UNIVERSITAT DE BARCELONA

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Memoria presentada por Claudia Ximena Restrepo Ortiz para optar al título de Doctor por la Universidad de Barcelona

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A mis padres, por creer siempre en mí
A Jean-Chris por dejarme SER
A mi bicho, por llenar mi mundo de muchas más posibilidades

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Cuando pensaba en esta parte de mi vida, en este pedacito de historia, no lograba conseguir ubicar el momento exacto donde todo comenzó. Podría decir que fue cuando me otorgaron la beca, o cuando llegue a Barcelona, o incluso un poco antes en Alicante, y yendo de más en más hacia atrás, me di cuenta que para mí, todo comienza de pie en la puerta de mi casa a 8 mil kilómetros de distancia, con una maleta llena de ilusiones para que el camino fuera más ameno, llena de recuerdos para no olvidar de donde vengo, llena de café para que el olor apaciguara la nostalgia, llena de mucho amor, para que en las noches no me atacaran los miedos y llena de mucha salsa para no hacerle caso a los fantasmas. La vida para mí, es simplemente un cúmulo de historias, todas ellas siempre llenas de sueños, metas, desafíos, frustraciones, alegrías y tristezas, pero sobre todo, para suerte mía, siempre está llena de personas. Hoy que me encuentro al final de este pedazo de historia, mi maleta solo está llena de agradecimientos, porque este camino jamás lo recorrí sola.

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vos. Pitufa, gracias por tu locura, por mostrarme siempre que el vaso está medio lleno y que la vida hay que vivirla, que ya habrá tiempo para lo demás. A todas las físicas y físicos que la vida me prestó, gracias por lo vivido, por todas las locuras de la universidad, por los paseos de rio y los viajes por el mundo, por lo años en Barcelona, por el cuidado y el cariño, y aunque muchos ya no hagan parte de mi vida, gracias por la historia. A mis biólogas locas, Lucu, Leidy, Caro, Karen, gracias por todo el tiempo vivido y compartido, porque a pesar de la distancia y lo años seguimos riendo, bailando, llorando y recochando. A Joha, por ser la primera persona en entrar conmigo al maravilloso mundo de la biología, por estar desde ese primer momento y hasta el final, por darme tantísimas lecciones de berraquera, y mostrarme que la mayor parte del tiempo, querer tiene poder.

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Informe del director

El Dr. Emilio Ortega Casamayor, Investigador Científic del Centre d'Estudis Avançats de Blanes (CSIC), i director de la Tesi Doctoral elaborada per Claudia Ximena Restrepo Ortiz i que porta per títol "***Biology and population ecology of uncultured archaea in natural environments analyzed by taxon-specific molecular markers***".

INFORMA

Que els treballs de recerca portats a terme per Claudia Ximena Restrepo Ortiz com a part de la seva formació pre-doctoral i inclosos a la seva Tesi Doctoral han donat lloc a dos articles SCI internacionals publicats, i un manuscrit adicional preparat per a ser enviat a una revista també d'àmbit internacional. A continuació es detalla la llista d'articles així com els índexs d'impacte (segons el SCI de la ISI Web of Knowledge) de les revistes on han estat publicats els treballs.

> *1. Restrepo-Ortiz C, EO Casamayor (**2013**) Environmental distribution of two widespread uncultured freshwater Euryarchaeota clades unveiled by specific primers and quantitative PCR. **Environ Microbiol Reports 5: 861-867***

L'índex d'impacte de la revista *Environmental Microbiology Reports* es de 3.264. Aquesta revista pertany a la categoria "Environmental Sciences" a la posició 35 de 216 revistes, quedant inclosa al grup de revistes del 1er quartil.

*2. Restrepo-Ortiz C, JC Auguet, EO Casamayor (**2014**) Unveiling spatio-temporal dynamics of planktonic SAGMCG-1 and segregation of ammonia-oxidizing thaumarchaeota ecotypes by newly designed primers and quantitative PCR. **Environ Microbiol 16: 689-700***

L'índex d'impacte del *Environmental Microbiology* al 2014 va ser de 6.201. Tenint en compte aquest índex d'impacte la revista ocupa el 12è lloc de la categoria ISI "Microbiology", quedant inclosa en les revistes del primer decil.

Alhora, FA CONSTAR

Que na Claudia Ximena Restrepo Ortiz ha participat activament en el desenvolupament del treball de recerca associat a cadascun d'aquests treballs així com en la seva elaboració a les diferents fases, participant en el plantejament inicial dels objectius i liderant l'optimització i aplicació de la metodologia, el processament de les dades, així com en la redacció dels articles i seguiment del procés de revisió dels mateixos.

Que cap dels co-autors dels articles abans esmentats ha utilitzat o be te present utilitzar implícita o explícitament aquests treballs per a l'elaboració de cap altra Tesi Doctoral.

Signat a Blanes, 1 desembre 2015

Dr. Emilio Ortega Casamayor

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Part II: Retrieval and integration database, design and optimization of specific CARD-FISH probes for SAGMCG-1 group

Biology and population ecology of uncultured Archaea in natural environments analyzed by taxon-specific molecular markers

1

Introducción

El trabajo pionero de Carl Woese en la década de 1970, basado en el RNA ribosómico (rRNA) para clasificar todas las formas de vida, condujo al sorprendente descubrimiento de *Archaea* (Woese and Fox, 1977). El dominio *Archaea* representa una tercera línea de descendencia evolutiva, un segundo linaje procariótico distinto del dominio *Bacteria*, así como de las células eucariotas (Figura 1.1). Los análisis bioquímicos y genéticos subsecuentes proporcionaron apoyo al sistema de reclasificación de Woese y específicamente a la idea de *Archaea* como una línea de células procariotas que en muchos aspectos, tenía lazos más estrechos con las células eucariotas que con las células bacterianas (Jones *et al.*, 1987; Woese, 1987; Woese *et al.*, 1990; Allers and Mevarech, 2005). Los primeros árboles filogenéticos de *Archaea* contenían sólo archaea cultivadas e incluían sólo dos phyla, *Crenarchaeota* y *Euryarchaeota* ambos considerados extremófilos (Woese *et al.*, 1990). La aplicación de esta metodología a una variedad de ambientes extremos y moderados, supuso el descubrimiento sorprendente que *Archaea* estaba presente como componente importante o incluso principal, no sólo en una gran variedad de hábitats extremos, sino también en hábitats extensos no extremos, tales como aguas oceánicas, suelos y sedimentos (DeLong, 1992, 2005; Barns *et al.*, 1994, 1996; DeLong *et al.*, 1994). La diversidad de secuencias del rRNA de archaea, en muchos casos, superó la estimación de la

diversidad que había sido obtenida de los cultivos y sugirió una diversidad fisiológica que iba mucho más allá de las arqueas cultivadas representadas por metanógenos, halófilos extremos, e hipertermófilos y, de igual forma, dio lugar a la idea de que los representantes de archaea eran ubicuistas (Olsen *et al.*, 1994; Delong, 1998).

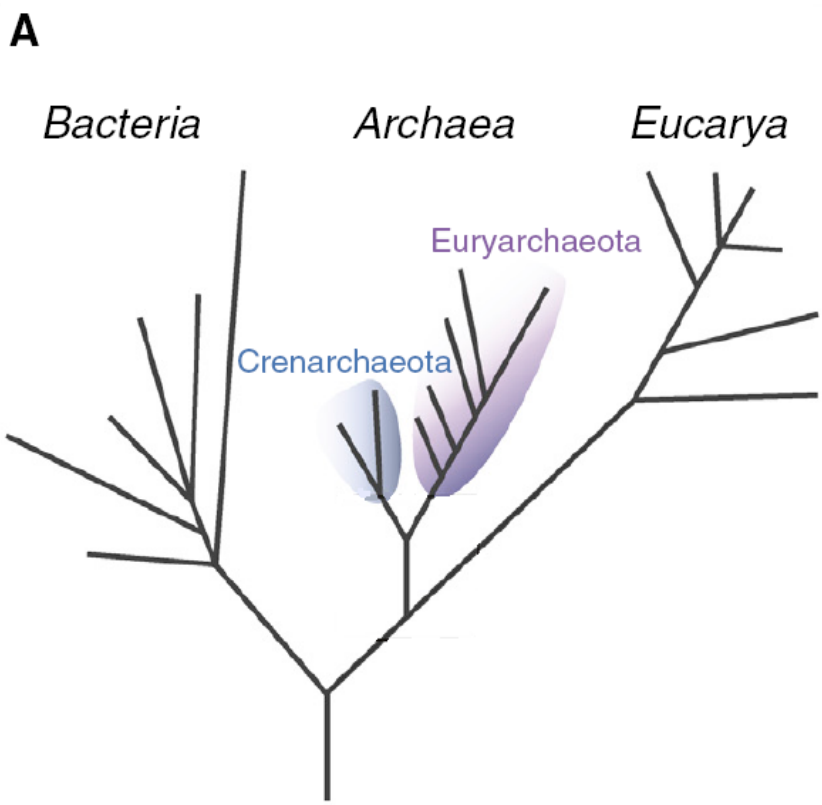

Figura 1.1: Árbol evolutivo de la vida. (A) Los tres dominios del árbol de la vida, tal como es concebido por Woese *et al*., 1990. Figura modificada de Eme and Doolittle, 2015.

Los nuevos genomas y secuencias del gen 16S rRNA disponibles han ampliado y reconfigurado radicalmente el árbol filogenético de Archaea, en el que se han propuesto gran variedad de nuevos linajes (Figura 1.2). Los nuevos linajes principales, *Korarchaeota*, representado por un genoma (Elkins *et al.*, 2008), *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008), que comprende todas las archaeas conocidas como oxidadoras de amonio (AOA, ammonia-oxidizing Archaea) y *Aigarchaeota* (Nunoura *et al.*, 2011),

representan colectivamente, junto con el phylum *Crenarchaeota*, un superphylum de archaea, denominado TACK (Guy and Ettema, 2011).

Posteriormente, un nuevo phylum denominado *Bathyarchaeota* (Meng *et al.*, 2014) se propuso dentro de TACK, y fue definido a partir del grupo MGC (*Miscellaneous Crenarchaeota Group*), que desempeña un papel ecológico en la remineralización de proteínas en los sedimentos marinos anóxicos (Lloyd *et al.*, 2013). También se han revelado varios linajes enigmáticos de pequeñas arqueas no cultivadas, con células alrededor de 400-500 nm y genomas entre 550 a 1000 genes (Baker *et al.*, 2010; Rinke *et al.*, 2013), de diversos entornos e incluso se ha argumentado a favor de la existencia de un superphylum denominado DPANN.

El superphylum DPANN abarca *Nanoarchaeota* (Waters *et al.*, 2003) único phylum de DPANN con representantes cultivados, así como los phylum *Nanohaloarchaeota* (Narasingarao *et al.*, 2012), el SAG (Ghai *et al.*, 2011) a partir de ambientes hipersalinos; *Parvarchaeota* (Baker *et al.*, 2010), *Aenigmarchaeota* y *Diapherotrites* (Rinke *et al.*, 2013), ambos definidos a partir de linajes dentro de SAG de aguas subterráneas de minas. Como tal, el superphylum DPANN representa una recopilación intrigante de phylum con preferencias fisiológicas y distribución ambiental diversas, que van desde las especies simbióticas y termófilas obligadas dentro del *Nanoarchaeota*, pasando por los acidófilos de *Parvarachaeota,* hasta los phylum no-extremófilos *Aenigmarchaeota* y *Diapherotrites*.

A pesar de que *Archaea* representa una fracción significativa de la biodiversidad de la Tierra, continúan siendo mucho menos comprendidas y estudiadas que las bacterias. Sin embargo, la capacidad de reconstruir los genomas completos de muestras ambientales mediante el rápido desarrollo de técnicas de single cell analysis y secuenciación masiva de alto rendimiento (Castelle *et al.*, 2015), promete una aceleración contundente en la comprensión de la diversidad y compleja historia evolutiva de este "tercer dominio de la vida", y sus vínculos con *Bacteria* y *Eukarya*.

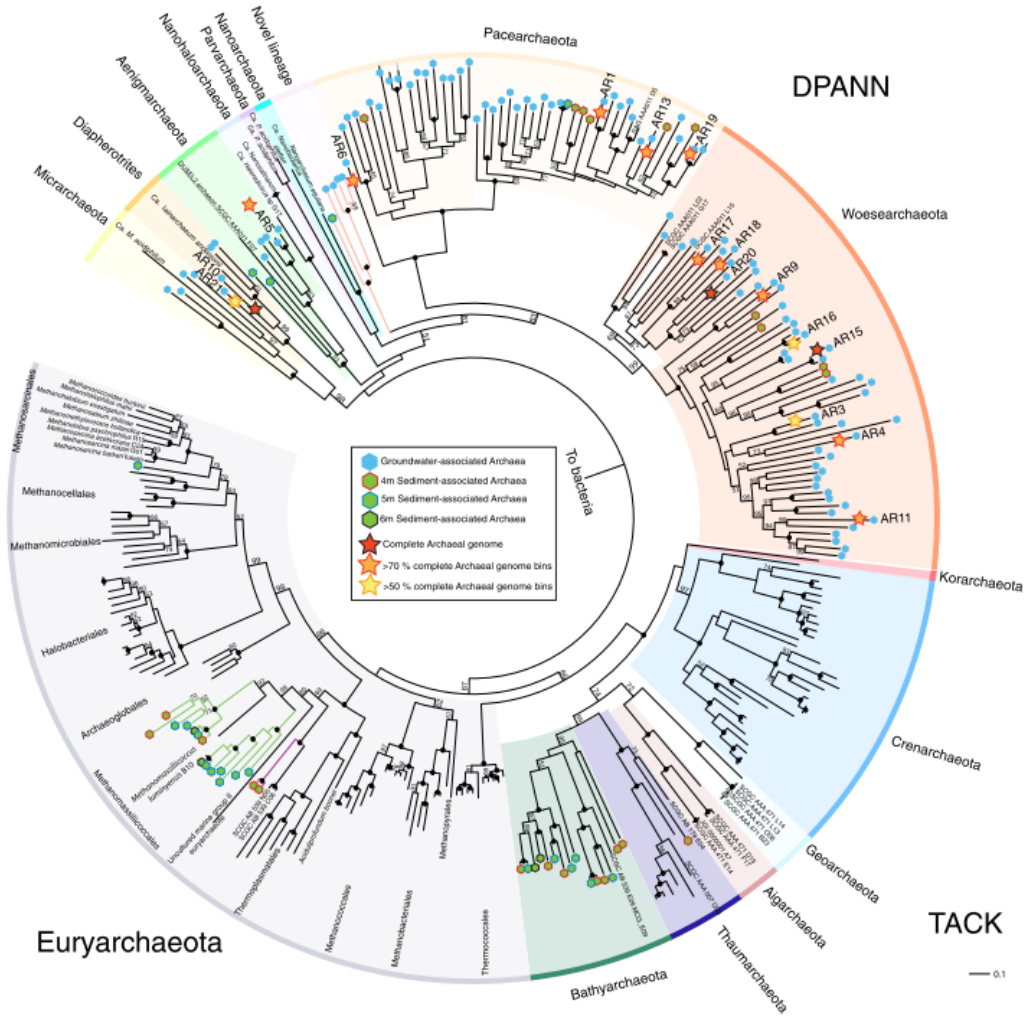

Figura 1.2: Filogenia de Máxima verosimilitud de los superphylum TACK y DPANN y el phylum *Euryarchaeota*. Figura obtenida de Castelle *et al.*, 2015.

1.1 Diversidad, abundancia y distribución de Archaea

El estudio de la biología y ecología de Archaea es actualmente uno de los temas de investigación en ecología microbiana más emocionantes y dinámicos. Las nuevas herramientas genéticas han permitido ampliar la ecología, diversidad metabólica y distribución de Archaea mucho más allá de lo inicialmente conocido, al presentar una amplia distribución y una diversidad inesperada (Schleper *et al.*, 2005; Chaban *et al.*, 2006; Auguet and Casamayor, 2008; Llirós *et al.*, 2008; Casamayor and Borrego, 2009). Actualmente está bien establecido que las arqueas están ampliamente extendidas en todos los ambientes (Figura 1.3). Algunos de estos ambientes, tales como los hipersalinos, sedimentos marinos, aguas dulces y suelos, resultan ser un componente clave y merecen mayor atención, ya que representan un modelo ambiental importante para el estudio de las archaea. Por ejemplo, se encontró que las fuentes hidrotermales presentan un alto número de linajes, lo que sugiere que podría ser el primer hábitat colonizado por este grupo de microorganismos (Auguet *et al.*, 2010).

Adicionalmente junto a este hábitat, los ambientes de agua dulce contienen las mayores reservas de diversidad de arqueas (Auguet *et al.*, 2010, 2011) y en consecuencia son prometedores para avanzar en su estudio en términos de composición, distribución, filogenias e importancia ecológica. Por el contrario, se observa que las comunidades de archaea encontradas en los suelos están más agrupadas filogenéticamente y su diversidad es el resultado de un alto número de filotipos estrechamente relacionados dentro de un limitado número de linajes (Auguet *et al.*, 2010).

Actualmente, debido a su gran abundancia y potenciales metabólicos, las archaea son reconocidas como un componente importante en lagos (Keough *et al.*, 2003; Lliros *et al.*, 2010) y suelos (Leininger *et al.*, 2006), e incluso compiten con *Bacteria* en las profundidades del océano (Karner *et al.*, 2001). Sin embargo, la importancia ecológica de todas las poblaciones de archaea presentes en los sistemas acuáticos y en suelos sigue sin ser entendida completamente (Barberán *et al.*, 2011, 2012; Vissers *et al.*, 2013).

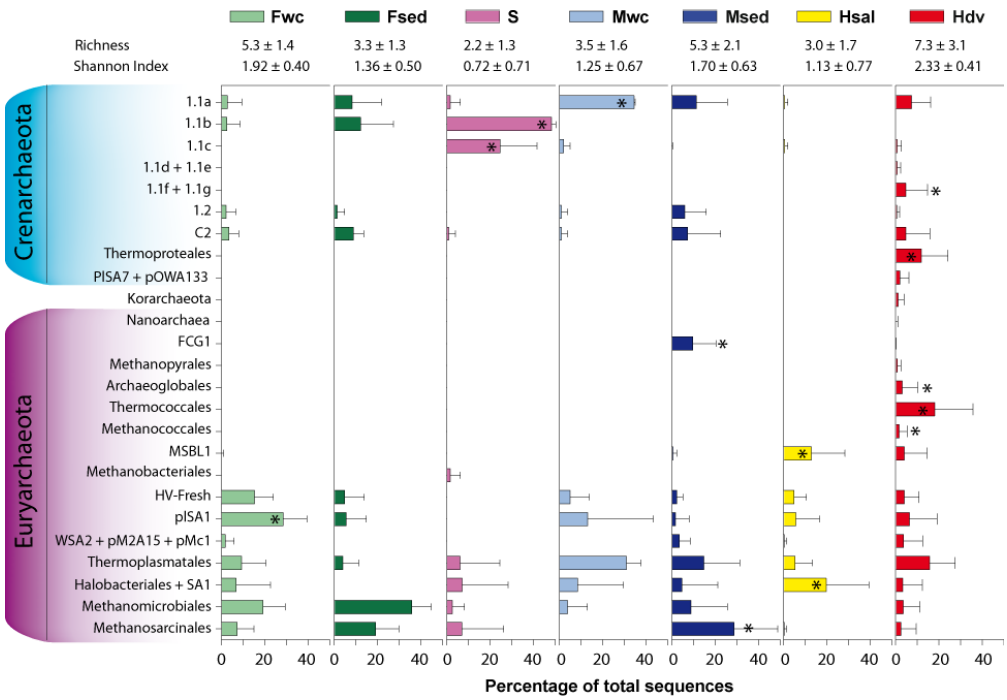

Figura 1.3: Proporción relativa de linajes de *Archaea* (basado en un metaanálisis de la abundancia de secuencias del gen rRNA 16S) dentro de cada uno de los siete hábitats identificados: sedimentos de agua dulce (Fsed), suelo (S), fuente hidrotermal (Hdv), hipersalinos (Hsal), plancton marino (Mwc), sedimento marino (Msed) y plancton de agua dulce (Fwc). Figura obtenida de Auguet *et al.*, 2010.

En esta tesis doctoral nos hemos centrado en el estudio de tres linajes, *Deep-Sea Euryarchaeotal Group* (DSEG), *Miscelaneous Euryarchaeotic Group* (MEG) y *South African Gold Mine Crenarchaeotic Group-1* (SAGMCG-1) (Takai *et al.*, 2001), particularmente representativos de ambientes de agua dulce oligotróficos (Auguet *et al.*, 2011, 2012; Auguet and Casamayor, 2013; Restrepo-Ortiz and Casamayor, 2013; Restrepo-Ortiz *et al.*, 2014). En la Tabla 1.1 se recogen las características principales y la evolución histórica en la nomenclatura de estos tres grupos. Afortunadamente, a medida que se ha avanzado en su estudio se ha ganado consenso en su nomenclatura y posicionamiento taxonómico.

Tabla 1.1: Caracteristicas principales de los grupos objeto de estudio durante esta tesis.

	SAGMCG-1 (Takai *et al.*, 2001)	**DSEG (Takai *et al.*, 2001)**	**MEG (Takai *et al.*, 2001)**
Superphylum	TACK (Guy and Ettema, 2011)	DPANN (Rinke *et al.*, 2013)	DPANN (Rinke *et al.*, 2013)
phylum	*Thaumarchaeota* (Brochier-Armanet *et al.*, 2008)	*Aenigmarchaeota* (Rinke *et al.*, 2013)	*Diapherotrites* (Rinke *et al.*, 2013)
Representante[1]	*Candidatus Nitrosotalea devanaterra* (Lehtovirta-Morley *et al.*, 2011)	*Candidatus Aenigmarchaeum subterraneum* (Rinke *et al.*, 2013)	*Candidatus Iainarchaeum andersonii* (Youssef *et al.*, 2014)
Sinonimias	Inicialmente estaba considerado dentro del grupo *Marine Group 1.1a* (MG1.1a)	- Lake Dagow sediment (LDS), (Glissmann *et al.*, 2004) - VALIII (Jurgens *et al.*, 2000) - HV-Fresh (Auguet *et al.*, 2010)	- Rice Cluster V (RC_V) (Großkopf *et al.*, 1998) - VALII/Eury4 (Jurgens *et al.*, 2000) - pISA1 (Auguet *et al.*, 2010)
Principales ambientes conocidos	Agua dulce Suelos	Agua dulce Suelos	Agua dulce Suelos
Metabolismo	Oxidadores de amonio	Desconocido	Desconocido

[1]Sólo SAGMCG-1 contiene un representante cultivado, los demás son genomas de single cell.

1.1.1 Diversidad y abundancia en lagos

Los hábitats de agua dulce se han convertido en un reservorio insospechado de diversidad (Galand *et al.*, 2006; Lliros *et al.*, 2010; Auguet *et al.*, 2011) y abundancia de archaea, que puede oscilar desde el 1% hasta el 20% del total de bacterioplancton (Pernthaler *et al.*, 1998; Glockner *et al.*, 1999; Keough *et al.*, 2003). La mayoría de los estudios se han realizado en lagos poco profundos (Auguet and Casamayor, 2008; Auguet *et al.*, 2011) o sólo la parte superior de la columna de agua (Boucher *et al.*, 2006; Hugoni *et al.*, 2013). En estas capas de agua, la comunidad de archaea que prevalece, a menudo es

Thaumarchaeota (Auguet *et al.*, 2011; Hugoni *et al.*, 2013), aunque los estudios de dinámica temporal entre invierno y verano no habían indicado ninguna tendencia en las abundancias máximas (Hugoni *et al.*, 2015). En lagos profundos, *Thaumarchaeota* tiende a dominar tanto en la capa oxiclina (Lliros *et al.*, 2010; Auguet *et al.*, 2012; Restrepo-Ortiz *et al.*, 2014) y haloclina (Lliros *et al.*, 2010; Comeau *et al.*, 2012). Sin embargo, la actividad potencial de *Thaumarchaeota* lacustres en relación a los cambios estacionales, salinidad y gradientes químicos sigue siendo poco conocida. Por ejemplo, rara vez son encontrados en aguas más profundas (es decir, en las zonas anóxicas y/o subóxicas y por encima de los sedimentos) (Lliros *et al.*, 2010; Vissers *et al.*, 2013), donde la metanogénesis realizada por *Euryarchaeota* es a menudo el proceso más importante (Lehours *et al.*, 2005; Lliros *et al.*, 2010). Por el contrario, en las capas más profundas de ambientes lacustres sí que se ha observado una gran diversidad de los *Euryarchaeota* afiliados a los grupos DSEG y MEG (Hugoni *et al.*, 2015). Estos grupos son muy diversos y se recuperan con frecuencia en lagos (Jurgens *et al.*, 2000; Glissmann *et al.*, 2004; Restrepo-Ortiz and Casamayor, 2013) y ríos (Galand *et al.*, 2006; Herfort *et al.*, 2009), lo que sugiere que desempeñan un papel funcional clave en hábitats de agua dulce (Barberán *et al.*, 2011).

Ahora que se acepta que la mayoría de los miembros de *Thaumarchaeota* son archaeas oxidadoras de amonio (AOA), estudios recientes muestran que este phylum prospera y compite activamente con bacterias oxidantes de amonio (AOB) en los sistemas acuáticos oligotróficos (Martens-Habbena *et al.*, 2009; Reed *et al.*, 2010). Sin embargo, su diversidad filogenética sugiere que probablemente tienen una variedad de metabolismos (Pester *et al.*, 2011; Stahl and de la Torre, 2012; Beam *et al.*, 2014), que podría permitir su adaptación a una gran diversidad de ambientes.

En general, los lagos proporcionan sistemas ideales para el estudio de las comunidades microbianas, y particularmente los estudios en lagos profundos permiten extender los conocimientos asociados a los procesos biogeoquímicos en los cuerpos de agua estratificados, proporcionando una variedad de nichos para el crecimiento y la diferenciación microbiana (Pouliot *et al.*, 2009).

1.1.2 Diversidad y abundancia en suelos

Los microorganismo presentes en los suelos desempeñan un papel importante en el mantenimiento de las funciones del ecosistema incluyendo los ciclos de los nutrientes y la productividad primaria (McGrady-Steed *et al.*, 1997; Griffiths *et al.*, 2011). La identificación de los factores que afectan la composición de estas comunidades es fundamental para predecir la respuesta de los ecosistemas a la perturbación y el cambio global (Martiny *et al.*, 2011). En los suelos, las archaeas son abundantes, relativamente diversas y ampliamente extendidas, y la composición de sus comunidades está fuertemente influenciada por los cambios en el ambiente del suelo (Leininger *et al.*, 2006). Por ejemplo, el pH es conocido por tener un efecto considerable en la actividad de las comunidades microbianas del suelo y los procesos biogeoquímicos que ellas median, ya que el pH afecta la forma química, la concentración y la disponibilidad de los sustratos e influye en el crecimiento y actividad de las células (Kemmitt *et al.*, 2006). Por otra parte, las concentraciones de amonio, resulta ser otro factor determinante en el crecimiento diferencial de las arqueas y bacterias oxidantes de amonio (AOA y AOB, respectivamente) en los suelos (Verhamme *et al.*, 2011).

En muchos suelos, el gen *amoA* de archaeas (que codifica para la subunidad α de la enzima amonio-monooxigenasa) superan en número a sus homólogos bacterianos (Leininger *et al.*, 2006). Generalmente, las AOA parecen dominar la oxidación de amonio en el suelo en condiciones de baja disponibilidad de nitrógeno, mientras que las AOB tienden a ser más competitivas en cantidades superiores de nitrógeno (Erguder *et al.*, 2009; Jia and Conrad, 2009; Gubry-Rangin *et al.*, 2010; Pratscher *et al.*, 2011).

1.2 Diversidad metabólica y participación en los ciclos biogeoquímicos

La diversidad metabólica y la función biogeoquímica de Archaea sigue siendo poco conocida. Hay evidencia de que al menos algunas poblaciones de Archaea, tales como *Marine Crenarchaeaota Group 1* (MGC1), son capaces de crecimiento quimioautotrofo o mixotrófico bajo ciertas condiciones, que se ilustra por su capacidad para llevar a cabo, independiente de la luz, la fijación de carbono (Wuchter *et al.*, 2003; Herndl *et al.*, 2005), la captación de aminoácidos (Ouverney and Fuhrman, 2000; Herndl *et al.*, 2005), y la absorción de dióxido de carbono (CO_2) (Hallam *et al.*, 2006). Sin embargo, se cree que la mayoría de MGC1 son autótrofos y utilizan fuentes de carbono inorgánicos tales como CO_2 y bicarbonato (Pearson *et al.*, 2001). Esta gama de posibles sustratos metabólicos se ha extendido además gracias al descubrimiento de un gen único que codifica la enzima clave para la nitrificación (Venter *et al.*, 2004) lo que sugiere que Archaea puede oxidar el amonio (NH_4^+).

A través de esta capacidad para metabolizar los sustratos inorgánicos como el CO_2 y NH_4^+, estos organismos desempeñan un papel central en los ciclos del carbono (C) y del nitrógeno (N), respectivamente. Los componentes clave en el ciclo global del carbono son sin embargo los metanógenos, los cuales descomponen la materia orgánica en condiciones anaeróbicas mediante la conversión de un número limitado de sustratos de carbono, como el CO_2, formiato, metanol, metilaminas y/o acetato, al metano (Thauer *et al*., 2008), contribuyendo así a la mineralización en el suelo o sedimentos. Por otra parte, el grupo MCG1 y 1.1b, desempeñan un papel importante en el ciclo de nitrógeno (Nicol and Schleper, 2006).

1.2.1 Participación en el ciclo del nitrógeno

La nitrificación representa la parte oxidativa del ciclo del N y se refiere al proceso de dos pasos, donde el amoníaco se oxida a nitrito y posteriormente a nitrato (Figura 1.4). Este proceso completa el ciclo redox del N, desde la forma más reducida a la más oxidada, y desempeña un papel clave en el presupuesto global de N en los ecosistemas de la Tierra. El primer paso de la nitrificación, fue descrito por Houzeau en 1872, y más tarde se atribuyó a la acción fermentativa de los microorganismos (Müller, 1873; Schloesing and

Muntz, 1877). Posteriormente, con el aislamiento de una bacteria oxidante de amoniaco, se confirmó el papel de las bacterias en la mediación de la etapa inicial de la vía de la nitrificación (Winogradsky, 1890). Hasta 2004, los científicos creían que sólo las bacterias mediaban el proceso de oxidación de amoniaco aeróbico. Sin embargo, el conocimiento científico acerca de la nitrificación y los organismos involucrados ha cambiado mucho en los últimos años, con la identificación de un conjunto de genes en *Archaea* que codifica para la enzima amonio monooxigenasa (*amoA*) (Venter *et al.*, 2004; Treusch *et al.*, 2005) y por el cultivo de la primera archaea amonio-oxidante *Nitrosopumilus maritimus* (Könneke *et al.*, 2005), ahora afiliado dentro del phylum, *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008).

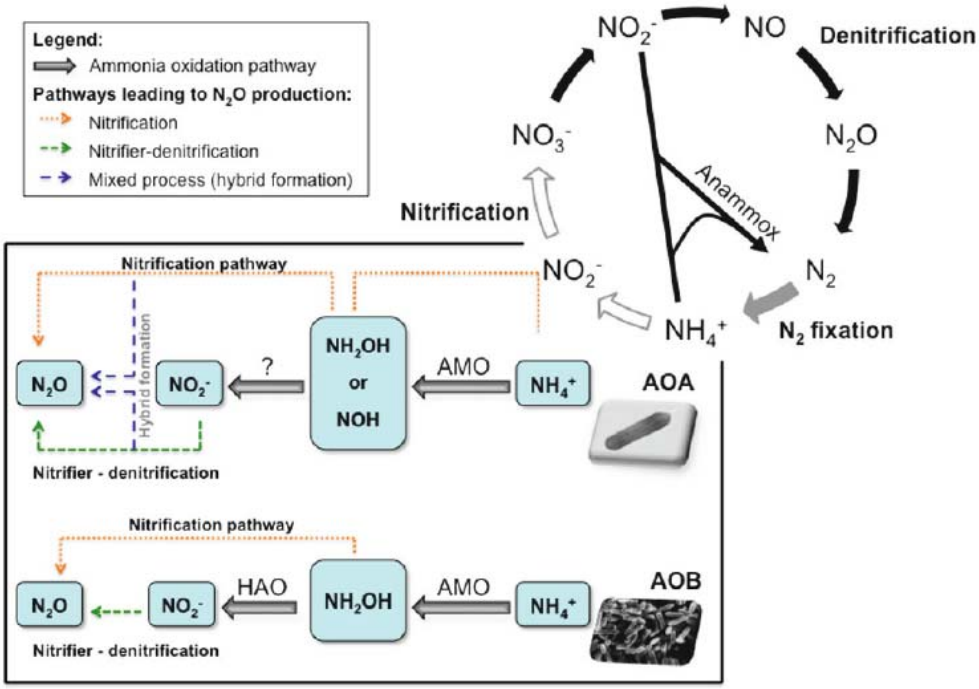

Figura 1.4: Ilustración esquemática de los procesos claves que intervienen en el ciclo del nitrógeno destacando las vías de oxidación aeróbica de amoniaco AOA y AOB dentro de la nitrificación. Figura modificada de Monteiro et al (2014).

La participación de *Thaumarchaeota* en la oxidación de amoniaco ha atraído la atención de numerosos grupos de investigación que reconocen a este phylum como un importante linaje de *Archaea*, que comprende un amplio

grupo de organismos ubicuos(Hallam *et al.*, 2006; Brochier-Armanet *et al.*, 2008; De La Torre *et al.*, 2008; Pester *et al.*, 2011). Más allá de eso, los estudios de diversidad y dispersión de las AOA, demostró que eran cosmopolitas, y la idea de que la actividad de este grupo de organismos contribuyen a la de ciclo N global ha sido generalmente aceptada (Francis *et al.*, 2005; Hallam *et al.*, 2006; Brochier-Armanet *et al.*, 2008; Pester *et al.*, 2012; Stahl and de la Torre, 2012). El aumento actual de estudios centrados en la fisiología celular, la ecología, la biogeoquímica, ecofisiología, genómica y, más recientemente, la proteómica está abriendo nuevas puertas en la investigación de estos nuevos AOA. Finalmente, el descubrimiento y cultivo de *Nitrosotalea devanaterra*, un Thaumarchaeota oxidador de amonio, confirma el papel central que este linaje representa en el ciclo del nitrógeno (Lehtovirta-Morley *et al.*, 2011).

1.2.2 Participación en el ciclo del carbono

El metano (CH_4) es la especie de orgánicos más abundantes en la atmósfera de la Tierra y el segundo gas más importante del efecto invernadero antropogénico después del dióxido de carbono (CO_2). La mayor parte del CH_4 atmosférico se forma a través de la actividad metabólica de las arqueas metanogénicas que demuestra el importante papel de estos organismos en el ciclo global del carbono. El ciclo global del carbono es complejo e incluye ciclos de CH_4 y CO_2 en entornos óxicos y anóxicos (Thauer *et al*., 2008). Las archaeas metanogénicas producen grandes cantidades de metano durante la metanogénesis en condiciones anaeróbicas que es consumido por bacterias y/o arqueas anaerobias oxidantes de metano, o directamente liberado a la atmósfera.

Los estudios realizados hasta la fecha han demostrado que *Marine Group II* (MG II) representan un componente abundante de la microbiota oceánica, muestran una gran variación estacional y espacial y una gran diversidad filogenética. Adicionalmente, ensayos a partir de biomarcadores específicos de lípidos de arqueas y análisis isotópicos, indican que MG II planctónicos tienen la capacidad de crecimiento autotrófico, corroborando la importancia metabólica global de estos organismos respecto a los elementos

claves en la transformación biogeoquímica del carbono (Hallam *et al.*, 2006b)

1.3 Detección de Archaea

1.3.1 Aproximaciones dependientes del cultivo microbiano

Hay dos estrategias establecidas para investigar la diversidad microbiana en los ecosistemas, los métodos cultivo-dependientes y los cultivo-independientes (Leuco *et al.*, 2007). Muchas especies de arqueas se han aislado con éxito de varios ambientes termófilos y mesófilos (Blöchl *et al.*, 1997; Koenneke *et al*., 2005), pero la mayoría de ellas son típicamente recalcitrantes al cultivo, sesgando así su detección a ciertos ambientes y especies. Por lo tanto, la verdadera diversidad de Archaea está altamente subestimada cuando nos aproximamos a los ecosistemas mediante metodologías basadas en el aislamiento en cultivo.

1.3.2 Aproximaciones independientes del cultivo microbiano

Debido a las limitaciones de los enfoques cultivo-dependientes, los estudios moleculares basados en la reacción en cadena de la polimerasa (PCR, polymerase chain reaction) son ampliamente utilizados para evaluar la diversidad de archaea en entornos naturales (Niederberger *et al*., 2012). Estas aproximaciones han desvelado una gran diversidad y la presencia extendida de Archaea en ambientes moderados (Chaban *et al.*, 2006), así como en ambientes extremos (Stetter, 1999). Una gran variedad de genes se puede utilizar como dianas para los métodos moleculares, pero el gen 16S ribosómico RNA (rRNA), que codifica para la subunidad ribosómica pequeña, se utiliza normalmente para los estudios de detección y diversidad de especies. Hay tres razones principales por las que este gen es más ventajoso que los demás: I. se asume que el gen 16S rRNA nunca ha sido transferido horizontalmente, II. toda la vida celular contiene al menos una copia, y III. algunas regiones en el gen evolucionan rápido y otras regiones evolucionan muy lentamente, permitiendo resolver las relaciones filogenéticas a varios niveles (Woese *et al.*, 1990).

Por otra parte, es posible cuantificar específicamente comunidades de microorganismos a través de PCR cuantitativa en tiempo real (qPCR) (Hultman *et al*., 2010; Stefanis *et al.,* 2013.) utilizando este u otros genes. La qPCR (Figura 1.5), permite la amplificación y cuantificación de genes específicos, lo que posibilita acceder a datos sobre la cuantificación genética de las poblaciones de estudio. Este método se ha convertido en una herramienta común en la investigación básica, así como en muchas otras áreas aplicadas (p.ej. industria, agricultura, medicina), las cuales se han visto beneficiadas por su sensibilidad, velocidad y especificidad. Los métodos que utilizan marcadores fluorescentes, como la qPCR, tienen la ventaja de ser muy sensibles y evitan posibles artefactos o secuencias inespecíficas presentes en el producto, por lo que la interpretación de los resultados suele ser más rápida y directa. Además su implementación requiere de una pequeña cantidad de muestra, lo cual resulta ideal en trabajos de campo.

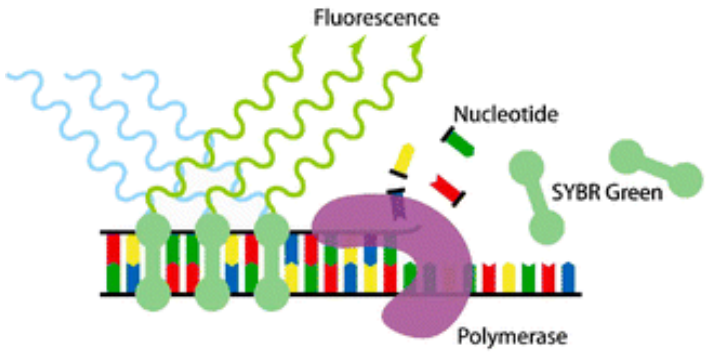

Figura 1.5: Formato esquemático para la PCR cuantitativa. Figura modificada de Guertler *et al*., 2009.

Sin embargo, este enfoque necesita del diseño de secuencias específicas para su uso (cebadores específicos) y este es el paso más laborioso y costoso (Lee *et al*., 2004) ya que es necesario un correcto diseño de estos cebadores que dé como resultado características ideales del cebador (p.ej., especificidad con el organismo diana, adecuado contenido en G-C, temperaturas de hibridación dentro del rango óptimo) que influyan positivamente en la eficiencia de la reacción. Otras posibles desventajas de esta técnica, son la presencia de inhibidores de la enzima polimerasa dentro de las muestras, la

dificultad para discernir entre células vivas o muertas y la sobreestimación de las abundancias, que se ha evidenciado en trabajos comparativos (Schouten *et al*., 2010; Xie *et al*., 2013; Lloyd *et al*., 2013).

A pesar de la importancia de esta metodología y nuestra comprensión de sus limitaciones, dos preguntas básicas persisten: (i) ¿Cómo podemos cuantificar con precisión las células de un grupo microbiano en particular?, y (ii) ¿Cómo podemos saber cuántos de estos organismos están potencialmente activos? Para poder abordar estas preguntas se hace necesaria una metodología complementaria basada en una aproximación microscópica. Desde que se desarrolló por primera vez (DeLong *et al*.,1989), la hibridación fluorescente *in situ* (FISH, fluorescence *in situ* hybridization), se ha convertido en una de las técnicas moleculares utilizadas más habitualmente en microbiología ambiental. La técnica FISH se puede usar para detectar, identificar y enumerar microorganismos ambientales sin necesidad de cultivarlos, por lo que se ha utilizado para ayudar a dilucidar la ecología microbiana de muchos hábitats, incluyendo el suelo, los sedimentos y medios acuáticos (Nakamura *et al*., 2006). Sin embargo, hay varios problemas en la aplicación de FISH, principalmente la sensibilidad es insuficiente debido al bajo número de moléculas diana en las células, baja permeabilidad de la sonda de las células, y la pobre eficiencia de hibridación de la sonda (Amann *et al*., 1997). Muchos métodos se han ideado para superar estos problemas (Amann *et al*., 2001) entre ellos la aplicación de CARD-FISH para aumentar la sensibilidad de detección.

En FISH y CARD-FISH, una sonda de oligonucleótidos con una secuencia específica se une directamente al RNA de los ribosomas (Amann *et al*., 1990). Para la aproximación FISH (Figura 1.6), esta sonda se une a un fluorocromo, permitiendo que las células den una señal positiva. Para CARD-FISH (Figura 1.7), la sonda se une a una enzima peroxidasa (HRP, horseradish peroxidase), que cataliza la deposición de muchas tiramidas fluorescentes, y mejora enormemente la intensidad de la fluorescencia.

En principio, estos métodos cuantifican células sólo viables, mediante imágenes de células intactas en lugar de moléculas individuales, tales como ADN o lípidos que pueden derivarse de restos celulares, y apuntan al rRNA, que se presume se degrada rápidamente después de la muerte celular (Davis *et al*., 1986). Sin embargo, las mediciones están sesgadas por poblaciones microbianas cuya secuencia de rRNA no coincide por completo con la sonda

o, en el caso de CARD-FISH, cuya pared celular es resistente a los procedimientos de permeabilización, tal como sucede en Archaea.

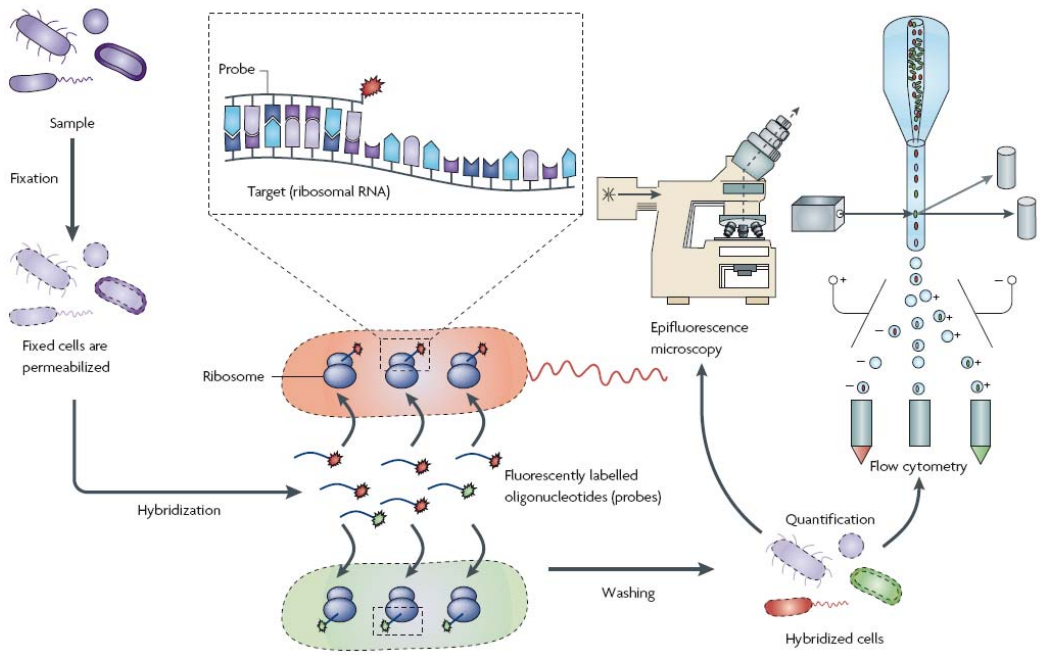

Figura 1.6: Pasos básicos de la hibridación fluorescente in situ. La muestra se fija primero para estabilizar las células y permeabilizar las membranas celulares. A continuación se añade la sonda oligonucleótida marcada y se deja hibridar con sus dianas intracelulares antes de que el exceso de sonda sea eliminado por el lavado. La muestra está entonces lista para la identificación de células individuales y su cuantificación ya sea por microscopía de epifluorescencia o citometría de flujo. Figura obtenida de Amann and Fuchs, 2014.

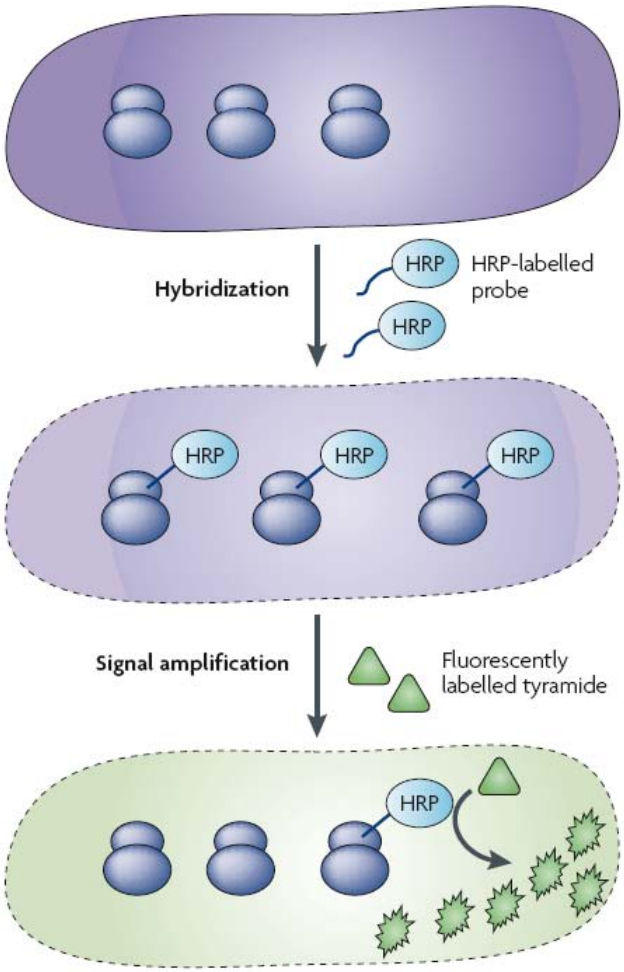

Figura 1.7: El principio de CARD-FISH (Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization), que combina el CARD de tiramidas marcadas con fluorescencia con la identificación de células individuales por FISH. La hibridación implica un único oligonucleótido que es reticulado covalentemente al marcador de peroxidasa de rábano picante (HRP, horseradish peroxidase). La amplificación de la señal con respecto a la conseguida con sondas que están etiquetados con un solo fluorocromo se basa en la radicalización de múltiples moléculas de tiramida por las HRP. Figura obtenida de Amann and Fuchs, 2014.

En esta tesis doctoral hemos explorado la diversidad y distribución de archaea típicamente planctónicas a nivel de poblaciones representativas, aplicando sondas moleculares especificas tanto para el gen 16S rRNA como para el gen funcional amoA y con métodos moleculares basados en qPCR y CARD-FISH. Los resultados desvelan una dinámica poblacional y una riqueza genética a nivel de poblaciones que había pasado desapercibida hasta fechas muy recientes. Los marcadores moleculares también nos han servido como trazadores de procesos biogeoquímicos relevantes en el ciclo del nitrógeno, identificando las condiciones ambientales que promueven la acumulación de estos microorganismos.

2

Objetivos

Los microorganismos del Dominio Archaea son un componente común del plancton que ha pasado, mayoritariamente desapercibido para la Limnología durante mucho tiempo. Sólo tras la aplicación de técnicas moleculares en ecología microbiana se ha descubierto su enorme riqueza biológica y abundancia tanto en ambientes acuáticos como terrestres, más allá del grupo de las archaea metanógenas. Estudios recientes han demostrado que los hábitats de agua dulce tienen la mayor diversidad filogenética y representan entornos prometedores para el descubrimiento de nuevos linajes de Archaea.

Uno de los ambientes que constituyen una fuente inexplorada de riqueza de nuevos filotipos de Archaea son los lagos alpinos, donde diferencias llamativas en la composición filogenética y abundancia de las arqueas se han reportado recientemente. Esto hace que los sistemas lacustres sean un modelo útil para comprender los factores ecológicos y la influencia de la heterogeneidad del hábitat sobre la composición de microorganismos Archaea, y para vincular los rasgos ecológicos y metabólicos de las arqueas con el funcionamiento del ecosistema. Sin embargo, poco se sabe sobre los reguladores ambientales y las limitaciones fisiológicas que produce la segregación del hábitat, ya que la baja cultivabilidad de la mayoría de estas archaea lacustres limitan seriamente un conocimiento más detallado de sus capacidades metabólicas y su función en el ecosistema. En consecuencia, esta tesis tiene como objetivo combinar microbiología ambiental, técnicas

moleculares y aproximaciones bioinformáticas para progresar en el conocimiento de la distribución ecológica y ecofisiología de filotipos representativos de las arqueas de agua dulce superando la limitación de su baja cultivabilidad.

Los resultados de esta tesis se han dividido en dos partes dependiendo del enfoque molecular y ecológico utilizados. La parte I incluye dos capítulos cuyos objetivos están centrados en el diseño y optimización de cebadores específicos de los genes 16S rRNA y subunidad alfa de la amonio-monooxigenasa (amoA) mediante PCR cuantitativa (qPCR) para el estudio exploratorio de la distribución y dinámica anual de los grupos de Archaea que predominan en ambientes lacustres oligotróficos y aeróbicos. La parte II contiene un capítulo cuyo objetivo busca el desarrollo de técnicas microscópicas de hibridación in situ fluorescente y sondas moleculares 16S rRNA específicas para la visualización in situ y cuantificación de thaumarchaeota *Nitrosotalea* spp. en suelos y ambientes lacustres. Los objetivos detallados, siguiendo la estructura de esta tesis, se indican a continuación:

Parte I: Distribución y dinámica anual de Archaea en ambientes lacustres oligotróficos y aeróbicos mediante qPCR

Cada vez hay más evidencia de que Archaea representan una fracción considerable del picoplancton en los ecosistemas acuáticos no extremos (entre 1-40% entre los diferentes sistemas acuáticos y hasta 37% en los lagos de alta montaña). Se puede deducir a partir de su ubicuidad y abundancia que estos microorganismos pueden tener un impacto sustancial en los ciclos biogeoquímicos y los flujos globales de energía. La diversidad de linajes de archaea que prosperan en estos entornos oligotróficos (SAGMGC-1 taumarchaeota, MEG y DSEG euryarchaeota), sugieren vínculos directos entre la segregación del linaje y los ciclos biogeoquímicos prevalentes que operan en estos lagos.

Esta parte se centra en cuantificar la abundancia del gen 16S rRNA (centrados principalmente en los grupos SAGMGC-1, MEG y DSEG) en un

conjunto de ambientes lacustres, para comparar la dinámica temporal y espacial, y para buscar los principales factores abióticos y bióticos que determinan la segregación del hábitat.

- **Capítulo 3** diseñar y optimizar cebadores específicos para el gen SAGMGC-1 16S rRNA y los diferentes ecotipos de archaea oxidadoras de amonio, AOA (gen amoA) con el fin de cuantificar mediante qPCR las distribuciones espacio-temporales a lo largo de una exploración anual en un lago profundo de alta montaña, donde las deposiciones atmosféricas son la principal fuente de nitrógeno reactivo.

- **Capitulo 4** explorar las diferencias espacio-temporales en el plancton superficial de un gran conjunto de lagos con gradientes ambientales marcados, mediante el diseño y optimización de cebadores específicos para el gen 16S rRNA de los grupos de euryarchaeota MEG y DSEG.

Parte II: Integración de bases de datos, diseño y optimización de sondas específicas de CARD-FISH para el grupo SAGMGC-1.

El descubrimiento y cultivo de la especie *Nitrosotalea devanaterra* (taumarchaeota acidofilico obligado) oxidante de amonio, perteneciente al grupo SAGMGC-1, ha ampliado el papel asignado a thaumarchaeota en el ciclo del nitrógeno. Este grupo es especialmente interesante, porque ha sido reconocido por su importancia ecológica, especialmente en suelos ácidos y aguas dulces oligotróficas.

Los estudios filogenéticos moleculares realizados en entornos naturales durante los últimos 20 años, han proporcionado una mejor comprensión de la diversidad y la filogenia de Archaea basado en el gen ribosómico RNA 16S. En la actualidad, se han desarrollado una amplia gama de técnicas moleculares de gran alcance basadas en este gen que impulsan estudios sobre la fisiología y ecología de Archaea.

CARD-FISH es una de las herramientas moleculares más importantes que combinada con microscopía de epifluorescencia permite cuantificar y mejorar nuestra comprensión de la distribución de los microorganismos in situ. Debido a su aumento de la sensibilidad y de los muchos avances técnicos recientes, el CARD-FISH se puede utilizar ahora no sólo para la detección de rRNA, sino también mRNA y genes funcionales codificados en el genoma microbiano. El diseño refinado de sondas moleculares específicas permite un estudio detallado a diferentes niveles taxonómicos.

- **Capítulo 5** construir una base de datos genética detallada del gen ribosómico 16S rRNA de Archaea utilizando SILVA y nuestros propios datos genéticos generados a partir de trabajos anteriores, con el fin de diseñar y optimizar sondas específicas de CARDFISH para el grupo taumarchaeota SAGMGC-1, combinando aproximaciones in silico, in vitro e in situ. Cuantificación selectiva de dicha población a lo largo de un gradiente ambiental.

Part I: Distribution and annual dynamics of Archaea in oligotrophic and aerobic freshwaters by qPCR

3

Targeting spatio-temporal dynamics of planktonic SAGMCG-1 and segregation of ammonia-oxidizing thaumarchaeota ecotypes by newly designed primers and quantitative PCR

Resumen

Mediante el diseño de nuevos cebadores específicos y el análisis de PCR cuantitativa se estudio la dinámica anual de tres diferentes ecotipos (amoA gen) de arqueas oxidadoras de amonio (AOA) y del grupo SAGMCG-1 (16S rRNA gen) (*Nitrosotalea*-like acuática Thaumarchaeota) en un lago oligotrófico profundo de alta montaña (lago Redon, Observatorio limnológico de los Pirineos, España). Se observaron distribuciones segregadas de las principales poblaciones de AOA, con picos de abundancia separados en tiempo y espacio, bajo concentraciones de amoníaco y condiciones de irradiación diferentes. Se encontró una fuerte correlación positiva entre las abundancias del gen 16S rRNA SAGMCG-1 y uno de los ecotipos de *amo*A, lo que propone al grupo SAGMCG-1 como potencial oxidador de amonio de agua dulce. También se observó predominio de ecotipos *Nitrosotalea*-like más *Nitrosopumilus*-like (MG 1.1a), y una dinámica anual diferente a la de los dos clados thaumarchaeotal. La fina escala de segregación en el espacio y tiempo de los diferentes ecotipos AOA indicó la presencia de especies AOA filogenéticamente cercanas pero ecológicamente distantes, adaptadas a las condiciones ambientales específicas. Queda por dilucidar cuáles serían esos reguladores ambientales.

Abstract[1]

The annual dynamics of three different ammonia-oxidizing archaea (AOA) ecotypes (*amo*A gene) and of the SAGMCG-1 (*Nitrosotalea*-like aquatic Thaumarchaeota) group (16S rRNA gene) were studied by newly designed specific primers and quantitative PCR analysis in a deep oligotrophic high mountain lake (Lake Redon, Limnological Observatory of the Pyrenees, Spain). We observed segregated distributions of the main AOA populations, peaking separately in time and space, and under different ammonia concentrations and irradiance conditions. Strong positive correlation in gene abundances was found along the annual survey between 16S rRNA SAGMCG-1 and one of the *amo*A ecotypes, suggesting the potential for ammonia oxidation in the freshwater SAGMCG-1 clade. We also observed dominance of *Nitrosotalea*-like ecotypes over *Nitrosopumilus*-like (MG 1.1a), and not the same annual dynamics for the two thaumarchaeotal clades. The fine scale segregation in space and time of the different AOA ecotypes indicated the presence of phylogenetically close but ecologically segregated AOA species specifically adapted to specific environmental conditions. It remains to be elucidated what would be such environmental drivers.

3.1 Introduction

Thaumarchaeota are ubiquitous microorganisms in marine and freshwaters, soils, sediments, and biofilms (see a recent metadata analysis by Fernandez-Guerra and Casamayor 2012), and represent a major reservoir of prokaryotic biomass (Prosser and Nicol, 2008). Initially classified as 'mesophilic Crenarchaeota', comparative genomics showed that they form a separate and deep-branching phylum within the Archaea (Brochier-Armanet et al 2008, Stahl and de la Torre 2012). Thaumarchaeota encompasses all previously known putative ammonia-oxidizing archaea (AOA), and other archaea with unknown energy metabolisms potentially linked to the nitrogen cycle (Pester et al, 2011) and to other biogeochemical cycles (see a recent review by Hatzenpichler 2012). In fact, the widespread distribution of putative Archaeal ammonia monooxygenase (*amo*A) genes in marine, terrestrial and freshwater

[1]See original publication in Restrepo-Ortiz *et al.,* (2014)

environments (Francis *et al.,* 2005, Zhang *et al.,* 2008, Auguet *et al.,* 2011), strongly suggests that AOA play a major role in global nitrification.

The SAGMCG-1 group, initially found in gold mines in Africa (Takai *et al.,* 2001), forms a monophyletic cluster within Thaumarchaeota closely related to the Marine Group 1.1a (Prosser and Nicol, 2008, Pester *et al.,* 2011). The recent discovery and cultivation of the chemolithotrophic, obligate acidophilic thaumarchaeal ammonia-oxidizing species *Nitrosotalea devanaterra* (Lehtovirta-Morley *et al.,* 2011) belonging to the SAGMCG-1 group, has enlarged the role assigned to thaumarchaeota in the nitrogen cycling. The SAGMCG-1 and *Nitrosotalea* clusters are especially interesting since only very recently it has been recognized its ecological importance, particularly in acidic soils (Gubry-Rangin *et al.,* 2011, Pester *et al.,* 2011) and oligotrophic freshwaters (Auguet and Casamayor 2008, 2013). The relative abundance, recurrent appearance, and significant correlations with nitrogen species suggest a key role of SAGMCG-1 in the N biogeochemical cycle of alpine lakes (Auguet *et al.,* 2011, 2012).

In the present study, we designed and tested specific primers for quantifying by qPCR the SAGMCG-1 16S rRNA gene and different AOA ecotypes (*amo*A gene) spatio-temporal distributions along an annual survey in the deep high-altitude Lake Redon, where atmospheric depositions are the main source of reactive nitrogen (Catalan *et al.,* 2006). Vertical positioning in the water column has been unveiled as a key factor to understand the ecology of different thaumarchaeotal clades in aquatic environments (Francis *et al.,*, 2005; Hallam *et al.,*, 2006; Mincer *et al.,*, 2007; Beman *et al.,*, 2008; Santoro *et al.,* 2010, Llirós *et al.,* 2010, Auguet *et al.,* 2012). We specifically quantified three freshwater thaumarchaeota populations in Lake Redon showing (i) the dominance of the *Nitrosotalea*-like (SAGMCG-1) populations over *Nitrosopumilus*-like (MG 1.1a), (ii) the differential dynamics of these two clades along the annual study, and (iii) the fine scale distribution in space and time of different AOA ecotypes.

3.2 Material and Methods

3.2.1 Study site and sample collection

The annual survey was carried out in Lake Redon, an alpine deep lake located in the central Spanish Pyrenees (42º38'34''N, 0º46'13''E, altitude 2240 m, maximum depth 73 m, surface 0.24 km2). The lake is oligotrophic with poor vegetation and soil development in the small catchment area (c. 1.5 km^2). It is located on the head of the valley, and atmospheric deposition is the main source of reactive nitrogen (Catalan *et al.*,, 1994, 2006). Samples were monthly collected between 2007 and 2008 from six depths (surface, 2 m, 10 m, 20 m, 35 m, and 60 m) on the deepest point of the lake using a 2L Niskin bottle. These depths covered different limnological characteristics of the lake (Auguet *et al.,* 2012, Camarero and Catalan 2012). Changes in temperature, oxygen, pH, nutrients, and chlorophyll *a* were used to follow the seasonal variability of physical, chemical and biological properties of the lake, and were measured as recently reported (Auguet *et al.,* 2012, where also additional field data can be found). The light extinction coefficient was calculated after Secchi disk depth data (Armengol *et al.,* 2003). Samples from surface waters of additional lakes within the Limnological Observatory (Auguet and Casamayor 2013) were used to test the primer sets performance and specificity. Samples were processed and DNA was extracted and purified as reported (Hervàs *et al.,* 2009, Demergasso *et al.,* 2008). A previous study with the same dataset had shown ammonia-oxidizing bacteria (AOB) below detection limits in the plankton of Lake Redon (Auguet *et al.,* 2012).

3.2.2 Specific primers and PCR conditions

Four sets of specific primers were designed and tested according to previously reported 16S rRNA and *amo*A thaumarchaeotal genes sequences available in GenBank from inland aquatic systems and soils (Appendix A Table A.S3.1). Multiple-sequence alignment was carried out with the software MAFFT (Katoh et al 2002) and consensus regions were identified as target specific-sites. Primers were designed using Primer3 (Rozen and Skaletsky 2000) with the following settings for optimal amplification in qPCR: (1) amplified PCR fragment < 400 bp, (2) primers of at least 20 bp

Table 3.1: Characteristics and specific targets of the newly designed primer sets developed in this study for quantitative PCR.

Primer name	Primer sequence (5´- 3´)	Target	Gene	Position	Expected lenght (bp)	%GC	Tm
SAGMCG1-274F	AGGAGAAGCCCGGAGATGGGT	SAGMCG-1 (Thaumarchaeota)	16S rRNA	274-294	192	61.91	58.73
SAGMCG1-446R	ATTAYCGCGGCGGCTGACAC		16S rRNA	446-465		60.0 - 65.0	57.87 - 60.11
Ntalea 1.1a -135F	GCTCGCAGTCGGTGCAGCATA	*Nitrosotalea* subcluster 1.1 (SF1OTU1)	*amo*A	135-155	411	61.91 - 66.67	59.85 - 62.11
Ntalea 1.1a -545R	GCACTAGCGCCTGCACCCAAA		*amo*A	545-525		57.14 - 61.91	57.66 - 60.25
Ntalea 1.1b-133F	ATGCTTTCAGTCGGTGCCGC	*Nitrosotalea* subcluster 1.1 (SF1OTU2)	*amo*A	133-152	400	60.0 - 65.0	58.27 - 60.93
Ntalea 1.1b-538R	CACCCGCACCTAGCGCGAC		*amo*A	538 - 520		68.42 - 73.68	57.71 - 60.46
Npumilus-420F	GCGGACCCACTAGAAACGGCA	*Nitrosopumilus* cluster (F5OTU3)	*amo*A	420-440	123	57.14 - 61.91	56.79 - 59.12
Npumilus-542R	ACCTGCACCTGCACCCAGTG		*amo*A	542-523		65.0 - 70.0	59.19 - 61.99

The *amo*A clusters nomenclature follows Pester *et al.*, (2012)

Table 3.2: PCR amplification conditions for the newly designed primer sets developed in this study.

Primer pairs	Use	Template (ng/µL)	No. of cycles	Cycling conditions					
				Denaturation		Annealing		Extension	
				Temp (ºC)	Time (s)	Temp (ºC)	Time (s)	Temp (ºC)	Time (s)
SAGMCG1-274F/ SAGMCG1-446R	Cloning	25	35	94	30	58	45	72	90
	qPCR	1	45	98	5	58	20	72	15
Ntalea 1.1a -135F/ Ntalea 1.1a -545R	qPCR	1	45	98	5	63	20	72	15
Ntalea 1.1b-133F/ Ntalea 1.1b-538R	qPCR	1	45	98	5	63	20	72	15
Npumilus-420F/ Npumilus-542R	qPCR	1	45	98	5	63	20	72	15

for better specific amplification and (3) maximum one degeneracy per primer. One of the primer sets (SAGMCG1 274F-446R, Table 3.1) specifically targeted the 16S rRNA gene of the SAGMCG-1 (*Nitrosotalea*-like) Thaumarchaeota group, a sister clade of the Marine Group (MG) 1.1a. In silico, the forward primer matched >75% of the available SAGMCG-1 sequences and showed a few unspecificities with the MG 1.1a. The reverse primer matched >88% of the SAGMCG-1 sequences and a few matches with 1.1b and 1.1c thaumarchaeal sequences and crenarchaeal group sequences. Combined, these primers had only the SAGMCG-1 cluster as potential target. The primers set was experimentally tested using both environmental clones previously available from the Limnological Observatory of the Pyrenees (LOOP) containing nearly-full 16S rRNA fragments of the SAGMCG-1 group, and natural samples from Lake Botornas, Lake Granotes, and Lake Redon collected in previous studies (Triadó-Margarit and Casamayor 2012, Auguet and Casamayor 2013). One ~192 bp length DNA fragment was observed in all the cases, as expected (Fig. 3.1).

The PCR product from the three lakes was cloned in TOPO TA cloning kit (Invitrogen) as previously reported (Ferrera *et al.,* 2004) following PCR Bconditions reported in Table 3.2, and 96 clones were sequenced using external facilities (http://www.macrogen.com). Phylogenetic analysis showed that all the sequences were placed within the SAGMCG-1 cluster. Additional cloning and sequencing with universal primers 21f-958r for the archaeal 16S rRNA gene was carried out when needed following Auguet *et al.,* (2011).

The three additional primers sets targeted the *amo*A gene of the three most abundant AOA populations previously found in the LOOP (OTU 1, 2, and 3, Fig. 3.2). The *amo*A gene sequences were highly conserved within each OTU allowing us to design the specific primers without the need of degenerated bases. The *amo*A OTU 1 was closely related to the *Nitrosotalea* 1.1 subcluster (Pester *et al.,* 2012), it matched 7 % of total *amo*A gene sequences found in the LOOP, and it was covered by the specific primer set Ntalea1.1a 135F-545R (Table 3.1).

The *amo*A OTU 2 was also closely related to the *Nitrosotalea* 1.1 subcluster, it matched 26 % of total *amo*A gene sequences found in the LOOP, and it was covered by primer set Ntalea1.1b 133F-538R. Finally, the *amo*A OTU 3 was closely related to the *Nitrosopumilus* cluster (Pester *et al.,* 2012), it matched 60 % of total *amo*A gene sequences in the LOOP, and it

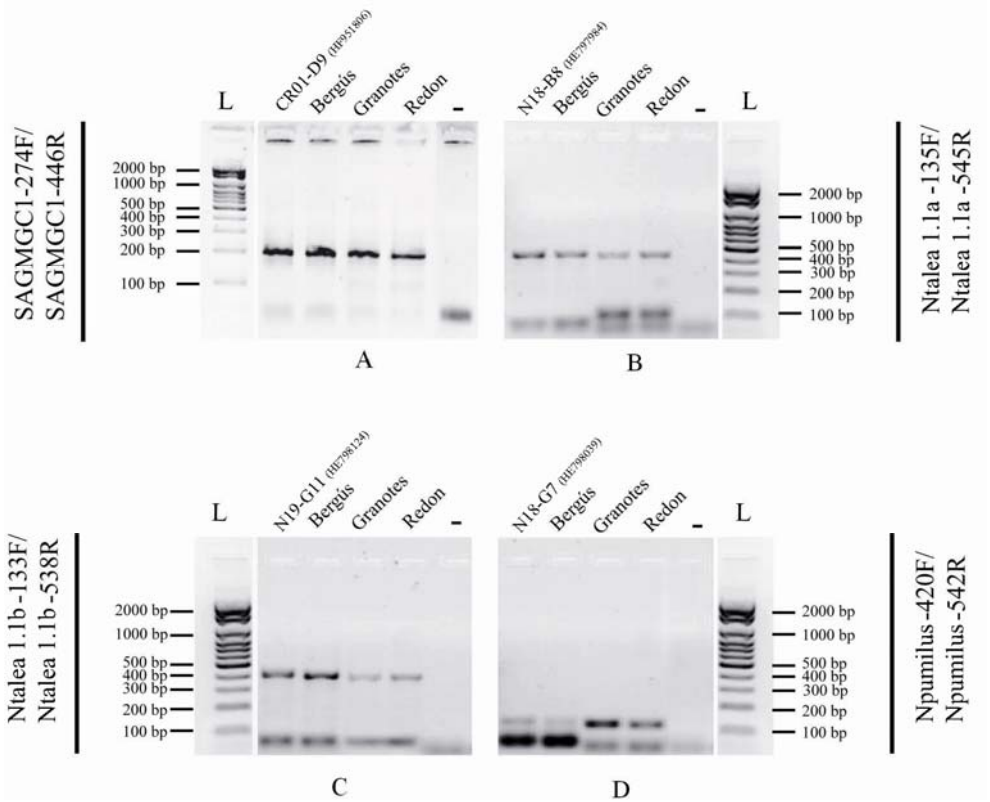

Figure 3.1: Agarose gel electrophoresis analysis of SAGMCG-1 16S rRNA and amoA genes amplified using different combinations of the newly designed specific primers in lakes Bergús, Granotes and Redon, and in clones containing the target sequences. Electrophoresis was carried out at 110 V for 45 min, and the gels were stained with ethidium bromide (2 mg ml^{-1}).

was covered by the primer set Npumilus 420F-542R. The coverage for these primers ranged between 67 and 89% of the aligned *amo*A gene dataset, and the specificity of primers sets was checked on the *amo*A database of Pester *et al.,* (2011). Each primers pair was very specific and matched only with sequences belonging to its own cluster (Fig. 3.2). Additionally, we experimentally tested the primers with environmental *amo*A clones and natural samples from the LOOP. Abundant PCR products of the expected size were obtained from all lake samples (Fig. 3.1), and not cross priming amplification was detected when different clones belonging to each *amo*A cluster were tested (Fig. 3.3).

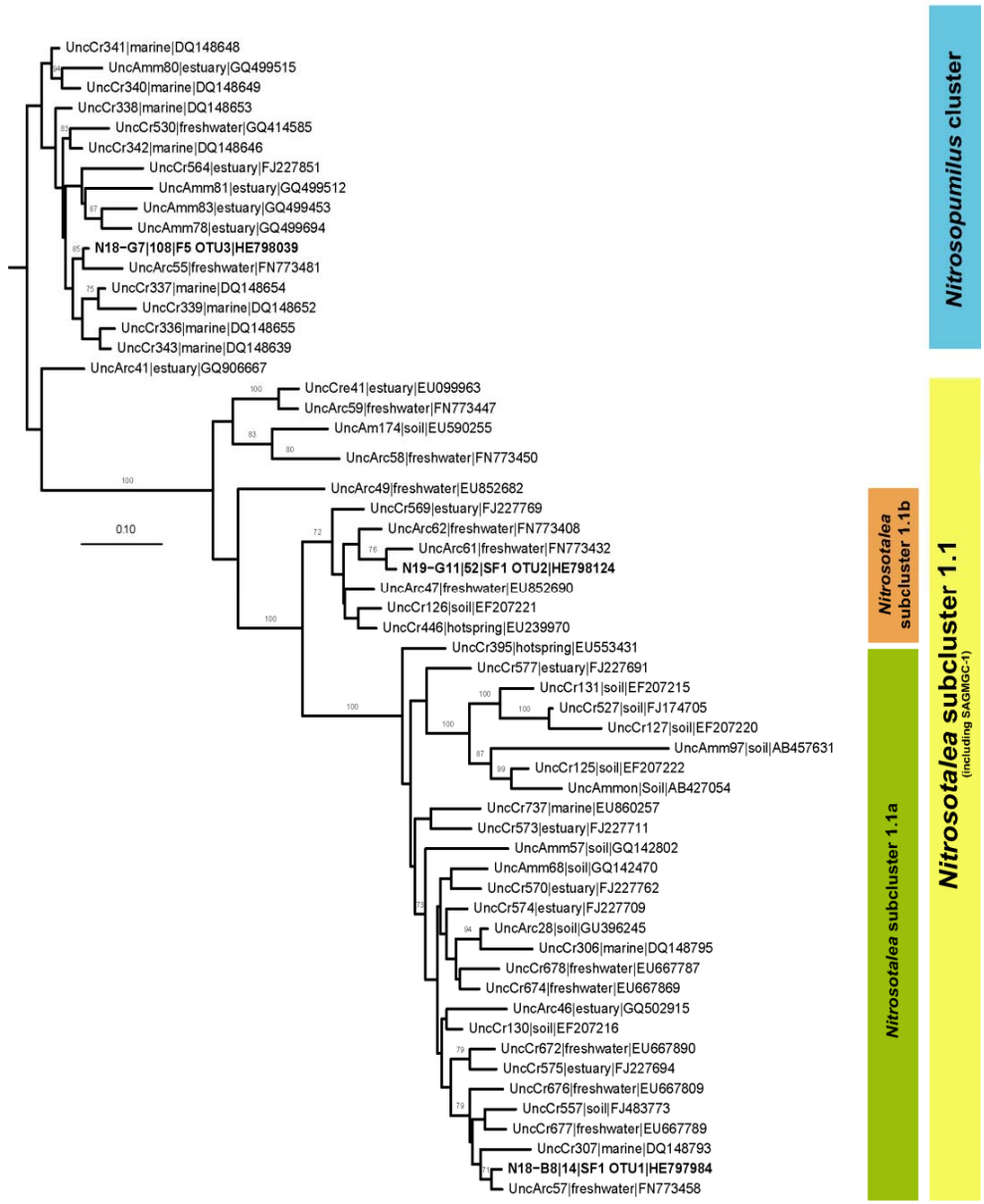

Figure. 3.2. Maximum-likelihood phylogenetic tree of the archaeal *amo*A gene for the SAGMCG-1 and MG 1.1a thaumarchaetal groups. The three AOA ecotypes shown in Figure 3.3 are highlighted. See more details in supplementary material Table A.S3.1. Scale bar, 10% estimated divergence.

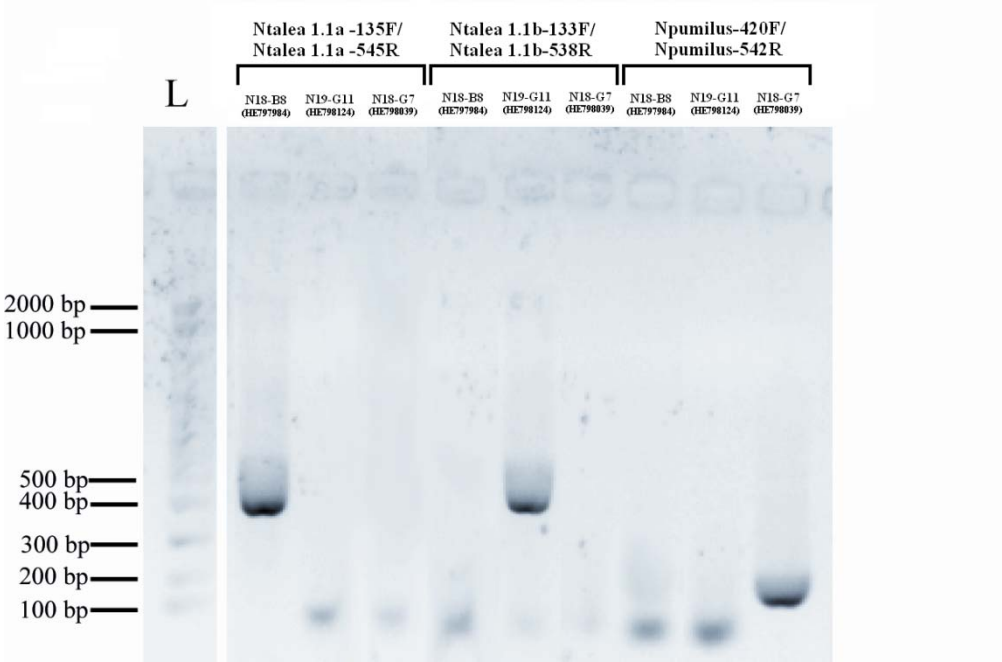

Figure 3.3: Agarose gel electrophoresis analysis of amoA gene amplification after different combinations of the newly designed specific primers and different clones containing target and non-target sequences. Negative results were obtained in all the combinations with non-target clones. Electrophoresis was carried out at 110 V for 45 min, and the gels were stained with ethidium bromide (2 mg ml−1).

3.2.3 Quantitative real-time PCR (qPCR) and data analysis

Abundances of the thaumarchaeota SAGMCG-1 16S rRNA and *amo*A genes were determined by qPCR amplification. The qPCR assays were run on 96-well white qPCR plates (Bio-Rad) in a DNA engine thermal cycler (Bio-Rad, Hercules, CA) equipped with a Chromo 4 real-time detector (Bio-Rad). The reaction mixture (20 µl) contained 10 µl of SsoFast EvaGreen supermix (Bio-Rad), 5 µl of template DNA (1 ng), 10 µM primers, and molecular biology-grade water (Sigma). The qPCRs were run for 2 min at 98°C, followed by 45 cycles as detailed in Table 3.2. Standard curves were obtained from environmental clones as follows: SAGMCG-1 16S rRNA clone CR01Pyr-D9 Lake Bergús (HF951806); *amo*A OTU1 clone N18- B8 Lake Aixeus (HE797984); *amo*A OTU2 clone N19-G11 Lake Roi (HE798124); and *amo*A

OTU3 clone N18-G7 Lake Muntanyó d'Arreu (HE798039). All reactions were run in triplicate with standard curves spanning from 10^2 to 10^7 copies of DNA. Optimal primer concentration (Table 3.2) produced amplification efficiency of 84-92% and r^2 value of 0.999. For all amplification reactions, melting curves from 65°C to 95°C were carried out after each run with an incremental increase in temperature of 0.5°C.The specificity of reactions was confirmed by melting-curve analyses and by agarose gel electrophoresis to identify unspecific PCR products, such as primer dimers or gene fragments of unexpected length (data not shown). Spearman rank (rs) correlations and multiple linear regression analysis were run in R (http://www.r-project.org/) with the corrgram and ggplot2 packages to investigate the relationships between gene abundance and environmental parameters.

3.2.4 Phylogenetic analyses

The 16S rRNA gene sequences were automatically aligned with the NAST aligner, clustered at identity threshold of 97% and imported into the Greengenes database (http://greengenes.lbl.gov/).The Archaea base frequency filter available in ARB (http://www.arb-home.de) was applied to exclude highly variable positions before sequences were added using the ARB parsimony insertion tool to the optimized tree provided by default. The *amo*A gene sequences were manually checked and clustered before multiple sequence alignment and phylogenetic inference by maximum likelihood as recently reported (Auguet *et al.*,, 2011).

3.3 Results

Figure 3.4 shows seasonal and vertical changes in water temperature (panel A), percentage of incident light (B), nitrogen compounds (C, D, and E), and chlorophyll-*a* (F), in the deep glacial stratified Lake Redon along one year. The lake has a dimictic regime, with mixing periods in late spring and autumn, and it is usually ice- and snow covered for about 6 months of the year, being the underlying water column mostly in the dark for several months in winter and spring. The thickness of the snow cover usually reaches several meters in April. During the ice-free season, light penetration into the water column was very high because of the high transparency due to low

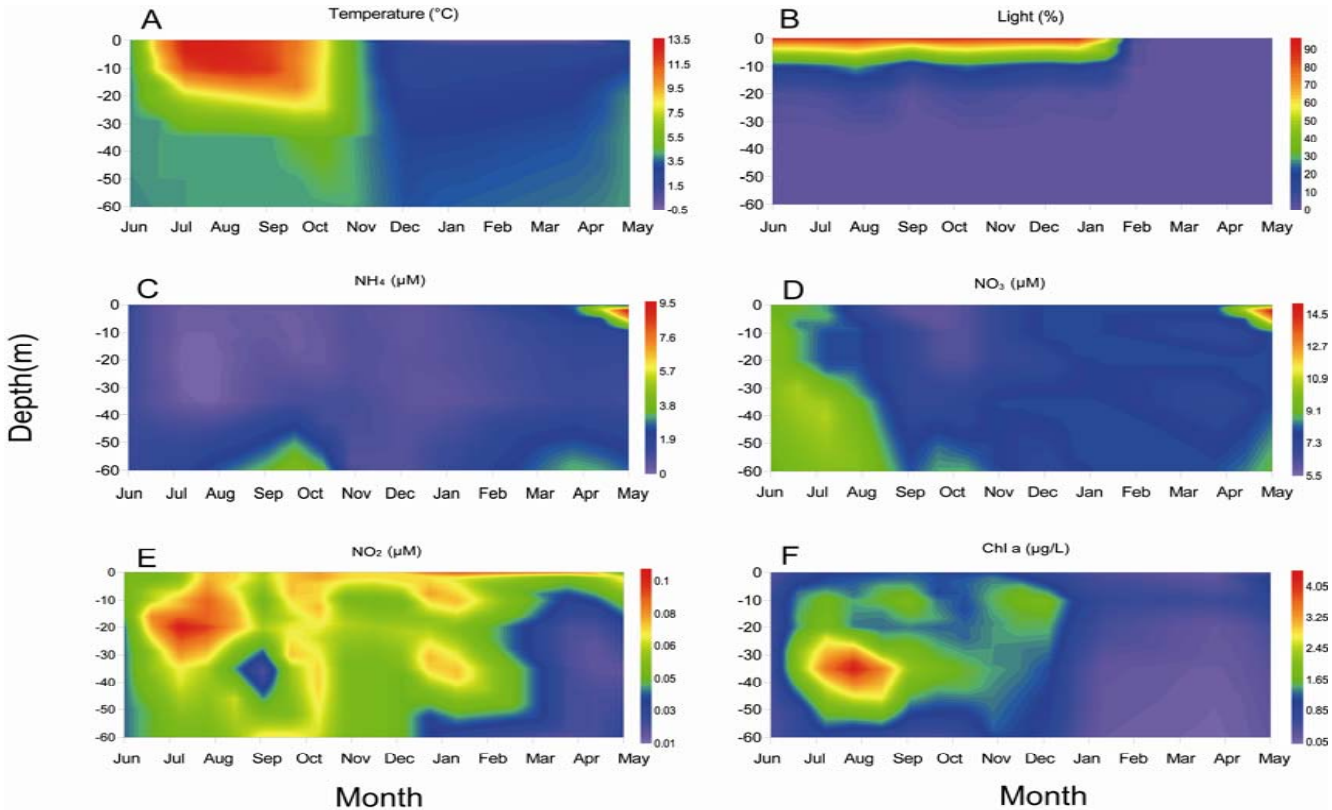

Figure 3.4: Annual variation in (**A**) water temperature, (**B**) irradiance (% of incident light), and concentrations of (**C**) ammonia, (**D**) nitrate, (**E**) nitrite, and (**F**) chlorophyll-*a*, along the vertical gradient in the deep alpine Lake Redon. The lake was covered by ice and snow from January to May-Jun

dissolved organic carbon (10-100 µM, Auguet el al. 2012) and low algae growth (i.e., low Chl *a* concentrations). Along the melting period, and as atmospheric nitrogen deposition (i.e., snow and rain) is the main source of reactive nitrogen in alpine areas (c. 30 fold higher than mean values measured in lake water, Auguet *et al.,* 2011, Camarero and Catalan 2012), maximal concentrations of released nitrate (c. 15 µM) and ammonium (c. 10 µM) were observed in surface waters. In addition, snow thawing in the catchment maintains a large water flow from the catchment into the lake. Additional minor peaks in nitrate and ammonia were observed in bottom waters in Sep-Oct and in April-May, most probably because of microbial mineralization and nitrification in the sediment. Interestingly, higher nitrate concentrations were also observed along the water column in early summer, and in general higher nitrite concentrations were observed in surface than in deep waters along the year.

The newly designed primers were used to follow the vertical and temporal changes of SAGMCG-1 and AOA ecotypes along the annual survey in Lake Redon. Amplification signal was obtained from all depths and dates examined. The SAGMCG-1 16S rRNA gene showed the largest variation in gene abundance spanning 5 orders of magnitude from <5 copies/mL lake water up to 8 x 10^4. Interestingly, we observed a bloom of this population during the dark period at c. 20 m depth, starting in January-February in deeper waters and reaching the highest concentration in early spring (Fig. 3.5A). Thus, SAGMCG-1 was more abundant during the winter stratification period when Lake Redon was in the dark covered by ice and snow, and gene concentrations substantially decreased after the snow melt period and during the summer stratification.

The analysis of the annual distribution pattern of abundance also unveiled segregated maxima for the different *amo*A populations, separated in time and space (Fig. 3.5 B, C and D). The *Nitrosotalea* 1.1b ecotype (SF1OTU2 in former works, Auguet *et al.,* 2013) was the most abundant and showed gene concentrations spanning 4 orders of magnitude ranging from <5 copies/mL lake water up to 1 x 10^4. A bloom of this population was found matching the bloom and annual dynamics of SAGMCG-1 (Fig. 3.5B). This fact strongly indicates that both SAGMCG-1 16S rRNA and Ntalea (*Nitrosotalea*-target) 1.1b primer sets targeted the same population. In fact, a strong correlation

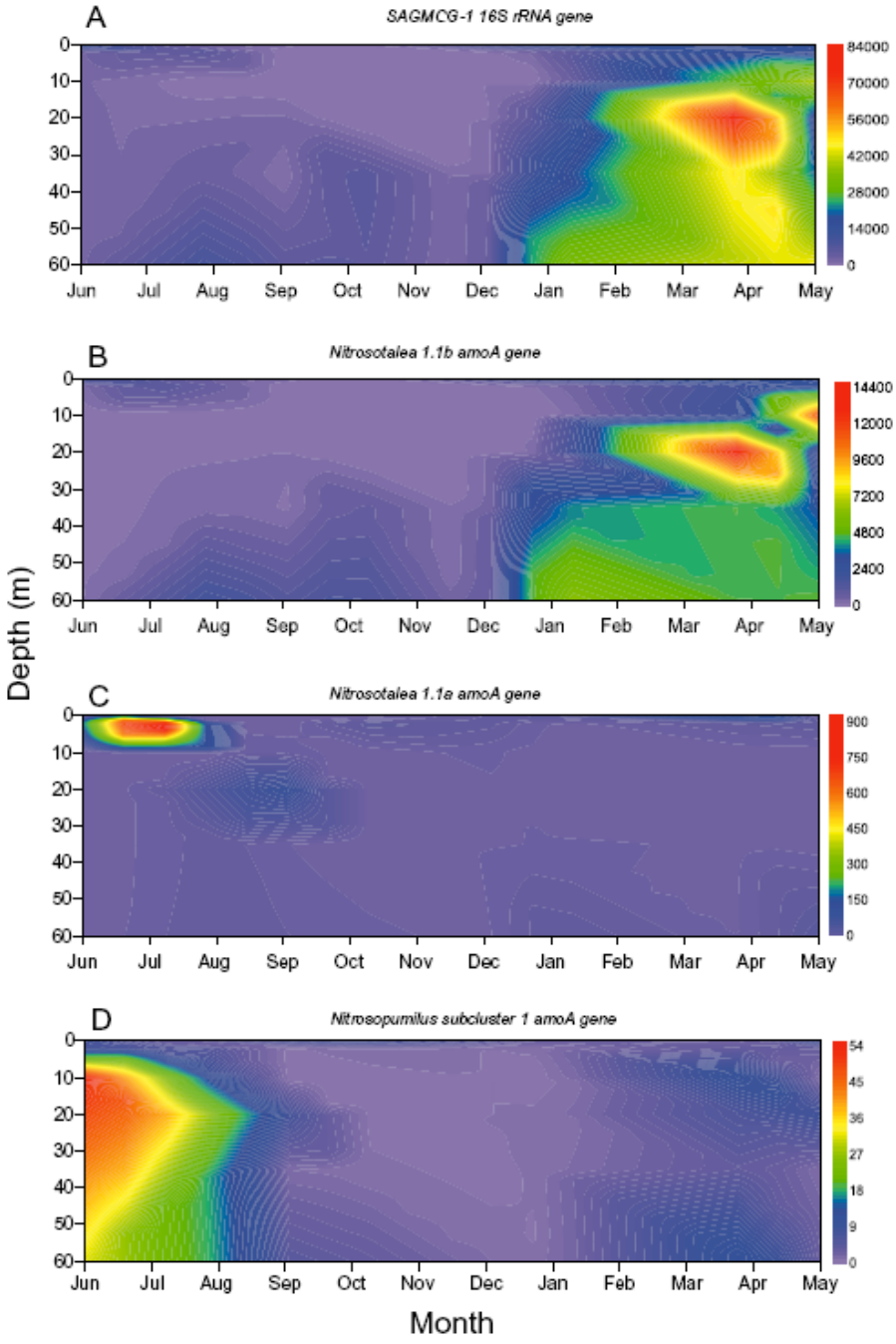

Figure 3.5: Annual spatio-temporal variation in the concentrations of specific AOA genes (gene copies/mL lake water) determined by qPCR for (**A**) SAGMCG-1 16S rRNA, (**B**) *Nitrosotalea* 1.1b *amo*A, (**C**) *Nitrosotalea* 1.1a *amo*A, and (**D**) *Nitrosopumilus amo*A, along the vertical gradient in the deep alpine Lake Redon.

(r_s = 0.82, P < 0.001, n = 44) was found between gene abundances of both populations after combining the whole dataset (Fig. 3.6A). Gene abundances of both SAGMCG-1 16S rRNA and *Nitrosotalea* 1.1b ecotype were positive and significantly correlated with nitrate (r_s=0.40 and 0.40, P < 0.01, n=44) and ammonium (r_s= 0.70 and 0.69, P < 0.01, n=44), and negative and significantly correlated with nitrite (r_s= -0.46 and -0.42, P < 0.01, n=44), chlorophyll-*a* (r_s= -0.70 and -0.69, P < 0.01, n=44), light (r_s= -0.63 and -0.60, P < 0.01, n=44) and water temperature (r_s= -0.35, P < 0.05, n=44). However, only nitrite and chlorophyll-*a* concentration significantly explained the variability in gene abundance after multiple linear regression analysis (P < 0.001 and P < 0.05, respectively).

Conversely to the *Nitrosotalea* 1.1b ecotype that bloomed during the dark period, the other two *amo*A ecotypes were substantially less abundant and showed the highest abundances immediately after the melting period but again with a marked spatial and temporal segregation. The *Nitrosotalea* 1.1a population (SF1OTU1 in former works, Auguet *et al.,* 2013) bloomed in surface waters (i.e., above 10 m) in July, and showed abundances in the lake spanning 3 orders of magnitude ranging from <5 copies/mL up to 9 x 10^2 (Fig. 3.5C). No significant relationship was found with the distribution of SAGMCG-1 (r_s = 0.01, P < 1, n = 44) (Fig. 3.6B), and the ratio SAGMCG-1 / Ntalea 1.1a was >100. Finally, the *amo*A gene abundance of *Nitrosopumilus* subcluster 1 population (F5OTU3 in former works, Auguet *et al.,* 2013) peaked in subsurface waters (i.e. 10 m and below) in June, and showed abundances in the lake spanning 2 orders of magnitude ranging from <5 copies/mL up to 50 (Fig. 3.5D). As expected, no significant relationship was found with the distribution of SAGMCG-1 (r_s = 0.004, P < 1, n = 44) (Fig. 3.6C). Any of the environmental variables significantly explained the variability (P < 0.05) in the *amo*A gene abundance of these two minor populations after multiple linear regression analysis.

Gene abundances of *Nitrosotalea* 1.1a and *Nitrosopumilus* were correlated (Spearman rank) positive and significantly only with nitrate (r_s=0.30 and 0.44, respectively, P < 0.01, n=44).

Finally, we explored the 16S RNA gene of depths July 2m and June 10m with universal archaeal primers (21f-958r), and >60 clone sequences were analyzed. These results were compared with the 16S rRNA gene sequences

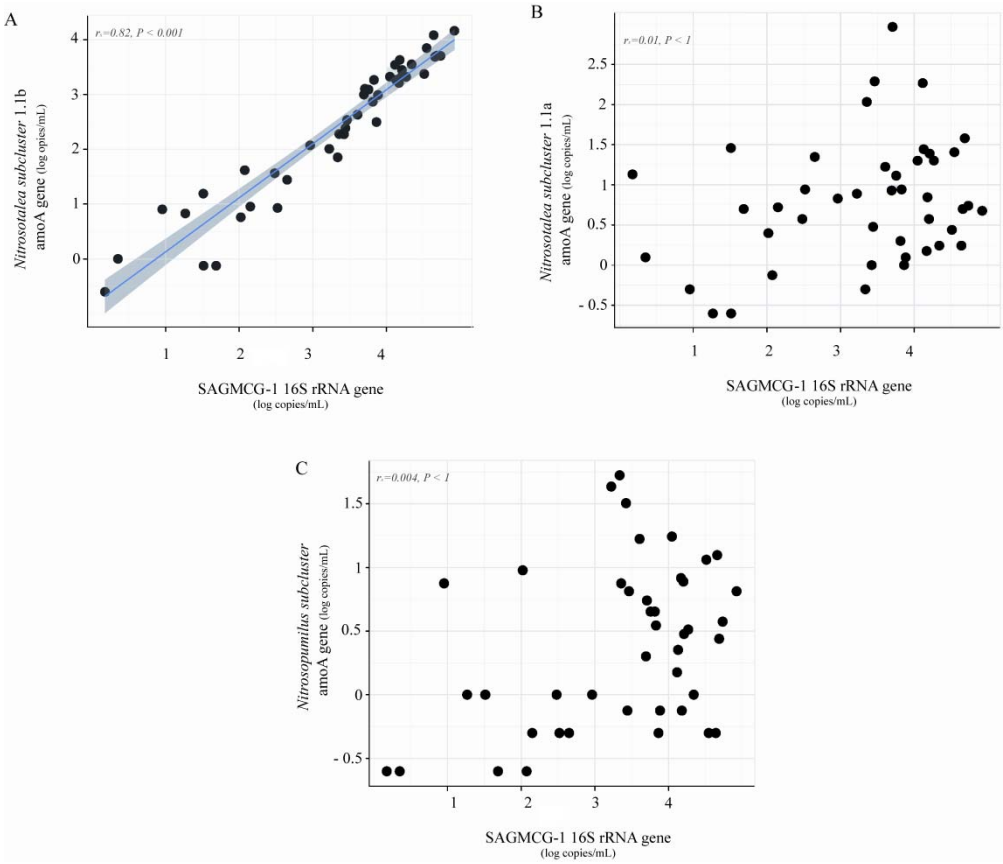

Figure 3.6: Relationships between abundances of SAGMCG-1 16S rRNA gene and the *amo*A gene of (**A**) *Nitrosotalea* subcluster 1.1b (PyrSF1OTU2), (**B**) *Nitrosotalea* subcluster 1.1a (PyrSF1OTU1), and (**C**) *Nitrosopumilus* subcluster 1 (PyrF5OTU3). Regression coefficients and associated *P* values are shown.

obtained from April 20 m (dark period) in a previous work (Auguet *et al.*, 2012) with accession numbers in GenBank HE589747 and HE589751 for representative OTUs (Table A.S3.1). All the 16S rRNA gene sequences from July 2m were SAGMCG-1 located within a subgroup that we tentatively named *Pyr-July light* (Fig. 3.7). In June 10m, 84% of the 16S rRNA sequences matched the SAGMCG-1 *Pyr-July light* cluster and 16% were *Nitrosopumilus*–like sequences (*Pyr-July subsurface,* Fig. 3.7). Interestingly, this *Pyr-July light* cluster was separated from the 16S rRNA gene sequences obtained from April 20 m and labeled as *Pyr-Apr dark* cluster in Figure 3.7

(90% identity to *Nitrosotalea devanaterra*). The ecologically segregated SAGMCG-1 ecotypes were therefore also phylogenetically separated.

3.4 Discussion

The environmental heterogeneity of Pyrenean Lakes in particular, and probably mountain lakes in general, is a very convenient natural framework to investigate the ecology and distribution of idiosyncratic ammonia-oxidizing archaea (AOA) and to explore the links with the nitrogen cycle. Previous studies on the seasonal changes in ribosomal (16S rRNA) and functional (ammonia monooxygenase, *amo*A) thaumarchaeotal genes showed dynamic changes in the diversity and abundance of AOA assemblages inhabiting oligotrophic mountain lakes (Auguet *et al.,* 2011, 2012, Vissers *et al.,* 2013). In addition, both correlation of AOA dynamics with changes in nitrogen species and absence of bacterial *amo*A gene counterparts suggested a key role of Archaea in the N biogeochemical cycling in alpine lakes (Auguet *et al.,* 2011, 2012, 2013).

In previous studies based on 16S rRNA gene cloning and sequencing and analysis of relative gene abundances, we had shown the coexistence of thaumarchaeotal MG 1.1a and SAGMCG-1 clades in the same lacustrine district but apparently segregated both vertically by distinctive positioning in the water column (Auguet *et al.,* 2012), and spatially among lakes by differential response to environmental drivers (Auguet and Casamayor 2013). All these results confirmed the potential ecological importance of the SAGMCG-1 cluster in oligotrophic waters. While members of the MG 1.1a have been largely implicated in nitrification (Konneke *et al.,*, 2005; Hallam *et al.,*, 2006; Blainey *et al.,*, 2011), for the SAGMCG-1 clade only a very recent study indicates that a cultured ammonia-oxidizing strain from an acid soil, *Nitrosotalea devanaterra*, contained the *amo*A gene (Lehtovirta-Morley *et al.,* 2011). In the present investigation, the correlational approach carried out using quantitative data strongly supports the potential for ammonia oxidation within the freshwater counterparts of the SAGMCG-1 cluster. However, we observed substantial differences in the ratio between the abundance of SAGMCG-1 16S rRNA gene and the amoA gene of the different *Nitrosotalea*-like ecotypes, being the 16S rRNA more abundant than the amoA gene detected in all the cases.

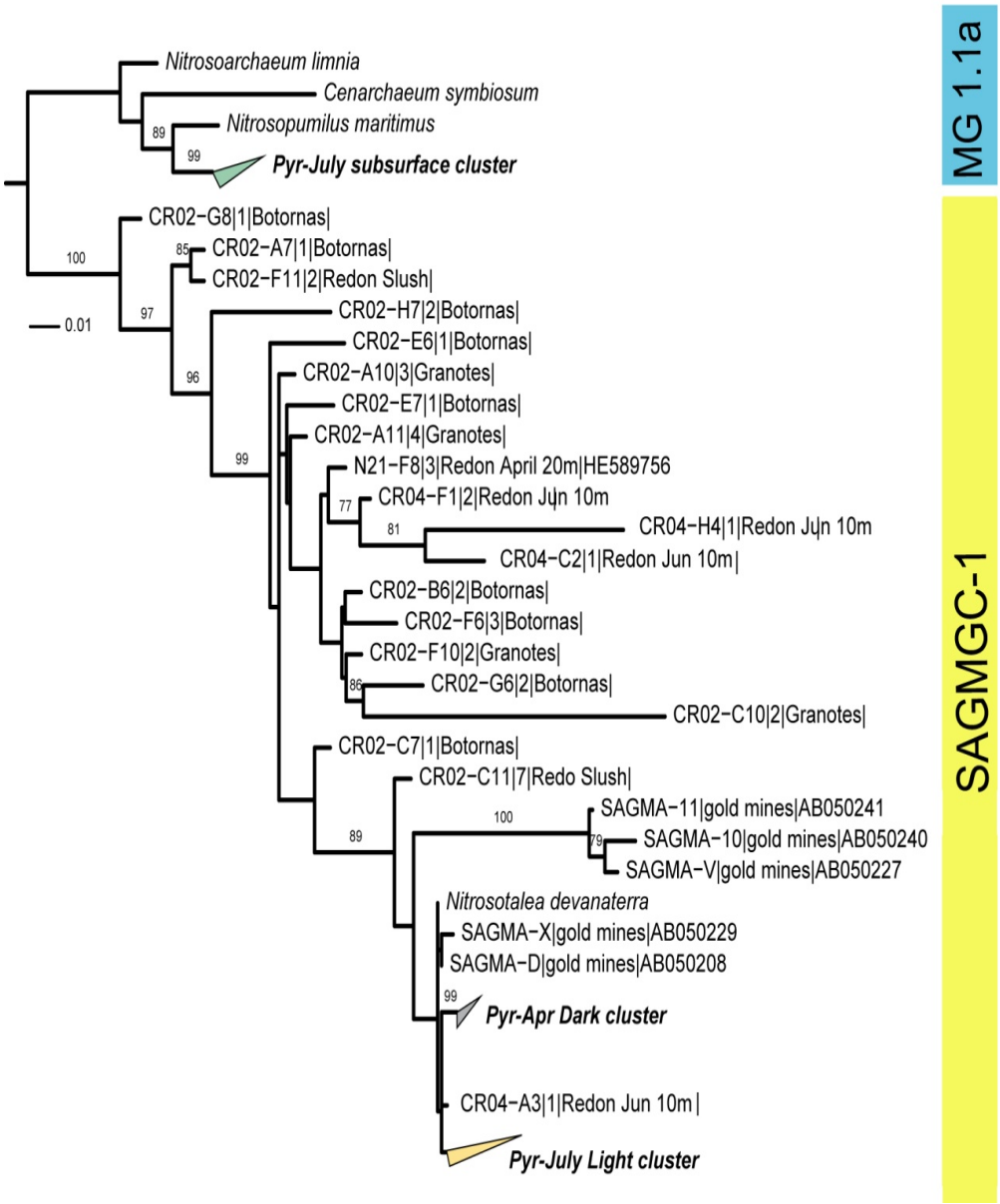

Figure 3.7: Maximum-likelihood phylogenetic tree of the Thaumarchaeota 16S rRNA gene sequences from the SAGMCG-1 and MG 1.1a groups. The three AOA ecotypes are highlighted (see text for details). See in more information in Table A.S3.1. Scale bar, 10% estimated divergence.

The primer for SAGMCG-1 did not shown preferential amplification for any of the two 16S rRNA clusters against the other ("light" vs. "dark" clusters, Fig 3.7). Thus, apparently only a few of the SAGMCG-1 had also *Ntalea* 1.1a amoA. Other explanations for the gene ratio SAGMCG-1/*Ntalea* rely on the number of gene copies per genome in each one of these populations, or that not all the SAGMCG had amoA, but these questions cannot be tested without cultures from the different ecotypes. Finally, additional ecotypes not covered by the amoA primer sets used here may also be present in this environment, and even different PCR efficiencies cannot be ruled out. Nevertheless, our results showed a completely different spatio-temporal distribution for the different freshwater AOA unveiling the existence of phylogenetically close but ecologically distinct AOA species.

At present, the ecological factors shaping the differential distribution of AOA ecotypes are, however, difficult to be properly established. Again, cultures from the different ecotypes for ecophysiological analyses in the laboratory would be desirable. However, two environmental factors deserve to be explored in more detail in future studies. The first factor is ammonia concentration, because changes in ammonia affinity may potentially explain AOA species distribution. In fact, the distribution of two distinct ecotypes of marine thaumarchaeota Group I (MGI) detected in the tropical Atlantic and the coastal Arctic (Sintes *et al.,* 2012), nicely matched medium (1-2 μM on average in the coastal Arctic) and low ammonia concentrations (< 0.01 μM in the deep Atlantic waters). In several Pyrenean mountain lakes explored, ammonia concentrations ranged between 0.3 and 2.3 μM (Auguet and Casamayor 2013) and concentrations in Lake Redon reached up to 4 μM both in bottom waters and close to the ice-cover in winter (Auguet *et al.,* 2012).

Thus, these mountain lakes are closer to the "medium" ammonia concentrations range than to the "low" range. However, ammonia concentrations during the blooms of the different AOA ecotypes in Lake Redon varied close to one order of magnitude from 0.2 μM in surface waters in June-July (where *Nitrosotalea* 1.1a and *Nitrosopumilus* bloomed) to 1.5 μM at 20 m in April (where *Nitrosotalea* 1.1b bloomed), and the influence of the in situ changes in ammonia concentration on thaumarchaeota populations dynamics in Lake Redon cannot be completely ruled out. Interestingly, the distribution of relatively high nitrite concentrations close to the air-water interface (0.05-0.1 μM) may also be related to both the high ammonia

concentrations provided by atmospheric deposition (Camarero and Catalan 2012) and the presence of active AOA cells, but this hypothesis certainly remains to be tested.

The second factor relies on differential photoinhibition, because inhibition by light potentially influences the distribution of ammonia oxidizers in aquatic environments and photoinhibition in AOA strains has been recently shown in laboratory cultures of *Nitrosopumilus maritimus* and *Nitrosotalea devanaterra* (Merbt *et al.,* 2012). Thus, the ecological effect of light intensity and/or quality is of particular interest since susceptibility to light applies to both nitrifying Bacteria (Horrigan *et al.*,, 1981; Olson, 1981; Guerrero and Jones 1996a, b) and Archaea (Mincer *et al.*,, 2007, Merbt *et al.,* 2012). Alpine lakes, especially those located at higher altitudes are exposed to high solar radiation due to the natural increase of irradiance with elevation (Catalan *et al.,* 2006). Furthermore, alpine lakes are among the most UV transparent aquatic ecosystems (Sommaruga & Augustin, 2006). Conversely, during the long ice cover period, the water column remains under an attenuated light regimen or in the dark. Therefore, differential spatial and temporal AOA species distribution might be expected along the annual cycle in Lake Redon. In addition, it has been shown that nitrite oxidizers (NOB) are more sensitive to light than AOB, whereas AOB may recover more rapidly from photoinhibition than NOB (Guerrero and Jones 1996b). AOB also became more photoresistant in the presence of higher ammonia concentrations, while NOB did not significantly change their light sensitivity after increasing nitrite concentration (Guerrero and Jones 1996b). If these results also apply to alpine AOA, the distribution of relatively high nitrite concentrations close to the air-water interface could be related to a differential sensitivity to light that deserves further investigations.

Overall, these results suggest a hypothetical active recycling of atmospheric reactive nitrogen by different AOA ecotypes that deserve further investigations for accurate testing of the hypothesis mentioned above. The distribution pattern of two of the three AOA ecotypes observed was consistent with the recently reported photoinhibition of two AOA strains under controlled conditions in the laboratory. However, a third AOA ecotype showed an accumulation peak close to the top of the lake in early summer, intriguingly suggesting an adaptation to the high solar irradiance and UV doses accounting in surface waters of Lake Redon. Thus, certainly there is

not a single parameter controlling AOA abundance and distribution in deep alpine lakes but rather a set of environmental variables intimately related to the limnology and the seasonal lake dynamics.

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4

Environmental distribution of two widespread uncultured freshwater Euryarchaeota clades unveiled by specific primers and quantitative PCR

Resumen

Se exploro la distribución ambiental de dos Euryarchaeota no cultivados de agua dulce (papel funcional desconocido), mediante el diseño de nuevos cebadores para PCR cuantitativa dirigidos al gen 16S rRNA de los clados MEG (siglas en ingles de Miscellaneous Euryarchaeota Group, que contiene los grupos pMC2A384 y VALII/Eury4) y DSEG (siglas en ingles de Deep-Sea Euryarchaeotal Groups, dirigidos al clúster denominado VALIII que contiene los grupos DHVE-3/DSEG, BC07-2A-27/DSEG-3, y DSEG-2). Se analizó el plancton superficial en verano de 28 lagos y adicionalmente Redon, un lago dimíctico profundo, se evaluo vertical y temporalmente para cubrir la variabilidad limnológica de temporada. Una gama trófica entre el intervalos de 0.2 y 5.2 mg/L de Chl a y pH 3.8 a 9.5, se exploró en altitudes entre 632 y 2.590 metros sobre el nivel del mar. Los cebadores mostraron ser altamente selectivos con un 85% de cobertura y 100% de especificidad. Sólo el pH explicó significativamente los cambios observados en la abundancia de los genes y el ambiente. En el lago Redon, DSEG floreció en profundas aguas estratificadas, tanto en verano y principios de la primavera, y MEG a profundidades intermedias durante el período de hielo cubierto. En general, MEG y DSEG mostraron una diferencial en la distribución ecológica, aunque los análisis de correlación no indicaron el acoplamiento de ambos Euryarchaeota con el fitoplancton (clorofila a). Sin embargo, una enigmática relación positiva y significativa se encontró entre DSEG y el presuntamente thaumarchaeota oxidante de amonio.

Abstract[1]

Quantitative environmental distribution of two widely distributed uncultured freshwater Euryarchaeota with unknown functional role, was explored by newly designed qPCR primers targeting the 16S rRNA gene of clades MEG (Miscellaneous Euryarchaeota Group containing the groups pMC2A384 and VALII/Eury4) and DSEG (Deep-Sea Euryarchaeotal Groups, targeting the cluster named VALIII containing the DHVE-3/DSEG, BC07-2A-27/DSEG-3, and DSEG-2 groups), respectively. The summer surface plankton of 28 lakes was analyzed and one additional dimictic deep lake, Lake Redon, was temporally and vertically surveyed covering seasonal limnological variability. A trophic range between 0.2 and 5.2 µg/L Chl *a*, and pH span from 3.8 to 9.5 was explored at altitudes between 632 and 2590 m above sea level. The primers showed to be highly selective with c. 85% coverage and 100% specificity. Only pH significantly explained the changes observed in gene abundances and environment. In Lake Redon, DSEG bloomed in deep stratified waters both in summer and early spring, and MEG at intermediate depths during the ice-cover period. Overall, MEG and DSEG showed a differential ecological distribution although correlational analyses indicated lack of coupling of both euryarchaeota with phytoplankton (chlorophyll *a*). However, an intriguing positive and significant relationship was found between DSEG and putative ammonia oxidizing Thaumarchaeota.

4.1 Introduction

After several years of environmental ribosomal gene surveys, a large number of uncultured mesophilic Euryarchaeota clades have been reported in the literature that contain ubiquitous microorganisms of unknown metabolism and unassigned functional roles in nature yet (see recent meta-analyses in Auguet *et al.,*, 2010, and Durbin and Teske, 2012). In addition, in some occasions such uncultured lineages hold different names, provided by different authors and at different databases, and lack a statistically robust branching (Auguet *et al.,*, 2010, Barberán *et al.,* 2011, Durbin and Teske, 2012), hindering a better understanding on the whole ecological potential and

[1]See original publication in Restrepo-Ortiz and Casamayor (2013)

natural history of Euryarchaeota. Analyzing samples containing natural enrichments of these targeted populations with taxon-specific molecular tools, functional genes, and metagenomic surveys will substantially improve the current knowledge on the ecology and physiological potential of these enigmatic groups, analogous to previous work carried out on the Thaumarchaeota (e.g., Schleper *et al.,* 2005, Konneke *et al.,,* 2005, Lehtovirta-Morley *et al.,* 2011). Unfortunately, in the case of the uncultured clades of Euryarchaeota with potential environmental importance, we are still far from what has been achieved for Thaumarchaeota in the last few years.

Although non-extremophile aerobic Euryarchaeota were initially described in marine environments (DeLong 1992, Fuhrman *et al.,* 1992) and were widely seen in different oceans and seas (mainly Groups II, III and IV; Bano *et al.,* 2004, Martin-Cuadrado *et al.,* 2008, Galand *et al.,* 2010), uncultured Euryarchaeota have also been frequently recovered from heterogeneous freshwater ecosystems (reviewed in Auguet *et al.,* 2010). Euryarchaeotal lineages are ubiquitous in continental aquatic ecosystems with a large and still heavily uncovered phylogenetic diversity (Barberán *et al.,* 2011), suggesting that they have key functional roles in freshwater habitats and have experienced large diversification processes (Auguet *et al.,* 2010). Since no cultured representatives of these lineages are available, their physiology, metabolism, and specific role in ecosystems functioning remain unknown. Most of these planktonic euryarchaeota are allocated in the SILVA database (http://www.arb-silva.de/) within both the clade MEG (Miscellaneous Euryarchaeota Group) containing the groups pMC2A384 (named after the hydrothermal vent studies of Takai and Horikoshi, 1999) and VALII/Eury4 (Jurgens *et al.,* 2000, Durbin and Teske 2012), on the one hand, and, on the other hand, the clade DSEG (Deep-Sea Euryarchaeotal Groups) targeting the cluster named VALIII (euryarchaeotal clades VAL name taken from the small boreal lake Valkea Kotinen studied by Jurgens *et al.,* 2000) which comprises the DHVE-3/DSEG, BC07-2A-27/DSEG-3, and DSEG-2 groups (Durbin and Teske 2012). For convenience, from now on in this paper we will follow the SILVA database nomenclature as clades MEG and DSEG (Fig.4.1).

Recently, we have carried out an environmental survey in the plankton of high-mountain lakes using “universal” primers for the archaeal 16S RNA gene, illustrating how these environments hold a rich archaeal community

dominated by Thaumarchaeota (mainly SAGMCG-1 and MG1.1a) and Euryarchaeota mostly from uncultured clades and Methanomicrobiales (Auguet and Casamayor 2008, 2013; Auguet *et al.*, 2011, 2012).

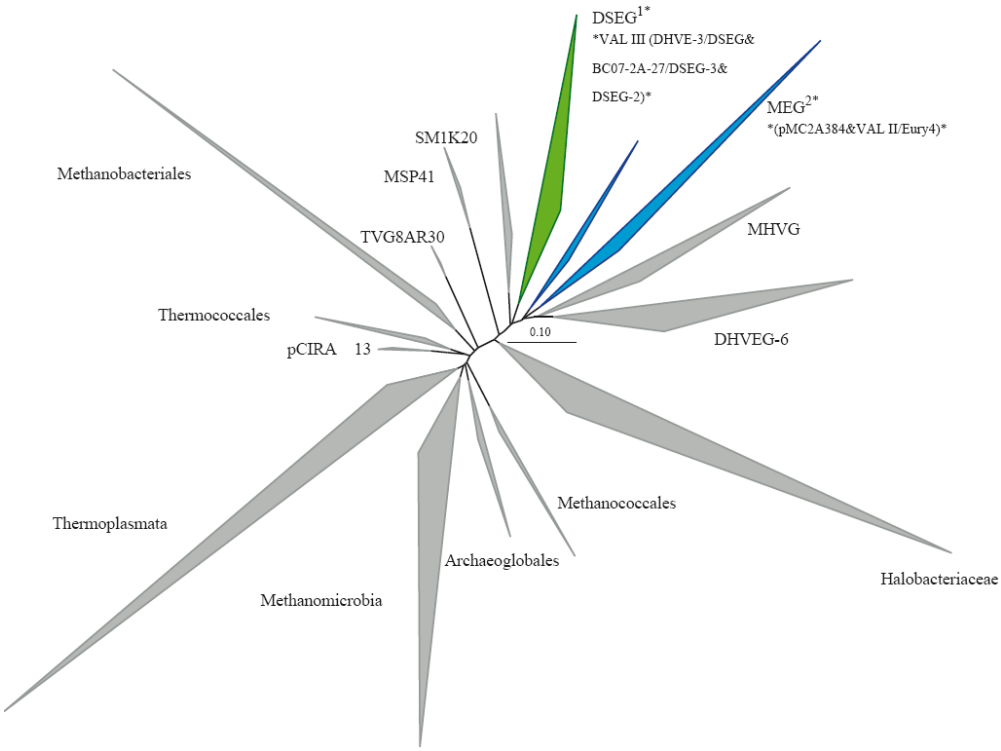

Figure 4.1: Phylogenetic context for the 16S rRNA gene of the Euryarchaeota DHVE and MEG targeted by specific primers in this study (SILVA ribosomal database 111 RefNR, July 2012). The clades are highlighted and equivalent clade nomenclature has been added following Durbin and Teske (2011, 2012). Scale bar, 10% estimated divergence.

High mountain lakes are globally distributed ecosystems with limited local anthropogenic disturbance; they are very sensitive indicators of global change that respond rapidly to environmental perturbations (Catalan *et al.*, 2006). Usually, they are low in ion concentrations and nutrient content, permanently cold, and experience marked seasonal changes in irradiance and high exposure to UV radiation during the ice-free period (Rose *et al.*, 2009). In the present study, we have designed and tested specific primers for

quantifying by qPCR the abundance of DSEG and MEG 16S rRNA gene in natural samples. In a companion paper, specific primers for freshwater thaumarchaeota (16S rRNA gene) and ammonia-oxidizing archaea (*amo*A gene) were optimized and tested (Restrepo-Ortiz *et al.*, 2014). We explored spatio-temporal differences in the surface plankton of a large set of lakes with marked environmental gradients, and along a temporal survey analyzing different dates and depths in the deep glacial stratified alpine Lake Redon located in the central Spanish Pyrenees (Catalan *et al.*, 2006, Auguet *et al.*, 2012). The correlational analyses indicated lack of coupling of euryarchaeotal populations with phytoplankton abundance and distribution (chlorophyll *a*) but an intriguing relationship between DSEG and putative ammonia oxidizing thaumarchaeota.

4.2 Material and Methods

4.2.1 Study site and sample collection

The annual survey was carried out in Lake Redon, an alpine deep lake located in the central Spanish Pyrenees (42º38'34''N, 0º46'13''E, altitude 2240 m, maximum depth 73 m, surface 0.24 km2). The lake is oligotrophic with poor vegetation and soil development in the small catchment area (c. 1.5 km^2). It is located on the head of the valley, and atmospheric deposition is the main source of reactive nitrogen (Catalan *et al.*,, 1994, 2006). Additionally, samples from 28 lakes sampled in summer 2008 and 2011 were used for an extensive spatial scrutiny of surface waters (first 1 m integrated). Samples collection, chemical and biological properties of the lakes, were measured as recently reported (Auguet *et al.*, 2012, where also additional field data can be found, and Restrepo-Ortiz *et al.*, 2014). Samples were processed and DNA was extracted and purified as reported (Hervàs *et al.*, 2009, Demergasso *et al.*, 2008).

4.2.2 Specific primers and PCR conditions

Specific primers for the clades MEG (Miscellaneous Euryarchaeota Group) and DSEG (Deep-Sea Euryarchaeotal Group) were designed and tested following the procedure recently reported (Restrepo-Ortiz *et al.*, 2014) on

16S rRNA genes sequences available in *SILVA* 111 RefNR ribosomal RNA (rRNA) (release July 2012) database (Appendix A Table A.S4.1) complemented with archaeal 16S rRNA gene from the Limnological Observatory of the Pyrenees (LOOP) (Auguet & Casamayor 2008, Auguet *et al.*, 2011, 2012, 2013). Multiple-sequence alignment was carried out with the software MAFFT (Katoh et al 2002) and consensus regions were identified as target specific-sites. Primers were designed using Primer3 (Rozen and Skaletsky 2000) with the following settings for optimal amplification in qPCR: (1) amplified PCR fragment < 400 bp, (2) primers of at least 20 bp for better specific amplification and (3) maximum one degeneracy per primer.

The PCR product was cloned in TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), as previously reported (Ferrera *et al.*,, 2004), 96 clones were sequenced using external facilities (http://www.macrogen.com). Additional cloning and sequencing with universal primers 21f-958r for the archaeal 16S rRNA gene was carried out when needed, following Auguet and colleagues (2011). The primers were experimentally tested both using environmental clones available from the Limnological Observatory of the Pyrenees (LOOP) containing nearly-full 16S rRNA fragments of the MEG and DSEG clades, and after cloning and sequencing of the PCR products obtained from up to three different lakes.

4.2.3 Quantitative real-time PCR (qPCR) and data analysis

Abundances of the euryarchaeota MEG and DSEG 16S rRNA gene were determined by qPCR amplification. The qPCR assays were run on 96-well white qPCR plates (Bio-Rad) in a DNA engine thermal cycler (Bio-Rad, Hercules, CA) equipped with a Chromo 4 real-time detector (Bio-Rad). The reaction mixture (20 µl) contained 10 µl of SsoFast EvaGreen supermix (Bio-Rad), 5 µl of template DNA (1 ng), 10 µM primers, and molecular biology-grade water (Sigma). Standard curves were obtained from environmental clones as follows: MEG 16S rRNA clone CR01-B6 (HF951784) and DSEG clone CR01-A5 (HF951773) both from Lake Montanyo. All reactions were run in triplicate with standard curves spanning from 10^2 to 10^7 copies of DNA. For all amplification reactions, melting curves from 65°C to 95°C were carried out after each run with an incremental increase in temperature of 0.5°C.The specificity of reactions was confirmed by melting-curve analyses

and by agarose gel electrophoresis to identify unspecific PCR products, such as primer dimers or gene fragments of unexpected length. Spearman rank (rs) correlations and multiple linear regression analysis were run in R (http://www.r-project.org/) with the corrgram and ggplot2 packages to investigate the relationships between gene abundance and environmental parameters.

4.2.4 Phylogenetic analyses

The 16S rRNA gene sequences were automatically aligned with the NAST aligner, clustered at identity threshold of 97% and imported into the *SILVA* 111 RefNR database (http://www.arb-silva.de/).The Archaea base frequency filter available in ARB (http://www.arb-home.de) was applied to exclude highly variable positions before sequences were added using the ARB parsimony insertion tool to the optimized tree provided by default.

4.3 Results and Discussion

4.3.1 Primers optimization and PCR conditions

Two potential specific primers sets were obtained for each euryarchaeotal clade (Table 4.1) that was tested experimentally at different annealing temperatures in different lakes samples (Fig. 4.2). Only the primer pairs MEG93F-392R and DSEG510F-725R produced a single fragment of the expected size, respectively (Fig. 4.2). These primer pairs did not shown unspecific matching in silico with any additional group. Thus, 4 mismatches were needed in the MEG primers to found unspecificities with eukaryotes, bacteria and halobacteria, and up to 3 mismatches in the DSEG primers to found unspecificities with other euryarchaeotal groups.

The new primers targeted on average 86% of the MEG and 84% of the DSEG 16S rRNA gene present in databases, respectively. Optimized amplification conditions are shown in Table 4.2. ARB-based phylogenetic analysis (Restrepo-Ortiz *et al.,* 2014) and pairwise comparison with available cloned sequences (c. 900 bp) from the LOOP dataset, showed all sequences placed within the respective MEG and DSEG clades, (Figs.4.3 and 4.4, respectively) indicating a good specificity for the new primers sets.

Table 4.1: Specific primers set designed in this study for qPCR analysis of the euryarchaeotal clades MEG and DSEG. The primer sets MEG 93f-392r, and DSEG 510f-725r showed the best performance and specificity, respectively (see Fig 4.2).

Primer name	Primer sequence (5´- 3´)	Target	Application	Position	Expected fragment (bp)	%GC	Tm
MEG-93F	TCGACGGACGGTGTACGGCT	MEG (Euryarchaeota-Halobacteria)	Clonning and quantitative PCR	93-112	296	65	59.97
MEG-392R	AGGTTTSGCGCCTGCTGCAT			392-373		60	59.9
MEG-410F	ATGCAGCAGGCGCGAAACCT	MEG (Euryarchaeota-Halobacteria)	Clonning	410-429	143	60	59.97
MEG-516R	CATGGCGGCTGGCACTGGTC			516-497		70	60.66
DSEG_6_510F	GCCAGCCGCCGCGGTAATAA	DSEG (Euryarchaeota-Halobacteria)	Clonning and quantitative PCR	510-529	216	65	60.18
DSEG_6_725R	GCYTTCGCCACAGGTGGTCC			725-706		65	58.19
DSEG_7_261F	ARGATGGGACTGCGGCGGAT	DSEG (Euryarchaeota-Halobacteria)	Clonning	261-280	270	60	58.2
DSEG_7_529R	TTATTACCGCGGCGGCTGGC			529-510		60	57.77

Table 4.2: Amplification conditions for the two newly developed qPCR primers for MEG and DSEG Euryarchaeota.

Primer pairs	Use	Template (ng/µL)	No. of cycles	Cycling conditions					
				Denaturation		Annealing		Extension	
				Temp (ºC)	Time (s)	Temp (ºC)	Time (s)	Temp (ºC)	Time (s)
MEG-93F/MEG-392R	Cloning	25	35	94	30	66	45	72	90
	qPCR	1	45	98	5	66	20	72	15
DSEG-510F/DSEG-725R	Cloning	25	35	94	30	58	45	72	90
	qPCR	1	45	98	5	58	20	72	15

The ammonia oxidizer thaumarchaeotal group SAGMCG-1 was studied using the specific 16S rRNA gene qPCR primers set 274F-446R recently described (Restrepo-Ortiz *et al.*, 2014). To explore the natural 16S rRNA gene abundances of freshwater euryarchaeota MEG and DSEG and thaumarchaeota SAGMCG-1, we combined both an extensive spatial scrutiny of surface waters (first 1 m integrated) from 28 lakes sampled in summer 2008 and 2011, and a spatio-temporal survey in the deep alpine Lake Redon.

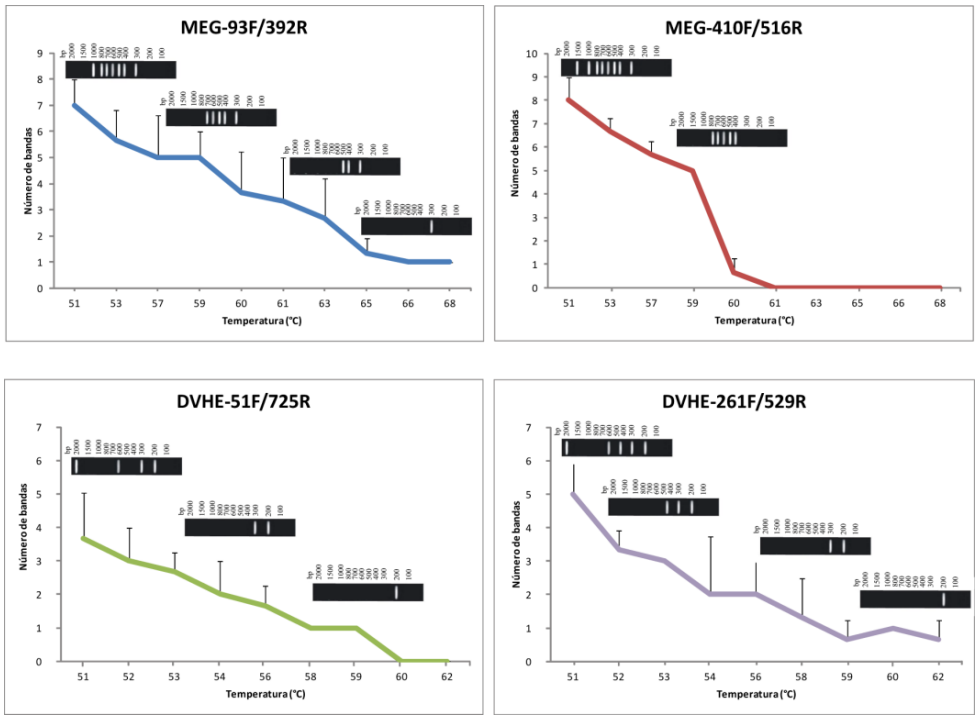

Figure 4.2: Specificity level (number of bands observed in an agarose gel) observed for the newly designed primers at different PCR annealing temperatures. Averaged values and error range for PCR products obtained from lakes Bergús, Llebreta, and Muntanyó d'Arreu. The primers sets MEG 93f-392r (expected PCR product size ~296 bp), and DSEG 510f-725r (~216 bp) showed the best performance and specificity.

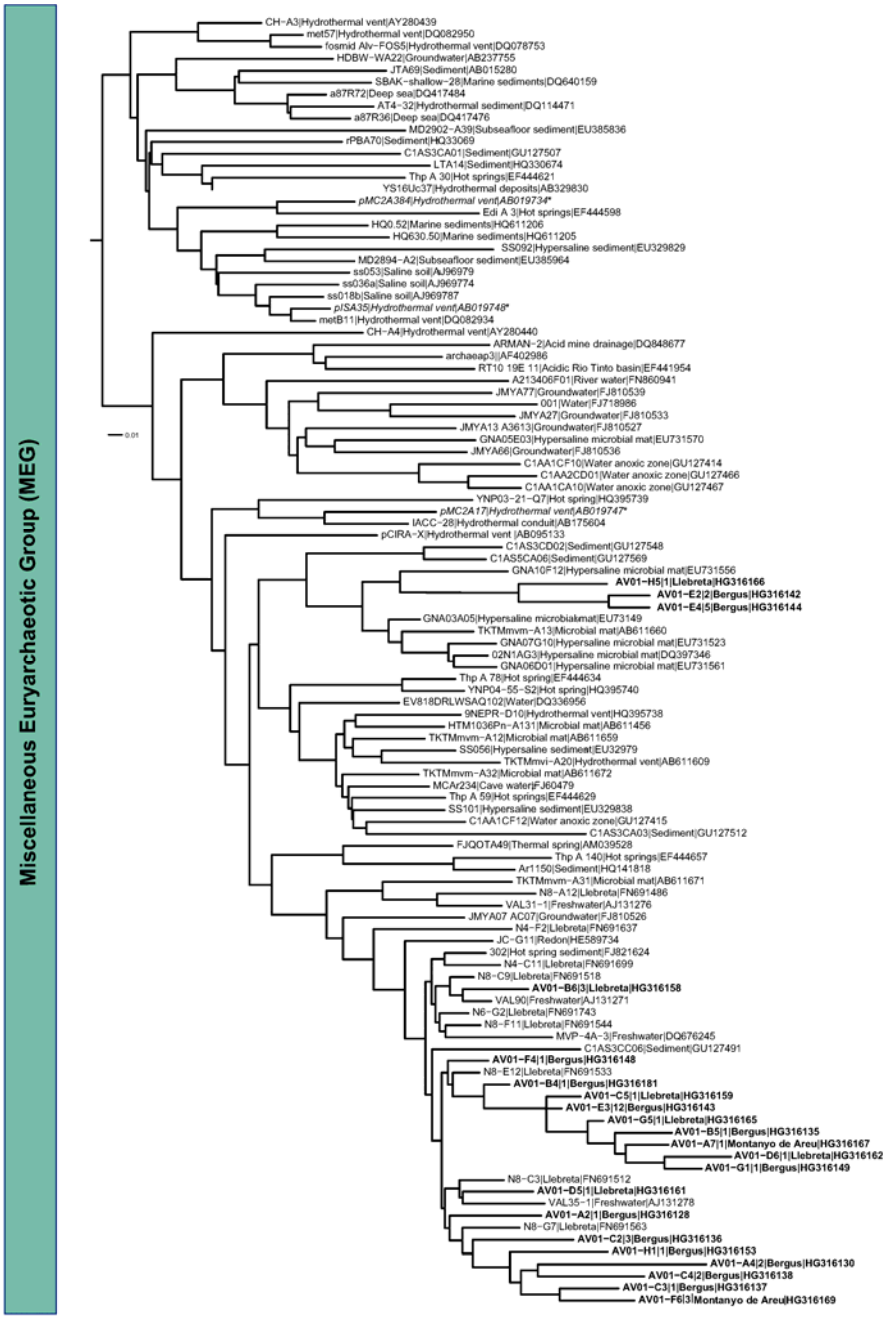

Figure 4.3: Maximum-likelihood phylogenetic tree for the 16S rRNA gene of the Euryarchaeota clade MEG (Miscellaneous Euryarchaeota Group comprising the clades pMC2A384 and VALII/Eury4; Durbin and Teske 2012) targeted by the specific primers set designed in this study. Scale bar, 10% estimated divergence.

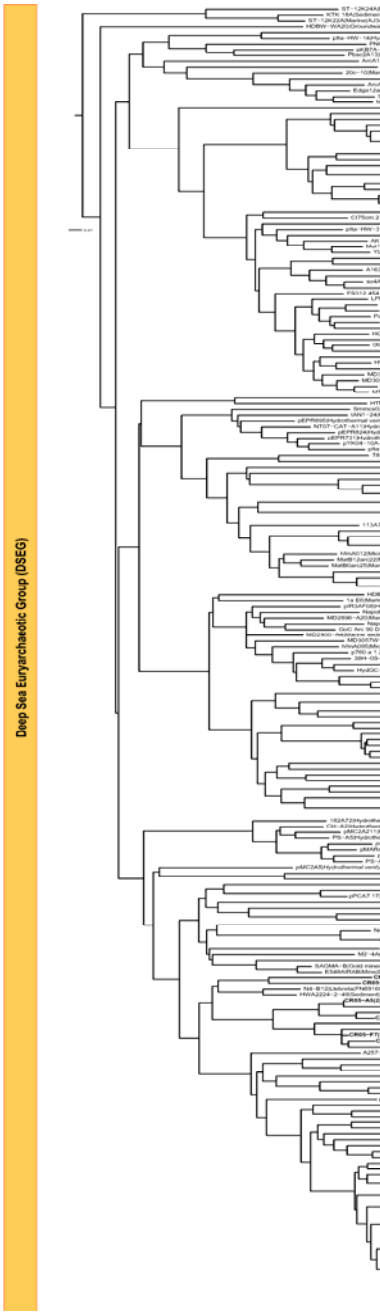

Figure 4.4:. Maximum-likelihood phylogenetic tree for the 16S rRNA gene of the Euryarchaeota clade DSEG (Deep-Sea Euryarchaeotal Groups, targeting the cluster named VALIII comprising the DHVE-3/DSEG, BC07-2A-27/DSEG-3, and DSEG-2 groups; Durbin and Teske 2011, 2012) targeted by the specific primers set designed in this study. Scale bar, 10% estimated divergence.

4.3.2 Spatial heterogeneity and environmental forcing

For the spatial scrutiny a set of lakes was selected covering a large environmental range of pH (3.8-9.5), temperature (4-31 °C), conductivity (3-1805 µS/cm), trophic status (i.e. Chl *a* concentrations, 0.43-5.18 µg/L), and altitudes above sea level from 632 to 2590 m (Table A.S4.2).Most of these lakes were shallow (i.e. <15 m depth), well mixed by wind, and not stratified. Interestingly, the 16S rRNA gene abundance for each archaeal population showed a spatially heterogeneous distribution and, on average, the highest abundance was found for DSEG (4.6×10^4; range <0.01-10^5 copies/mL) followed by MEG (1.1×10^4; range 10^1-10^5 copies/mL) and SAGMCG-1 (4.3×10^3; range 10^{-1}-10^4 copies/mL) (Table A.S4.2). A MDS ordination analysis grouped the lakes in different clusters of lakes based on gene abundances of each archaeal population (Fig. 4.5). Cluster I contained lakes dominated by clade DSEG (i.e., >88% of all three archaeal gene abundances) with minor contributions by MEG (< 8%) and SAGMCG-1 (< 4%). Conversely, the cluster III was dominated by clade MEG (>96 % of all three populations) with DSEG essentially absent and minor presence of SAGMCG-1 (< 4%).

In between, cluster II covered a gradient from lakes with quite similar abundances of DSEG and MEG to lakes with abundant MEG (70-90%) and minor contribution of DSEG (10-20%). Finally, four lakes were separated from the rest, i.e., the most acidic lakes (i.e., Pica Palomera, Aixeus, and Certascan; pH<5.7) having the highest proportion of AOA (45% to 93%), and Lake Muntanyó d'Arreu with a high abundance of both DSEG and SAGMCG-1 (c. 45% each) and substantial presence of MEG (c. 10%). Overall, there were no significant effects on the abundance of any of the populations by the spatial and morphometric lake variables tested (altitude, and $A_{lake}/A_{catchment}$ ratio), either by Chl *a*, temperature or conductivity (Spearman rank correlations, $p>0.1$). The changes in the abundance of the different archaeal populations were only explained by changes in pH (PERMANOVA, $p<0.01$, $R2= 0.198$).

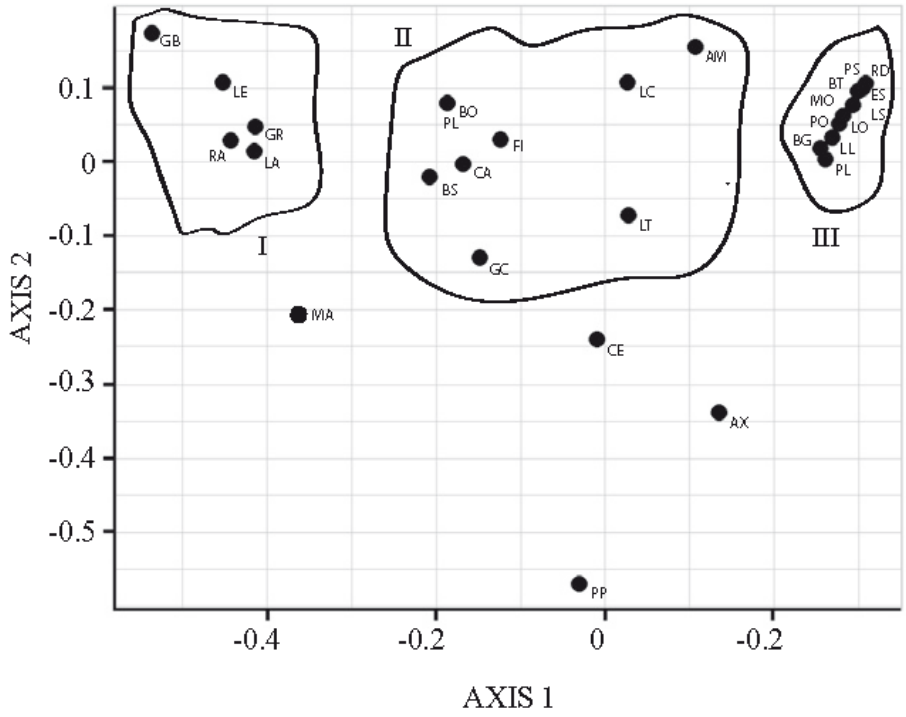

Figure 4.5: MDS ordination analysis grouping the different lakes in clusters according to the Euryarchaeota DSEG and MEG, and Thaumarchaeota SAGMCG-1 16S rRNA gene abundances. Lakes code in Table A.S4.2.

4.3.3 Spatio-temporal variations in deep Lake Redon

Lake Redon has a dimictic regime, with mixing periods in late spring and in autumn, and remains covered by snow for about 6 months of the year. The ice-cover thickness usually reaches several meters during the maximum in April, leaving the underlying water in the dark for several months in winter and spring. The surface sample corresponded both to the air-water interface during the ice free period (Auguet and Casamayor 2008), and the slush (mixture of snow and ice) present on the top of the lake during winter and most spring (Llorens-Mares *et al.*, 2012). Peaks in nitrate and ammonia were observed in surface waters during the melting period, and in bottom waters in Sep-Oct and in April-May (Table A.S4.3), and see more details in Restrepo-Ortiz *et al.*, 2014), most probably because of microbial mineralization and nitrification in the sediment. Interestingly, higher nitrate concentrations were

also observed along the water column in early summer, and higher nitrite concentrations were in general observed along the year in surface waters than at the bottom. During the ice-free season, light penetration into the water column was very high because of the high transparency due to low dissolved organic carbon (10-100 µM, Auguet el al. 2012) and low algae growth (i.e., low Chl *a* concentrations).

The newly designed primers were used to follow the vertical and temporal changes of DSEG and MEG euryarchaeota in Lake Redon. Amplification signal was obtained from most depths and dates examined (Table A.S4.3). Again, the DSEG 16S rRNA gene showed the largest variation in abundance spanning 6 orders of magnitude from 2 copies per mL of lake water up to 5 x 10^5. Interestingly, we observed blooms of this population in deep waters (60 m) during the annual stratification periods both in summer and in early spring, and in the slush layers when Lake Redon was covered by ice and snow (Fig. 4.6). DSEG gene concentrations substantially decreased after snow melting and during the mixing periods.

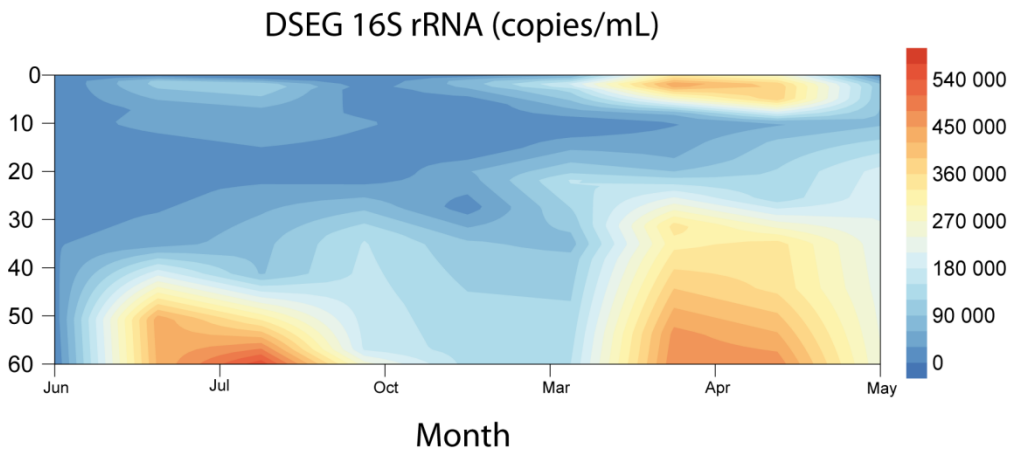

Figure 4.6: Spatiotemporal variation for abundance of the specific 16S rRNA gene for Euryarchaeota DSEG population in Lake Redon.

Conversely, MEG 16S rRNA gene abundances ranged 4 orders of magnitude from 1 up to 10^4 copies per mL of lake water, and a bloom of this population was found during the ice-cover period both at intermediate depths (35 m) and

in the slush layers (Fig. 4.7). MEG gene concentrations were low during summer stratification and the mixing periods. Overall, after multiple linear regression analysis, negative and significant ($P<0.01$) relationships were observed between nitrite and chlorophyll *a* concentrations, and the vertical variability of the euryarchaeotal 16S rRNA gene abundances along the temporal survey.

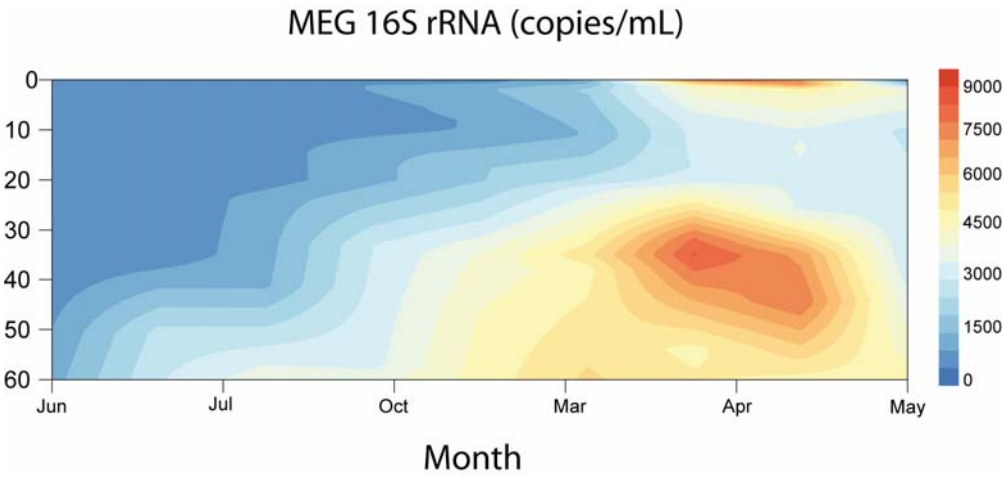

Figure 4.7: Spatiotemporal variation for abundance of the specific 16S rRNA gene for the Euryarchaeota MEG population in Lake Redon.

These results were also compared with the 16S rRNA gene abundance distribution of SAGMCG-1 recently reported for Lake Redon (Restrepo-Ortiz *et al.*, 2014, and Table A.S4.3), and significant relationships among the abundances of all three populations were observed. However, when we pooled together populations abundance from Lake Redon and from the 28 remaining lakes, a significant and positive correlation was found only between DSEG and SAGMCG-1 ($rs=0.82$, $p<0.001$) but not between MEG and SAGMCG-1 ($rs=0.01$, $p>0.9$) (Fig. 4.8). The positive and strongly significant correlation was also observed for DSEG and SAGMCG-1 without the data from Lake Redon ($rs=0.25$, $p<0.01$). Thus, the correlational analysis strongly suggested a close relationship between the unknown euryarchaeotal

DSEG clade and the thaumarchaeotal ammonia oxidizing SAGMCG-1 clade that certainly deserves further and more detailed explorations.

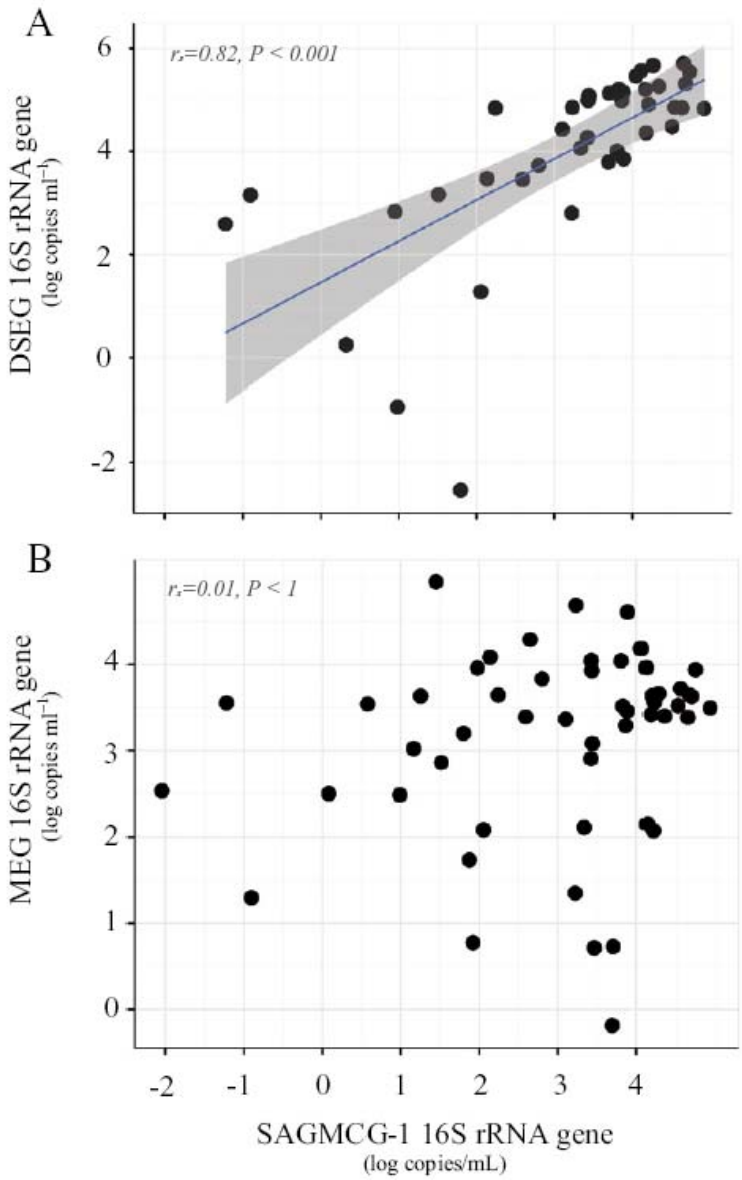

Figure 4.8: Correlational analysis for DSEG/SAGMCG-1 and MEG/SAGMCG-1 specific 16S rRNA gene along the complete dataset analysed.

4.3.4 Environmental role of uncultured Euryarchaeota: the big gap of knowledge

In cold and mesophilic environments, methanogens and extreme halophiles (Haloarchaea) have been studied for a long time and have well known functional roles. Methanogens are active key players in anoxic waters and sediments (Garcia *et al.,* 2000), and Euryarchaeota show prevalence over other archaeal phyla for high salinity environments such as the MSBL-1 candidate order, the Halobacteriaceae, and the DHVEG-6 clade (van der Wielen 2005, Auguet *et al.,* 2010, Casamayor et al 2013). The most widely encountered uncultured euryarchaeotal lineages found in freshwater environments are the MEG and DSEG lineages, along with the Marine benthic Group-D (MBG-D) (Galand *et al.,* 2006, Barberán *et al.,* 2011, Borrel *et al.,* 2012). Although the metabolism of the MBG-D members remains unknown, and they were previously labeled as non-methanogens (Jiang *et al.,* 2008), phylotypes of this group are found in sites with high methane concentrations (Borrel *et al.,* 2012, and references therein) and freshwater places where anaerobic methane oxidation processes are detected, such in Lake Cadagno (Schubert *et al.,* 2011). In the case of MEG and DSEG, their environmental distribution did not offer consistent clues to assign them a putative environmental role. DSEG had been previously reported mostly in sediments both marine and freshwater and microbial mats (70% of the OTUs present in SILVA database), and in hydrothermal vents (12%), and hypersaline and freshwater plankton (6% each). MEG had previously been more often recovered from freshwater systems (30%), sediments and microbial mats (25%), and hotsprings and hypersaline systems (c. 20% each). A recent analysis on the natural history of these two groups indicated very large phylogenetic diversity in these clades far from being reasonably sampled and much higher than in Methanobacteriales and Cenarchaeales (Barberán *et al.,* 2011). Both groups accumulated higher phylogenetic diversity in oxic freshwaters and sediments than in soils (Barberán *et al.,* 2011). Unfortunately, cultivation has remained elusive for these new euryarchaeal groups present in aerobic freshwater habitats that are distantly related to their extremophilic counterparts. There is therefore a big gap in the current knowledge of the ecology and metabolism of mesophilic and cold Euryarchaeota beyond methanogens and halophiles.

Overall, we observed a large range of variation in the 16S rRNA gene abundances of the different euryarchaeotal populations examined, but unfortunately most of the environmental parameters measured did not provide a satisfactory explanation for the changes in the abundances observed. Only pH appeared as the strongest gradient controlling the abundance of the euryarchaeotal and thaumarchaeota populations. Together with salinity, pH has been shown as one of the major drivers of microbial community structure (Fierer and Jackson 2006, Newton *et al.*, 2007, Auguet and Casamayor 2013, Triadó-Margarit and Casamayor 2012), but this parameter does not provide clues to infer a potential metabolic role in situ. Interestingly, the correlational analyses indicated lack of coupling of such euryarchaeota with phytoplankton (chlorophyll *a*), but an intriguing positive and significant relationship between DSEG and putative ammonia oxidizing thaumarchaeota abundances. Previous studies on marine planktonic Euryarchaeota suggested either a putative anaerobic respiration physiology (Martin-Cuadrado *et al.*, 2008) or the potential to carry out a photoheterotrophic metabolism by light-capturing proteorhodopsins to gain a competitive advantage (Frigaard *et al.*, 2006). In the case of Lake Redon, we could rule out both strategies because the freshwater populations were found blooming in aerobic cold waters and in the dark. However, the correlation found with ammonia oxidizing microorganisms may shed some light to guide future studies. Whether this is related to the potential of DSEG Euryarchaeota to perform any key metabolic step in the global nitrogen cycling remains to be shown, but the correlational analysis and seasonal environmental distribution between freshwater AOA and DSEG strongly suggests that they share a closely related ecological niche.

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Part II: Retrieval and integration database, design and optimization of specific CARD-FISH probes for SAGMCG-1 group

5

Design, evaluation and optimization of 16S rRNA SAGMCG-1 Oligonucleotide Probe for Catalyzed Reporter Deposition – Fluorescence In Situ Hybridization

Resumen

El estudio del RNA ribosómico (rRNA) enfocado a la evolución y ecología microbianas se ha convertido en una parte integral de la microbiología ambiental. Gracias al diferente grado de conservación de la secuencia del rRNA, diferentes sondas oligonucleótidas pueden ser diseñadas con especificidades que van desde el nivel de especie, hasta el de reinos o incluso dominios. Cuando estas sondas se marcan con la enzima peroxidasa de rábano picante (HRP), pueden ser utilizadas para identificar las células microbianas individuales directamente por hibridación *in situ* fluorescente con una elevada sensibilidad. A partir de análisis comparativos de secuencias del gen 16S rRNA hemos localizado los sitios específicos para el grupo SAGMCG-1 de Thaumarchaeota, incluyendo la especie *Nitrosotalea devanaterra*. Oligonucleótidos complementarios a estas regiones fueron evaluados como potenciales sondas moleculares para la diferenciación del linaje SAGMCG-1. Las condiciones de hibridación se optimizaron mediante diferentes concentraciones de formamida en el tampón de hibridación. La intensidad de fluorescencia y los efectos sobre la visualización de células mejoraron sensiblemente mediante el diseño de dos fragmentos ayudantes adyacentes para la sonda. La hibridación mediante CARD-FISH utilizando cultivos de *N. devanaterra*, permitió ajustar in vitro la especificidad de la sonda, y la hibridación de células completas con derivados de sondas fluorescentes en muestras de suelos seleccionadas permitió la identificación y cuantificación satisfactoria de células microbianas individuales in situ. Las

sondas diseñadas y optimizadas en este trabajo serán de gran utilidad para el estudio *in situ* de la distribución y dinámica de estas poblaciones oxidadoras de amonio microbianas estrechamente vinculadas al ciclo biogeoquímico del nitrógeno.

Abstract[1]

The ribosomal-RNA (rRNA) approach to microbial evolution and ecology has become an integral part of environmental microbiology. Based on the conservation of rRNA, oligonucleotide probes can be designed with specificities that range from the species level to the level of phyla or even domains. When these probes are labelled with the enzyme horseradish peroxidase (HRP), they can be used to identify single microbial cells directly by fluorescence in situ hybridization. Based on comparative analyses of 16S rRNA sequences we have located sites specific for the SAGMCG-1 group of Thaumarchaeota, including the species *Nitrosotalea devanaterra*. Oligonucleotides complementary to these signature regions were evaluated as potential nucleic acid probes for the differentiation of SAGMCG-1 lineage. Hybridization conditions were optimized by the addition of increasing formamide concentrations to the hybridization buffer. The fluorescence intensity was enhanced and the effects on the cell visualization were improved by design of two adjacent helpers for the probe. CARD-FISH hybridization using *N. devanaterra* demonstrated high probe specificity in vitro, and whole cell hybridization with fluorescent probe derivatives allowed the identification and quantification in selected soil samples of individual microbial cells in situ. The probes will be useful for determinative studies and for the *in situ* monitoring of population distribution and dynamics in microbial communities closely related to the N biogeochemical cycling.

5.1 Introduction

Thaumarchaeota were initially reported as marine group I Archaea and were considered as members of the crenarchaeotal phylum based on 16S ribosomal RNA gene phylogeny (DeLong, 1992; Fuhrman *et al*., 1992). However, subsequent studies using comparative genomics revealed that Thaumarchaeota form a separate and deep-branching phylum within the Archaea (Brochier-Armanet *et al*., 2008; Spang *et al*., 2010). Recent metadata analysis have confirmed that the thaumarchaeotal phylum is highly diverse

[1]Restrepo-Ortiz *et al.*, **Manuscript in preparation**

and it is present in a wide variety of ecosystems as marine, freshwater, soil, sediments, biofilms and hot springs environments (Fernàndez-Guerra and Casamayor, 2012; Erguder *et al*., 2009; Hatzenpichler, 2012). This phylum comprises not only ammonia-oxidizing archaea (AOA) but also environmental sequences representing microorganisms of unknown metabolism (Pester *et al*., 2011). The discovery and cultivation of the chemolithotrophic obligate acidophilic thaumarchaeal ammonia-oxidizing species *Nitrosotalea devanaterra* (Lehtovirta-Morley *et al*., 2011) belonging to the SAGMCG-1 group (monophyletic cluster within Thaumarchaeota) has enlarged the role assigned to thaumarchaeota in the nitrogen cycling. The SAGMCG-1 and *Nitrosotalea* clusters are especially interesting because its ecological importance, particularly in oligotrophic freshwaters (Auguet and Casamayor, 2008; 2013) and acidic soils (Gubry-Rangin *et al*., 2011; Pester *et al*., 2011). The relative abundance, recurrent appearance and significant correlations with nitrogen species suggest a key role of SAGMCG-1 in the N biogeochemical cycle of oligotrophic alpine lakes (Auguet *et al*., 2011; 2012, Restrepo-Ortiz *et al.,* 2014).

In the last 20 years, great attention has been paid to the optimization of Molecular Biological Tools (MBTs) for a rapid and accurate estimation of microbial communities with different metabolic capabilities. Quantitative polymerase chain reaction (qPCR) has been largely used for quantification of microbial communities, and several optimized protocols are currently available and widely applied for the quantification of the 16S rRNA gene (Ding and He, 2012 and Justé *et al.,* 2008). However, recent studies showed that the sole use of qPCR might introduce some biases in the quantification, mainly due to the loss of DNA during the sampling procedure and/or nucleic acid extraction (Matturro *et al.,* 2012 and Ding and He, 2012). In situ-hybridization assays have been suggested as confirmatory methodologies, being independent from the nucleic acid extraction and able to exactly provide consensus enumeration of cells (Matturro *et al.,* 2012, Matturro *et al.,* 2013a and Pernthaler *et al.,* 2002).

Fluorescence in situ hybridization (FISH) is a modern method of molecular biology for the phylogenetic identification and the enumeration of microorganisms in natural environments without prior cultivation (Amann and Fuchs, 2008). Numerous fluorescently labeled oligonucleotide probes have been designed (Amann *et al.,* 1995 and Daims *et al.,* 1999) and FISH

has been successfully applied to a wide range of environmental samples such as soil (Barra Caracciolo *et al.,* 2005 and Hahn *et al.,* 1992), roots (Assmus *et al.,* 1995 and Watt *et al.,* 2006), sludge (Chu *et al.,* 2005 and Wagner *et al.,* 1994), biofilms (Mobarry *et al.,* 1996 and Schramm *et al.,* 1996), and sediments (MacGregor *et al.,* 2001 and Pernthaler and Amann, 2004). Regarding environmental samples, the major drawbacks of the FISH technique are (i) weak signal intensity due to low rRNA contents, starvation of target cells, or signal quenching; (ii) inhibited target accessibility; and (iii) high levels of background fluorescence (Amann *et al.,* 1995, Marras *et al.,* 2002, Pernthaler *et al.,* 2001 and Wagner *et al.,* 2003).

To overcome these limitations and to improve the detection of target cells, catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) was introduced into the molecular ecology toolbox (Pernthaler *et al.,* 2002). Since then, the application of CARD-FISH to environmental samples was reported to significantly amplify fluorescent signals relative to monolabeled probes, and thus to substantially increase the detection rates of investigated microorganisms (Eickhorst and Tippkötter, 2008, Ferrari *et al.,* 2006 and Ishii *et al.,* 2004). The increase in signal intensity is based on the enzymatic deposition of a large number of fluorescently labeled tyramine molecules during tyramide signal amplification (TSA). Following in situ hybridization with horseradish peroxidase (HRP)-labeled oligonucleotide probes, HRP "activates" the fluorescently labeled tyramides in presence of hydrogen peroxide, and therefore catalyses the covalent binding of tyramides to tyrosines in the target cells (Bobrow *et al.,* 1989, Speel *et al.,* 1999 and Van Gijlswijk *et al.,* 1997). The applications of CARD-FISH provides clarification not only in rRNA-targeted phylogenetic identification but also in linking microbial phylogeny to physiology and metabolic activity. CARD-FISH is one of the most important molecular tools for enhancing our understanding of environmental microorganisms. Presently, the major challenge of using CARD-FISH is no longer the staining technique itself, but rather the design and application of new probes within the context of a particular unknown microbial assemblage.

The interest and purpose of this chapter were to specifically address the design and optimization of 16S rRNA SAGMCG-1 oligonucleotide probes for CARD-FISH method and the optimization of a CARD-FISH protocol for specific and optimal detection of the SAGMCG-1 Thaumarchaeota group.

5.2 Material and Methods

5.2.1 Design and check of probes

5.2.1.1 Retrieval of 16S rRNA sequences from databases

Sequences with signature targets were found out in ARB (http://arb-home.de/) (Ludwig, Strunk *et al.,* 2004), with the public available 16S rRNA database. Highly related sequences of several typical SAGMCG-1 sequences were searched using blastn (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul, Madden *et al.,* 1997), and the sequences obtained from former studies in the Pyrenees lakes (Auguet *et al.,* 2012, Restrepo-Ortiz *et al.,* 2014), were imported into ARB database as well. Those sequences closely related to known SAGMCG-1 sequences were chosen for further phylogenetic analysis and probe design.

5.2.1.2 Constructing phylogenetic tree

The 16S rRNA gene sequences were automatically aligned with the Nearest Alignment Space Termination (NAST) algorithm (DeSantis *et al.,* 2006), clustered at 97% identity threshold, and imported into the Silva database (http://www.arb-silva.de/) using "ARB parsimony (Quick add marked)" with filter "Positional variability by parsimony for Archaea".
Neighbor joining (NJ), maximum parsimony (MP), maximum likelihood (ML) (all three are embedded in ARB) and posterior probability (using external program MrBayes) trees were calculated for SAGMCG-1 group sequences.

5.2.1.3 Design of probes, helpers and competitors

Probes designs were made in ARB, using a PT-server built from all sequences in the database. Newly designed probes were checked using a PT-server with all available 16S rRNA sequences and analyses of the percent catches and the specificity of the probe were carried out. The SAGMCG-1 group (*Nitrosotalea devanaterra*-like) was marked in the phylogenetic tree within ARB, and with the PROBE_DESIGN tool we look for all possible signature sequences that were diagnostic for the selected species. The tool

excludes potential probe sequences, which contain self-complementary regions with more than three nucleotides. The GC-content of probe sequences influences their melting behavior. By default this parameter is set in PROBE_DESIGN between 50 to 100% to ensure a tight binding but usually it ranges between 50 to 70%.

To increase the specificity of probes that show only one mismatch with non-target sequences, it may be required to construct one or several competitor oligonucleotides. Such competitors are designed to perfectly match with the non-target sequence at the homologous site, and they are subsequently synthesized without fluorescent label.

Helpers (Fuchs, Glöckner *et al.,* 2000) are longer than probes, on average 21nt, and thus have higher annealing temperature. They are either adjacent to the probe binding site, or complementary to the site that the probe targets according to the secondary structure of the 16S rRNA. Ideally, helpers should cover all of the sequences that are targeted by the probe.

During hybridization, the competitor and helpers are added to the buffer at an equal concentration as the labeled probe.

5.2.1.4 in silico Probe specificity

Analysis of target and specificity of the probe was carried out with the programs TestProbe 3.0 from silva (http://www.arb-silva.de/?id=650), probeChek and probeBase (Loy *et al.,* 2007) from Divison of Microbial Ecology (DOME, http://www.microbial-ecology.net/). In mathFISH program (http://mathfish.cee.wisc.edu/index.html) (Yilmaz *et al.,* 2011), complete thermodynamic analysis for probe-target pair, including reaction free energy change of hybridization series ($\Delta G^{o'}s$), overall free energy change of hybridization ($\Delta G^{o}_{overall}$), probe alignment, hybridization efficiency and melting formamide concentration (Yilmaz *et al.,* 2004, Yilmaz *et al.,* 2006), were carried out. Moreover formamide curves (Yilmaz *et al.,* 2007), mismatch analysis (Yilmaz *et al.,* 2008), competitor model (Hoshino *et al.,* 2008), and accessibility of the 16S rRNA (Fuchs *et al.,* 1998) were done with the following hybridization conditions: 1M Na+ concentration, 260nM probe concentration and two hybridization temperatures 46ºC (temperature from MPI protocol) and 35ºC (temperature from CEAB protocol). Finally we evaluated the melting temperature, using the nearest neighbor method (NN

Tm, calculated using 50 mM NaCl and 50 μM oligo). This method can be performed on-line using websites such as Oligo Calc (Kibbe WA.2007, http://www.basic.northwestern.edu/biotools/oligocalc.html).

These analyzes were conducted using partial sequences of *Nitrosotalea devanaterra* (target), *Nitrosopumilus maritimus* (non-target, competitors test) and sequences from the study by Auguet *et al.,* 2012 (environmental samples target). Once carried out these analyses, probes that exhibit less optimal conditions were discarded, such as low stringency and hybridization, high unspecificity, low %GC and melting temperature (Tm) and low accessibility of the 16S rRNA.

5.2.1.5 Probe synthesis

The synthesis was conducted by biopolymer synthesis service of biomers.net, a partner of the SILVA database project.

5.2.2 Empirical probe testing and optimization

5.2.2.1 Sample sources

For probes testing and optimization using helpers and competitors, three types of samples were used:

1) AOA enrichment cultures of *Nitrosotalea devanaterra*, which was cultured in acidic (pH 4.5) aqueous medium as described by Lehtovirta-Morley *et al.,* (2011), and *Nitrosopumilus maritimus*, which was grown in HEPES-buffered, synthetic medium (pH 7.6) (Martens-Habbena *et al.,* 2009). Both strains were grown aerobically in 100-ml quartz flasks containing 50 mL inorganic growth medium. The samples were provived by the Craibstone Estate, Scotland's Rural College (SRUC), Aberdeen.

2) Soil samples coming from pH-manipulated plots at the Scottish Agricultural College on Craibstone Estate, NE Scotland (Grid ref. NJ 867112) in August 2006. The soil plots have been maintained at 0.5 pH unit intervals in the range 4.5–7.5 for the past 40 years, by the addition of lime or aluminium sulphate (Lehtovirta *et al.,* 2009). Cells were extracted from the

soil matrix using a Nycodenz density gradient (Murayama *et al.,* 2001). Samples were sent from SRUC previously fixed in 4% paraformaldehyde (PFA), and once in the Blanes laboratory cells were collected by vacuum filtering through a polycarbonate membrane (Millipore, 25 mm diameter, 0.2 μm pore size).

3) Finally, freshwater samples from different lakes of the Limnological Observatory of the Pyrenees (LOOP; Spanish Pyrenees; 42°33′N, 00°53′E) within the protected area of the Aigüestortes National Park. Samples were collected as stored as described in Auguet *et al.,* 2011.

5.2.2.2 Protocol Standardization

The first step taken was the standardization of the CARD-FISH protocol. For this purpose, parallel tests between the protocols for samples on membrane filters used in the MPI (Pernthaler *et al.,* 2004) and the CEAB-CSIC were run. CARD-FISH was done using the ARCH915 (positive control) and NON338 (negative control) probes (Table 5.1), following the procedure as described in Table 5.2. The standardized and complete protocol is shown in the Appendix B and it was completed after the experimental formamide curve with the test and specificity of the newly design probes plus helpers and competitors.

Table 5.1: CARD-FISH probes used in this study.

Probe	Target	Sequence (5′ – 3′)	Formamide concentration (%)		Reference
			35 °C	46 °C	
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	40	35	Medina-Sanchez 2005, Raskin *et al.,* 1994
NON 338	Antisense of EUB338	ACTCCTACGGGAGGCAGC	55	35	Wallner *et al.,* 1993

Table 5.2: Description of the CARD-FISH protocols by the Max Planck Institute (MPI) and the Center for Advanced Studies of Blanes (CEAB-CSIC). (RT: room temperature)

Stage	*Description*	
	MPI protocol	*CEAB protocol*
Embedding	• Boil low gelling point agarose (0.1%, gel strength should be approx. 1000 g cm^{-2}) and fill the agarose in a pre-warmed petri dish and let it cool down to 35-40°C. • Dip filter with both sides in the agarose and place it face-down (shiny side with bacteria down!) onto a parafilm covered, even surface (e.g. glass plate), let dry; Temperature for drying is not crucial, anything between 20 and 50°C is fine. • Remove filters from surface by soaking in 80 – 96% ethanol. • Let the filter air dry on a piece of tissue paper.	• Dip filters in 0.1% low-gelling-point agarose at 35 to 40 °C. • Place filters face down onto parafilm, let them dry at 35 to 40°C. • Remove filters by soaking them in 96% ethanol (RT). • Let air dry on a piece of tissue paper
Inactivation of endogenous peroxidases[1]	• Incubate in 0.01 M HCl for 10-20 min. at RT • Wash filters well in excess MilliQ water and 96% ethanol and let the filter air dry on a paper.	• Incubate in 0.01 M HCl for 10 min. at RT. • Wash filters quickly in 1X PBS • Wash filters well in excess MilliQ water • Dehydrate in 96% ethanol at RT and let the filter air dry
Permeabilization	• Incubate filter in 100 ml of fresh lysozyme solution in a small petri dish (10 mg ml-1 in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0) for 60 min. at 37°C. • Wash in excess MilliQ water.	• Incubate filter in fresh lysozyme solution (10 mg ml-1 in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0) for 60 min. at 37°C. • Wash 3 times in MilliQ water and 96% ethanol. • Let air dry • Incubate filter fresh achromopeptidase solution (60U ml-1 in 5M NaCl; 0,1M tris-HCl, pH 8.0) for 30 min. at 37°C. • Wash 3 times in MilliQ water and 96% ethanol. • Let air dry
	•	

Hybridization	• Prepare a humidity chamber by inserting a tissue in a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer. • Mix 300 Gl hybridization with probe working solution (50 ng DNA µl-1) in a ratio 300:1 (i.e. 1 Gl) to 100:1 (i.e. 3 Gl) depending on sample. • Dip each filter completely into the hybridization solution and place filters face-up onto a parafilm covered glass slide; spread the rest of the solution evenly onto the filters. Close tube firmly and keep the tube in a horizontal position. • Incubate at 46°C for 2-3 h (coastal water) or overnight for oligotrophic/open ocean water samples.	• Prepare a humidity chamber by inserting a tissue in a tupperware and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer. • Mix (100:1 [vol/vol]) hybridization buffer and probe solution (50ng/µl) at a suitable quantity to dip filters after adjusting the piston. • Put a drop of about 50-100 µl of the hybridization solution on each piece of filter and place filters in petri dish and turn it into the humidity chamber (Tupperware). Close tupper firmly and keep in a horizontal position. • Incubate filters at 35°C for 4 h on a rotation shaker.
Washing	• Transfer filter to prewarmed (48°C) washing buffer by immersing the whole slide in the buffer => filter will swim off. • Transfer filters to 1 x PBS (do not let filter run dry!) and incubate for 15 min at RT. • To remove excess liquid, dab filter on blotting paper, but do not let filter run dry!	• Wash filters in prewarmed washing buffer for 10 min at 37°C. Do not air dry filters after washing. • Transfer filters to 1 x PBS and incubate for 15 min at RT. • To remove excess liquid, dab filter on blotting paper, but do not let filters dry.

CARD	• Prepare a moisture chamber by inserting a piece of tissue paper in a 50 ml tube and soak it with 2 ml water. • Prepare a fresh solution of H2O2 (0.15% in PBS), keep it cool. • Mix amplification buffer with H2O2 solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient) and add fluorescently labeled tyramide [1 mg dye ml-1] and mix well, keep in the dark (The volume of labeled tyramide added strongly depends on the nature of the sample, start with 1000:1; if the signal is not sufficient: in-/decrease the ratio of added tyramide). • Dip filter completely in the amplification mix, place filter sections face-up on a parafilm covered glass slide and spread the rest of the amplification mix over the filters. • Put the slides into the humidity chamber and incubate at 46°C for up to 45 min in the dark. • To remove excess liquid, dab filter on blotting paper and incubate in 1 x PBS for 5-10 min at RT in the dark (or: 15 min at 46°C on a shaker). Wash filters thoroughly in excess with deionized water. Therefore use a Büchner funnel and MQ water to create a gyre in the funnel. Then wash filters thoroughly twice in excess in 96% ethanol (1-2 min), let completely air dry in the dark before counterstaining with DAPI.	• Prepare a moisture chamber by inserting a piece of tissue paper in a 50 ml tube and soak it with 2 ml water. • Prepare a fresh solution of H2O2 (0.15% in PBS), keep it cool. • Mix amplification buffer with H2O2 solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient) and add 3µL fluorescently labeled tyramide [1 mg dye ml-1] and mix well, keep in the dark. • Dip filters completely in the amplification mix • Incubate in substrate mix for 15 min at 42°C in the dark. • To remove excess liquid, dab filter on blotting paper and incubate in 1 x PBS for 15 min at RT in the dark. • Wash filters in excess MilliQ water and thoroughly twice in excess in 96% ethanol (1 min). • Let air dry.
Mounting	• For counterstaining put filter sections on a glass plate, cover with app. 50 8l of DAPI solution (1 8g ml-1), and incubate for 3 min. Afterwards wash filter sections subsequently for 1 min. in distilled H2O and for 1 min. in 80% ethanol to remove unspecific staining. Let air-dry. • Samples are mounted in a 4:1 mix of Citifluor and Vecta Shield. The filter sections have to be completely dry before embedding, otherwise part of the cells might detach during inspection.	• Counterstain filters by soaking them with DAPI solution (1 µg/ml FC),samples are mounted in a 4:1 mix of Citifluor and Vecta Shield. • The filter sections have to be completely dry before embedding, otherwise part of the cells might detach during inspection.

[1] in the MPI protocol, this stage is performed after permeabilization

5.2.2.3 Probe testing and optimization

Different experiments were conducted for the evaluation and optimization of the probe. The procedures were as shown in Table 5.3.

Table 5.3: Experiments performed for the evaluation of the probes

Sample	CARD-FISH	
	Test	Objective
NItrosotalea devanaterra strain	• newly desings probes • Formamide curve (15-80 %FA) • double labeling with newly desing probe and ARCH 915. • newly desing probe and helpers • double labeling with newly desing probe plus helpers and ARCH 915.	• functionality probe • optimization of the probe and protocol • probe efficiency and specificity • helpers efficiency • helpers efficiency
NItrosopumilus maritimus strain	• newly desing probe and helpers • newly desing probe, helpers and competitor	• probe specificity • competitor efficiency
Soils 4.5-7.5 pH	• newly desing probe and helpers • double labeling with newly desing probe plus helpers and ARCH 915.	• probe efficiency in complex samples
Freshwater	• newly desing probe and helpers • double labeling with newly desing probe plus helpers and ARCH 915.	• probe efficiency in environmental samples

5.2.2.4 Cell counts

The filters were observed under a fluorescence microscope Zeiss Axio Imager with a lamp X-Cite 120 and a camera attached AxioCamMrm with software AxioVision image acquisition PC. A set of filters suitable for DAPI (Zeiss filter set 01 BP365 / 12 FT396 LP397), Alexa-Fluor 488 and Alexa Fluor 594 (Zeiss filter sets 09 FT510 BP450-490 LP515, or 24 DBP485 / 20 DFT500 / 600 BP515-540 + LP610). The filters were examined at x1000 magnification under the fluorescence microscope Zeiss Axio Imager, at least 500 cells were counted in a number of visual fields known, to establish overall abundance (counts DAPI) provided by setting the number of hybridized probe in the respective cells in order to estimate the abundance and percent hybridization of the labeled group.

ImageJ program (http://imagej.nih.gov/ij/index.html, National Institutes of Health, USA) was used to calculate the efficiency of the helpers. The intensity of light emitted by the probe with and without helpers was compared by analyzing the microscopic images.

5.3 Results and Discussion

5.3.1 Defining the SAGMCG-1 clade

A total of 257 sequences were collected for the construction of a phylogenetic tree and subsequent analyzes and design of specific probes for CARD-FISH (NCBI accession numbers of the 16S rRNA gene sequences are shown in Table A.S5.1). Phylogenetic analysis confirmed that SAGMCG-1 is a monophyletic clade with very high bootstrap support (100%) and was consistently separated from Marine Group I (MG1). Within SAGMCG-1, most sequences were recovered from freshwater environments, and in lower proportion soils and gold mines, and were affiliated to *Nitrosotalea devanaterra*. The remaining sequences were grouped in a different cluster coming from different environments (Fig. 5.1). This fact suggests the presence of two phylogenetic clades within SAGMCG-1, candidatus *Nitrosotalea* (165 sequences) and Miscellaneous SAGMCG-1 (92 sequences).

5.3.2 Targeting of the probes

Potential target regions for specific SAGMCG-1 group probes were positions 357-374, 362-378, and 530-547, based on *Escherichia coli* 16S rRNA positions numbering. Three different probes were developed and chosen for further investigation: probe SAG1_357, SAG1_362 and SAG1_530 (Table 5.5). In probeBase and probeCheck programs, specificity and novelty of the probes was verified, confirming that these three probes targeted the SAGMCG-1 clade and that probes sequences had not been previously designed. Likewise, it was confirmed that there were no previous registration of any probe specifically designed to this group, including *Nitrosotalea devanaterra*. For the newly designed probe SAG1_530 (Table 5.5), TestProbe identified 221 sequences without mismatches, 93.2% (206

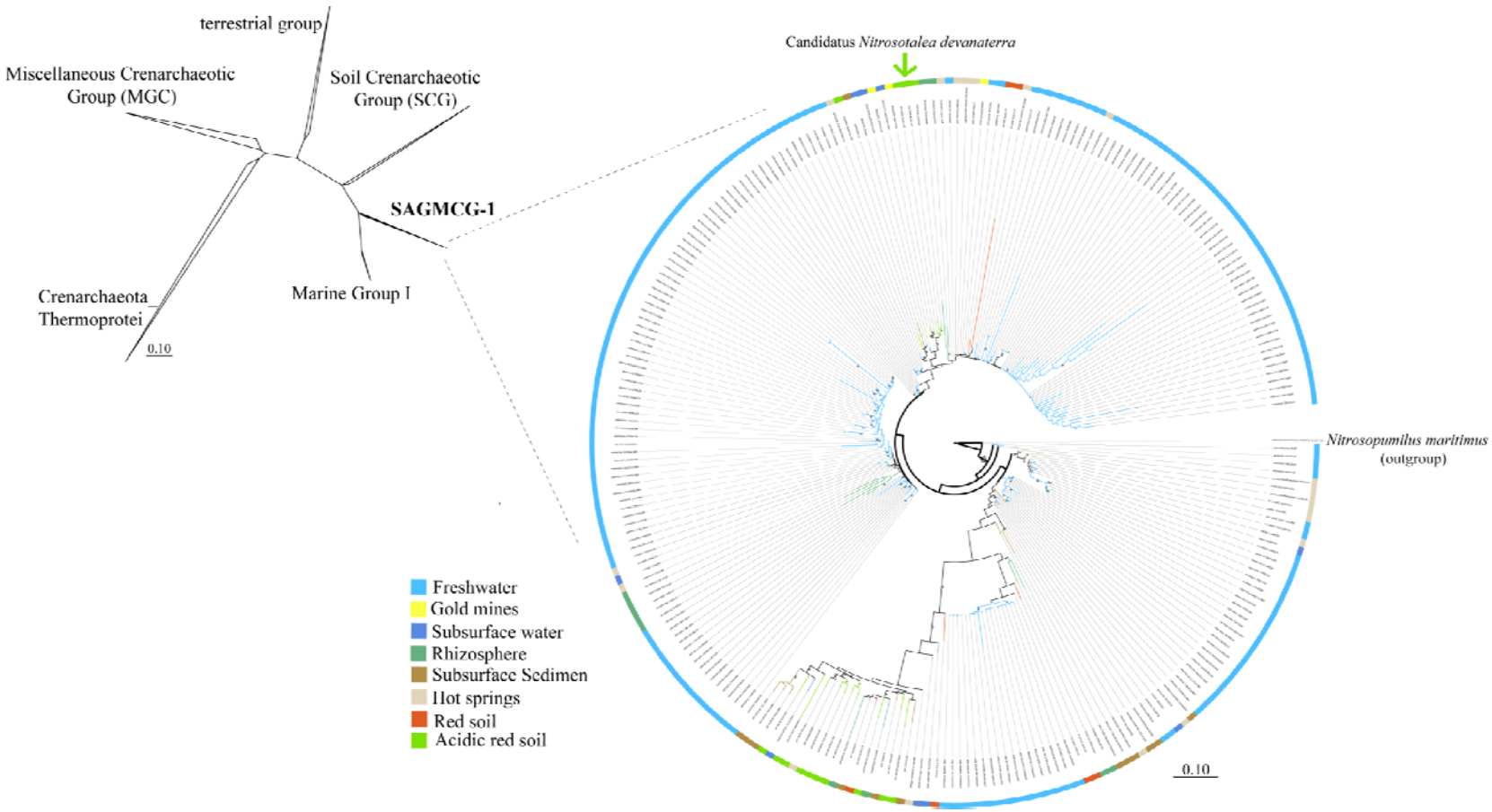

Figure 5.1: SAGMCG-1 maximum-likelihood (ML) phylogenetic tree with environmental features mapped as outer ring.

sequences) corresponding to the target group and the rest (6.8%, 15 sequences) to other groups, and 976 sequences allowing one mismatch. For the SAG1_362 probe (Table 5.5), it was identified 210 sequences without mismatches, 96.2% (202 sequences) corresponding to the target group and 8 sequences (3.8%) to other groups, and 333 sequences allowing one mismatch. Finally for the SAG1_357 probe (Table 5.5), it was identified 192 sequences without mismatches, 89% (172 sequences) to the target group and 20 sequences (11%) to other groups, and 344 sequences allowing one mismatch. All percentages and identity of the target and non-target groups are detailed in Table 5.4. Moreover, as shown in Table 5.5, the probes do not cover the entire SAGMCG-1 clade. The targeted non-SAGMCG-1 sequences belonged to the MG1 clade, Miscellaneous Crenarchaeota group (MCG), Soil Crenarchaeotic Group (SCG), HDBA, Terrestial_group, C_Termoprotein and ArcC-u-cDO6 (Table 5.4).

A useful probe may contain one or more mismatches to non-target sequences, ideally located in the middle of the target string. It may be possible to centralize the mismatch by checking variations proposed by the probe design program. If a non-target sequence contains only a single weak mismatch to the target string, it may be necessary to design a competitor probe. Except for MG1 clade which is closely related to SAGMCG-1 clade, is equally abundant and may share a common environment (Auguet *et al.,* 2011), the SAGMCG-1 probes can be still considered to be specific enough. In order to solve the problem with non-target group (MG1), competitors were designed. These competitors are perfectly complementarily to non-target organism with the target sequence mismatch. Oligonucleotide probes can distinguish single base mismatches, although it depends on the sequence and the position of the mismatch (Amann *et al.,* 1990). Even if single base mismatches are not distinguishable, the use of competitor probes makes this possible (Hoshino *et al.,* 2008., Kubota *et al.,* 2006., Lin *et al.,* 2006., Manz *et al.,* 1992). However, contrary to the review by Amann and Fuchs (2008), the use of unlabelled competitor probes does not always improve the specificity in the phylogenetic group-specific probes, and in some cases can actually cause further probing inconsistencies (Barr *et al.,* 2010). Although the probes SAG1_530 and SAG1_362 show more specificity (respect to the number of target and non-target sequences) as compared to the probe

Table 5.4: Unspecificity of the CARD-FISH probes specific for SAGMCG-1 designed in this study

Taxonomic group	Total No. sequences	SAG1_357		SAG1_362		SAG1_530	
		No. Sequences target	Target into the group (%)	No. Sequences target	Target into the group (%)	No. Sequences target	Target into the group (%)
SAGMCG-1	257	172	67	202	79	206	80.20
MG1	1039	4	0.38	4	0.38	12	1.15
MCG	1605	12	0.74	0	0	0	0
C_Termoprotein	456	2	0.43	0	0	0	0
Terrestrial_group	140	1	0.71	1	0.71	0	0
SCG	252	0	0	0	0	1	0.39
HDBA	2	1	50	1	50	0	0
HM187555	1	0	0	1	100	1	100
ArcC-u-cDo6	3	0	0	0	0	1	33.30
Verrucomicrobia	2539	0	0	1	0.04	0	0

Table 5.5: Description of the CARD-FISH probes specific for SAGMCG-1 designed in this study.

Name Probe	Sequence probe 5'-3'	Position	GC%	Length (n)	TARGET PROBE[1]	
					SAGMCG-1 sequences	other groups sequences
SAG1_357	TTGCTAAGGTTTCTCGCC	357-374	50	18	172 (90.1%)	19 (9.9%)
SAG1_362	CACATTGCTAAGGTTTC	362-378	41	17	202 (96.2%)	8 (3.8%)
SAG1_530	TCGAGGTGCTGGTATTAC	530-547	50	18	206 (93.2%)	15 (6.8%)

[1] from the total percentage of the probe sequences matches based on the full SILVA databases SSU 114 Ref NR

SAG1_357, when analyzing the design of competitors, we saw that the SAG1_357 probe was more useful. Only one competitor was required to SAG1_357 probe, since the variation of a single mismatch was enough to restrict hybridization with non-target organisms. Conversely, for SAG1_530 and SAG1_362 probes, three and two competitors respectively were necessary (Table 5.6.).

Table 5.6: Competitors designed and used in this study

Name Probe	Name Competitor	[1]Sequence Competitor 5'-3'
SAG1_357	cSAG1_357	TTGCAAAGGTTTCTCGCC
SAG1_362	cSAG1_362_1	CACATTGCGAAGGTTTC
	cSAG1_362_2	CACATTGCAAAGGTTTC
SAG1_530	cSAG1_530_1	TCGAGGTGCTGGTTTTAC
	cSAG1_530_2	TCGGGGTGCTGGTATTAC
	cSAG1_530_3	TCGAGGAGCTGGTATTAC

[1] The mismatch of each competitor with the target sequences is shown in red.

We carried out the analysis of the effect of the competitors on the formamide dissociation profile of the probe with both target (SAGMCG-1) and non-target (MG1) using mathFISH. For the three competitors set (a total of 6 competitors), it was found that the probe was a perfect match to target, and has 1 mismatch to non-target. Competitor was a perfect match to non-target and has 1 mismatch to target.

5.3.3 Characterization of designed probes and associated parameters

The hybridization probes efficiencies can be defined as mainly a function of two factors, the thermodynamic affinity of the probe to the target site and the accessibility of the target site (Yilmaz and Noguera, 2004). Thermodynamic affinity is defined by the three thermodynamic components of affinity ($\Delta G^{o}_{1,}$ ΔG^{o}_{2} and ΔG^{o}_{3}), and can be combined assuming equilibrium to give the predictable $\Delta G°_{overall}$, which describes the stability of the DNA/RNA hybrid and can be correlated with the brightness of a hybridized probe (Yilmaz *et al.,* 2010). The values of thermodynamic components of affinity for the three probes designed in this study are given in Table 5.7.

Table 5.7: Complete thermodynamic analysis for probe-target pair, including reaction free energy change of hybridization series ($\Delta G^{o'}$s), overall free energy change of hybridization ($\Delta G^{o}_{overall}$), probe alignment, hybridization efficiency and melting formamide concentration ([FA]m).

Thermodynamic values	Probe					
	SAG1_357		SAG1_362		SAG1_530	
	46ºC	35ºC	46ºC	35ºC	46ºC	35ºC
ΔG^{o}_{1} (kcal/mol)	-19.4	-23.7	-14.4	-18.4	-17.7	-22.0
ΔG^{o}_{2} (kcal/mol)	0.6	-0.1	0.7	0.4	1.4	1.1
ΔG^{o}_{3} (kcal/mol)	-3.3	-6.7	-8.3	-9.8	-4.1	-5.8
$\Delta G^{o}_{overall}$ (kcal/mol)	-15.9	-16.6	-6.0	-8.4	-13.5	-16.1
[FA]m	31.8%	47.7%	-80.0%	-18.2%	20.5%	39.6 %
Hybridization Efficiency[1]	0.9999	1.0000	0.0030	0.1838	0.9977	1.0000
Probe alignment	PROBE--3'CCGCTCTTTGGAATCGTT5' .....|||||||||||||||||| TARGET-5'GGCGAGAAACCTTAGCAA3'		PROBE--3'CTTTGGAATCGTTACAC5' .....||||||||||||||||| TARGET-5'GAAACCTTAGCAATGTG3'		PROBE--3'CATTATGGTCGTGGAGCT5' .....|||||||||||||||||| TARGET-5'GTAATACCAGCACCTCGA3'	

[1] At 0% formamide

The SAG1_357 probe exhibited a range of $\Delta G°_{overall}$ recommended due to low risk for both false negatives and false positives hybridization signals (Fig. 5.2A) and high hybridization efficiency, in both temperatures. SAG1_530 probe also had a high hybridization efficiency and theoretical optimal for avoiding false positives in both temperatures, but with an increased risk of false negative at 46ºC (Fig. 5.2B). Lastly, SAG1_362 probe showed high risk of false negative and almost no chance of hybridization (Fig. 5.2B), whereby the assumption of equilibrium was not valid for this probe and the kinetics of the reactions will also affect hybridization efficiency. However, kinetic limitations can be eliminated or minimized with extended incubation periods or with the presence of formamide in the hybridization buffer (Yilmaz and Noguera, 2004).

For the accessibility of the target site, it can be regarded as the ability of the probe to reach the target, regardless of whether hybridization will occur or not. The 16S rRNA in situ accessibility for oligonucleotide probes has been systematic studied for *Escherichia coli* (Fuchs *et al.,* 1998). Now it is possible to evaluate the percentage of accessibility of the probe into the secondary structure of the 16S rRNA, thanks to this color-coded map that gives a distribution of probe-conferred cell fluorescence in six set brightness classes (classes I to VI). According to *E.coli* accessibility map, the newly designed probes in this study, had different percentages of accessibility: the probe SAG1_357 showed the best result (class II, 61-80%), followed by SAG1_530 (class III, 41-60%) and finally SAG1_362 (class IV, 21-40%) (data not shown, see Fuchs *et al.,* 1998 for maps of the accessibility sites).

Moreover, the oligonucleotide probes are also often limited by low signal intensities, an impermeability of the cell periphery, a low cellular rRNA content and low melting temperature (Tm). Empirical observations showed that probes with Tm ≥57°C have a greater chance of success, and that the melting behaviour of oligonucleotide probes depends on temperature, the composition of the hybridization buffer, oligonucleotide sequence (GC-content, a higher level indicates a higher melting temperature), and probe length (Manz *et al.,* 1992). If the Tm of the probe is <57°C, the Tm can be raised by increasing the probe length, often a one or two base extension is sufficient. Therefore, a balance between these parameters, can lead to succeed in the hybridization probe.

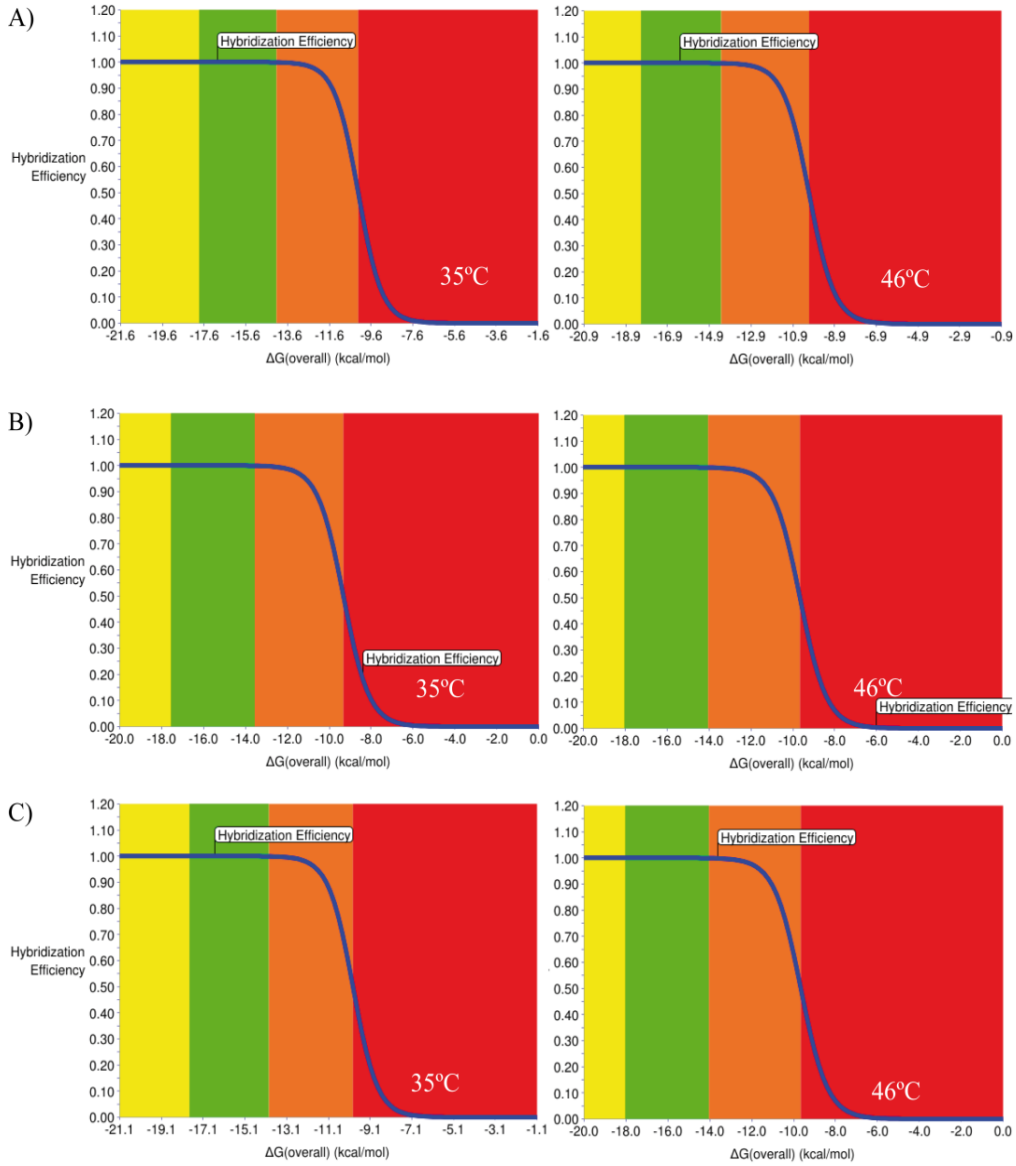

Figure 5.2. Hybridization efficiency curves of the three newly design probes for SAGMCG-1 group whit the *Nitrosotalea devanaterra* 16S rRNA gene, partial sequence. A) SAG1_357, B) SAG1_362 and C) SAG1_530. The curves are based on a color coding, the sigmoid curve provides a recommended guideline for the optimization of sensitivity and specificity for perfect matches only. The color coded recommendations are 1) Green: Recommended $\Delta G^{o}_{overall}$ range due to low risk for both false negatives and false positives. 2) Orange: Theoretical optimal for avoiding false positives, but with an increased risk of false negative. 3) Red: Strongly recommended against due to high risk of false negative. 4) Yellow: Very low risk of false negative, but increased risk of false positive due to excessive probe affinity.

SAG1_357 and SAG1_530, both with a length of 18 nucleotides, showed values below recommended: a GC-content 50% and a Tm to 48ºC and 42ºC respectively (Table 5.5). The third probe (SAG1_362), with a length of 18 nucleotides, exhibits a 41% GC and a 37ºC Tm (Table 5.5). Consequently, we proceeded to increase the length of the probe (18/17 to 20/19 nucleotides) to obtain values that allowed us to reach an optimal result of hybridization. However, after checking the probe with additional nucleotides in the ARB database, it was found that this procedure compromised the specificity of the probes. Therefore, improvement in hybridization efficiency must be performed after protocol optimization.

5.3.4 Empirical probe testing

In CARD-FISH, an oligonucleotide probe with a taxon-specific sequence binds directly to rRNA. For CARD-FISH, the probe is bound to a large horseradish peroxidase (HRP) enzyme, which catalyzes the deposition of many fluorescent tyramides, enhancing the fluorescence intensity. HRP is far too large (~40 kDa) to diffuse freely into cells (Braithwaite *et al.,* 1976), so cells must first be permeabilized by partially degrading their cell walls.

Before applying CARD-FISH, permeabilization protocols must be optimized for the group of interest. This is often laborious because the range for "optimum" permeabilization is very narrow. Even though some species can be detected without any treatments (Ishii *et al.,* 2004), most species cells need to be pretreated for probe penetration. The two most visible and significant differences between the MPI and CEAB protocols were: 1) Permeabilization (CEAB: lysozyme + achromopeptidase), and 2) annealing (Tm), washing and amplification temperatures (see Table 5.1). Enzymatic treatments using lysozyme, achromopeptidase, proteinase K, and pseudomurein endopeptidase are often employed for permeabilization. Lysozyme is the most commonly used enzyme for treatment many bacteria are permeabilized by lysozyme treatment; however, because several species are resistant to lysozyme (Chassy and Giuffrida, 1980), and because lysozyme only partly digests the murein multilayers of fixed cells (Sekar *et al.,* 2003), some bacteria cannot be permeabilized by lysozyme treatment alone. Simultaneous treatment with achromopeptidase and lysozyme is effective for lysis of these bacteria. Sekar *et al.,* (2003) introduced

achromopeptidase treatment following lysozyme treatment for permeabilization of Actinobacteria. Achromopeptidase hydrolyzes lysyl peptide bonds (Trebesius *et al.,* 1994), and lysozyme treatment likely improves the accessibility of achromopeptidase to the peptide bonds. This treatment is used for permeabilization of many microbial cells, including Archaea (Ishii *et al.,* 2004). Counting cells resulted in 100% efficiency of ARCH915 and NON338 (negative control) zero cells, for CEAB protocol and no sign of MPI protocol. These results were reproduced both for the *Nitrosotalea devanaterra* strain (1.86 $x10^6$ cells / mL DAPI and ARCH915), and for environmental samples from high mountain lakes (8.65 $x10^5$ cells/mL DAPI and 1.55 $x10^4$ cells/mL ARCH915, data not shown). This comparison showed the CEAB protocol as the best method of optimizing specific probes for SAGMG-1 group and confirmed achromopeptidase treatment as the best method for permeation in *Archaea*.

After the *in silico* tests results, the probes were evaluated for their potential to hybridize to whole cells. Probes SAG1_362 and SAG1_530 not yielded fluorescent signals after hybridization and were not investigated further. The remaining CARD-FISH compatible SAG1_357 probe was best suited resulting in clear signals and hybridization efficiency of 100%, whit DAPI and SAG1_357 counts of 2.01 $x10^6$ cells per ml (data not shown). Therefore, optimization procedures were performed only using SAG1_357 probe.

Accessibility of target sites to probes improved by the use of unlabeled helper probes has specific limitations, their design is often impossible for probes with broader specificities. In this work, however, it was possible to design of the two adjacent helpers for SAG1_357 probe (5′ and 3′ adjacent) with the purpose of solving the problem of GC-content and effectively increase both the efficiency and the signal probe. As noted previously, the helpers are longer than probes, on average 21 nt, and cover all of the sequences that are targeted by the correspondent probe. The sequences are given as follow: SAG1_357_h5' TGCTGCGCCCCATAGGGCCTC and SAG1_357_h3 ACCYAGTCGTGCTTTCGCACA.

5.3.5 Formamide dissociation profile

Once designed and evaluated, the next step was probe stringency optimization. This process usually involves hybridizing the probe to pure cultures of target organisms and non-target organisms (with the fewest mismatches to the probe sequence) at a range of stringencies. Stringency can be adjusted via a number of parameters, such as temperature and formamide (denaturant) concentration. The objective is to determine the range of stringencies (formamide concentrations) at which the probe specifically hybridizes to the target organisms but not to the non-target organisms. The most desirable hybridization stringency often occurs at the point immediately before the target cell fluorescence begins to decrease ("drop-off point"). At this formamide concentration, hybridization to the non-target organism should be low or absent. We obtained probe denaturation profiles in *Nitrosotalea devanaterra* strain with a formamide series of ten concentrations: 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65% on a volume by volume basis (v/v). Theoretical vs. experimental profiles are shown in Figure 5.3. As expected, increasing formamide creates a sigmoid-like loss of signal as the efficiency of target capture decreases (Figure 5.3).

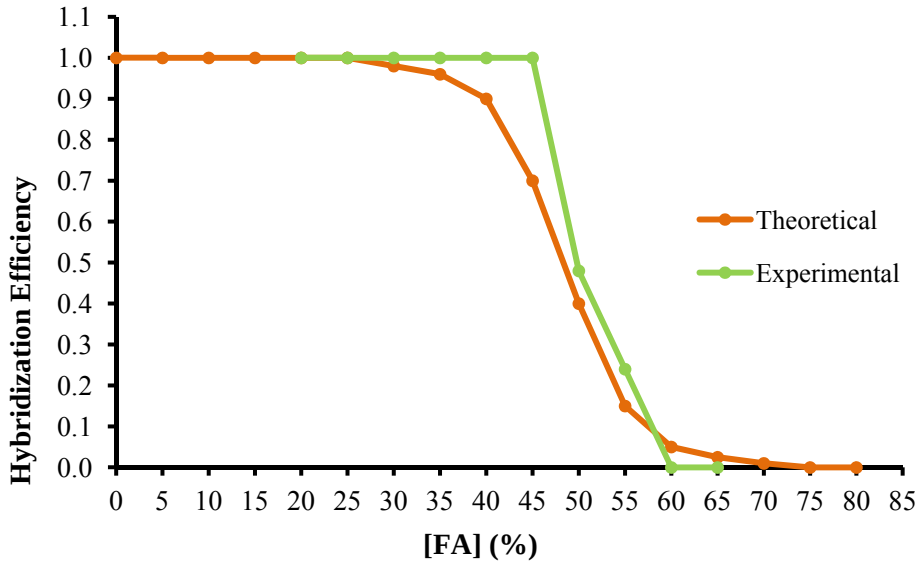

Figure 5.3: Graphical display of theoretical vs. experimental formamide dissociation profile for SAG1_357 probe in *Nitrosotalea devanaterra* strain at 35ºC hybridization temperature. The plot of hybridization efficiency as a function of formamide concentration.

For SAG1_357 probe with a full sigmoid trend, there is a general increase of signal with increasing formamide at lower formamide concentrations, as exemplified by the perfect match probe, which may be due to the removal of structural kinetic limitations by formamide as in FISH (Yilmaz and Noguera, 2004). The gradual loss of signal at higher stringency creates a formamide concentration (45% in the Figure 5.3), where the signal from perfect match is easily detectable, and thereby allowing mismatch discrimination as desired. Consequently, using the efficient experimentation and data acquisition set up, we were able to obtain equilibrium denaturations with formamide (45%) and temperature (35ºC) as denaturants during hybridization for SAG1_357 probe.

5.3.6 Probe, helpers and competitors evaluation

Specificities and sensitivities of fluorescently derivatives of probe SAG1_357 was evaluated by hybridization to fixed cells of reference *Nitrosotalea devanaterra* (target) and *Nitrosopumilus maritimus* (non-target) strains, using the conditions predetermined for optimum hybridization. Under in situ conditions, excellent specificity was observed at this formamide stringency (45% FA) which gave well-fluorescing signals (100% DAPI and SAG1_357 counts). However, to achieve optimal probe sensitivity we had to use helpers. Without helpers, only light spots by SAG1_357 probe can be seen in the cell instead of whole cell, which makes cell counting difficult (Fig. 5.4A).

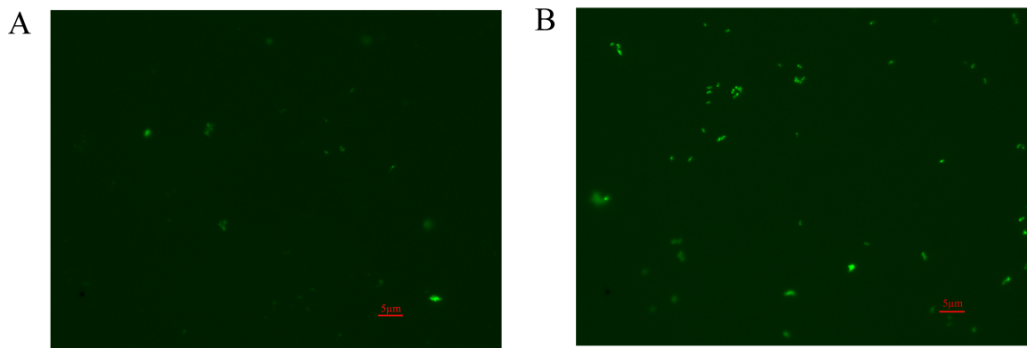

Figure 5.4: Comparison of the effect of helper in CARD-FISH. (A) *Nitrosotalea devanaterra* cells with HRP-linked SAG1_357 probe without helpers, hybridized at 45% formamide. (B) *Nitrosotalea devanaterra* cells with HRP-linked SAG1_357 probe with helpers SAG1_357_h5' and SAG1_357_h3', hybridized at 45% formamide. Exposition time: 879ms.

While with helpers, the fluorescence intensity is enhanced very significantly (Fig. 5.5), and was sensitive enough to clearly visualize the small rod-shaped cells (0.33 ± 0.01 µm wide and 0.89 ± 0.05 µm long, Lehtovirta-Morley *et al.*, 2011) (Fig.5.4B). Thus, helpers do not only greatly enhance the signal intensity, but also have effects on the cell visualization and probably on specificity. Moreover, probe SAG1_357 showed non-specific binding to the non-target organism *N. maritimus,* and hybridization tests with/without helpers and competitors did not give results positives. Although the *in silico* analysis showed mismatch with non target organism, and thus there is a potential for non-specific hybridization with other groups.

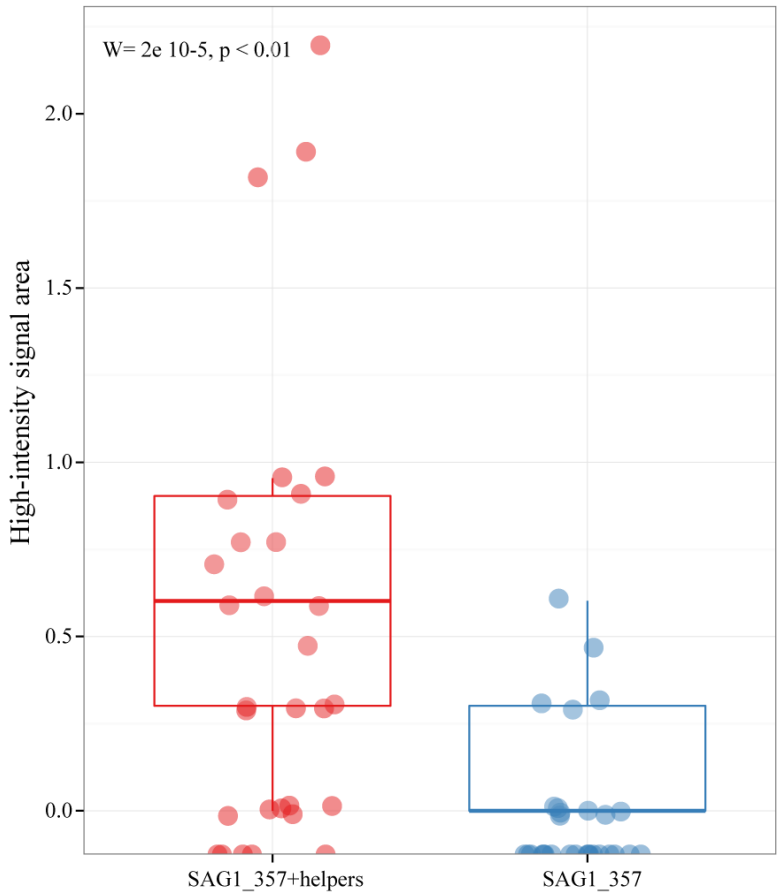

Figure 5.5: Evaluation of helper effects in the intensity-signal of SAG1_357 probe on CARD-FISH yield with non parametric Wilcoxon test.

As mentioned above, *in silico* analysis results could give as false positive or negative of the probe, because sequences shorter or just mistakes in sequencing. Still, further analysis in environmental samples will be necessary in order to be sure the SAG1_357 probe specificity, the influence or not the helpers and the usefulness or otherwise of specific competitors designed. Also, any CARD-FISH signal with NON 338 (negative control) of all samples wasn't detected and autofluorescence was easy to distinguish from CARD-FISH signal.

5.3.7 Nitrosotalea devanaterra, detection and quantification in complex samples

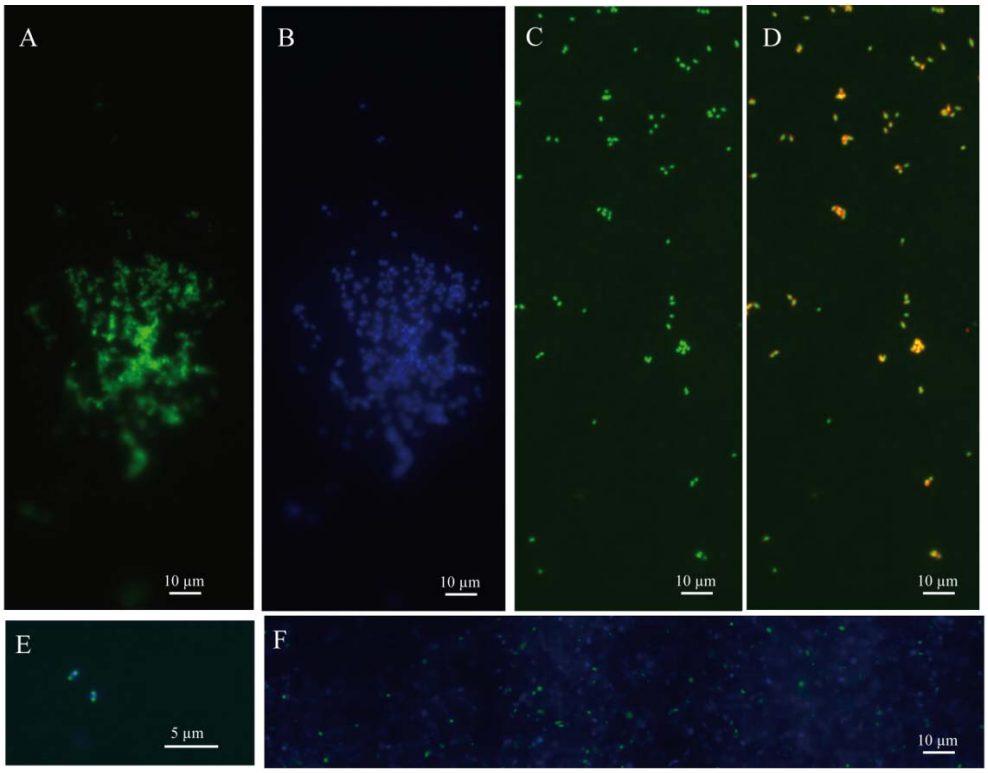

Figure 5.6: Morphology and dominance of *Nitrosotalea devanaterra* in pure culture and an acidic soil samples. CARD-FISH images with (A) SAG1_357 probe labeled with Alexa 488 (green) of *N. devanaterra* strain, (B) DAPI (blue) of *N. devanaterra* strain, (C) *N. devanaterra* strain with SAG1_357 probed labeled Alexa 488, (D) Doubly hybridization SAG1_357 labeled with Alexa 488 (green) and ARCH915 labeled Alexa 594 (red) probes of *N. devanaterra* strain, (E) DAPI (blue) and SAG1_357 (green) of *N. devanaterra* strain, (F) DAPI (blue) and SAG1_357 (green) of *N. devanaterra* at 4.5 pH soil sample.

Nitrosotalea was detected in all samples used, both pure culture and complex samples from acid soils. Once again, the performance of the probe showed 100% efficiency supported by DAPI counts and double hybridization of SAG1-357 and ARCH915 probes (Figure 5.6AD). Additionally, in Figure 5.6E it could be confirmed the previous observation by Lehtovirta-Morley *et al.,* (2011) in the *N. devanaterra* culture, where ribosomes were concentrated in the poles and the genome at the center of the cell.

5.3.8 Evaluation of the probes in natural samples

In natural complex samples, *N.devanaterra* can be found in many acidic soils but not in all of them (Figure 5.6F). Significant negative correlation (r=-0.84, P<0.001) (Figure 5.7) was found between pH and the number of *N. devanaterra* cells, and also in signal intensity. At pH 4.5 and 5.0, were found that the 69% (1.61x105 cells per ml) and 70% (2.44 x105 cells per ml) respectively, of the DAPI abundance corresponded to *N. devanaterra* cells (Figure 5.7). This result is consistent with previous findings by Lehtovirta-Morley *et al.,* (2011) where low pH was shown to be a major factor for the presence of *N.devanaterra*.

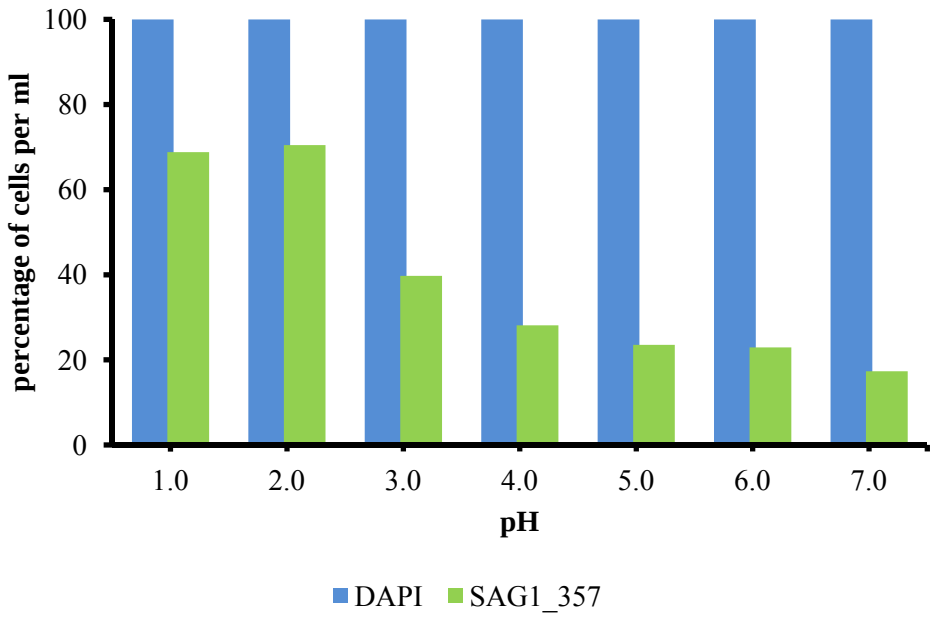

Figure 5.7: Quantification of *Nitrosotalea devanaterra* cells in acidic soils samples.

Interestingly, higher fluorescence was observed in low pH than in high pH complex samples, no matter with or without helpers. These results suggest that cells fluorescence can be modulate by pH. This is in agreement with a previous study showing the change in fluorescence lifetime by pH (Ogikubo *et al.,* 2011).

Assessments in freshwater samples, carried out on samples from high mountain lake in the Pyrenees, did not produce any positive result. In order to rule out any methodological problem, permeabilization tests were performed with the ARCH915 probe. In this case, it was found that the best protocol for archaea pemeabilizacion was still the lysozyme and achromopeptidasa protocol. Although SAG1_357 probe assays were performed increasing the incubation time (5 hours and overnight) and hybridacion temperatures, no positive results were found. One possible reason is we were under detection limits since the volume of filtered water was only 14 mL. It remains to be tests on samples of different volumes filtered in order to determine the minimum volume detection and efficiency of the SAG1_357probe.

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Visión general

6

Discusión general

Los microorganismos Archaea son un componente común en diversos hábitats del planeta. Diversos grupos de Archaea no cultivadas tienden a ocupar diferentes nichos ecológicos, gracias a un amplio potencial metabólico y fisiológico que desconocemos mayoritariamente y que suponen un continuo desafío al que la ecología microbiana debe enfrentarse. Gracias al trabajo desarrollado en esta tesis doctoral, que combina aproximaciones ecológicas y de la microbiología tradicional así como aproximaciones moleculares modernas, se ha logrado una mejor comprensión de la dinámica poblacional de tres grupos de Archaea relevantes en ambientes lacustres aportando herramientas sofisticadas para expandir el conocimiento actual existente sobre la biología y ecología poblacional de microorganismos Archaea en ambientes naturales.

6.1 Identificación de patrones, moduladores ambientales y distribución ecológica de Archaea en ambientes naturales

6.1.1 Concentraciones de amonio

La heterogeneidad ambiental de los lagos de los Pirineos en particular, y probablemente de los lagos de alta montaña en general, ha resultado ser un modelo ambiental muy conveniente para estudiar y explorar la ecología y distribución de poblaciones específicas de Archaea y sus relaciones con el ciclo del nitrógeno (N). Estudios previos de los genes estructurales (ribosómico 16S rRNA) y funcionales (*amo*A) de Thaumarchaeota a lo largo de variaciones estacionales, mostraron cambios sustanciales en la dinámica de la diversidad y abundancia de las poblaciones de AOA en los lagos oligotróficos alpinos (Auguet *et al.*, 2011, 2012, Vissers *et al.*, 2013). Adicionalmente, la correlación hallada entre la dinámica de las especies de AOA con los cambios en la dinámica del N, y la coexistencia de los clados de Thaumarchaeota MG 1.1a, ampliamente implicado en la nitrificación (Konneke *et al.*, 2005; Hallam *et al.*, 2006; Blainey *et al.*, 2011) y SAGMCG-1, recientemente sugerido como actor clave dentro del ciclo del N (Lehtovirta-Morley *et al.*, 2011), apuntaban hacia un papel relevante de las Archaea en el ciclo del nitrógeno. De manera más específica, esta tesis doctoral identifica al clado SAGMCG-1 como el más relevante dentro del plancton de ambientes lacustres (Auguet *et al.*, 2012, Auguet and Casamayor 2013, Restrepo-Ortiz *et al.*, 2014). Curiosamente, estos estudios previos no detectaron la presencia del gen amoA de bacterias, sugiriendo una exclusión competitiva de AOB en este tipo de ambientes oligotróficos (Auguet *et al.*, 2011, 2012, 2013). Sin embargo, estudios más recientes han detectado presencia de este gen mayoritariamente en zonas profundas del lago Redon (Sala-Faig *et al.*, en preparación) y sugieren una diferente distribución temporal-espacial entre AOA y AOB que merece un estudio más detallado.

En la presente investigación, el estudio correlacional realizado apoya fuertemente el potencial de oxidación de amonio dentro de los homólogos de agua dulce del clúster SAGMCG-1. Sin embargo, se observaron diferencias sustanciales en la relación entre la abundancia del gen 16S rRNA de SAGMCG-1 y el gen amoA de los diferentes ecotipos de *Nitrosotalea* spp., siendo el gen 16S rRNA más abundante que el gen *amo*A en todos los casos.

En consecuencia, se podría inferir que sólo unos pocos representantes del clado SAGMCG-1 contiene el gen amoA de *Ntalea* 1.1a o, alternativamente, que SAGMCG-1 contiene varias copias del gen 16S rRNA. Sin embargo, y pese a al número de cuestiones que surgen a partir de estos resultados, podemos mostrar que existe una distribución espacio-temporal completamente variada para los diferentes ecotipos de AOA en agua dulce, revelando la existencia de distintas especies de AOA filogenéticamente cercanas pero ecológicamente distantes. Sin embargo, desconocemos que aspectos fisiológicos o metabólicos pueden ser los responsables de esa segregación entre ecotipos. Sintes y colegas (2013) plantearon la hipótesis que la biogeográfica y la distribución de grupos AOA con respecto a la profundidad, podrían estar relacionados con las diferencias en la disponibilidad de amoníaco en ambientes marinos. Curiosamente se encontró una separación espacial a lo largo de la columna de agua entre ecotipos de *Nitrosopumilus* que se ha relacionado con aspectos fisiológicos de alta y de baja afinidad por el amonio. Recientemente se observó que, en general, los AOA en la superficie y profundidad de aguas Atlánticas exhiben relaciones de decaimiento frente a la distancia y que siguen la regla de Rapoport de manera similar a como lo hacen las comunidades bacterianas y los macroorganismos (Sintes *et al.*, 2015). Esto podría afectar fuertemente a la composición de las comunidades de AOA, lo que apoya la importancia de este nutriente en la conformación de la comunidad amoniaco-oxidante. A pesar de no definir con precisión los nichos ecológicos, en la presente investigación se pudo evidenciar esta diferenciación de nichos, confirmando la idea de que thaumarchaeota se compone de diferentes ecotipos de AOA, según lo propuesto anteriormente para lagos (Auguet *et al.*, 2012; Auguet and Casamayor, 2013) y la costa Mediterránea (Hugoni *et al.*, 2013; Sintes *et al.*, 2013). Sin embargo, se necesita más investigación para aclarar las adaptaciones fisiológicas y el papel de los diferentes ecotipos de AOA en la oxidación de amoníaco.

6.1.2 Fotoinhibición

Los efectos ecológicos de la intensidad y/o la calidad de la luz, son de particular interés ya que la susceptibilidad a la luz aplica tanto a las bacterias nitrificantes (Horrigan et al, 1981; Olson, 1981; Guerrero y Jones 1996a, b)

como a las arqueas (Mincer *et al.,* 2007, Merbt *et al.,* 2012). Los lagos alpinos, especialmente los ubicados en las zonas altas están expuestos a alta radiación solar debido al aumento natural de la radiación con la elevación (Catalán *et al.,* 2006). Además, estos lagos se encuentran entre los ecosistemas acuáticos más transparentes y por tanto con mayor incidencia de luz en la columna de agua (Sommaruga y Augustin, 2006). Por el contrario, durante el largo período de cobertura de hielo, la columna de agua se mantiene bajo un régimen de luz atenuada o en completa oscuridad. Por lo tanto, es de esperar una distribución espacial y temporal diferencial de las especies de AOA a lo largo del ciclo anual. Por otra parte, se ha demostrado que los oxidantes de nitrito (NOB) son más sensibles a la luz que AOB, y la recuperación a la fotoinhibición es más rápida en los AOB (Guerrero y Jones 1996b, Merbt *et al.,* 2012). Si estos resultados aplican también a los AOA de los lagos alpinos, la distribución de las concentraciones relativamente altas de nitrito cerca de la interfase aire-agua podría estar relacionada con una sensibilidad diferencial a la luz. En general, los resultados de esta tesis sugieren un posible reciclaje activo de nitrógeno atmosférico reactivo por diferentes ecotipos de AOA. Además, el patrón de distribución de dos de los tres ecotipos AOA observados fue consistente con la fotoinhibición descrita recientemente de dos cepas AOA bajo condiciones controladas en el laboratorio (Merbt *et al.,* 2012). Sin embargo, un tercer ecotipo AOA mostró un pico de acumulación menor cerca de la superficie del lago a principios de verano, lo que sugiere curiosamente una posible adaptación a la alta radiación solar. El estudio de este ecotipo resulta por tanto especialmente interesante.

Mientras que en los AOA el mecanismo bioquímico de fotoinhibición no se entiende completamente, en los AOB se sabe que la luz desnaturaliza la enzima amonio monooxigenasa (Hyman y Arp, 1992) que conduce a una inactivación completa de la oxidación de amonio en cultivos (French *et al.,* 2012, Merbt *et al.,* 2012). Otras posibles causas detrás de esta inhibición están relacionadas con la turbidez, que puede tanto promover la nitrificación como proteger a los nitrificantes de la fotoinhibición y limitar la competencia de sustrato con el fitoplancton.

6.1.3 El pH

La adaptación al pH es un importante motor de diversificación en Thaumarchaeota, como se ha podido observar comparando las asociaciones entre el pH y una serie de otros factores ambientales con la diversificación en Thaumarchaeota, que a su vez indican un fuerte efecto del pH en la distribución de las especies de AOA en los ecosistemas terrestres (Nicol *et al.*, 2008; Gubry-Rangin *et al.*, 2011). En general, las AOA son las principales impulsoras de la oxidación de amoniaco en muchos suelos, particularmente aquéllos con bajo pH (Gubry-Rangin *et al.*, 2010; Stopnišek *et al.*, 2010). En los lagos de los Pirineos y en los estudios microscópicos de distribución de *N. devanaterra* que hemos realizado en suelos, el gradiente de pH apareció como un factor clave de control de la abundancia de las poblaciones de thaumarchaeota. De hecho, junto con la salinidad (Auguet *et al.*, 2010), el pH se ha mostrado como uno de los principales moduladores de la estructura de la comunidad microbiana en general (Fierer y Jackson 2006, Newton *et al.*, 2007, Auguet y Casamayor 2013, Triadó-Margarit y Casamayor 2012). Sin embargo, este parámetro por sí solo no proporciona pistas para inferir el papel potencial metabólico in situ de estos microorganismos, pero sí que condiciona el estado químico del NH^{+3}/NH^{+4} y la adaptación fisiológica para oxidarlo biológicamente necesaria a bajos pH. Es este aspecto, la adaptación de las arqueas a bajos rangos de pH, adquiere un importante papel en la nitrificación de los suelos, ya que eran conocidas las limitaciones de las bacterias para asimilar el amonio a bajos pH (Prosser and Nicol, 2008).

6.2 Diseño y optimización de marcadores moleculares a nivel de población.

La combinación de microautoradiografía con sustratos marcados isotópicamente y técnicas microscópicas como CARD-FISH está ofreciendo nuevos avances en el conocimiento del potencial metabólico de microorganismso no cultivados. Por ejemplo, para la detección específica de captación de L-asp (aspartic acid) por bacterias y archaeas, en diferentes capas de profundidad del Atlántico Norte (Teira *et al.*, 2006), o mediciones de absorción de leucina en células individuales del bacterioplancton marino

(Sintes and Herndl, 2006). Los métodos que utilizan marcadores fluorescentes, como la qPCR y el CARD-FISH, tienen la ventaja de ser muy precisos y evitar posibles artefactos o secuencias inespecíficas presentes en el producto, por lo que la interpretación de los resultados suele ser más rápida y directa. Sin embargo, estos enfoques necesitan del diseño de secuencias específicas para su uso (cebadores y sondas específicas) y éste es el paso más laborioso y costoso (Lee *et al.*, 2004) ya que es necesario un correcto diseño que dé como resultado características ideales de la sonda o cebador (p.ej., especificidad con el organismo diana, adecuado contenido en G-C, temperaturas de hibridación dentro del rango óptimo) que influyan positivamente en la eficiencia de la reacción.

Desde que se inicia el proceso del diseño de cebadores y sondas específicas para qPCR y CARD-FISH, respectivamente, hasta que se logra su optimización y eficiencia óptima, nos encontramos con diversos inconvenientes que deben irse solucionando de manera progresiva y secuencial. Una de las primeras barreras para el diseño de cebadores y sondas, es la disponibilidad de una buena y diversa base de datos de secuencias del gen 16S rRNA para el taxón microbiano de interés. En nuestro caso, el grupo SAGMG-1 hasta hace poco tiempo era muy poco conocido. En un principio este grupo comprendía solo secuencias provenientes de la exploración de minas de oro de África y desde fechas muy recientes la mayoría de secuencias pertenecen a los trabajos realizados en los lagos de alta montaña de los Pirineos. Estos trabajos extensivos, mediante técnicas moleculares como clonning, qPCR, y secuenciación combinadas con análisis bioinformático, nos permitieron dar visibilidad y generar un particular interés sobre SAGMG-1 dentro de los ambientes de lagos oligotróficos, además de ampliar enormemente la base de datos del gen 16S rRNA para este grupo.

Gracias a estos estudios, uno de los resultados metodológicos más relevantes de esta investigación es el desarrollo de nuevos cebadores de qPCR específicos para los grupos más representativos de los lagos oligotróficos de los Pirineos, testados y optimizados, que permitieron analizar la distribución poblacional in situ de tres poblaciones específicas de Archaea. En primer lugar, el grupo ya mencionado SAGMG-1 del phylum Thaumarchaeota que presenta una amplia distribución espacial, al igual que una distribución vertical y temporal en el lago Redon y, en segundo lugar, dos linajes dentro del phylum Euryarchaeota los MEG y DSEG. Por otra

parte, el descubrimiento y cultivo de la especie acidofílica presente en suelos *Nitrosotalea devanaterra* del grupo SAGMCG-1 Thaumarchaeota, puso de manifiesto que este género tiene un papel relevante dentro del ciclo del nitrógeno. Nuestros trabajos buscaban demostrar que SAGMCG del plancton de lagos oligotróficos también podrían tener el potencial de estar implicados en el ciclo del N en ambientes lacustres. Con esta premisa en mente, se diseñaron también cebadores de qPCR específicos del gen funcional *amo*A para estos tres ecotipos del grupo SAGMG-1, que nos permitió evaluar la dinámica y dilucidar la relación con el gen SAGMG-16S rRNA.

A pesar que la qPCR respresenta una metodología ampliamente utilizada en la investigación básica, por su alta sensibilidad, rapidez y operatividad con pequeños volúmenes de muestra, tiene también como desventajas, la presencia de inhibidores dentro de las muestras, la dificultad para discernir entre células vivas o muertas y la sobreestimación de las abundancias, que se ha evidenciado en trabajos comparativos (Schouten *et al.*, 2010; Xie *et al.*, 2013; Lloyd *et al.*, 2013), lo que pone en duda la completa fiabilidad de esta metodología para el análisis específicamente cuantitativo de la distribución y ecología poblacional con muestras complejas en ambientes naturales.

En el caso de CARD-FISH, uno de los grandes inconvenientes para la optimización de sondas especialmente en el caso de Archaea está relacionado con la permeabilización de la pared celular. La mayoría de las paredes celulares de archaea están rodeadas por una capa superficial (Capa S) gruesa, integrada por proteínas o glicoproteínas, que se auto ensamblan rodeando toda la superficie de la célula. Las proteínas de la capa S pueden diferir marcadamente incluso entre especies relacionadas y pueden representar hasta el 10-15% del contenido proteínico total de una célula (Pum *et al.*, 1991). Por lo tanto, un método eficaz para permeabilizar estas paredes implica necesariamente la digestión parcial de esta capa proteica compleja y heterogénea. A lo largo de mucho tiempo, diversas metodologías han sido propuestas, tales como la proteinasa K, la lisozima, SDS, conjunto de enzimas que se potencializan unas a las otras como el dúo lisozima-acromopeptidasa, entre otras, y todos estos mecanismos han dado regularmente resultados positivos en diversas poblaciones de Archaea de diferentes ambientes. Sin embargo, también se han descrito diversos problemas en los métodos de permeabilización debido a la resistencia de

varias especies de archaea a estas enzimas, por lo que, como se evidencia en esta tesis, es siempre necesario optimizar cuidadosamente los pasos previos que permitan la correcta permeabilización de las células. Este proceso resulta a menudo difícil y costoso, porque no existe una investigación a fondo que haya estudiado todas las formas de permeabilización en diferentes poblaciones de Archaea. En esta tesis doctoral, evaluamos diferentes estrategias de permeabilización, y comprobamos que el tratamiento simultáneo con acromopeptidasa y lisozima (Sekar *et al.,* 2003) es efectiva para la lisis de archaea de agua dulce y suelos, así como para los cultivos de *Ntalea* y *Npumilus*, y que funciona perfectamente en filtros de policarbonato tanto para sondas generales (ARCH915) como especificas (SAG1_357).

Otras desventajas conocidas de esta metodología están relacionadas con el tamaño de la sonda, la termo-estabilidad, la sensibilidad de la RNasa, el tiempo de análisis y el coste. Aun así, las sondas oligonucleótidas tienen grandes ventajas sobre sondas de ARN, que incluyen mayor estabilidad, mejor disponibilidad, mayor penetración celular, y mejor reproducibilidad y especificidad (Polak *et al.,* 1998). El tamaño y la termo-estabilidad de las sondas fue un inconveniente con el que nos encontramos en esta investigación, puesto que, a pesar de tener un alto porcentaje de especificidad, la sonda contenía una baja concentración de contenido G-C, lo cual afecta de manera directa las temperaturas de hibridación de las sondas y por ende su eficiencia. Una posible solución a este problema pasa por el alargamiento (adición de un par de nucleótidos) de la sonda, lo que potencialmente puede incrementa su contenido G-C y mejorar la termo-estabilidad. Sin embargo, en nuestro caso, el alargamiento de la sonda trajo como consecuencia una importante reducción en la eficiencia y especificidad de la sonda, por lo que la optimización de ésta se hizo a partir de secuencias oligonucleotidas adyacentes a la sonda, llamadas helpers, que permiten una mejor hibridación en la célula e incrementa la eficiencia de la señal fluorescente y optimización de la temperatura de hibridación. Después de los diferentes ensayos en el cultivo de *Ntalea*, *Npumilus* y en muestras ambientales complejas, se pudo confirmar que los helpers no solo mejoraron significativamente la intensidad de la señal de la sonda, sino también tuvieron efectos sobre la visualización celular, como pudo ser evidenciado tras observar la morfología y estructura de las células individuales de *Ntalea* descrita por Lehtovirta *et al.,* (2011).

Una vez superada la barrera de la permeabilización de la pared celular y teniendo en cuenta la dificultad del diseño y optimización de las sondas oligonucleótidos, el CARD-FISH resulta ser una de las metodologías más adecuadas para mejorar nuestra comprensión de la ecología de los microorganismos, debido a su mayor sensibilidad y los muchos avances técnicos recientes que se pueden acoplar para la detección del rRNA, ARNm y genes funcionales. Gracias a la sonda SAG1_357 diseñada en esta investigación es posible ahora expandir el abanico de herramientas moleculares para el análisis de la biología y ecología de este linaje en particular, por ejemplo, a través de su combinación con técnicas de microautoradiografía. Adicionalmente, los recuentos efectuados permitieron confirmar hipótesis planteadas en anteriores trabajos, donde se atribuía al pH un importante efecto controlador de la distribución y abundancia de *Ntalea* en suelos.

En conclusión, para concentrarse en un población específica relevante y profundizar en las diferencias genéticas y fenotípicas, la distribución global, diversidad, ecología y fisiología, se hace absolutamente necesaria la combinación de técnicas moleculares basadas en amplificación selectiva, como la qPCR, con el CARD-FISH y con la aplicación de las sondas especificas con otras técnicas complementarias de FISH ya disponibles. Por un lado, Clone-FISH, una técnica sencilla y rápida compatible con una amplia variedad de vectores de clonación, de gran utilidad para la validación de sondas y el cribado de bibliotecas de clones (Schramm *et al.*, 2002). Clone-FISH permitiría por tanto un cribado muy eficiente de bibliotecas de clones del gen 16S rRNA completo a la búsqueda de aquéllos que contengan la secuencia diana de interés y así mejorar las bases de datos y en consecuencia el posterior diseño de sondas. Por otro lado, Gene-FISH, que permite la vinculación de genes activos con la identidad de las células en muestras ambientales, aplicado exitosamente con sondas dirigidas al gen *amoA* de Taumarchaeota en muestras de agua de mar de Namibia (Moraru *et al.*, 2010). Esta interacción de técnicas, con seguridad, proporcionará respuestas a preguntas básicas de quién, cuánto, dónde, cómo y porqué grupos determinados de Archaea evolucionan y ocupan diferentes nichos ecológicos exitosamente. Será entonces cuando la ecología microbiana mejore su comprensión sobre los patrones de distribución de los

microorganismos al igual, o incluso con mayor resolución, que lo que la ecología general ha desarrollado para animales y plantas.

7

Conclusiones

- La heterogeneidad ambiental de los lagos de alta montaña, resulta ser un modelo ambiental muy conveniente para estudiar y explorar la ecología y distribución de poblaciones específicas de Archaea y sus relaciones con los ciclos biogeoquímicos.

- El grupo SAGMGC-1 tiene un importante potencial ecológico sobre el ciclo del nitrógeno en ambientes oligotróficos y suelos.

- SAGMGC es más abundante durante el periodo de estratificación de invierno cuando el lago Redon está cubierto por el hielo y la nieve, y su abundancia disminuye después del período de deshielo y durante la estratificación estival.

- Existe una distribución espacio-temporal completamente variada para los diferentes ecotipos de AOA en agua dulce, revelando la existencia de distintas especies de AOA filogenéticamente cercanas pero ecológicamente distantes.

- Se sugiere un reciclaje activo de nitrógeno atmosférico reactivo por diferentes ecotipos de AOA. Existe un patrón de distribución en los ecotipos de AOA que entre otros factores podría estar relacionado con fotoinhibición.

- El análisis de correlación y la distribución estacional entre los AOA y DSEG de agua dulce, sugiere fuertemente que comparten un nicho ecológico, lo que puede ser clave para entender la funcionalidad ecológica de este grupo.

- Los cebadores específicos de qPCR diseñados, herramienta muy útil para la evaluación de la dinám los grupos SAGMGC-1, MEG y DSEG.

- La sonda SAG1_357 diseñada para CARD-FISH resulta ser una importante herramienta para el análisis de la biología y ecología del grupo SAGMGC-1, y puede ser aplicada en diversos ambientes y combinada con metodologías que exploren aspectos metabólicos y funcionales como la microautoradiografía.

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Appendix A

Supplementary material

Table A.S3.1: Accession numbers for the 16S rRNA and amoA genes sequences used for designing specific primers targeting SAGMCG-1 and AOA ecotypes.

Phylogenetic group	NCBI accession numbers	Habitat	Nº sequences	Reference
16S rRNA				
SAGMCG-1	FN691526, FN691554, FN691736, HHE589628, HE589631, HE589632, HE589633, HE589634, HE589652, HE796156, HE796159, HE796176, HE796165, HE795996, HE796074, HE795996, HE796074, HE795998, HE795999, HE796053, HE796011, HE796020, HE796084, HE796054, HF951779, HF951837, HF951841, HF951806, HF951833, HF951785, HF951823, HF951817, HF951775, HF951809, HF951810, HF951788, HF951780, HF951815, HF951772, HF951797, HF951822, HF951799, HF951771, HF951827, HF951789, HF951778, HF951819, HF951836, HF951790, HF951828, HF951824, HF951822, HF951774, HF951795, HF951796, HF951816, HF951776, HF951808, HF951834, HF951800	Freshwater	60	1, 2, 3
	AB050241, AB050227, AB050240, AB050229, AB050208	gold mines	5	7
	DQ223189, DQ223191, DQ223195, DQ223196	subsurface water	4	4
	AB243798, AB243797, AB243800, AB243807, AB243811	Rice passy soils	5	5
	AB294259, AB294260, AB294261, AB294262, AB294263	stream	5	6
Archaeal amoA gene				
Nitrosotalea 1.1a (SF1OTU1**)**	HE797963, HE797977, HE797978, HE797980, HE797984, HE797985, HE797991, HE797992, HE797998, HE798001, HE798002, HE798047, HE798087, HE79809	Soil/Freshwater	14	13

Table A.S3.1: Continued

Nitrosotalea 1.1b (SF1OTU2)	HE797967, HE797968, HE797969, HE797970, HE797971, HE797972, HE797973, HE797974, HE797975, HE797976, HE797979, HE797981, HE797982, HE797983, HE797986, HE797987, HE797988, HE797989, HE797990, HE797993, HE797994, HE797995, HE797997, HE797999, HE798000, HE798003, HE798009, HE798012, HE798016, HE798021, HE798027, HE798031, HE798032, HE798035, HE798044, HE798057, HE798075, HE798084, HE798090, HE798096, HE798098, HE798099, HE798112, HE798113, HE798124, HE798129, HE798131, HE798142	Soil/Freshwater	52	3
Nitrosopumilus (F5OTU3)	HE797996, HE798005, HE798006, HE798007, HE798008, HE798010, HE798011, HE798013, HE798014, HE798015, HE798017, HE798018, HE798019, HE798022, HE798023, HE798024, HE798025, HE798026, HE798028, HE798029, HE798033, HE798034, HE798036, HE798037, HE798038, HE798039, HE798040, HE798041, HE798043, HE798045, HE798042, HE798046, HE798048, HE798049, HE798050, HE798051, HE798052, HE798054, HE798055, HE798053, HE798058, HE798059, HE798060, HE798061, HE798062, HE798063, HE798064, HE798066, HE798067, HE798068, HE798065, HE798069, HE798070, HE798071, HE798072, HE798073, HE798074, HE798076, HE798079, HE798080, HE798082, HE798085, HE798086, HE798093, HE798095, HE798100, HE798101, HE798102, HE798104, HE798105, HE798106, HE798107, HE798108, HE798109, HE798110, HE798111, HE798114, HE798115, HE798116, HE798117, HE798118, HE798119, HE798120, HE798121, HE798123, HE798125, HE798122, HE798126, HE798127, HE798128, HE798130, HE798132, HE798133, HE798135, HE798136, HE798137, HE798134, HE798138, HE798139, HE798140, HE798141, HE798143, HE798144, HE798145	Freshwater	108	3

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Table A.S4.1: NCBI accession numbers of the 16S rRNA gene sequences used to design the new and specific primers for Euryarchaeotal clades MEG and DSEG in combination with the Pyrenean 16S rRNA gene dataset.

Phylogenetic group	NCBI accession numbers	Habitat	Nº sequences	Reference
16S rRNA				
MEG	EU385964, EU385836, FJ821624, GU127491, GU127415, GU127512, GU127548, GU127569, GU127466, GU127467, GU127414, GU127507, HQ141818, HQ330674, HQ330695	sediment	15	1, 2
	AM039528, EF444657, EF444629, EF444634, EF444598, EF444621,	hot springs	6	3, 4
	AB019747, AB019748, AB019734, AB175604, AB611671, AB611672, AB611609, AB611659, AB611456, AB611660, DQ078753, DQ082934, DQ082934, DQ082950, DQ640159, DQ417476, DQ417484, HQ395738, HQ395740, HQ395739, HQ611206, HQ611205,	Marine	22	5, 6, 7, 8, 9, 10, 11
	DQ336956, DQ848677, FJ718986	Mines	3	12, 13
	FN691563, FN691512, FN691533, FN691743, FN691544, FN691518, FN691699, FN691637, FN691486, DQ676245, AJ131278, AJ131271, AJ131276, HG316127, HG316128, HG316129, HG316130, HG316131, HG316132, HG316133, HG316134, HG316135, HG316136, HG316137, HG316138, HG316139, HG316140, HG316141, HG316142, HG316143, HG316144, HG316145, HG316146, HG316147, HG316148, HG316149, HG316150, HG316151, HG316152, HG316153, HG316154, HG316155, HG316156, HG316157, HG316158, HG316159, HG316160, HG316161, HG316162, HG316163, HG316164, HG316165, HG316166, HG316167, HG316168, HG316169, HG316170, HG316171	Freshwater	58	14, 15, 16
	DQ397346, EU329838, EU329793, EU329829, EU731561, EU731523, EU731499, EU731556, EU731570,	hypersaline	9	17, 18, 19
	FJ810526, FJ810536, FJ810527, FJ810539, FJ810533,	Groundwater	5	20
	FJ604792	Cave water	1	21
	AJ969774, AJ969787, AJ969793	Saline soil	3	22

Table A.S4.1: Continued

DSEG	AB019743, AB019744, AB019745, AB019746, AB111479, AB175601, AB213092, AB235346, AB237753, AB237754, AB247870, AB239075, AB293222, AB301858, AB301864, AB301866, AB301867, AB301873, AB301975, AB301978, AB329763, AB329770, AB426447, AB496482, AB600437, AB600440, AB600443, AB600445, AB600446, AB600447, AB600450, AB600453, AB600455, AB600456, AB600457, AB600459, AB600460, AB600461, AB600462, AB600464, AB611354, AB611623, AB622735, AB622748, AB629607, AF526943, AF526944, AF526948, AY280438, AY592520, AY592549, AY555816, JN798471, JN798473, JN798477, JN798485, AY280447, AY280448, AY354122, AY592049, AY592491, AY800211	deep-sea hydrothermal vent	62	5, 23, 6, 24, 25, 26, 27, 28, 29, 30, 7, 31, 32, 33, 34
	AB049035, AB177269	deep-sea	2	35, 36
	AB050206	gold mines	1	37
	FN691627, FN691653, FN691654, FN691689, FN691703, GU135471, GU135472, GU135473, GU135474, HM244100, HM244101, HM244105, HM244116, HM244122, HM244129, HM244130, HM244133, HM244138, HM244169, HM244174, HM244207, HM244212, HM244218, HM244219, HM244222, HM244223, HM244233, HM244238, HM244279, HM244303, HM244351, HM244370, HQ267295, HQ692057, HG316172, HG316173, HG316174, HG316175, HG316176, HG316177, HG316178, HG316179, HG316180, HG316181, HG316182, HG316183, HG316184, HG316185, HG316186, HG316187, HG316188, HG316189, HG316190, HG316191, HG316192, HG316193, HG316194, HG316195, HG316196, HG316197, HG316198, HG316199, HG316200, HG316201, HG316202, HG316203, HG316204, HG316205, HG316206, HG316207, HG316208, HG316209, HG316210, HG316211, HG316212, HG316213, HG316214, HG316215, HG316216, HG316217, HG316218	Freshwater	81	14, 38, 39, 40
	AJ133623, AJ299185, AJ347789, AJ347790, AM229256, EF208735, EF367461, EF367558, EF367683, EF444628, EF687554, EF687618, EF687644, EU048601, EU048649, EU048655, EU048658, EU329794, EU329799, EU329818, EU329832, EU385987, EU385997, EU420701, EU635927, EU681930, FJ264519, FJ264821, FJ351032, FJ455960, FJ484292, FJ487457, FJ487459, FJ487468, FJ487470, FJ649527, FJ685735, FJ685741, FJ902689, FJ902690, FJ902712, FN428825, FN554054, FN554067, FN820413, FR682481, GQ410800, GQ410917, GQ848374, GQ926247, GQ926263, GQ926303, GQ927568, GQ927573, GQ927590, GQ927591, GQ927604, GQ927671, GQ927693, GQ927703, GQ994181, GQ994204, GQ994242, GQ994243, GQ994346, GU190971, GU190973, GU190974, GU190975, GU190977, GU190979, GU190980, GU190982, GU190984, GU190987, GU190988, GU190990, GU190994, GU270126, GU270173, GU363066, GU363070, GU363074, GU363078, HM998544, HM998547, HQ611207, HQ611209, HQ611210, HQ611211	Marin sediments	88	41, 42, 43, 44, 4, 45, 46, 18, 47, 48, 49, 50, 51, 52, 53
	DQ103681, DQ103684, DQ133424, DQ640160, DQ640161, DQ640163, DQ641735, DQ910086, DQ925866, JF747739, JF747743, JF747745, JF747747	Hypersaline	13	54, 55, 9, 56, 57
	HQ395737, JF935162, JF935172	Hot spring	3	11

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Table A.S4.2. Environmental parameters of the different lakes analysed in this study and specific 16S rRNA gene abundances for the Euryarchaeota DSEG and MEG, and Thaumarchaeota SAGMCG-1 populations. Standard deviations < 10% applies for all the qPCR data. N/A not available; N/D not detected.

Lake	Altitude (m.a.s.l.)	Alake/Acatch	pH	Temp (ºC)	Cond (µs/cm)	Chl-a (µg/L)	MEG (copies/mL)	DSEG (copies/mL)	SAGMCG-1 (copies/mL)
Pica Palomera (PP)	2308	0.072	3.80	8.3	29.6	1.43	6	<0.01	83
Aixeus (AX)	2370	0.042	4.97	8.3	49.9	0.60	54	<0.01	75
Certascan (CE)	2335	0.135	5.67	4.3	9.1	0.20	121.5	19	113.75
Bergus (BG)	2449	0.064	6.28	13.0	10.6	1.02	306.25	0.125	9.75
Romedo de Dalt (RD)	2114	0.050	6.31	4.5	5.8	2.82	340.75	<0.01	0.01
Plan (PL)	2188	0.217	6.35	19.0	7.6	0.92	1570.25	0.0025	62.5
Podo (PO)	2450	0.139	6.80	13.0	5.1	0.87	1047.25	<0.01	14.5
Lloseta (LT)	2480	0.001	7.18	12.0	15.6	0.67	40136.5	7133	7767.75
Gelat Colomers (GC)	2590	n/a	7.25	9.5	3.3	0.30	10940.25	10173.75	6396
Llong Colomers (LC)	2135	n/a	7.25	13.0	7.5	1.04	12064.5	2984.25	136.75
Llong Lliat (LL)	2140	0.153	7.45	17.0	91	0.94	19300.25	<0.01	442.75
Llong (LO)	2000	0.006	7.46	16.5	10.9	0.74	9043	<0.01	94.5
Llauset (LA)	2190	0.057	7.47	10.7	60.4	1.08	2298	26655.5	1261.25
Botornat (BO)	2340	0.013	7.50	10.0	12.7	1.12	317.75	<0.01	1.25
Llebreta (LE)	1620	0.001	7.55	11.3	30.6	1.84	4359.75	69347.25	174
Redo AT (RA)	2150	0.019	7.60	13.0	10.6	0.87	15102.25	288683.75	11197
Cavallers (CA)	1784	n/a	7.75	15.0	7.6	0.43	2439.5	2886.25	389.75
Filia (FI)	2140	0.009	7.79	8.7	133.3	1.70	6751	5345	631.25
Estanya (ES)	670	n/a	7.88	26.0	1805	0.44	4251.5	<0.01	18
Basturs (BS)	632	n/a	7.88	31.0	293	0.93	11025	18636.5	2682
Gerber (GB)	2170	0.038	7.90	4.9	23.4	1.26	19.75	1430.75	0.125
Llosa (LS)	2480	0.013	7.90	17.0	15.7	2.07	90327	<0.01	28
Aiguamog (AM)	1429	n/a	8.02	13.0	60.4	0.74	3558.75	391	0.05
Bassa Oles (BA)	1600	0.015	8.02	15.2	154	3.76	48127.5	70944.25	1703.25
Pois (PS)	2056	0.026	8.19	8.1	67.2	2.86	3443.75	<0.01	3.75
Granotes (GR)	2330	0.277	9.14	18.0	4.1	5.18	8433.5	97440.75	2724.25

Table A.S4.3: Environmental parameters for the different dates and depths analysed in Lake Redon and specific 16S rRNA gene abundances for the Euryarchaeota DSEG and MEG, and Thaumarchaeota SAGMCG-1 populations. Standard deviations < 10% applies for all the qPCR data. N/A not available; N/D not detected.

Depth (m)	Jun-07	Jul-07	Aug-07	Sep-	Oct-07	Nov-	Dec-	Mar-08	Apr-08	May-08
	MEG (copies/mL)									
SML/Slus	118.37	0.65	/	/	/	/	/	141.15	9114.75	/
2	5.175	5.35	/	/	/	/	/	1203.02	4541.97	3568.8
10	129.32	35.4	/	/	/	/	/	721.525	3296.87	2410
20	6.925	22.375	/	/	/	/	/	1938.67	3107	2598.17
35	63.075	152.425	/	/	2853.27	/	/	4281.8	8653.07	2501.3
60	798.9	3429.25	/	/	3245.12	/	/	5197.17	4371.95	4222.97
	DSEG (copies/mL)									
SML/Slus	/	6184.8	/	/	683.9	/	/	N/A	368277	/
2	/	133112.	/	/	1457.6	/	/	120696	463333	81451.3
10	11460.	49284.5	/	/	/	/	/	N/A	30183.9	69991.8
20	296.1	638.8	/	/	1.8	/	/	96701.2	67630.9	158962.
35	23632	43525.1	/	/	138675	/	/	22915.3	347918	182133
60	N/A	568750	/	/	160253.	/	/	71176.1	504000	202936.
	SAGMCG-1 (copies/mL)									
SML/Slus	15923.	4941.7	13065.	118.8	9.0	/	/	13482.2	13065.8	/
2	2911.3	5091.7	6519.0	1.6	32.6	/	447.4	2770.3	18532.8	16243.3
10	2171.7	/	105.1	2279.2	18.6	/	330.9	32.8	32592.6	43811.6
20	/	1670.7	0.0	918.2	2.1	/	141.6	7313.7	83900.6	14762.0
35	/	/	4052.6	5703.7	7670.6	/	48.4	15168.5	54340.2	21972.5
60	2651.1	/	11135.	118.8	6758.5	/	303.1	35174.7	45754.9	48697.0
	DOC (µM)									
SML/Slus	66.6	66.6	83.3	75.0	100.0	66.6	66.6	50.0	91.7	/
2	58.3	66.6	75.0	66.6	83.3	/	58.3	41.7	83.3	1.4
10	66.6	58.3	58.3	83.3	83.3	/	50.0	33.3	41.7	0.7
20	58.3	50.0	58.3	58.3	75.0	/	50.0	25.0	50.0	0.6
35	50.0	50.0	50.0	50.0	50.0	/	50.0	25.0	33.3	0.6
60	50.0	58.3	50.0	41.7	41.7	/	50.0	25.0	8.3	0.5
	NH_4^+ (µM)									
SML/Slus	1.4	0.3	0.4	0.4	0.4	0.9	0.6	0.8	2.8	/
2	0.2	0.2	0.3	0.3	0.3	0.8	0.5	0.7	2.6	15
10	0.3	0.2	0.2	0.4	0.2	0.9	0.5	0.7	1.8	8
20	0.2	0.1	0.1	0.6	0.2	0.8	0.6	0.7	1.5	8
35	0.7	0.3	0.1	0.8	1.1	1.1	0.6	0.5	1.4	8
60	1.6	1.7	2.6	3.4	4.6	0.7	0.7	1.4	3.4	9
	NO_3^- (µM)									
SML/Slus	9	9	7	6	6	7	8	8	8	/
2	9	9	7	6	6	7	8	8	8	15
10	8	8	8	7	6	7	8	8	7	8
20	9	8	8	7	6	7	7	8	8	8
35	8	11	9	7	7	8	8	8	7	8
60	8	10	10	8	9	8	7	8	8	8.8
	NO_2^- (µM)									
SML/Slus	0.05	0.05	0.08	0.05	0.08	0.07	0.07	0.09	0.08	/
2	0.13	0.05	0.08	0.05	0.08	0.07	0.07	0.07	0.05	0.07
10	0.13	0.07	0.09	0.04	0.08	0.05	0.05	0.08	0.03	0.05
20	0.13	0.10	0.09	0.06	0.07	0.06	0.06	0.06	0.02	0.03
35	0.09	0.07	0.06	0.02	0.08	0.05	0.05	0.08	0.02	0.02
60	0.04	0.09	0.08	0.07	0.07	0.05	0.05	0.03	0.02	0.03

Table A.S4.3: Continued

	Chla (µg L^{-1})									
SML/Slush	1.03	1.10	1.24	1.05	1.15	0.70	0.45	0.16	0.35	/
2	1.12	1.20	1.31	1.13	1.37	0.93	0.50	0.22	0.97	1.21
10	1.88	1.39	1.92	1.03	1.57	1.93	0.70	0.54	2.10	0.85
20	2.08	1.23	1.36	1.22	1.43	1.62	0.50	0.17	2.02	0.46
35	3.29	4.44	2.26	1.67	1.42	1.28	0.23	0.12	1.98	0.32
60	0.91	0.70	0.53	0.40	1.19	0.98	0.16	0.07	0.57	0.21
Depth (m)	**Jun-07**	**Jul-07**	**Aug-07**	**Sep-07**	**Oct-07**	**Nov-07**	**Dec-07**	**Mar-08**	**Apr-08**	**May-07**
	Temperature (°C)									
SML/Slush	4	13	13	12	10	4	1	0	0	/
2	9	13	13	12	10	4	2	1.6	1.9	2
10	8.3	11.4	12.7	12.4	9.6	4.1	1.9	2.2	2.5	3.1
20	6.7	7.4	8.2	9	9.5	4.1	2.5	2.6	2.9	3.9
35	4	4	4	4	5	4	3	3	3.3	4
60	4	4	4	4	4	4.1	3.2	3.4	3.7	4
	4	13	13	12	10	4	1	0	0	
	Oxygen (µM)									
SML/Slush	/	/	/	/	/	/	/	/	/	/
2	300	272	272	272	288	316	353	403	322	10.4
10	300	284	262	272	294	300	362	316	303	9.6
20	316	328	319	310	294	300	378	350	269	8.8
35	303	297	303	300	297	303	336	297	291	8.9
60	275	256	222	222	206	300	325	228	172	7
	Bacterial abundance (×10^5 cell mL^{-1})									
SML/Slush	5.9	13.4	6.4	5.2	6.0	4.6	4.8	6.4	5.1	
2	3.4	15.2	6.4	5.3	5.8	6.2	4.1	2.3	3.3	
10	4.9	11.6	7.3	6.3	5.1	5.7	4.9	2.6	3.7	
20	3.2	20.4	11.3	12.1	5.4	5.4	4.5	2.2	3.6	
35	5.6	21.5	19.2	11.4	5.1	6.4	3.7	2.1	3.3	
60	9.3	14.5	16.4	8.8	7.6	7.0	5.2	2.8	4.0	
	Archaeal abundance (×10^3 cell mL^{-1})									
SML/Slush	10.5	45.2	3.2	26.9	8.7	21.0	11.0	197.4	91.2	
2	9.5	44.9	22.4	10.5	3.9	17.5	15.0	10.9	34.4	
10	15.0	34.9	15.7	24.7	2.0	15.9	13.9	12.8	10.9	
20	25.0	215.7	40.4	134.6	3.2	11.6	10.6	4.0	10.9	
35	28.4	195.9	71.8	116.7	2.0	18.3	20.3	20.5	2.5	
60	62.8	194.9	53.9	74.5	19.0	15.3	17.3	22.5	10.2	

Table A.S5.1: Accession numbers for the 16S rRNA gene sequences used for the construction of phylogenetic trees and designing specific primers targeting SAGMGC-1.

Member's accession number	**Ambient**	**Nº sequences**	**Reference**
JN227488	stream *Nitrosotalea devanaterra*	1	15
HM745411	Acid mine effluent	1	10
FJ584409, FJ584402, FJ584381, FJ584389	Acidic red soil	4	11
JF917253, JF917266, JF917258, JF917255, JF917264, JF917262, JF917244, JF917251, JF917263	Acidic soil	9	14
HQ671235, HQ671234, HQ671233	Amazon soil	3	13
DQ190071, DQ190067	Aquifer	2	9
GQ302597, GQ302604	Cold spring	2	8
EF032790	cyanobacterial mat	1	28
DQ837284	Donana coastal aquifer	1	19
AY186060, AY186061	Ferromanganese deposit	2	4
AB050227, AB050229 AB050240	Gold mines	3	26
EF444676	Hot springs	1	23
EU370091	Oyser shell	1	20
FJ174729, FJ174736, FJ174728, FJ174735, FJ174732, FJ174731	Red soil	6	18
EF020413, EF020446, EF020370, EF020399, EF020420, EF020435, EF020862, EF021125, EF021182, EF021965, EF021167	Rhizosphere	11	7
AB550811, JN397659	River	2	6, 17
GU135485, HM187557, HM187528, HM187541, HM187544, HM187547, HM187548	Sediment	7	5; 16
AB262708, JF799613, EU306967, EU307071, EU307080, EU307057	Soil	6	21, 26, 27
AB294260, AB294259, AB294262, AB294261	Stream	4	12
DQ337056, DQ223191, DQ223190, DQ223192, DQ223194	Subsurface water	5	22
AJ535128	Uranium mill tailings	1	24
FJ936634, FJ936687	Volcano mud	2	25

Table A.S4.3: Continued

FN691484, FN691499, FN691526, FN691554, FN691580, FN691667, FN691667, FN691709, FN691728, HE589623, HE589623, HE589628, HE589631, HE589632, HE589633, HE589633, HE589634, HE589644, HE589652, HE589653, HE589658, HE589663, HE589669, HE589670, HE589670, HE589670, HE589676, HE589676, HE589677, HE589677, HE589680, HE589680, HE589684, HE589686, HE589688, HE589692, HE589693, HE589700, HE589707, HE589713, HE589721, HE589722, HE589735, HE589747, HE589751, HE589756, HE589757, HE795996, HE795998, HE796011, HE796018, HE796020, HE796053, HE796054, HE796055, HE796074, HE796156, HE796159, HE796165, HE796176, HE796269, HE796271, HE796292, HE796316, HE796320, HE796324, HE796338, HE796369, HE796382, HE796386, HE796394, HE796401, HE796566, HE796575, HE796581, HE796620, HE796624, HE796625, HE796632, HE796636, HE796639, HE796650, HE796658, HF951771, HF951775, HF951795, HF951796, HF951799, HF951800, HF951815, HF951816, HF951823, HF951824, HF951825, HF951827, HF951834, HF951836, HF951841, HF951843, HF951844, HF951845, HF951847, HF951852, HF951853, HF951857, HF951858, HF951859, HF951861, HF951874, HF951875, HF951878, HF951879, HF951881, HF951888, HF951890, HF951893, HF951896, HF951899, HF951904, HF951906, HF951908, HF951913, HF951914, HF951917, HF951919, HF951920, HF951921, HF951926, HF951927, HF951938, HF951940, HF951941, HF951943, HF951946, HF951947, HF951948, HF951951, HF951952, HF951953, HF951957, HF951959, HF951961, HF951964, HF951965, HF951969	Freshwater	182*	1, 2, 3, 23

*35 sequences have not yet been published in the NCBI database

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Appendix B

CARD-FISH optimized protocol

CARD-FISH protocol for samples on membrane filters using SAG1_357 probe.

a. *Embedding*

1. Dip filters in 0.1% low-gelling-point agarose at 35 to 40 °C.
2. Place filters face down onto parafilm, let them dry at 35 to 40°C.
3. Remove filters by soaking them in 96% ethanol (RT).
4. Let air dry on a piece of tissue paper

b. *Inactivation of endogenous peroxidases*

1. Incubate in 0.01 M HCl for 10 min. at RT.
2. Wash filters quickly in 1X PBS
3. Wash thoroughly with ddH2O
4. Let the filter air dry

c. *Permeabilization*

1. Incubate filter in fresh lysozyme solution for 60 min. at 37°C.

Stock Reagent	Final concentration
0,5M EDTA (pH 8,0)	0,05M
1M tris-HCl (pH 7,4)	0,1M
Lysozyme	10mg/ml
ddH2O	

2. Wash 3 times in ddH2O
3. Wash in 96% ethanol.
4. Let air dry
5. Incubate filter fresh achromopeptidase solution for 30 min. at 37°C.

Stock Reagent	Final concentration
5M NaCl	0,01M
0,1M tris-HCl (pH 8)	0,01M
Achromopeptidase	60U/ml
ddH2O	

6. Wash 3 times in ddH2O
7. Wash in 96% ethanol.
8. Let air dry

Note: for repetitions and trials with different probes on a single sample, cut into triangular pieces filter (maximum 12 is recommended, otherwise the filter sample will be very small).

d. *Hybridization in humidity chamber*

1. Prepare a humidity chamber by inserting a tissue in a tupperware and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer.
2. Mix (100:1:1:1 [vol/vol]) hybridization buffer, probe solution (50ng/µl), competitor solution (50ng/µl) and helpers solution (50ng/µl) at a suitable quantity to dip filters after adjusting the piston.

Hybridization buffer (final volumen 20ml)

Stock Reagent	Volume	Final concentration
5M NaCl	3,6ml	0,9M
1M tris-HCl (pH 7,4)	0,4ml	20mM
20% SDS[1]	20µl	0,02%
Blocking reagent	2ml	1%
Formamide	9ml	45%
Dextran sulfate	2g	10%
ddH2O	5ml	

Note: It is very important to note that the addition of SDS has to be after all other reagents, to prevent precipitates.

Blocking reagent (final volumen 20ml)

Stock Reagent	Volume	Final concentration
Maleic acid	2,32g	100mM
NaCl	1,75g	150mM
ddH2O	16ml	
NaOH	to pH 7,5	
ddH2O	4ml	
Blocking reagent	2g	10%

3. Put a drop (about 50-100 µl enough to cover the piece of filter) of the hybridization solution on each piece of filter and place it in petri dish and turn it into the humidity chamber (Tupperware). Close tupper firmly and keep in a horizontal position.
4. Incubate filters at 35°C for 4 h on a rotation shaker.

e. *Washing*

1. Wash filters in prewarmed washing buffer for 10 min at 37°C.

Washing buffer (final volumen 50ml)

Stock Reagent	Volume	Final concentration
5M NaCl	160µl	
0,5M EDTA (pH 8,0)	0.5ml	5mM
1M tris-HCl (pH 7,4)	1ml	20mM
20% SDS	25µl	0,02%
ddH2O	to 50ml	

2. Transfer filters to 1 x PBS (do not let filters run dry) and incubate for 15 min at RT.
3. To remove excess liquid, dab filter on blotting paper, but do not let filters dry.

f. *Amplification (CARD)*

1. Prepare a moisture chamber by inserting a piece of tissue paper in a 50 ml tube and soak it with 2 ml water.
2. Prepare a fresh solution of H2O2 (0.15% in PBS), keep it cool.

Stock Reagent	Volume (µl)
1X PBS	995
H2O2 30%	5

3. Mix amplification buffer with H2O2 solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient) and add 3µL fluorescently labeled tyramide [1 mg dye ml-1] and mix well, keep in the dark.

Amplification buffer (final volumen 40ml)

Stock Reagent	Volume	Final concentration
20X PBS	2ml	
Blocking reagent	400µl	0,1%
5M NaCl	16ml	2M
Dextran sulfate	4g	10%
ddH2O	To 40ml	

4. Dip filters completely in the amplification mix
5. Incubate in substrate mix for 15 min at 42°C in the dark.
6. To remove excess liquid, dab filter on blotting paper and incubate in 1 x PBS for 15 min at RT in the dark.
7. Wash filters in excess ddH2O (1 min) and thoroughly twice in 96% ethanol (1 min).
8. Let air dry.

Note: Filter the amplification buffer before adding the tyramide to mix.

g. *Mounting*

1. Counterstain and mounted filters by soaking them with DAPI/Citifluor and Vecta Shield (4:1 [vol/vol] mix) solution: 2µl DAPI (1000x), 750µl citifluor and 250µl VectaShied.

Note: The filter piece have to be completely dry before embedding, otherwise part of the cells might detach during inspection.

Supporting information

For others formamide and NaCl concentrations, volumes are indicated in the following tables (Pernthaler et al. 2004):

Table 1: Volumes of formamide and water for 20 ml of hybridization buffer

% formamide in hybridization buffer	ml formamide	ml water
0	0	14
5	1	13
10	2	12
15	3	11
20	4	10
25	5	9
30	6	8
35	7	7
40	8	6
45	9	5
50	10	4
55	11	3
60	12	2
65	13	1
70	14	0

Table 2: NaCl concentration in the washing buffer for washing at 48°C after hybridization at 46°C

% formamide in hybridization buffer	[NaCl] in M (final concentration)	µl 5 M NaCl in 50 ml
0	0.900	9000
5	0.636	6300
10	0.450	4500
15	0.318	3180
20	0.225	2150
25	0.159	1490
30	0.112	1020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
55	0.020	100
60	0.014	40
65	-	-
70	-	-

Table 3: NaCl concentration in the washing buffer for washing at 35°C after hybridization at 35°

% formamide in hybridization buffer	[NaCl] in M final concentration	µl 5 M NaCl in 50 ml
20	0.145	1350
25	0.105	950
30	0.074	640
35	0.052	420
40	0.037	270
45	0.026	160
50	0.019	90
55	0.013	30
60	0.009	0
65	0,008	0
70	0,005	0

Appendix C

Original publications

environmental microbiology

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Targeting spatiotemporal dynamics of planktonic SAGMGC-1 and segregation of ammonia-oxidizing thaumarchaeota ecotypes by newly designed primers and quantitative polymerase chain reaction

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Summary

The annual dynamics of three different ammonia-oxidizing archaea (AOA) ecotypes (*amo*A gene) and of the SAGMGC-1 (*Nitrosotalea*-like aquatic Thaumarchaeota) group (16S rRNA gene) were studied by newly designed specific primers and quantitative polymerase chain reaction analysis in a deep oligotrophic high mountain lake (Lake Redon, Limnological Observatory of the Pyrenees, Spain). We observed segregated distributions of the main AOA populations, peaking separately in time and space, and under different ammonia concentrations and irradiance conditions. Strong positive correlation in gene abundances was found along the annual survey between 16S rRNA SAGMAGC-1 and one of the *amo*A ecotypes suggesting the potential for ammonia oxidation in the freshwater SAGMAGC-1 clade. We also observed dominance of *Nitrosotalea*-like ecotypes over *Nitrosopumilus*-like (Marine Group 1.1a) and not the same annual dynamics for the two thaumarchaeotal clades. The fine scale segregation in space and time of the different AOA ecotypes indicated the presence of phylogenetically close but ecologically segregated AOA species specifically adapted to specific environmental conditions. It remains to be elucidated what would be such environmental drivers.

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Introduction

Thaumarchaeota are ubiquitous microorganisms in marine and freshwaters, soils, sediments and biofilms (see a recent metadata analysis by Fernàndez-Guerra and Casamayor, 2012), and represent a major reservoir of prokaryotic biomass (Prosser and Nicol, 2008). Initially classified as 'mesophilic Crenarchaeota', comparative genomics showed that they form a separate and deep-branching phylum within the Archaea (Brochier-Armanet *et al.*, 2008; Stahl and de la Torre, 2012). Thaumarchaeota encompasses all previously known putative ammonia-oxidizing archaea (AOA), and other archaea with unknown energy metabolisms potentially linked to the nitrogen cycle (Pester *et al.*, 2011) and to other biogeochemical cycles (see a recent review by Hatzenpichler, 2012). In fact, the widespread distribution of putative archaeal ammonia monooxygenase (*amo*A) genes in marine, terrestrial and freshwater environments (Francis *et al.*, 2005; Zhang *et al.*, 2008; Auguet *et al.*, 2011) strongly suggests that AOA play a major role in global nitrification.

The SAGMGC-1 group, initially found in gold mines in Africa (Takai *et al.*, 2001), forms a monophyletic cluster within Thaumarchaeota closely related to the Marine Group (MG) 1.1a (Prosser and Nicol, 2008; Pester *et al.*, 2011). The recent discovery and cultivation of the chemolithotrophic obligate acidophilic thaumarchaeal ammonia-oxidizing species *Nitrosotalea devanaterra* (Lehtovirta-Morley *et al.*, 2011) belonging to the SAGMGC-1 group has enlarged the role assigned to thaumarchaeota in the nitrogen cycling. The SAGMGC-1 and *Nitrosotalea* clusters are especially interesting because only very recently, it has been recognized by its ecological importance, particularly in acidic soils (Gubry-Rangin *et al.*, 2011; Pester *et al.*, 2011) and oligotrophic freshwaters (Auguet and Casamayor, 2008; 2013). The relative abundance, recurrent appearance and significant correlations with nitrogen species suggest a key role of SAGMGC-1 in the N biogeochemical cycle of oligotrophic alpine lakes (Auguet *et al.*, 2011; 2012).

In the present study, we designed and tested specific primers for quantifying by quantitative polymerase chain reaction (qPCR) the SAGMGC-1 16S rRNA gene and different AOA ecotypes (*amo*A gene) spatiotemporal

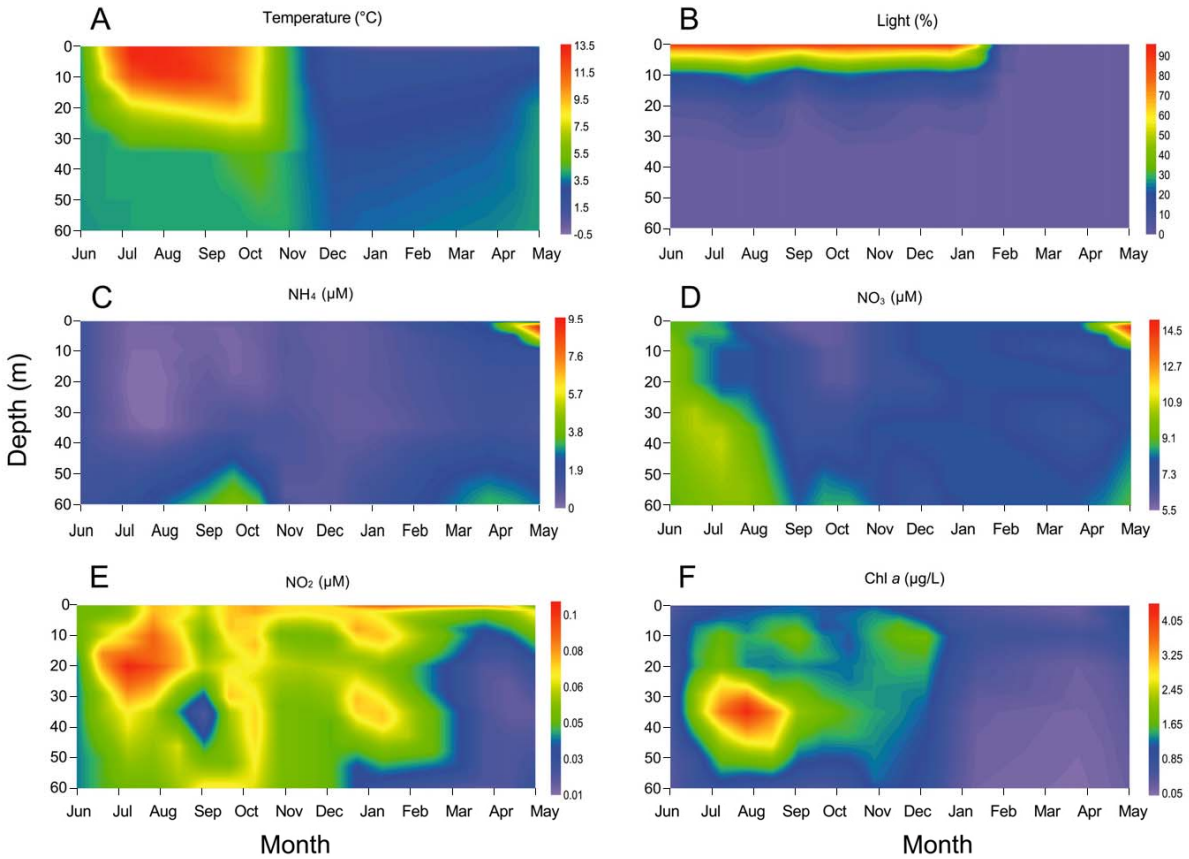

Fig. 1. Annual variation in (A) water temperature, (B) irradiance (% of incident light), and concentrations of (C) ammonia, (D) nitrate, (E) nitrite and (F) chlorophyll-*a* along the vertical gradient in the deep alpine Lake Redon. The lake was covered by ice and snow from January to May–June.

distributions along an annual survey in the deep high-altitude Lake Redon, where atmospheric depositions are the main source of reactive nitrogen (Catalan *et al.*, 2006). Vertical positioning in the water column has been unveiled as a key factor to understand the ecology of different thaumarchaeotal clades in aquatic environments (Francis *et al.*, 2005; Hallam *et al.*, 2006; Mincer *et al.*, 2007; Beman *et al.*, 2008; Llirós *et al.*, 2010; Santoro *et al.*, 2010; Auguet *et al.*, 2012). We specifically quantified three freshwater thaumarchaeota populations in Lake Redon showing (i) the dominance of the *Nitrosotalea*-like (SAGMGC-1) populations over *Nitrosopumilus*-like (MG 1.1a), (ii) the differential dynamics of these two clades along the annual study, and (iii) the fine-scale distribution in space and time of different AOA ecotypes.

Results

Figure 1 shows seasonal and vertical changes in water temperature (A), percentage of incident light (B), nitrogen compounds (C, D and E) and chlorophyll-*a* (F) in the deep glacial stratified Lake Redon along 1 year. The lake has a dimictic regime, with mixing periods in late spring and autumn, and it is usually ice- and snow-covered for about 6 months of the year, being the underlying water column mostly in the dark for several months in winter and spring. The thickness of the snow cover usually reaches several meters in April. During the ice-free season, light penetration into the water column was very high because of the high transparency due to low dissolved organic carbon (10–100 µM, Auguet *et al.*, 2012) and low algae growth (i.e. low Chl *a* concentrations). Along the melting period and as atmospheric nitrogen deposition (i.e. snow and rain) is the main source of reactive nitrogen in alpine areas (c. 30-fold higher than mean values measured in lake water, Auguet *et al.*, 2011; Camarero and Catalan, 2012), maximal concentrations of released nitrate (c. 15 µM) and ammonium (c. 10 µM) were observed in surface waters. In addition, snow thawing in the catchment maintains a large water flow from the catchment into

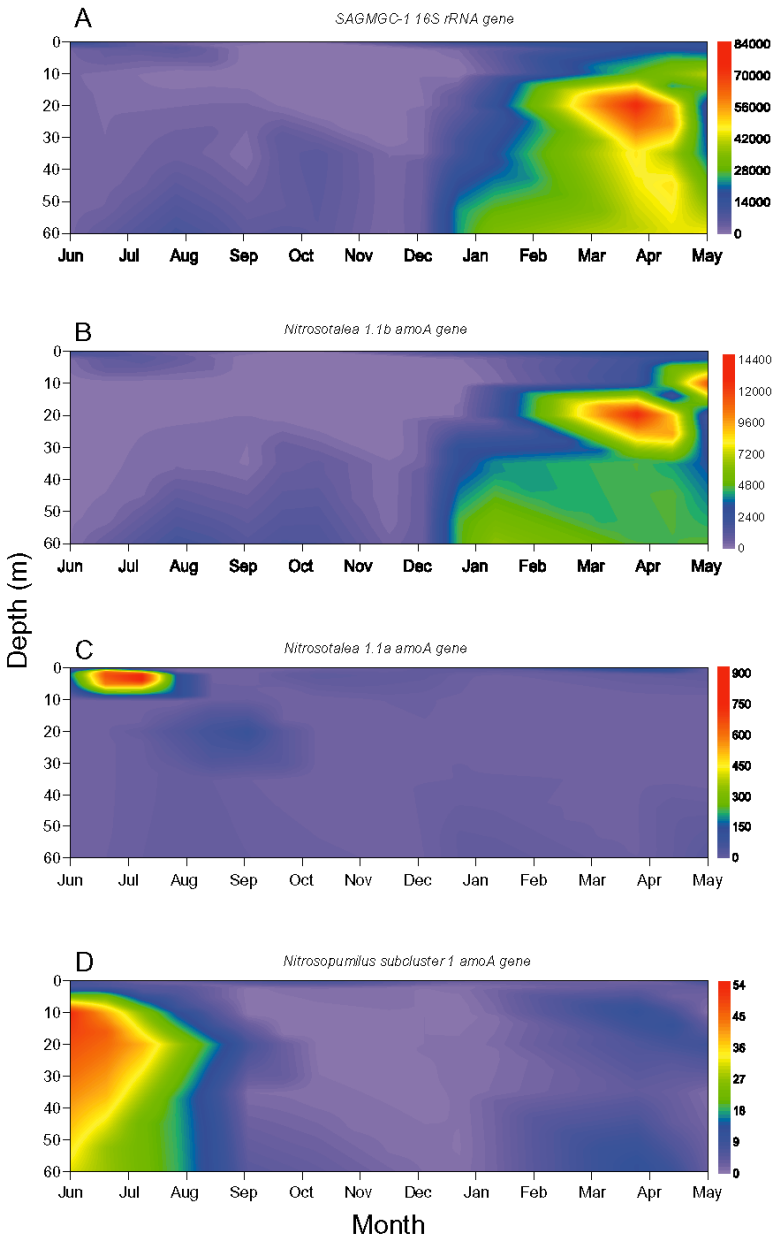

Fig. 2. Annual spatiotemporal variation in the concentrations of specific AOA genes (gene copies/ml lake water) determined by qPCR for (A) SAGMGC 16S rRNA, (B) *Nitrosotalea* 1.1b *amo*A, (C) *Nitrosotalea* 1.1a *amo*A, and (D) *Nitrosopumilus amo*A, along the vertical gradient in the deep alpine Lake Redon.

the lake. Additional minor peaks in nitrate and ammonia were observed in bottom waters in September to October and in April to May most probably because of microbial mineralization and nitrification in the sediment. Interestingly, higher nitrate concentrations were also observed along the water column in early summer, and in general, higher nitrite concentrations were observed in surface than in deep waters along the year.

The newly designed primers were used to follow the vertical and temporal changes of SAGMGC-1 and AOA ecotypes along the annual survey in Lake Redon. Amplification signal was obtained from all depths and dates examined. The SAGMGC-1 16S rRNA gene showed the largest variation in gene abundance spanning five orders of magnitude from <5 copies/ml lake water up to 8×10^4. Interestingly, we observed a bloom of this population during the dark period at c. 20 m depth, starting in January to February in deeper waters and reaching the highest concentration in early spring (Fig. 2A). Thus, SAGMGC-1 was more abundant during the winter strati-

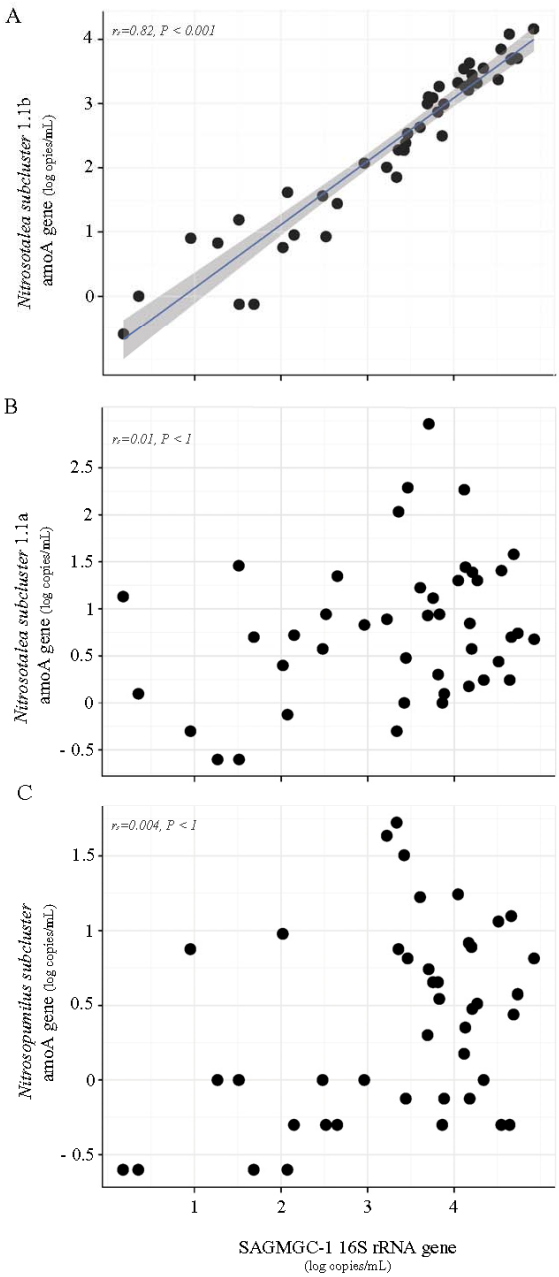

Fig. 3. Relationships between abundances of SAGMGC-1 16S rRNA gene and the *amo*A gene of (A) *Nitrosotalea* subcluster 1.1b (PyrSF1OTU2), (B) *Nitrosotalea* subcluster 1.1a (PyrSF1OTU1) and (C) *Nitrosopumilus* subcluster 1 (PyrF5OTU3). Regression coefficients and associated *P* values are shown.

fication period when Lake Redon was in the dark covered by ice and snow, and gene concentrations substantially decreased after the snowmelt period and during the summer stratification.

The analysis of the annual distribution pattern of abundance also unveiled segregated maxima for the different *amo*A populations separated in time and space (Figs. 2 B, C and D). The *Nitrosotalea* 1.1b ecotype (SF1OTU2 in former works, Auguet and Casamayor, 2013) was the most abundant and showed gene concentrations spanning four orders of magnitude ranging from <5 copies/ml lake water up to 1×10^4. A bloom of this population was found matching the bloom and annual dynamics of SAGMGC-1 (Fig. 2B). This fact strongly indicates that both SAGMGC-1 16S rRNA and Ntalea (*Nitrosotalea*-target) 1.1b primer sets targeted the same population. In fact, a strong correlation ($r_s = 0.82$, $P < 0.001$, $n = 44$) was found between gene abundances of both populations after combining the whole data set (Fig. 3A). Gene abundances of both SAGMGC-1 16S rRNA and *Nitrosotalea* 1.1b ecotype were positive and significantly correlated with nitrate ($r_s = 0.40$ and 0.40, $P < 0.01$, $n = 44$) and ammonium ($r_s = 0.70$ and 0.69, $P < 0.01$, $n = 44$), and negative and significantly correlated with nitrite ($r_s = -0.46$ and -0.42, $P < 0.01$, $n = 44$), chlorophyll-*a* ($r_s = -0.70$ and -0.69, $P < 0.01$, $n = 44$), light ($r_s = -0.63$ and -0.60, $P < 0.01$, $n = 44$) and water temperature ($r_s = -0.35$, $P < 0.05$, $n = 44$). However, only nitrite and chlorophyll-*a* concentration significantly explained the variability in gene abundance after multiple linear regression analysis ($P < 0.001$ and $P < 0.05$ respectively).

Conversely to the *Nitrosotalea* 1.1b ecotype that bloomed during the dark period, the other two *amo*A ecotypes were substantially less abundant and showed the highest abundances immediately after the melting period but again with a marked spatial and temporal segregation. The *Nitrosotalea* 1.1a population (SF1OTU1 in former works, Auguet and Casamayor, 2013) bloomed in surface waters (i.e. above 10 m) in July and showed abundances in the lake spanning three orders of magnitude ranging from <5 copies/ml up to 9×10^2 (Fig. 2C). No significant relationship was found with the distribution of SAGMGC-1 ($r_s = 0.01$, $P < 1$, $n = 44$) (Fig. 3B), and the ratio SAGMGC-1/Ntalea 1.1a was >100. Finally, the *amo*A gene abundance of *Nitrosopumilus* subcluster 1 population (F5OTU3 in former works, Auguet and Casamayor, 2013) peaked in subsurface waters (i.e. 10 m and below) in June and showed abundances in the lake spanning two orders of magnitude ranging from <5 copies/ml up to 50 (Fig. 2D). As expected, no significant relationship was found with the distribution of SAGMGC-1 ($r_s = 0.004$, $P < 1$, $n = 44$) (Fig. 3C). Any of the environmental variables significantly explained the variability ($P < 0.05$) in the *amo*A gene abundance of these two minor populations after multiple linear regression analysis. Gene abundances of *Nitrosotalea* 1.1a and *Nitrosopumilus* were correlated (Spearman rank) positive and significantly only with nitrate ($r_s = 0.30$ and 0.44, respectively, $P < 0.01$, $n = 44$).

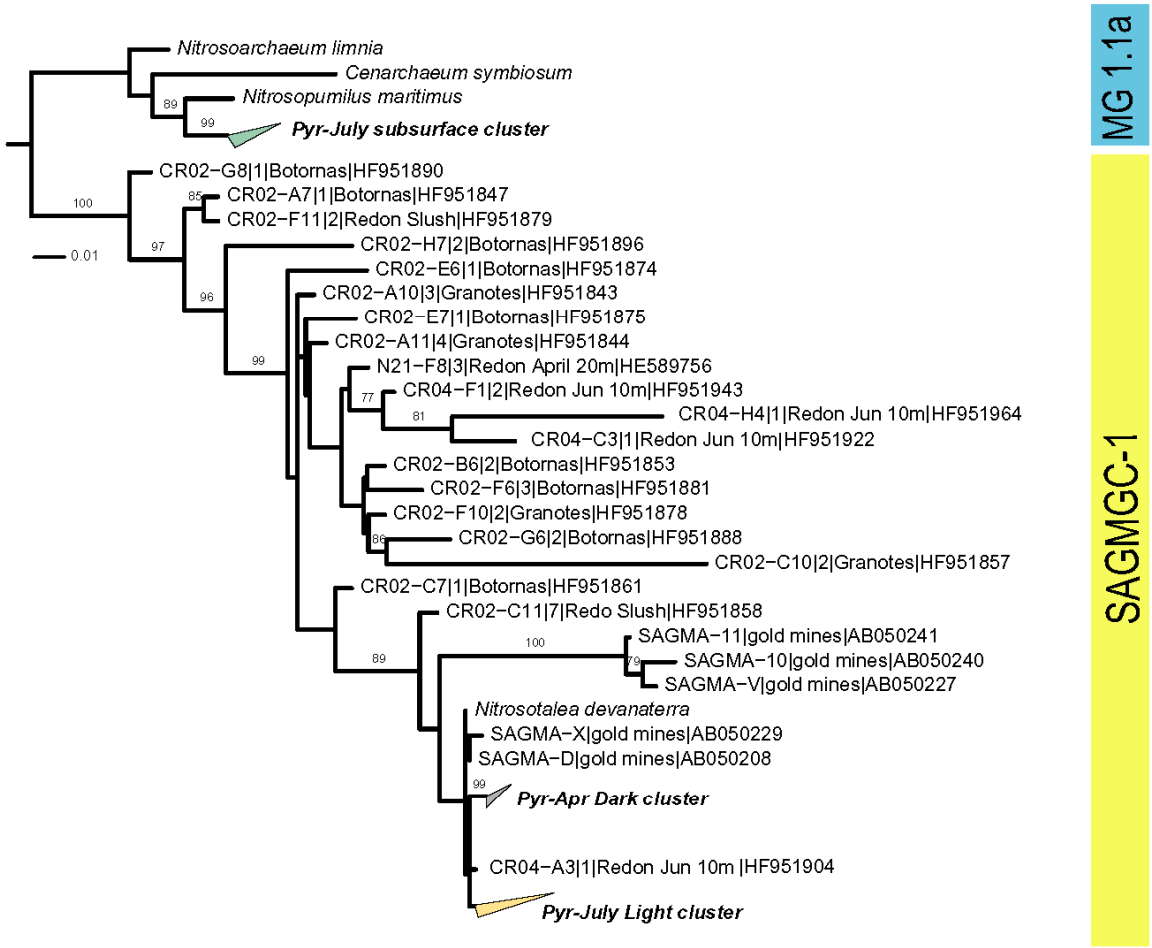

Fig. 4. Maximum-likelihood phylogenetic tree of the Thaumarchaeota 16S rRNA gene sequences from the SAGMAGC-1 and MG 1.1a groups. The three AOA ecotypes are highlighted (see text for details). See expanded tree in Fig. S1 and more information in Table S1. Scale bar, 10% estimated divergence.

Finally, we explored the 16S RNA gene of depths July 2 m and June 10 m with universal archaeal primers (21f-958r), and >60 clone sequences were analysed. These results were compared with the 16S rRNA gene sequences obtained from April 20 m (dark period) in a previous work (Auguet *et al.*, 2012) with accession numbers in GenBank HE589747 and HE589751 for representative Operational Taxonomic Units (OTUs) (Fig. S1 and Table S1). All the 16S rRNA gene sequences from July 2 m were SAGMGC-1 located within a subgroup that we tentatively named *Pyr-July light* (see Fig. 4, and expanded tree in Fig. S1). In June 10 m, 84% of the 16S rRNA sequences matched the SAGMGC-1 *Pyr-July light* cluster, and 16% were *Nitrosopumilus*-like sequences (*Pyr-July subsurface,* Figs 4 and S1). Interestingly, this *Pyr-July light* cluster was separated from the 16S rRNA gene sequences obtained from April 20 m and labelled as *Pyr-Apr dark* cluster in Fig. 4 (90% identity to *Nitrosotalea devanaterra*). The ecologically segregated SAGMGC-1 ecotypes were therefore also phylogenetically separated.

Discussion

The environmental heterogeneity of Pyrenean Lakes in particular, and probably mountain lakes in general, is a very convenient natural framework to investigate the ecology and distribution of idiosyncratic AOA and to explore the links with the nitrogen cycle. Previous studies on the seasonal changes in ribosomal (16S rRNA) and functional (*amo*A) thaumarchaeotal genes showed dynamic changes in the diversity and abundance of AOA assemblages inhabiting oligotrophic mountain lakes (Auguet *et al.*, 2011; 2012; Vissers *et al.*, 2013). In addition, both correlation of AOA dynamics with changes in

nitrogen species and undetected bacterial *amo*A gene counterparts suggested a key role of Archaea in the N biogeochemical cycling in alpine lakes (Auguet *et al.*, 2011; 2012; Auguet and Casamayor, 2013).

In previous studies based on 16S rRNA gene cloning, sequencing, and analysis of relative gene abundances, we had shown the coexistence of thaumarchaeotal MG 1.1a and SAGMGC-1 clades in the Pyrenean lacustrine district but apparently segregated both vertically by distinctive positioning in the water column (Auguet *et al.*, 2012) and spatially among lakes by differential response to environmental drivers (Auguet and Casamayor, 2013). All these results confirmed the potential ecological importance of the SAGMGC-1 cluster in oligotrophic waters. While members of the MG 1.1a have been largely implicated in nitrification (Konneke *et al.*, 2005; Hallam *et al.*, 2006; Blainey *et al.*, 2011), for the SAGMGC-1 clade only a very recent study indicates that a cultured ammonia-oxidizing strain from an acid soil, *Nitrosotalea devanaterra*, contained the *amo*A gene (Lehtovirta-Morley *et al.*, 2011). In the present investigation, the correlational approach carried out using quantitative data strongly supports the potential for ammonia oxidation within the freshwater counterparts of the SAGMGC-1 cluster. However, we observed substantial differences in the ratio between the abundance of SAGMGC-1 16S rRNA gene and the *amo*A gene of the different *Nitrosotalea*-like ecotypes, being the 16S rRNA more abundant than the *amo*A gene detected in all the cases. The primer for SAGMGC-1 did not show preferential amplification for any of the two 16S rRNA clusters against the other ('light' vs. 'dark' clusters, Fig. 4). Thus, apparently only a few of the SAGMGC had also Ntalea 1.1a *amo*A. Other explanations for the observed gene ratio SAGMGC/Ntalea rely on the number of gene copies per genome in each one of these populations, or that not all the SAGMCG had amoA, but these questions cannot be tested without cultures from the different ecotypes. Finally, additional ecotypes not covered by the amoA primer sets used here may also be present in this environment, and even different PCR efficiencies cannot be ruled out. Nevertheless, our results showed a completely different spatiotemporal distribution for the different freshwater AOA unveiling the existence of phylogenetically close but ecologically distinct AOA species.

At present, the ecological factors shaping the differential distribution of AOA ecotypes are, however, difficult to be properly established. Again, cultures from the different ecotypes for ecophysiological analyses in the laboratory would be desirable. However, two environmental factors deserve to be explored in more detail in future studies. The first factor is ammonia concentration because changes in ammonia affinity may potentially explain AOA species distribution. In fact, the distribution of two distinct ecotypes of marine thaumarchaeota Group I (MGI) detected in the tropical Atlantic and the coastal Arctic (Sintes *et al.*, 2013) nicely matched medium (1–2 μM on average in the coastal Arctic) and low ammonia concentrations (<0.01 μM in the deep Atlantic waters). In several Pyrenean mountain lakes explored, ammonia concentrations ranged between 0.3 and 2.3 μM (Auguet and Casamayor, 2013), and concentrations in Lake Redon reached up to 4 μM both in bottom waters and close to the ice cover in winter (Auguet *et al.*, 2012). Thus, these mountain lakes are closer to the 'medium' ammonia concentrations range than to the 'low' range. However, ammonia concentrations during the blooms of the different AOA ecotypes in Lake Redon varied close to one order of magnitude from 0.2 μM in surface waters in June to July (where *Nitrosotalea* 1.1a and *Nitrosopumilus* bloomed) to 1.5 μM at 20 m in April (where *Nitrosotalea* 1.1b bloomed), and the influence of the in situ changes in ammonia concentration on thaumarchaeota populations dynamics in Lake Redon cannot be completely ruled out. Interestingly, the distribution of relatively high nitrite concentrations close to the air-water interface (0.05-0.1 μM) may also be related to both the high ammonia concentrations provided by atmospheric deposition (Camarero and Catalan, 2012) and the presence of active AOA cells, but this hypothesis certainly remains to be tested.

The second factor relies on differential photoinhibition because inhibition by light potentially influences the distribution of ammonia oxidizers in aquatic environments, and photoinhibition in AOA strains has been recently shown in laboratory cultures of *Nitrosopumilus maritimus* and *Nitrosotalea devanaterra* (Merbt *et al.*, 2012). Thus, the ecological effect of light intensity and/or quality is of particular interest because susceptibility to light applies to both nitrifying bacteria (Horrigan *et al.*, 1981; Olson, 1981; Guerrero and Jones, 1996a,b) and Archaea (Mincer *et al.*, 2007; Merbt *et al.*, 2012). Alpine lakes, especially those located at higher altitudes are exposed to high solar radiation because of the natural increase of irradiance with elevation (Catalan *et al.*, 2006). Furthermore, alpine lakes are among the most UV-transparent aquatic ecosystems (Sommaruga and Augustin, 2006). Conversely, during the long ice cover period, the water column remains under an attenuated light regimen or in the dark. Therefore, differential spatial and temporal AOA species distribution might be expected along the annual cycle in Lake Redon. In addition, it has been shown that nitrite oxidizers (NOB) are more sensitive to light than ammonia-oxidizing bacteria (AOB), whereas AOB may recover more rapidly from photoinhibition than NOB (Guerrero and Jones, 1996b). AOB also became more photoresistant in the presence of higher ammonia concentrations, while NOB did not significantly change their light sensitivity after increasing nitrite concentration

(Guerrero and Jones, 1996b). If these results also apply to alpine AOA, the distribution of relatively high nitrite concentrations close to the air-water interface could be related to a differential sensitivity to light that deserves further investigations.

Overall, these results suggest a hypothetical active recycling of atmospheric reactive nitrogen by different AOA ecotypes that deserve further investigations for accurate testing of the hypothesis mentioned earlier. The distribution pattern of two of the three AOA ecotypes observed was consistent with the recently reported photoinhibition of two AOA strains under controlled conditions in the laboratory. However, a third AOA ecotype showed an accumulation peak close to the top of the lake in early summer, intriguingly suggesting an adaptation to the high solar irradiance and UV doses accounting in surface waters of Lake Redon. Thus, certainly, there is not a single parameter controlling AOA abundance and distribution in deep alpine lakes but rather a set of environmental variables intimately related to the limnology and the seasonal lake dynamics.

Material and methods

Study site and sample collection

The annual survey was carried out in Lake Redon, an alpine deep lake located in the central Spanish Pyrenees (42°38′34″N, 0°46′13″E, altitude 2240 m, maximum depth 73 m, surface 0.24 km2). The lake is oligotrophic with poor vegetation and soil development in the small catchment area (c. 1.5 km^2). It is located on the head of the valley, and atmospheric deposition is the main source of reactive nitrogen (Catalan *et al.*, 1994; 2006). Samples were monthly collected between 2007 and 2008 from six depths (surface, 2, 10, 20, 35 and 60 m) on the deepest point of the lake using a 2 L Niskin bottle. These depths covered different limnological characteristics of the lake (Auguet *et al.*, 2012; Camarero and Catalan, 2012). Changes in temperature, oxygen, pH, nutrients and chlorophyll *a* were used to follow the seasonal variability of physical, chemical and biological properties of the lake and were measured as recently reported (Auguet *et al.*, 2012, where also additional field data can be found). The light extinction coefficient was calculated after Secchi disk depth data (Armengol *et al.*, 2003). Samples from surface waters of additional lakes within the Limnological Observatory (Auguet and Casamayor, 2013) were used to test the primer sets performance and specificity. Samples were processed, and DNA was extracted and purified as reported (Demergasso *et al.*, 2008; Hervàs *et al.*, 2009). A previous study with the same data set had shown AOB below detection limits in the plankton of Lake Redon (Auguet *et al.*, 2012).

Specific primers and PCR conditions

Four sets of specific primers were designed and tested according to previously reported 16S rRNA and *amo*A thaumarchaeotal genes sequences available in GenBank from inland aquatic systems and soils (Table S1). Multiple-sequence alignment was carried out with the software MAFFT (Katoh *et al.*, 2002), and consensus regions were identified as target-specific sites. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) with the following settings for optimal amplification in qPCR: (i) amplified PCR fragment <400 bp, (ii) primers of at least 20 bp for better specific amplification and (iii) maximum one degeneracy per primer.

One of the primer sets (SAGMGC1 274F-446R, Table 1) specifically targeted the 16S rRNA gene of the SAGMGC-1 (*Nitrosotalea*-like) Thaumarchaeota group, a sister clade of the Marine Group (MG) 1.1a. In silico, the forward primer matched >75% of the available SAGMGC sequences and showed a few unspecificities with the MG 1.1a. The reverse primer matched >88% of the SAGMGC sequences and a few matches with 1.1b and 1.1c thaumarchaeal sequences and crenarchaeal group sequences. Combined, these primers had only the SAGMGC-1 cluster as potential target. The primer set was experimentally tested using both environmental clones previously available from the Limnological Observatory of the Pyrenees (LOOP) containing nearly full 16S rRNA fragments of the SAGMGC group and natural samples from Lake Botornas, Lake Granotes and Lake Redon collected in previous studies (Triadó-Margarit and Casamayor, 2012; Auguet and Casamayor, 2013). One ~ 192 bp length DNA fragment was observed in all the cases, as expected (Fig. S2). The PCR product from the three lakes was cloned in TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), as previously reported (Ferrera *et al.*, 2004), following PCR conditions reported in Table 2, and 96 clones were sequenced using external facilities (http://www.macrogen.com). Phylogenetic analysis showed that all the sequences were placed within the SAGMGC-1 cluster. Additional cloning and sequencing with universal primers 21f-958r for the archaeal 16S rRNA gene was carried out when needed, following Auguet and colleagues (2011).

The three additional primers sets targeted the *amo*A gene of the three most abundant AOA populations previously found in the LOOP (OTU 1, 2 and 3, see Fig. 5). The *amo*A gene sequences were highly conserved within each OTU allowing us to design the specific primers without the need of degenerated bases. The *amo*A OTU 1 was closely related to the *Nitrosotalea* 1.1 subcluster (Pester *et al.*, 2012), it matched 7% of total *amo*A gene sequences found in the LOOP, and it was covered by the specific primer set Ntalea1.1a 135F-545R (Table 1). The *amo*A OTU 2 was also closely related to the *Nitrosotalea*

Table 1. Characteristics and specific targets of the newly designed primer sets developed in this study for quantitative PCR.

Primer name	Primer sequence (5′- 3′)	Target	Gene	Position	Expected length (bp)	%GC	Tm
SAGMGC1-274F	AGGAGAAGCCCGGAGATGGGT	SAGMGC-1	16S rRNA	274–294	192	61.91	58.73
SAGMGC1-446R	ATTAYCGCGGCGGCTGACAC	(Thaumarchaeota)	16S rRNA	446–465		60.0–65.0	57.87–60.11
Ntalea 1.1a-135F	GCTCGCAGTCGGTGCAGCATA	*Nitrosotalea* subcluster 1.1	*amo*A	135–155	411	61.91–66.67	59.85–62.11
Ntalea 1.1a-545R	GCACTAGCGCCTGCACCCAAA	(SF1OTU1)	*amo*A	545–525		57.14–61.91	57.66–60.25
Ntalea 1.1b-133F	ATGCTTTCAGTCGGTGCCGC	*Nitrosotalea* subcluster 1.1	*amo*A	133–152	400	60.0–65.0	58.27–60.93
Ntalea 1.1b-538R	CACCCGCACCTAGCGCGAC	(SF1OTU2)	*amo*A	538–520		68.42–73.68	57.71–60.46
Npumilus-420F	GCGGACCCACTAGAAACGGCA	*Nitrosopumilus* cluster	*amo*A	420–440	123	57.14–61.91	56.79–59.12
Npumilus-542R	ACCTGCACCTGCACCCAGTG	(F5OTU3)	*amo*A	542–523		65.0–70.0	59.19–61.99

The *amo*A clusters nomenclature follows Pester and colleagues (2012).

Table 2. PCR amplification conditions for the newly designed primer sets developed in this study.

				Cycling conditions					
				Denaturation		Annealing		Extension	
Primer pairs	Use	Template (ng/μL)	No. of cycles	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)
SAGMGC1-274F/SAGMGC1-446R	Cloning	25	35	94	30	58	45	72	90
	qPCR	1	45	98	5	58	20	72	15
Ntalea 1.1a-135F/Ntalea 1.1a-545R	qPCR	1	45	98	5	63	20	72	15
Ntalea 1.1b-133F/Ntalea 1.1b-538R	qPCR	1	45	98	5	63	20	72	15
Npumilus-420F/Npumilus-542R	qPCR	1	45	98	5	63	20	72	15

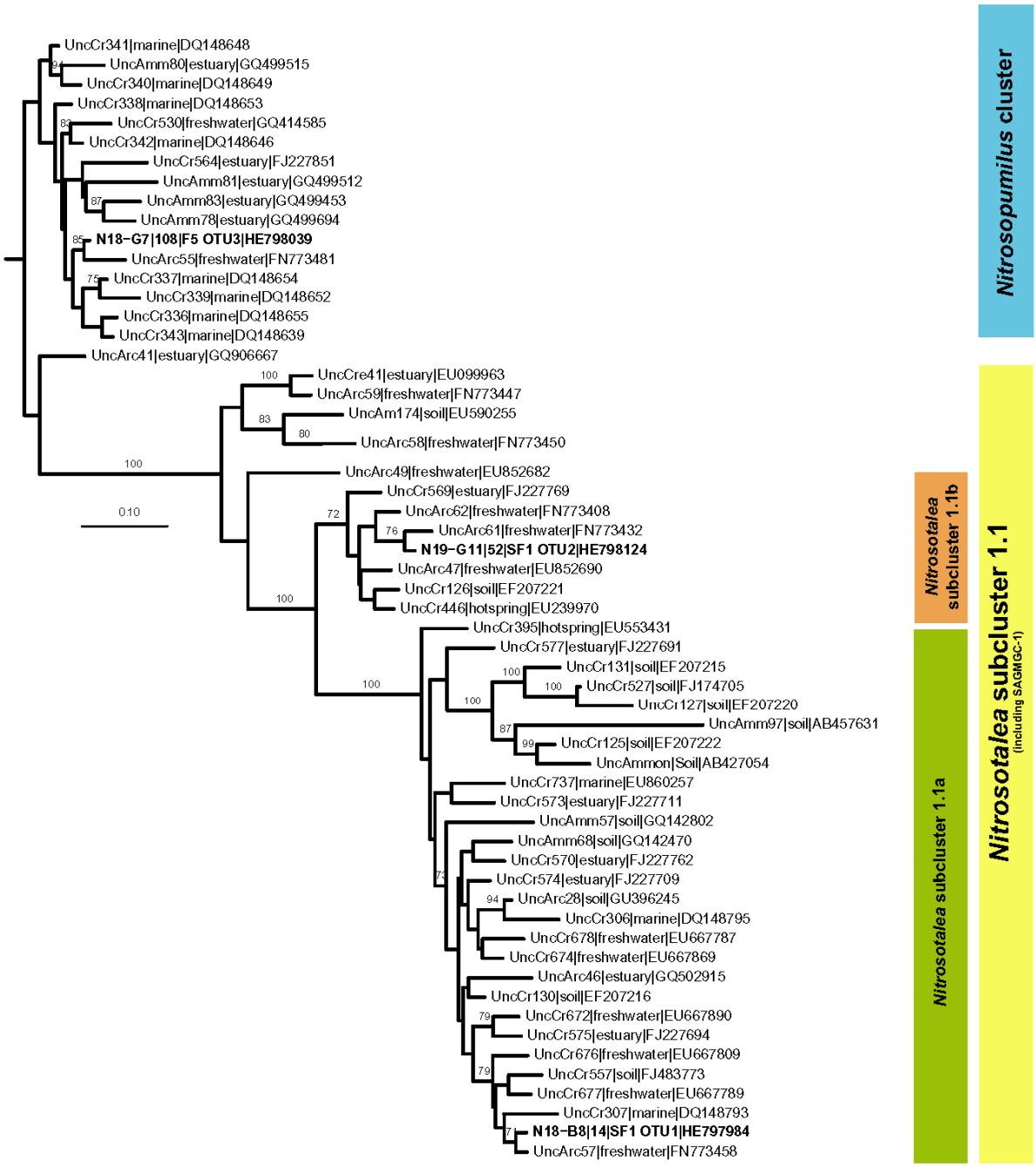

Fig. 5. Maximum-likelihood phylogenetic tree of the archaeal *amo*A gene for the SAGMGC-1 and MG 1.1a (*Nitrosopumilus* cluster) thaumarchaetal groups. The three AOA ecotypes shown in Fig. 2 are highlighted. See more details in Table S1. Scale bar, 10% estimated divergence.

1.1 subcluster, it matched 26% of total *amo*A gene sequences found in the LOOP, and it was covered by primer set Ntalea1.1b 133F-538R. Finally, the *amo*A OTU 3 was closely related to the *Nitrosopumilus* cluster (Pester *et al.*, 2012), it matched 60% of total *amo*A gene sequences in the LOOP, and it was covered by the primer set Npumilus 420F-542R. The coverage for these primers ranged between 67% and 89% of the aligned *amo*A gene

data set, and the specificity of primer sets was checked on the *amo*A database of Pester and colleagues (2011). Each primers pair was very specific and matched only with sequences belonging to its own cluster (Fig. 5). Additionally, we experimentally tested the primers with environmental *amo*A clones and natural samples from the LOOP. Abundant PCR products of the expected right size were obtained from all samples (Fig. S2). Cross priming amplification was not detected between non-targeted clones and specific *amo*A primers (see an example in Fig. S3).

qPCR and data analysis

Abundances of the thaumarchaeota SAGMGC-1 16S rRNA and *amo*A genes were determined by qPCR amplification. The qPCR assays were run on 96-well white qPCR plates (Bio-Rad, Hercules, CA, USA) in a DNA engine thermal cycler (Bio-Rad) equipped with a Chromo 4 real-time detector (Bio-Rad). The reaction mixture (20 μl) contained 10 μl of SsoFast EvaGreen supermix (Bio-Rad), 5 μl of template DNA (1 ng), 10 μM primers and molecular biology-grade water (Sigma, St Louis, MO, USA). The qPCRs were run for 2 min at 98°C, followed by 45 cycles, as detailed in Table 2. Standard curves were obtained from environmental clones as follows: SAGMGC-1 16S rRNA clone CR01Pyr-D9 Lake Bergús (HF951806); *amo*A OTU1 clone N18-B8 Lake Aixeus (HE797984); *amo*A OTU2 clone N19-G11 Lake Roi (HE798124); and *amo*A OTU3 clone N18-G7 Lake Muntanyó d'Arreu (HE798039). All reactions were run in triplicate with standard curves spanning from 10^2 to 10^7 copies of DNA. Optimal primer concentration (Table 2) produced amplification efficiency of 84–92% and r^2 value of 0.999. For all amplification reactions, melting curves from 65 to 95°C were carried out after each run, with an incremental increase in temperature of 0.5°C.The specificity of reactions was confirmed by melting curve analyses and by agarose gel electrophoresis to identify unspecific PCR products, such as primer dimers or gene fragments of unexpected length (data not shown). Spearman rank (r_s) correlations and multiple linear regression analysis were run in R (http://www.r-project.org/) with the corrgram and ggplot2 packages to investigate the relationships between gene abundance and environmental parameters.

Phylogenetic analyses

The 16S rRNA gene sequences were automatically aligned with the NAST aligner, clustered at identity threshold of 97% and imported into the Greengenes database (http://greengenes.lbl.gov/). The Archaea base frequency filter available in ARB (http://www.arb-home.de) was applied to exclude highly variable positions before sequences were added using the ARB parsimony insertion tool to the optimized tree provided by default. The *amo*A gene sequences were manually checked and clustered before multiple sequence alignment and phylogenetic inference by maximum likelihood as recently reported (Auguet *et al.*, 2011).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Maximum-likelihood phylogenetic tree for the 16S rRNA gene of all SAGMAGC-1 sequences found in NCBI (search Nov 2012). GenBank accession numbers are shown for each sequence. Scale bar, 10% estimated divergence.
Fig. S2. Agarose gel electrophoresis analysis of SAGMGC-1 16S rRNA and *amo*A genes amplified using different combinations of the newly designed specific primers in lakes Bergús, Granotes and Redon, and in clones containing the target sequences. Electrophoresis was carried out at 110 V for 45 min, and the gels were stained with ethidium bromide (2 mg ml^{-1}).
Fig. S3. Agarose gel electrophoresis analysis of *amo*A gene amplification after different combinations of the newly designed specific primers and different clones containing target and non-target sequences. Negative results were obtained in all the combinations with non-target clones. Electrophoresis was carried out at 110 V for 45 min, and the gels were stained with ethidium bromide (2 mg ml^{-1}).
Table S1. Accession numbers for the 16S rRNA and amoA genes sequences used for designing specific primers targeting SAGMGC-1 and AOA ecotypes.

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Environmental distribution of two widespread uncultured freshwater Euryarchaeota clades unveiled by specific primers and quantitative PCR

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Summary

Quantitative environmental distribution of two widely distributed uncultured freshwater Euryarchaeota with unknown functional role was explored by newly designed quantitative PCR primers targeting the 16S rRNA gene of clades Miscellaneous Euryarchaeota Group (MEG, containing the groups pMC2A384 and VALII/Eury4) and Deep-Sea Euryarchaeotal Groups (DSEG, targeting the cluster named VALIII containing the DHVE-3/DSEG, BC07-2A-27/DSEG-3 and DSEG-2 groups), respectively. The summer surface plankton of 28 lakes was analysed, and one additional dimictic deep alpine lake, Lake Redon, was temporally and vertically surveyed covering seasonal limnological variability. A trophic range between 0.2 and 5.2 μg l^{-1} Chl *a*, and pH span from 3.8 to 9.5 was explored at altitudes between 632 and 2590 m above sea level. The primers showed to be highly selective with c. 85% coverage and 100% specificity. Only pH significantly explained the changes observed in gene abundances and environment. In Lake Redon, DSEG bloomed in deep stratified waters both in summer and early spring, and MEG at intermediate depths during the ice-cover period. Overall, MEG and DSEG showed a differential ecological distribution although correlational analyses indicated lack of coupling of both Euryarchaeota with phytoplankton (chlorophyll *a*). However, an intriguing positive and significant relationship was found between DSEG and putative ammonia oxidizing thaumarchaeota.

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Introduction

After several years of environmental ribosomal gene surveys, a large number of uncultured mesophilic Euryarchaeota clades have been reported in the literature that contain ubiquitous microorganisms of unknown metabolism and unassigned functional roles in nature yet (see recent meta-analyses in Auguet *et al.*, 2010, and Durbin and Teske, 2012). In addition, in some occasions such uncultured lineages hold different names, provided by different authors and at different databases, and lack a statistically robust branching (Auguet *et al.*, 2010; Barberán *et al.*, 2011; Durbin and Teske, 2012), hindering a better understanding on the whole ecological potential and natural history of Euryarchaeota. Analysing samples containing natural enrichments of these targeted populations with taxon-specific molecular tools, functional genes and metagenomic surveys will substantially improve the current knowledge on the ecology and physiological potential of these enigmatic groups, analogous to previous work carried out on the Thaumarchaeota (e.g. Konneke *et al.*, 2005; Schleper *et al.*, 2005; Lehtovirta-Morley *et al.*, 2011). Unfortunately, in the case of the uncultured clades of Euryarchaeota with potential environmental importance, we are still far from what has been achieved for Thaumarchaeota in the last few years.

Although non-extremophile aerobic Euryarchaeota were initially described in marine environments (DeLong, 1992; Fuhrman *et al.*, 1992) and were widely seen in different oceans and seas (mainly groups II, III and IV; Bano *et al.*, 2004, Martín-Cuadrado *et al.* 2008, Galand *et al.*, 2010), uncultured Euryarchaeota have also been frequently recovered from heterogeneous freshwater ecosystems (reviewed in Auguet *et al.*, 2010). Euryarchaeotal lineages are ubiquitous in continental aquatic ecosystems with a large and still heavily uncovered phylogenetic diversity (Barberán *et al.*, 2011), suggesting that they have key functional roles in freshwater habitats and have experienced large diversification processes (Auguet *et al.*, 2010). Because no cultured representatives of these lineages are available, their physiology, metabolism and specific role in ecosystems functioning remain unknown. Most of these planktonic Euryarchaeota are allocated in the SILVA database (http://www.arb-silva.de/) within both the clade Miscellaneous Euryarchaeota Group (MEG)

Table 1. Amplification conditions for the two newly developed qPCR primers for MEG and DSEG Euryarchaeota.

					Cycling conditions					
					Denaturation		Annealing		Extension	
Primers set sequence (5′-3′)	Primers set	Method	Template (ng DNA)	Num. of cycles	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)
TCGACGGACGGTGTACGGCT/ AGGTTTSGCGCCTGCTGCAT	MEG-93F/ MEG-392R	Cloning	25	35	94	30	66	45	72	90
		q PCR	1	45	98	5	66	20	72	15
GCCAGCCGCCGCGGTAATAA/ GCYTTCGCCACAGGTGGTCC	DSEG-510F/ DSEG-725R	Cloning	25	35	94	30	58	45	72	90
		q PCR	1	45	98	5	58	20	72	15

containing the groups pMC2A384 (named after the hydrothermal vent studies of Takai and Horikoshi, 1999) and VALII/Eury4 (Jurgens *et al.*, 2000; Durbin and Teske, 2012) on the one hand, and on the other hand, the clade Deep-Sea Euryarchaeotal Group (DSEG) targeting the cluster named VALIII (euryarchaeotal clades VAL name taken from the small boreal lake Valkea Kotinen studied by Jurgens *et al.*, 2000) that comprises the DHVE-3/DSEG, BC07-2A-27/DSEG-3 and DSEG-2 groups (Durbin and Teske, 2012). For convenience, from now on, in this paper, we will follow the SILVA database nomenclature as clades MEG and DSEG (Fig. S1).

Recently, we have carried out an environmental survey in the plankton of high-mountain lakes using 'universal' primers for the archaeal 16S RNA gene illustrating how these environments hold a rich archaeal community dominated by Thaumarchaeota (mainly SAGMGC and MG1.1a) and Euryarchaeota mostly from uncultured clades and Methanomicrobiales (Auguet and Casamayor, 2008; 2013; Auguet *et al.*, 2011; 2012). High mountain lakes are globally distributed ecosystems with limited local anthropogenic disturbance; they are very sensitive indicators of global change that respond rapidly to environmental perturbations (Catalan *et al.*, 2006). Usually, they are low in ion concentrations and nutrient content, permanently cold, and experience marked seasonal changes in irradiance and high exposure to ultraviolet radiation during the ice-free period (Rose *et al.*, 2009). In the present study, we have designed and tested specific primers for quantifying by quantitative PCR (qPCR) the abundance of DSEG and MEG 16S rRNA gene in natural samples. In a companion paper, specific primers for freshwater thaumarchaeota (16S rRNA gene) and ammonia-oxidizing archaea (*amo*A gene) were optimized and tested (Restrepo-Ortiz *et al.*, 2014). We explored spatiotemporal differences in the surface plankton of a large set of lakes with marked environmental gradients and along a temporal survey analysing different dates and depths in the deep glacial stratified alpine Lake Redon located in the central Spanish Pyrenees (Catalan *et al.*, 2006; Auguet *et al.*, 2012). The correlational analyses indicated lack of coupling of euryarchaeotal populations with phytoplankton abundance and distribution (chlorophyll *a*) but an intriguing relationship between DSEG and putative ammonia-oxidizing thaumarchaeota.

Results and discussion

Primers optimization and PCR conditions

Specific primers for the clades MEG and DSEG were designed and tested following the procedure recently reported (Restrepo-Ortiz *et al.*, 2014) on 16S rRNA genes sequences available in *SILVA* 111 RefNR ribosomal RNA (rRNA) (release July 2012) database (Table S1) complemented with archaeal 16S rRNA gene from the Limnological Observatory of the Pyrenees (LOOP) (Auguet and Casamayor, 2008; 2013; Auguet *et al.*, 2011; 2012). Two potential specific primer sets were obtained for each euryarchaeotal clade (Table S2) that were tested experimentally at different annealing temperatures in different lakes samples (Fig. S2). Only the primer pairs MEG93F-392R and DSEG510F-725R produced a single fragment of the expected size, respectively (Fig. S2). These primer pairs did not show unspecific matching *in silico* with any additional group. Thus, four mismatches were needed in the MEG primers to found unspecificities with eukaryotes, bacteria and halobacteria, and up to three mismatches in the DSEG primers to found unspecificities with other euryarchaeotal groups. The new primers targeted on average 86% of the MEG and 84% of the DSEG 16S rRNA gene present in databases, respectively. Optimized amplification conditions are shown in Table 1, and the qPCR assays were run as described in Restrepo-Ortiz and colleagues (2014). The primers were experimentally tested both using environmental clones available from the LOOP containing nearly full 16S rRNA fragments of the MEG and DSEG clades, and after cloning and sequencing of the PCR products obtained from up to three different lakes (Fig. S2). ARB-based phylogenetic

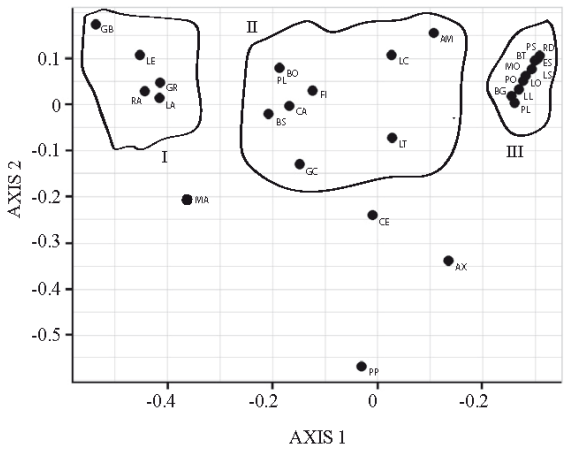

Fig. 1. MDS ordination analysis grouping the different lakes in clusters according to the Euryarchaeota DSEG and MEG, and Thaumarchaeota SAGMGC 16S rRNA gene abundances. Lakes code in Table S3.

analysis (Restrepo-Ortiz *et al.*, 2014) and pairwise comparison with available cloned sequences (c. 900 bp) from the LOOP dataset showed all sequences placed within the respective MEG and DSEG clades (Figs. S3 and S4, respectively), indicating a good specificity for the new primer sets. The ammonia-oxidizing thaumarchaeotal group SAGMGC was studied using the specific 16S rRNA gene qPCR primers set 274F-446R recently described (Restrepo-Ortiz *et al.*, 2014). To explore the natural 16S rRNA gene abundances of freshwater euryarchaeota MEG and DSEG and thaumarchaeota SAGMGC, we combined both an extensive spatial scrutiny of surface waters (first 1 m integrated) from 28 lakes sampled in summer 2008 and 2011, and a spatiotemporal survey in the deep alpine Lake Redon. Environmental parameters were measured as recently reported (Auguet *et al.*, 2012), and DNA samples were processed as reported (Demergasso *et al.*, 2008; Hervàs *et al.*, 2009).

Spatial heterogeneity and environmental forcing

For the spatial scrutiny, a set of lakes was selected covering a large environmental range of pH (3.8–9.5), temperature (4–31°C), conductivity (3–1805 μS cm ^{1}), trophic status (i.e. Chl *a* concentrations, 0.43–5.18 μg l^{-1}) and altitudes above sea level from 632 to 2590 m (Table S3). Most of these lakes were shallow (i.e. < 15 m depth), well mixed by wind and not stratified. Interestingly, the 16S rRNA gene abundance for each archaeal population showed a spatially heterogeneous distribution, and on average, the highest abundance was found for DSEG (4.6×10^4; range < 0.01–10^5 copies ml^{-1}) followed by MEG (1.1×10^4; range 10^1–10^5 copies ml^{-1}) and SAGMGC (4.3×10^3; range 10^{-1} to 10^4 copies ml^{-1}) (Table S3). An multidimensional scaling (MDS) ordination analysis grouped the lakes in different clusters of lakes based on gene abundances of each archaeal population (Fig. 1). Cluster I contained lakes dominated by clade DSEG (i.e. > 88% of all three archaeal gene abundances) with minor contributions by MEG (<8%) and SAGMGC (<4%). Conversely, the cluster III was dominated by clade MEG (> 96% of all three populations) with DSEG essentially absent and minor presence of SAGMGC (<4%). In between, cluster II covered a gradient from lakes with quite similar abundances of DSEG and MEG to lakes with abundant MEG (70–90%) and minor contribution of DSEG (10–20%). Finally, four lakes were separated from the rest, i.e. the most acidic lakes (i.e. Pica Palomera, Aixeus and Certascan; pH <5.7) having the highest proportion of ammonia-oxidizing archaea (AOA) (45–93%), and Lake Muntanyó d'Arreu with a high abundance of both DSEG and SAGMGC (c. 45% each) and substantial presence of MEG (c. 10%). Overall, there were no significant effects on the abundance of any of the populations by the spatial and morphometric lake variables tested (altitude and Alake/Acatchment ratio), either by Chl *a*, temperature or conductivity (Spearman rank correlations, $P > 0.1$). The changes in the abundance of the different archaeal populations were only explained by changes in pH [permutational multivariate analysis of variance (PERMANOVA), $P < 0.01$, R2 = 0.198].

Spatiotemporal variations in deep Lake Redon

Lake Redon (42°38′34″N, 0°46′13″E, altitude 2240 m, maximum depth 73 m, surface 0.24 km^2) has a dimictic regime, with mixing periods in late spring and in autumn, and remains covered by snow for about 6 months of the year. The ice-cover thickness usually reaches several metres during the maximum in April, leaving the underlying water in the dark for several months in winter and spring. Lake Redon was sampled in June, July and October 2007, and March, April and May 2008 (Table S4) collecting samples from six depths (surface, 2-10-20-35-60 m) that captured the different limnological characteristics of the lake (Camarero and Catalan, 2012). The surface sample corresponded both to the air-water interface during the ice-free period (Auguet and Casamayor, 2008), and the slush (mixture of snow and ice) present on the top of the lake during winter and most spring (Llorens-Marés *et al.* 2012). Peaks in nitrate and ammonia were observed in surface waters during the melting period, and in bottom waters in September–October and in April–May (Table S4, and see more details in Restrepo-Ortiz *et al.*, 2014) most probably because of microbial mineralization and nitrification in the sediment. Interestingly, higher nitrate concentrations were also observed along the water column in

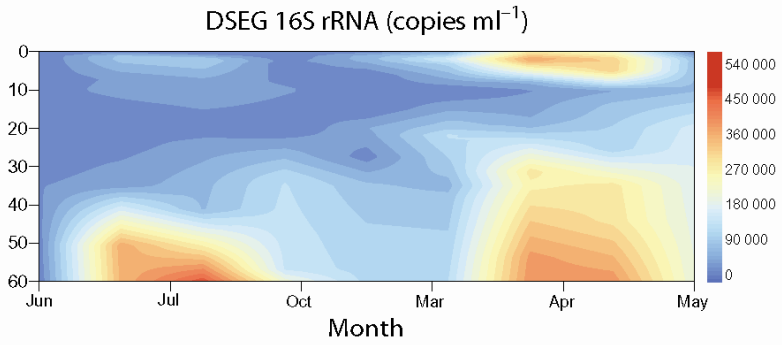

Fig. 2. Spatiotemporal variation for abundance of the specific 16S rRNA gene for Euryarchaeota DSEG population in Lake Redon.

early summer, and higher nitrite concentrations were in general observed along the year in surface waters than at the bottom. During the ice-free season, light penetration into the water column was very deep by the high water transparency because of both low dissolved organic carbon (10–100 μM, Auguet *et al.*, 2012) and low algae growth (i.e. low Chl *a* concentrations).

The newly designed primers were used to follow the vertical and temporal changes of DSEG and MEG Euryarchaeota in Lake Redon. Amplification signal was obtained from most depths and dates examined (Table S4). Again, the DSEG 16S rRNA gene showed the largest variation in abundance spanning five orders of magnitude from 2 copies per ml of lake water up to 5×10^5. Interestingly, we observed blooms of this population in deep waters (60 m) during the annual stratification periods both in summer and in early spring, and in the slush layers when Lake Redon was covered by ice and snow (Fig. 2). DSEG gene concentrations substantially decreased after snow melting and during the mixing periods. Conversely, MEG 16S rRNA gene abundances ranged four orders of magnitude from 1 up to 10^4 copies per ml of lake water, and blooms of this population were found during the ice-cover period both at intermediate depths (35 m) and in the slush layers (Fig. 3). MEG gene concentrations were low during summer stratification and the mixing periods. Overall, after multiple linear regression analysis, negative and significant ($P < 0.01$) relationships were observed between nitrite and chlorophyll *a* concentrations, and the vertical variability of the euryarchaeotal 16S rRNA gene abundances along the temporal survey. These results were also compared with the 16S rRNA gene abundance distribution of SAGMGC recently reported for Lake Redon (Restrepo-Ortiz *et al.*, 2014; Table S4), and significant relationships among the abundances of all three populations were observed. However, when we pooled together populations abundance from Lake Redon and from the 28 remaining lakes, a significant and positive correlation was found only between DSEG and SAGMGC (rs = 0.82, $P < 0.001$) but not between MEG and SAGMGC (rs = 0.01, $P > 0.9$) (Fig. 4). The positive and strongly significant correlation was also observed for DSEG and SAGMA without the data from Lake Redon (rs = 0.25, $P < 0.01$). Thus, the correlational analysis strongly suggested a close relationship between the unknown euryarchaeotal DSEG clade and the thaumarchaeotal ammonia oxidizing SAGMGC clade that certainly deserves further and more detailed explorations.

Environmental role of uncultured Euryarchaeota: the big gap of knowledge

In cold and mesophilic environments, methanogens and extreme halophiles (Haloarchaea) have been studied for

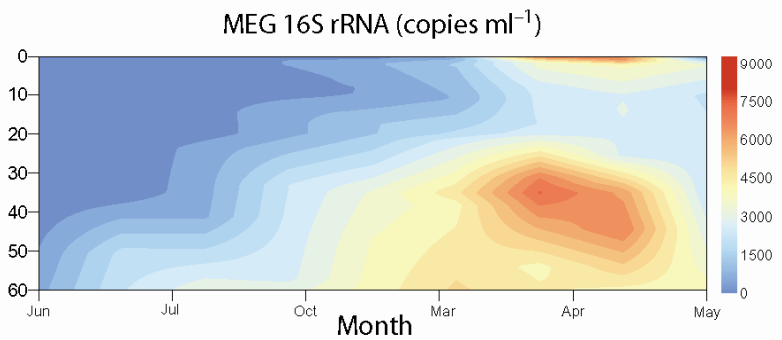

Fig. 3. Spatiotemporal variation for abundance of the specific 16S rRNA gene for the Euryarchaeota MEG population in Lake Redon.

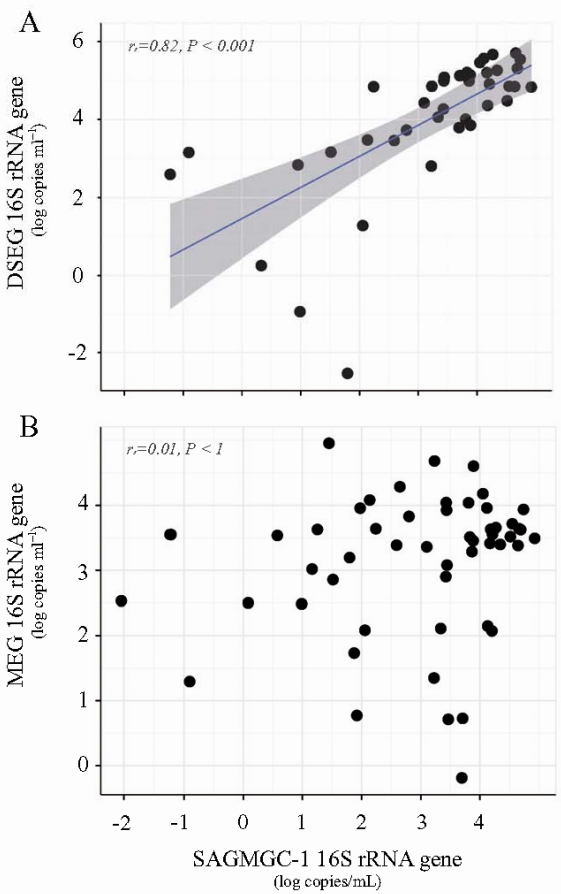

Fig. 4. Correlational analysis for DSEG/SAGMGC and MEG/SAGMGC specific 16S rRNA gene along the complete dataset analysed.

a long time and have well-known functional roles. Methanogens are active key players in anoxic waters and sediments (Garcia *et al.*, 2000), and Euryarchaeota show prevalence over other archaeal phyla for high-salinity environments such as the MSBL-1 candidate order, the Halobacteriaceae and the DHVEG-6 clade (van der Wielen *et al.*, 2005; Auguet *et al.*, 2010; Casamayor *et al.*, 2013). The most widely encountered uncultured euryarchaeotal lineages found in freshwater environments are the MEG and DSEG lineages, along with the Marine Benthic Group D (MBG-D) (Galand *et al.*, 2006; Barberán *et al.*, 2011; Borrel *et al.*, 2012). Although the metabolism of the MBG-D members remains unknown and they were previously labelled as non-methanogens (Jiang *et al.*, 2008), phylotypes of this group are found in sites with high methane concentrations (Borrel *et al.*, 2012, and references therein), and freshwater places where anaerobic methane oxidation processes are detected, such in Lake Cadagno (Schubert *et al.*, 2011). In the case of MEG and DSEG, their environmental distribution did not offer consistent clues to assign them a putative environmental role. DSEG had been previously reported mostly in sediments both marine and freshwater, and microbial mats (70% of the Operational Taxonomic Units present in SILVA database), and in hydrothermal vents (12%), and hypersaline and freshwater plankton (6% each). MEG had previously been more often recovered from freshwater systems (30%), sediments and microbial mats (25%), and hot-springs and hypersaline systems (c. 20% each). A recent analysis on the natural history of these two groups indicated very large phylogenetic diversity in these clades far from being reasonably sampled and much higher than in Methanobacteriales and Cenarchaeales (Barberán *et al.*, 2011). Both groups accumulated higher phylogenetic diversity in oxic freshwaters and sediments than in soils (Barberán *et al.*, 2011). Unfortunately, cultivation has remained elusive for these new euryarchaeal groups present in aerobic freshwater habitats that are distantly related to their extremophilic counterparts. There is therefore a big gap in the current knowledge of the ecology and metabolism of mesophilic and cold Euryarchaeota beyond methanogens and halophiles.

Overall, we observed a large range of variation in the 16S rRNA gene abundances of the different euryarchaeotal populations examined, but unfortunately, most of the environmental parameters measured did not provide a satisfactory explanation for the changes in the abundances observed. Only pH appeared as the strongest gradient controlling the abundance of the euryarchaeotal and thaumarchaeota populations. Together with salinity, pH has been shown as one of the major drivers of microbial community structure (Fierer and Jackson, 2006; Newton *et al.*, 2007; Triadó-Margarit and Casamayor, 2012; Auguet and Casamayor, 2013), but this parameter does not provide clues to infer a potential metabolic role *in situ*. Interestingly, the correlational analyses indicated lack of coupling of such Euryarchaeota with phytoplankton (chlorophyll *a*), but an intriguing positive and significant relationship between DSEG and putative ammonia-oxidizing thaumarchaeota abundances. Previous studies on marine planktonic Euryarchaeota suggested either a putative anaerobic respiration physiology (Martín-Cuadrado *et al.* 2008) or the potential to carry out a photoheterotrophic metabolism by light-capturing proteorhodopsins to gain a competitive advantage (Frigaard *et al.*, 2006). In the case of Lake Redon, we could rule out both strategies because the freshwater populations were found blooming in aerobic cold waters and in the dark. However, the correlation found with ammonia-oxidizing microorganisms may shed some light to guide future studies. Whether this is related to the potential of DSEG Euryarchaeota to perform any key metabolic step in the global nitrogen cycling remains to be shown, but the correlational analysis and

seasonal environmental distribution between freshwater AOA and DSEG strongly suggests that they share a closely related ecological niche.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylogenetic context for the 16S rRNA gene of the Euryarchaeota DHVE and MEG targeted by specific primers in this study (SILVA ribosomal database 111 RefNR, July 2012). The clades are highlighted and equivalent clade nomenclature has been added following Durbin and Teske (2011; 2012). Scale bar, 10% estimated divergence.

Fig. S2. Specificity level (number of bands observed in an agarose gel) observed for the newly designed primers at different PCR annealing temperatures. Averaged values and error range for PCR products obtained from lakes Bergús, Llebreta and Muntanyó d'Arreu. The primers set MEG 93f-392r (expected PCR product size ~ 296 bp) and DSEG 510f-725r (~ 216 bp) showed the best performance and specificity.

Fig. S3. Maximum-likelihood phylogenetic tree for the 16S rRNA gene of the Euryarchaeota clade MEG (Miscellaneous Euryarchaeota Group comprising the clades pMC2A384 and VALII/Eury4; Durbin and Teske, 2012) targeted by the specific primers set designed in this study. Scale bar, 10% estimated divergence.

Fig. S4. Maximum-likelihood phylogenetic tree for the 16S rRNA gene of the Euryarchaeota clade DSEG (Deep-Sea Euryarchaeotal Groups, targeting the cluster named VALIII comprising the DHVE-3/DSEG, BC07-2A-27/DSEG-3 and DSEG-2 groups; Durbin and Teske, 2011; 2012) targeted by the specific primer set designed in this study. Scale bar, 10% estimated divergence.

Table S1. NCBI accession numbers of the 16S rRNA gene sequences used to design the new and specific primers for Euryarchaeotal clades MEG and DSEG in combination with the Pyrenean 16S rRNA gene dataset.

Table S2. Specific primers set designed in this study for qPCR analysis of the euryarchaeotal clades MEG and DSEG. The primer sets MEG 93f-392r, and DSEG 510f-725r showed the best performance and specificity, respectively (see Fig S2).

Table S3. Environmental parameters of the different lakes analysed in this study and specific 16S rRNA gene abundances for the Euryarchaeota DSEG and MEG, and Thaumarchaeota SAGMGC populations. Standard deviations < 10% applies for all the qPCR data. N/A not available; N/D not detected.

Table S4. Environmental parameters for the different dates and depths analysed in Lake Redon and specific 16S rRNA gene abundances for the Euryarchaeota DSEG and MEG, and Thaumarchaeota SAGMGC populations. Standard deviations < 10% applies for all the qPCR data. N/A not available; N/D not detected.