The global average percentage of oxidation in the three lakes was 38 ± 23%, which determines a range of percentage oxidation from 33% to 43% with a 95% confidence level. From the metadata analysis presented in Fig. 1, a balance between AOM rate from unamended tests and CH_4 production rates previously reported gives a percentage of oxidation of 22 ± 26%, which is within the inferior values determined from our experiments. Altogether, our results combined with literature data give a percentage of CH_4 oxidation from 29% to 34% with a 95% confidence level, suggesting that AOM in lake sediments, might be a more important actor of the global CH_4 cycling than previously estimated.

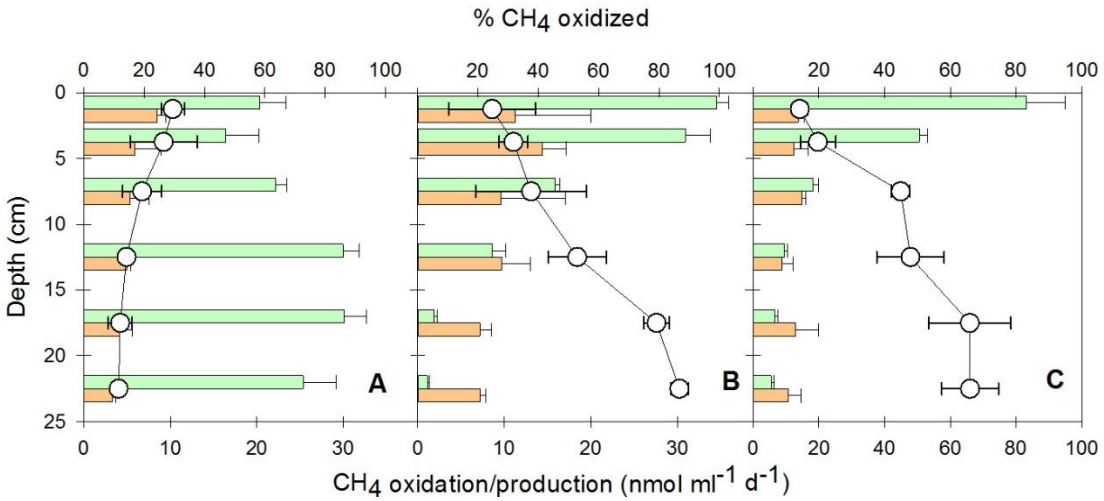

Fig. 3: CH_4 oxidation rate (AOMR; orange bars), net CH_4 production rate (NMPR; green bars), and the percentage of CH_4 that is oxidized through AOM (Open circles). Percentage of AOM was determined from AOMR and total CH_4 production rate (NMPR+AOMR); A, Doughnut Lake; B, Vault Lake; C, Dagow Lake.

In Vault Lake and Dagow Lake, a clear and similar trend was observed with higher NMPR in the top section of the cores and higher percentage of CH_4 oxidation in the deeper sections of the cores. Doughnut Lake showed the opposite trend. It has been demonstrated that the amount of CH_4 produced from freshwater sediments would be limited by the amount of labile organic matter and likely by landscape-scale factors[23]. Doughnut Lake was likely formed by thaw of yedoma-type permafrost, which is loess-dominated permafrost with high ice and organic carbon

contents of several to tens of meters deep[24-26]. Currently, Doughnut Lake doesn't appear to have active thermokarst along its margins, but ancient carbon might be still decomposed throughout the sediment profile, below the 25 cm core sampled. Another hypothesis to explain the profiles observed in Doughnut Lake might be subject a higher sedimentation rates than in Vault Lake and Dagow Lake, as it has been shown that high sedimentation rates; i.e 5–11 cm yr^{-1}, rapidly push organic matter to deep sediment layers, where its labile fraction fuels methanogenesis[23].

To confirm that the rates measured through incubation matched the actual conditions prevailing in the sediments, the production/oxidation rates presented on Fig. 3 were used to feed the diffusion/reaction model (Fig. 2). The model fitted relatively well the profiles of CH_4 concentration in the three lakes (Fig. 4), with a CH_4 diffusion coefficient of 1.52 10^{-5}, 1.11 10^{-5} and 2.52 10^{-5} cm^2 s^{-1}, for Doughnut, Vault and Dagow lakes, respectively. These CH_4 diffusion coefficients are within the range reported in the literature from diffusion in sediments (2.3 – 6.3 10^{-6} cm^2 s^{-1}; ref. 27) to CH_4 diffusion in water (8.7 10^{-6} – 1.31 10^{-5}; ref. 28). The correlation coefficient (R^2) of the model for Doughnut, Vault and Dagow lakes were 0.98, 0.86 and 0.97, respectively, which indicate that the rates determined from laboratory incubations matched probably well with the actual conditions prevailing in the sediments.

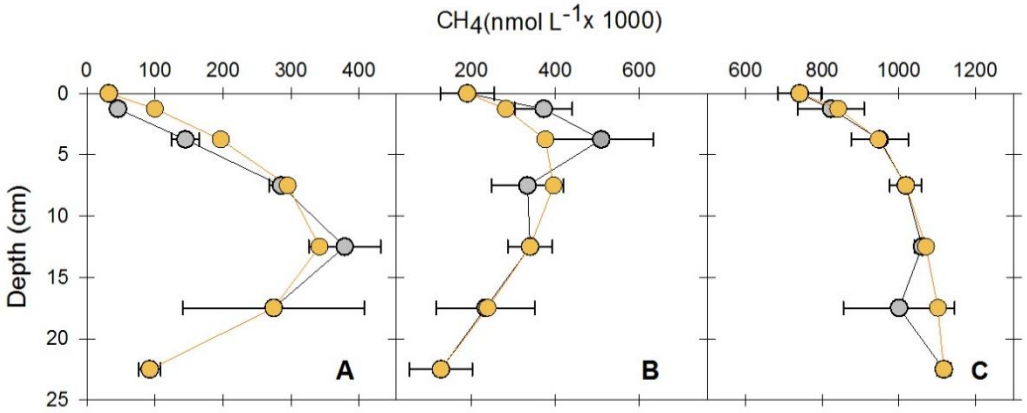

Fig. 4: Diffusion/reaction model fitting (●) and dissolved CH_4 concentration (●) observed in sediment cores in; (A) Doughnut, (B) Vault and (C) Dagow lakes. Error bars show 1 standard deviation.

We also measured the isotopic signature of CH_4 and CO_2 along the sediment cores, by determining the $\delta^{13}C$ for both, CH_4 and CO_2 (Fig. 5). The lower $\delta^{13}C$-CH_4 values ranged from -61.3 to -66.7 $^0/_{00}$ for Dagow and Doughnut lakes respectively, which are consistent with the CH_4

production isotopic signatures[21,29]. The higher δ^{13}C-CH_4 values were -36.9, -49.8 and -59.7 $^{0}/_{00}$ for Doughnut, Vault and Dagow lakes, respectively, all of them found in the first 2.5 cm of the sediment cores (Fig. 5). The δ^{13}C-CH_4 signatures in the superficial sediments for Doughnut and Vault sediments indicate a clear enrichment due to CH_4 oxidation, which agrees with the maximum AOMR found in the upper section of sediment cores. The δ^{13}C-CH_4 profiles observed in Doughnut and Vault lakes are in agreement with previous reports[22]. The δ^{13}C-CO_2 profiles in the three lakes exhibited a distinguishable symmetry compared to δ^{13}C-CH_4. These results are in agreement with Conrad et al.[21] who suggested a higher acetoclastic methenogenesis in the upper layer of lake sediments, and hydrogenotrophic methanogenesis in deeper sediment layers; i.e. below 10 cm depth.

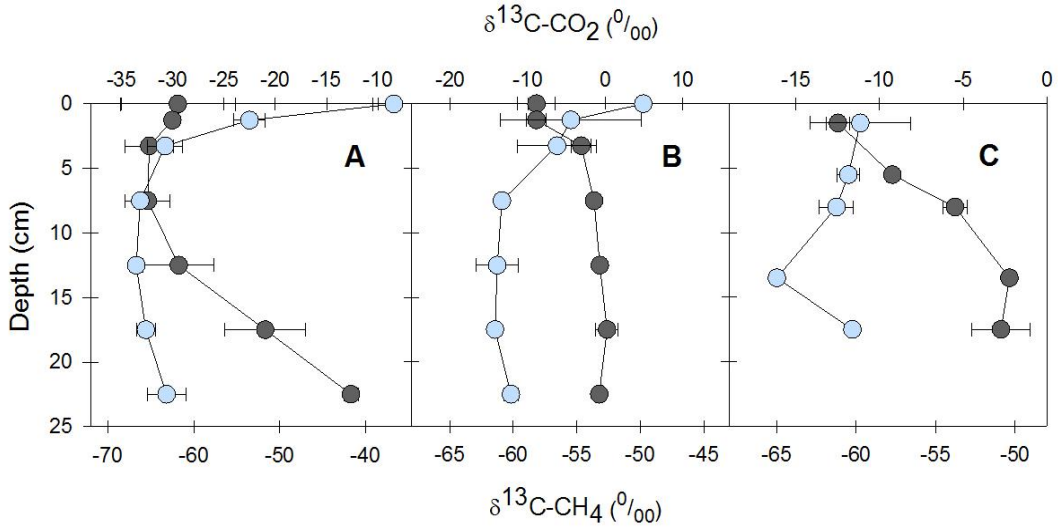

Fig. 5: Profiles of δ^{13}C-CH_4 (●) and δ^{13}C-CO_2 (●) of Doughnut (A), Vault (B) and Dagow (C) lakes.

Sediment samples were also tested for aerobic methanotrophy in vials flushed with zero air and spiked with CH_4. The aerobic methanotrophic rates (AMR) observed ranged from 102 to 266 nmol mL^{-1} d^{-1}, observed in the middle section of Dagow lake core and close to the bottom of the Doughnut Lake core, respectively (results not shown). These rates are within the rates reported in the literature for similar lake sediments and freshwater ecosystems[5,30,31]. In addition, we found a linear correlation between AOMR and AMPR (Fig 6), which suggests that aerobic methanotrophs might be directly or indirectly involved in AOM. The latter is in agreement with a previous report of AOM by the denitrifying methanotroph *Candidatus Methylomirabilys oxyfera*[32], and correlation between abundance of aerobic methanotrophs and AOM previously reported in anaerobic lake sediments[11,33].

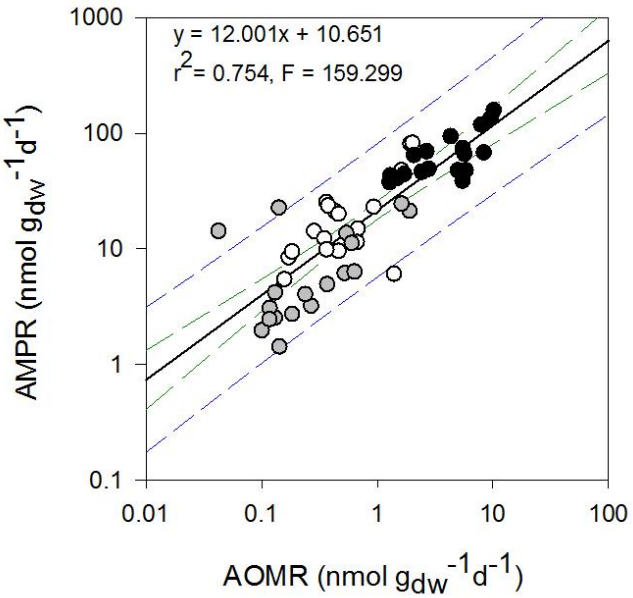

Fig. 6: Linear correlation between AOMR and AMPR, from Doughnut (○), Vault (◍) and Dagow (●) lakes incubation tests, n = 54.

We explored the potential electron acceptors involved in AOM. The highest value of dissolved SO_4^{2-} , i.e., 1 mM, was observed in sediments of Vault Lake (results not shown). Beal et al. (2011)[34] reported previously a strong decoupling of AOM and sulfate reduction at sulfate levels below 1 mM, which suggests that AOM observed in the three lakes was probably not coupled to sulfate reduction. In addition, no opposite trend of SO_4^{2-} and dissolved CH_4 concentrations was observed, as in previous studies where sulfate was suggested as electron acceptor[22,35]. The concentrations of NO_3^- and NO_2^- in the sediments of the three lakes were very low and no correlation was found between their concentration and AOMR, which suggests AOM is not coupled to denitrification either. Our results do not allow any conclusion on the possible electron acceptor coupled to the observed AOM. Potential electron acceptor may have been humic substances, as previously suggested[2,17], Fe (III), and/or Mn (IV).

Conclusion

Anaerobic oxidation of CH_4 was observed, with no exogenous electron acceptor, in the surface sediments of 12 lakes and at all depths along three lakes sediments core profiles. This evidence collected from a span of freshwater environments, endorses the widespread distribution of AOM as a common feature of freshwater anoxic environments, which has been suggested but

not formally established before. Altogether, previous reports and our study combine a total dataset of 120+ AOM rates, ranging from 0 to 43 nmol mL^{-1} d^{-1}, which represent a percentage of CH_4 oxidation from 29% to 34% of the total CH_4 produced in sediments. Our estimations were obtained from laboratory incubations, but a diffusion/reaction model correlated well field measurements, and suggests that CH_4 production/oxidation observed during incubations mimic correctly the actual processes taking place in lake sediments. Additional evidences showed that no exogenous electron acceptor was required for AOM to occur and that aerobic methanotrophy might be involved in the process. This evidence does not unveil the mystery surrounding AOM but contributes to constrain better that process, as a major and widely distributed piece of the global biogeochemical carbon puzzle.

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6.5 Anaerobic oxidation of methane by aerobic methanotrophs in arctic lake sediments

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Abstract

Anaerobic oxidation of methane (AOM) is a biological process that plays an important role by reducing the CH_4 emissions from a wide range of ecosystems. Some microorganisms have been identified to be involved in AOM process. In sulfate-rich environments, anaerobic methanotrophic (ANME) archaea have been found in syntrophic association with sulfate-reducing bacteria to perform AOM. Additionally, *Methylomirabilis oxyfera*, a member of NC10 phylum and *Candidatus Methanoperedens nitroreducens*, an ANME-type archea were both identified as capable of independent AOM using nitrate (NO_3^-) as the terminal electron acceptor. However, some studies have also suggested aerobic methanotrophs, especially *Methylobacter* species to be active in O_2 limited and anoxic conditions. This study sought to identify microorganisms involved in AOM in sub-Arctic lake sediments using DNA- and phospholipid fatty acid- (PLFA) based stable isotope probing. Results indicated that aerobic methanotrophs belonging to the genera *Methylobacter* might be involved in AOM in sediments further challenging our understanding on CH_4 oxidation in anaerobic lake sediments.

Introduction

Methane (CH_4) is an important greenhouse gas biologically produced in anoxic environments, such as sediments of aquatic ecosystems. The CH_4 flux from sediment to the sediment-water interface is reduced by anaerobic oxidation of methane (AOM). This has been demonstrated to occur in anaerobic sediments and soils (Blazewicz et al., 2012; Boetius et al., 2000; Reeburgh, 1976; Schubert et al., 2011; Zehnder and Brock, 1980), and to significantly constrain biological CH_4 emissions to the atmosphere (Reeburgh, 2007; Sivan et al., 2011; Smemo and Yavitt, 2011).

Methanogen-like archaea called anaerobic methanotrophic (ANME) archaea have been found in syntrophic association with sulfate-reducing bacteria to perform AOM in sulfate-rich environments, such as the marine environment (Hoehler et al. 1994; Boetius et al. 2000; Valentine, 2002). With few exceptions such as areas prone to acid rain or in watersheds containing acid SO_4^{-2} soils and/or rocks, SO_4^{-2} concentrations in most freshwater systems are typically too low for the process to be thermodynamically favorable (Smemo and Yavitt, 2007; Alperin and Reeburgh 1984). Anaerobic iron (Fe^{+3}) and manganese (Mn^{+4}) reduction coupled to AOM is a more favorable reaction which has been demonstrated to occur and to be a significant process in freshwater ecosystems (Nordi et al., 2013; Sivan et al., 2011). However, little is known about the microorganisms involved in AOM coupled to Fe^{+3} and Mn^{+4}. One study revealed that the most abundant microorganisms in the marine sediments after incubations of AOM linked to Fe^{+3} and Mn^{+4} were affiliated with the marine benthic group D and ANME (Beal et al., 2009).

AOM coupled to denitrification is also thermodynamically more favorable than AOM linked to sulfate-reduction. AOM coupled to denitrification was first demonstrated in enriched-cultures (Islas-Lima et al., 2004; Raghoebarsing et al., 2006) and has since been reported in lake sediments with a high input of NO_3^- (Deutzmann and Schink, 2011). *Methylomirabilis oxyfera*, a member of NC10 phylum, has been described to carry out AOM by an intra-aerobic pathway of nitrite (NO_2^-) reduction (Ettwig et al., 2010). More recently, it was revealed that *Candidatus Methanoperedens nitroreducens*, an ANME-type archeae, is capable of independent AOM through reverse methanogenesis using nitrate (NO_3^-) as the terminal electron acceptor, by containing genes encoding for NO_3^- reductase laterally transferred from a bacterial donor (Haroon et al., 2013). Although NO_3^- and NO_2^- are sparse in most anoxic lake sediments (Smemo and Yavitt,

2007), populations of NC10 bacteria have been detected, although in low abundance, in both oxic and semi-oxic sediment layers of Lake Washington (Beck et al. 2013) and in anoxic sediments of Lake Constance (Deutzmann et al., 2014). Interestingly, in Lake Constance, NC10 type was detected along with the traditional aerobic methanotrophs (Deutzmann et al., 2014).

Recently, some studies have found aerobic methanotrophs, especially *Methylobacter*, in anoxic zones of lakes (Biderre-Petit et al., 2011; Blees et al., 2014; Kalyuzhnaya et al., 2013) suggesting that some *Methylobacter* species are active in O_2 limited and anoxic conditions, further challenging the long-term dogma of "strictly" aerobic nature of these organisms (Chistoserdova, 2015; Kalyuzhnaya et al., 2013).

In this study, we used DNA- and phospholipid fatty acid- (PLFA) based stable isotope probing (SIP), quantitative (q)-PCR, and gene-targeted metagenomics to track carbon derived from $^{13}CH_4$ through the active anaerobic microbial community. This study sought to identify microorganisms involved in AOM in sub-Arctic lake sediments incubated without the addition of external electron acceptors. Results indicate that aerobic methanotrophs belonging to the genera *Methylobacter* might be responsible for oxidizing about 30% of CH_4 in anaerobic conditions in sediments.

Material and Methods

Sample collection and physical and chemical analysis

Vault Lake is a thermokarst lake formed in yedoma permafrost with an actively expanding thaw bulb (Heslop et al. 2015). The main morphological and limnological characteristics were described by Sepulveda-Jauregui et al. (2015).

In March 2013 three sediment cores were collected from the center of Vault Lake. The cores were collected in polycarbonate tubes (6.5 cm diameter) with overlaying water, with a gravity corer (Alaska, USA) and sealed without headspace. Cores were immediately transported to the laboratory and stored in the dark at 4°C until processing and analysis, within 24 h of collection.

Sediment cores were analyzed and processed as reported in Martinez-Cruz et al. (in press). Briefly, we opened the cores vertically in half, one half of each core was immediately sealed with four layers of O_2 and moisture-barrier film (Krehalon PC101, Filcon, Clare, Michigan, USA) for impeding gas exchange and was stored at 4°C until initiation of incubations tests. The other half was subsampled for physicochemical analysis. Martinez-Cruz et al. (in prep.) demonstrated higher AOM rates in the top 2.5 cm of sediment cores from Vault Lake and two other sub-arctic and temperate lakes. This study is focused on the top 2.5 cm of Vault Lake sediment cores.

Dissolved nitrate (NO_3^-) and nitrite (NO_2^-) in pore water were determined according to standard methods (APHA, 1999), detection limit 1 μM. Sulfate (SO_4^{2-}) concentration was measured with ion chromatograph (150/4.0mm ID column, eluent 3.2mM Na_2CO_3, 1 mM $NaHCO_3$, detection limit 1 mM; ECD Alaska, USA).

Stable isotope probing incubations

Stable isotope probing (SIP) incubations were conducted by homogenizing 200 mL of sediments from the top 2.5 cm section of the cores and transferring them into 130 mL of anaerobic water while flushing with ultrahigh purity N_2 (99.999%, AirGas, USA) to keep anaerobic conditions and generate a homogeneous slurry (60% of wet sediment). 30 mL of the homogeneous slurry was transferred to each of the six sterile serum 50 mL bottles. The bottles were kept in anaerobic conditions by flushing ultrahigh purity N_2 (99.999%) to each bottle for several minutes. Once sediments were placed in the bottles, they were immediately sealed with blue butyl rubber stoppers (Bellco, USA) and aluminum crimp caps. Finally, each bottled was spiked with 2 mL of 99.9% of $^{13}CH_4$ (99 atom % ^{13}C, Sigma-Aldrich, USA). Incubation were carried out in darkness at 4 ± 2 °C; the annual mean temperature of boreal lakes (Sepulveda-Jauregui, 2014).

Incubations were assessed every 30 days for CH_4, CO_2 and O_2 concentration with a Shimadzu GC-2014 gas chromatograph (Kyoto, Japan) and for stable isotopes ratios ^{13}C:^{12}C (CH_4 and CO_2) with a Picarro G2201-i Analyzer (Picarro Inc., California, USA). The equipment reported in the delta notation vs. Vienne Pee Dee Belemnite standard (V-PDB, see below). Three killed controls were prepared by sterilizing 50 mL bottles with 30 mL of homogenous slurry from each section of the core at 121 °C for 20 min, we flushed ultrapure N_2 to change the headspace. Finally, we spiked the AOM killed controls with 2 mL of $^{13}CH_4$ (99 atom % ^{13}C, Sigma-Aldrich, USA). All

incubations were carried out for 204 days. Samples were destructively harvested at four time points by freezing at -80 °C: t_0 at the beginning (day 0) of the incubation, t_1 at day 80, t_2 at day 142, and t_3 at the end of the incubation (day 204).

AOM rates were calculated from the linear decrease of $^{13}CH_4$ concentration against time. We determined $^{13}CH_4$ concentration, based on equation (1).

$$\delta^{13}C = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{PDB} - 1 \right) \times 1000 \text{ }^{\circ}/_{\circ\circ} \quad (1)$$

Where, $\delta^{13}C$ is the isotopic fraction measured by the equipment, ^{13}C and ^{12}C are the sample concentrations (mg g_{dw}^{-1}), and PDB is the Vienne Pee Dee Belemnite standard = 0.0112372. Given that we determined the total CH_4 concentration in the headspace, the ^{12}C can be expressed as the difference between the total and the $^{13}CH_4$ concentration and the equation (1) is modified and simplify as follows:

$$^{13}C = \frac{TC}{\frac{1}{\left(\frac{\delta^{13}C}{1000\ ^{\circ}/_{\circ\circ}}+1\right)\times PDB}+1} \quad (2)$$

DNA extraction, density gradient centrifugation and Q-PCR

DNA was extracted from 0.58 ± 0.08 g of SIP-incubated sediment in duplicate for each serum bottle using the Bio101 Fast DNA Spin Kit for soil (MP Biomedicals, Solon, OH, USA). Prior to running density gradient centrifugation, we evaluated the incorporation of the ^{13}C into the DNA, by determining the $\delta^{13}C$-DNA in an elemental analyzer (Finnigan DeltaPlus XP, Thermo Scientific) coupled to a Costech ECS4010 Elemental Analyzer (Costech Scientific, Valencia, California, USA) at the University of Alaska Stable Isotope Facility, Fairbanks, Alaska, USA. All $\delta^{13}C$-DNA values are expressed relative to PDB. In the samples where the ^{13}C-DNA labelling was found, density gradient centrifugation and Q-PCR was run according to He et al. (2012). Briefly, samples of 5 mg DNA were combined with cesium trifluoroacetate (CsTFA, GE Healthcare, UK) solution with a starting BD of 1.60 g ml^{-1} and placed in Beckman polyallomer Quick-Seal centrifuge tubes (13 ¥ 51 mm), sealed, and spun in a NVT 100 rotor in an Optima L-100 XP ultracentrifuge (Beckman Coulter) at 45 600 r.p.m. and 25°C for 72 h. Gradients were

fractionated into 20 fractions. Sample DNA was precipitated from fractions with isopropanol overnight at -20°C, and then centrifuged; resulting pellets were washed twice with isopropanol and resuspended in nuclease free water (Sigma-Aldrich, USA). The distribution of DNA in gradient fractions ranging in BD from 1.586 to 1.654 g ml^{-1} was determined by Q-PCR as described previously (Leigh et al., 2007). After the range of fractions containing ^{13}C-labelled DNA was identified, fractions were combined to constitute compiled 'heavy' fraction (^{13}C-DNA) for sequencing.

Illumina sequencing and analyses of 16S rRNA genes

The V4 region of the 16S rRNA gene was amplified using Illumina fusion primers as described by Caporaso et al. (2012). PCR output for all samples was normalized using a Life Technologies SequalPrep Normalization plate. The normalized products were pooled. After Ampure clean up, QC and quantitation the pool was loaded on a standard v2 MiSeq flow cell and sequenced in a 2x250bp format using custom V4 sequencing and index primers (see Caporaso et al.) and a MiSeq 500 cycle reagent cartridge (v2). Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ with Illumina Bcl2fastq v1.8.4.

Sequences were processed using the mothur software package, version 1.33.3 as previously described (Kozich et al., 2013; Uhlik et al., 2012). Briefly, the reads were processed by removing primers and tags, and sequences that had an average quality score of above 25 and read length of no longer than 275 nt. Identical sequences were grouped and representatives aligned against the SILVA database. A further pre-clustering screening step was applied to reduce sequencing noise by clustering reads differing by only 1 bp per 100 nt of each read. Chimeric sequences were detected and removed using the mothur-implementation of Uchime (Edgar, 2010). The remaining high-quality reads were used to generate a distance matrix and clustered using a furthest-neighbor algorithm. Operational taxonomic units were defined by 97% similarity, and were assigned using the RDP Naïve Bayesian Classifier (80% confidence threshold). The nucleotide sequences have been deposited to the NCBI Short Read Archive under the accession number of SRP005485. Representative sequences from OTUs which remained unclassified at any taxonomic level after analysis were further investigated using the RDP SeqMatch tool to determine the closest type strain.

Because incorporation of ^{13}C into the DNA was previously analyzed for sequenced samples, no ^{12}C- control samples were sequenced.

Phospholipid Fatty Acids (PLFA) analysis

Triplicate subsamples of approximately 2 g freeze-dried sediment from AOM $^{13}CH_4$ incubations at t_0, t_2 and t_3 were extracted using a modified Bligh-Dyer method as described by Wu and colleagues (2009). The phospholipid ester-linked fatty acid methyl esters were dissolved in hexane for GC-FID and GC-combustion-isotope ratio mass spectrometry (GC-CIRMS) analysis as described by He et al. (2015). All $\delta^{13}C$ values were corrected for derivatization using an isotopic mass balance equation (Eq. 3) as described by Maxfield et al., (2006).

$$\delta^{13}CPLFA = (n + 1)\, n \times \delta^{13}CFAME - \delta^{13}CMeOH \quad (3)$$

Where n is the number of carbon atoms, $\delta^{13}CPLFA$ is the $\delta^{13}C$ value of the PLFA, $\delta^{13}CFAME$ is the $\delta^{13}C$ value of the PLFA after derivatization, $\delta^{13}CMeOH$ is the $\delta^{13}C$ value of the methanol used for methylation (−52.5 ‰).

Results

Methane oxidation rates and SIP labelling

The autoclaved control showed no AOM rates (Fig. 1A), suggesting that the observed CH_4 oxidation is a biological process. The mean AOM rate was 1.76 ± 0.20 μg $g_{dw}^{-1}d^{-1}$ (mean ± SD) and almost 50% of the total $^{13}CH_4$ was oxidized (Fig. 1A). The stable isotopic ratios $\delta^{13}C$ for both, CH_4 and CO_2 showed the oxidation of $^{13}CH_4$. We observed CH_4 production throughout the incubation time course, which, together with the AOM, indicates that $^{13}CH_4$ may be diluted by consumption and $^{12}CH_4$ production. Despite the CH_4 dilution, the $^{13}CH_4$ concentration ranged from 55-100% of the total CH_4 in the headspace. Taking into account the total CH_4 being produced (3.74 ± 0.15 μg $g_{dw}^{-1}d^{-1}$), approximately 32% of the produced CH_4 was anaerobically oxidized.

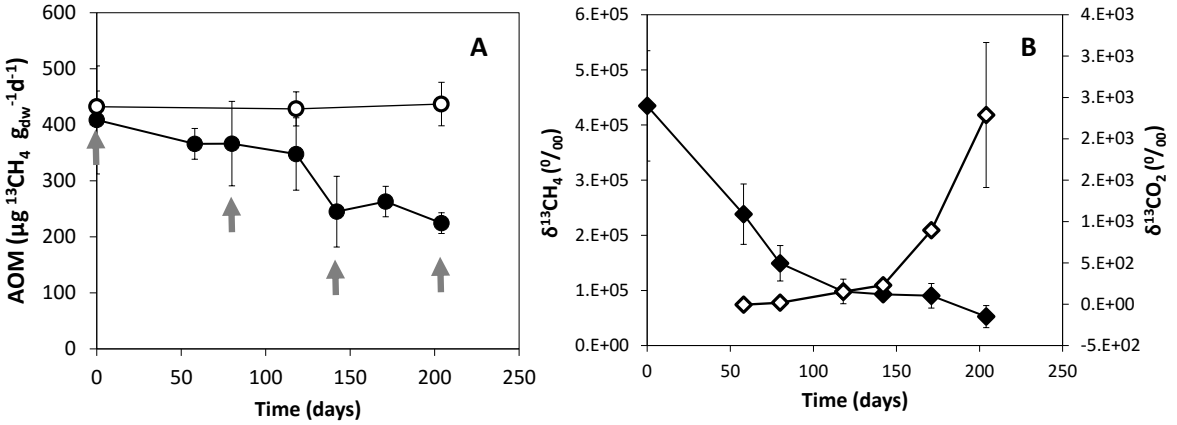

Figure 1. Stable isotopic probing for anaerobic oxidation of methane (AOM) in Lake Vault sediments. A) Methane consumption along the incubation time in lake sediments (closed circles) and sterile control (open circles), the arrows show the time points when samples for DNA and PLFAs samples were collected. B) Stable isotopes ratios $\delta^{13}C$ of CH_4 (closed diamonds) and CO_2 (open diamonds).

The concentration of SO_4^{2-} and NO_2^- in the initial pore water was below detection limits (1 mM and 1 µM respectively). Whereas the concentration of NO_3^- was 1.8 µM, which is below the concentration reported for AOM coupled to denitrification (Ettwig et al., 2009).

The DNA and PLFAs samples were analyzed for ^{13}C, and a strong labeling was found only at the end of the incubation series (t_3, table 1). When examined for ^{13}C enrichment, PLFAs extracted from sediments at t_3 showed the highest enrichment. The PLFA marker with the most ^{13}C-label was 16:1ω9t shifting from an initial value of 14.3 at t_0 to 14137 ‰ at t_3. The ^{13}C in 16:1ω9c/7c was also strongly enriched, shifting from 14.2 at t_0 to 8856 ‰ at t_3. The $\delta^{13}C$ in PLFAs 16:0, 18:1ω9c, 18:2ω6c/6t and 18:3ω6/3ω3 also showed increasing enrichment with the time, showing the highest $\delta^{13}C$ incorporation at t_3 (204 days; Fig. 2).

Table 1. Stable isotope ratios $\delta^{13}C$-DNA and $\delta^{13}C$-PLFAs along the stable isotopic probing AOM incubation. ND – Not determined.

Sample	**$\delta^{13}C$-DNA (‰ PDB)**	**$\delta^{13}C$-PLFA (‰ PDB)**
t_0	-31.81	-29.88
t_1	-28.92	ND
t_2	-26.06	-24.00
t_3	1.46	166.49

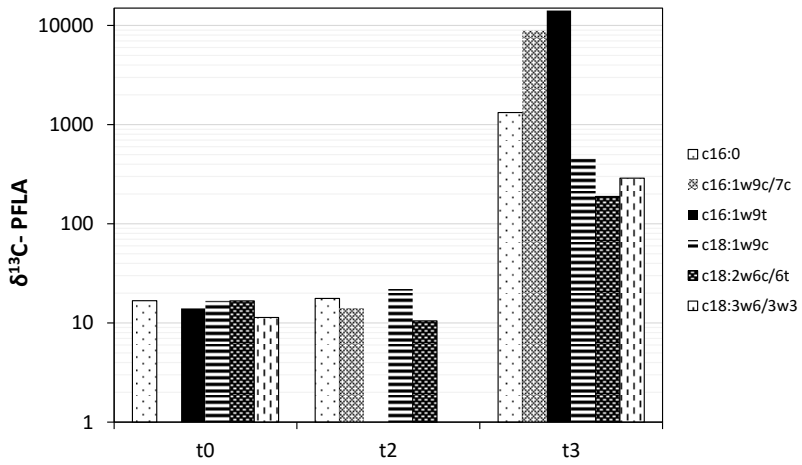

Figure 2. Stable isotopic ratios $\delta^{13}C$ values for different PLFA at different times along the stable isotope probing (SIP) AOM incubations (t_0= initial time, t_2= 142 and t_3=204 days).

Based on the stable isotope ratios $\delta^{13}C$-DNA (table 1), density gradient centrifugation was ran on DNA from t_0, t_2, and t_3. Q-PCR targeting bacterial and archaeal 16S rRNA genes in fractions from density gradient centrifugation indicated that significant quantities of bacterial ^{13}C-DNA were present at t_3, and lesser detectable quantities were present at t_2 (Fig 3 A and B). No ^{13}C-DNA was detected in archaeal 16S rRNA genes (Fig 3B).

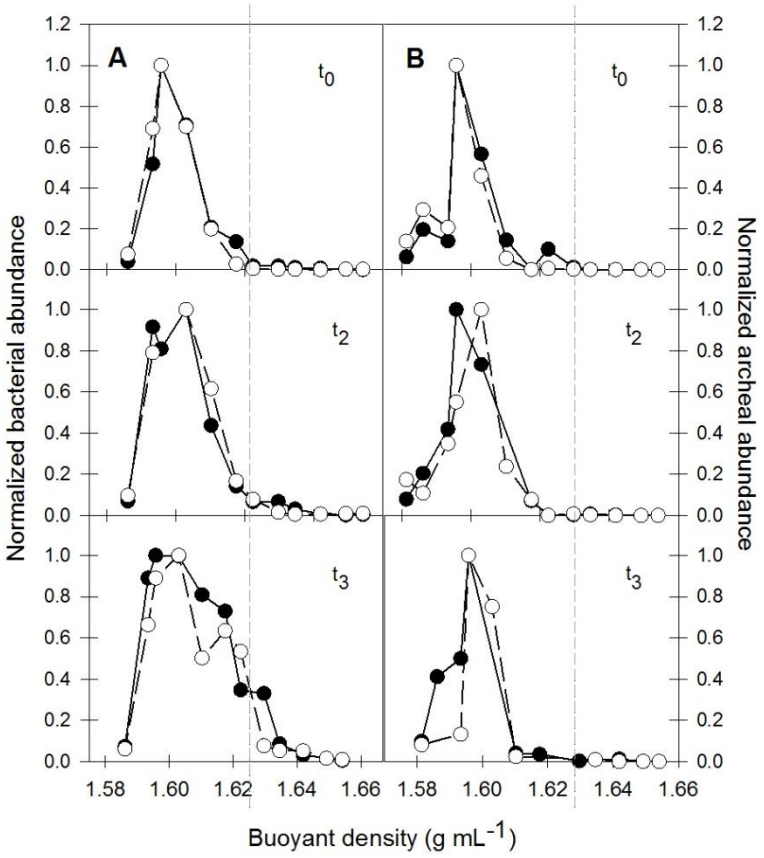

Figure 3. Normalized bacterial (A) and archaeal (B) abundances recovered from the density gradient centrifugation ran in duplicate from samples collected along the AOM-SIP incubation. Open and closed circles represent the two sequenced replicates.

The community of the heavy fraction at t_0 was represented by a total of 2047 OTUs (97% sequence identity), while t_2 was represented by a total of 1926 OTUs and t_3, which was the DNA with the highst ^{13}C label, was represented by a total of 1833 OTUs for each replicate. In the heavy fractions of both, t_0 and t_2, 37.4% and 28.8% of the total the OTUs respectively, showed greatest sequence similarity (97%) to Proteobacteria. Similarly, in the labelled fractions of t_3 40.1% of the total the OTUs showed greatest sequence similarity (97%) to the 16S rRNA gene of Proteobacteria phylum, of which Gammaproteobacteria and Methylococcaceae were the dominant class and family respectively, and *Methylobacter* was most prominently genus. At t_3, other OTUs revealed greatest sequence similarity (97%) to the 16S rRNA gene of Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria and Chloroflexi phyla, representing (11.3, 8.4, 6.2, 2.7 and 2.4 % of the total OTUs, respectively). All previous mentioned phyla were present in the sequences of the fresh sediments (Fig 4). *Ralstonia* was identified as most abundant genus

(97% sequence identity) in heavy-labeled DNA at t_0 and t_2; however, although it is present at t_3, the most dominant genus at t_3 is *Methylobacter*, which is a known aerobic methanotroph. The other dominant OTU present at all analyzed times, showed greatest sequence similarity (97%) to the 16S rRNA gene of *Methylophilu*s, a methylotroph commonly present in sediments. Representative sequences from OTUs which remained unclassified at any taxonomic level at t_3 after analysis were further investigated for NC10 pylum with no matches. However, other dominant OTUs revealed greatest sequence similarity (97%) to the 16S rRNA gene of genus *Syntrophus* and *Albidiferax*, among others.

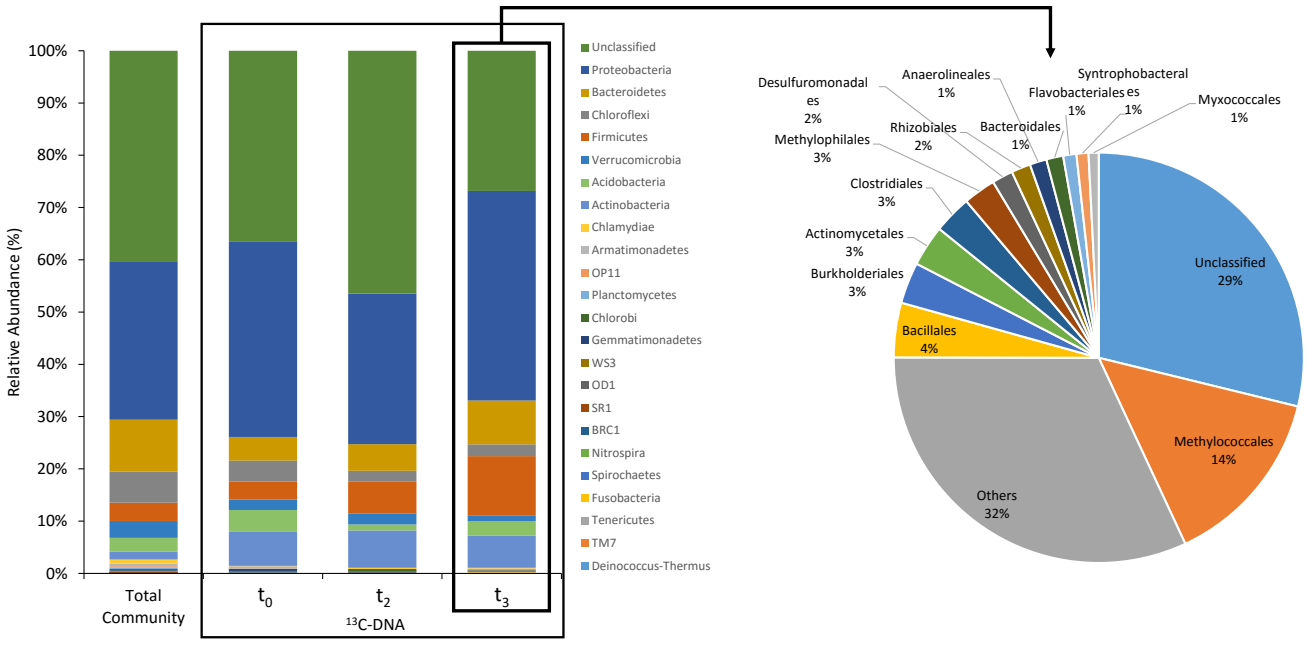

Figure 4. The histogram shows the taxonomic distribution of 16S rRNA gene sequences, t_0, t_2 and t_3 represent the sequences corresponding to the heavy fraction after running in a density gradient centrifugation. The pie chart shows the order distribution of 16S rRNA gene sequences from t_3, which comprised the more heavily labeled DNA.

Discussion

This study sought to determine the microorganisms involved in AOM in lake sediments in actual conditions. Therefore, our AOM incubations were not amended with any electron acceptors, favoring no one specific AOM pathway. Under the natural conditions of sediments of Vault Lake, it is improbable that AOM was coupled to denitrification because NO_3^- and NO_2^- concentrations were close to or lower than the detection limit (1 μM). SO_4^{2-} concentrations were

also detected at < 1 mM, at which has been reported a strong decoupling of AOM and sulfate reduction (Beal et al., 2011).

In order to elucidate the microorganisms involved in AOM in Vault Lake sediments, we tracked the $^{13}CH_4$ in AOM incubations of the lake sediments followed by DNA- and phospholipid fatty acid- (PLFA) based stable isotope probing. We observed $^{13}CH_4$ oxidation along the AOM incubation (fig. 1), however we found significant ^{13}C label in DNA and PLFA primarily only at t_3. This suggests that AOM might be carried out by different, potentially unexplored pathways in the same sediment sample. For instance, Kellerman et al. (2012) demonstrated that anaerobic methane-oxidizing archeae (mainly ANME-1) oxidize CH_4 but assimilate inorganic carbon. Hence it is not likely to find the ^{13}C label in DNA nor PLFAs, until the $^{13}CO_2$ concentration is high enough to be available for autotrophic assimilation.

The most ^{13}C enriched PLFA was 16:1ω9t, followed by 16:1ω9c/7c and 16:0, all of them have commonly been affiliated to aerobic methanotrophs type I (Hanson and Hanson, 1996; Monhanty et al., 2006). The presence of O_2 in our incubations after 204 d is unlikely due to the incubation conditions that does not allow atmospheric air to get inside the serum bottles. From t_2 to t_3, 0.17 mg of CH_4 was oxidized, in aerobic conditions and despising the O_2 consumed by aerobic heterotrophs, aerobic methanotrophs would require at least 0.5 mL of O_2 or 2.3 mL of ambient air. In the conditions our incubations were carried out, it is not possible to have such a volume of air inside the bottles after such a long time (142 – 204 d). Altogether our results suggest: (i) that aerobic methanotrophs might be involved in AOM in sediments of Vault Lake, or (ii) that there might be some cross feeding trough methylotrophs holding the same PLFAs. In a $^{13}CH_4$ labelling study of AOM in a microbial mat from the Black Sea, Blumenberg et al. (2005) found greater ^{13}C uptake into lipids of the sulfate reducing bacteria, archaea lipids were also labelled, and interestingly, 16:1ω9 appeared labelled as well, getting more ^{13}C enriched along the incubation. The latter suggests that methanotrophs type I might play a role in AOM in both, ocean and lake sediments. The PLFA 18:1ω9c, which was labeled but in much less proportion than the latter, often occur in type II methanotrophs (Nold et al., 1999), supporting the possibility for aerobic methanotrophs to play a role in AOM pathway. The PLFAs 18:2ω6c/6t and 18:3ω6/3ω3, which were the less labeled, have been reported for fungi (Green and Scow, 2000), which might indicate the fate of the CH_4-derived carbon.

The DNA-SIP analysis, followed by Q-PCR and gene-targeted metagenomics, indicated that *Methylobacter* was the main genera involved in AOM at t_3. The genera *Methylobacter* corresponds to type I methanotrophs which cell morphology is cocci, ellipsoidal or fat rods (Hanson and Hanson 1996), lending support to the PLFA-SIP findings. Active Methylobacter-type methanotrophs have been previously found in anoxic and oxic zones in different lakes. *Methylobacter* has been detected in the upper sediments (0-1 cm) from an arctic lake in Alaska to be active by aerobically oxidizing the CH_4 (He et al., 2012). In Québec, Canada, several thermokarstic ponds were analyzed for active communities, finding type I methanotrophs, mainly, Methylobacter-type in both, anoxic and oxic zones (Crevecoeur et al., 2015). Moreover, sequences of *Methylobacter* have previously been detected in anoxic but not oxic zones in the deep South-Alpine Lake Lugano, coinciding with the maximum CH_4 oxidizing potential zone 40 m below the chemocline (Blees et al., 2014), suggesting that aerobic methanotrophs can be more active in anoxic water layers. Methylobacter-type methanotrophs, were also found in anoxic zones in Lake Pavin (Biderre-Petit et al., 2011). Likewise, *Methylobacter*, together with Methylotenera-types methanotrophs, were identified as the dominant genera in incubations with low initial O_2 concentration (15 μM, Hernandez et al., 2015). Previous studies showed that *Methylobacter* genome encodes respiratory NO_3^- and NO_2^- reductases too, in addition, it contains genes predicted to encode functions essential to N_2 fixation (Kalyuzhnaya et al., 2015). The potential role of N_2 fixation by *Methylobacter* is not clear yet (Chistoserdova, 2015), but the ability of Methylobacter to denitrify, might be the key of its presence in anaerobic environments. Nevertheless, Blees et al. (2014) estimated the concentrations of NO_3^- and NO_2^- too low to account for the observed CH_4 oxidation occurring at O_2 concentrations below detection limit. In our study, NO_3^- and NO_2^- concentrations were also too low to expect AOM to occur linked to denitrification. Altogether, this evidence for aerobic CH_4 oxidizers activity in anaerobic environments, points towards the gap in our understanding about CH_4 oxidation in anaerobic lake sediments, but strongly suggests *Methylobacter* as a participant in CH_4 oxidation in anoxic sediment or water layers.

Sequences of *Methylophilus* were also abundant in ^{13}C-DNA of t_2 and t_3. *Methylophilus* is an obligate methylotroph that uses methanol as the sole source of carbon and energy (Vries et al., 1990; Bratina et al., 1992). He et al., (2012) reported *Methylophilu*s to be active during aerobic CH_4 oxidation in sediments of Alaskan lakes, attributed to the cross-feeding due to an excess of

extracellular methanol produced by manipulation of the sediment. However, its presence in other enriched cultures suggested that *Methylophilus*, might play an important role in the microbial food web processing carbon from CH_4 oxidation in arctic and subarctic lakes (He et al., 2012). Sequences of *Ralstonia* were also abundant in the heavy fraction mainly at t_2 and in less proportion at t_3. Species of *Ralstonia*, such as *R. eutropha* H16, in the absence of environmental O_2, can switch to anaerobic respiration by using alternative electron acceptors such as NO_3^- and NO_2^- through a complete denitrification pathway (Pohlmann et al., 2007). *Ralstonia* can fix CO_2 via the Calvin-Benson-Bassham (CBB) cycle (Bowien and Kusian, 2002), thus it might have the ^{13}C label due to the incorporation of $^{13}CO_2$ to its system. Since NC10 phylum has previously been identified as responsible for AOM in lake sediments (Deutzmann et al., 2014), we also investigated the unclassified sequences for NC10 phylum in the heavy fraction of t_3. However, we did not find NC10 phylum labelled, which indicates that AOM linked to denitrification possibly did not occur in Vault Lake sediments. Some of the most abundant "unclassified" sequences corresponded to stricly anerobic cross-feeders, i.e (i) Genus *Syntrophus* which can oxidize fatty acids in the presence of H_2/formate-utilizing methanogenic or sulfate-reducing partner bacteria (Jackson et al., 1999; Schocke and Schink, 1999) and (ii) Genus *Albidiferax*, which has been found in Fe, Mn, and arsenic-rich environments, it is also known to reduce Fe while oxidizing acetate (Akob et al., 2014; Ghosh et al., 2014; Kotik et al., 2013; Lu et al., 2013) . The latter may suggest the occurrence of iron reduction which has also been coupled to AOM.

Interestingly, no ^{13}C-labeled archaeal DNA was detected in any of our samples. Although this does not necessarily exclude archaea as potential anaerobic methane oxidizers, it does encourage the use of different techniques for elucidating the main actors of AOM in lake sediments.

Conclusions

The evidence obtained from DNA- and phospholipid fatty acid- (PLFA) based SIP in AOM incubations of arctic lake sediments, indicated that aerobic methanotrophs type I belonging to genera Methylobacter were involved in the AOM process. Methylobacter-type methanotrophs have been previously reported as "strict" aerobic methanotroph, but they have also found active in anaerobic conditions. In addition, the ^{13}C label from AOM appeared only at the final time of the AOM incubation, although $^{13}CH_4$ was oxidized constantly along the incubation, suggesting

that more than one pathway of AOM may co-occur in sediments of Vault Lake. By this technique, no archaea were found actively participating on AOM. The ^{13}C label was also found in *Methylophilus*, *Ralstonia* and *Syntrophus* genera, and in PLFA of fungi, showing a feasible fate of the C being incorporate by AOM to the food web. Some iron reducing genus were also found, which indicates that AOM coupled to iron-reduction might be a feasible pathway in Vault Lake sediments.

This study strongly encourages the investigation of metabolic pathways of aerobic methanotrophs in anoxic conditions, and suggests that AOM is being carried out by several microorganisms following different pathways, making the task of elucidating the AOM process much more complex.

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Chapter 7

Conclusions

This thesis comprised a broad study of the methane cycling in arctic, temperate and tropical lakes. In this chapter, general conclusions of the study, proof of hypotheses, and future research on CH_4 cycling are presented.

7.1 Conclusions

Throughout this thesis it was possible to investigate the methane production and oxidation in both water and sediments of arctic, temperate and tropical lakes. In arctic lakes, not only the CH_4 production, but also aerobic methanotrophy are dependent on the permafrost soil type in which the lakes are located. In lakes formed in yedoma-type permafrost, the carbon cycling is active, far below the sediment/water interface, promoting CH_4 formation meters to tens of meters below the sediment water interface in the anaerobic thaw bulb. However, aerobic methanotrophy can substantially mitigate the increase in CH_4 emission predicted by permafrost thawing (section 6.2). On the contrary, the anaerobic oxidation of methane (AOM) in surficial lake sediments seems to be equally active, regardless of permafrost soil type, trophic state and limnology; mitigating about 38% of the total CH_4 produced by methanogens in superficial lake sediments (section 6.3 and 6.4).

The evidence obtained from DNA- and phospholipid fatty acid- (PLFA) based stable isotope probing in AOM incubations of arctic lake sediments, pointed to the aerobic methanotrophs belonging to genera *Methylobacter* to be involved in the AOM process. This is not the first study suggesting a "strict" aerobic methanotroph to be involved in AOM, but it is the first one giving a direct evidence of it. However, the pathway in which *Methylobacter* oxidizes CH_4 in anaerobic conditions is still unknown. This study, also suggests that more than one pathway of AOM may co-occur in lake sediments (section 6.5).

Despite those findings, much about AOM remains uncertain; for instance, the role of sulfate and nitrates, as potential final electron acceptors in AOM in lake sediments is unclear. Incubations with and without those electron acceptors interchangeably showed AOM activity, with no significant difference (section 6.3). In addition, the concentration of sulfates and nitrates

in sediments in natural conditions, make them unlikely to be involved in AOM (section 6.4), leaving open the possibility that many other candidates may have play a significant role in AOM.

Finally, but not less important, the development of a non-invasive, portable method based on TDLAS to determine CH_4 production/oxidation rates in the field (section 6.1), allowed the study of 30 arctic lakes during two different seasons (section 6.2). The later highlights the importance of using portable, precise and practical methods in studies involving large amount of ecosystems.

7.2 Proof of hypotheses

The hypotheses addressed in this thesis were:

1. A method based on infrared Tunable Diode Laser Absorption Spectroscopy (TDLAS) combined with incubations in equilibration cells allows for the field determination of the CH_4 production/oxidation rates with several benefits compared to standard methods.

   This hypothesis was accepted. A non-invasive portable method based on TDLAS to determine CH_4 production/oxidation rates was developed. This method allows the measurement of CH_4 concentration through the headspace of incubation cells in which methanogenic/methanotrophic activity tests are performed, allowing the faster determination of production/oxidation rates faster compared to traditional gas chromatograph (GC) methods, at a similar equipment cost.

2. Aerobic methanotrophy in northern lakes is highly variable and depends primarily on dissolved CH_4 concentration, which in turn is a function of the permafrost soil type in which these lakes are located.

   This hypothesis was accepted. A study of the spatial and temporal variation of methanotrophic activity in 30 Alaskan lakes showed that thermokarst (thaw) lakes formed in yedoma-type permafrost have significantly higher CH_4 oxidation rates compared to other thermokarst and non-thermokarst lakes formed in non-yedoma permafrost environments. This observation was explained by the fact that thermokarst lakes formed in yedoma-type permafrost receive large quantities of terrestrial organic carbon from permafrost thaw and

subsidence of the surrounding landscape into the lake, promoting the CH_4 formation and enhancing the whole CH_4 cycling.

3. AOM is a widespread process among northern lakes, independent of lake trophic state and water physicochemical regime.

   This hypothesis was accepted. A significant AOM potential activity was observed in the sediments of the 15 Alaskan lakes, distributed along a north-south transect, that were sampled, independently from the lake trophic state and the water column physicochemical regime.

4. The addition of SO_4^{-2} or NO_3^- stimulates AOM, suggesting they are effective electron acceptor in lakes.

   This hypothesis was rejected. AOM incubations with sediment from 15 Alaskan lakes amended with SO_4^{-2}, NO_3^- or left unamended, showed no significant difference in AOM rates. Thus, the role of SO_4^{-2} and NO_3^- as final electron acceptors for AOM remains uncertain.

5. A significant fraction of the CH_4 produced in the anaerobic sediments of lakes is anaerobically oxidized with no added electron acceptor.

   This hypothesis was accepted. AOM and methanogenic profiles in sediment cores of three Alaskan lakes demonstrated that anaerobic CH_4 oxidation represent about 38% of the total CH_4 produced by methanogens in superficial lake sediments.

6. The identification of microorganisms involved in AOM in lake sediments is possible by using stable isotopic probing (SIP) techniques.

   This hypothesis was accepted. By using DNA and PLFAs-SIP in lake sediments after AOM incubations, the possible participation of aerobic methanotrophs in AOM was evidenced. A better approach would involve the utilization of RNA-SIP to corroborate this result.

7.3 Future research

- It was suggested that aerobic methanotrophs in arctic lakes, could substantially mitigate the increase in CH_4 emission predicted by permafrost thawing (section 6.2). However, it is necessary to determine the extent to which aerobic methanotrophy will mitigate CH_4 emission under global warming scenarios that stimulate the permafrost thawing and CH_4 production.
- Our evidence confirms that AOM is a widespread process and a significant piece of the global biogeochemical carbon puzzle, oxidizing an important portion of CH_4 before reaching the water column (sections 6.3 and 6.4). Therefore, the AOM in lakes, needs to be taken into account in future estimations and models of the global CH_4 budget.
- Despite our findings, the mystery surrounding AOM is still unbroken and many enigmas remain. Among them, the possible electron acceptors for AOM in lake sediments, other than sulfates, nitrates, need to be tested (sections 6.3 and 6.4). Iron and Manganese are some other possible electron acceptors that have been recently taken into account for this purpose, however, humic substances, pyruvate and chlorite have still not been analyzed.
- The founding of the "strict" aerobic methanotrophs belonging to genera *Methylobacter* involved in AOM (section 6.5), strongly encourages the investigation of metabolic pathways of aerobic methanotrophs in anoxic conditions.
- In section 6.5, it was suggested that AOM is carried out by several microorganisms following different pathways, making the task of elucidating the AOM process much more complex. It was also proved that SIP techniques might be useful but not enough for that purpose (section 6.5). Therefore, a better strategy to determine the different pathways and microorganisms involved in AOM in lake sediments needs to be developed.

- The method presented in section 6.1, extended the range of studied lakes in a short period. Therefore, the development of more and better portable methods to determine CH_4 production/oxidation rates in the field, is required in order to increase the knowledge regarding the carbon cycling in freshwater ecosystems. This thesis comprised a broad study of the methane cycling in arctic, temperate and tropical lakes. In this chapter, general conclusions of the study and the need of future research on CH_4 cycling are presented.

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