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CARACTERIZACIÓN MOLECULAR DE LA COMUNIDAD METANOTRÓFICA EN SUELOS

T E S I S

Que presenta

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“La mayor necesidad del hombre es una ecología equilibrada”

Isaac Asimov

“La ciencia se compone de errores, que a su vez, son los pasos hacia la verdad”

Julio Verne

MÉXICO
GOBIERNO DE LA REPÚBLICA

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Abreviaturas

AMO	Amonio Monooxigenasa
AOB	Amonnia Oxidising Bacteria –Bacterias que Oxidan Amonio
MOB	Methane Oxidising Bacteria –Bacterias que Oxidan Metano
OTU	Operational Taxonomic Unit –Unidad Taxonómica Operacional
PCR	Polymerase Chain Reaction –Reacción en Cadena de la Polimerasa
pMMO	particulate Methane Monooxygenase –Metano Monooxigenasa unida a membrana
*pmo*A	Gen de la pMMO que codifica para un polipéptido de 27 kDa
ppmv	Partes por millón (por volumen)
RuMP	Ribulosa monofosfato
sMMO	Soluble Methane Monooxygenase –Metano Monooxigenasa Soluble
sp.	Especie
spp.	Especies (plural)
USC	Upland Soil Cluster –Grupo de secuencias *pmo*A de suelos de bosques
JR-2, JR-3	Jasper Ridge clusters –Grupo de secuencias *pmo*A de suelos de pastizal

Resumen

Se investigó la capacidad de oxidación de metano y la composición de las comunidades oxidantes de metano (MOB) en tres suelos del exlago de Texcoco con diferente nivel de salinidad (Tex-S1, Tex-S2 and Tex-S3) y en dos suelos agrícolas de Alcholoya y Chiapas.
Las MOB se identificaron con base en la detección y análisis comparativo de secuencias del gen pmoA el cual codifica para una subunidad de la pMMO, una enzima clave en el proceso de oxidación aerobia de metano. Las secuencias de los suelos arables indicaron que las MOB detectadas fueron metanótrofos de los tipo I (Gammaproteobacteria) y II (Alphaproteobacteria). Las secuencias del suelo de Alcholoya se relacionaron cercanamente con *Methylocaldum, Methylocapsa* y grupos con alta afinidad al metano, JR-2, JR-3, USC-γ and USC-α, qye gab sudi detectados previamente en suelos no inundados que oxidant bajas concentraciones de metano. En el suelo de Chiapas se encontraron MOB cercanamente relacionadas al grupo JR-2 y metanótrofos tipo I que no se afiliaron a grupos conocidos. En los suelos del exlago de Texcoco, sólo se detectaron Gammaproteobacterias; se encontró un grupo nuevo de secuencias relacionadas con AOB *Nitrosococcus*-like (>90% de las clonas secuenciadas). *Crenothrix polyspora*, *Methylocystis*, JR-2 y USC-γ se detectaron en el suelo no salino, alcalino del exlago de Texcoco (Tex-S1). JR-2 y *Methylomicrobium* se detectaron en los suelos extremos salino-alcalinos del exlago de Texcoco (Tex-S2 y Tex-S3 respectivamente). Esta información sugiere que en Tex-S1 la comunidad metanotrófica es más diversa que en Tex-S2 y Tex-S3, del exlago de Texcoco.

Abstract

The methane oxidation capacity and the composition of communities of aerobic methane-oxidizing bacteria (MOB) was studied in three soils from the former lake Texcoco-Mexico with different level of salinity (Tex-S1, Tex-S2 and Tex-S3) and also in two agricultural soils from the Alcholoya and Chiapas. MOB were identified on the basis of the detection and comparative sequence analysis of the *pmo*A gene, that encodes a subunit of the pMMO, a key enzyme in the aerobic methane oxidation process. The sequences from the arable soils showed commonly detected MOB, where methanotrophs from types I (Gammaproteobacteria) and II (Alphaproteobacteria) were found. The sequences in Alcholoya soil were closely related to *Methylocaldum, Methylocapsa* and methane high affinity clusters, JR-2, JR-3, USC-γ and USC-α, which have been previously detected in upland soils that oxidize low concentrations of methane. In the Chiapas soil, it was found MOB closely related to JR-2 cluster and type I methanotrophs unnafiliated to a known cluster. In the soils of the former lake Texcoco, just Gammaproteobacteria were detected; a novel group of sequences related to the *Nitrosococcus*-like clade were detected (>90% of sequenced clones). *Crenothrix polyspora*, *Methylocystis*, JR-2 and USC-γ sequences were detected in the unsaline, alkaline soil from the former lake Texcoco (Tex-S1). JR-2 and *Methylomicrobium* sequences were detected in the extreme saline-alkaline soils of the former lake Texcoco (Tex-S2 and Tex-S3 respectively). These data suggest that the methanotrophic community was more diverse in the Tex-S1 than in extreme soils of the former lake Texcoco.

Introducción

Definido por Alexander (1994) como el material exterior, poco compacto de la superficie terrestre, el suelo representa el centro de organización de los ecosistemas terrestres, así como la zona más importante en términos de calidad y cantidad del recurso alimentario humano.

Representa el hábitat más dinámico en la tierra y a pesar de su importancia central para la vida, el conocimiento en cuanto a su funcionamiento y el papel de su biodiversidad es incompleto (Hinsinger et al. 2009).

Entre las actividades biológicas que los suelos llevan a cabo se incluyen muchos de los procesos que gobiernan el ecosistema globalmente, especialmente los ciclos del carbono, nitrógeno y fósforo. Los microorganismos obtienen energía y nutrientes a partir de las sustancias orgánicas, reintegrando a su vez productos metabólicos que promueven la continuidad de los diferentes ciclos.

Las bacterias están entre los organismos más fascinantes del planeta, son los actores silenciosos que hacen posible la vida en la tierra. Con el surgimiento de la secuenciación, se han dado pasos agigantados en cuanto al conocimiento del potencial que tienen estos diminutos organismos: enzimas y rutas metabólicas.

El estudio de las bacterias inició con su cultivo en el laboratorio y estudiando sus propiedades bioquímicas, a fin de predecir qué procesos metabólicos pueden realizar. Actualmente con la ayuda de herramientas utilizadas en biología molecular, como la clonación y el análisis de metagenomas, se pueden estudiar o predecir estos procesos sin necesidad de cultivos, sobretodo teniendo en cuenta que sólo el 1% o menos de los microorganismos de suelo se puede cultivar (Hirsch et al. 2010).

Recientemente, se investigó la emisión de gases de efecto invernadero en dos suelos agrícolas procedentes de los Estados de Chiapas e Hidalgo (Ruíz-Valdiviezo et al. 2010; Serrano-Silva et al. 2011), encontrándose que éstos oxidan metano, uno en mayor proporción que otro. La tasa de oxidación de metano de suelos provenientes de Chiapas fue de 1.8 – 8.2 μg C kg^{-1} $día^{-1}$ (Ruíz-Valdiviezo et al. 2010) y la tasa de oxidación de metano de suelos provenientes de Hidalgo fue de 0.07 ng C kg^{-1} $día^{-1}$ (Serrano-Silva et al. 2011). Aunque esta diferencia pudo deberse a otros factores no determinados, por ejemplo el efecto de fertilizantes nitrogenados usados regularmente en estos suelos (orgánico, inorgánico, si es fuente de amonio o fuente de nitrato) o la aplicación de pesticidas químicos, una posible explicación de estos resultados es que las comunidades metanotróficas sean diferentes entre ellos.

Otros suelos de interés, son los del exlago de Texcoco. Su importancia se debe a que son casi únicos en el mundo por sus características fisicoquímicas (Dendooven et al. 2010); de modo que hay varias publicaciones enfocadas hacia el estudio de sus propiedades fisicoquímicas y la dinámica de nutrientes como C y N (Beltrán-Hernández et al. 1999; Luna-Guido et al. 2000; Luna-Guido et al. 2001; Ramírez-Fuentes et al. 2002; Vega-Jarquín et al. 2003) y más recientemente hacia las poblaciones de bacterias y arqueas (Alcántara-Hernández et al. 2009a; Alcántara-Hernández et al. 2009b; Valenzuela-Encinas et al. 2008; Valenzuela-Encinas et al. 2009). Con respecto a las comunidades de metanótrofos en suelos del exlago de Texcoco no hay reportes previos.

En este estudio se utilizaron técnicas independientes de cultivo (PCR, clonación y secuenciación) para abordar la pregunta de ¿qué metanótrofos están en suelos agrícolas y suelos del exlago de Texcoco?.

1. Marco conceptual

1.1 El suelo como ecosistema fundamental para la vida

El estudio de los ecosistemas es fundamental para el manejo de los recursos naturales ya que pone en evidencia las interacciones que vinculan a los sistemas bióticos (donde el hombre es parte integral), con los sistemas físicos. El suelo, región en la que se sustenta la vida vegetal y de la cual las plantas obtienen soporte mecánico y muchos de sus nutrientes (Alexander 1994), es el mejor ejemplo para evidenciar la fuerte interacción entre los microorganismos y los elementos edáficos: materia mineral (45%), agua (20 – 30%), aire (20 – 30%), materia orgánica (5%) y organismos vivos.

Actualmente, existe una creciente apreciación del papel de los organismos en el funcionamiento de los ecosistemas y en cómo esas funciones proveen servicios que son vitales para el bienestar humano. De modo general, el suelo influye y a su vez es influenciado por los componentes de otros ecosistemas, al servir como centro regulador dinámico, sustrato físico para el desarrollo de actividades antrópicas y soporte para el almacenamiento de una gran diversidad de especies, lo que hace de este ecosistema un punto de intersección fundamental para el mantenimiento de la vida (González-Sáyer 2012).

1.1.1 Suelos agrícolas

Los suelos agrícolas se definen como tierras utilizadas ampliamente para la producción de alimentos y fibra (Anderson et al. 1976). La agricultura desempeña un papel vital en la seguridad alimentaria, la reducción de la pobreza y el desarrollo sustentable. Sin embargo, diversas prácticas de manejo de suelos agrícolas contribuyen a las emisiones de gases de efecto invernadero. Por ejemplo, el uso no controlado de fertilizantes nitrogenados

aumentan las emisiones de óxido nitroso (N_2O).

Dependiendo de la humedad, aireación y disponibilidad de N entre otros, los suelos de agricultura pueden ser fuentes o sumideros de metano (CH_4). En suelos arables (aireados) la oxidación de CH_4 es típicamente mayor que la producción de este gas (metanogénesis) y su potencial como sumideros varía de 1 a 2 kg CH_4 ha^{-1} $año^{-1}$ (Jarecki et al. 2008).

1.1.2 Suelos salinos

Estos suelos son afectados por sales solubles en agua que determinan sus propiedades físicas, químicas y biológicas (García 2003); la acumulación de sales depende de la cantidad de arcillas, regímenes de precipitación y evaporación, lavado y drenaje de los suelos.

García (2003), clasifica los suelos salinos en:

- Suelos Salinos: las concentraciones de sales solubles –principalmente cloruros, sulfatos y nitratos de Na^+, Ca^{2+} y Mg^{2+}– son muy altas para el crecimiento y producción óptimos de cultivos. El nivel crítico para determinar si un suelo es salino, corresponde a una conductividad electrolítica (CE) en el extracto de saturación de 4 dS m^{-1} o mayor.

- Suelos Sódicos: predomina el ión Na^+ en el complejo de cambio y alto pH. Para que un suelo sea considerado sódico se ha establecido el límite del 15% de sodio intercambiable.

- Suelos Alcalinos: con elevados contenidos de cationes básicos (Na^+, Ca^{2+}, Mg^{2+} y K^+); el pH generalmente es > 7.0.

1.1.3. El exlago de Texcoco

Los suelos del exlago de Texcoco son salino-alcalinos. Se formaron por la deposición de cenizas volcánicas en el exlago; su pH es > 8.5 (9.8 – 10.4). El drenaje natural es pobre y el nivel freático de 0.5 a 1 m debajo de la superficie. La evaporación rápida y la precipitación de 705 mm anuales mantienen la salinidad del suelo (22 – 150 dS m^{-1}) (Valenzuela-Encinas 2009). En cortas distancias (< 10 m) hay grandes variaciones en las características del suelo (Dendooven et al. 2010).

Desde finales de 80s los suelos del exlago de Texcoco son drenados artificialmente de manera parcial para remover el exceso de sales e irrigados con efluentes residuales. Para controlar su erosión, desde comienzos de 70s, se han introducido las especies vegetales *Distichlis spicata* (un pasto halotolerante) y *Tamarix* spp. (Luna-Guido et al. 2000; Dendooven et al. 2010).

Por sus características, estos suelos son considerados únicos en el mundo (Valenzuela-Encinas 2009; Dendooven et al. 2010).

1.2 El metano y su papel como gas de efecto invernadero

El metano (CH_4) es el principal hidrocarburo presente en la atmósfera, con una concentración promedio de 1.7 ppmv. Después del vapor de agua y el dióxido de carbono (CO_2), el CH_4 es de los gases de efecto invernadero más importantes. A pesar de su corto tiempo de residencia (aproximadamente 10 años), el CH_4 tiene la capacidad de absorber radiación infrarroja 20 a 30 veces más eficientemente que el CO_2, lo que lo hace un potente gas de efecto invernadero. Además, es muy reactivo químicamente y está involucrado en los cambios en la composición química de la atmósfera (Le Mer & Roger 2001).

Lelieveld et al. (1993) predijeron que el incremento en concentración de CH_4 en la atmósfera disminuye la concentración de radicales hidroxilo (OH) y así aumenta el tiempo

de vida de CH_4 en la atmósfera. Sin embargo, al disminuir las emisiones de CH_4 habría una retroalimentación positiva sobre la disminución de CH_4 en la atmósfera como resultado de un incremento en las concentraciones de radicales OH.

1.2.1 El ciclo global del metano

El CH_4 es oxidado fotoquímicamente en la atmósfera o biológicamente en ecosistemas acuáticos y terrestres. Los océanos, pastizales y desiertos constituyen sumideros importantes de CH_4 mientras que los humedales, suelos agrícolas y de ganadería, y otras fuentes como rellenos sanitarios son las principales fuentes.

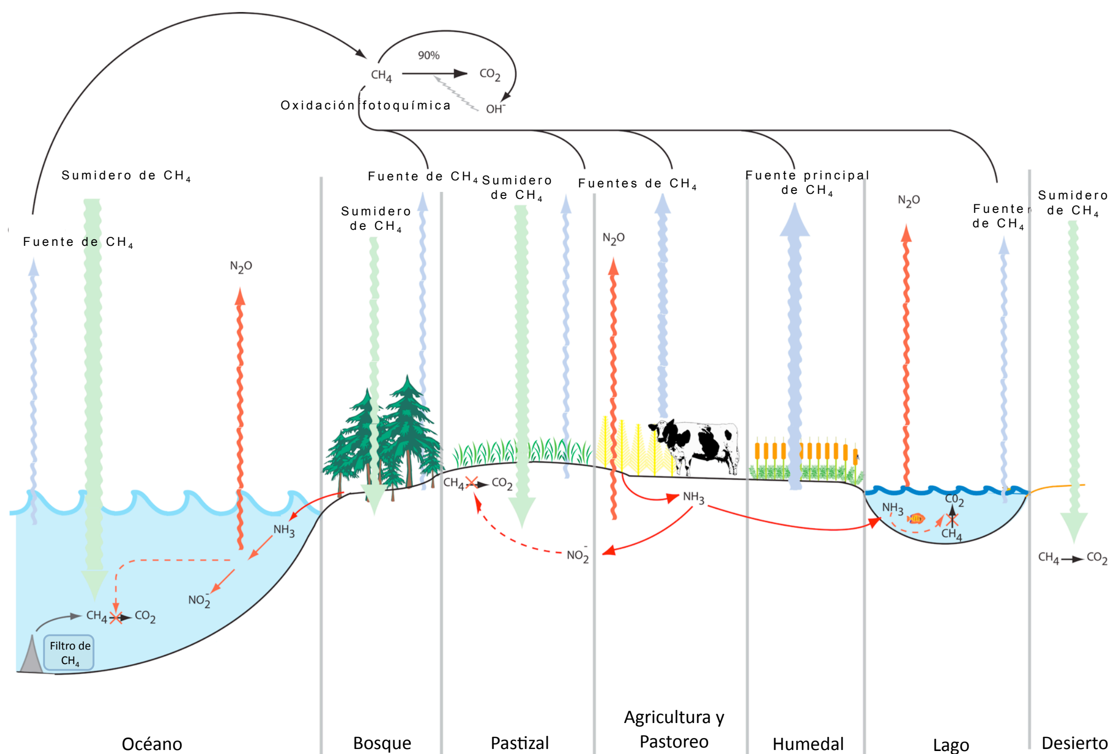

Figura 1. Ciclo global del metano (Tomada de Ward et al. 2004).

1.3 Suelos como sumidero de metano

Se estima que la concentración de CH_4 en la atmósfera se ha incrementado de 0.715 ppmv desde la era preindustrial, hasta 1.774 ppmv en 2005 (IPCC 2008). El único sumidero biológico para el CH_4 atmosférico es su oxidación por metanótrofos (bacterias metanotróficas u oxidantes de CH_4) y bacterias y arqueas anaerobias (Hanson & Hanson 1996) que contribuyen con la destrucción de más del 15% del CH_4 a nivel global (Singh 2011).

Los factores fisicoquímicos como temperatura, humedad y pH, junto con las prácticas antrópicas como la agricultura, afectan a las comunidades biológicas de los suelos y sus funciones (SBSTTA 2001). La estructura de las comunidades microbianas del suelo depende de las propiedades fisicoquímicas de éste, los exudados de raíz y las prácticas agrícolas (Yousuf et al. 2012).

Aunque son muchos los reportes que describen claramente las estructura de las comunidades microbianas del suelo, así como sus múltiples funciones allí (Nannipieri et al. 2003), éstas siguen siendo consideradas como “cajas negras”.

1.4 Bacterias oxidadoras de metano (MOB) –Metanótrofos

La oxidación biológica de CH_4 se realiza por microorganismos metanotróficos, ya sean bacterias aerobias, bacterias anaerobias como Candidatus ‘*Methylomirabilis oxyfera’* que oxida el CH_4 de forma acoplada a la reducción de NO_2^- (Ettwig et al. 2010), o arqueas anaerobias como Candidatus ‘*Methanoperedens nitroreducens’*, que oxida CH_4 y utiliza NO_3^- como aceptor final de electrones (Haroon et al. 2013). Incluso en ambientes altamente salinos (hasta 292 g L^{-1}) también se han reportado arqueas que oxidan CH_4 de forma acoplada a la reducción de SO_4^{2-} (Maignien et al. 2013).

Aunque la capacidad de oxidar CH_4 se ha detectado en dos especies de levaduras:

Rhodotorula glutinis, y *Sporobolomyces roseus*, y en el hongo *Penicillium jahtinellum* (Higgins et al. 1981), como se dijo, los metanótrofos son principalmente bacterias y arqueas. Casi todos los metanótrofos conocidos pueden usar no sólo CH_4, sino también metanol como fuente de carbono y energía, aunque no todas las bacterias que utilizan metanol pueden oxidar CH_4.

Las bacterias oxidadoras de CH_4 o metanotróficas aerobias, tienen un metabolismo altamente especializado; utilizan el CH_4 como única fuente de carbono y energía, por lo que juegan un papel importante en el ciclo global del CH_4. Se han encontrado de manera ubicua en muchos ambientes incluyendo suelos (arrozales, bosques de pradera y caducifolios), sedimentos de agua dulce, sedimentos marinos, pantanos, lodos activados, turberas, superficies de minas de carbón, aguas termales y ambientes fríos, tales como la Antártida y otros ambientes de extrema salinidad y pH (Murrell et al. 1998; Lin et al. 2004; Bowman 2006).

Dichas bacterias, tienen un sitema complejo de membranas intracitoplasmáticas (Figura 2) y constituyen un grupo diverso que hasta el momento, está representado por 21 géneros en los phyla Proteobacteria (clases alfa y gamma) y Verrucomicrobia (Hakemian & Rosenzweig 2007).

Actualmente, el número de géneros y especies metanotróficas conocidas está en crecimiento. La razón de esto, en parte, se debe a nuevos aislamientos de diversos ambientes y a nuevas técnicas utilizadas para su análisis. El reciente descubrimiento de diversos metanótrofos confirma que este grupo de bacterias es más diverso de lo que se pensaba. Dentro de los más recientes se han reportado bacterias filamentosas: *Crenothrix polyspora* y *Clonotrix fusca* (Conrad 2009), así como metanótrofos termoacidófilos dentro del phylum Verrucomicrobia (Dunfield et al. 2007).

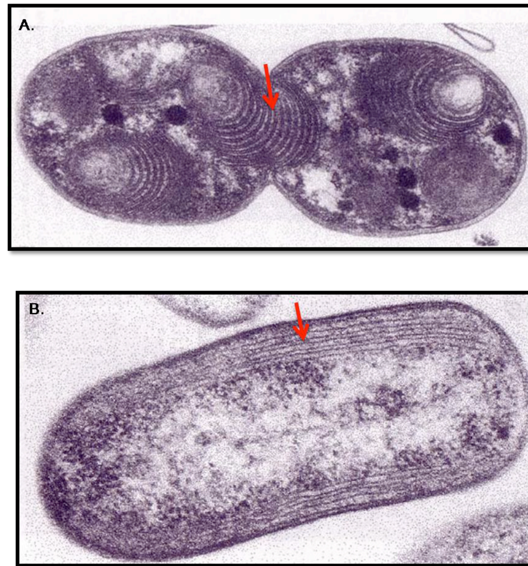

Figura 2. Microfotografías electrónicas de membranas intracitoplasmáticas (ICM intracytoplasmic membranes) organizadas como vesículas (A. Metanótrofos Tipo I) y en la periferia de la célula (B. Metanótrofos Tipo II). (Tomada de Green 1992).

1.4.1 Clasificación de las bacterias metanotróficas

De acuerdo con su morfología celular, ultraestructura, filogenia y ruta de asimilación de C, tradicionalmente los metanótrofos se dividen en dos grupos: Tipo I y Tipo II (Dumont & Murrell 2005a; Bowman 2006). Su clasificación taxonómica ha sido reportada por Nazaries et al. (2013) y se muestra en la Tabla 1.

Por otro lado, dentro de las bacterias que oxidan CH_4, también se encuentran las bacterias oxidadoras de amonio, que cooxidan CH_4 (Hanson & Hanson 1996) como *Nitrosococcus* y *Nitrospira* (Jiang & Bakken 1999).

Tabla 1. Taxonomía de bacterias metanotróficas (Nazaries et al. 2013).

Dominio: *Bacteria*/ Reino: *Eubacteria*/ Phylum: *Proteobacteria*				Phylum: *Verrucomicrobia*
Clase: *Gammaproteobacteria* Orden: *Methylococcales*			Clase: *Alphaproteobacteria* Orden: *Rhizobiales*	Clase: *Verrucomicrobiae* Orden: *Methylacidiphilales*
Familia: *Methylococcaceae*			Familia: *Methylocystaceae*	Familia: *Methylacidiphilaceae*
Methylobacter	***Methylomonas***	***Methylocaldum***	***Methylocystis***	***Methylacidiphilum***
Methylobacter bovis	*Methylomonas aurantiaca*	*Methylocaldum gracile*	*Methylocystis echinoides*	*Methylacidiphilum infernorum*
Methylobacter chroococcum	*Methylomonas fodinarum*	*Methylocaldum szegediense*	*Methylocystis heyeri*	*Methylacidiphilum fumarolicum*
Methylobacter luteus	*Methylomonas methanica*	*Methylocaldum tepidum*	*Methylocystis hirsute*	*Methylacidiphilum kamchatkensis*
Methylobacter marinus	*Methylomonas rubra*		*Methylocystis methanolicus*	
Methylobacter psychrophilus	*Methylomonas scandinavica*	***Methylococcus***	*Methylocystis minimus*	
Methylobacter tundripaludum	*Methylomonas paludis*	*Methylococcus capsulatus*	*Methylocystis parvus*	
Methylobacter vinelandii	*Methylomonas koyamae*	*Methylococcus thermophiles*	*Methylocystis pyriformis*	
Methylomicrobium			*Methylocystis rosea*	
Methylomicrobium agile	***Methylosarcina***	***Methylogaea***	*Methylocystis bryophila*	
Methylomicrobium album	*Methylosarcina fibrata*	*Methylogaea oryzae*	***Methylosinus***	
Methylomicrobium buryatense	*Methylosarcina lacus*		*Methylosinus sporium*	
Methylomicrobium pelagicum	*Methylosarcina quisquiliarum*		*Methylosinus trichosporium*	
Methylohalobius				
Methylohalobius crimeensis	***Methylosphaera***		**Familia: *Beijerinckiaceae***	
Methylosoma	*Methylosphaera hansonii*		***Methylocapsa***	
Methylosoma difficile			*Methylocapsa acidiphila*	
Methylothermus	***Methylovulum***		*Methylocapsa aurea*	
Methylothermus thermalis	*Methylovulum miyakonense*		***Methylocella***	
Methylothermus subterraneus	***Methylomarinum***		*Methylocella palustris*	
Clonothrix	*Methylomarinum vadi*		*Methylocella silvestris*	
Clonothrix fusca	***Crenothrix***		*Methylocella tundra*	
	Crenothrix polyspora		***Methyloferula***	
			Methyloferula stellata	

Código de colores de metanótrofos extremófilos/extremotolerantes: naranja = psicrófilos (crecen entre 5–10°C, pero no ≥ 20°C); púrpura = haloalcalófilos (crecen en 12% NaCl y a pH de 9–11); amarillo = halófilos (crecen en 15% NaCl); azul = termófilos (crecen a > 45°C); verde = acidófilos (crecen a pH de 3.8–5.5); gris: termoacidófilos (crecen a 60°C y a pH = 2). Adaptado de Trotsenko & Murrell (2008).

Basándose en su afinidad por el CH_4, las bacterias metanotróficas también se han clasificado en de alta y de baja afinidad (Bender & Conrad 1992). Los metanótrofos de baja afinidad se han descrito y estudiado ampliamente (Nazaries et al. 2013).

Los metanótrofos de alta afinidad pueden oxidar CH_4 a concentraciones atmosféricas (~1.7 ppmv). Sin embargo, no se han aislado hasta este momento y la información que se tiene de ellos está basada sólo en el uso de biomarcadores como *pmo*A (gen que codifica para un polipéptido de 27 kDa de la pMMO).

En suelos de bosques y pastizales se han encontrado varios grupos de secuencias *pmo*A, relacionadas filogenéticamente con proteobacterias α- y γ, que se presume están involucradas en la oxidación de metano a baja concentración, como USC-α, USC-γ (Knief et al. 2003), JR-1, JR-2 y JR-3 (Horz et al. 2005).

1.4.2 Filogenia de las bacterias metanotróficas

La filogenia molecular tiene un papel fundamental en la clasificación e identificación de microorganismos en función de sus relaciones evolutivas (Ludwig & Schleifer 1994). Los análisis de secuencias de los genes codificantes del RNA ribosomal se utilizan cotidianamente para la construcción de árboles filogenéticos de todo tipo de especies. Esto se debe en gran medida al hecho de que los rRNA son abundantes, distribuidos universalmente en las células (Ingraham et al. 1983), conservados y homólogos (Pace et al. 1986).

Otro enfoque para el estudio de las relaciones filogenéticas entre organismos metanotróficos es el uso del gen funcional *pmo*A (Figura 3), que codifica para la subunidad β de la hidroxilasa pMMO. La filogenia de *pmo*A es consistente con la del gen 16S rRNA (Murrell et al. 1998; Costello & Lidstrom 1999).

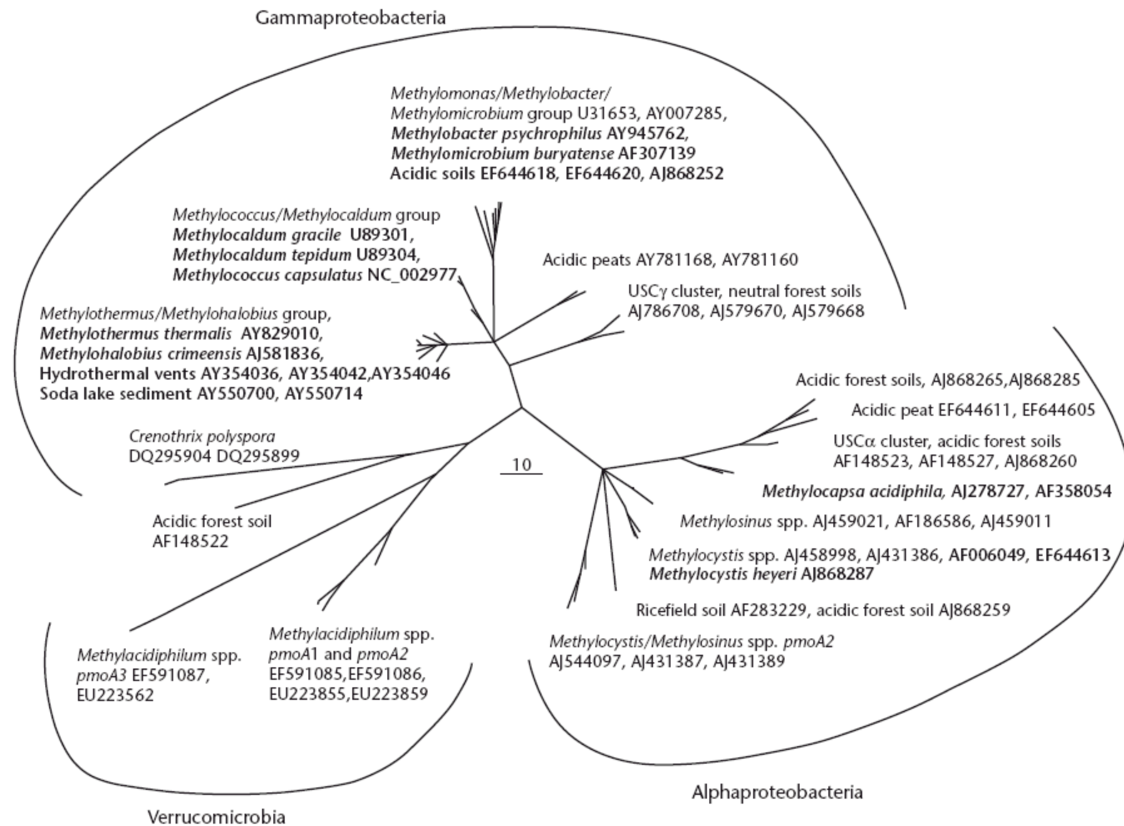

Figura 3. Árbol filogenético por máxima verosimilitud, de bacterias metanotróficas, basado en secuencias parciales del gen *pmo*A que codifica para una subunidad de MMO. Contiene secuencias de cultivos puros y obtenidas de distintos ambientes por técnicas independientes de cultivo. La barra de error representa 0.1 cambios por posición nucleotídica (Tomado de Dunfield 2009).

1.5 Oxidación aerobia de metano

Los metanótrofos pueden utilizar el CH_4, ya que tienen la enzima metano monooxigenasa (MMO) que oxida el metano a metanol. Después de la conversión de metano a metanol, este último es oxidado a formaldehído por una pirrolquinolina quinona (PQQ) dependiente de la metanol deshidrogenasa (MDH) (Hanson & Hanson 1996). Dependiendo del tipo de microorganismo, éstos asimilan el C al nivel de formaldehído, ya sea por la ruta de la ribulosa monofosfato (RuMP) o por la vía de la serina, o el formaldehído puede ser oxidado completamente a CO_2, con lo que se genera poder reductor para el metabolismo celular (Figura 4). De este modo, los metanótrofos oxidan el CH_4 a CO_2 o lo incorporan a su

biomasa.

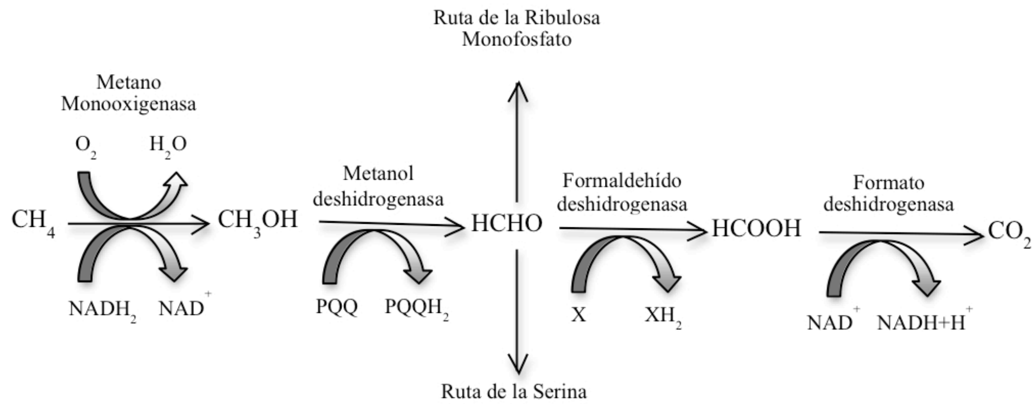

Figura 4. Oxidación aerobia de metano y rutas de asimilación de formaldehído en metanótrofos (Modificada de McDonald et al. 2005).

1.6 Metano monooxigenasa (MMO)

La MMO es la enzima clave de la oxidación de CH_4 en metanótrofos y puede estar libre en citoplasma (soluble – sMMO) o unida a membrana (particulada – pMMO) dependiendo de la especie de metanótrofo. La sMMO se encuentra sólo en algunos metanótrofos mientras que la pMMO se encuentra en todos los metanótrofos conocidos excepto en *Methylocella* spp. y *Methyloferula* spp. (Dumont & Murrell 2005b; Vorobev et al. 2011).

Las pMMO y sMMO parecen no estar relacionadas evolutivamente y no tienen similitudes ni en su estructura ni en su secuencia (Semrau et al. 1995). Además, tienen diferente especificidad por sustrato, sMMO tiene una baja especificidad y puede cooxidar una amplia gama de alcanos, alquenos y compuestos aromáticos (Sullivan et al. 1998), mientras que la pMMO tiene una especificidad más alta por sustratos y sólo puede cooxidar unos pocos alcanos de cadena corta y amonio (Murrell et al. 2000).

La pMMO comparte varias similitudes con la enzima amonio monooxigenasa (AMO) de bacterias oxidadoras de amonio, entre las que destacan: alto grado de identidad en las

secuencias de aminoácidos, sustratos muy similares (CH_4 y NH_3) y tener un papel crucial en el metabolismo celular. Los metanotrófos y las bacterias oxidadoras de amonio pueden oxidar tanto el CH_4 como el amonio, sin embargo, pueden obtener energía sólo a partir de la oxidación del metano y amonio respectivamente (Bourne et al. 2001).

1.6.1 Metano monooxigenasa unida a membrana (pMMO)

Aunque la pMMO es la forma predominante de MMO, no ha sido tan bien estudiada como la sMMO, debido a la dificultad de purificar la proteína en su forma activa. Sin embargo, se ha logrado establecer que el complejo pMMO consiste de dos componentes: la hidroxilasa (pMMOH) integrada por tres subunidades (α, β y γ, de aproximadamente 47, 24 y 22 kDa respectivamente) y una reductasa putativa (pMMOR) constituida por dos subunidades de 63 y 8 kDa (Basu et al. 2003).

Los genes que codifican para la pMMO de varios metanótrofos se han clonado y secuenciado. Su organización en el genoma y sintenia se muestran en la Figura 5; la transcripción del operón es dependiente de un promotor σ^{70} antes de pmoC (Gilbert et al. 2000).

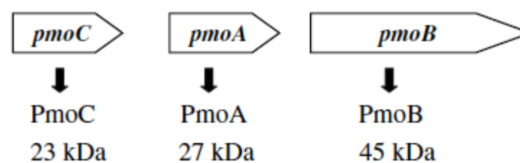

Figura 5. Clúster de genes de pMMO de metanótrofos (Tomada de McDonald et al. 2005).

1.6.2 Metano monooxigenasa soluble (sMMO)

La sMMO es más estable que la pMMO y se ha purificado de varios metanótrofos, por lo que ha sido bien estudiada. Se conforma por tres componentes principales, una hidroxilasa ($\alpha_2\beta_2\gamma_2$) que contiene el sitio activo, una reductasa dependiente de NADH (MmoC) que

transporta electrones a la hidroxilasa y una proteína de acople (proteína B, MmoB).

El clúster de genes que codifican las sMMO está organizado como mmoXYBZDC (Figura 6). El grupo mmoX, Y y Z codifica las subunidades α, β y γ de la hidroxilasa, mientras que *mmo*B codifica la proteína B, *mmo*C codifica la reductasa y *mmo*D codifica un péptido de función desconocida.

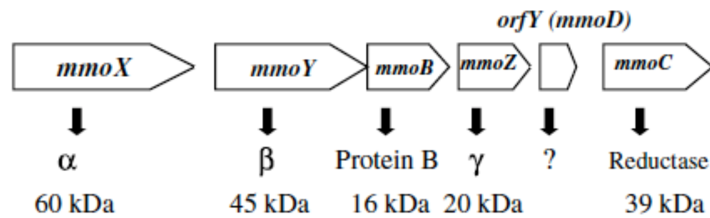

Figura 6. Clúster de genes para sMMO de metanótrofos (Tomada de McDonald et al. 2005).

1.7 Significancia ecológica de los metanótrofos

Como se mencionó anteriormente, el CH_4 contribuye al calentamiento global debido a su capacidad eficiente como gas de efecto invernadero -que atrapa el calor- en comparación con el CO_2. Los metanótrofos, a través de la actividad eficiente de metano monooxigenasas, son los principales actores en la remoción del CH_4 de la atmósfera y por lo tanto, en la mitigación del calentamiento global. Además, la distribución cosmopolita de metanótrofos, es una indicación de su papel significativo en la oxidación de CH_4 y el ciclo global del carbono.

Se han propuesto varios enfoques para aumentar la actividad de los metanótrofos con el fin de disminuir la cantidad de CH_4 emitido a la atmósfera (p. e., cubiertas y filtros biológicos) (Scheutz et al. 2009; Jiang et al. 2010; Chiemchaisri et al. 2012).

1.8 Estudio de metanótrofos en suelos

1.8.1 Estrategias basadas en cultivo

Los metanótrofos son microorganismos de crecimiento muy lento, lo que dificulta su cultivo en medios convencionales, aunado a que los microorganismos asociados pueden agotar los nutrientes en placas de agar. Además, los metanótrofos aislados de muestras ambientales, pueden no corresponder a organismos dominantes en la población original, sino sólo ser reflejo de las condiciones usadas para el enriquecimiento y el aislamiento.

Esta dificultad para cultivar y aislar metanótrofos ha llevado a usar métodos independientes de cultivo para su estudio en el ambiente.

1.8.2 Técnicas independientes de cultivo

La aplicación de técnicas basadas en el análisis de DNA ha incrementado el conocimiento que se tiene sobre la diversidad microbiana en ambientes naturales y está generando el desarrollo de nuevas herramientas diagnósticas.

El desarrollo de las técnicas conocidas como independientes de cultivo incluyen, desde el monitoreo de microorganismos mediante el uso de tinciones especializadas y técnicas como bioluminiscencia y análisis de ácidos grasos (PLFA), hasta la hibridación con sondas especificas *in situ* (FISH) (Hirsch et al. 2010) y la amplificación de genes marcadores mediante la técnica de reacción en cadena de la polimerasa (PCR, Polymerase Chain Reaction).

La técnica de reacción en cadena de la polimerasa (PCR) ha permitido el estudio de la ecología y diversidad de metanótrofos, para lo cual, se utilizan iniciadores que permiten la amplificación del gen 16S rDNA (la región del DNA que codifica para la subunidad

ribosómica 16S) y genes funcionales que codifican enzimas únicas en su metabolismo (Murrell et al. 1998).

Debido a la extensa base de datos de secuencias del 16S rDNA y a la naturaleza conservada de estas secuencias, se han diseñado sondas e iniciadores de PCR para detectar el 16S rDNA de diferentes géneros o especies de metanótrofos en muestras ambientales. Sin embargo, debido a que la filogenia de estos micoorganismos es muy diversa, al estar ubicados dentro de las α- y γ-proteobacterias, así como en el phylum Verrucomicrobia, es difícil diseñar iniciadores o sondas que detecten todos los metanótrofos.

Para el estudio de metanótrofos, los genes funcionales incluyen *pmo*A, *mmo*X y *mxa*F, que codifican subunidades de la metano monooxigenasa particulada (pMMO), metano monooxigenasa soluble (sMMO) y metanol deshidrogenasa (MDH) respectivamente (Dumont & Murrell 2005a).

El gen *pmo*A está presente en todos los metanótrofos conocidos, excepto en *Methylocella* spp. y en *Methyloferula* sp., por lo que es el blanco más frecuente en estudios de ecología molecular de metanótrofos. Existe una base de datos grande de secuencias *pmo*A que permite identificar un metanótrofo en función de la secuencia de sus genes, y también, se han reportado varios juegos de iniciadores para amplificar este gen (Dumont & Murrell 2005a).

Por ello en el presente estudio se utiliza como biomarcador el gen funcional *pmo*A para analizar la diversidad de las comunidades metanotróficas en dos suelos agrícolas y en tres con distinto nivel de salinidad.

2. Justificación, Objetivos e Hipótesis

2.1 Justificación

Los suelos agrícolas no inundados (arables) constituyen sumideros importantes de metano, pues son responsables de la oxidación de entre 1 y 2 kg CH_4 atmosférico ha^{-1} $año^{-1}$ (Jarecki et al. 2008). En México, más del 11% de la superficie territorial se usa para agricultura (SAGARPA 2008). Teniendo en cuenta que la diversidad de metanótrofos no se conoce completamente, sobre todo aquellos que oxidan metano a bajas concentraciones (i.e., a concentración ambiental ~1.7 ppm), es importante caracterizar las comunidades metanotróficas en suelos agrícolas. Por otro lado, considerando la amplia distribución de metanótrofos en distintos ambientes y que el suelo del exlago de Texcoco, un ambiente único, ya que su pH y su salinidad son muy altos (Dendooven et al. 2010), está cambiando, debido que se ha construido un sistema de drenaje para eliminar la salinidad y bajar el pH (Valenzuela-Encinas 2009), es indispensable caracterizar la comunidad oxidante de metano en estos suelos, ya que se desconoce. Además de ser tan importantes en el ciclo global del metano y por tanto, en el control de la temperatura del planeta, el estudio de bacterias metanotróficas se ha enfocado en su capacidad de cooxidar una gama de compuestos alifáticos, heterocíclicos y aromáticos (p. e. tricloroetileno, cloformo y tetracloroetileno) teniendo aplicación en el campo de la biorremediación (Bowman 2006).

2.2 Objetivos

2.2.1 General

Analizar la diversidad de las comunidades metanotróficas, en dos suelos agrícolas y en tres con distinto nivel de salinidad, a partir del análisis de las secuencias de librerías del gen funcional *pmo*A de cada suelo.

2.2.2 Específicos

1. Obtener y caracterizar fisicoquímicamente muestras de suelos agrícolas y suelos del exlago de Texcoco con distinto nivel de salinidad.

2. Conocer el potencial de oxidación de metano en suelos agrícolas y suelos del exlago de Texcoco con distinto nivel de salinidad.

3. Amplificar, construir librerías y analizar las secuencias del gen funcional *pmo*A de bacterias metanotróficas, usando como molde el DNA metagenómico de los suelos.

4. Analizar la composición de la comunidad oxidante de metano de los suelos en estudio.

2.3 Hipótesis

Si la actividad oxidadora de metano en el suelo está en función de la diversidad de microorganimos metanotróficos presentes en él, entonces se esperaría que los suelos con mayor capacidad oxidadora tuvieran una mayor riqueza y abundancia de estas bacterias que suelos con menor actividad.

3. Materiales y métodos

3.1 Sitios de muestreo, recolección de muestras y análisis de suelos

Se tomaron muestras de suelo de tres sitios ubicados en el exlago de Texcoco (Estado de México, México). En cada uno de ellos se determinó la conductividad electrolítica (CE), buscando que ésta fuera diferente en cada sitio. El primer suelo, Tex-S1, (19° 28.64 N, 98° 58.21 O) tuvo una baja CE < 1 dS m^{-1}, el segundo, Tex-S2, (19°30.22 N, 98°59.42 O) una mediana CE < 20 dS m^{-1} y el tercero, Tex-S3, (19°30.77 N, 98°59.42 O) una alta CE < 100 dS m^{-1} (Tabla 4).

Las principales sales en estos suelos son NaCl (0.1 – 12 % p/v) y Na_2CO_3 (0.25 – 10.5 % p/v), aunque $Ca+^2$ y $Mg+^2$ también son cationes importantes (Gutiérrez-Castorena et al. 2005; Fernández-Buces et al. 2006).

Las muestras de suelo del exlago de Texcoco se obtuvieron en temporada de lluvias. Se delimitaron tres áreas diferentes de ca. 400 m^2 en cada sitio, se descartaron los dos primeros cm de suelo y se hizo un muestreo compuesto de 20 submuestras de la capa de 2 – 15 cm del suelo de cada área. Al final, se obtuvieron tres muestras de cada uno de los suelos Tex-S1, Tex-S2 y Tex-S3.

Los suelos agrícolas se obtuvieron del municipio de Suchiapa, perteneciente a la Depresión Central de Chiapas (16º 37' N, 93º 05' O) y de la localidad de Alcholoya en el municipio de Acatlán, estado Hidalgo (20º 09' N, 98º 26' O). El primero se encuentra a 491 msnm, en un clima cálido sub-húmedo (ACw) con lluvias en verano, temperatura media de 26 ºC y precipitación anual media de 990 mm. Los suelos son cambisoles y tradicionalmente se han cultivado maíz y frijol (Ruíz-Valdiviezo et al. 2010); sin embargo, desde 2008 se empezó a cultivar el Piñón Mexicano (*Jatropha curcas* L.) con el propósito de utilizar su aceite como

sustituto de combustible diesel (Herrera et al. 2010).

Alcholoya se encuentra a 2120 msnm, presenta un clima templado con inviernos secos (Cwb), temperatura media de 14ºC y precipitación anual media de 600 mm; se cultiva principalmente maíz, frijol, cebada y maguey (López-Valdez et al. 2011).

El muestreo de los suelos agrícolas se hizo de la misma manera que los suelos del exlago de Texcoco y se mantuvieron las tres réplicas para cada sitio. Por consiguiente, se manejaron y analizaron 15 muestras de suelo en total para este estudio.

Para determinar las características fisicoquímicas, los suelos fueron secados a la sombra de 1 a 3 días y tamizados en una malla de 5 mm. La caracterización de los suelos se hizo de acuerdo a los métodos previamente descritos (Tabla 2). Se tomaron submuestras de 20 g de suelo fresco de cada sitio y se guardaron a -80ºC para la extracción de DNA.

Tabla 2. Métodos de caracterización fisicoquímica de suelos.

Característica	***Método***
pH	Potenciómetro (Thomas, 1996)
Humedad	Método Gravimétrico
Textura	Densímetro de Bouyoucos. (Gee & Bauder, 1986)
Conductividad Electrolítica (CE)	Conductímetro de cuatro anillos – Conductronic
Capacidad de Retención de Agua (CRA)	Método Gravimétrico
N total	Método Kjeldahl. (Bremner, 1996)
C orgánico - C inorgánico	Equipo analizador de carbono orgánico total (TOC) Shimatzu

3.2 Dinámicas de oxidación de metano en suelos

Se ajustó el contenido de humedad al 50% de la CRA en cada suelo muestreado (n = 5) con

120 ml. Las botellas se taparon con septos de caucho y se sellaron con casquillos de aluminio.

El potencial de oxidación de CH_4 en los suelos se determinó con varios ensayos variando la concentración de CH_4 en el espacio de cabeza, desde ~2 ppm hasta ~80,000 ppm. Se utilizó un estándar de CH_4 con un 99% de pureza que se diluyó hasta la concentración deseada. El CH_4 se agregó después de remover un volumen igual de aire en cada unidad experimental.

Se prepararon muestras de suelo estéril que se incubaron de la misma manera que los tratamientos. Se incubaron triplicados de cada sitio (n = 9) a temperatura ambiente (~25ºC) y en oscuridad. La concentración de CH_4 se analizó inyectando 1 ml del espacio de cabeza en un cromatógrafo de gases Agilent Technology 4890D adaptado con un detector de ionización de flama (FID –flame ionization detector), como lo describe Serrano-Silva et al. (2011). Se tomaron muestras del espacio de cabeza los días 0, 1, 3, 7, 14 y 21 de la incubación.

Para el análisis estadístico de los datos se hizo una regresión lineal y se obtuvieron las pendientes con el paquete estadístico SAS (SAS Institute 1989) con un nivel de significancia del 5%.

3.3 Extracción y evaluación de la calidad del DNA

El DNA total se extrajo de 0.5 g de suelo por triplicado (1.5 g). La extracción se hizo utilizando una técnica modificada de Guo et al. (1997) (Valenzuela-Encinas et al. 2008), que consiste en una lisis directa de células con agitación mecánica, choque térmico y detergentes. Los componentes orgánicos del suelo se removieron con pirofosfato de sodio 0.15 M y los residuos de sales con un buffer de fosfatos pH 8 (Ceja-Navarro et al. 2010).

La calidad y el rendimiento del DNA se verificó por electroforesis en geles de agarosa (0.8%) y se documentó en un transiluminador de luz UV (Gel Doc 2000, Bio-Rad

La calidad y el rendimiento del DNA se verificó por electroforesis en geles de agarosa (0.8%) y se documentó en un transiluminador de luz UV (Gel Doc 2000, Bio-Rad Laboratories Inc., Carlsbad, CA, USA) después de teñirse con bromuro de etidio (0.5 μg ml^{-1}). El DNA se almacenó a -20ºC hasta usarse para las PCRs –polymerase chain reactions.

3.4 Amplificación por PCR de genes funcionales de metanótrofos

Para la amplificación del gen funcional *pmo*A de la enzima metano monooxigenasa unida a membrana (pMMO), se utilizó el protocolo de PCR anidada propuesto por Horz et al. (2005), con los iniciadores A189f en combinación con A682r (Holmes et al. 1995), mb661r (Costello & Lidstrom 1999) o A650r (Bourne et al. 2001) (Tabla 3). Se hizo una primera PCR de 20 ciclos con los iniciadores A189f-A682r utilizando un protocolo de "towch-down" de 62 ºC-52 ºC disminuyendo 0.5 ºC por ciclo. De esta primera PCR se tomó una alícuota de 0.25 µl para la segunda PCR (múltiple) de 25 ciclos, donde se usaron los iniciadores A189f-mb661r/A650r.

Para la detección de metanótrofos en los que no se ha detectado pMMO, i.e., *Methylocella* spp. y *Methyloferula* sp., se utilizaron iniciadores para el gen *mmo*X de la enzima metano monooxigenasa soluble (sMMO): mmoX206f y mmoX886r (Hutchens et al. 2004) (Tabla 3).

Se prepararon reacciones de PCR en 25 µl con 100 ng de DNA, los iniciadores adecuados 0.5 µM, buffer de PCR 1× (Invitrogen Life Technologies, Sao Paulo, Brazil); $MgCl_2$ 2.5 mM; dATP, dCTP, dGTP y dTTP 200 µM cada uno; 0.5 U de Taq DNA polimerasa (Invitrogen Life Technologies) y 20 µg de BSA (bovine serum albumin). Se prepararon tres reacciones por cada muestra de DNA y se corrieron en un termociclador FTGRAD2D (TECHNE DUXFORT, Cambridge, UK) con los protocolos de la Tabla 3. Los productos de PCR se visualizaron en geles de agarosa (1.5%), se mezclaron y se purificaron para la clonación.

3.5 Clonación y secuenciación

Los productos de PCR de tamaño esperado se insertaron en el vector de clonación pGEM con el kit pGEM T-Easy Vector Cloning System I (Promega, Madison, WI, USA). Se transformaron células de *Escherichia coli* TOP10 químicamente competentes, preparadas a pequeña escala (Sambrook & Russell 2001).

Las clonas positivas (color blanco) se seleccionaron por α-complementación del gen de la β-galactosidasa en placas de agar Luria Broth (LB) con X-Gal 2 $\mu g\ ml^{-1}$ (Invitrogen Life Technologies, Carlsbad, CA, USA) y ampicilina 50 $\mu g\ ml^{-1}$. El DNA clonado se amplificó por PCR de colonia, con los iniciadores M13f y M13r (Tabla 3). Las secuencias de nucleótidos se determinaron con un secuenciador de DNA 3730X (Applied Biosystems, Foster City, CA, USA) en Macrogen (Macrogen Inc., Seoul, Korea) usando el iniciador M13f.

3.6 Análisis de secuencias parciales del gen *pmo*A

Las secuencias de nucleótidos se alinearon con otras en bases de datos públicas (http://blast.ncbi.nlm.nih.gov/Blast.cgi) usando BLASTX (Altschul et al. 1997). Se editaron manualmente cuando fue necesario, con el programa SEAVIEW (Galtier et al. 1996). Se verificó si habían secuencias quimeras dividiéndolas en dos fragmentos iguales y haciendo un BLAST de cada fragmento; si los porcentajes de identidad cuando se comparan con cepas de referencia son parecidos, se descartan como quimeras (Pester et al. 2004).

Se verificó el marco de lectura abierto u ORF (Open Reading Frame) trasladando las secuencias de nucleótidos a aminoácidos con la herramienta en línea BCM Search Launcher tool (http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.htm), y se alinearon con CLUSTALW (Chenna et al. 2003).

Se obtuvo una matriz de distancia en el programa DNADIST del paquete PHYLIP versión 3.67 (Felsestein 1993) y se utilizó Mothur (v.1.25.1) (Schloss et al. 2009) para generar las OTUs (Operational Taxonomic Units) con 94 % de similitud entre secuencias (Antony et al. 2010). La reconstrucción filogenética se realizó por el método de máxima verosimilitud en PhyML (Guindon & Gascuel 2003). El modelo de substitución nucleotídica que mejor se ajusta a los datos determinó en jModelTest (Posada 2008), empleando el criterio de información de Akaike (AIC). La robustez de las agrupaciones en el árbol, se evaluó con la prueba de bootstrap después de 1000 réplicas. El árbol filogenético se visualizó y editó en FIGTREE (v.1.3.1) (Rambaut 2009). Para la asignación taxonómica se generaron matrices de similitud e identidad utilizando el programa MatGAT (v.2.02) (Campanella et al. 2003).

3.7 Riqueza de especies y diversidad de las comunidades metanotróficas

El análisis de diversidad de las comunidades metanotróficas en los suelos de este estudio, se realizó con el software Mothur (v.1.25.1) (Schloss et al. 2009). La cobertura, probabilidad de que cualquier clona analizada sea diferente de otra analizada previamente, se calculó con el método de Good (Good 1953). El índice de Shannon (Magurran 1988) se usó para medir la diversidad de la comunidad mientras que el estimador Chao1 (Chao 1984) para determinar la riqueza de la comunidad.

Para comparar las librerías del gen funcional *pmo*A en cada suelo, se hizo una prueba ponderada (weighted) en el servidor de UniFrac (http://bmf.colorado.edu/unifrac) (Lozupone et al. 2006). UniFrac es una herramienta filogenética que mide la distancia entre comunidades basado en los diferentes filotipos presentes en una topología.

En la Figura 7 se muestra un esquema global por etapas, de la estrategia experimental que se planteó para lograr los objetivos de este estudio.

Tabla 3. Lista de iniciadores usados en este estudio.

Iniciador	Secuencia (5´→3´)	Producto (pb)	Protocolo de amplificación	Referencia
A189f [a] A682r	GGNGACTGGGACTTCTGG GAASGCNGAGAAGAASGC	 531	94ºC/420s; 94°C/45s; 62–52°C/60s; 72°C/60s (20 ciclos); 72ºC/420s; 4ºC/∞	Holmes et al. (1995)
A189f [b] mb661r/ A650r	GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC ACGTCCTTACCGAAGGT	 508 501	94ºC/420s; 94°C/45s; 56°C/60s; 72°C/60s (25 ciclos); 72ºC/420s; 4ºC/∞	Costello & Lidstrom (1999) Bourne et al. (2001)
206f [c] 886r	ATCGCBAARGAATAYGCSCG ACCCANGGCTCGACYTTGAA	 700	94ºC/420s; 94°C/45s; 60°C/60s; 72°C/60s (35 ciclos); 72ºC/420s; 4ºC/∞	Hutchens et al. (2004)
M13f [d] M13r	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	 750	94ºC/600s; 94°C/45s; 55°C/90s; 72°C/60s (25 ciclos); 72ºC/420s; 4ºC/∞	Horz et al. (2005)

N(nucleótido); B(T, C o G); M(A o C); R(A o G); S(C o G); W(A o T); Y(C o T)

[a] Iniciadores usados en la primera vuelta de PCR del gen *pmo*A; después de cada ciclo la temperatura de alineamiento se disminuyó en 0.5ºC hasta alcanzar 52ºC

[b] Iniciadores usados en la segunda vuelta de PCR del gen *pmo*A

[c] Iniciadores para el gen *mmo*X

[d] Iniciadores usados para PCR de colonias con inserto, y posteriormente para la secuenciación de fragmentos de DNA

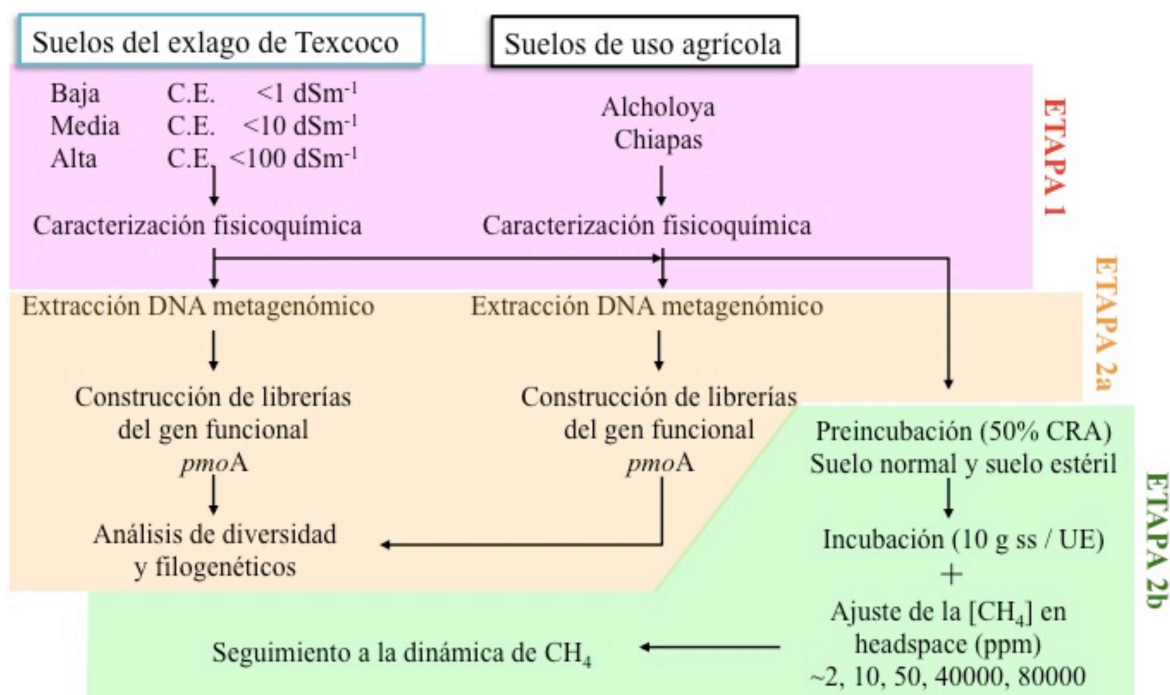

Figura 7. Estrategia experimental.

3.8 Análisis de secuencias *pmo*A-like

Las secuencias nucleotídicas similares a *pmo*A (*pmo*A-like) relacionadas cercanamente con el clado parecido a *Nitrosococcus* (*Nitrosococcus*-like), recuperadas de los suelos del exlago de Texcoco se tradujeron a aminoácidos (PmoA) y se alinearon con el programa MUSCLE (Edgar 2004). Se seleccionaron secuencias al azar y se alinearon con otras de la base de datos, de los genes *pmo*A (de la enzima metano monooxigenasa unida a membrana –pMMO) y *amo*A (de la enzima Amonio Monooxigenasa –AMO) para identificar motifs (regiones conservadas de aminoácidos) propios de pMMO y AMO.

3.9 Números de acceso de las secuencias nucleotídicas

Las secuencias *pmo*A y *pmo*A-like obtenidas se depositaron en la base de datos del GeneBank con los números de acceso KC122266–KC122354, KF995731–KF995732 y KJ026961–KJ026966.

4. Resultados y discusión

4.1 Caracterización fisicoquímica de los suelos

En la Tabla 4 se muestran los resultados de la caracterización físicoquímica de los suelos del exlago de Texcoco de baja (Tex-S1), mediana (Tex-S2) y alta (Tex-S3) conductividad electrolítica (CE), así como de los suelos agrícolas tomados para la obtención de librerías del gen funcional *pmo*A por clonación.

La baja conductividad electrolítica (CE) del suelo Tex-S1 (0.7 dS m^{-1}) lo hace no salino, alcalino (pH: 8.5); los suelos Tex-S2 y Tex-S3 de mediana y alta CE son salino-alcalinos. Los suelos agrícolas son no salinos, uno de pH neutro, Alcholoya (pH 6.7) y el otro ácido, Chiapas (pH 5.6).

Los suelos agrícolas son de textura franca, Tex-S1 franco-arenoso, mientras que Tex-S2 y Tex-S3 de textura arcillosa. Esta propiedad física de los suelos puede favorecer la oxidación de CH_4 en los primeros, ya que el gas puede difundirse más fácilmente en ellos.

Los suelos del exlago de Texcoco tienen altos contenidos de carbono orgánico, comparados con los suelos agrícolas. El C puede provenir de la irrigación con efluentes residuales; esta condición puede favorecer a las arqueas metanogénicas (que producen CH_4) y posteriormente a los metanótrofos (que consumen CH_4).

Tabla 4. Características de los suelos del exlago de Texcoco y suelos agrícolas de Alcholoya y Chiapas.

Suelo	CE [a] (dS m^{-1})	pH	C orgánico (g kg^{-1} suelo)	C Inorgánico	N Total	CRA [b]	Arcillas	Limos	Arenas	Textura
Tex-S1[c]	0.7 (0.1) [d]	8.5 (0.4)	32.8 (5.3)	7.3 (0.2)	1.9 (0.3)	855 (20)	130 (12)	250 (23)	620 (31)	Franco-arenoso
Tex-S2 [e]	9.0 (2.7)	10.3 (0.1)	22.2 (1.4)	3.7 (0.8)	1.3 (0.1)	1046 (37)	480 (74)	340 (44)	180 (50)	Arcilloso
Tex-S3 [f]	84.8 (5.3)	10.3 (0.2)	16.7 (1.0)	6.5 (0.6)	0.9 (0.2)	1120 (71)	650 (55)	270 (26)	80 (76)	Arcilloso
Alcholoya	0.7 (0.3)	6.7 (0.2)	11.1 (1.1)	ND [h]	1.0 (0.2)	846 (47)	160 (25)	350 (44)	490 (31)	Franco
Chiapas [g]	0.8 (0.2)	5.6 (0.2)	20.7 (5.3)	ND [h]	1.9 (0.1)	895 (4)	230 (25)	330 (23)	440 (32)	Franco

[a] Conductividad Electrolítica, [b] Capacidad de retención de agua, [c] Suelo de Texcoco con baja CE, [d] Valores entre paréntesis: desviación estándar (*n*=3), [e] Suelo de Texcoco con mediana CE, [f] Suelo de Texcoco con alta CE, [g] Datos tomados de Ruiz-Valdiviezo et al. (2010), [h] ND: no detectado.

4.2 Potencial de oxidación de metano en los suelos

La presencia de metanótrofos en ambientes hipersalinos era dudosa debido a las dificultades en el aislamiento y en ensayos de oxidación de CH_4 en estos ambientes (Conrad 1996). Sin embargo, se ha demostrado el consumo de CH_4 en lagos salados, lagos carbonatados y lagos extremamente salino alcalinos (Khmelenina et al. 2000, Lin et al. 2004, 2005). De igual manera, también se ha demostrado la presencia de metanótrofos en ambientes alcalinos e hipersalinos por Lin et al. (2005), Sorokin & Kuenen (2005) y Eshinimaev et al. (2008).

El contenido de humedad en los suelos de este estudio se ajustó al 50% de la capacidad de retención de agua (CRA). En las Figuras 8 a 12 se muestran las dinámicas de oxidación de CH_4 en suelos incubados con diferentes concentraciones de CH_4 en el espacio de cabeza: CH_4 atmosférico (2.0 - 2.5 ppm), 10 - 12 ppm, 50 - 65 ppm, 35000 - 45000 ppm y 75000 - 85000 ppm. Se calcularon las tasas de oxidación de CH_4 en cada condición (Tabla 5).

Se determinó que la oxidación de CH_4 fue biológica y que los factores abióticos no afectaron la concentración del CH_4, ya que en los tratamientos estériles no hubo disminución significativa del gas.

Los suelos agrícolas oxidaron CH_4 a concentración atmosférica (< 2.5 ppmv), no así los suelos del exlago de Texcoco (Tabla 5). Resultados similares en los suelos agrícolas se reportaron previamente por Ruíz-Valdiviezo et al. (2010) en suelos de Chiapas, y por Serrano-Silva et al. (2011) en suelos de la localidad de Alcholoya.

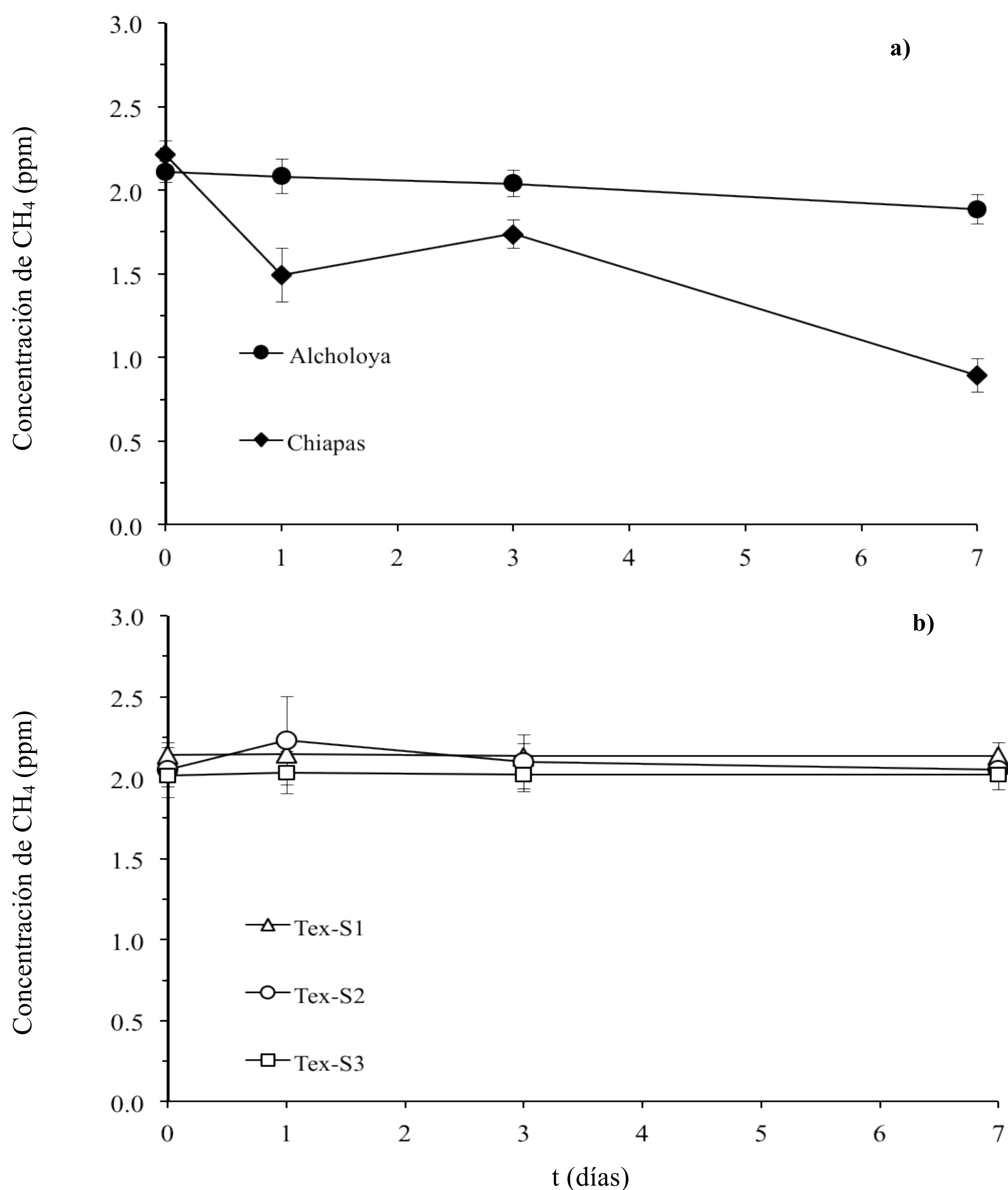

Figura 8. Oxidación de CH_4 en (a) suelos agrícolas Alcholoya y Chiapas, y (b) suelos del exlago de Texcoco, Tex-S1 (baja CE), Tex-S2 (mediana CE) y Tex-S3 (alta CE), incubados con 2.0 - 2.5 ppmv de CH_4 en el espacio de cabeza.

El suelo agrícola de Chiapas oxidó el CH_4 cuando se incubó con ~11 ppmv (Tabla 5).

Tabla 5. Tasa de emisión de CH_4 (mg CH_4 kg^{-1} $día^{-1}$) en suelos del exlago de Texcoco y agrícolas de Alcholoya y Chiapas, incubados con bajas concentraciones de CH_4 durante siete días y altas concentraciones de CH_4 durante 21 días.

Suelo	Tasa de emisión de CH_4 (mg CH_4 kg^{-1} suelo $día^{-1}$)				
Rango [CH_4] (ppm)	2—2.5	10—12	50—65	35000-45000	75000-85000
TXS1[a]	-0.00001 C [d]	1.36E-7 C	-0.00632 C	-2.51 B	-33.06* A
TXS2 [b]	-0.00007 C	-0.00008 B	-0.00363 B	-0.36 D	-12.26 C
TXS3 [c]	0.00001 C	0.00003 B	-0.00346 B	-0.78 C	-11.08 D
Alcholoya	-0.00024 B	-0.00009 B	-0.02194* A	-3.24* A	-27.67* B
Chiapas	-0.00157* A	-0.00155* A	-0.00329 B	-0.89 C	-6.82 E
EEE	0.00007	0.00017	0.00193	0.30	1.83

EEE: Error Estándar del Estimado ($p < 0.05$).

[a] Suelo de Texcoco con baja CE, [b] Suelo de Texcoco con mediana CE, [c] Suelo de Texcoco con alta CE, [d] Valores con la misma letra en mayúscula son significativamente diferentes entre suelos (columnas) * La emisión fue altamente significativa.

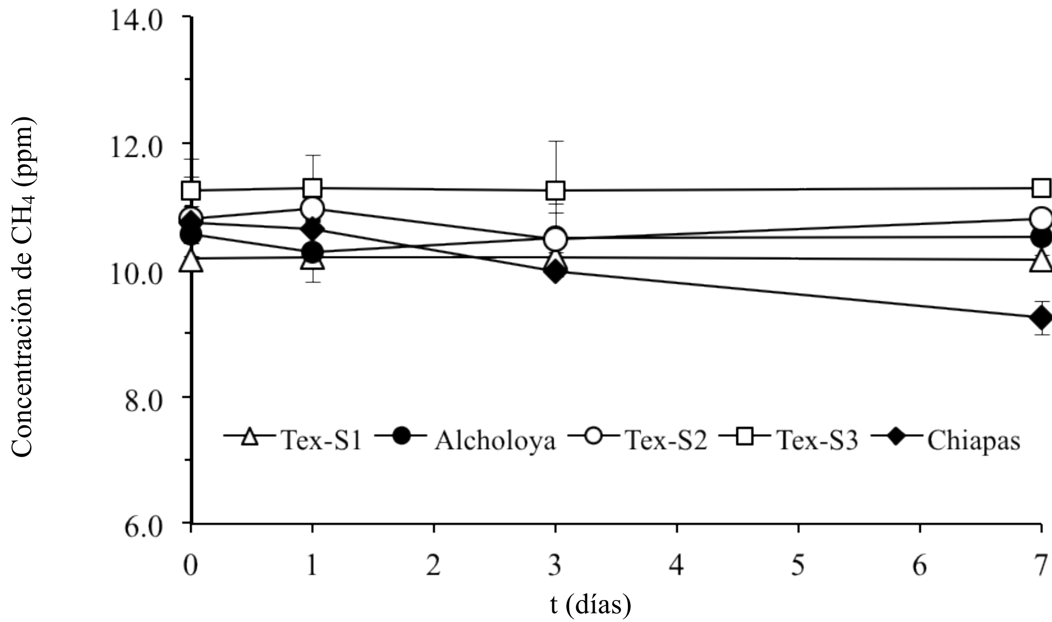

Figura 9. Oxidación de CH_4 en suelos agrícolas Alcholoya y Chiapas, y del exlago de Texcoco, Tex-S1, Tex-S2 y Tex-S3, incubados con 10 - 12 ppm de CH_4.

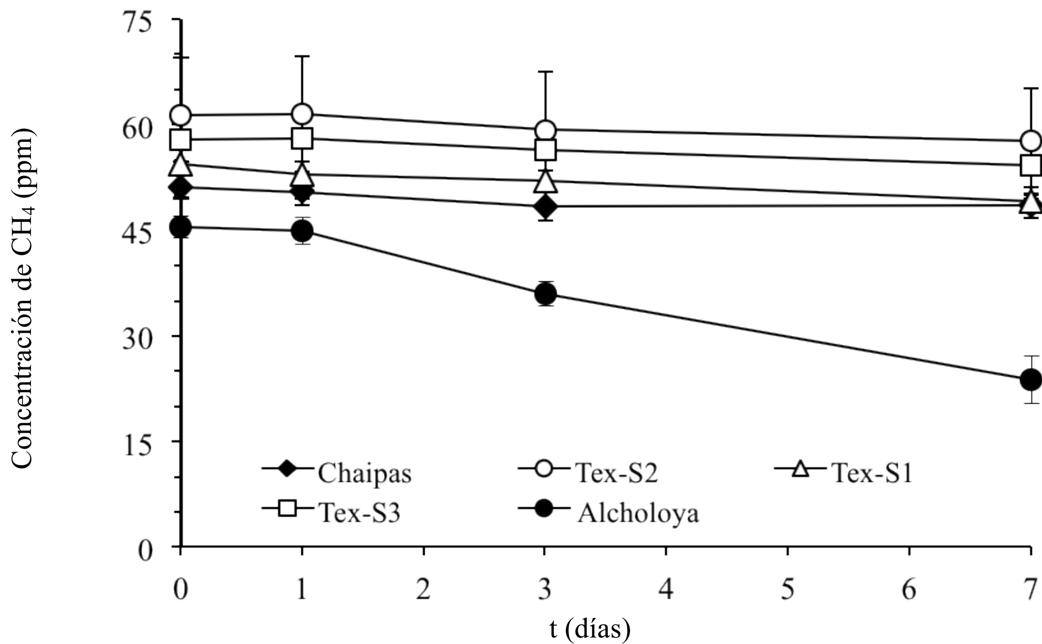

Figura 10. Oxidación de CH_4 en suelos agrícolas Alcholoya y Chiapas, y del exlago de Texcoco, Tex-S1, Tex-S2 y Tex-S3, incubados con 50 - 65 ppmv de CH_4.

Al incrementar la concentración de CH_4 en el espacio de cabeza a ~45 ppmv, el suelo agrícola de Alcholoya oxidó el gas de manera significativa.

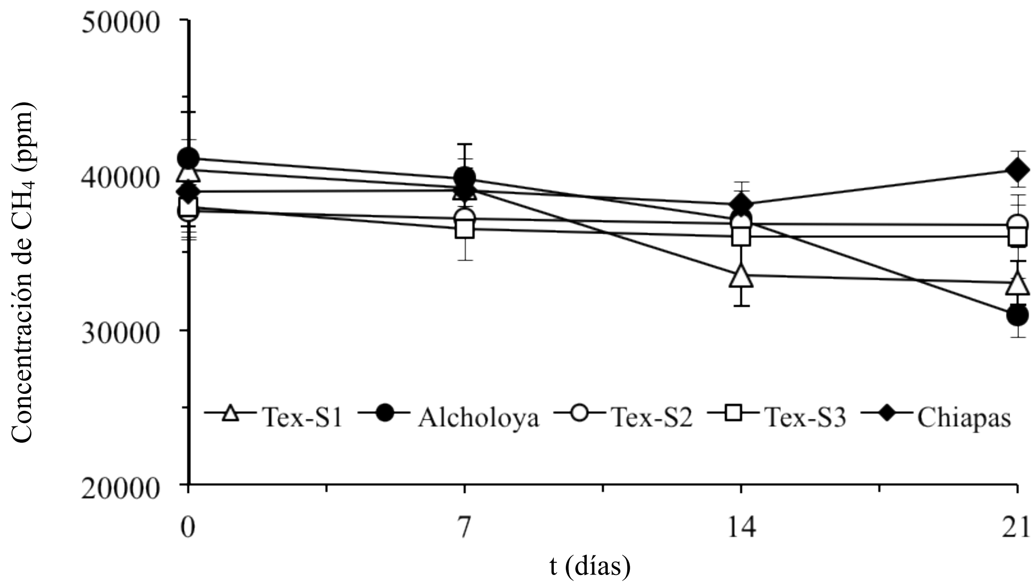

Figura 11. Oxidación de CH_4 en suelos agrícolas y del exlago de Texcoco, incubados con 35000 - 45000 ppmv de CH_4 en el espacio de cabeza.

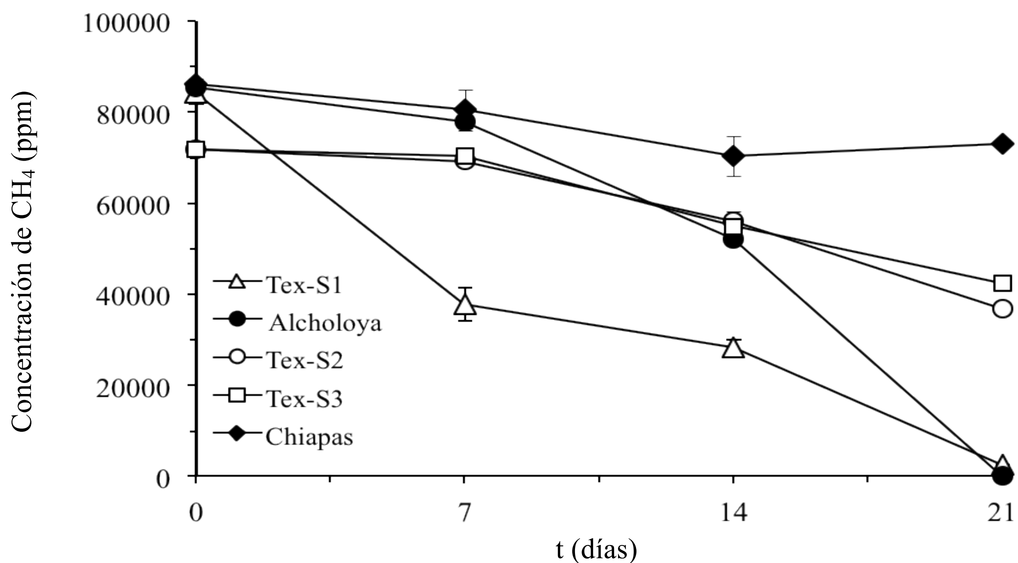

Figura 12. Oxidación de CH_4 en suelos agrícolas y del exlago de Texcoco, incubados con 75000 - 85000 ppmv de CH_4 en el espacio de cabeza.

El suelo agrícola de Alcholoya y el suelo del exlago de Texcoco de baja conductividad electrolítica (CE) Tex-S1, oxidaron el CH_4 cuando se incubaron bajo una atmósfera de ~40000 ppmv.

Al ser incubados con 75000 - 85000 ppmv de CH_4, los suelos de este estudio provenientes del exlago de Texcoco y agrícolas, oxidaron el CH_4 (Tabla 5). Aparentemente el incremento en la concentración de CH_4 favorece la actividad de los metanótrofos de baja afinidad en los suelos (Bender & Conrad 1992; Le Mer & Roger 2001).

La menor velocidad de oxidación de CH_4 fue encontrada en el suelo agrícola de Chiapas incubado con una alta concentración de CH_4 (7.5 - 8.5%) (Tabla 5). Los resultados de la Tabla 5 indican que la actividad metanotrófica en el suelo agrícola de Chiapas se ve favorecida cuando se incuba con bajas concentraciones de CH_4 (2 -10 ppm) y disminuye al incrementar la concentración del gas (> 50 ppm). Esto sugiere que las enzimas involucradas en la oxidación de CH_4 por metanótrofos en estos suelos, difieren de las de los demás suelos y probablemente, se expresan mejor a bajas concentraciones de sustrato (Baani & Liesack 2008).

Las tasas de oxidación de CH_4 en los suelos del exlago de Texcoco con baja CE (Tex-S1) y el suelo agrícola de Alcholoya fueron similares (Tabla 5). Alcholoya consumió casi todo el CH_4 en el espacio de cabeza (~8.5%) en 21 días, mientras que Tex-S1 consumió el 97% en este periodo. La mayor actividad metanotrófica en Tex-S1 fue durante los días 0 y 7, mientras que en Alcholoya hubo una fase de adaptación (lag) en la primera semana de incubación (Figura 12). La actividad metanotrófica en Tex-S1 se estimuló más rápido que en Alcholoya, esto sugiere que la composición de las comunidades es diferente en los dos suelos.

Los suelos del exlago de Texcoco con mediana y alta CE (Tex-S2 y Tex-S3) tuvieron el más bajo consumo de CH_4 comparado con los demás, en donde sólo el 49 y 41%, del CH_4 respectivamente, se consumió en 21 días. Similares al suelo agrícola de Alcholoya, estos

suelos necesitaron una semana de adaptación hasta detectar actividad metanotrófica (Figura 12).

Existe poca información reportada en relación a las tasas de consumo de CH_4 en suelos salino alcalinos. Aunque el nivel de salinidad en los suelo del exlago de Texcoco Tex-S3 (85.1 dS m^{-1}) y Tex-S2 (10.4 dS m^{-1}) es mayor que en un suelo salino (3.2 mS cm^{-1}) de la gran Rivera China (Zhang et al. 2011), la oxidación de CH_4 en los suelos del exlago de Texcoco (12.8 - 15.0 mg CH_4 kg^{-1} d^{-1}) fue mayor que en el de China (14 - 24 µg CH_4-C m^{-2} h^{-1} considerando una densidad aparente de 0.93 g cm^{-3} y 20 cm de profundidad).

Los metanótrofos haloalcalófilos están adaptados para vivir en esos ambientes (Khmelenina et al. 2000; Sorokin et al. 2000); es posible que cuando se aplican al suelo agua y substratos (CH_4 y O_2), la actividad metanotrófica se estimula.

Han et al. (2009), reportaron una tasa de oxidación de 3.0 µmol CH_4 g^{-1} suelo húmedo d^{-1} o 48 µg CH_4 g^{-1} suelo húmedo d^{-1} en un suelo alcalino (pH 9.4) de una mina de carbón en China; ésta es mayor que la encontrada en los suelos del exlago de Texcoco Tex-S3 y Tex-S2 (8.2 y 9.8 µg CH_4 g^{-1} suelo húmedo d^{-1}, respectivamente). Sin embargo, el suelo alcalino usado en ese estudio está constantemente expuesto a altas concentraciones de CH_4 (3000 ppm), lo que podría explicar la mayor tasa de oxidación de CH_4.

Khmelenina et al. (2000) reportaron una tasa de oxidación de CH_4 de 33.2 nmol ml^{-1} d^{-1} en sedimentos del lago Khuzhirta (pH 10.2 de baja salinidad (Lin et al. (2004)) y de 18.2 nmol ml^{-1} d^{-1} en sedimentos del lago Gorbunka (pH 9.5 y 4% de salinidad (Lin et al. (2004)). Asumiendo una densidad aparente del sedimento de 1.77 g ml^{-1} (Avnimelech et al. 2001), la oxidación de CH_4 en esos sedimentos (0.30 y 0.16 µg CH_4 g^{-1} sedimento d^{-1}) fue menor que la encontrada en Tex-S3 y Tex-S2. A pesar del pH y salinidad altos en los suelos del exlago de Texcoco, la disponibilidad de O_2 en estos suelos pudo haber favorecido una mayor actividad metanotrófica en comparación con los sedimentos de lagos salino alcalinos. El alto contenido de C orgánico (Tabla 4) en los suelos del exlago de Texcoco, es

otro factor que pudo favorecer la oxidación de CH_4 ya que los materiales orgánicos son substrato para la producción de CH_4 cuando los suelos se inundan y un aumento en la concentración de CH_4 también conducirá a un aumento en su oxidación.

4.3 Análisis de secuencias *pmo*A y *pmo*A-like

Se obtuvieron en total 309 secuencias parciales del gen *pmo*A (438 pb) de los suelos del exlago de Texcoco y suelos agrícolas de este estudio (Tabla 6). Se recuperaron 63 secuencias de Tex-S1, 85 de Tex-S2, 78 de Tex-S3, 33 de Alcholoya y 50 de Chiapas. Para la definición de OTU se utilizó un criterio del 6 % de diferencia entre las secuencias nucleotídicas *pmo*A (Schloss et al. 2005; Antony et al. 2010). Las secuencias se agruparon en 30 OTUs (Tabla 6).

4.3.1 Verificación de secuencias quimera

Después de que se verificaron las OTUs para detectar quimeras, de acuerdo como lo describen Pester et al. (2004), se comprobó que los porcentajes de identidad cuando se compararon con cepas de referencia fueron similares. No se obtuvieron secuencias quimera (información verificada el 09-Abril-2014).

4.3.2 Análisis de rarefacción e índices de diversidad

Para determinar si la comunidad metanotrófica estuvo representada por cada librería, se construyeron curvas de rarefacción (Figura 13). En el suelo del exlago de Texcoco de mediana CE (Tex-S2) se logró la asíntota con 85 secuencias. A pesar de que no se obtuvo una asíntota completa en los demás suelos, la cobertura (diversidad capturada) en las librerías fue > 90% (Tabla 6).

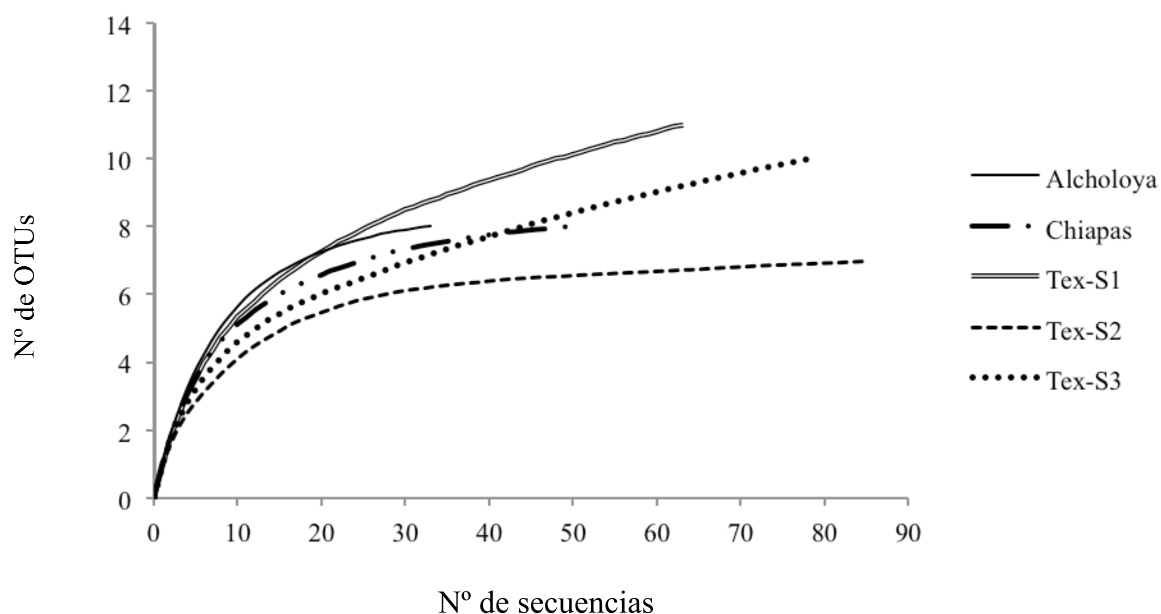

Figura 13. Curvas de rarefacción de metanótrofos en suelos agrícolas de Alcholoya y Chiapas, y suelos del exlago de Texcoco de baja CE (Tex-S1), mediana CE (Tex-S2) y alta CE (Tex-S3).

La comunidad metanotrófica en los suelos de Alcholoya, Chiapas y Tex-S1 fue más diversa (valores más bajos de D) que la de suelos salino-alcalinos del exlago de Texcoco Tex-S2 y Tex-S3 (valores más altos de D) (Tabla 6). Además, Tex-S2 y Tex-S3 tienen comunidades menos equitativas o especies dominantes (valores más bajos de H) (Tabla 6).

Tabla 6. Índices de diversidad en suelos agrícolas de Alcholoya y Chiapas y suelos del exlago de Texcoco.

Suelo	*Nº secs*	*Nº OTUs*	*Cobertura*	*Chao1*	*Shannon (H)*	*Simpson (D)*
Alcholoya	33	8	0.97	8 (0,8)	1.9 (1.7,2.1)	0.14 (0.1,0.2)
Chiapas	50	8	0.98	8 (0,8)	1.8 (1.6,2.0)	0.18 (0.1,0.2)
Tex-S1[a]	63	11	0.94	14 (11,34)	1.9 (1.7,2.1)	0.19 (0.1,0.3)
Tex-S2[b]	85	7	0.99	7 (0,7)	1.4 (1.2,1.6)	0.35 (0.2,0.4)
Tex-S3[c]	78	10	0.95	13 (10,33)	1.6 (1.4,1.9)	0.24 (0.2,0.3)

[a] Suelo del exlago de Texcoco de baja CE, [b] Suelo del exlago de Texcoco de mediana CE, [c] Suelo del exlago de Texcoco de alta CE.

El anális Unifrac separó las comunidades metanotróficas de los suelos agrícolas y de los suelos del exlago de Texcoco, lo que sugiere que fueron diferentes (Figura 14). Los suelos de mediana y alta CE del exlago de Texcoco (Tex-S2 y Tex-S3) tienen comunidades metanotróficas similares; la similitud en su actividad metanotrófica concuerda con esto.

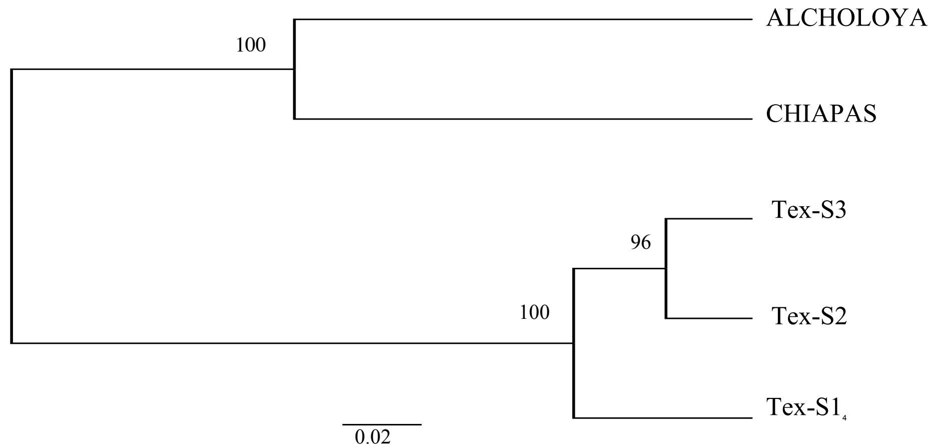

Figura 14. Comparación de comunidades metanotróficas en suelos agrícolas de Alcholoya y Chiapas, y suelos del exlago de Texcoco. Tex-S1: de baja CE, Tex-S2: de mediana CE; Tex-S3: de alta CE. La barra de escala representa 2 % de divergencia entre librerías.

4.3.3 Análisis filogenético de secuencias *pmo*A

En los suelos del exlago de Texcoco se encontraron, en su gran mayoría, metanótrofos tipo I (Gammaproteobacteria) (Figura 15; Tabla 7). Esto concuerda con lo reportado previamente para ambientes salinos y alcalinos (saline ponds y soda lakes) (Trotsenko & Khmelenina 2002; Semrau et al. 2010).

Lin et al. (2004), estudiaron la estructura de la comunidad metanotrófica y las poblaciones activas en sedimentos de los lagos salinos Suduntuiskii Torom y Gorbunka. Analizaron la diversidad de secuencias del fragmento del gen codificante del 16S rRNA y los genes funcionales *pmo*A y *mmo*X, utilizando la técnica DNA-SIP (stable isotope probing). La mayoría (50%) de las secuencias 16S rRNA y todas (100%) las secuencias *pmo*A

detectadas en la fracción pesada de DNA (13C-DNA) fueron metanótrofos tipo I en ambos lagos salinos.

Los metanótrofos en sedimentos de lagos alcalinos (soda lakes) parecen estar restringidos a unos pocos géneros como *Methylomicrobium* y *Methylobacter* (Sorokin & Kuenen 2005; Antony et al. 2012).

Se detectó un filotipo del clado *Crenothrix* en el suelo del exlago de Texcoco de baja CE, Tex-S1, (TXS1_211) (Figura 15, Tabla 7). Filotipos similares a éste han sido recuperados de suelos de bosque (Levine et al. 2011). *Crenothrix polyspora* es una bacteria filamentosa oxidadora de CH_4, que se caracteriza por formar conglomerados de color marrón-amarillento que contaminan tuberías de agua (Völker et al. 1977; Stoecker et al. 2006). Puede ser que TXS1_211 haya sido introducido a los suelos del exlago de Texcoco a través del sistema de irrigación con aguas residuales domésticas, instalado para disminuir su salinidad y que se haya adaptado a las condiciones de Tex-S1 (i.e., pH 8.5; 0.7 dS m^{-1}).

También se detectaron filotipos que oxidan CH_4 a concentración atmosférica (~1.7 ppmv) de los clados USC-γ (TXS1_153) y JR-2 (TXS1_229) en Tex-S1 (Figura 15, Tabla 7). Las secuencias del clado USC-γ se han detectado frecuentemente en suelos de pH > 6.0 (Knief et al. 2003; Bissett et al. 2012). Filotipos de este clado (USC-γ) también se detectaron en el suelo de Alcholoya, pH 6.7, (AL_235) y en el suelo del exlago de Texcoco de mediana CE, Tex-S2, pH 10.3, (TXS2_274), lo que sugiere que los filotipos del clado USC-γ son metanótrofos neutrófilos o alcalófilos, como lo reporta Knief et al. (2003).

En lagos alcalinos (soda lakes) y ambientes marinos se han aislado metanótrofos halófilos y alcalófilos del género *Methylomicrobium* (Nakamura et al. 2007; Jensen et al. 2008; Semrau et al. 2010). Se encontró un filotipo (TXS3_337) del clado *Methylomicrobium* en el suelo del exlago de Texcoco de alta CE, Tex-S3, (Figura 15, Tabla 7). Las especies de *Methylomicrobium* spp. son metanótrofos que se encuentran frecuentemente en ambientes de alta salinidad, (i.e., *M. alcaliphilum* 20Z se aisló de sedimentos de lagos salinos de Asia

Central –Tuva soda lakes) y han sido reportados como metanótrofos adaptados a ecosistemas con alto pH y alta salinidad (Khmelenina et al. 1997). La habilidad de M. *alcaliphilum* para oxidar CH_4 depende de la concentración de sales y del pH con valores óptimos de pH 9.0 y 0.7 M de NaCl (Khmelenina et al. 1997).

Los filotipos recuperados del suelo agrícola de Alcholoya se agruparon con secuencias de metanótrofos en clados reportados previamente (Figura 15, Tabla 7). Estos clados incluyen metanótrofos Tipo I (Gammaproteobacteria) como USC-γ, JR-2 y JR-3, y también metanótrofos Tipo II (Alfaproteobacteria) como USC-α. Se postula que los clados USC (Upland Soil Clusters) –α y –γ, y JR (Jasper Ridge clusters) –2 y –3, están conformados por metanótrofos de suelos arables que consumen CH_4 a concentración atmosférica y aún no se han podido aislar en cultivo puro (Holmes et al. 1999; Knief et al. 2003; Horz et al. 2005; Lüke & Frenzel 2011). Los filotipos del clado USC-α están relacionados con otros recuperados de suelos de bosque de pinos y abetos (Degelmann et al. 2010), suelos de pasto y pino (Singh et al. 2009), un glaciar en deshielo en el sureste de Groenlandia (Bárcena et al. 2011) y suelos de bosque natural y deforestados (Dörr et al. 2010), que consumen CH_4 atmosférico.

También se recuperaron filotipos del clado *Methylocaldum* (AL_348 y AL_12B) en el suelo de Alcholoya. Estos conforman un grupo de metanótrofos termotolerantes y termófilos que están ampliamente distribuidos en la naturaleza (Bodrossy et al. 1997). Se han encontrado en aguas termales, lodos activados, suelos arables, residuos de ensilaje y estiércol (Trotsenko et al. 2009). *Methylocaldum gracile* por ejemplo, puede crecer a 20°C y *Methylocaldum tepidum* a 30°C, pero la temperatura óptima para los dos es de 42°C y crecen a una temperatura máxima de 47°C (Bowman 2006). Estos metanótrofos tienen una cápsula gruesa como la de los quistes de *Azotobacter*, diferente de los quistes típicos de metanótrofos (Bowman 2006; Trotsenko et al. 2009).

Los filotipos que se recuperaron del suelo de Chiapas son metanótrofos Tipo I (Gammaproteobacteria), sin embargo, la mayoría de ellos no se afiliaron a ningún clado

específico previamente reportado, excepto por JR-2 (CH_119) conformado por metanótrofos de suelos arables que consumen CH_4 a concentración atmosférica y aún no se han podido aislar en cultivo puro (Horz et al. 2005; Lüke & Frenzel 2011). Los metanótrofos identificados en este suelo (Chiapas) son similares a los de suelos en los que se ha encontrado oxidación de CH_4: pastizales (Horz et al. 2005; Zhou et al. 2008; Judd 2011), suelos de pradera alpina en la Meseta Tibetana (Zheng et al. 2012), arrozales fertilizados con urea, superfosfato, cloruro de potasio y residuos de cosecha (Zheng et al. 2008) y suelos de cobertura de relleno sanitario (Henneberger et al. 2012).

Filotipos del clado USC-α no se detectaron en el suelo de Chiapas. Es posible que los metanótrofos en este clado no estén presentes o podrían representar filotipos que son más sensibles que los de USC-γ a monoterpenos producidos por *Jatropha curcas* L., cultivada en estos suelos. Se ha reportado que la raíz de algunas especies de *Jatropha* producen monoterpenos (Pertino et al. 2007) y estos inhiben la oxidación de CH_4 en suelos de bosque (Amaral & Knowles 1998; Maurer et al. 2008). Degelman et al. (2010) reportaron que varios filotipos del clado USC-α se afectaron por monoterpenos e indicaron que algunos de ellos pudieron ser más sensibles que otros del mismo clado.

4.4 Diversidad de la comunidad metanotrófica en los suelos de este estudio

Aunque no se detectaron secuencias parciales del gen *mmo*X en los suelos de este estudio, no se descarta la presencia de *Methylocella*, *Methyloferula* o cualquier género metanotrófico que contenga la forma soluble de la metano monooxigenasa (sMMO), es probable que su abundancia haya sido muy baja para ser detectada con la técnica usada (amplificación seguida de clonación).

Los metanótrofos encontrados en suelos agrícolas de este estudio, Chiapas (100 %) y la mayoría en Alcholoya (69.7 %), son de Tipo I. Esto podría estar vinculado con las bajas concentraciones de CH_4 que normalmente se espera encontrar en esos suelos (< 2 ppmv).

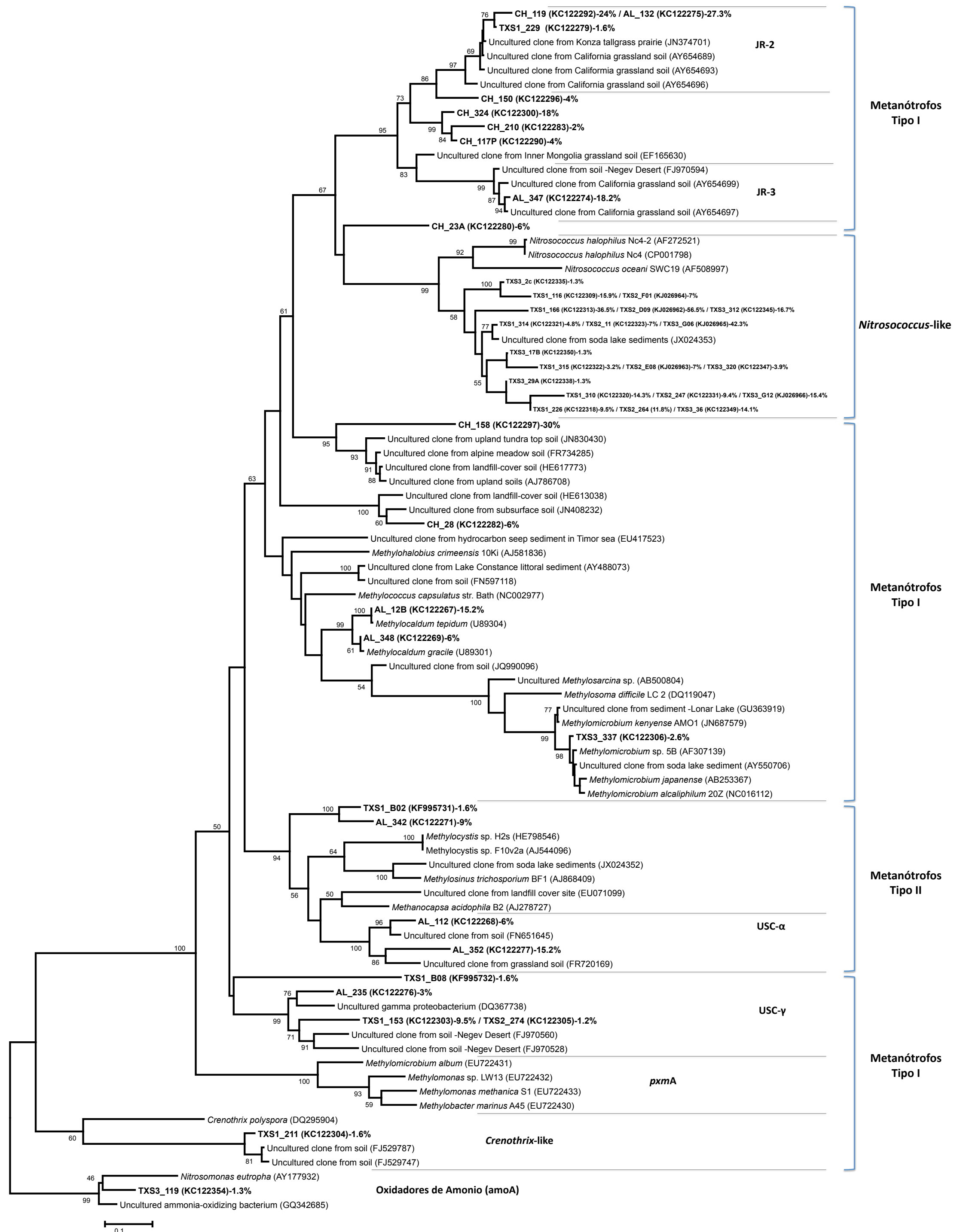

Figura 15. Arbol filogenético derivado de secuencias parciales del gen pmoA por el método de Máxima Verosimilitud a partir de muestras de suelo. Clonas TXS1, de suelo del exlago de Texcoco con baja CE (0.6−0.7 dS m^{-1}), TXS2 de mediana CE (5.9−10.8 dS m^{-1}) y TXS3 con alta CE (79.3−89.9 dS m^{-1}); AL, secuencias del suelo agrícola de Alcholoya y CH, del suelo agrícola de Chiapas. Secuencias de referencia de metanótrofos cultivados y no cultivados se obtuvieron del GenBank. las OTUs se obtuvieron con 6% de diferencia en nucleótidos. El árbol se construyó con un modelo GTR y parámetro gamma a = 0.9310. Se muestran los valores de Bootstrap > 50%. La barra de escala representa 10% de divergencia entre secuencias.

Tabla 7. Afiliación más cercana de secuencias *pmo*A de suelos del exlago de Texcoco y suelos agrícolas de Alcholoya y Chiapas.

Suelo OTU Representativa	Nº Clonas por OTU	Afiliación más cercana	% Similitud [a]	Grupo metanotrófico	% Clonas en la librería
Tex-S1 [b]					
TXS1_211	1	Bacteria no cultivada –soil (FJ529787)	93.4	*Crenothrix*-like	1.6
TXS1_229 [e]	1	Bacteria no cultivada –Konza tallgrass prairie (JN374701)/ –California grassland soil (AY654689)	96.6	JR-2 (Tipo Ic)	1.6
TXS1_B02	1	*Methylocystis* sp. F10V2a (AJ544096)/ *Methylocystis bryophila* (HE798546)	70.8	Tipo II	1.6
TXS1_B08 [f]	1	Bacteria no cultivada –sediment Lake Constance (AY488073)/ –Negev Desert soil (FJ970560)	65.5	Tipo I	1.6
TXS1_153 [f]	6	Bacteria no cultivada –California grassland soil (AY654697)	98.2	USC-γ (type Ic)	9.5
TXS1_314 [g]	3	Bacteria no cultivada –sediment soda lake (JX024353)	78.5	*Nitrosococcus*-like	4.8
TXS1_315 [g]	2	*Nitrosococcus oceani* SWC19 (AF508997)	74.2	*Nitrosococcus*-like	3.2
TXS1_226 [g]	6	*Nitrosococcus oceani* SWC19 (AF508997)	72.8	*Nitrosococcus*-like	9.5
TXS1_310 [g]	9	*Nitrosococcus oceani* SWC19 (AF508997)	73.5	*Nitrosococcus*-like	14.3
TXS1_116 [g]	10	*Nitrosococcus oceani* SWC19 (AF508997)	71.2	*Nitrosococcus*-like	15.9
TXS1_166 [g]	23	*Nitrosococcus oceani* SWC19 (AF508997)	74.4	*Nitrosococcus*-like	36.5
Tex-S2 [c]					
TXS2_274 [f]	1	Bacteria no cultivada –Negev Desert soil (FJ970560)	80.4	USC-γ (Tipo Ic)	1.2
TXS2_F01 [g]	6	*Nitrosococcus oceani* SWC19 (AF508997)	72.1	*Nitrosococcus*-like	7.0
TXS2_E08 [g]	6	*Nitrosococcus oceani* SWC19 (AF508997)	72.8	*Nitrosococcus*-like	7.0
TXS2_11 [g]	6	Bacteria no cultivada –sediment soda lake (JX024353)	79.2	*Nitrosococcus*-like	7.0
TXS2_247 [g]	8	*Nitrosococcus oceani* SWC19 (AF508997)	73.5	*Nitrosococcus*-like	9.4
TXS2_264 [g]	10	*Nitrosococcus oceani* SWC19 (AF508997)	74.7	*Nitrosococcus*-like	11.8
TXS2_D09 [g]	48	*Nitrosococcus oceani* SWC19 (AF508997)	74.2	*Nitrosococcus*-like	56.5
Tex-S3 [d]					
TXS3_337	2	*Methylomicrobium buryatense* (AF307139)/ Bacteria no cultivada –sediment soda lake (AY550706)	97.7	*Methylomicrobium* (Tipo Ia)	2.6
TXS3_119	1	Bacteria no cultivada –ammonia-oxidizing (GQ342685)	88.6	AOB-like	1.3
TXS3_2c	1	*Nitrosococcus oceani* SWC19 (AF508997)	74.4	*Nitrosococcus*-like	1.3
TXS3_17B	1	*Nitrosococcus oceani* SWC19 (AF508997)	74.7	*Nitrosococcus*-like	1.3
TXS3_29A	1	*Nitrosococcus oceani* SWC19 (AF508997)	74.0	*Nitrosococcus*-like	1.3
TXS3_320 [g]	3	*Nitrosococcus oceani* SWC19 (AF508997)	74.2	*Nitrosococcus*-like	3.9
TXS3_36 [g]	11	*Nitrosococcus oceani* SWC19 (AF508997)	72.8	*Nitrosococcus*-like	14.1
TXS3_G12 [g]	12	*Nitrosococcus oceani* SWC19 (AF508997)	73.3	*Nitrosococcus*-like	15.4
TXS3_312 [g]	13	*Nitrosococcus oceani* SWC19 (AF508997)	74.4	*Nitrosococcus*-like	16.7
TXS3_G06 [g]	33	Bacteria no cultivada –sediment soda lake (JX024353)	78.5	*Nitrosococcus*-like	42.3

Alcholoya					
AL_235	1	Bacteria no cultivada –Negev Desert soil (DQ367738)	85.8	USC-γ (Tipo Ic)	3.0
AL_348	2	*Methylocaldum gracile* (U89301)	99.3	Tipo Ib	6.1
AL_12B	5	*Methylocaldum tepidum* (U89304)	99.1	Tipo Ib	15.1
AL_342	3	*Methylocapsa acidiphila* B2 (AJ278727)	75.6	Tipo II	9.1
AL_112	2	Bacteria no cultivada –soil (FN651645)	92.0	USC-α (Tipo II)	6.1
AL_352	5	Bacteria no cultivada –soil (FN651645)	80.8	USC-α (Tipo II)	15.1
AL_347	6	Bacteria no cultivada –California grassland soil (AY654697)	98.2	JR-3 (Tipo Ic)	18.2
AL_132 [e]	9	Bacteria no cultivada –Konza tallgrass prairie (JN374701)/ –California grassland soil (AY654689)	96.6	JR-2 (Tipo Ic)	27.3
Chiapas					
CH_210	1	Bacteria no cultivada –Mongolia grassland soil (EF165630)	80.8	Tipo I	2.0
CH_117P	2	Bacteria no cultivada –Mongolia grassland soil (EF165630)	82.0	Tipo I	4.0
CH_23A	3	Bacteria no cultivada –Mongolia grassland soil (EF165630)	74.4	Tipo I	6.0
CH_28	3	Bacteria no cultivada –subsurface soil (JN408232)	88.1	Tipo I	6.0
CH_150	5	Bacteria no cultivada –California grassland soil (AY654696)	84.0	Tipo I	10.0
CH_324	9	Bacteria no cultivada –Mongolia grassland soil (EF165630)	83.1	Tipo I	18.0
CH_119 [e]	12	Bacteria no cultivada –California grassland soil (AY654693)	92.5	JR-2 (Tipo Ic)	24.0
CH_158	15	Bacteria no cultivada –landfill cover soil (HE617773)	74.4	Tipo I	30.0

[a] Generado con el programa MATGAT (v.2.02), [b] Suelo del exlago de Texcoco de baja Conductividad Electrolítica (CE), [c] Suelo del exlago de Texcoco de mediana CE, [d] Suelo del exlago de Texcoco de alta CE, [e] OTUs compartidas entre Tex-S1, Alcholoya y Chiapas, [f] OTUs compartidas entre Tex-S1 y Tex-S2 y Alcholoya, [g] OTUs compartidas entre Tex-S1, Tex-S2 y Tex-S3.

En estudios ambientales y con cultivos continuos, se ha encontrado que los metanótrofos Tipo I (Gammaproteobacteria) son competitivamente dominantes sobre los Tipo II (Alfaproteobacteria) en ambientes con bajas concentraciones de CH_4 (Macalady et al. 2002; Knief & Dunfield 2005; Zheng et al. 2008).

Para la definición de OTU se utilizó un criterio del 6 % de diferencia entre las secuencias *pmo*A (DNA). Se encontraron ocho OTUs diferentes en el suelo agrícola de Alcholoya (Tabla 7). La mayoría de los metanótrofos encontrados en este suelo (69.7 %) son similares (80.8 – 98.2 %) a metanótrofos que oxidan CH_4 a concentración atmosférica: JR-2 (27.3 %), JR-3 (18.2 %), USC-α (21.2 %), USC-γ (3.0 %); mientras que el 21.2 % son metanótrofos similares (> 99 %) a *Methylocaldum* y una menor proporción (9.1 %) similar

(75.6 %) a *Methylocapsa*.

En el suelo agrícola de Chiapas también se encontraron ocho OTUs (Tabla 7). Sin embargo, la mayoría de las secuencias no pudieron afiliarse a clados previamente descritos y su similitud con bacterias no cultivadas fue relativamente baja (74.4 – 88.1 %). Una gran proporción de metanótrofos en este suelo (24 %) fue altamente similar (92.5 %) a metanótrofos del clado JR-2 de suelos de pastizal en California, USA, que oxidan CH_4 a concentración atmosférica (Horz et al. 2005).

En el suelo del exlago de Texcoco con la más baja salinidad, Tex-S1 (0.7 dS m^{-1}) se encontraron cinco OTUs de metanótrofos altamente similares (93.4 – 98.2 %) a otros de clados previamente reportados (JR-2, USC-γ y *Crenothrix*). Sin embargo, seis OTUs (84.2 % de las secuencias), se afiliaron al clado *Nitrosococcus* con bajos porcentajes de similitud en su secuencia de DNA (71.2 – 74.4 %) a *Nitrosococcus oceani* SWC19, Gammaproteobacteria marina, oxidadora de amonio (Ward et al. 2000).

Se encontró una OTU (TXS2_274) en el suelo del exlago de Texcoco de mediana salinidad, Tex-S2 (9.0 dS m^{-1}), similar (80.4 %) a una bacteria no cultivada, del clado USC-γ, recuperada de suelos del Desierto de Negev con actividad metanotrófica. Seis OTUs más (98.8 % de las secuencias recuperadas) se afiliaron al clado *Nitrosococcus* con bajos porcentajes de similitud en su secuencia de DNA (71.2 – 74.7 %) a *Nitrosococcus oceani* SWC19, Gammaproteobacteria marina, que se reporta como oxidadora de amonio (Ward et al. 2000).

En el suelo del exlago de Texcoco con alta salinidad, Tex-S3, (84.8 dS m^{-1}), se encontró una OTU altamente similar (97.7 %) al alcalófilo *Methylomicrobium buryatense* (AF307139) y a una bacteria no cultivada (AY550706) recuperada de sedimentos del lago alcalino Transbaikal en Rusia (Lin et al. 2004). Similarmente a los demás suelos del exlago de Texcoco, siete OTUs (96.1 % de las secuencias recuperadas) en Tex-S3 se afiliaron al clado *Nitrosococcus* con bajos porcentajes de similitud en su secuencia de DNA (72.8 –

74.7 %) a *Nitrosococcus oceani* SWC19. Un alto porcentaje (42.3 %) de metanótrofos en este suelo (Tex-S3) fueron similares (78.5 %) a metanótrofos recuperados de sedimentos de "lonar lake", un ecosistema con moderada salinidad y alto pH en India (Antony et al. 2012).

Una OTU fue similar (88.6 y 80.4 % respectivamente) a una bacteria oxidadora de amonio recuperada de agua de lagos de la meseta tibetana (Hu et al. 2010) y a *Nitrosomonas eutropha* (Avrahami et al. 2003) recuperada de aguas altamente salinas en California "mono lake" (Ward et al. 2000).

4.5 Conservación de aminoácidos y motifs en secuencias PmoA-like

Casi el 93% (212) de las secuencias recuperadas de los suelos del exlago de Texcoco (226), se relacionaron más cercanamente con la única Gammaproteobacteria oxidadora de amonio, *Nitrosococcus*, que con bacterias oxidadoras de metano previamente reportadas (Figura 16). Estas secuencias se agruparon en un clado nuevo (*Nitrosococcus*-like) con alto valor de soporte por el método de máxima verosimilitud (99%), como se muestra en la Figura 16. Resultados similares han sido reportados previamente por Judd (2011), con secuencias recuperadas de suelos de pastizal (Sevilleta Grassland Soil – SEV y Shortgrass Steppe – SGS). Rojas-Oropeza (2012), en su estudio de Mineralización del N en el suelo salino sódico del exlago de Texcoco, encontró resultados similares al analizar el gen *pmo*A en muestras con salinidades de 34, 63 y 134 dS m^{-1}; poca diversidad, Gammaproteobacterias y vecinas de *Nitrosococcus oceani*. Los genes *amo*A reportados por Rojas-Oropeza (2012) en suelos del exlago de Texcoco se agruparon dentro de las Betaproteobacterias.

Las enzimas amonio monooxigenasa (AMO) de bacterias oxidadoras de amonio y metano monooxigenasa unida a membrana (pMMO) de bacterias oxidadoras de metano son similares en estructura y función; i.e., ambas contienen Cu, las dos pueden oxidar amonio y metano, comparten residuos aminoacídicos altamente conservados y se relacionan

evolucionariamente (Holmes et al. 1995; 1999; Conrad 1996; Hanson & Hanson 1996; Horz et al. 2005).

Se revisaron los residuos aminoacídicos y motifs de secuencias PmoA y AmoA de referencia y PmoA-like de este estudio (Holmes et al. 1999; Ricke et al. 2004; Horz et al. 2005) y se encontró que las secuencias tienen firmas de ambas enzimas (Figura 16). Dado esto, el gran número de secuencias *pmo*A-like y que en los suelos agrícolas de Alcholoya y Chiapas no se detectó este tipo de secuencias, se descartó un posible artefacto de la PCR para amplificar *pmo*A/coamplificar *amo*A. Los iniciadores para amplificar genes *pmo*A, utilizados (Bourne et al. 2001; Horz et al. 2005), producen altos rendimientos de *pmo*A y se detectaron dos nuevos clados (JR-2 y JR-3) por Horz et al. (2005).

En los péptidos PmoA-like recuperados de los suelos del exlago de Texcoco (clado *Nitrosococcus*-like), se identificaron 17 residuos diagnósticos PmoA y 5 residuos que fueron exclusivos del clado *Nitrosococcus*-like propuesto, i.e., residuos 25, 26, 51, 57 y 119 (Figura 16). Estos péptidos se compararon con aquellos de secuencias de ambientes salinos/alcalinos como "soda lake sediments" (Lin et al. 2004; 2005; Bodrossy et al. 2003), "movile cave" un ecosistema de Rumania cerca del Mar Negro, inusual con mucho CH_4 (1–2%) y poco O_2 (7–10%) en la atmósfera (Hutchens et al. 2004) y suelos tropicales alcalinos de relleno sanitario (Chang et al. 2010); se encontró que los cinco residuos firma fueron exclusivos del clado *Nitrosoccocus*-like (Figura 16).

La conservación de residuos aminoacídicos funcionales y diagnósticos (Figura 16) sugiere que las secuencias *pmo*A-like TXS1, TXS2 y TXS3 del clado *Nitrosococcus*-like codifican para la enzima metano monooxigenasa en lugar de la amonio monooxigenasa (Holmes et al. 1999; Tukhvatullin et al. 2001; Ricke et al. 2004; Horz et al. 2005). La topología de las monooxigenasas ha sido determinada (Holmes et al. 1995; Horz et al. 2005; Lüke & Frenzel 2011), sin embargo, parece reflejar la relación filogenética de los organismos, pero no su preferencia por metano o por amonio como substrato (Holmes et al. 1999).

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                    10        20        30        40        50
N.europaea : GDWDFWMDWKDRQWWPVVTPIVGITYCSAIMYYLWVNYRQPFGATLCVVCLLIGEWL :  57
N.multifor : GDWDFWLDWKDRQWWPVVTPIVGITYCAAIMYYLWVNYRLPFGATLCIVCLLVGEWL :  57
N.oceani   : GDWDFWVDWKDRRFWVTVVPIVSVAYPAAAQAFFWEKFRLPFGATLVTLGVLAGEWA :  57
N.watsonii : GDWDFWVDWKDRRFWVTVVPIVSVAYPAAAQAFFWEKFRLPFGATLVTLGLLAGEWA :  57
N.halophil : GDWDFWVDWKDRRMWVTVAPIVAIAYPAAMQAFFWEKFRLPFGATIVTLGVLAGEWA :  57
TXS1_116   : GDWDFWTDWKDRRMWVTVAPIVAIAYPAAMQAFLWEKFRLPFGATIVILGVLAGEWA :  57
TXS1_27P   : GDWDFWLDWKDRRMWVTVAPIVAIAYPAAMQAFLWEKFRLPFGATIVILGVLAGEWA :  57
TXS2_13    : GDWDFWTDWKDRRMWVTLLPIVAIAYPAAMQAFLWEKFRLPFGATIVILGVLLGEWA :  57
TXS3_2b    : GDWDFWTDWKDRRMWVTVAPIVAIAYPAAMQAFLWEKFRLPFGATIVILGVLLGEWA :  57
Clone1     : GDWDFWTDWKDRRLWVTVAPIVSITFPAAVQACLWYRYRLPFGAVVCVLGLLLGEWV :  57
Clone2     : GDWDFWTDWKDRRLWVTVAPIVSITFPAAVQAMLWWRYRLPFGAVLCILGLLLGEWI :  57
Clone3     : GDWDFWTDWKDRRLWVTVVPIVAVTFPAAVQACLWGRYRLPWGATICVLGLLFGEWI :  57
M.capsulat : GDWDFWSDWKDRRLWVTVTPIVLVTFPAAVQSYLWERYRLPWGATVCVLGLLLGEWI :  57
M.tepidum  : GDWDFWSDWKDRRLWVTVTPIVLVTFPAAIQAWAWERFRNPWGATIAVLALLFGEWV :  57
M.trichosp : GDWDFWVDWKDRRMWPTVVPILGVTFAAAAQAFFWENFKLPFGATFAVSGLLIGEWI :  57
M.sp.SC2   : GDWDFWIDFKDRRMWPTVTPIVAMCFAAAAQSFFWTRFRLPIGATTVVLALLIGEWI :  57

              60        70        80        90       100       110
N.europaea : TRYWGFYWWSHYPINFVTPGIMLPGALMLDFTLYLTRNWLVTALVGGGFFGLLFYPG : 114
N.multifor : TRFWGFYWWSHYPMNFVFPSTMIPGALVMDTVLLLTRNWMITALVGGGAFGLLFYPG : 114
N.oceani   : NRYFNFVGFTYFPINFVWPTILLPMALFLDAMLAISKSYGLTAVVGGLMYGLLMYPA : 114
N.watsonii : NRYFNFVGFTYFPINFVWPTILLPMALFLDAMLAISKSYGLTAVVGGLMYGLLMYPA : 114
N.halophil : NRYYNFYGFTYFPINFVWPAILVPMALFLDAVLVLTKSYGLTAIVGGLFYGLLLYPA : 114
TXS1_116   : NRYFNFYGWTYFPIGFVWPAVLVPMALVLDTLLMASKSYGLTAITGGLFFGLLLYPS : 114
TXS1_27P   : NRYFNFYGWTYFPINFVWPAVLVPMALVLDTILLLSRSYGLTAIVGGLFFGLLLYPA : 114
TXS2_13    : NRYFNFYGWTYFPINFVWPAVLVPMALVLDTVLLLTRSYGLTAVVGGLFFGLLFYPA : 114
TXS3_2b    : NRYFNFYGWTYFPINFVWPAVLVPMALVLDTVLLLTRSYGLTAVVGGLFFGLLFYPA : 114
Clone1     : NRYLNFWGWTYFPVNFCFPSNLMPGAIVLDVVLMLSNSMTLTAVVGGMAWGLLFYPG : 114
Clone2     : NRYLNFWGWTYFPVNFVFPSNLMPGAIVLDVILMLSGSMTLTAVLGGLGYGLLFYPG : 114
Clone3     : NRYFNFWGWTYFPVNFVFPSQLIPSAIILDVVLLLSNSFTFTAVAGAMGWGLIFYPS : 114
M.capsulat : NRYFNFWGWTYFPINFVFPASLVPGAIILDTVLMLSGSYLFTAIVGAMGWGLIFYPG : 114
M.tepidum  : DRYFNFWGWTYFPINFVFPAVLVPGAIILDTFLMLSGSYLFTAIVGXMAWGLVFYPG : 114
M.trichosp : NRYCNFWGWTYFPISLVFPSALVVPALWLDIIMLLSGSYVITAVVGSLGWGLLFYPN : 114
M.sp.SC2   : NRYDNFWGWTFFPINLVFPSALIPMGFWLDIVLMISGSWLVTALLGGLGWGLLFYPI : 114

                120       130       140       150       160
N.europaea : NWPIFGPTHLPIVVEGTLLSMADYMGHIYVRTGTPEYVRHIEQGSLRTFGGHTTV : 169
N.multifor : NWTIFGPTHLPLVAEGVLLSVADYTGFIYVRTGTPEYVRLIEQGSLRTFGGHTTV : 169
N.oceani   : NWPLLSAFHVPAEYNGVVMSLADIMGYQYVRTGTPEYIRMVEKGTLKTFGKDVVP : 169
N.watsonii : NWPLLSAFHVPAEYNGVVMSLADVAGYQYVRTGTPEYIRMVEKGTLRTFGKDVVP : 169
N.halophil : NWPLLAQFHVPVEYNGLVMSIADIMGYHYVRTGTPEYIRMVEKGTLRTFGKDVVP : 169
TXS1_116   : NWPLLSVYHVPVEYNGMVMSVADVMGYHYIRTGTPEYIRLVEKGTLRTFGKDVAP : 169
TXS1_27P   : NWPLLAAFHVPVEYNGLVMSVADVMGYHYVRTGTPEYIRLVEKGTLRTFGKDVAP : 169
TXS2_13    : NWPLLAAFHVPVEYNGLVMSVADVMGYHYVRTGTPEYIRLVEKGTLRTFGKDVAP : 169
TXS3_2b    : NWPLLAAFHVPVEYNGLVMSVADVMGYHYVRTGTPEYIRLVEKGTLRTFGKDVAP : 169
Clone1     : NWPIIAPLHIPVEYNGMMFTLADLQGYHYVRTGTPEYIRMVEKGTLRTFGKDVAP : 169
Clone2     : NWPIIAPLHVPVEYNGMMMTLADLQGYHYVRTGTPEYIRMVEKGTLRTFGKDVAP : 169
Clone3     : NWPVIAPLHVPVEYNGMMMTLADLQGYHYVRTGTPEYIRMVEKGTLRTFGKDVAP : 169
M.capsulat : NWPIIAPLHVPVENNGMLMSIADIQGYNYVRTGTPEYIRMVEKGTLRTFGKDVAP : 169
M.tepidum  : NWPVIAPLHVPVEYNGMLMSIADIQGYNYVRTGTPEYIRMVEKGTLRTFGKDVXP : 169
M.trichosp : NWPAIAALHQATEQHGQLMSLADLVGFHFVRTSMPEYIRMVERGTLRTFGKEVVP : 169
M.sp.SC2   : NWPVLAQYHQAAEIDGVLLTLADLIGFNYVRTGTPEYIRMVERGTLRTFGKDVVP : 169
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Figura 16. Identificación de aminoácidos conservados y diagnósticos en secuencias PmoA y AmoA (Holmes et al. 1999; Ricke et al. 2004, Horz et al. 2005). Las secuencias *Nitrosococcus* AmoA/PmoA-like se recuperaron de suelos salino alcalinos del exlago de Texcoco y se alinearon con AmoA de Betaproteobacteria, AmoA de Gammaproteobacteria, y PmoA de Gammaproteobacteria y Alphaproteobacteria. Se muestran las secuencias de *Nitrosomonas europaea* ATCC 19718 (N.europaea, NP841017), *Nitrosospira multiformis* ATCC 25196 (N.multifor, YP411498), *Nitrosococcus watsonii* C-113 (N.watsonii, AF153344), *Nitrosococcus oceani* ATCC 19707 (N.oceani, AAB57809), *Nitrosococcus halophilus* Nc4 (N.halophil, YP003526243), *Methylococcus capsulatus* str. Bath (M.capsulat, AAB49821), *Methylocaldum tepidum* (M.tepidum, AAC04382), *Methylosinus trichosporium* OB3b (M.trichosp, AAA87220) y *Methylocystis* sp. SC2 (M.spSC2, CAE48352). Las clonas 1−3 son secuencias PmoA de bacterias no cultivadas de ambientes salinos o alcalinos. Clona 1HM_25 from soda lake sediment (Clone1, AAS91065), Clona mvpb13.2 de Romania:Movile Cave (Clone2, AAR04324), Clona 0507-M40-6 de un suelo alcalino de relleno sanitario (Clone3, ACB28545). Se obtuvo el alineamiento en CLUSTALW y la figura se editó con GENEDOC. Los residuos universalmente conservados en las secuencias AmoA/PmoA se muestran marcados en negro, los conservados en PmoA/*Nitrosococcus*-like en gris con letras blancas y los de *Nitrosococcus*-like en gris con letras negras

5. **Conclusiones**

La medición de la acitividad metanotrófica y la presencia del gen funcional *pmo*A, permitió determinar que los suelos agrícolas y del exlago de Texcoco de este estudio, tienen potencial para oxidar CH_4.

Las comunidades metanotróficas en suelos del exlago de Texcoco son diferentes de las de suelos agrícolas de Alcholoya y Chiapas.

La composición de las comunidades metanotróficas en suelos del exlago de Texcoco reveló que (i) La riqueza fue mayor en el suelo de baja salinidad (Tex-S1) que en los suelos de mediana (Tex-S2) y alta (Tex-S3) salinidad y (ii) un grupo grande de secuencias relacionadas a *Nitrosococcus* dominaron las comunidades metanotróficas en cada suelo.

La mayoría de metanótrofos que se encontraron en los suelos del exlago de Texcoco fueron metanótrofos Tipo I (Gammaproteobacteria).

La disminución en la salinidad puede haber favorecido la diversidad metanotrófica en el suelo del exlago de Texcoco de baja salinidad, Tex-S1.

Las especies microbianas con las secuencias del clado *Nitrosococcus*-like detectadas, podrían estar participando en la oxidación de CH_4 en los suelos del exlago de Texcoco, sin embargo, se necesitan más experimentos para confirmar que estas bacterias cooxidan CH_4.

6. Recomendaciones y Perspectivas

En este trabajo se determinó que los suelos de este estudio son capaces de oxidar metano y se detectó el gen funcional *pmo*A, involucrado en la oxidación de metano por bacterias aerobias; sin embargo, el estudio de las secuencias de DNA no prueba que los microorganismos detectados estén activos en cada ambiente. Queda pendiente determinar cuáles de los genes detectados están activos y bajo qué condiciones.

Se encontró un grupo de secuencias relacionadas con bacterias que oxidan amonio, que pueden estar involucradas en la oxidación de metano en los suelos del exlago de Texcoco; es recomendable hacer más estudios para su identificación y comprobar su actividad metanotrófica.

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ORIGINAL PAPER

Changes in methane oxidation activity and methanotrophic community composition in saline alkaline soils

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Abstract The soil of the former Lake Texcoco is a saline alkaline environment where anthropogenic drainage in some areas has reduced salt content and pH. Potential methane (CH_4) consumption rates were measured in three soils of the former Lake Texcoco with different electrolytic conductivity (EC) and pH, i.e. Tex-S1 a >18 years drained soil (EC 0.7 dS m^{-1}, pH 8.5), Tex-S2 drained for ~10 years (EC 9.0 dS m^{-1}, pH 10.3) and the undrained Tex-S3 (EC 84.8 dS m^{-1}, pH 10.3). An arable soil from Alcholoya (EC 0.7 dS m^{-1}, pH 6.7), located nearby Lake Texcoco was used as control. Methane oxidation in the soil Tex-S1 (lowest EC and pH) was similar to that in the arable soil from Alcholoya (32.5 and 34.7 mg CH_4 kg^{-1} dry soil day^{-1}, respectively). Meanwhile, in soils Tex-S2 and Tex-S3, the potential CH_4 oxidation rates were only 15.0 and 12.8 mg CH_4 kg^{-1} dry soil day^{-1}, respectively. Differences in CH_4 oxidation were also related to changes in the methane-oxidizing communities in these soils. Sequence analysis of *pmo*A gene showed that soils differed in the identity and number of methanotrophic phylotypes. The Alcholoya soil and Tex-S1 contained phylotypes grouped within the upland soil cluster gamma and the Jasper Ridge, California JR-2 clade. In soil Tex-S3, a phylotype related to *Methylomicrobium alcaliphilum* was detected.

Keywords Methane oxidation · Methane monooxygenase · Saline alkaline soils · *pmo*A

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Introduction

The soil of the former Lake Texcoco is a unique extreme environment, considered a soda desert with anthropogenic influence, located near one of the biggest cities in the world, Mexico City. Large parts are saline-alkaline with pH often >10 and electrolytic conductivity (EC) >150 dS m^{-1} with large variations in soil characteristics over short distances (<10 m) (Dendooven et al. 2010). The major salts in the soils of the former Lake Texcoco are NaCl and Na_2CO_3, but Ca^{2+} and Mg^{2+} are also important cations (Luna-Guido et al. 2000). Reported values for calcium carbonate range from 0.25 to 10.5 % (Gutiérrez-Castorena et al. 2005; Fernández-Buces et al. 2006), while salinity in NaCl ranges from 0.1 to 12 % (w/v). Some halophilic/halotolerant vegetation as *Distichlis spicata, Suadera nigra* and *Tamarix aphylla* can be found, but large parts remain bare due to salt crusts (Luna-Guido et al. 2000; Betancur-Galvis et al. 2012). For more than 30 years, parts of the former lake bed have been drained artificially and irrigated with sewage effluents to reduce the salt content and to vegetate the area (Luna-Guido et al. 2000).

Saline alkaline ecosystems, such as soda lakes and soda deserts, were thought to be inhabited by a small number of

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organisms restricted to few phyla; however, cultivation-independent approaches have shown a diverse and unique community involved in the biogeochemical processes (Jones et al. 1998; Valenzuela-Encinas et al. 2009). These organisms, such as methanogens, methanotrophs, phototrophs, denitrifiers, sulphur oxidizers, sulphate reducers and syntrophs, shape complex microbial food webs that affect the fluxes of greenhouse gases (Antony et al. 2012).

The methane (CH_4) emitted from soil contributes to the 'greenhouse effect' (Smith et al. 2003). It is mostly produced under strict anaerobic conditions. However, the soil is not only a source of CH_4, but it can also be a sink. Little information exists about CH_4 oxidation in saline alkaline soils as it is assumed that high ionic salt concentrations might inhibit the oxidation process by moisture stress (Dalal et al. 2008). The existence of methanotrophs in hypersaline and alkaline habitats was questioned previously (Conrad et al. 1995). However, methane oxidation activity has been found in water column and sediment samples from soda lakes (Khmelenina et al. 2000; Lin et al. 2005), and haloalkaliphilic and haloalkalitorerant methanotrophic bacteria have been also isolated from these extreme environments (Khmelenina et al. 1997; Sorokin and Kuenen 2005; Eshinimaev et al. 2008).

The methanotrophs, a specialized group of Bacteria, are mostly responsible for CH_4 oxidation (Trotsenko and Murrell 2008; Semrau et al. 2010). The key enzyme of methanotrophs is methane monooxygenase (MMO) that converts methane to methanol (Murrell et al. 2000; Hakemian and Rosenzweig 2007; Trotsenko and Murrell 2008). Two types of this enzyme have been described so far: a membrane bound version (pMMO) and a cytoplasmic soluble version (sMMO). Nearly all aerobic methanotrophs described possess pMMO, with the exception of the genera *Methylocella* and *Methyloferula* in which only sMMO has been detected (Dedysh et al. 2005; Vorobev et al. 2011). Thus, a gene encoding for pMMO polypeptides, i.e. *pmo*A, has been widely used as a molecular marker to characterize methanotrophic communities in environmental samples (Dumont and Murrell 2005). McDonald et al. (2008) reviews molecular ecology techniques for methanotrophs.

Methane-oxidizing bacteria described so far belong to the Gammaproteobacteria, Alphaproteobacteria and Verrucomicrobia (Semrau et al. 2010). Gammaproteobacterial methanotrophs are classified as type I and include families such as Methylococcaceae and Crenotrichaceae. Meanwhile, those belonging to Alphaproteobacteria are considered as type II, such as phylotypes from Methylocystaceae and Beijerinckiaceae (Lüke et al. 2010). The phylum Verrucomicrobia is represented by the genus *Methylacidiphilum*, apparently restricted to extreme thermoacidophilic environments (Op den Camp et al. 2009). Likewise, ammonium-oxidizing Gammaproteobacteria can also co-oxidize CH_4 (Jones and Morita 1983; Bedard and Knowles 1989).

The methanotrophic activity in saline alkaline soils of the former Lake Texcoco has not been determined yet and the potential communities involved in the process remain unknown. Additionally, the artificial drainage system in the area enables the study of a gradient in soil salinity and pH. The aim of this study was to investigate the CH_4 oxidation capacity and the potential methane-oxidizing communities in three soils of the former Lake Texcoco with different electrolytic conductivity and pH, i.e. Tex-S1, a >18 years drained soil (EC 0.7 dS m^{-1}, pH 8.5); an alkaline soil with medium EC, Tex-S2 (EC 9.0 dS m^{-1}, pH 10.3) and an alkaline soil with high salinity, Tex-S3 (EC 84.8 dS m^{-1}, pH 10.3). An arable soil from nearby Alcholoya (EC 0.7 dS m^{-1}, pH 6.7) was used as external control to compare CH_4 oxidation capacity and the microbial community structure using partial *pmo*A sequences.

Materials and methods

Soil sampling

Three sites located in the former Lake Texcoco (State of Mexico, Mexico) were sampled. The three locations were characterized by a different EC. The first Texcoco soil (Tex-S1) had a low EC (0.6–0.7 dS m^{-1}), the second (Tex-S2) a medium EC (5.9–10.8 dS m^{-1}) and the third (Tex-S3) a high EC (79.3–89.9 dS m^{-1}) (Table 1). More details of the characteristics of the Texcoco soil can be found in Dendooven et al. (2010). Soil samples were collected from three plots at the three different sites on July 16th 2010 in the rainy season. Three different areas of ca. 400 m^2 were delimited at each site and 20 soil samples were taken from the 0–15 cm soil layer. The 0–2 cm topsoil layer of each soil core was discarded so that the 2–15 cm layer was kept. The 20 cores taken in each plot were pooled. Nine soil samples were obtained, i.e. three soil samples from three sites.

The arable soil was sampled in Alcholoya, a hamlet of Acatlan, State of Hidalgo, Mexico (2080 m above sea level). Details of the sampling site can be found in Serrano-Silva et al. (2011). Three different areas of ca. 400 m^2 were delimited and 20 soil samples were taken from the 0–15 cm soil layer. The 20 cores taken in each plot were pooled so that three soil samples were obtained. The agricultural soil of Alcholoya had shown a methane-oxidizing capacity of 0.09 ng CH_4 kg^{-1} dry soil day^{-1} when incubated with atmospheric CH_4 concentration (ca. 2 ppm) (Serrano-Silva et al. 2011).

The field-based replication was maintained in the laboratory study. As such, 12 soil samples were obtained, i.e.

Table 1 Characteristics and potential CH_4 oxidation rates (mg CH_4 kg^{-1} dry soil day^{-1}) of soils of the former Lake Texcoco and the arable soil of Alcholoya

Soil	Localization	EC (dS m^{-1})[a]	pH	Organic C (g kg^{-1} soil)	Inorganic C (g kg^{-1} soil)	Total N (g kg^{-1} soil)	WHC[b] (g kg^{-1} soil)	Clay (g kg^{-1} soil)	Silt (g kg^{-1} soil)	Sand (g kg^{-1} soil)	USDA textural classification	CH_4 oxidation[c]
Tex-S1[d]	N 19°28.64 W 98°58.21	0.7 (0.1)[e]	8.5 (0.4)	32.8 (5.3)	7.3 (0.2)	1.9 (0.3)	855 (20)	130 (12)	250 (23)	620 (31)	Sandy loam	32.5
Tex-S2[f]	N 19°30.22 W 98°59.42	9.0 (2.7)	10.3 (0.1)	22.2 (1.4)	3.7 (0.8)	1.3 (0.1)	1046 (37)	480 (74)	340 (44)	180 (50)	Clay	15.0
Tex-S3[g]	N 19°30.77 W 98°59.42	84.8 (5.3)	10.3 (0.2)	16.7 (1.0)	6.5 (0.6)	0.9 (0.2)	1120 (71)	650 (55)	270 (26)	80 (76)	Clay	12.8
Alcholoya[h]	N 20°09 W 98°26	0.7 (0.3)	6.7 (0.2)	11.1 (1.1)	ND[i]	1.0 (0.2)	846 (47)	160 (25)	350 (44)	490 (31)	Loam	34.7

[a] Electrolytic conductivity

[b] Water holding capacity

[c] CH_4 oxidation rate (mg CH_4 kg^{-1} dry soil day^{-1}) for soil incubated in serum closed vials with 7.5–8.5 % CH_4 in the headspace for 21 days

[d] Texcoco soil with low EC

[e] Values between parenthesis are standard deviations ($n = 3$)

[f] Texcoco soil with medium EC

[g] Texcoco soil with high EC

[h] Data derived from Serrano-Silva et al. (2011)

[i] ND: below detection level

from the four soils in triplicate. A sub-sample of 20 g soil was taken from each sample and stored at −80 °C until extracted for DNA. The remaining soil was dried at environmental temperature, passed through a 5-mm sieve and characterized as described in Conde et al. (2005).

Methane oxidation activity

Ten grams of sieved soil from each sampling site ($n = 4$) and each plot ($n = 3$) was adjusted to 50 % water holding capacity (WHC) and placed in 120 ml serum bottles sealed with butyl rubber septa. Ten millilitres of the headspace was extracted and replaced with 10 ml CH_4 (99 % purity). Triplicates of each soil were incubated in the dark at 25 °C. Sterile soil samples were incubated in the same way to determine if abiotic factors affected the CH_4 concentration in the headspace. The CH_4 concentration in the headspace was determined with an Agilent Technology 4890D gas chromatograph (GC) fitted with a flame ionization detector. Details of the settings of the GC can be found in Serrano-Silva et al. (2011).

Extraction of total DNA

Total DNA was extracted from 0.5 g soil in triplicate and pooled (1.5 g soil) by using a modified method based on direct cell lysis technique described by Guo et al. (1997). Organic components in soil were removed with sodium pyrophosphate (Ceja-Navarro et al. 2010) and chemical cellular lysis with lysozyme was replaced by mechanical lysis using a BeadBeater with glass beads with various sizes (Cullen and Hirsch 1998). Further details can be found in Valenzuela-Encinas et al. (2008). The DNA was recovered in 200 µl bi-distilled water. The DNA-yield was quantified on an agarose gel (0.8 %) electrophoresis and UV transillumination (Gel Doc 2000, Bio-Rad Laboratories Inc., Carlsbad, CA, USA) after staining with ethidium bromide (0.5 µg ml^{-1}). The DNA extracted was stored at −20 °C until used for PCR amplification.

PCR amplification

All DNA samples obtained were used as template for PCR amplification. The primers A189 (5′ GGN GAC TGG GAC TTC TGG 3′) in combination with either the A682 (5′ GAA SGC NGA GAA GAA SGC 3′) (Holmes et al. 1995), mb661 (5′ CCG GMG CAA CGT CYT TACC 3′) (Costello and Lidstrom 1999) or A650 primer (5′ ACG TCC TTA CCG AAG GT 3′) (Bourne et al. 2001) were used to amplify *pmo*A. The amplification procedure was done via the semi-nested PCR technique as described by Horz et al. (2005). Briefly, a first PCR was done with the 5′ primer A189 and the 3′ primer A682 in a "touch-down" protocol from 62 to 52 °C. After each cycle, the annealing temperature was decreased by 0.5 °C until it reached 52 °C. Aliquots of the first round of PCR (0.25 µl) were used as the template in the second round of PCR using the 5′ primer A189 and the two 3′ primers mb661 and A650 in a multiplex PCR setting. The PCR mixture (25 µl) contained 100 ng DNA with the appropriate primers at 0.5 µM; 1× PCR Buffer (Invitrogen Life Technologies, Sao Paulo, Brazil); 2.5 mM $MgCl_2$; dATP, dCTP, dGTP and dTTP each at a concentration of 200 µM; 1 U Taq DNA polymerase (Invitrogen Life Technologies) and 20 µg bovine serum albumin. The reactions were done in a Touchgene Gradient FTGRAD2D (TECHNE DUXFORT, Cambridge, UK) with an initial denaturation at 94 °C for 10 min, followed by 30 cycles of: 94 °C for 1 min, 55 °C for annealing (A189/mb661 and A189/A650) for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Three PCRs were done per soil sample. The PCR products of three subsamples were mixed and analysed on an agarose gel (1.5 %) electrophoresis and UV transillumination after staining with ethidium bromide.

Cloning and sequencing

The methanotrophic bacterial community was characterized by cultivation-independent retrieval of the partial *pmo*A gene. The obtained PCR products of the expected size (~500 bp) were inserted into the pGEM cloning plasmid with the pGEM T-Easy Vector Cloning System I kit (Promega, Madison, WI, USA). Positive clones were detected by the appearance of white colonies in LB plates containing 1.6 mg of X-Gal spread on the surface (Invitrogen Life Technologies, Carlsbad, CA, USA) and 50 µg ml^{-1} ampicillin. Plasmid inserts were amplified with M13F and M13R primers. Nucleotide sequences of cloned genes were determined with a 3730X DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Macrogen sequencing service (Macrogen Inc., Seoul, Korea) using the M13F primer. Overall, 253 clones were sequenced.

Representative *pmo*A and coamplified *amo*A nucleotide sequences obtained were submitted to the GenBank database under accession numbers KC122266–KC122354, KF995731–KF995732 and KJ026961–KJ026966.

Phylogenetic analysis of the *pmo*A partial sequences

Nucleotide sequences were compared with entries in the NCBI database using BLASTX (Altschul et al. 1997). Sequences were checked for chimeras as described by Pester et al. (2004). SEAVIEW (Galtier et al. 1996) was used when manual editing of the nucleotides was necessary. Sequences were aligned with CLUSTALW (Chenna

et al. 2003) using the translated amino acid configuration to keep the analogous codon positions lined up. The molecular sequence data were analysed by maximum likelihood using the general time reversible model of substitution with PhyML, and 1000 replicates were calculated to determine the reliability of the phylogenetic reconstruction (Guindon and Gascuel 2003). The phylogenetic tree was visualized and edited using FIGTREE (v.1.3.1) (Rambaut 2007). OTUs were generated using Mothur v.1.25.1 (Schloss et al. 2009) at a 94 % sequence similarity cut-off for nucleotide sequence of the *pmo*A gene (Antony et al. 2010). Similarity matrices were calculated based on the nucleotide alignment using the program MatGAT (v.2.02) (Campanella et al. 2003).

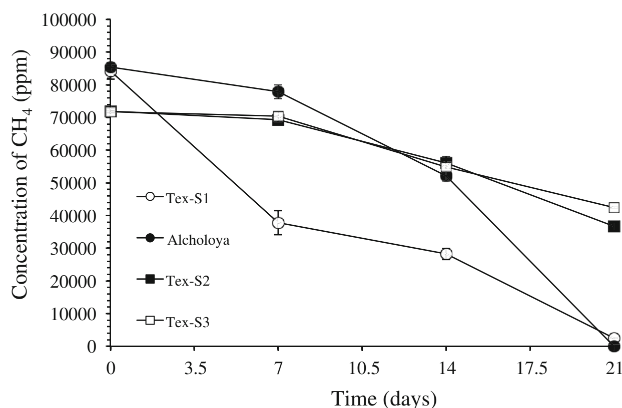

Fig. 1 Change in headspace methane concentration with time for the arable soil of Alcholoya and soils of the former Lake Texcoco: Tex-S1 with low EC (0.6–0.7 dS m^{-1}), Tex-S2 with medium EC (5.9–10.8 dS m^{-1}) and Tex-S3 with high EC (79.3–89.9 dS m^{-1}). Data are the means of triplicate gas analyses

Results and discussion

Methane oxidation in soils

Difficulties in isolating methanotrophs and unsuccessful assays to measure CH_4 oxidation by gas chromatography questioned the presence of methanotrophs in hypersaline environments (Conrad et al. 1995). Since then, however, consumption of CH_4 has been demonstrated in brine lakes, soda lakes and in extremely alkaline and saline lakes (Khmelenina et al. 2000; Lin et al. 2004; 2005). Methanotrophic bacteria have been isolated and culture-independent studies have demonstrated the presence of methanotrophs in alkaline and hypersaline environments (Lin et al. 2005; Sorokin and Kuenen 2005; Eshinimaev et al. 2008). The extreme soil of the former Lake Texcoco is a unique environment where salinity and pH are highly correlated with the composition of the bacterial and archaeal communities (Valenzuela-Encinas et al. 2008, 2009, 2012).

Samples of the four soils: three of the former Lake Texcoco with different salinity and one arable soil were incubated in triplicate with high CH_4 concentration (7.5–8.5 % CH_4 in headspace) (Fig. 1). The methanotrophic activity in these soils was assayed and the potential CH_4 oxidation rates measured after 21 days (Table 1). The results showed that the microbial community metabolized CH_4, and no abiotic factor affected the concentration of CH_4 as no CH_4 oxidation occurred in the sterilized soils.

Methane oxidation rates in the soil of the former Lake Texcoco with low EC (Tex-S1) and the arable soil were similar. The Alcholoya soil consumed nearly all CH_4 added to the headspace (8.5 % of CH_4 in headspace) within 21 days, whereas that Tex-S1 consumed 97 % (Fig. 1). The highest methanotrophic activity in Tex-S1 was between days 0 and 7, while in Alcholoya soil there was a lag or adaptation phase in the first week of the incubation (Fig. 1). The methanotrophic activity in Tex-S1 was stimulated faster than in the Alcholoya soil, so this suggested that the methanotrophic community composition was different in these two soils. The soils of the former Lake Texcoco with medium EC (Tex-S2) and high EC (Tex-S3) showed lower consumption of CH_4 than the other soils; just 49 % of the added CH_4 was consumed in Tex-S2 and 41 % in Tex-S3 within 21 days. Similar to the Alcholoya soil, the Tex-S2 and Tex-S3 soils needed a week of incubation with 7.5 % CH_4 in the headspace, before methanotrophic activity was detected.

There is little information about CH_4 uptake rates in saline alkaline soils. Even though the level of salinity in soils of the former Lake Texcoco with high EC (85.1 dS m^{-1}) and medium EC (10.4 dS m^{-1}) was more than of a saline soil (EC 3.2 mS cm^{-1}) from the Yellow River in China (Zhang et al. 2011), the CH_4 oxidation rate in the saline alkaline soils of the former Lake Texcoco (12.8–15.0 mg CH_4 kg^{-1} dry soil day^{-1}) (Table 1) was higher than that in soil from the Yellow River China (14–24 μg CH_4-C m^{-2} h^{-1} considering a bulk density of 0.93 g cm^{-3} and 20 cm of depth). The haloalkaliphilic methanotrophs have adapted to live in those environments (Khmelenina et al. 2000; Sorokin et al. 2000); it is possible that when water and substrates (CH_4 and O_2) are applied, methanotrophic activity is stimulated.

Han et al. (2009) reported an CH_4 oxidation rate of 3.0 μmol CH_4 g^{-1} wet soil day^{-1} or 48 μg CH_4 g^{-1} wet soil day^{-1} in an alkaline soil (pH 9.4) from a Chinese coal mine, which is higher than that found in Tex-S3 and Tex-S2 soils (8.2 and 9.8 μg CH_4 g^{-1} wet soil day^{-1}, respectively). However, the alkaline soil used in the study of Han et al. (2009) was constantly exposed to high (3000 ppm)

CH_4 concentrations, which might explain the higher CH_4 oxidation rate.

Khmelenina et al. (2000) reported a CH_4 oxidation rate of 33.2 nmol ml^{-1} day^{-1} in sediments from soda lakes at Khuzhirta [pH 10.2 low salinity (Lin et al. (2004)] and of 18.2 nmol ml^{-1} day^{-1} in sediments of soda lakes [pH 9.5 and 4 % salinity (Lin et al. (2004)] at Gorbunka. Assuming a bulk density of the sediment of 1.77 g ml^{-1} (Avnimelech et al. 2001), the CH_4 oxidation rates in these sediments (0.30 and 0.16 µg CH_4 g^{-1} sediment day^{-1}) were lower than those found in the soils Tex-S3 and Tex-S2. Despite the higher pH and salinity in the Texcoco soils than in the soda lake sediments, the availability of O_2 in our soils might have favoured a higher aerobic methanotrophic activity. The organic C content in the Texcoco soils was high, which is another factor that might have favoured high CH_4 oxidation rates, as organic material is a substrate for CH_4 production when soils are flooded.

Methanotrophic communities in the soils of the former Lake Texcoco

Detection of *pmo*A partial sequences was also difficult. A first set of primers targeting the *pmo*A gene (A189/mb661) was used and no detectable amplification was achieved for the three soils of the former Lake Texcoco. A nested PCR protocol was then used to detect the aerobic methane-oxidizing communities as previously reported by Horz et al. (2005). The primer sets used in this study (A189/A682, mb661, A650) have been reported to generate high yields of *pmo*A amplicons (Bourne et al. 2001; Horz et al. 2005). Horz et al. (2005) detected two new clades (JR-2, JR-3) using semi-nested PCR and by using all these primers. Additionally, an arable soil was used as control to validate our experimental methods, i.e. to verify the single amplification of *pmo*A gene partial sequences. The soil samples from Alcholoya had been previously studied and showed CH_4 oxidation capacity (0.09 ng CH_4 kg^{-1} dry soil day^{-1}) when incubated with atmospheric CH_4, i.e. 1.7 ppm (Serrano-Silva et al. 2011).

A total of 253 putative *pmo*A partial gene sequences were obtained in this study. Twenty-seven sequences were obtained from the Alcholoya soil, 63 from the soil of the former Lake Texcoco with low EC, 85 from the medium EC and 78 from the high EC. These sequences were grouped in 22 OTUs based on a 94 % threshold of nucleotide sequence similarity using the furthest neighbour algorithm (Schloss et al. 2009; Antony et al. 2010). The methanotrophic communities in soils of the former Lake Texcoco (Tex-S1, Tex-S2 and Tex-S3) were difficult to identify. Despite nested PCR protocol with specific *pmo*A primers (mb661 and A650) to avoid *amo*A (*Nitrosococcus*-like ammonia oxidizers) co-amplification, a high number of *amo*A sequences (>92 % of clones) mainly in the soils with medium and high EC (Tex-S2 and Tex-S3) were found (Fig. 2). The results indicate a low number of methanotrophs with little diversity in the soils of the former Lake Texcoco. The methanotrophic diversity in saline alkaline environments, mainly soda lakes, seems restricted to a few prominent genera (Sorokin and Kuenen 2005; Antony et al. 2012).

The Texcoco soil with low EC (Tex-S1) had a higher number of OTUs than the other Texcoco soils with medium and high EC (Table 2). The decrease in pH and salinity, the addition of nutrients to soil through flooding with residual effluents and the growth of a halotolerant vegetation (Luna-Guido et al. 2000) might have favoured the presence of methanotrophs in Tex-S1. However, the arable soil from Alcholoya had more OTUs than the Texcoco soils (Table 2). The lower methanotrophic activity in saline alkaline soils of the former Lake Texcoco (Tex-S2 and Tex-S3) compared to that in the arable soil appears to agree with the limited diversity of the *pmo*A gene found in those soils.

Partial sequences of *mmo*X were not detected in soils of this study. The abundance of *Methylocella*, *Methyloferula*, or any other genera containing sMMO, might have been too low to be detected.

Phylogenetic analysis of the partial *pmo*A sequences

Most of the methanotrophs found in the Texcoco soils belonged to type I (Gammaproteobacteria) (Fig. 2). The methanotrophs in sediments of soda lakes seem restricted to a few prominent genera, mainly type I—Gammaproteobacteria, such as *Methylomicrobium* and *Methylobacter* (Sorokin and Kuenen 2005; Antony et al. 2012). It has been reported that methane-oxidizing communities of saline and alkaline environments (saline ponds and soda lakes) are dominated by type I methanotrophs (Trotsenko and Khmelenina 2002; Semrau et al. 2010). Lin et al. (2004) examined the methanotrophic community structure and the active population in soda lake sediments from the lake Suduntuiskii Torom and lake Gorbunka by analysing sequences of DNA encoding for 16S rRNA and functional genes (*pmo*A and *mmo*X) diversity and by using stable isotope probing. Most of the DNA sequences encoding for 16S rRNA (50 %) and *pmo*A (100 %) genes detected in the ^{13}C-DNA fraction were type I methanotrophs in sediments of both soda lakes (Lin et al. 2004).

In Tex-S1, a *Crenothrix*-like genotype, was found (Fig. 2; Table 2). Similar genotypes have been retrieved from deciduous forest soil (Levine et al. 2011). *Crenothrix polyspora* is a filamentous methane-oxidizing bacterium, characterized by brownish-beige mass developments, contaminating water pipes (Völker et al. 1977; Stoecker

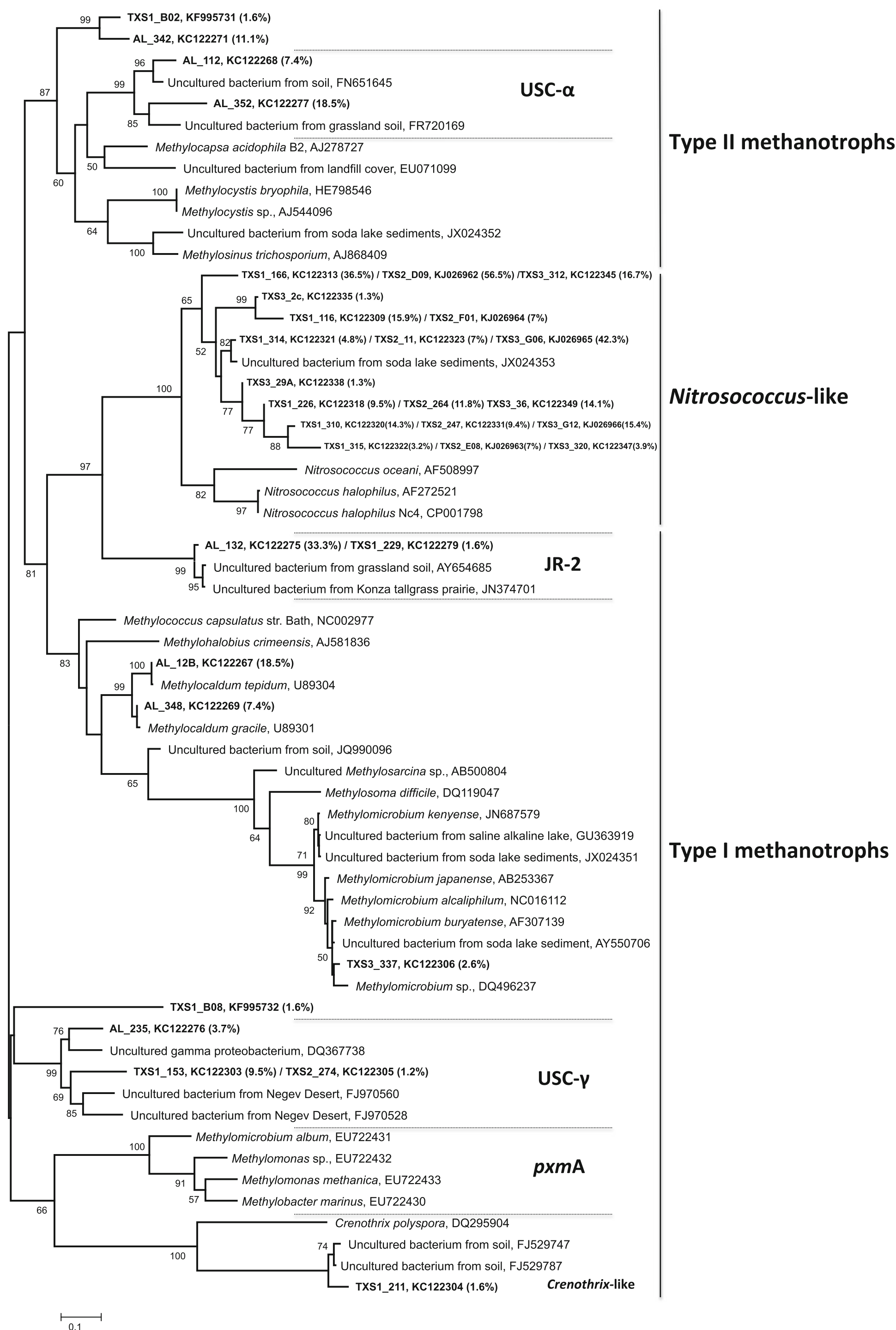

Fig. 2 Maximum-likelihood tree derived from the nucleotide sequences of *pmo*A genes from different soil samples. Clones TXS1 obtained from Texcoco soil with low EC (0.6–0.7 dS m^{-1}), TXS2 from medium EC (5.9–10.8 dS m^{-1}) and TXS3 with high EC (79.3–89.9 dS m^{-1}). AL are sequences from the Alcholoya arable soil. Reference sequences from cultured and uncultured bacteria were obtained from the GenBank. OTUs with 6 % difference were used for the analysis. Bootstrap values ≥50 % are mentioned. The percentages for abundance of different *pmo*A clone types are indicated in parenthesis. The *scale bar* represents 10 % sequence divergence

et al. 2006). Genotypes that oxidize atmospheric concentration of CH_4 belonging to the upland soil clusters (USC)-γ and JR-2 clades were also detected in Tex-S1 (Fig. 2; Table 2). Sequences belonging to the USC-γ clade have frequently been detected in soils with pH > 6.0 (Knief et al. 2003; Bissett et al. 2012). USC-γ phylotypes were detected in the Alcholoya soil (pH 6.7) and in the Texcoco soil with low (pH 8.5) and medium EC (pH 10.3). This suggests that the USC-γ phylotypes are neutrophilic or alkaliphilic methanotrophic bacteria as stated by Knief et al. (2003). Both, halophilic and alkaliphilic *Methylomicrobium* methanotrophs have been isolated from soda lakes and marine environments (Nakamura et al. 2007; Jensen et al. 2008; Semrau et al. 2010). A *Methylomicrobium*-like phylotype was found in the Texcoco soil with high EC (Tex-S3) (Fig. 2; Table 2). *Methylomicrobium*

Table 2 Closest affiliation of *pmo*A sequences from the saline alkaline soils of the former lake Texcoco and the arable soil of Alcholoya

Representative OTU	Closest affiliation (accession number)	Similarity (%)[a]	Methanotrophic group affiliation (type)	Per cent of clones in the library[g]
Soil				
Tex-S1[b]				
TXS1_211	Uncultured bacterium, soil (FJ529787)	93.4	*Crenothrix*-like	10.0 (1)
TXS1_153[f]	Uncultured bacterium, Negev desert (FJ970560)	80.4	USC-γ (type Ic)	60.0 (6)
TXS1_229[e]	Uncultured bacterium, grassland soil (AY654685)	96.3	JR-2 (type Ic)	10.0 (1)
TXS1_B02	*Methylocystis bryophila* (HE798546)	70.8	Type II	10.0 (1)
TXS1_B08	Uncultured gamma proteobacterium clone pmoAg3 (DQ367738)	67.1	USC- γ (type Ic)	10.0 (1)
Tex-S2[c]				
TXS2_274[f]	Uncultured bacterium, Negev desert (FJ970560)	80.4	USC-γ (type Ic)	100 (1)
Tex-S3[d]				
TXS3_337	Uncultured bacterium, soda lake sediment (AY550706)	97.7	*Methylomicrobium* (type Ia)	100 (2)
Alcholoya				
AL_112	Uncultured bacterium, Soil (FN651645)	91.8	USC-α (type II)	7.4 (2)
AL_235	Uncultured bacterium, Negev desert (FJ970560)	81.7	USC-γ (type Ic)	3.7 (1)
AL_12B	*Methylocaldum tepidum* (U89304)	98.9	Type Ib	18.5 (5)
AL_352	Uncultured bacterium, grassland soil (FR720169)	80.4	USC-α (type II)	18.5 (5)
AL_132[e]	Uncultured bacterium, grassland soil (AY654685)	95.4	JR-2 (type Ic)	33.3 (9)
AL_342	*Methylocapsa acidiphila* B2 (AJ278727)	75.3	Type II	11.1 (3)
AL_348	*Methylocaldum gracile* (U89301)	99.8	Type Ib	7.4 (2)

[a] Calculated with MATGAT (v.2.02) program

[b] Texcoco soil with low EC

[c] Texcoco soil with medium EC

[d] Texcoco soil with high EC

[e] OTUs shared within Alcholoya and Tex-S1

[f] OTUs shared within Tex-S1 and Tex-S2

[g] Number of clones in the library in parenthesis

spp. are methanotrophs frequently found in saline environments, for example *M. alcaliphilum* 20Z was isolated from sediments of Tuva soda lakes (Central Asia) and represents a typical methanotrophic bacteria adapted to high-pH and high-salinity ecosystems (Khmelenina et al. 1997). The methane oxidation activity of *M. alcaliphilum* depended on salt concentration and pH with an optimum at pH 9.0 and 0.7 M NaCl (Khmelenina et al. 1997). Although specific primers for methanotrophs containing only sMMO were not used and partial sequences of *mmo*X were not detected, the presence of *Methylocella*, *Methyloferula* or other genera containing sMMO cannot be ruled out in the saline alkaline soils of the former Lake Texcoco.

The sequences obtained from the arable soil, i.e. Alcholoya, were located within previously described *pmo*A clusters (Fig. 2). The described clades included groups belonging not only to the methanotrophs type I, such as USC-γ and JR-2, but also to the group USC-α from the methanotrophs type II (Table 2). Most clones (33 %) of the Alcholoya soil were found within the JR-2 cluster, while 26 % were closely related to *Methylocaldum*-like clade (Table 2). It has been postulated that USC-α, USC-γ and Jasper Ridge cluster JR-2 represent yet uncultivated methanotrophic bacteria from upland soils consuming atmospheric CH_4 (Holmes et al. 1999; Knief et al. 2003; Lüke and Frenzel 2011). Methanotrophs within the *Methylocaldum*-like clade, detected also in the Alcholoya soil, are a group of methanotrophs that are widely distributed in nature (Bodrossy et al. 1997). Their habitats are thermal springs, activated sludges, arable soils, silage wastes and manure (Trotsenko et al. 2009). *Methylocaldum gracile* can grow at 20 °C and *Methylocaldum tepidum* at 30 °C, both

optimally at 42 °C and at a maximum of 47 °C (Bowman 2006). Both have a thick capsule resembling *Azotobacter*-type cysts, unlike typical cysts of methanotrophs (Bowman 2006; Trotsenko et al. 2009). Phylotypes within the USC-α clade were related to phylotypes that were retrieved from beech and spruce forest soils (Degelmann et al. 2010), pasture and pine soils (Singh et al. 2009), a receding glacier forefield in southeast Greenland (Bárcena et al. 2011) and natural forest and afforested soils (Dörr et al. 2010). All of them consumed CH_4 at atmospheric concentrations.

Conclusions

The obtained CH_4 oxidation activity and the *pmo*A sequences retrieved in this study showed that an aerobic methanotrophic community exists in all the soils studied. Our results suggest that increased salt content and pH reduced the methane oxidation activity. The methanotrophic communities in the saline alkaline soils of the former Lake Texcoco differed from those found in the arable Alcholoya soil by using *pmo*A as a functional marker. The presence of only type I methanotrophs (Gammaproteobacteria) in the alkaline saline Texcoco soils and the sequences obtained, such as those of *Methylomicrobium* sp., were similar to those retrieved from other saline alkaline environments (sediment soda lakes). Several other sequences from the Texcoco libraries (>92 %) related to the *Nitrosococcus*-like clade were co-amplified.

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Methanogenesis and Methanotrophy in Soil: A Review[*1]

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ABSTRACT

Global warming, as a result of an increase in the mean temperature of the planet, might lead to catastrophic events for humanity. This temperature increase is mainly the result of an increase in the atmospheric greenhouse gases (GHG) concentration. Water vapor, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are the most important GHG, and human activities, such as industry, livestock and agriculture, contribute to the production of these gases. Methane, at an atmospheric concentration of 1.7 μmol mol^{-1} currently, is responsible for 16% of the global warming due to its relatively high global warming potential. Soils play an important role in the CH_4 cycle as methanotrophy (oxidation of CH_4) and methanogenesis (production of CH_4) take place in them. Understanding methanogenesis and methanotrophy is essential to establish new agriculture techniques and industrial processes that contribute to a better balance of GHG. The current knowledge of methanogenesis and methanotrophy in soils, anaerobic CH_4 oxidation and methanotrophy in extreme environments is also discussed.

Key Words: anaerobic CH_4 oxidation, biological production, global warming, methanogenic archaea, methanotrophic bacteria

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INTRODUCTION

The rising temperature of earth's atmosphere and oceans, known as global warming (GW), is mainly the result of an increase in the concentration of greenhouse gases (GHG) in the atmosphere since the beginning of the 20th century, and this increase is mostly due to anthropogenic activities (IPCC, 2007; Houghton, 2009). Global warming is one of the major threats to the environment because of the resulting climate change.

Oxygen (O_2) and nitrogen (N_2) are the principal components of the atmosphere at concentrations of 21% and 78%, respectively, but they do not absorb or emit thermal radiation (Seinfeld, 2011). However, water vapor and less abundant components, such as carbon dioxide (CO_2) (390 μmol mol^{-1}), methane (CH_4) (1.7 μmol mol^{-1}) and nitrous oxide (N_2O) (0.3 μmol mol^{-1}) are known as greenhouse gases, because of their long atmospheric lives and their relatively high thermal absorption capacities. The global warming potential (GWP), a quantification of the averaged relative radiative forcing impacts of a particular greenhouse gas, has been set as 1 for CO_2, and, in consequence, the values for CH_4 and N_2O are 25 and 298, respectively, for a 100 year time horizon (IPCC, 2007). Besides water vapor, the most important greenhouse gas is CO_2, but CH_4 and N_2O are also important contributors to GW (Seinfeld, 2011).

Greenhouse gases in the atmosphere play an important role for life on earth. It has been calculated that the average temperature of earth, which is 15 °C nowadays, would be −15 °C or −18 °C if the GHG were not present, which would severely limit life on earth (Seinfeld, 2011).

Biological or non-biological, and natural or anthropogenic processes are involved in GHG cycling. Soils, depending on the conditions, can be a source or a sink of CO_2, CH_4 and N_2O. Unaltered natural soils are principally a sink for CH_4 and N_2O and sequester as much carbon (C) as they emit CO_2, but due to human activities, mainly agriculture, soils are often a source for GHG (Christiansen *et al.*, 2012). In 2005, agriculture accounted for 5 100–6 100 Tg CO_2-equivalents $year^{-1}$, or 10%–12% of total global GHG: 60% of total global N_2O emissions, 50% of CH_4 emissions and less than 1% of CO_2 emissions (Smith, 2012).

After CO_2, CH_4 is the second most important GHG

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produced as a result of human activity. Methane is responsible for approximately 16% of the greenhouse effect (Aydin *et al.*, 2010). Methane concentration in the atmosphere remained stable for thousands of years (0.715 μmol mol^{-1}), but recently, due to human activities, it has more than doubled (1.774 μmol mol^{-1}) (IPCC, 2007). Nowadays its concentration in the atmosphere increases at a rate of 0.003 μmol mol^{-1} year^{-1} (Butenhoff and Khalil, 2007).

Whalen (2005) estimated a total annual emission of CH_4 from natural and anthropogenic sources of 600 Tg year^{-1}. Natural sources account for 40% of global CH_4 emissions, whereas 60% comes from anthropogenic sources (Karakurt *et al.*, 2012). Around 70% of atmospheric CH_4 originates from biogenic sources (Conrad, 2009). Biological processes in anoxic environments are considered the main biogenic source of atmospheric CH_4 (Houghton *et al.*, 2001) and produced by a group of microorganisms (methanogens) belonging to the domain archaea.

Anthropogenic sources of CH_4 are the result of agriculture, energy, waste and industrial production, with agriculture and energy production the most important. Waste, energy, industry and agriculture contribute 20.61%, 28.65%, 0.10%, and 50.63% of the calculated annual CH_4 emission, respectively (Karakurt *et al.*, 2012) (Fig. 1). Landfills are responsible for an annual emission of 3%–10% of the total global emissions (Bogner and Matthews, 2003) and are among the largest anthropogenic sources of CH_4 in the United States (USEPA, 2007).

CH_4 PRODUCTION IN SOILS

Most of CH_4 production in soils has been attributed to anaerobic methanogenesis when anoxic microsites are formed, which serve as a refuge for methanogenic archaea (Watanabe *et al.*, 2007), or in flooded soils where a low redox potential required for CH_4 production is present (Dalal *et al.*, 2008). However, several authors reported CH_4 production from oxic environments (Von Fischer and Hedin, 2007). Methane is used as a C and energy source by methanotrophic microorganisms (Smith *et al.*, 2003) and this process contributes up to 10% of global CH_4 oxidation (Lowe, 2006).

While forests are considered important sinks of CH_4 (Grunwald *et al.*, 2012), wetlands are the major biological source (Conrad, 2009), contributing 145 Tg CH_4 year^{-1} (Norina, 2007). Flooded rice fields, mostly in the humid tropics, are among the largest sources of atmospheric CH_4 with an estimated contribution of approximately 5%–19% of total global emissions (IPCC, 2007). Their contribution is increasing as the production of rice (*Oryza* sp.), a staple food in Asia and becoming more popular in the world, to satisfy an increasing demand of a growing world population (Nguyen and Ferrero, 2006).

Northern-latitude soils are of great importance in the global budget of CH_4 as they contain one-third of the global organic C pool (Post *et al.*, 1982), although recent studies suggest that even more C might be stored in these regions (Jungkunst, 2010). As these soils are water saturated during summer, there is a large CH_4 production due to anaerobic metabolism. Because of this, it is expected that in the Arctic the effects of the observed and predicted climate change will be stronger than the global average (Trenberth *et al.*, 2007) and increase with time (Wuebbles and Hayhoe, 2002).

Kammann *et al.* (2009) reported that significant amounts of CH_4 were produced even after homogenization of soil samples, where the anoxic microsites were destroyed. This has led to some authors to hypothesize that methanogens are not the sole source of CH_4 in

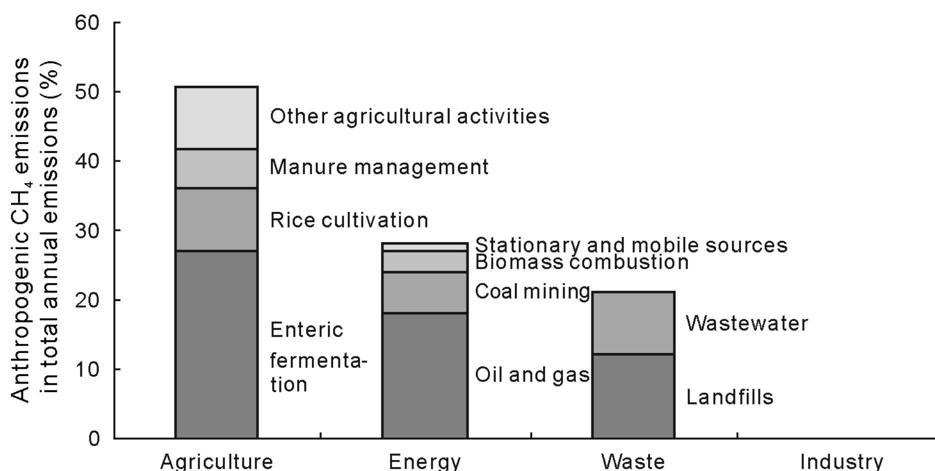


Fig. 1 Anthropogenic methane (CH_4) emissions by sectors and sources in 2010 (Karakurt *et al.*, 2012; Yusuf *et al.*, 2012).

oxic soils, but the possibility of non-microbial CH_4 formation in soils has to be considered (Von Fischer and Hedin, 2007). Hurkuck *et al.* (2012) conducted an experiment in which CH_4 release from soil increased with increasing temperature and organic C content, and also with the addition of water to dried soils. Some of the temperatures tested (up to 70 °C) were beyond the limits of the known enzymatic activity of methanogens, therefore excluding the possibility of microbial production of CH_4. This suggests the existence of an unknown chemical process that produces CH_4 under oxic conditions in soil. Recently, abiotic formation of CH_4 under highly oxidative ambient conditions has been described and it might be important in soils (Althoff *et al.*, 2010). The naturally available compounds of ascorbic acid, ferrihydrite and hydrogen peroxide are involved, but the reaction pathway is still unknown.

Keppler *et al.* (2006) demonstrated, by using stable C isotopes, that CH_4 can be produced *in situ* in living terrestrial plants and plant litter under oxic conditions by some hitherto unknown process and its impact on global warming is significant, *i.e.*, 62–236 Tg year^{-1} for living plants and 1–7 Tg year^{-1} for plant litter. This process might explain the elevated CH_4 mixing ratios observed recently above evergreen forests in tropical regions indicating an additional tropical source of CH_4 (Houghton *et al.*, 2001; Keppler *et al.*, 2006).

Mechanisms for CH_4 emissions from soil

Possible mechanisms for CH_4 emission from soil to the atmosphere include i) diffusion of dissolved CH_4 along the concentration gradient, ii) release of CH_4-containing gas bubbles (ebullition), and iii) transport *via* the aerenchyma of vascular plants (plant-mediated transport) (Fig. 2). These three mechanisms rule the spatial and temporal variations in CH_4 production (Lai, 2009).

The first process, diffusion, takes place because of the formation of a CH_4 concentration gradient from deeper soil layers, where the production of CH_4 is large, to the atmosphere, while oxidation of CH_4 occurs in upper layers (10%–40% in rice paddies) (Krüger *et al.*, 2002; Lai, 2009). Diffusion is a slow process compared to the other two transport mechanisms, *i.e.*, ebullition and plant-mediated transport, but it is biogeochemically important because it extends the contact between CH_4 and methanotrophic bacteria in the upper aerobic layer, promoting CH_4 oxidation (Whalen, 2005).

The second process, ebullition, takes place when CH_4 production is large. Gas bubbles are formed and emigrate to the surface. As this process is fast, CH_4 oxidation is absent or negligible (Conrad, 1989). In a bog or a paddy field, the high CH_4 content of the bubbles reaching the surface is an indicator of the high CH_4 concentration in the root zone (Smith *et al.*, 2003). In Vercelli, northern Italy, the concentrations of CH_4 in bubbles in a rice paddy ranged from 0.025 to 0.15 mol CH_4 mol^{-1} (2.5%–15% CH_4), and increased with time (Marik *et al.*, 2002). Similar results were found by Krüger *et al.* (2002) at the same site. They also found 0.01–0.04 mol CH_4 mol^{-1} in the plant stems, concentrations substantially lower than those in the bubbles. Thus, in plants adapted to flooded environments, the concentration gradient formed from the root zone to the air outside, through the plant stems, promotes diffusion of CH_4 to the atmosphere (Smith *et al.*, 2003).

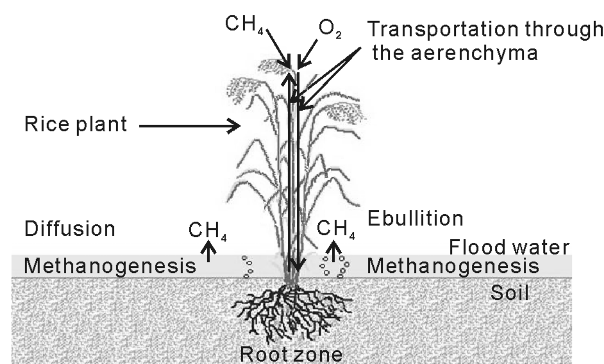


Fig. 2 Methane (CH_4) transportation mechanisms in rice fields. The vascular structure named aerenchyma is used for the plant to transport oxygen (O_2) to the root and submerged organs and also serves for the diffusion of CH_4 from the rhizosphere to the atmosphere (plant mediated transport). Modified from Lai (2009).

The third process, plant mediated transport, takes place through an internal system of continuous air spaces named aerenchyma, a structure which is developed by vascular plants to adapt to flooded environments (Lai, 2009). The basic function of this structure is to transport the O_2 necessary for root respiration and cell division in submerged organs (Joabsson *et al.*, 1999), but it is also used for CH_4 transportation from the rhizosphere to the atmosphere, bypassing the aerobic, CH_4-oxidizing layers (Brix *et al.*, 1992; Smith *et al.*, 2003; Whalen, 2005). This process involves two major mechanisms: molecular diffusion and bulk flow (Joabsson *et al.*, 1999). The gradient of CH_4 concentration formed inside the aerenchyma conduits is the driving force for CH_4 diffusion from the peat root zone to the aerial parts of the plant.

The other plant-mediated transport mechanism, bulk transportation, involves the migration of CH_4 along the plant, also through the aerenchyma structure, from the leaves to the rhizome and back to the atmosphere through old leaves or horizontal rhizomes

connected to other shoots. The driving force for this process is a pressure gradient generated by differences in temperature or water vapor pressure between the internal air spaces in plants and the surrounding atmosphere (Brix *et al.*, 1992). This is a very efficient and rapid mechanism of CH_4 transportation and, in consequence, it is responsible for most of CH_4 emissions ($>$ 95%) to the atmosphere from rice paddies (Banker *et al.*, 1995).

Factors affecting CH_4 fluxes in a soil

Dalal *et al.* (2008) mentioned that the factors controlling CH_4 production in soil are anaerobic conditions and redox potential, electron acceptors, substrate availability, temperature, diffusion, water availability and water table, soil pH and salinity, fertilizer and manure additions and amendments, trace metals, competitive inhibition, vegetation, plant species and cultivars, and elevated CO_2 concentrations.

The concentration and type of organic matter and the concentration of O_2 are considered the determining factors for CH_4 production (Dalal *et al.*, 2008). Generally, nitrogen (N) and phosphorus (P) limitations are not frequently observed in methanogenesis (Bachoon and Jones, 1992).

The presence of some compounds can be unfavorable for methanogenesis. Sulfate-rich anaerobic environments inhibit the activity of methanogens because of the competition with sulfate-reducers for substrates, such as hydrogen (H_2) and acetate (Muyzer and Stams, 2008) and the toxicity of sulfide formed during anaerobiosis (Paula Jr. and Foresti, 2009).

Effect of chemical fertilizers and floodwater depth

The type and the mode of application of a fertilizer affect CH_4 emissions from a flooded soil. In a rice paddy field with 30 cm water depth, CH_4 emission was lower in plots treated with a mixture of urea and Nimin (Godrej Agrovet Limited, Bombay) than in plots with no fertilizer and prilled urea alone (Sethunathan *et al.*, 2000). Nimin is known to inhibit autotrophic oxidation of NH_4^+ to NO_2^- (Sahrawat and Parmar, 1975; Patra and Chand, 2009).

N-Fertilizer additions

There are a number of studies investigating the effect of N-fertilizers addition on CH_4 emissions in different agricultural systems with different results. Zou *et al.* (2005) found in a 3-year field experiment in rice paddies that CH_4 emissions were reduced when urea was applied, while N_2O emissions significantly increased. In flooded soils, CH_4 emissions were lower when ammonium sulfate was used as a fertilizer compared to urea, and this was explained by the competition of sulfate-reducing bacteria with methanogens (Bufogle *et al.*, 1998). In another work in a rice field, CH_4 emissions decreased when fertilized with nitrate and this was due to an increase in the redox potential by the NO_3^- added (Jugsujinda *et al.*, 1995). Hou *et al.* (2000) reported that, when urea and manure were used as fertilizers, CH_4 production could be prevented and the reduction of N_2O to N_2 would be encouraged by maintaining the soil redox potential between -100 to 200 mV. In studies conducted in paddy fields applied with ammonium sulfate or potassium nitrate, the former significantly reduced CH_4 emissions compared to the latter (Liou *et al.*, 2003; Snyder *et al.* 2009).

Depth to the water table

In natural northern wetlands, the type of vegetation and the depth to the water table are the factors that have an important effect on CH_4 emissions, with the latter being more important (Smith *et al.*, 2003). Several authors reported a positive correlation between CH_4 emissions and the depth to the water table (von Arnold *et al.*, 2005).

Type and activity of vegetation

Methanogenic microorganisms use root exudates as easily degradable substrates. At the same time, as mentioned before, some plants may transport CH_4 and oxygen from the air to the root zone, *via* aerenchyma. This leads to the possibility of CH_4 oxidation in the rhizosphere (Le Mer and Roger, 2001; Philippot *et al.*, 2009). Therefore, plant activity plays an important role in the regulation of CH_4 fluxes in peatlands with a deep water table, and this has to be considered in the prediction of CH_4 emissions when climatic conditions change (Norina, 2007).

Temperature and CO_2 concentration

An elevated CO_2 concentration and high temperature increased methanogenesis and decreased CH_4 oxidation in tropical rice soils (Das and Adhya, 2012). It is expected that CH_4 emissions from these soils will increase with increasing atmospheric CO_2 concentration and temperature due to global warming (Das and Adhya, 2012). This is related to a decrease in soil redox potential and an increase in C substrates like acetate, favoring the proliferation of methanogens. Additionally, the methanotrophic bacterial population decreases as the redox potential decreases in a CO_2 enriched environment (Das and Adhya, 2012). However, the reported data were derived from short-term

incubation experiments under highly controlled conditions. Long-term studies simulating field conditions including rice plants are needed to expand our knowledge of methanogenesis and methanotrophy when both temperature and CO_2 concentrations increase.

Organic matter

Addition of fresh organic materials (rice straw and other cellulosic materials) to a rice soil enhanced CH_4 production and emission as they served as C substrates for soil microorganisms. Composted organic sources (Azolla compost, blue green algae (BGA) compost, and farmyard manure) had less effect on the production of CH_4 (Favoino and Hogg, 2008). Application of poultry manure resulted in low emission of CH_4 as its high sulfur content inhibited methanogenic microorganisms (Sethunathan *et al.*, 2000).

Influence of floodwater regimes

In non-flooded soils, methanogenic activity is generally low as the redox potential is not favorable for methanogens (van Cleemput *et al.*, 1983). In the 1960s, the shift from continuous flooding to mid-season drainage irrigation significantly reduced the amount of CH_4 emissions from rice paddies in China (Zhang *et al.*, 2011).

Effect of pesticide application

There are a number of reports about the inhibitory effect of pesticides on CH_4 production. Chakraborty *et al.* (2002) reported that Tara-909 (Dimethoate 300 g kg^{-1}, Emulsifiers, solvents and auxiliaries 700 g kg^{-1}; Sarabhai Chemicals Ltd., India.), a pesticide extensively used in rice paddy fields, strongly inhibited methanogenesis *in vitro*. Mohanty *et al.* (2004) found a significant inhibition of CH_4 fluxes, both plant-mediated and ebullition, in a rice flooded field by the herbicide butachlor (N-butoxymethyl-2-chloro-2′,6′-diethyl acetanilide) applied at 1 kg active ingredient ha^{-1}. The organochlorine insecticide dichlorodiphenyltrichloroethane (DDT) and carbofuran, a carbamate insecticide, are known to inhibit CH_4 production in rice paddies (Kumaraswamy *et al.*, 2000).

Process of methanogenesis

Biological production of CH_4 can only starts when some chemical agents with a high redox potential, *i.e.*, molecular oxygen, nitrate, iron (III) (Fe^{3+}), manganese (IV), sulphate, and possibly humic acids, have been reduced completely (Garcia *et al.* 2000; Dalal *et al.* 2008), as the reduction of these alternative electron acceptors supplies more energy than methanogenesis (Zehnder and Stumm, 1988). In natural wetlands and flooded rice fields, as well as in lake sediments, where a methanogenic enriched microbial population can be found, such low redox potentials are commonly found as a result of prolonged water logging (Smith *et al.*, 2003) and consequently CH_4 production is large (Conrad, 2009).

Methane production is a four-steps process and several groups of microorganisms are involved: 1) hydrolysis of macromolecules and polymers, 2) acidogenesis, 3) acetogenesis, and 4) methanogenesis by archaea, some using acetate as substrate while others using CO_2, H_2, formate or methylated compounds (Le Mer and Roger, 2001; Chandra *et al.*, 2012). The rate of methanogenesis depends on how rapidly hydrogen and/or acetate are supplied by other organisms (Topp and Pattey, 1997).

According to Segers and Leffelaar (1996) the following simple mathematical model describes methanogenesis as a function of the factors that rule the process:

$$R_{\mathrm{MP}} = I \times C \times F \tag{1}$$

where R_{MP} is the methane production rate; I is anaeration inhibition function, which is 1 in complete anaerobic conditions and 0 under aerobic conditions; C is the anaerobic C-mineralization rate; and F is the fraction of the anaerobically degraded C, which is transformed into CH_4.

A basic assumption underlying Eq. 1 is that the availability of organic matter controls CH_4 production. The presence and concentration of alternative electron acceptors explains variations in F (Segers and Leffelaar, 1996).

Microbiology and biochemistry of methanogenesis

Methanogens can be found in a number of environments including marine sediments, digestive tract of almost all animals including humans, ruminants, insects and termites, freshwater habitats, volcanic springs, hydrothermal vents, high saline environments and anaerobic sewage digesters (Garcia *et al.*, 2000).

Methanogenic microorganisms are strictly anaerobes; therefore their activity takes place only at low redox potentials, which occurs when soil is waterlogged (Smith *et al.*, 2003). Methanogens are less competitive thermodynamically for electron donors than the bacterial reducers of nitrate, Fe^{3+} and sulfate in soil (Ma and Lu, 2011). Methanogens depend on other organisms to produce suitable substrates and reducing conditions. Therefore methanogens can only function as members of a microbial community (Topp and Pattey, 1997).

The taxonomy of methanogens has been recently reviewed by Nazaries *et al.* (2013). They are now classified in the domain archaea that includes the most ancient microorganisms. Thirty two genera have been identified, grouped in 12 families, 6 orders and 3 classes, within the phylum *Euryarchaeota*.

Some methanogens are extremophilic tolerant. For instance, the genera *Methanothermobacter*, *Methanothermus*, *Methanothermococcus*, *Methanocaldococcus*, *Methanotorris* and *Methanopyrus* are extreme thermophiles that grow at > 80 °C, while *Methanohalobium*, *Methanohalophilus* and *Methanolobus* are extreme halophiles that grow at 4.3 mol L^{-1} NaCl (Nazaries *et al.*, 2013).

Methanogens have been divided into five groups according to the substrate they use (Garcia *et al.*, 2000; Le Mer and Roger, 2001): i) hydrogenotrophs ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$); ii) formatotrophs ($4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$); iii) acetotrophs ($CH_3COOH \rightarrow CO_2 + CH_4$); iv) methylotrophs ($4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$, $4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$); and v) alcoholotrophs ($4CH_3CHOHCH_3 + CO_2 \rightarrow CH_4 + 4CH_3COCH_3 + 2H_2O$).

According to Norina (2007), methanogens that use acetate, mainly as C source, contribute 70%–90% to CH_4 production, while other substrates like H_2/CO_2 and formate contribute 10%–30%. However, this will vary with the environment studied (Garcia *et al.*, 2000). Methanogens that reduce CO_2 to CH_4 facilitate redox reactions with electrons provided by proton reducing acetogens *via* interspecies H_2 transfer (Whalen, 2005). The reduction of other compounds, such as methanol and methylamine, is not considered quantitatively important in most of the environments (Conrad *et al.*, 2006).

In freshwater sediments and flooded paddy soils, *ca.* 70% of biogenic CH_4 produced, comes from acetate cleavage by acetoclastic methanogens as evidenced by CO_2 reduction by hydrogenotrophic methanogens (Conrad, 2005). In general, acetoclastic methanogenic archaea dominate in flooded paddy soils (Conrad *et al.*, 2008).

All methanogens use NH_4^+ as N source, although the ability to fix molecular N and the N fixation gene (*nif*) is present in four orders of methanogens, *i.e.*, *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* and *Methanosarcinales* (Leigh, 2005).

Methanogens have several unique characteristics including novel co-factors involved in the CH_4-producing pathway, and unusual membrane lipids. Trace quantities of nickel, iron and cobalt are required by all methanogens (Garcia *et al.*, 2000). Most methanogens are mesophilic, able to function in temperature ranging from 20 to 40 °C, but methanogens can be found in the range from 4 to 110 °C (Garcia *et al.*, 2000). The pH range of most methanogens is 6.0–8.0, but there have been reports of moderately acidophilic methanogens that grow best in a pH range from 5.6 to 6.2, and alcaliphilic methanogens that can function in a pH from 8.0 to 9.2 (Garcia *et al.*, 2000).

OXIDATION OF CH_4

Methane undergoes chemical and photochemical oxidations in the atmosphere and stratosphere, and their products, mainly the hydroxyl radical, have a direct or indirect effect on the global warming (Saarnio *et al.*, 2009). However, biological oxidation of CH_4 is of great importance for the global CH_4 balance.

Biological CH_4 oxidation is done by methanotrophic microorganisms (methanotrophs), either aerobic methanotrophic bacteria or a consortium of anaerobic archaea in association with anaerobic bacteria (anaerobic CH_4 oxidation) (Ettwig *et al.*, 2010).

Methanotrophs can be found in a variety of environments where an interface between oxic and anoxic conditions exists (Wendlandt *et al.*, 2010), *i.e.*, soils, rice paddies, peat bogs, wetlands, marine or freshwater sediments and columns, sewage sludge and groundwater, acidic peatlands, landfills, alkaline soda lakes, hot springs, cold environments, and even from highly acidic and thermophilic environments (Semrau *et al.*, 2010). However, optimal activity of most methanotrophs has been found in environments with near neutral pH, temperature in the mesophilic range (*ca.* 25 °C) and low salinity (Le Mer and Roger, 2001).

In some environments, such as sediments and rice paddies, the generation of CH_4 is large, but the net flux is attenuated by an intense low-affinity methanotrophic activity (Conrad, 2009). It is currently considered that 80% of the CH_4 produced in soil by methanogenic archaea is consumed by methanotrophic bacteria at the soil surface, to use as a C and energy source (Hanson and Hanson, 1996; Conrad *et al.*, 2007).

Considering differences in the concentration of CH_4 in soils and sediments, two types of kinetics have been proposed: low and high affinity (Nayak *et al.*, 2007). Low affinity CH_4 oxidation which shows a K_m (a constant which is equal to the value of the substrate (CH_4) concentration, at which half of the maximum enzymatic speed is reached) value in the µmol L^{-1} range, is performed by methanotrophs that are present in all CH_4-producing soils (Reay, 2003). High affinity CH_4 oxidation, performed by methanotrophs with a K_m

value in the range of nmol L^{-1}, enables oxidation at atmospheric concentration (Lau *et al.*, 2007).

The complex intracellular membrane structures that methanotrophic bacteria possess have been used to differentiate taxonomically from other bacteria (Whittenbury *et al.*, 1983). Pure cultures were obtained for the first time in 1906 after years of unsuccessful attempts (Hanson and Hanson, 1996).

Biochemistry and microbiology of methanotrophy

A large number of studies have been published about the phylogeny and biochemistry of low-affinity methanotrophs that can be cultivated in laboratory. Very little information, however, is available about high-affinity methanotrophs, which are the major oxidizers of atmospheric CH_4, as they are very difficult to cultivate in the laboratory (Maxfield *et al.*, 2008).

Methanotrophic bacteria belong to the group of Methylotrophs, which are aerobic bacteria, capable of metabolizing one-C compounds more reduced than formic acid, *e.g.*, CH_4, methanol, formaldehyde, methylated amines, halomethanes, and methylated compounds containing sulfur (Hanson and Hanson, 1996). However, the possibility that they could use other organic substrates more complex than CH_4, such as acetate, has been reported (Semrau *et al.*, 2011).

Methanotrophs were initially grouped into three types: Type I, Type II, and Type X. Type I methanotrophs use the ribulose monophosphate (RuMP) pathway for C assimilation and Type II the serine pathway. Type X methanotrophs have characteristics of both types, but use both the RuMP pathway and ribulose 1, 5-biphosphate for C assimilation. They were, however, reclassified within Type I (Hanson and Hanson, 1996).

More recently, a classification of methanotrophs has been proposed based on the 16S rRNA gene sequences. Type I methanotrophs are grouped within the *Gammaproteobacteria* while Type II within the *Alphaproteobacteria*. Methanotrophs have been classified within 2 phyla, 3 orders and 4 families, and 21 genera and 56 species have been identified. Some are extremophilic/tolerant species (Nazaries *et al.*, 2013). *Methylobacter psychrophilus*, *Methylomonas scandinavica* and *Methylovulum miyakonense* are psychrophiles that grow at 5–10 °C but not above 20 °C; *Methylohalobius crimeensis* is an haloalkaliphile which grows at 12% NaCl and pH 9–11; *Methylosoma difficile* is an halophile that grows at 15% NaCl; *Methylocaldum gracile*, *Methylocaldum szegediense*, *Methylocaldum tepidum*, *Methylococcus capsulatus*, and *Methylococcus thermophiles* are thermophiles that grow at > 45 °C; *Methylocystis heyeri*, *Methylocystis bryophila*, *Methylocapsa acidiphila*, *Methylocapsa aurea*, *Methylocella palustris*, *Methylocella silvestris*, *Methylocella tundra*, and *Methyloferula stellate* are acidophiles that grow at pH 3.8–5.5; and *Methylacidiphilum infernorum*, *Methylacidiphilum fumarolicum*, and *Methylacidiphilum kamchatkensis* are thermoacidophiles that grow at 60 °C and at pH 2 (Nazaries *et al.*, 2013).

Several techniques, such as cloning, denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism (T-RFLP), microarrays, and the techniques of stable isotope probing (SIP) based on DNA, RNA, and phospholipid fatty acids (PLFA), have been used to characterize methanotrophic communities from different environments (Conrad, 2009).

Three functional genes, *pmoA*, *mmoX*, and *mxaF*, which encode for subunits of particulate CH_4 monooxygenase (MMO), soluble MMO, and methanol dehydrogenase, respectively, have been used to detect and identify methanotrophs in a number of environments (Shukla *et al.*, 2009). The *pmoA* gene has been widely used in the characterization of methanotrophic microbial populations in soils as it can be found in almost all methanotrophs and it is phylogenetically comparable with the 16S rRNA gene and even more informative as it gives more resolution in the definition of the phylogenetic tree (Rastogi *et al.*, 2009).

Metabolic pathway for CH_4 oxidation

Three intermediates are involved in the biological oxidation of CH_4 to CO_2, methanol, formaldehyde, and formate. The enzymatic complex MMO initiates the oxidation of CH_4. There are two different types of MMO located in different regions inside the methanotrophic cell, one is solubilized in the cytoplasm (sMMO) and the other is attached to the membrane in a particulate form (pMMO).

While pMMO has been found in almost all methanotrophs, except in the genera *Methylocella* and *Methyloferula*, only a few strains have sMMO (Murrell *et al.*, 2000; Stein *et al.*, 2012). The enzyme sMMO is not constitutive and the inducer for its genetic expression is a Cu to biomass ratio ≤ 2.5 mmol g^{-1} cell. When this ratio is > 2.5 mmol g^{-1} cell, pMMO is synthesized (Stanley *et al.*, 1983). An extended description of each of these enzymes can be found in Murrell *et al.* (2000), Semrau *et al.* (2010), Smith and Murrell (2010), and Wendlandt *et al.* (2010).

The product of the initial oxidation of CH_4 is methanol, which is further oxidized to formaldehyde by methanol dehydrogenase, which is a periplasmic enzyme containing pyrroloquinoline quinone (PQQ) as

a prosthetic group. Formaldehyde is a cytotoxic compound and its oxidation to formate and then to CO_2 is a key step in the oxidation of CH_4 to CO_2 as it provides most of the reducing power needed for the initial oxygenation of CH_4 (Hanson and Hanson, 1996; Wendlandt *et al.*, 2010). Finally, formate is oxidized to CO_2 by formate dehydrogenase (FDH), a NAD^+ dependent enzyme present in all known methanotrophs. Various types of FDH enzymes have been described (Chistoserdova *et al.*, 2004).

Factors affecting methane oxidation in soil

Populations of methanotrophs in soils are affected by a number of environmental factors, mostly temperature, pH, type and concentration of N sources, and variations in concentration of CH_4 and O_2 (Hanson and Hanson, 1996). The extent of the effects depends on whether Type I or Type II predominates. For example, Type I strains predominate in nutrient rich environments, whereas Type II is able to survive in environments where N is scarce (Ho *et al.*, 2013).

Atmospheric CO_2 concentration

Several studies of different ecosystems have indicated that CH_4 consumption is reduced at elevated concentrations of CO_2 (Phillips *et al.*, 2001; Dubbs and Whalen, 2010), but the mechanisms involved are not well understood. It is possible that CO_2 enrichment affects the size or activity of the CH_4-oxidizing microbial community or compete for O_2 as a result of a higher organic C content (Phillips *et al.*, 2001).

Temperature

Several authors considered that the effect of temperature on microbial CH_4 oxidation is lower than that on CH_4 production, and this could be due to the strong affinity of some methanotrophs for CH_4 (Borken *et al.*, 2006). However, contradictory results have been reported. Castro *et al.* (1995) reported that at low temperatures, between 5 and 10 °C, CH_4 oxidation was affected by temperature, but no such effect was found between 10 and 20 °C. In environments with high CH_4 concentrations, *e.g.*, landfill cover soils (Kallistova *et al.* 2005) and composts (Jäckel *et al.* 2005), a pronounced effect of temperature on CH_4 oxidation was found.

It is expected that the microbial community structure changes when the temperature changes. Horz *et al.* (2005) reported that methanotrophic type II population decreased when the temperature increased, with a subsequent change in the rate of CH_4 oxidation. Bender and Conrad (1995), however, did not detect a clear correlation between CH_4 oxidation and temperature.

Soil water content

Soil moisture content is a critical factor for CH_4 oxidation (Gebert *et al.*, 2003; Jugnia *et al.*, 2008). In general, CH_4 oxidation decreases when soil water content increases (Werner *et al.*, 2006) and this could be due to the low solubility and diffusion of O_2 and CH_4 in the soil water-filled pores (Del Grosso *et al.*, 2000).

Several authors reported that at soil moisture contents $< 20\%$ water holding capacity (WHC) CH_4 oxidation rates decreased sharply (Bender and Conrad, 1995; Jäckel *et al.*, 2001). Otherwise, at high moisture contents, methanogenesis is favored due to lower diffusion of O_2. Net CH_4 oxidation has been reported in environments where the water-filled pore space is $>$ 60%, which could be attributed to the presence of aerobic microsites and/or anaerobic CH_4 oxidation (Khalil and Baggs, 2005). Some studies using ^{13}C-labeled CH_4 have shown oxidation and production of CH_4 occurring at the same time in wet soils (Khalil and Baggs, 2005).

After rainfall events, methanotrophic activity is stimulated in deserts and semiarid regions, and CH_4 oxidation increases (McLain and Martens, 2004). In regions where water is abundant, such as tropical forests, CH_4 oxidation is promoted in the dry season, most likely due to an increase in oxygen concentration and a faster diffusion of CH_4 into the soil solution (Kiese *et al.*, 2003).

Aeration and soil texture

The rate of CH_4 oxidation is related to the diffusion of CH_4 and O_2 from the atmosphere to the soil and this depends on the texture and the degree of compaction (bulk density) of a soil (Del Grosso *et al.*, 2000; Castaldi *et al.*, 2006). Methane diffusion is considered the limiting factor for CH_4 oxidation in soil (Templeton *et al.*, 2006).

Higher rates of CH_4 oxidation were found in a sandy soil than in a clayey soil and this is congruous with the idea that diffusivity is higher when pore size is higher (sandy soil) (Boeckx *et al.*, 1997).

O_2 and CH_4 concentrations

Oxygen is a determinant factor for CH_4 oxidation as aerobic CH_4 oxidation is the most important process for CH_4 consumption in soil. In turn, O_2 diffusion depends on soil porosity and water content (Mancinelli, 1995). More O_2 diffusion occurs when soil porosity increases, while increased water content inhibits O_2 diffusion.

In studies with soils and sediments, CH_4 oxidation

and the number of methanotrophs increased with increased CH_4 concentrations (Chan and Parkin, 2001; Khalil and Baggs, 2005) and this is most likely due to an increased diffusion of CH_4 (Chan and Parkin, 2001). However, the concentration below which no CH_4 oxidation occurs was lower for soils (< 0.1 to 0.4 µmol mol^{-1}) than sediments (2–3 µmol mol^{-1}) (Born *et al.*, 1990). It is likely that a population of high affinity methanotrophs is favored in soils and a population of low affinity methanotrophs in sediments.

In a recent experiment, Reim *et al.* (2012) found that methanotrophic diversity changed importantly along the microprofile of a flooded paddy soil. They conducted an experiment incubating the top 3-mm of the soil, supplying CH_4 from below and air from above. This created a counter-gradient from high O_2/low CH_4 in shallower layers to low oxygen/high CH_4 concentrations in deeper layers. In a few days, a methanotrophic community was developed, which oxidized virtually all the supplied CH_4. The methanotrophic community structure was analyzed along the profile. They found that type Ib methanotrophs, *e.g.*, *Methylococcus* and *Methylocaldum*, dominated in the oxic-anoxic interface between 1.0 and 2.0 mm depth, while *Methylobacter* (type Ia) dominated in the upper 0.5 mm. *Methylosarcina* was found at the surface zone with high O_2 and low CH_4 concentrations. It was concluded that differences in O_2 and CH_4 concentrations within the µmol L^{-1} range drive changes in methanotrophic populations in the oxic/anoxic interface.

Soil pH

The pH range for methanotrophic activity in soils is wide. There are reports of methanotrophic activity in acid (< 3.5) (Benstead and King, 2001) and alkaline conditions (pH 9.5) (Saari *et al.*, 2004). However, the highest CH_4 oxidation rates have been found in soils with pH close to 7.0 (Mosier and Delgado, 1997).

Salinity

Salinity is reported to affect about 7% of the soils around the world (Zhang *et al.*, 2011) and little is known about CH_4 production and oxidation in saline soils. Several studies showed that salinity affects CH_4 oxidation in soils (Price *et al.*, 2004; Saari *et al.*, 2004). However, Zhang *et al.* (2011) reported that methanotrophic bacteria isolated from saline soils showed higher tolerance to increasing salt concentrations than those isolated from a non-saline soil.

Osmotic pressure (Schnell and King, 1996) and/or the inhibitory effects of NH_4^+ and Cl^- ions are the major limiting factors for microbial activity (Price *et al.*, 2004), but CH_4 solubility decreases with increasing salt concentrations (Trotsenko and Khmelenina, 2002).

Soil N and N fertilizers

Ammonium and CH_4 oxidizers compete for O_2 to use it as an electron acceptor. The enzyme MMO, which initiates the oxidation of CH_4, is capable of binding to the NH_4^+ and react with it, as NH_4^+ and CH_4 are similar in size and structure (Schimel, 2000). For this reason, NH_4^+ fertilizers are considered to inhibit CH_4 oxidation (Bykova *et al.*, 2007).

However, contradictory results have been found with different soils under different conditions. There have been reports of no effect (Kiese *et al.*, 2003), a negative effect (Hutsch *et al.*, 1994), and a positive effect (Jacinthe and Lal, 2006) of NH_4^+ on CH_4 oxidation. The explanation for this behavior is based on the fact that the different types of methanotrophs, *i.e.*, I and II, respond in a different way to NH_4^+. While type II methanotrophs (*e.g.*, *Methylocystis* sp.) are inhibited by N fertilizer addition, type I methanotrophs (*e.g.*, *Methylomicrobium* or *Methylobacter*) are stimulated (Mohanty *et al.*, 2006). Consequently, the inhibitory effect of NH_4^+ fertilizer will be lower or absent in the presence of a diverse methanotrophic community (Kravchenko *et al.*, 2002). Another factor to consider is that in some soils, such as landfill cover or paddy rice soils, the consumption of NH_4^+ by nitrifiers is relatively rapid, with the subsequent reduction or elimination of the inhibitory effect of NH_4^+ on CH_4 oxidation (Cai and Mosier, 2000).

Hutsch *et al.* (1994) reported that NO_3^--fertilizer application had no effect on CH_4 oxidation. King and Schnell (1994) found an inhibitory effect of nitrite on CH_4 oxidation in two pure cultures of *Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3b.

Other soil nutrients and amendments

Methane monooxygenase needs copper as a cofactor (Cook and Shiemke, 1996), and the amount necessary for optimal activity vary within the community of methanotrophs. Type II methanotrophs are more active than Type I when Cu concentration is low. When Cu concentration is high, the activity of Type I methanotrophs increases (Myronova *et al.*, 2006). There are no conclusive results about the effect of addition of K to a soil on methanotrophic activity (Sanhueza *et al.*, 1994), although Jagadeesh Babu *et al.* (2006) reported a stimulating effect.

The addition of various organic materials, such as crop residues, affects CH_4 oxidation in different ways, depending on the C:N ratio. When the C:N ratio of

the residues is low, *i.e.*, high N content, CH_4 oxidation decreases. The increased release of NH_4^+ increases NH_4^+ oxidation inhibiting CH_4 oxidation. On the contrary, when the residue is characterized by a high C:N ratio, CH_4 oxidation rate is not affected (Wendlandt *et al.*, 2010). There are some studies in which the introduction of earthworms into a soil significantly increased CH_4 oxidation (Moon *et al.*, 2010).

Herbicides and pesticides

At ambient or low CH_4 concentrations, the methanotrophic activity in soil is inhibited by various herbicides and pesticides (Boeckx *et al.*, 1998; Prieme and Ekelund, 2001). However, this has not been observed at high concentrations of CH_4, such as in landfill cover soils (Boeckx *et al.*, 1998). This has been attributed to Cu^{2+} complexation with herbicides and/or pesticides as Cu^{2+} becomes unavailable as a co-factor for MMO enzyme (Wendlandt *et al.*, 2010).

Prieme and Ekelund (2001) suggested that the lower CH_4 oxidation rates in arable soils compared to forest soils could be partly due to the presence of pesticides. The long-term use of the herbicides, *i.e.*, atrazine and metolachlor, altered the soil community structure (Seghers *et al.*, 2005), but not the abundance of methanotrophs, so CH_4 oxidation rates were similar. An inhibition in production and oxidation of CH_4 was observed when butachlor (*N*-butoxymethyl-2-chloro-2′,6′-diethyl acetanilide), a commonly used herbicide in rice cultivation, was used even at very low concentrations. Mohanty *et al.* (2004) mentioned that the inhibition of CH_4 oxidation was due to the reduction in the population of methanotrophic bacteria that produce soluble MMO.

The effect of fumigants on CH_4 oxidation depends on the specific fumigant used and the fumigation history of the soil. In some cases, CH_4 oxidation rate increases but in others decreases. Chloropicrin, for instance, had a negative effect on CH_4 oxidation regardless of the fumigation history of the soil (Wendlandt *et al.*, 2010).

Methanotrophy in extreme environments

Some methanotrophic bacteria have been isolated from environments with pH values as low as 1 or as high as 11, temperatures from 0 to 72 °C and salinities up to 30% (Dunfield, 2009). However, little is known about the mechanisms these organisms have developed to cope with these conditions (Jiang *et al.*, 2010).

Extremophilic methanotrophic bacteria have been isolated from acidic northern peatlands, cold oceanic sediments, acidic soils, hypersaline lakes, salterns, warm composts, hydrothermal vents, geothermally heated sediments and alkaline soda lakes (Dunfield, 2009). Depending on the factor that defines the environment, extremophilic methanotrophs can be classified as thermophiles, psychrophiles, acidophiles, alkaliphiles and halophiles (Hanson and Hanson, 1996; Dunfield, 2009; Jiang *et al.*, 2010).

Methanotrophic thermophiles

Since 1970, methanotrophic bacteria that grow best or are active > 40 °C have been isolated (thermophiles or thermo-tolerants, respectively). For instance, *Methylococcus capsulatus* Bath was isolated from a hot spring (Whittenbury *et al.*, 1970), and *Methylocaldum* spp. (Bodrossy *et al.*, 1997) and *Methylothermus thermalis* were isolated from a Japanese hot spring (Tsubota *et al.*, 2005).

Bodrossy *et al.* (1997) reported the isolation of two methanotrophic bacteria with optimum growth temperatures above 40 °C, *i.e.*, the thermo-tolerant strain LK6 and the moderately thermophilic strain OR2, isolated from an agricultural soil and from the effluent of an underground hot spring, respectively. Both strains were found to be phenotypically related to *Methylococcus thermophiles*, but according to their 16S gene sequences they are markedly different with 8% divergence. With these two isolates and also with the inclusion of *Methylomonas gracilis* and *Methylococcus thermophilus*, the authors propose to establish a new genus, *Methylocaldum*.

The isolation and initial characterization of a strain of a highly thermoacidophilic obligately methanotroph from an acidic hot spring in Kamchatka, Russia, have been reported (Islam *et al.*, 2008). It grows optimally at pH 3.5 and at a temperature of 55 °C, that situates it as the most thermoacidophilic methanotroph known so far and it belongs to the *Verrucomicrobia* phylum.

Methanotrophic acidophiles

Methanotrophy is also present in some acidic environments, although methanotrophs do not grow well at pH < 5.0 (Dedysh *et al.*, 1998). Peatlands and acidic forests are important sources of atmospheric CH_4. In these acidic environments two genera of acidophilic/acid tolerant methanotrophs: *Methylocella* and *Methylocapsa* (Dedysh *et al.*, 2002), and more recently some acidophilic/acid tolerant species of *Methylocystis* (Dedysh *et al.*, 2007) were isolated. *Methylocapsa acidiphila* and *Methylocella palustris* (Dedysh *et al.*, 2002), both belonging to *Alphaproteobacteria*, play an important role in CH_4 oxidation in mildly to extremely acidic soils.

Dunfield *et al.* (2007) described an extremely acidophilic methanotroph that grows optimally at pH 2.0–2.5. This bacterium is phylogenetically more related to the *Verrucomicrobia* than the *Proteobacteria* phylum and it is supposed to use a novel methylotrophic pathway. Little is known about the phylum *Verrucomicrobia* because it includes a diverse group of mostly uncultured bacteria. This fact makes us think of a greater diversity of methanotrophic bacteria in acidic environments than previously thought. A more extended description of this bacterium can be found in Dunfield *et al.* (2007) and a detailed description of methanotrophic diversity in acidic northern wetlands and acidic peatlands in Chen *et al.* (2007) and Dedysh (2009).

Methanotrophic halophiles and alkaliphiles

Although the extension of saline soils is increasing due to irrigation, little is known about CH_4 oxidation in these environments and the contribution of these soils to the global CH_4 balance has not been taken into account for the calculations of soil CH_4 sink budget (Zhang *et al.*, 2011).

Salinity is known to affect both microbial metabolism and plant productivity. In rice paddy fields increased salt concentrations result in lower plant yields and changes in the soil microbial population (Tilak *et al.*, 2005). The size of the methanotrophic bacterial population is an indicator of the extent of CH_4 oxidation capacity in saline or alkaline environments, in particular in rain-fed paddy fields (Carini *et al.*, 2005).

Soda lakes are extreme environments with a high pH and salinity. The East African Rift Valley and central Asian Soda Lakes are important examples of continental soda lakes, which comprise about 80% of all inland lake water by volume (Trotsenko and Khmelenina, 2002). Despite the extreme conditions of these environments, a large microbial diversity and representatives of the main phyla of prokaryotes have been found (Zavarzin *et al.*, 1999). Methane oxidation also occurs in brine lakes and soda lakes (Trotsenko and Khmelenina, 2002). *Methylomicrobium alcaliphilum*, isolated from Tuva soda lakes (Trotsenko and Khmelenina, 2002) and *Methylomicrobium buryatense*, isolated from soda lakes in Russia (Kaluzhnaya *et al.*, 2001), are examples of alkaliphilic and alkaliphilic-alkalitolerant methanotrophs, respectively.

Most of the known halophilic and alkaliphilic methanotrophs belong to the genera *Methylomicrobium* and *Methylohalobius*. Based on molecular techniques, however, other genera should be included in the methanotrophic diversity in soda lakes (Trotsenko and Khmelenina, 2002; Dunfield, 2009; Jiang *et al.*, 2010).

Methanotrophic psychrophiles

Methanotrophic psychrophiles, *i.e.*, methanotrophs that are able to grow at low temperatures, play an important role in the Arctic CH_4 balance because of the activity of methanogenic archaea in these environments. However, very little is known about the impact of permafrost on the global CH_4 budget (Waldrop *et al.*, 2009).

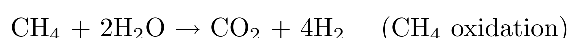
In various studies using molecular approaches, the presence of almost all known methanotrophs in cold environments has been demonstrated. Among these environments are underground waters, northern taiga and tundra soils, polar lakes, and permafrost sediments. Surprisingly, the occurrence of methanotrophic activity even at temperatures below zero has been demonstrated. However, the mechanisms involved in the survival and adaptation of methanotrophs in these environments are still unknown (Trotsenko *et al.*, 2005).

Methylobacter psychrophilus isolated from a Siberian tundra soil (Omel'chenko *et al.*, 1996), *Methylosphaera hansonii* isolated from an Antarctic lake (Bowman *et al.*, 1997), and *Methylomonas scandinavica* isolated from ground water of Sweden (Kalyuzhnaya *et al.*, 1999) were the first three psychrophilic methanotrophs isolated. They belong to methanotrophs of type I that grow best from 5 to 15 °C and have a low DNA G+C content (43–54%) (Dunfield, 2009). Wartiainen *et al.* (2006a) isolated a Gram-negtive, rod shaped, pale-pink pigmented methanotrophic bacterium, *Methylobacter tundripaludum*, from an Arctic wetland soil in Svalbard, that grows best at 23 °C, but also grows at 10 °C. From the same environment another methanotroph belonging to type II, *Methylocystis rosea*, was isolated. It is a Gram-negative, pink-red pigmented, polymorphic rod that can grow in the temperature range of 5–37 °C, with an optimal growth at 27 °C (Wartiainen *et al.*, 2006b). Other psychrotolerant methanotrophs that have been isolated and characterized as new genera and species are *Methylocella palustris*, *M. silvestris*, *M. tundrae* and *Methylocapsa acidiphila* (Trotsenko *et al.*, 2005).

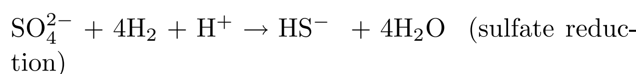
Anaerobic oxidation of CH_4

Although anaerobic oxidation of CH_4 (AOM) has been described since 1980, it is not well understood so far, but it is considered to contribute substantially to the reduction of CH_4 globally (Orphan *et al.*, 2002). It is estimated that $> 50\%$ of the gross annual production of CH_4 in the oceans is consumed by anaerobic

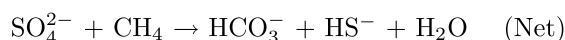
methanotrophs, before it diffuses to the atmosphere (Offre *et al.*, 2013). The mechanisms proposed for this process are reverse methanogenesis, acetogenesis, and methylogenesis (Caldwell *et al.*, 2008). The most investigated mechanism is the reverse reaction of methanogenesis, which takes place when sulfate-reducing bacteria (SRB) deplete the concentration of hydrogen, thus CH_4 concentration becomes higher than that of hydrogen, making the reverse reaction thermodynamically possible, *i.e.*, oxidation of CH_4 to CO_2 (Caldwell *et al.*, 2008; Wendlandt *et al.*, 2010). This process is also called sulfate-dependent CH_4 oxidation, which is done by archaea in a syntrophic association with SRB and the formation of hydrogen is a key step (Valentine and Reeburgh, 2000). One mechanism proposed for this process is as follows:



$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2 \quad (CH_4 \text{ oxidation})$$

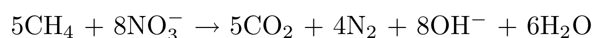


$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O \quad (\text{sulfate reduction})$$



$$SO_4^{2-} + CH_4 \rightarrow HCO_3^- + HS^- + H_2O \quad (\text{Net})$$

On the other hand, a process of AOM coupled to nitrate reduction denitrification, has been described. In this process CH_4 is used as an electron donor for the needed reduction power (Islas-Lima *et al.*, 2004). The following equation has been proposed:



$$5CH_4 + 8NO_3^- \rightarrow 5CO_2 + 4N_2 + 8OH^- + 6H_2O$$

where

$$\Delta G = -960 \text{ kJ mol}^{-1}$$

where ΔG is the standard Gibbs free energy change.

A novel process has been reported by Ettwig *et al.* (2010) in which an anaerobic bacterium, *Candidatus Methylomirabilis oxyfera*, is believed to oxidize CH_4 coupled to nitrite reduction to dinitrogen in pure culture. There is evidence that this bacterium does not use reverse methanogenesis to oxidize CH_4 but a variant of the typical aerobic methanotrophic pathway. This process involves an unknown enzyme that reduces nitric oxide directly to dinitrogen bypassing the formation of N_2O. No archaea has been found to be involved in this process, so this bacterium isolated recently is supposed to be capable of doing both, CH_4 oxidation and nitrite reduction. It is an anaerobic bacterium and has the characteristic of generating O_2 by the reduction of nitric oxide, which is further used to oxidize CH_4. This is a novel process of biochemical O_2 generation which has a geochemical and evolutionary importance (Ettwig *et al.*, 2010).

Anaerobic CH_4-oxidizing archaea

Archaea are considered of great importance in the global biogeochemical cycles. Two essential processes for nutrient cyclings are performed exclusively by archaea: methanogenesis and sulfate-dependent AOM (Offre *et al.*, 2013). Anaerobic oxidation of CH_4 in marine sediments is done mainly by anaerobic methanotrophic archaea (ANME), but the possibility of the participation of other microorganisms exists (Strous and Jetten, 2004).

Anaerobic methanotrophic archaea, along with various methanogenic archaea, are strict anaerobes of a single taxonomic class, *Methanomicrobia*, which belong to *Euryarchaeota*. According to some studies based on the 16S rRNA gene sequence, three sequence clusters have been defined, ANME-1, ANME-2, and ANME-3, which do not form a monophyletic lineage (Offre *et al.*, 2013). These archaea are frequently found in a syntrophic association with sulfate-reducing *Deltaproteobacteria*. In a recent study, however, it is mentioned that ANME-2 organisms performed AOM coupled to dissimilatory sulfate reduction, without the participation of bacteria (Milucka *et al.*, 2012).

Methanotrophic archaea can be found in anoxic environments where CH_4 and sulfate are present, *i.e.*, the sulfate-CH_4 transition zone (SMTZ). Archaea, due to their physiological diversity, can be found in environments with different physicochemical characteristics, such as marine benthic zones, anoxic water columns, hydrothermal vents, soils, aquifers, and freshwater habitats (Offre *et al.*, 2013). Anaerobic methanotrophic archaea have also been found in extreme environments, *i.e.*, at temperatures up to 95 °C in hydrothermal sediments, at a pH of 4 in CO_2-vented sediments, in alkaline fluids of carbonate chimneys with pH values of 9–11 and temperatures up to 70 °C, and at elevated salt concentrations (Knittel and Boetius, 2009).

It is believed that AOM initiates with the reversal of the last reaction in methanogenesis, the reduction of methyl-coenzyme M (CoMS-CH_3) which is catalyzed by methyl-coenzyme M reductase (MCR). This hypothesis is supported by the findings of *mcrA* genes being found in sediments from AOM zones or enrichment cultures, and these genes could be assigned to ANME-1 and ANME-2 (Knittel and Boetius, 2009). Phylogenies based on the *mcrA* gene are now getting more importance as they may help to elucidate the diversity of methanogenic and methanotrophic archaea.

Mechanisms, bioenergetics, and ecology of associ-

ated microorganisms of AOM are described in detail by Caldwell *et al.* (2008), Knittel and Boetius (2009), and Offre *et al.* (2013).

CONCLUSIONS

In spite of the recent advances in the research on methanogenesis and methanotropy and the role of soils in the CH_4 cycle, further investigations are required to expand our knowledge about these processes to establish new environmentally friendly techniques. Additionally we need more knowledge about the role of the different ecosystems in the global CH_4 balance. Examples of research fields that should be further explored are: long-term field studies on rice paddy, the two recently proposed processes of CH_4 production under oxic conditions (the non-microbial methanogenic chemical process and CH_4 production by terrestrial plants), the microbiology of high affinity methanotrophs by using new molecular biology techniques, and the processes of AOM.

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