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AMMONIA-OXIDIZING ARCHAEA FROM HIGH ARCTIC SOILS

Tese de Mestrado

Ricardo Jorge Eloy Alves

MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada por Dr. Tim Urich (UNIVIE)
e Prof. Dr. Rogério Tenreiro (FCUL-BioFIG)

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Summary/Sumário

As regiões árcticas e boreais cobrem 22% da superfície terrestre e englobam toda uma variedade de ecossistemas particularmente sensíveis a alterações ambientais, entre ecossistemas terrestres (Chapin III *et al*., 2000; Sala *et al*., 2000). O Árctico está actualmente a sofrer alterações dramáticas, previstas de aumentar drasticamente durante o presente século em resultado do aumento das temperaturas que desproporcionalmente afecta estas regiões (IPCC, 2007). Alterações na estrutura e funcionamento destes ecossistemas têm um enorme impacto no clima, não só à escala local e regional, como também à escala global (Chapin III *et al*., 2000; Post e*t al*., 2009). Adicionalmente, estimativas recentes indicam que a quantidade de carbono armazenado nos solos *permafrost* das regiões árcticas e boreais é duas vezes superior ao carbono presente na atmosfera e mais de três vezes superior a todo o carbono armazenado na totalidade da biomassa florestal, perfazendo 50% do carbono orgânico subterrâneo (Tarnocai *et al*., 2009; Kuhry *et al*., 2010). O aumento dos processos de decomposição e respiração em função do aumento das temperaturas está previsto constituir um importante *feedback* positivo do aquecimento global, através da libertação na atmosfera do carbono contido nestes reservatórios, incluindo aquele contido nas fracções mais recalcitrantes do solo (Biasi *et al*., 2005; Schuur *et al*., 2008 e 2009).

A produtividade e respiração dos ecossistemas árcticos são geralmente limitadas pelo efeito conjunto das baixas temperaturas, reduzida disponibilidade de nutrientes e reduzidos *inputs* externos de nutrientes (Jonasson e Michelsen, 1996; Jonasson e Shaver, 1999). No entanto, foi demonstrado que o aumento das temperaturas leva à redução desta limitação através da estimulação dos processos de decomposição e mineralização, com consequente libertação de nutrientes armazenados na matéria orgânica do solo (Binkley *et al*., 1994; Mack *et al*., 2004; Biasi *et al*., 2008). Este fenómeno está previsto induzir um ciclo de *feedbacks* positivos na actividade microbiana, levando a desequilíbrios na reciclagem de nutrientes e, em última instância, exercendo extensas alterações no ciclo do carbono e no funcionamento geral dos ecossistemas árcticos, a curto e longo prazos (Shaver *et al*., 1992; Callaghan *et al*., 2004).

O azoto é geralmente considerado o principal nutriente limitante em solos do Árctico (Shaver e Chapin III, 1980; Jonasson e Michelsen, 1996; Nordin *et al*., 2004) e a sua disponibilidade no ambiente depende sobretudo dos processos de decomposição e de transformação de compostos azotados catalisados por microrganismos (Canfield *et al*., 2010). Apesar da enorme diversidade taxonómica e funcional dos microrganismos envolvidos nestes processos, bem como do facto que a composição das comunidades microbianas afecta, e é afectada pelo funcionamento geral dos ecossistemas (Reed e Martiny, 2007; Strickland *et al*., 2009), as populações microbianas e o seu papel nos ciclos biogeoquímicos do Árctico permanecem virtualmente por caracterizar. Estudos recentes verificaram, de facto, variações espaciais e temporais nas comunidades microbianas nestas regiões (Neufeld e Mohn, 2005; Zak e Kling, 2006; Wallenstein *et al*., 2007; Chu *et al*., 2010; Schütte *et al*., 2010), bem como em função de aumentos na temperatura e na deposição de nutrientes (Campbell *et al*., 2010). Foram também efectuadas semelhantes observações relativamente a alguns grupos funcionais especificamente envolvidos no ciclo do azoto, nomeadamente variações nas populações de microrganismos fixadores de azoto (Deslippe *et al*.,

2005; Deslippe e Egger, 2006; Walker *et al*., 2008) e de bactérias desnitrificantes (Walker *et al*., 2008). No entanto, outros grupos funcionais cruciais, tais como os responsáveis pela nitrificação, permanecem por caracterizar, apesar de este processo ter já sido extensamente documentado em solos do Árctico, sendo reconhecidamente dependente das condições ambientais (Booth *et al*., 2005).

A nitrificação é um processo exclusivamente microbiano, consistindo na conversão sequencial da amónia (NH_3) a nitrito (NO_2^-), por intermédio de microrganismos oxidantes de amónia, e subsequente conversão do NO_2^- a nitrato (NO_3^-), por microrganismos oxidantes de nitrito. Foi também demonstrado que este processo é responsável pelo consumo de cerca de metade da quantidade líquida de azoto mineralizado anualmente em solos do Árctico, chegando aos 100% em alguns anos (Giblin *et al*., 1991). Adicionalmente, o processo de nitrificação está, directa- e indirectamente, envolvido nas emissões do gás óxido nitroso (N_2O) (Conrad, 1996), o qual possui um potencial enquanto gás do efeito estufa 300 vezes superior ao do dióxido de carbono (CO_2), sendo considerado a principal substância responsável pela degradação da camada de ozono no séc. XXI (Ravishankara *et al*., 2009). Foi de facto demonstrado que a nitrificação contribui com a maior parte do N_2O produzido em alguns solos, incluindo no Árctico, comparativamente ao processo canónico de desnitrificação (Ma *et al*., 2007; Siciliano *et al*., 2009).

A oxidação da amónia é realizada tanto por bactérias como por árqueas, apesar da contribuição relativa destes grupos permanecer por esclarecer (Nicol e Schleper, 2006; Prosser e Nicol, 2008). Durante mais de um século, as bactérias oxidantes de amónia (AOB, *ammonia-oxidizing bacteria*) foram consideradas as principais responsáveis por este processo na natureza, embora recentemente tenha sido demonstrado que árqueas pertencentes ao novo filo Thaumarchaeota (Brochier-Armanet *et al*., 2008; Spang *et al*., 2010) podem deter um papel fundamental na nitrificação, dado possuírem genes que codificam o enzima amónia monooxigenase e dado estarem amplamente distribuídas, sendo extremamente abundantes em vários ambientes, por vezes ordens de magnitude mais abundantes que as AOB (Francis *et al*., 2005; Leininger *et al*., 2006; Wuchter et al., 2006; Caffrey *et al*., 2007; Lam *et al*., 2007). Enquanto várias propriedades fisiológicas e metabólicas foram já descritas nas AOB, apenas recentemente foi demonstrado que as árqueas oxidantes de amónia (AOA, *ammonia-oxidizing archaea*) são efectivamente capazes de oxidar amónia de forma autotrófica em diversos ambientes, tais como em ambientes marinhos (Könneke *et al*., 2005; Walker *et al*., 2010; Blainey *et al*., 2011), em fontes termais (de la Torre *et al*., 2008; Hatzenpichler *et al*., 2008) e em diversos solos (Offre *et al*., 2009 e 2011; Pratscher *et al*., 2011; Tourna *et al*., 2011; Xia *et al*., 2011; Zhang *et al*., 2011). Diversos estudos indicam que as AOA estão geralmente mais activas em ambientes oligotróficos e/ou com baixos níveis de pH, oxigénio e azoto (Lam *et al*., 2007; Nicol *et al*., 2008; Erguder *et al*., 2009; Di *et al*., 2009; Di *et al*., 2010; Gubry-Rangin *et al*., 2010; Molina *et al*., 2010; Verhamme *et al*., 2011). No entanto, a associação directa com estes factores ambientais e com um metabolismo autotrófico permanece restrita a poucas linhagens de AOA, apesar da diversidade taxonómica e ubiquidade das AOA sugerirem que estas possam deter um potencial metabólico e fisiológico muito mais vasto. De facto, foi demonstrado que a recentemente isolada taumárquea *Nitrososphaera viennensis* possui requerimentos nutricionais distintos, podendo inclusivamente ser cultivada sob concentrações de

amónia relativamente elevadas, comparativamente às outras AOA caracterizadas (Tourna *et al*., 2011). Outras evidências sugerem ainda que algumas AOA podem de facto possuir metabolismos heterotróficos ou mixotróficos (Ingalls *et al*., 2006; Schleper e Nicol, 2011).

A hipótese de que a ecologia e evolução das árqueas é conduzida pela sua adaptação a condições de stress energético crónico (Valentine, 2007) leva à sugestão de que os ecossistemas árcticos poderão constituir ambientes favoráveis às árqueas, dadas as condições resultantes das baixas temperaturas, reduzida disponibilidade de nutrientes, ciclos drásticos de congelamento-descongelamento e verões curtos intercalados por longos períodos com reduzida luz solar. De facto, a maioria das populações e diversidade das árqueas estão presentes em ambientes frios (Cavicchioli, 2006), nos quais árqueas pertencentes ao filo Thaumarchaeota, potencialmente capazes de oxidar amónia, estão amplamente distribuídos (Preston *et al*., 1996; Nicol *et al*., 2005; Koch *et al*., 2007; Nakagawa *et al*., 2007; Ayton *et al*., 2009; Kalanetra *et al*., 2009). Para mais, foram já identificadas grupos de Thaumarchaeota em solos da Sibéria (Ochsenreiter *et al*., 2003) e em solos *permafrost* do Árctico canadiano (Steven *et al*., 2007 e 2008), bem como outros grupos de árqueas relacionados com este filo detectados em solos de Svalbard (Høj *et al*., 2005, 2006 e 2008). Curiosamente, foram também identificadas taumárqueas em vários solos de tundra alpina, nos quais não foram detectadas AOB (Nemergut *et al*., 2008). Apesar da sugestão de que as AOA poderão estar geralmente presentes em solos sujeitos a baixas temperaturas, as regiões árcticas são, não obstante, extremamente heterogéneas, o que poderá ser reflectido nas comunidades microbianas presentes, inclusivamente nas comunidades de AOA. De facto, o Árctico engloba uma grande variedade de ecossistemas terrestres, os quais impõem variáveis pressões selectivas às populações de microrganismos presentes, em resultado das interacções entre vegetações distintas, processos de crioturbação e diferentes propriedades geo-físico-químicas do solo, frequentemente em reduzidas escalas espaciais.

Com base no pressuposto de que os solos do Árctico poderão constituir ambientes favoráveis às árqueas e tendo em conta a importância fundamental dos processos responsáveis pela reciclagem do azoto nestes ecossistemas, o presente trabalho teve como objectivo a caracterização das comunidades de AOA em distintos solos do Árctico, relativamente às propriedades físico-químicas dos solos e ao potencial envolvimento das AOA no processo de nitrificação. Doze amostras de solo recolhidas principalmente em Spitsbergen (Svalbard), mas também em Tazovskiy (Sibéria) e em Zackenberg (Gronelândia), foram analisadas através de uma abordagem polifásica, com recurso a análises moleculares do gene *amoA* (que codifica a subunidade A da amónia monooxigenase; geralmente utilizado como marcador molecular de organismos oxidantes de amónia), determinação de taxas brutas de nitrificação através do método de diluição do isótopo ^{15}N e culturas de enriquecimento de AOA em laboratório.

Com base na análise filogenética da sequência aminoacídica traduzida a partir de genes *amo*A, foi observada uma extensa diversidade de AOA distribuída em vários solos de tundra distintos, ao contrário das AOB, as quais foram frequentemente indetectáveis. A diversidade de AOA identificada englobou a maioria das linhagens filogenéticas conhecidas, com predominância de grupos associados à vasta linhagem dominante em solos e sedimentos. Os seis grupos principais de AOA identificados estavam distribuídos entre os diferentes solos de acordo com o tipo de

ecossistema (i.e. habitat), independentemente da localização geográfica, revelando também uma elevada especificidade local dos grupos individuais. Análises estatísticas multivariadas baseadas nas proporções relativas dos filotipos de AOA e num vasto conjunto de parâmetros ambientais mostraram que a composição das comunidades de AOA estava parcialmente associada ao conteúdo em azoto do solo, em particular ao NO_3^-, bem como às condições hídricas dos solos. No entanto, a separação geral entre populações distintas de AOA não pareceu estar unicamente associada a nenhuma das propriedades individuais dos solos, revelando uma relação aparentemente multifactorial entre as AOA e o ambiente, frequentemente em pequenas escalas filogenéticas e espaciais.

A detecção de AOA em concomitância com taxas de nitrificação mensuráveis em solos onde as AOB estavam aparentemente ausentes, sugeriu que as AOA poderão ser efectivamente responsáveis pela oxidação da amónia implicada nesse processo. As diferentes taxas de nitrificação medidas nesses solos, onde aparentemente as AOA eram os procariotas oxidantes de amónia dominantes, sugeriram que as diferentes populações de AOA residentes diferem no seu potencial nitrificante, tanto sob as condições nativas de azoto no solo, como após adição de reduzidas quantidades de NH_4^+. Curiosamente, as diferentes populações residentes de AOA, dominadas por filotipos distintos, aparentaram estar associadas a diferentes taxas de nitrificação. As taxas de nitrificação mais elevadas foram detectadas em solos com elevado teor hídrico, onde as populações de AOA eram dominadas por filotipos associados a um grupo basal da linhagem predominante em solos/sedimentos, ou associados a um grupo previamente detectado em ambientes geotérmicos, pertencente à linhagem predominantemente marinha. As taxas de nitrificação foram geralmente mais baixas em solos com baixo teor hídrico, dominados pelo grupo de AOA mais abundante e amplamente distribuído em solos, independentemente da presença ou ausência de AOB. Estes resultados sugeriram, não só uma diferenciação dos nichos ocupados pelas AOA, como também uma baixa redundância funcional entre os diferentes grupos. Adicionalmente, a análise de inúmeras culturas de enriquecimento de AOA em laboratório, iniciadas a partir de diferentes solos de Spitsbergen, confirmou que as populações de AOA presentes, pelo menos em alguns solos de turfeira, tundra de musgos e de *frost boils*, são efectivamente capazes de oxidar amónia, em particular aquelas que incluíam membros de um grupo basal não-caracterizado da linhagem de solos/sedimentos, detectados quase exclusivamente nos locais de turfeira.

De um modo geral, os resultados obtidos pelo presente estudo sugerem que os solos do Árctico podem ser colectivamente considerados enquanto "*hotspots*" de diversidade de AOA, nos quais estes organismos podem deter um papel crucial no processo de nitrificação. Adicionalmente, várias indicações suportam a ideia de que a diversidade destes organismos pode, de facto, representar um potencial fisiológico e metabólico superior ao geralmente suposto, com diferentes grupos de AOA detendo papéis funcionais não-redundantes, nomeadamente no Árctico. Dada a importância da regulação do ciclo do azoto no funcionamento dos ecossistemas árcticos, estas observações enfatizam a necessidade de compreender a dinâmica das populações de AOA, bem como de outros grupos de microrganismos envolvidos nestes processos, sobretudo no âmbito das respostas destes ecossistemas a alterações climáticas.

Ammonia-oxidizing archaea from High Arctic soils

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Arctic ecosystems are of crucial importance for the climatic balance of the globe. Therefore basic knowledge about the microbial groups involved in their biogeochemical processes is required for a better understanding of their functioning and their responses to environmental changes. Nitrogen has been shown to be a major limiting nutrient for ecosystem productivity and respiration in Arctic soils and its availability is strongly regulated by nitrification, the biological conversion of ammonia to nitrate, via nitrite. Yet, the microbial communities involved in this process remain largely uncharacterized in Arctic soils, in particular the ammonia-oxidizing archaea (AOA), which have been recently found to be abundant and widespread in most other environments. The present study aimed at characterizing the AOA communities in several distinct Arctic tundra soils in relation to soil properties and potential role in nitrification. Twelve soils collected mainly from Spitsbergen (Svalbard) and also from Tazovskiy (Siberia) and Zackenberg (Greenland) were analysed through a polyphasic approach, coupling molecular analysis of the *amo*A gene (encoding subunit A of ammonia monooxygenase), determination of gross nitrification rates by a ^{15}N pool dilution method and enrichment of archaeal nitrifiers in laboratory cultures. Based on phylogenetic analyses of amoA sequences, AOA were shown to be diverse and widespread throughout a variety of distinct tundra soils, in contrast to ammonia-oxidizing bacteria (AOB), which were often undetectable. The six identified clusters of AOA were distributed according to their habitat, independent of geographic location. Multivariate analyses showed that AOA distribution was partially associated with the nitrogen composition of the soil, particularly with nitrate. However, no single soil property could be directly associated with overall differences in AOA communities, which showed an apparent multifactorial association with the environment, often at small scales. *In situ* gross nitrification was measurable in all soils, with the highest rates detected in wet soils, namely in the two tundra fen sites and in a moss tundra site. The wet moss tundra soil was dominated by an AOA group affiliated with a geothermal cluster within the marine lineage, and in one of the tundra fen sites a basal AOA soil cluster co-occurred

with AOB, while in the other fen site only AOB were detected. Nitrification activity was invariably lower in dryer soils dominated by typical soil AOA groups, regardless of co-detection of AOB. Gross nitrification rates measured in different soils where no AOB were detected showed that the distinct resident AOA populations differed in their potential to nitrify under *in situ* or low NH_4^+ amendment conditions. Furthermore, laboratory enrichment cultures with AOA indicated that archaeal populations from Arctic soils are capable of ammonia oxidation, in particular those affiliated with the soil cluster that dominated the fen peat soil. Together, these observations suggest that Arctic soils can be collectively regarded as "hotspots" of AOA diversity and that these organisms might harbour a greater physiological and niche versatility than generally assumed, possibly playing an important role in nitrification in Arctic terrestrial ecosystems.

Subject category: microbial population and community ecology
Keywords: ammonia-oxidizing archaea; nitrification; Arctic ecology; soil; diversity; multivariate analysis; ^{15}N pool dilution; enrichment cultures

Introduction

Arctic and boreal regions cover 22% of the land surface of the planet and encompass a variety of ecosystems that are outstandingly sensitive to environmental changes among terrestrial ecosystems (Chapin III *et al*., 2000; Sala *et al*., 2000). The Arctic is currently undergoing dramatic changes that are predicted to increase drastically in the following century, as a result of the rising temperatures disproportionally affecting these regions (IPCC, 2007). Changes in structure and functioning of these ecosystems have a great impact on climate, not only on local and regional scales, but also on a global scale (Chapin III *et al*., 2000; Post *et al*., 2009). Furthermore, recent estimates indicate that the carbon stored in permafrost soils in Arctic and boreal regions is more than twice the atmospheric carbon and more than three times the carbon stored in the global forest biomass, comprising approximately 50% of the total belowground organic carbon (Tarnocai *et al*., 2009; Kuhry *et al*., 2010). Increased microbial decomposition and respiration following rising temperatures is predicted to become a major positive feedback to climate warming through release of carbon from this sink into the atmosphere, including also carbon from the more recalcitrant fractions (Biasi *et al*., 2005; Schuur *et al*., 2008 and 2009).

Ecosystem productivity and respiration in the Arctic is generally constrained by low temperatures coupled to low nutrient availability and low external inputs of nutrients (Jonasson and Michelsen, 1996; Jonasson and Shaver, 1999). However, it has been shown that increases in temperature are likely to relieve nutrient limitation by, directly or indirectly, stimulating higher rates of decomposition and mineralization and consequently releasing nutrients stored in the soil organic matter (Binkley *et al*., 1994; Mack *et al*., 2004; Biasi *et al*., 2008). This phenomenon is expected to trigger a positive feedback loop of microbial activity, leading to imbalances in the turnover of nutrients and ultimately exerting profound changes in carbon cycling and ecosystem functioning in

the short- and long-terms (Shaver *et al*., 1992; Callaghan *et al*., 2004; Campbell *et al*., 2010; Xu *et al*., 2011).

A vast body of evidence indicates that nitrogen is generally the major limiting nutrient in Arctic soils (Shaver and Chapin III, 1980; Jonasson and Michelsen, 1996; Nordin *et al*., 2004) and its availability is dependent on decomposition processes and transformations of nitrogen compounds catalysed by microorganisms (Canfield *et al*., 2010). Despite the great taxonomic and functional diversity of these microorganisms and the fact that microbial community composition affects, and is affected by ecosystem functioning (Cavigelli and Robertson, 2000; Hortz *et al*., 2004; Reed and Martiny, 2007; Strickland *et al*., 2009), microbial populations and their role in the Arctic biogeochemical cycles remain greatly unexplored. Previous studies have indeed described spatial and temporal variations in microbial community composition across Arctic soils (Neufeld and Mohn, 2005; Zak and Kling, 2006; Wallenstein *et al*., 2007; Chu *et al*., 2010; Schütte *et al*., 2010) and as a response to increases in temperature and nutrient deposition (Campbell *et al*., 2010). Similar observations were made for few specific functional groups involved in the nitrogen cycle, namely variations in diazotroph (Deslippe *et al*., 2005; Deslippe and Egger, 2006; Walker *et al*., 2008) and denitrifier (Walker *et al*., 2008) communities in relation to location, vegetation cover and also in response to fertilization and changes in temperature. However, other crucial functional groups, such as nitrifiers, remain uncharacterized, even though nitrification has been extensively documented in Arctic soils (e.g. Nadelhoffer *et al*., 1991; Binkley *et al*., 1994) and is known to be strongly regulated by the environmental conditions (Booth *et al*., 2005).

Nitrification, the conversion of ammonia (NH_3) to nitrate (NO_3^-), via nitrite (NO_2^-), is catalysed by ammonia- and nitrite-oxidizing microorganisms and has been shown to consume approximately half of the nitrogen annually mineralized in some Arctic soils, reaching 100% consumption levels in individual years on a net process basis (Giblin *et al*., 1991). In addition, nitrification is also strongly involved in the emissions of nitrous oxide (N_2O) gas (Conrad, 1996), which has a warming potential over 300 times as high as carbon dioxide (CO_2), being considered the dominant ozone-depleting substance in the 21st century (Ravishankara *et al*., 2009). Nitrification indirectly regulates N_2O production through control over the availability of the substrates for denitrification (NO_3^- and NO_2^-) but also directly during the ammonia oxidation step and through nitrifier denitrification (Wrage *et al*., 2001; Öquist *et al*., 2007). It has indeed been shown that nitrification directly contributes most of the N_2O produced in some soils (Martikainen, 1985; Davidson *et al*., 1986; Mathieu *et al*., 2006), including in the Arctic (Ma *et al*., 2007; Siciliano *et al*., 2009), in comparison to other sources such as denitrification.

The autotrophic oxidation of ammonia is performed by both bacteria and archaea, although their relative contributions to nitrification in natural environments remains unclear (Nicol and Schleper, 2006; Prosser and Nicol, 2008). For over a century, ammonia-oxidizing bacteria (AOB) had been assumed to be the main responsible for this process in nature. However, in recent years it has been shown that archaea belonging to the newly defined phylum Thaumarchaeota (Brochier-Armanet *et al*., 2008; Spang *et al*., 2010) potentially play a major role in nitrification, given their wide distribution and high abundance in several environments, often being orders of magnitude more abundant than

AOB (Francis *et al*., 2005; Schleper *et al*., 2005; Leininger *et al*., 2006; Wuchter *et al*., 2006; Caffrey *et al*., 2007; Lam *et al*., 2007). It has been shown that ammonia-oxidizing archaea (AOA) are indeed capable of autotrophic ammonia oxidation in diverse environments, including in marine waters and sediments (Könneke *et al*., 2005; Walker *et al*., 2010; Blainey *et al*., 2011), hotsprings (de la Torre *et al*., 2008; Hatzenpichler *et al*., 2008; Reigstad *et al*., 2008) and soils (Offre *et al*., 2009 and 2011; Pratscher *et al*., 2011; Tourna *et al*., 2011; Xia *et al*., 2011; Zhang *et al*., 2011a). Additionally, several studies indicate that AOA appear to be generally more active in oligotrophic environments and under low nitrogen, low pH and low oxygen conditions (He *et al*., 2007; Lam *et al*., 2007; Hatzenpichler *et al*., 2008; Nicol *et al*., 2008; Erguder *et al*., 2009; Di *et al*., 2009 and 2010; Martens-Habbena *et al*., 2009; Gubry-Rangin *et al*., 2010; Molina *et al*., 2010; Verhamme *et al*., 2011). On the other hand, direct associations with these environmental factors and clear evidence for autotrophic carbon metabolism are restricted to few characterized AOA lineages and the ecophysiology and functional diversity of AOA remains largely uncharacterized (Pester *et al*., 2011). This greatly contrasts with the repertoire of physiological and metabolic properties described for the AOB during the last decades, including nitrifier denitrification (i.e. nitrifier denitrification) and usage of exogenous organic compounds (Geets *et al*., 2006; Arp *et al*., 2007). Nonetheless, the taxonomic diversity of AOA and their ubiquity in the environment strongly suggest that they might harbour a far greater metabolic and physiological potential. For example, the recently isolated thaumarchaeon *Nitrososphaera viennensis* has been shown to grow under higher nitrogen concentrations and different nutrient requirements than other isolated or enriched AOA (Tourna *et al*., 2011) and further evidence additionally suggests that at least some AOA might also be capable of heterotrophic or mixotrophic growth (Ingalls *et al*., 2006; Pester *et al*., 2011; Schleper and Nicol, 2011).

It has been hypothesized that the ecology and evolution of archaea is driven by their adaptation to chronic energy stress (Valentine, 2007), which is expected to be a common feature in Arctic terrestrial ecosystems as a result of the low temperatures, low nutrient availability, drastic freeze-thaw cycles and the short active seasons intercalated by long periods of low light availability. In fact, the largest proportion and greater diversity of archaea are present in cold environments (Cavicchioli, 2006) and lineages particularly affiliated with the thaumarchaeal ammonia-oxidizers are present in several low-temperature environments (Preston *et al*., 1996; Koch *et al*., 2007; Nakagawa *et al*., 2007; Ayton *et al*., 2009; Kalanetra *et al*., 2009). Moreover, culture-independent studies identified different thaumarchaea groups in soils from Siberia (Ochsenreiter *et al*., 2003) and in permafrost samples from the Canadian Arctic (Steven *et al*., 2007 and 2008), while other related groups of archaea were found in different Arctic soils from Svalbard (Høj *et al*., 2005, 2006 and 2008) and in Finnish boreal peatlands (Putkinen *et al*., 2009). Interestingly, thaumarchaea were also found across several alpine tundra sites, where no AOB were detected (Nemergut *et al*., 2008). Despite suggestions of the general occurrence of AOA in cold soils, Arctic landscapes are remarkably heterogeneous, which is likely to be reflected in the residing microbial communities. These regions comprise a great diversity of soil ecosystems, characterized by the interplay between distinct vegetation covers, cryoturbation processes and different geo- and

physicochemical properties, which in turn impose variable selective pressures upon microbial communities, often on very small scales.

Under the assumption that Arctic soils might constitute favourable environments for AOA and given the critical importance of nitrogen cycling processes in these ecosystems, the present work aimed at characterizing the AOA communities in several distinct high Arctic sites in relation to the soil properties and potential role in nitrification. In addition, a vast array of selective laboratory enrichment cultures was set up in order to further analyse the physiology and nitrifying potential of the AOA populations present in different tundra soils.

Materials and methods

Sampling sites description

Soil samples were collected from three distant Arctic geographic locations: Spitsbergen, in the Svalbard archipelago (78ºN), the main study-site; Zackenberg, in the eastern coast of Greenland (74ºN) and Tazovskiy, in western Siberia (67°N). Sampling in Spitsbergen was performed in the beginning of August of 2009, while sampling in Zackenberg and Tazovskiy was performed in the end of August of 2009. The sampling sites covered some of the most common Arctic tundra landscapes: moss, shrub and tussock dominated tundra, fen wetlands and cryoturbated soils, such as frost boils, each characterized by distinct geomorphologies, water regimes and vegetation type.

Moss and tussock tundra samples were collected from Longyearbyen and the Advent valley in Spitsbergen, respectively. The Longyearbyen site consisted of complex moss tundra located on a shallow slope with active solifluction. This process is characterized by the flow of water-saturated soil after the melting of the surface ice layer, where drainage is constrained by the underlying permafrost (as described in Høj *et al.,* 2006). In this site, this phenomenon resulted on a landscape with a “striped” appearance, where dryer hollows were intercalated by waterlogged ridges with more abundant vegetation, comprising mostly mosses. Vascular plants were also present, mainly on the dryer areas where mosses were sparsely distributed. Two samples were collected from the dry hollows and one wet soil sample was collected from a ridge adjacent to one of the sampled hollows (approximately 2 m apart). At the Advent valley, one sample was collected from a dry tussock tundra soil on an elevation along the river dominated by sedges of the genus *Eriophorum*, where dwarf-shrubs were also present.

Shrub tundra samples were collected from the Hottelneset peninsula in Spitsbergen, approximately 2 km northwest of Longyearbyen, and from Zackenberg and Tazovskiy. The Hottelneset site was located on a dry plateau close to the shore of the peninsula and it was dominated by dwarf shrubs of the *Dryas* genus, with lichens also occurring. The Zackenberg site consisted of typical shrub tundra with a pronounced moss layer, dominated by the genera *Salix*, *Dryas* and *Cassiope* (Ertl S., personal communication). One sample was collected at Hottelneset and two replicate samples at Zackenberg. At Tazovskiy, two distinct samples were collected from cryosols at close-by sites: one from a buried organic soil layer (Ajj horizon) on the lower part of a slopped hillside dominated by *Betula nana* and *Salix glauca* shrubs; and another from a mineral

layer (Ag horizon) on the top of a dry hill with more diverse and shorter vegetation, such as *Vaccinium* shrubs (Schnecker J., personal communication).

Tundra fen peat samples were collected from the shore of Lake Solvatnet, a protected bird sanctuary just outside the Ny-Ålesund settlement in Spitsbergen, and from the shore of a small lake at Knudsenheia, a marine terrace approximately 3 km northwest from Ny-Ålesund. The Solvatnet site consisted of a typical tundra fen wetland, which has been described before (Høj *et al.,* 2005, 2006 and 2008). The area surrounding the lake was covered with a dense waterlogged moss layer with frost boil formations of about 1 m in diameter and heavily influenced by Barnacle geese (*Branta leucopsis*) grubbing and Svalbard reindeer (*Rangifer tarandus plathyrynchus*) grazing. The Knudsenheia site was overall similar to the Solvatnet site, although consisting of a more complex system with frost boils of approximately 3 m in diameter. Animal influence was also evident from the animal dejections present in the area, although not as intense as in the Solvatnet site. Two replicate peat profiles were collected from each site.

Mineral soil upwellings were sampled from frost boils in the vicinity of the Solvatnet and Knudsenheia peat sampling sites. Frost boils (or non-sorted circles) are a form of patterned ground caused by cryoturbation, ubiquitous to the Arctic tundra. During this process, differential frost heave lead to the upwelling of deeper mineral soil, resulting in the formation of patches of barren or sparsely vegetated areas, usually surrounded by dense vegetation (Daanen *et al.*, 2008; Walker *et al.*, 2004 and references therein). In Solvatnet, the frost boils were covered with occasional small moss patches and abundant animal dejections, while at Knudsenheia the surface consisted essentially of bare mineral soil and rock fragments without any vegetation. One pooled sample was collected from the surface of each of the frost boils.

Sampling procedure

In Spitsbergen, moss, shrub and tussock tundra soil cores were collected with a 20 cm-long hand-held corer and stored in sealed clean plastic bags. Cores were transported in cooling bags and processed within few hours at the University Centre in Svalbard. After removal of the surface vegetation, the core was divided longitudinally and the undisturbed interior was sampled with sterilized metal spatulas. Samples were collected from the top 5 cm of every core and additionally at 15 cm depth from the Longyearbyen cores only.

The Zackenberg samples were collected at a depth between 5-10 cm, while the Tazovskiy organic and mineral samples were collected from dug pits, approximately at a depth between 30-35 cm and 5-15 cm, respectively. These samples were immediately stored in sterile cryotubes containing RNA*later*, kept at 4 ºC for 14 days and frozen at -20 ºC until further processing.

Fen peat samples were collected by cutting peat blocks of approximately 15x15 cm and variable height, depending on the depth of the underlying rocky layer. Plots with areas of 9 m^2 and 4 m^2 were set up at Solvatnet and Knudsenheia, respectively, and triplicate peat blocks were collected at random locations within each plot. At the laboratory, peat profiles were divided by top (aerobic) layer and one or two lower (anaerobic) layers, according to the distinguishable horizons. After separation, the anaerobic layers were quickly transferred to clean plastic bags, flushed with

dinitrogen and sealed. Triplicate peat layers were pooled and shredded by hand inside sealed bags before further processing.

Frost boils were sampled by digging a small pit of approximately 5 cm deep down to the underlying rocky layer. Samples were collected with sterilized metal spatulas and transferred into sterile 50 mL Falcon tubes. Three samples from unevenly distributed locations within each frost boil were collected and immediately pooled in the collection tubes. Both peat and frost boil soil samples were transported in cooling bags until processing at the laboratory in Ny-Ålesund.

All samples for molecular analyses were transferred to sterile cryotubes, flash-frozen and transported in a dry-shipper container until arrival at the laboratory in Vienna, where they were stored at -80 °C until analysis. Bulk soil samples for physicochemical analysis, nitrification measurements and enrichment cultures were stored at 4 °C and processed within approximately 15 days after sampling.

Soil physicochemical parameters

Soil gravimetric water content (moisture) was measured in duplicate or triplicate for each sample by drying 2 g mineral soil or 10 g peat at 80 ºC for 48 h. Values were calculated as percentage of fresh soil weight.

Soil pH was measured *in situ* with a pH electrode or at the laboratory, in the case of the dry soils. The later were performed in a suspension of 2 g soil in 4 mL milli-Q water. All measurements were done at least in duplicate.

Ammonium, nitrate and nitrite concentrations in the soil were determined as described in Hood-Nowotny *et al.* (2010) after extraction with either KCl (1 M) or $CaSO_4$ (10 mM). The slurries containing 1 g sieved soil or grinded peat and 10 mL extractant were incubated for 30 min with vigorous shaking prior to filtering with ash-free paper filters. Briefly, NH_4^+ was measured from the $CaSO_4$ extracts after oxidation to chloroamine by sodium dichloroisocyanuric acid, with subsequent formation of a green indophenol in the presence of phenolic compounds in an alkaline media. The absorbance was measured photometricaly at 660 nm and the concentration calculated from a series of 2-fold dilutions of a fresh NH_4Cl solution ranging from 0.014 to 1.750 mg NH_4^+-N L^{-1}. Nitrate was measured after extraction with KCl, by reduction to NO_2^- in acidic vanadium (III) chloride medium, directly coupled with the Griess reaction. The absorbance was measured photometrically at 540 nm and the concentration calculated from a series of 2-fold dilutions of a fresh KNO_3 solution ranging from 0.02 to 5 mg NO_3^--N L^{-1}. Nitrite from both extracts was measured with the Griess method and the concentrations were calculated from 8 dilutions of a fresh $NaNO_2$ solution ranging from 0.028 to 0.280 mg NO_2^--N L^{-1}.

Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured from the $CaSO_4$ extracts using a TOC/TN analyser (Shimadzu TOC-V CPH E200V with TNM-1 220V unit and ASI-V autosampler; Shimadzu, Vienna, Austria).

The dissolved inorganic nitrogen (DIN) was calculated as the sum of NH_4^+, NO_3^- and NO_2^-, while the dissolved organic nitrogen (DON) values were obtained by subtracting the DIN from the TDN value.

In situ and potential gross nitrification measurement

Gross nitrification rates were measured by a ^{15}N pool dilution assay. For each sample, 2 g sieved soil or 1 g peat were incubated in plastic vials with 0.5 mL $K^{15}NO_3$ (0.5 mM, 10 at % ^{15}N) at 15 ºC. This temperature is similar to the highest values measured at the region during the warm season (Westermann *et al*., 2011). For potential gross nitrification measurements, a solution of NH_4Cl was added to a final concentration between 1.7 and 2.5 mM. Five replicates for each sample were incubated for 4 or 24 h, for determination of the starting and ending time point, respectively. Reactions were stopped by addition of 15 mL KCl (2 M) and shaken for 1 h, following filtration through ash-free paper filters. The NH_4^+ initially present in the extracts was removed by conversion to gaseous NH_3 at high pH by addition of 100 mg MgO and incubation of the open vials for 3 days with frequent shaking. The NO_3^- pool was subsequently converted to NH_4^+ by addition of 0.5 g of the reducing catalyst Devarda's alloy and the NH_3 produced was isolated by microdiffusion into acid traps during a 5 days incubation. Each of the acids traps consisted of an ash-free filter paper disc containing 7.5 µL $KHSO_4$ (2.5 M) wrapped in Teflon tape. The acid traps were prepared for isotopic analysis by drying in a desiccator and subsequent transfer of the filter to tin capsules. Isotopic analyses and nitrification rates were performed as described in Inselbacher *et al*. (2007). ^{15}N enrichment was measured by continuous flow isotope ratio MS (IRMS) using an elemental analyser (EA 1110, CE Instruments, Milan, Italy). The elemental analyser was interfaced via a ConFlo II device (Finnigan MAT, Bremen, Germany) to the gas isotope ratio mass spectrometer (DeltaPLUS, Finnigan MAT).

AOA enrichments cultures set-up and nitrification activity determination

Soil samples from the top layer of all Svalbard sites and lower layers from the Longyearbyen sites only were used to inoculate 54 initial enrichment cultures. Four enrichment cultures were initiated from each soil by inoculating 1 g soil in sterile plastic vials with 20 mL of medium. Each of the four parallel cultures was incubated at 20 or 32 ºC, with either 0.2 or 0.5 mM NH_4Cl. Additionally, six cultures were incubated in 40 mL of medium and inoculated with 2 g of soil from either the Longyearbyen dry sites or Knudsenheia frost boil soils. One culture from each of these pairs was incubated at 20 ºC and the other at 32 ºC, with all six cultures amended with 0.5 mM NH_4Cl.

Fresh water medium (FWM) consisted of NaCl (1 g L^{-1}), $MgCl_2 \cdot 6H_2O$ (0.4 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.1 g L^{-1}), KH_2PO_4 (0.2 g L^{-1}) and KCl (0.5 g L^{-1}), FeNaEDTA solution (7.5 µM), sodium bicarbonate (2 mM) and 1 mL non-chelated trace element mixture (Könneke *et al*., 2005; Tourna *et al*., 2011). Additionally, 1 mL vitamin solution and $NaNO_2$ (0.1 mM) were added to the medium, and the pH was adjusted to 7.5. All solutions were prepared with milli-Q water and autoclaved or filter-sterilized in the case of heat-sensitive compounds. Streptomycin (50 µg mL^{-1}) was used as the default antibiotic in all cultures to selectively enrich for archaea.

Ammonia concentration in the enrichment cultures was measured at several time-points with the method previously described and used as indication of presence of potential ammonia-oxidizers. After a first enrichment stage, cultures where NH_4^+ was consumed were sub-cultured in a second stage with 20% inoculum in 20 mL fresh medium (30 cultures in total). In this second stage of

enrichment, sub-cultures with the same inoculum were incubated under the same different conditions as the initial enrichment step, with the exception that temperatures of 14 and 20 ºC were used. One of the initial enrichment cultures was used to inoculate eight additional sub-cultures incubated along a temperature gradient (17, 20, 32 and 37 ºC). This batch of sub-cultures was initiated with 10% inoculum in 20 mL of medium and incubated in pairs at each temperature, with either 0.2 mM or 0.5 mM. Cultures with high NH_4^+ consumption during the second stage of enrichment were used as inocula for a third stage of sub-culturing with 0.5 mM NH_4Cl under different temperatures and antibiotic treatments (32 cultures in total). In the third enrichment stage, nine sub-cultures with the same inoculum were incubated at 4, 20 and 28 ºC in groups of three, with each of the individual cultures treated with either streptomycin (50 µg mL^{-1}), ampicillin (100 µg mL^{-1}) or lysozyme. Sub-cultures with streptomycin incubated at 20 ºC were inoculated at 20% of the total volume (20 mL) and the remaining cultures with 7.5% inoculum. Lysozyme treatment was performed as described by Repaske (1956). The method is based on the principle that lysozyme, when co-adjuvated with EDTA (etylenediamine tetraacetic acid) and buffered by TRIS (tris(hydroxymethyl)aminomethane), will induce the lysis of both Gram positive and Gram negative bacteria. Briefly, the inocula were incubated with lysozyme (16.7 mg mL^{-1}), EDTA (0.9 mM, pH 7.5) and TRIS (100 µM, pH 8) for 30 min, and subsequently diluted in FWM up to a total volume of 20 mL.

The conversion of NH_4^+ to NO_2^- was verified in the third stage of enrichment culturing by measuring the concentration of both substrate and end-product at different time-points. Three hundred µL from each culture were centrifuged for 20 min in order to precipitate particulate matter, and 200 µL or 20 µL of the supernatant were respectively used for the photometric determination of NH_4^+ and NO_2^-, according to the protocols previously described. Control incubations without inocula were performed in parallel to the enrichment cultures under all conditions tested.

DNA extraction

DNA was extracted from 0.3 - 0.5 g mineral soil or from 0.2 g peat with the FastDNA® Spin Kit for Soil coupled with lysis in a FastPrep® instrument (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer protocols. Prior to extraction from the soil samples stored in RNA*later*, 1 g of each sample was washed three times with 1x PBS in a proportion of 1:5, resuspended in lysis buffer and transferred to Lysing matrix E tubes, followed by the same lysis and extraction protocols. Additional extraction steps with phenol:chloroform:isoamyl alcohol were also performed after the bead-beating step and before combining with the binding matrix, similar to what has been described by Abell *et al.* (2009). In parallel to the kit-based extractions, an optimized phenol:chloroform-based extraction method was performed for the peat samples (Urich *et al.*, 2008; Tveit *et al*., in preparation). Briefly, the peat samples were grinded in liquid nitrogen and 0.2 g of the resulting powder was transferred into a Lysing matrix E tube, following the lysis protocol mentioned above. Extraction was performed in the presence of phenol:chloroform and a potassium phosphate/CTAB buffer, with subsequent washing with chloroform:isoamyl alcohol and precipitation of the nucleic acids with PEG8000.

The DNA extracted from soils was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA yields were in the range of 85-495 ng/µL with an average of 250 ± 144 ng/µL (mean ± standard deviation) and an $A_{260/280}$ ratio of 1.67 ± 0.21.

DNA was extracted from enrichment cultures by collecting the cells from 1 mL of culture after centrifugation. Cell lysis was performed as previously described, followed by a standard phenol:chloroform extraction method and precipitation with PEG8000, similar to what has been described by Griffiths *et al*. (2000).

PCR, cloning and sequencing

Primers Arch-amoA-7F (5'-ATGGTCTGGBTDAGAMG-3') and Arch-amoA-638R (5'-GCRGCCATCCATCTRTA-3') were designed based on the alignment of nearly full-length *amo*A gene sequences from all the current isolated or enriched AOA, long environmental metagenomic sequences available in the GenBank database and unpublished sequences from a metagenomic fosmid clone library from soil (Bartossek *et al*., unpublished). Two mismatches with the *amo*A gene of *Candidatus* Cenarchaeum symbiosum were allowed in the reverse primer, prioritizing the amplification of soil-derived sequences by reducing the number of degenerated bases. These primers anneal to the same priming regions as the widely used primer pairs Arch-amoAF/Arch-amoAR (Francis *et al.*, 2005) and CrenamoA23f/CrenamoA616R (Tourna *et al.,* 2008), even though they have shorter sequence lengths and include additional degenerated bases to increase coverage. The positions on the primers' names were given taking the soil fosmid clone 54d9 *amo*A sequence as a reference (Treusch *et al.*, 2005). Primer specificity was checked with the BLAST algorithm at the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and analysis with the IDT OligoAnalyzer 3.1 web application (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) showed identical melting temperatures desirable for specific amplification. A gradient PCR was further performed within a range of 50 to 60 ºC to determine the optimal annealing temperature of the primer pair.

After testing different dilutions of extracted DNA for inhibitory effects by co-extracted compounds, either 30 or 60 ng of template DNA were used in each 50 µL PCR reaction, containing: 1.25 U of GoTaq® Flexi DNA Polymerase, 1 x Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 µM of each primer. Thermal conditions for the archaeal *amo*A PCR were as follows: 5 min initial denaturing step at 95 °C, followed by 35 cycles of 45 sec denaturing at 95 °C, 45 sec annealing at 55 °C and 45 sec extension at 72 °C, with a final extension step of 10 min at 72 °C. Bacterial *amo*A PCR was done with the primer pair amoA-1F/amoA-2R (Rotthauwe *et al.*, 1997) under the same conditions as for the archaeal *amo*A PCR, with the difference that only 30 sec were used for each of the denaturing, annealing and extension steps. PCR amplifications from the enrichment cultures were performed as above, with 3 µL of extracted DNA as template. Genomic DNA of *N. viennensis* was used as a positive control for the archaeal *amo*A, while genomic DNA of *Nitrosomonas europaea* and *Nitrosospira multiformis* were used in parallel as positive controls for the β-proteobacterial *amo*A. All PCR products were verified on 1.5% agarose gels stained with ethidium bromide under UV light.

The cloning PCR procedure followed the protocol above, with the exception that only 30 cycles were applied, and triplicate or quadruplicate reactions were performed in order to minimize PCR drift bias. Pooled PCR products were column-purified with the NucleoSpin® Extract II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the PCR clean-up protocol on the manufacturer's manual. Specific amplicons from the peat sample were gel-purified with the same kit according to the gel extraction protocol, given the co-amplification of unspecific products during PCR.

Clean archaeal *amo*A gene amplicons (ca. 630 bp) were cloned in TOP10 chemically competent *Escherichia coli* cells with the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Clones were selected for sequencing after confirmation of the correct insert size by M13 colony PCR and visualization on agarose gel electrophoresis. Plasmid extraction and sequencing of all clones were processed by LGC Genomics (Berlin, Germany).

RFLP fingerprinting

Restriction fragment length polymorphism (RFLP) analysis of the archaeal *amo*A gene was performed as an exploratory screening of general patterns in the AOA communities from the different soils and to allow the selection of samples for further molecular analysis. Peat samples were excluded from this analysis given the difficulty in obtaining a specific *amo*A PCR product with the concentration required for the digestion reaction. Archaeal *amo*A amplicons from enrichment cultures were also analysed by RFLP in order to assess potential differences in AOA communities growing under different conditions.

Selection of the restriction endonuclease was based on the *in silico* analysis of several selected *amo*A sequences from distinct groups of AOA available on GenBank, using the pDRAW32 (AcaClone Software, http://www.acaclone.com/) and TRiFLe packages (Junier *et al*., 2008). Even though the latter was primarily designed for T-RFs (terminal restriction fragments) analysis, it has proven useful to screen enzyme candidates based on multiple sequence alignments prior to the analysis with pDRAW32. The enzyme *Hpy*CH4V was selected given its ability to discriminate both between main groups of archaeal *amo*A genes and between closely related sequences within the same group. A similar result was obtained by Abell *et al.* (2009) based on clone library sequences and the same enzyme was successfully used for T-RFLP fingerprinting of AOA communities in sediments.

Amplification of *amo*A genes for RFLP analysis was performed as described above, followed by visual estimation of the PCR products' concentration based on their relative band intensity on the agarose gel. Similar amounts of PCR product from each sample were digested in 50 µL reactions, containing 1.5 U *Hpy*CH4V and 1x NEBuffer 4 (New England Biolabs GmbH, Frankfurt, Germany). Digestion reactions were performed at 37 ºC for 2 h, followed by 20 min at 65 ºC to inactivate the enzyme. Full reaction volumes were loaded and visualized on a 2.5 % agarose gel under UV light. The *amo*A of *N. viennensis* was used as a digestion control by comparison to the *in silico* predicted pattern and an additional column-purified amplicon of the same gene was used as control for gel artefacts derived from the PCR reaction mixture.

RFLP fingerprinting of enrichment cultures was performed as above, with the exception that *amo*A amplicons were column-purified prior to analysis and 100 ng of each clean PCR product was used as template for the digestion reaction. Electrophoresis and visualization of the products were performed on a 4% agarose gel.

Phylogenetic analysis

Obtained archaeal *amo*A gene sequences were imported into BioEdit Sequence Alignment Editor 7.0.9.0 software package (Hall, 1999), manually checked for sequencing errors and aligned with the CLUSTALW multiple alignment program (Thompson *et al.*, 1994) implemented in BioEdit. Sequence identity was verified by comparison with the GenBank database using the Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990). A total of 191 translated sequences with 197 amino acid residues were obtained from the soil samples and clustered into operational taxonomic units (OTUs) at 98, 97.5 and 97% amino acid sequence identity levels with the software mothur (Schloss *et al.,* 2009). An amoA amino acid sequence identity cut-off value of 97.5% was selected to define the AOA phylotypes and subsequently used for the phylogenetic and phylotype-based analyses. Representative clones of each phylotype were selected with mothur for the phylogeny reconstruction, with addition of their best BLASTX matches with the GenBank database, reference sequences and representatives of other main AOA lineages.

The best-fit model of amino acid substitution was selected with ProtTest (Abascal *et al*., 2005) and the Le and Gascuel substitution model (LG, Le and Gascuel, 2008) with uneven amino acid frequencies and site variation with four γ-rate categories was selected. Maximum-likelihood phylogenetic tree and bootstrap support values were calculated with PhyML 3.0 (Guindon and Gascuel, 2003; Guindon *et al.*, 2010) and the resulting tree was manually edited with the Tree Explorer included in the MEGA version 5 software package (Tamura *et al.*, 2011). The tree was manually rooted with the hyperthermophilic AOA lineage as the outgroup. The identity of the *amo*A sequences amplified from the enrichment cultures was verified by aligning with all the environmental clones, re-calculation of the OTU clusters at the same amino acid identity level and comparison with the phylogenetic tree previously calculated.

Diversity analysis

Heatmaps of phylotype relative abundance, corrected Chao1 richness estimator (S_{chao1}) and Shannon (*H'*) and Simpson (*D*) diversity indices were calculated with mothur for the selected amoA phylotype definition. Rarefaction analyses with a re-sampling without replacement approach were performed with mothur in order to estimate the coverage of the clone libraries. The Faith's phylogenetic diversity index (PD, Faith, 1992) was calculated with mothur as a divergence-based measure of α–diversity. The PD index estimates the proportion of branch length in a tree leading to the organisms in a community relative to the total branch length, thus being independent from *a priori* phylotype definitions (Lozupone and Knight, 2008).

The weighted UniFrac distance metric was used to compare and test how significantly different the AOA communities were. Like PD, UniFrac is a divergence-based approach that measures the

difference in overall community composition between any pair of communities based on the fraction of unique branch length in a phylogenetic tree leading to their collective sequence composition. The weighted version of UniFrac is a quantitative β–diversity measure that takes into account abundance information and, like the unweighted version, can also be used as a significance test of community dissimilarity by using Monte Carlo simulations (Lozupone and Knight, 2005; Lozupone *et al*., 2007). Selection of the Jones-Taylor-Thornton model of amino acid substitution with uneven amino acid frequencies and site variation with four γ-rate categories (JTT, Jones *et al*., 1992), and phylogenetic tree construction for the UniFrac and PD calculations were done as described for the phylogenetic analysis. UniFrac pairwise distances' calculation, unweighted pair group method with arithmetic averages (UPGMA) hierarchical clustering with jackknife analysis and UniFrac dissimilarity significance tests were performed with the Fast UniFrac online application (Hamady *et al*., 2010).

Multivariate statistical analysis

Several multivariate analyses based on distinct statistical assumptions were performed in order to assess potential relationships between AOA phylotype distribution and measured environmental variables. Multivariate analyses included only the data collected from Svalbard, since not all environmental parameters were available from the Siberia and Greenland samples. Given the uneven scaling of the environmental parameters measured, all values were Log(X+1) transformed prior to statistical analyses, with the exception of pH. Phylotype abundance data were standardized by the number of total counts, in order to express their relative abundances.

The BIO-ENV analysis implemented in the PRIMER 6 software (version 6.1.13 for Windows, PRIMER-E Ltd, Plymouth, UK) was used as an exploratory non-parametric approach, to infer potential associations between AOA phylotype distribution and measured environmental parameters. The BIO-ENV algorithm compares two (dis)similarity matrices derived from biotic and abiotic data, respectively, and expresses their degree of association by calculating the weighted Spearman's rank correlation coefficient (ρ_w). This method is independent from *a priori* biological or statistical assumptions and retains maximum freedom and flexibility when testing individual variables (Clarke and Ainsworth, 1993). Comparisons were based on Bray-Curtis similarity and Euclidean distance matrices calculated from AOA phylotype relative abundance data and environmental parameters, respectively. Comparisons were also performed between the environmental data distance matrix and unweighted and weighted UniFrac distance matrices. Monte Carlo permutation tests were used to assess whether the set of abiotic variables matched the phylotype distribution better than random. Since BIO-ENV is a function of the rank similarities on which non-metric multidimensional scaling (NMDS) relies on, NMDS plots were calculated as a complementary representation of the relations between AOA communities in the different soils. Plots were calculated with PRIMER 6, based on the Bray-Curtis similarity matrix of phylotype data.

Correlation analyses were performed with the XL Toolbox add-in for Microsoft® Excel® (version 2.80, http://xltoolbox.sourceforge.net/).

Canonical correspondence analysis (CCA) biplots of AOA phylotype distribution in response to

environmental variables were calculated with CANOCO (version 4.5 for Windows, Biometris-Plant Research International, Wageningen, The Netherlands). This approach determines the extent of community structure that can be explained by the environment, by plotting the phylotype distribution in an ordination space constrained by a small number of axes representing linear combinations of environmental variables (Ramette, 2007). Several simulations of manual forward selection were performed in CANOCO and only the models including the maximum set of environmental variables yielding overall significant canonical axes were analysed and represented as phylotype-environment biplots. The scaling in the final CCA biplots was focused on inter-species (phylotypes) relations.

Results and discussion

Sampling sites and soil characteristics

The location and characteristics of the sampling sites, as well as the measured physicochemical properties of the soil at the time of sampling are summarized in Table 1. Twelve soil samples were collected mainly from Spitsbergen (9 samples), but also from Tazovskiy (2 samples) and Zackenberg (1 sample), and were categorized according to their ecosystem type: shrub, tussock and moss tundra, frost boils and tundra fens. The physicochemical properties of the soils varied greatly, even between soils from similar tundra ecosystems or close-by sites.

The mineral soils had low moisture content, with the exception of a moss tundra site that was flooded at the time of sampling, leading to further separation of the moss tundra samples into “dry” (Lon-mt2 and Lon-mt3) and “wet” (Lon-mt1) soils. The fen wetland sites were water-saturated probably due to the high water table fed by the surrounding watersheds and also as a result of the low evapotranspiration levels caused by the moss layer. Although the frost boils were located in the fen areas, the lack of a continuous deep moss layer is likely to have contributed to higher moisture losses and consequently dryer soils.

Soil pH values ranged between 3.7 and 8.4, with the lowest values observed in the soils dominated by vascular plants, in particular shrubs. The only truly acidic samples were the organic and mineral layers from the Tazovskiy cryosols (3.8 and 3.7, respectively). The pH from the fen peat and frost boils was invariably within the neutral range (7.3 - 7.7), with also close-to neutral values measured in the moss tundra soils, even though with considerable variation between the three sites (7.7, 6.6 and 8.4 in the Lon-mt1, Lon-mt2 and Lon-mt3 sites, respectively).

The DOC content of the predominantly mineral soils was within 38 - 85 µg C g^{-1} dw soil, with the lowest values measured in the moss tundra soils (38 - 41 µg C g^{-1} dw soil). Expectedly, the fen peat soil samples contained DOC levels within a much higher range (465 - 939 µg C g^{-1} dw soil), as expected in soils mainly composed by decomposing organic matter.

The concentration of extractable dissolved nitrogen (TDN) was generally low in the mineral soils (10 - 30 µg N g^{-1} dw soil), with much higher values measured in the fen peat soils (139 - 192 µg N g^{-1} dw soil). Yet, the relative contribution of the different nitrogen chemical forms to the total nitrogen pools was extremely variable in relation to the TDN. In the shrub, tussock and moss tundra soils, NH_4^+ comprised most of the nitrogen present (50 - 95%), usually followed by the DON (2 - 41%)

Table 1 Sampling sites location, characteristics and measured soil physicochemical properties

Sample	*Site*	*Ecosystem type*	*Geographic coordinates*	*Moisture* % *fw soil*	*pH*	*DOC* $\mu g\ C\ g^{-1}$ *dw soil*	*TDN* $\mu g\ N\ g^{-1}$ *dw soil*	NH_4^+ $\mu g\ N\ g^{-1}$ *dw soil*	NO_3^- $\mu g\ N\ g^{-1}$ *dw soil*	*DON* $\mu g\ N\ g^{-1}$ *dw soil*	*C/N*	NO_3^-/NH_4^+
Hot-st	Hottelneset *(Svalbard)*	*Dryas*-type shrub tundra	N78°15'0" E15°26'53"	20.9	5.7	76.36	7.76	3.88	0.73	3.14	9.85	0.19
Adv-tt	Adventalen *(Svalbard)*	*Eriophorum*-type tussock tundra	N78°10'26" E16°1'29"	24.7	6.8	57.59	13.58	11.07	0.41	2.09	4.24	0.04
Lon-mt1	Longyearbyen *(Svalbard)*	Moss tundra with active solifluction	N78°13'14" E15°37'6'	41.1	7.7	50.35	6.01	4.29	0.35	1.37	8.38	0.08
Lon-mt2				19.9	8.4	40.63	12.86	12.22	0.40	0.24	3.16	0.03
Lon-mt3			N78°13'16" E15°37'5"	25.8	6.6	38.19	5.80	3.32	0.21	2.28	6.58	0.06
Sol-tf	Solvatnet *(Svalbard)*	Tundra fen wetland peat	N78°55'33" E11°56'37"	90.1	7.6	938.09	191.94	90.47	70.51	30.95	4.89	0.78
Sol-fb		Frost boil mineral upwelling		34.3	7.7	84.82	30.30	6.11	19.30	4.89	2.80	3.16
Knu-tf	Knudsenheia *(Svalbard)*	Tundra fen wetland peat	N78°56'33" E11°49'3"	90.7	7.3	464.90	138.47	43.81	83.46	11.21	3.36	1.91
Knu-fb		Frost boil mineral upwelling		17.1	7.6	48.91	10.30	6.07	2.48	1.75	4.75	0.41
Taz-stol	Tazovskiy *(Siberia)*	Shrub tundra buried organic layer	N67°25'35" E78°38'31"	52.5	3.8	*n.a.*	*n.a.*	46.17	< 0.01[a]	*n.a.*	*n.a.*	< 0.01
Taz-stml		Shrub tundra mineral layer		10.7	3.7	*n.a.*	*n.a.*	37.79	< 0.01[a]	*n.a.*	*n.a.*	< 0.01
Zac-st	Zackenberg *(Greenland)*	Shrub tundra	N74°29'52" W20°28'48"	*n.a. (dry)*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*

Abbreviations: DOC, dissolved organic carbon; DON, dissolved organic nitrogen; TDN, total dissolved nitrogen; C/N, dissolved organic carbon to total dissolved nitrogen ratio; dw, dry weight; fw, fresh weight; *n.a.*, not available.

[a] Below the detection limit

and very low proportions of NO_3^- (3 - 9%). Among these soils, the Hottelneset shrub and Lon-mt3 moss tundra soils appeared to have considerably higher DON proportions (41% and 39%, respectively). In contrast, the relative levels of NO_3^- were much higher in the fen peat and frost boil soils (24 - 64%), accounting for the majority of the nitrogen pool in the Solvatnet frost boil and Knudsenheia fen peat (64% and 60%, respectively). The lower relative DON levels in the peat soils might result from higher turn-over rates of the organic nitrogen through potentially high mineralization activity. Nitrite was below the detection limit in all soils tested. Animal activity seemed to have a considerable effect on the nitrogen pool in these soils, as observed from the higher levels of nitrogen, particularly NO_3^-, in the sites where geese and reindeer dejections were abundant. The proportion of NO_3^--N was especially high in the sample collected from the frost boil in the Solvatnet bird sanctuary, when compared to other mineral soils. Other studies have indeed shown a high accumulation of NO_3^- in Arctic soils under strong animal influence, namely under bird cliffs (Odasz, 1994). The vegetation type also appeared to be generally associated with NO_3^- concentrations, as suggested by the lower levels observed in soils where vascular plants were abundant. It has been previously shown that various common Arctic plant types preferentially take up inorganic nitrogen, in some cases strongly discriminating in favour of NO_3^- over other nitrogen species (Atkin, 1996; Nordin *et al*., 2004; Clemmensen *et al*., 2008). Together with animal faeces deposition, the nitrogen preferences of the vegetation might contribute considerably to the differences in the NO_3^-/NH_4^+ ratios observed (Table 1) and constitute major regulation mechanisms over the relative abundance of the different nitrogen species in these soils.

In situ and potential gross nitrification rates

Gross nitrification rates under *in situ* nitrogen conditions were measurable in all soils, with considerable variation, even between soils from close-by sites (Table 2). The rates measured in the mineral soils (0.42 - 6.95 µg N g^{-1} dw soil d^{-1}) were within the lower range of those detected with the same technique in other Arctic and alpine soils. However, these values were not fundamentally different from rates measured in moderate (non-cold) soils (Booth *et al.*, 2005). While nitrification has been documented in Arctic soils under low temperatures (Nadelhoffer *et al*., 1991; Binkley *et al.*, 1994) and has also been suggested to occur beneath a high Arctic glacier (Wynn *et al*., 2007), this process is known to increase with temperature up to an optimal range between 20 and 37 ºC (Stark, 1996; Tourna *et al*., 2008). It is thus possible that the similarity between the gross nitrification rates measured here, as well as in other Arctic regions, and those measured in warmer soils might partially result from the uniform incubation temperatures across studies. Despite the fact that the incubation temperature used here (15 ºC) is not uncommon in Svalbard during the warm season (Westermann *et al*., 2011), it is higher than the average temperatures and might lead to an overestimation of the usual *in situ* nitrification rates in these regions. On the other hand, gross nitrification rates in the fen peat soils were much higher (17.84 - 49.22 µg N g^{-1} dw soil d^{-1}). This is expected in highly organic soils when comparing values on a dry weight basis and also assuming higher substrate availability for nitrification, as a result of the potentially higher mineralization activity. Among the mineral soils, gross nitrification rates were generally lower in the drier soils (0.42

Table 2 *In situ* and potential gross nitrification rates after a 24 h incubation at 15 ºC. Potential nitrification rates were measured after amendment with NH_4^+ up to approximately 2 mM. Rate values are given as the mean and standard deviation of 3 to 5 replicates

Ecosystem type	*Sample*	*Gross nitrification rate* $\mu g\ N\ g^{-1}\ dw\ soil\ d^{-1}$	
		In situ	*Potential*
Shrub tundra	Hot-st	1.94 ± 0.47	5.20 ± 1.31
Tussock tundra	Adv-tt	1.62 ± 1.08	1.05 ± 0.40
Moss tundra	Lon-mt1	6.95 ± 2.43	0.24 ± 0.33
	Lon-mt3	0.42 ± 0.24	0.41 ± 0.12
Frost boil	Knu-fb	0.50 ± 0.30	2.05 ± 0.61
	Sol-fb	0.55 ± 0.30	0.66 ± 0.59
Tundra fen	Knu-tf	49.22 ± 12.44	47.42 ± 30.56
	Sol-tf	17.84 ± 10.69	131.13 ± 26.09

- 1.94 µg N g^{-1} dw soil d^{-1}), particularly in the frost boil and dry moss tundra soils (0.42 - 0.55 µg N g^{-1} dw soil d^{-1}). In contrast, *in situ* gross nitrification was substantially higher in the wet moss tundra soil (6.95 ± 2.43 µg N g^{-1} dw soil d^{-1}). Although the effect of moisture on nitrification was not directly addressed, the higher rates measured in the waterlogged moss tundra and fen sites suggested that moisture might have a positive effect on nitrification, in contrast to what has been reported for other Arctic soils (Chapin, 1996). However, the nitrification process measured here might have benefited from better aeration under the incubation conditions, compared to the *in situ* conditions.

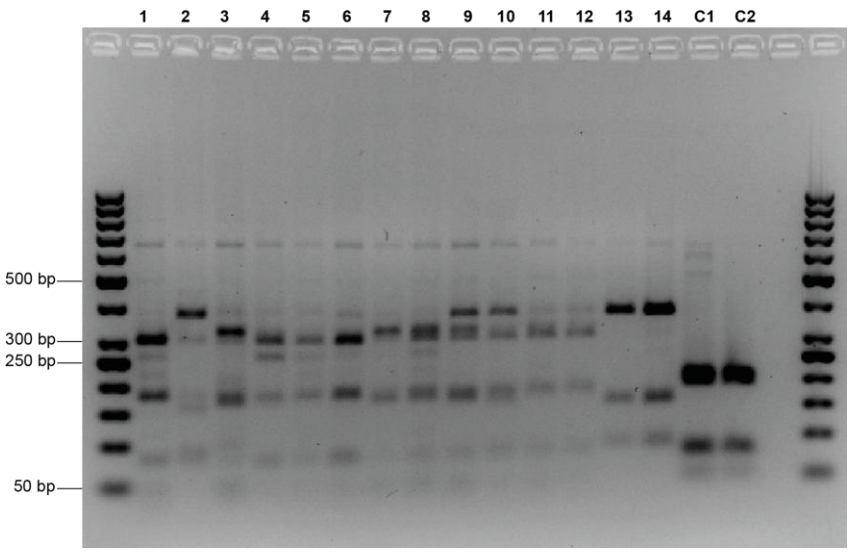
In half of the soils, namely in the Adv-tt, Sol-fb, Lon-mt3 and Knu-tf samples, potential gross nitrification rates, as measured after amendment with NH_4^+ to a final concentration of approximately 2 mM, were similar to the rates under *in situ* conditions (Table 2). On the other hand, potential nitrification rates were over 7-fold higher in the Sol-tf peat soil and 3- to 4-fold higher in the Hot-st shrub tundra and Knu-fb frost boil soils, showing a variability in the response to NH_4^+ amendment that was irrespective of the soil nature. In contrast, gross nitrification in the wet moss tundra soil (Lon-mt1) was almost completely inhibited under amended conditions (Table 2), which was particularly surprising given the low amount of NH_4^+ added.

Analysis of amo*A genes from archaeal and bacterial ammonia-oxidizers*

Archaeal *amo*A could be amplified by PCR from the top soil layer of all sites, with the exception of the Solvatnet fen soil, using the newly designed primer set based on *amo*A genes from all cultivated AOA and available long environmental sequences. In contrast, β-proteobacterial *amo*A genes could not be amplified from any of the shrub tundra soils, either from Spitsbergen, Tazovskiy or Zackenberg, or in the tussock and wet moss tundra soils from Spitsbergen (e.g. Spitsbergen soils in Figure 6). Not surprisingly, no archaeal or β-proteobacterial *amo*A genes were detected in the anoxic lower peat layers of the fen sites, since ammonia oxidation by all known AOA and AOB is an aerobic process. The presence of AOA in the Knudsenheia fen top soil, while being undetectable in the Solvatnet fen top soil, together with the presence of potential AOB in the top layers of both sites, was further supported by metatranscriptomic analyses of the same soils by Tveit *et al*. (in

preparation). Even though AOA or AOB detection could not be clearly explained by the properties of the soils, the generally lower pH values in the shrub and tussock tundra soils might be involved in the prevalence of AOA. In turn, the much higher DOC, NH_4^+ and DON concentrations in the Solvatnet fen site could in turn favour AOB dominance, in accordance with previous reports (Könneke *et al*., 2005; Nicol *et al*., 2008; Erguder *et al*., 2009; Martens-Habbena *et al*., 2009; Di *et al*., 2010; Verhamme *et al*., 2011).

RFLP fingerprinting of archaeal *amo*A amplicons revealed considerable pattern diversity between samples, with no consistent trend in relation to geographic location or soil type (Figure 1). This was evident from the different patterns observed between frost boils and between the three moss tundra samples collected from nearby locations. On the other hand, the two Tazovskiy samples showed the same pattern, despite being originated from distinct soil horizons at different locations. Comparison between the 5 cm and 15 cm depth samples from each of the Longyearbyen sites did not show substantial differences in pattern, with the possible exception of the Lon-mt2 site. The pattern observed in the lower layer from this site indeed appeared to show additional bands in relation to the top layer, which is in agreement with previous studies reporting variations in AOA diversity with soil depth (e.g. Höfferle *et al*., 2010). However, it was not possible to discriminate whether this apparent difference might have been an artefact from differences in amount of template digested or actually have reflected different AOA community compositions. The *amo*A amplified from the two replicate Zackenberg samples showed exact pattern matching, indicating a good reproducibility of the phylotypes detected. The overall similar patterns observed between the Tazovskiy and Hottelneset samples and between the Adventalen and Zackenberg samples suggest the presence of common AOA communities in distinct shrub tundra soils and in shrub and tussock tundra soils, regardless of geographic location. Despite the differences, the Longyearbyen samples



Cultures and conditions:

1 – Adv-tt
2 – Hot-st
3 – Sol-fb
4 – Knu-fb
5 – Lon-mt1 (5 cm deep)
6 – Lon-mt1 (15 cm deep)
7 – Lon-mt2 (5 cm deep)
8 – Lon-mt2 (15 cm deep)
9 – Lon-mt3 (5 cm deep)
10 – Lon-mt3 (15 cm deep)
11 – Zac-st (1)
12 – Zac-st (2)
13 – Taz-stml
14 – Taz-stol

Controls:

C1 – Non-purified *N. viennensis amo*A
C2 – Purified *N. viennensis amo*A

Figure 1 RFLP fingerprinting of archaeal *amo*A from soil samples. Purified and non-purified ddigested *amo*A genes from *N. viennensis* were used as control reactions. Main DNA ladder band sizes are indicated on the left-hand side of the picture.

showed considerable gene pattern overlap with the Adventalen, Zackenberg and Knudsenheia frost boil samples.

AOA diversity analysis

A total of 191 archaeal *amo*A gene sequences were obtained from 10 of the 11 soil samples where archaeal *amo*A could be detected, with an average of 19 ± 3 clones analysed *per* sample (Table 3). Given the complete overlap of the *amo*A RFLP patterns between the two Tazovskiy samples (Figure 1), only the Taz-stol sample was selected for clone library construction. Comparisons between AOA phylotype definitions of 97, 97.5 and 98% levels of maximum amino acid identity showed that: a 98% level resulted in a high number of unique and rare sequences, inflating the number of "rare" phylotypes within most samples to an extent that would greatly impede further comparisons between them; a 97% level resulted in the clustering of big groups of sequences dominating different samples, which would form distinct clusters at a slightly higher identity level (i.e. 97.5%). For instance, a level of ≥ 97.5% identity defined two groups of sequences comprising 86 and 82% of the clones from the Knu-fb and Sol-fb soils, respectively, while at ≥ 97% identity these groups would be clustered together, masking potential differences between these communities (data not shown). In order to preserve these subtle differences without greatly overestimating the intra-sample sequence diversity, a level of ≥ 97.5% amino acid identity was selected as the most parsimonious threshold, at least for the present dataset.

The number of AOA phylotypes observed (S_{obs}) at ≥ 97.5% amino acid identity level ranged between 1 and 7, while the S_{chao1} predicted richness estimator exceeded the observed value in some cases (Table 3). Despite the variability in the S_{obs} and S_{chao1} values between samples, the intra-sample richness of some communities was apparently underestimated, independently of the ecosystem type or number of sequences, as seen by the rarefaction analysis (Figure 2). In approximately half of the soils, the rarefaction curves nevertheless showed that most of the AOA richness was covered, despite the low number of clones sequenced (Figure 2). The shrub and

Table 3 Number of AOA phylotype richness observed (S_{obs}) and estimated (S_{chao1}), Shannon (H') and Simpson (D) diversity indices at ≥ 97.5% amino acid identity level, and Faith's phylogenetic diversity index (PD)

Ecosystem type	*Sample*	*No. of clones*	*Observed phylotype richness (S_{obs})*	*Estimated phylotype richness (S_{chao1})*	*Shannon index (H')*	*Simpson index (D)*	*Phylogenetic diversity index (PD)*
Shrub-tussock tundra	Hot-st	20	3	3	0.69	0.58	0.15
	Zac-st	20	3	3	0.52	0.72	0.06
	Taz-stol	20	1	1	0	1	0.03
	Adv-tt	17	2	2	0.22	0.88	0.05
Moss tundra	Lon-mt1	19	4	7	0.61	0.70	0.41
	Lon-mt2	22	4	4	0.86	0.54	0.09
	Lon-mt3	22	7	9	1.63	0.20	0.20
Frost boil	Sol-fb	17	3	3	0.58	0.68	0.14
	Knu-fb	22	4	7	0.55	0.74	0.11
Tundra fen	Knu-tf	12	3	4	0.57	0.68	0.10

tussock tundra soils showed overall lower phylotype richness when compared to most other soils, with the Tazovskiy buried organic layer being the only soil where only a single phylotype was detected. Even though the estimated richness of the Adventalen tussock tundra soil is in agreement with the number of phylotypes observed, the rarefaction curve does not level at that value, indicating that additional diversity was not detected (Figure 2). The wet and one of the dry moss tundra soils (Lon-mt1 and Lon-mt3, respectively), as well as one frost boil (Knu-fb) had the highest estimated richness values (S_{chao1} = 7, 9 and 7 phylotypes, respectively), and rarefaction analyses suggested that, at least in the case of the Lon-mt1 and Knu-fb samples, the values observed might have still underestimated the true richness present (Figure 2). This also appeared to be the case of the Knudsenheia fen site, which was clearly undersampled (Figure 2). The rarefaction curve of the phylotype-richest soil (Lon-mt3) supported the richness observed as a well-covered estimation for this site (Figure 2). Additional rarefaction of the non-parametric richness estimator S_{chao1} was performed, as suggested by Hughes *et al*. (2001), and the resulting richness estimation curves confirmed the trends observed for all soils with the re-sampling rarefaction approach (data not shown).

The Shannon (*H'*) and Simpson (*D*) diversity indices did not directly reflect the relative phylotype richness of the samples, indicating that the AOA phylotypes detected were not evenly distributed within the communities (Table 3). Both indices yielded similar relative values, with minor differences reflecting their intrinsic weighting of the dominant and rare phylotypes (Hill *et al*., 2003). Among all soils, AOA communities in the dry moss tundra soils (Lon-mt2 and Lon-mt3) had the highest phylotype diversity. This observation might be partially due to the terrain characteristics at these two sites, given their location on a slope with active solifluction, which can possibly promote AOA

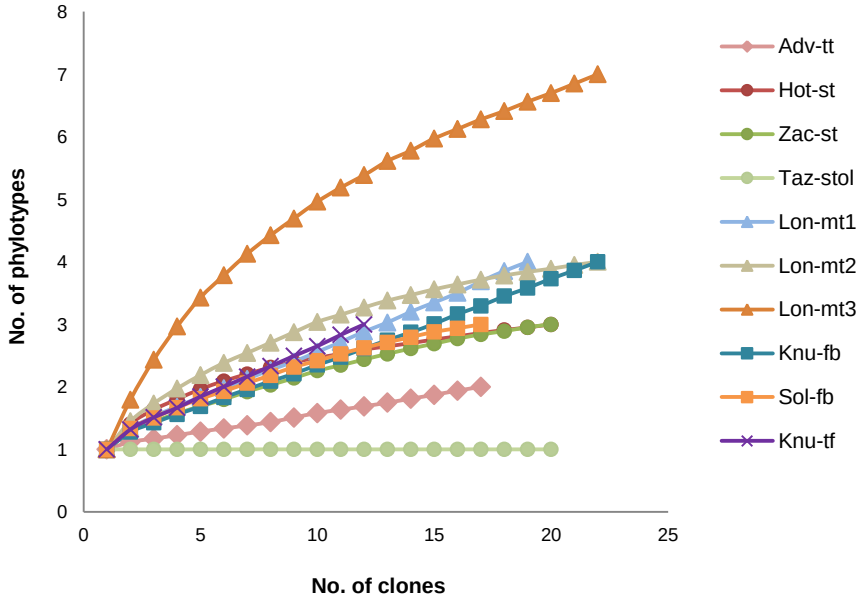


Figure 2 Rarefaction curves of the phylotype richness observed in the clone libraries from the 10 soils analysed. Phylotypes were defined at ≥ 97.5% amino acid identity level. Different symbols represent distinct soils: (◇) tussock tundra; (○) shrub tundra; (△) moss tundra; (□) frost boils and (**x**) tundra fen.

immigration from upstream soils. The more diverse vegetation cover and overall moderate physicochemical properties of these soils, in comparison to the other sites, might in turn allow the colonization and permanence of more diverse AOA communities. The lowest diversity was observed in shrub and tussock soils, in particular in the Taz-stol and Adv-tt samples, reflecting, not only the low richness detected in these sites, but also low evenness in community composition. However, the diversity estimated for the Adv-tt sample was likely biased by the underestimation of the phylotype richness mentioned above. On the other hand, the Tazovskiy sites had the only truly acidic pH values measured, which are known to be a major stress factor for microbial growth (Boot *et al*., 2002) and thus being prone to strongly select restricted microbial populations capable of coping with this environmental pressure. Therefore, it seems possible that the lower pH of these soils might be involved in the selection of a more limited AOA diversity. Even though the wet moss tundra (Lon-mt1) and Knudsenheia frost boil (Knu-fb) samples had the same observed richness as the Lon-mt2 sample, their diversity was considerably lower. This indicated low evenness in the community composition of the Knu-fb and Lon-mt1 soils, suggesting the prevalence of a dominant group of AOA. Similar diversity trends were obtained with the PD index, with the shrub and tussock tundra soils showing the lowest values, while the highest were observed in moss tundra soils (Table 3). In contrast to the phylotype-based diversity measures, the Lon-mt2 sample showed a relatively low phylogenetic diversity index (PD), probably as a result of the close phylogenetic relatedness of the AOA present. Despite the intermediate Shannon and Simpson indices of the Lon-mt1 community, in comparison to the other communities, the PD index was disproportionally higher as a result of the higher divergence between the AOA lineages present.

The pairwise weighted UniFrac metric was used to determine the significance of the overall dissimilarity between different AOA communities. The selection of the weighted UniFrac metric was due to two main reasons: first, given the low number of clones sequenced for some of the samples, there is a higher probability of not detecting rare taxa that might strongly influence the fraction of unique branch length leading to that community. Therefore, a (significance) test that relies on presence/absence data (i.e. unweighted UniFrac) is potentially more biased when applied to a small dataset. On the other hand, when abundance information is taken into account (i.e. weighted UniFrac), more weight will be given to the most abundant taxa and thus compensating, to some extent, the biases introduced by missing rare information. Second, significance analysis with the unweighted UniFrac algorithm is sensitive to whether the data has been dereplicated or not, implying that OTUs must be selected in order to obtain a true qualitative measure (Lozupone and Knight, 2008). This imposition hinders the circumvention of an *a priori* OTU definition, compromising the independency of the analysis intended here. The weighted UniFrac significance test showed a separation of the AOA communities between four major distinct groups with considerable segregation according to type of tundra ecosystem, but without an obvious geographic patterning, either between Svalbard, Siberia and Greenland soils, or within Svalbard soils. Both the fen peat (Knu-tf) and the wet moss tundra (Lon-mt1) soils harboured completely distinct AOA communities, with an overall phylogenetic composition that was significantly different from every other soil at a high level of confidence ($P \leq 0.001$). In turn, the Hottelneset and Tazovskiy shrub tundra soils (Hot-

st and Taz-stol, respectively) shared phylogenetically related communities ($P > 1$) that were significantly different from those present at the other sites ($P \leq 0.001$). The frost boils, the Zackenberg shrub, Adventalen tussock and Longyearbyen dry moss tundra sites appeared to harbor phylogenetically related AOA populations, which were not significantly different in any of the pairwise comparisons ($P > 1$).

AOA phylogenetic analysis

The phylogenetic analysis of the translated *amo*A sequences revealed a remarkable breadth of diversity spanning through the major known lineages of AOA, with the exception of the hyperthermophilic group (Figure 3). The archaeal *amo*A genes detected were distributed between four main groups and followed a clustering pattern that reflected the type of tundra ecosystem (i.e. habitat), similar to the overall community separation observed with the weighted UniFrac significance test. Furthermore, some AOA phylotypes showed great local specificity even within the same group (Figure 3), particularly the dominant phylotypes present in the frost boils, fen peat and wet moss tundra sites. In turn, the communities present in the dry moss, shrub and tussock tundra sites showed substantial overlapping. Despite the broad phylogenetic diversity of the AOA identified, each individual population was clearly dominated by a single phylotype. This uneven distribution of the AOA groups between sites was reflected on the general low intra-sample diversity observed when both phylotype richness and evenness were considered.

Most of the AOA identified were affiliated with the broad soil/sediment lineage, while only two related phylotypes were affiliated with the lineage generally associated with marine environments, to which *Nitrosopumilus maritimus* and *Candidatus* C. symbiosum belong (Prosser and Nicol, 2008). The AOA affiliated with the soil/sediments-like lineage clustered within three major groups: a widespread and abundant group (group A), encompassing three separate clusters that included the dominant phylotypes in the frost boils, tussock-shrub and dry moss tundra, respectively; a second group (group B) containing a shrub tundra dominated cluster and a low abundant phylotype present in the moss tundra; and a deep-branching group (group C) comprising the tundra fen cluster, also weakly detected in the frost boils (Figure 3). The greatest proportion and diversity of sequences were associated with the AOA soil group A (Figure 3) represented by the reference fosmid clone 54d9 (Treusch *et al*., 2005), which has been previously suggested to be the most abundant and widespread across the globe (Bates *et al*., 2010). Curiously, the group A phylotypes dominating and exclusively detected in the Solvatnet frost boil (FFB-2 and FFB-3), as well as one of the dominant phylotypes in tussock and shrub tundra soils, also present in the moss tundra (S-T-DM) were closely related to sequences from cold or high altitude environments. These included soils from the Mount Everest plateau (Zhang *et al*., 2009), the Inner Mongolia steppe (Zhang *et al*., 2011b), alpine soils (Nicol *et al*., unpublished) and sediments from a lake in the Tibetan plateau (Jiang *et al*., unpublished). This was also the case of the FP-2 phylotype affiliated with the basal group C, which was only detected in the Knudsenheia fen peat. The majority of the sequences retrieved from the dry moss and shrub tundra soils were related with sequences detected in soils potentially subject to high nitrogen inputs, such as those under agricultural practice. In fact, the phylotypes dominating or

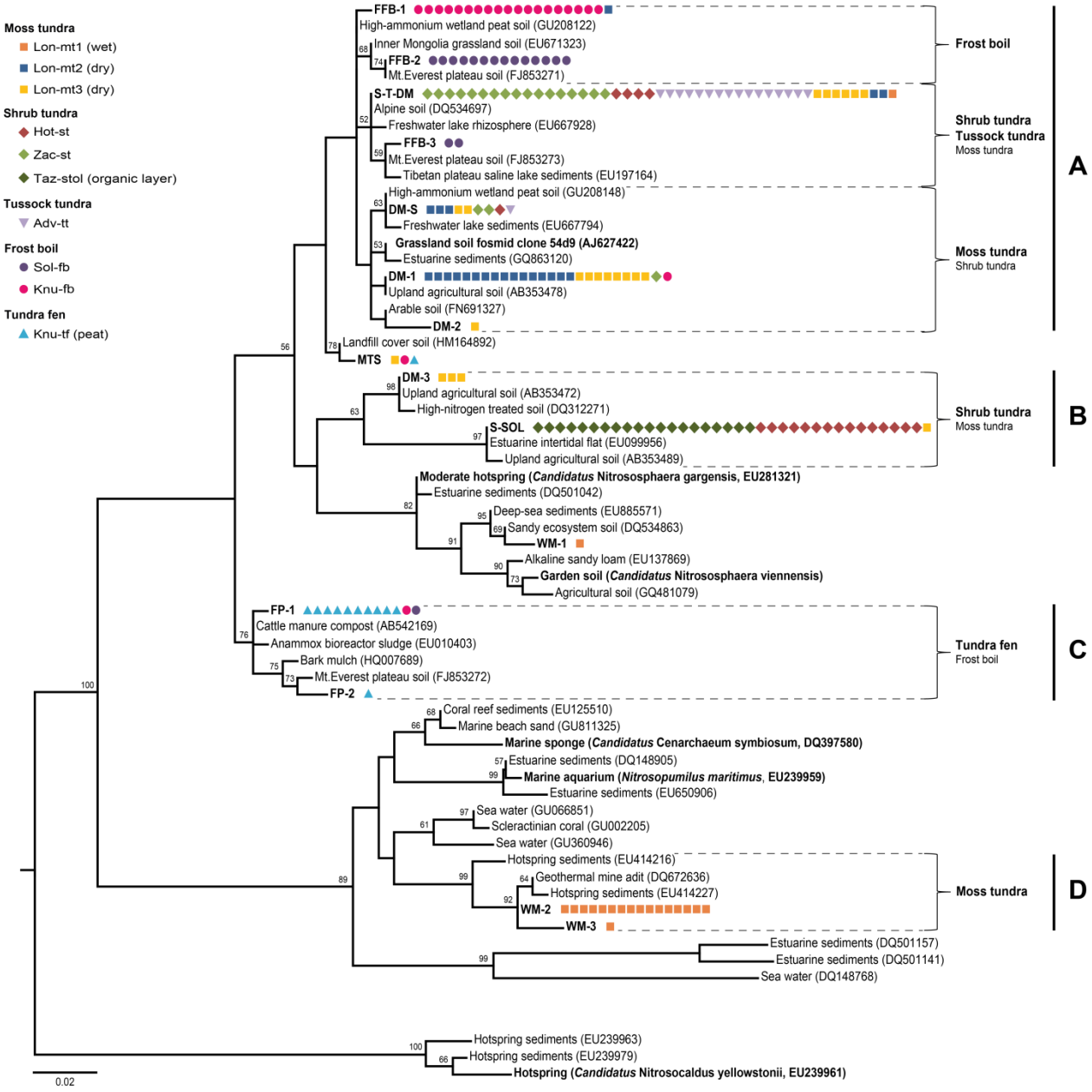


Figure 3 Maximum-likelihood phylogenetic tree of archaeal amoA based on the alignment of 62 sequences, including a representative of each phylotype (≥ 97.5% amino acid identity), closest non-redundant BLASTX hits with GenBank, sequences from all the available isolated or enriched species and representatives of major known lineages. Pairwise distances of 197 amino acid positions were calculated with the LG substitution model with uneven amino acid frequencies and site variation (four γ rate categories). The percentage of bootstrap replicates supporting the taxa clusters with values > 50% (1000 replicates) are represented close to the nodes. Phylotypes from this study, isolates, enrichment cultures and the soil fosmid clone 54d9 are indicated in bold. Reference sequences are named according to "Environmental source (accession number)". Clones from identical environments (shrub, tussock and moss tundra, frost boils and tundra fen) are indicated with the same symbol and colour-coded according to individual samples. Capital letters in bold on the right-hand side of the tree represent the main groups detected. Environment names inside each group refer to phylotype clusters according to their major environmental sources in this study.

exclusively detected in the dry Longyearbyen sites (DM-1, DM-2 and DM-3) and in the Hottelneset and Tazovskiy sites (S-SOL) were mostly related to sequences from upland agricultural soils (Jia and Conrad, 2009; Fan *et al*., unpublished) or soils under high nitrogen treatment (Avrahami *et al*., unpublished). The S-SOL phylotype was also related to a sequence retrieved from an estuarine intertidal flat (Magalhães *et al*., 2009), which might have been originated by terrestrial soil runoff from the agricultural areas along the river. Furthermore, a low abundant phylotype in both dry moss and shrub tundra (DM-S) and the dominant phylotype in the Knudsenheia frost boil (FFB-1) were associated with sequences from a high ammonium wetland peat soil (Höfferle *et al*., 2010). Only one phylotype represented by a single sequence amplified from the wet moss tundra soil (WM-1) was affiliated with the recently characterized *Nitrososphaera* clade, which includes the only current soil isolate *N. viennensis* (Tourna *et al*., 2011) and the enriched moderate thermophile *Candidatus* Nitrososphaera gargensis (Hatzenpichler *et al*., 2008). Interestingly, the group C AOA phylotype dominating the Knudsenheia tundra fen peat was associated with sequences from anoxic and/or highly organic and nitrogen-rich waste decomposing environments, such as the granular sludge from an anammox (anaerobic ammonia oxidation) bioreactor (Bae *et al*., 2010) and cattle manure compost (Yamamoto *et al*., unpublished). This was consistent with the expected low oxygen availability and the high DOC and TDN concentrations measured in the Knudsenheia tundra fen peat.

The only two phylotypes belonging to the marine/sediment lineage (WM-2 and WM-3) were exclusively detected in the waterlogged moss tundra soil, where they comprised 89% of the sequences amplified. Although this lineage is generally associated with marine environments, it encompasses a broad diversity of groups that have been detected in several other environments, including soils, hotsprings and freshwater systems (Prosser and Nicol, 2008). Surprisingly, these phylotypes were related to sequences from geothermal environments with temperatures up to 95 ºC (Spear *et al*., 2007; Reigstad *et al*., 2008), suggesting that closely related archaea might possess an astonishing range of physiological adaptations that allow them to thrive in both cold soils and geothermal springs or that physiologically distinct archaea might harbor similar AMO proteins.

AOA distribution in relation to environmental factors

The clustering of the AOA phylotypes within group A (Figure 3) appeared to reflect the overall similarity of the soil properties at the sites were this group dominated (Table 1). The habitat-specific clustering of AOA phylotypes within this group, despite of geographic location, suggested that specific environmental factors might have been involved in the partitioning observed. For instance, the frost boil-specific cluster (Figure 3) could only be associated with the particularly higher NO_3^--N and lower NH_4^+-N relative proportions, as well as with higher NO_3^-/NH_4^+ ratios (Table 1). In turn, the soils dominated by the tussock-shrub cluster (Figure 3) were characterized by generally lower pH values (Table 1). Additionally, and in contrast to other soils dominated by phylotypes within group A, these were the only sites densely covered by vascular plants (see "*Sampling sites description*" under "Materials and methods"), which are known to influence the communities of N-cycling microorganisms, including nitrifiers (Patra *et al*., 2006). Plants interact with the microbiota through

several mechanisms (Chapman *et al*., 2005; Skiba *et al*., 2011) and litter composition in particular has indeed been shown to influence Arctic bacterial and fungal communities (Wallenstein *et al*., 2007; Campbell *et al*., 2010). However, the convergence of AOA communities present in sites with distinct dominant vegetation (i.e. shrub vs. tussock) suggests that possible effects are likely to be related to competition for the available nitrogen or to plant-microbiota interactions within the rhizosphere. Despite the fact that both Hot-st and Taz-stol samples were originated from shrub tundra soils, the almost complete overlap between their AOA communities within group B was surprising, since the Hot-st sample was collected from a dry top soil layer while Taz-stol was collected from a moisty buried organic soil layer. Although extensive comparisons between the two samples were hindered by the limited environmental data available for the Taz-stol soil, the pH values measured in both soils were the lowest among the soils analysed (Table 1). This further emphasized the possible involvement of pH in the selection of the AOA groups present in shrub tundra soils, especially those affiliated with group B. This was also supported by the identical pH values and exact matching of the *amo*A RFLP patterns in the two Tazovskiy samples (Figure 1), regardless of the different soil depth and fundamentally different nature of the soils, i.e. organic vs. mineral (see "*Sampling sites description*" under "Materials and methods"). Only one soil (Lon-mt2) had a pH value within the alkaline range (8.4), which, unlike in the soils with lower pH values, was apparently not reflected on the resident AOA population, since all phylotypes in this soil were also present in soils with neutral pH values. In contrast to the gradual effect of soil pH observed on bacterial communities (Fierer and Jackson, 2006), it appeared that AOA were not equally affected by pH levels, at least within the pH range measured here. Instead, these observations suggest that acidic conditions have a stronger selective effect on AOA community composition leading to the selection of specific groups, rather than acting as a general regulator upon AOA diversity, in similarity to what has been reported by Nicol *et al*. (2008). The extent of the phylogenetic diversity observed in soils with similar pH values indeed indicated that other factors have a stronger selective effect on overall AOA community composition.

The AOA populations in the two dry moss tundra contrasted remarkably from those in the adjacent wet moss tundra over scales as small as 2 m, although all sites shared the same ecosystem and similar pH, DOC and nitrogen compositions. The only distinctive features of the wet site dominated by group D, in comparison to the dry sites dominated by group A, were the higher abundance of vegetation and the higher soil moisture. Although moisture have indeed been shown to influence AOA community composition in incubation experiments (Gleeson *et al*., 2010; Szukics *et al*., 2010), it is nevertheless probable that these drastic differences in AOA community composition resulted from factors not covered in the present study.

Tundra fen peatlands are fundamentally different ecosystems than tundra mineral soils, since they consist mostly of slowly decomposing organic matter, often under water saturation conditions and low oxygen availability. Additionally, the thick moss layer present in these sites has a strong effect on the thermal and hydrological balance of the underlying soils, contributing also to distinct soil chemistries and nutrient cycling processes, when compared to mineral soils dominated by vascular plants (Jonasson and Shaver, 1999; Gornall *et al*., 2007; Turetsky *et al*., 2008). Hence, it was not surprising that the Knudsenheia top peat layer harboured a very distinct and almost

exclusive AOA population (group C), possibly adapted to the highly organic carbon- and nitrogen-rich conditions of the peat soil (Table 1). It seems likely that organisms from this group are capable of nitrifying under very different conditions than previously described for AOA, possibly harbouring fundamentally distinct physiologic and metabolic properties that allow them to thrive in this habitat.

Potential relations between AOA community composition and the environment were further investigated using constrained (i.e. CCA) and non-constrained (i.e. BIO-ENV and NMDS) multivariate methods based on the relative abundance of the AOA phylotypes. Despite potential biases associated with small sequence sample size and diversity coverage, the dominance of most AOA populations in this study by a single phylotype suggested that the expected increase in diversity as a result of a bigger dataset would be unlikely to diminish significantly the relative contribution of the dominant phylotypes, consequently exerting little effect on the relations between the communities. The BIO-ENV analysis showed that the NO_3^-/NH_4^+ ratio and NO_3^- concentration had the best association with AOA community composition among the individual environmental parameters, with high rank correlation coefficients (ρ_w = 0.636 and 0.546, respectively; Table 4). Moisture content and TDN also showed a considerable degree of association (ρ_w = 0.429 and 0.328, respectively), with all the remaining variables tested showing low relative ρ_w values. When all possible combinations between the six non-reducible variables were taken into account in the BIO-ENV analysis, the combination between NO_3^- and moisture, or between these two and DOC,

Table 4 BIO-ENV analyses based on the weighted Spearman's rank correlation coefficient (ρ_w), showing the association between AOA community composition and environmental variables. In the first model, ρ_w was calculated for every individual variable (k = 1), while in the second it was calculated for the best ten combinations between all six non-reducible variables (k = 6). The Monte Carlo permutation test was used to calculate the probability of the rank correlations to match the communities' composition better than random (1000 randomizations)

Individual factors (k = 1)	*Spearman's coefficient (ρ_w)**	*Combined factors (k = 6)*	*Spearman's coefficient (ρ_w)***
NO_3^-/NH_4^+	0.636	NO_3^- + Moisture	0.728
NO_3^-	0.546	NO_3^- + Moisture + DOC	0.709
Moisture	0.429	NO_3^- + Moisture + DON	0.566
TDN	0.328	NO_3^- + DOC	0.557
DIN	0.173	NO_3^-	0.546
DON	0.161	NO_3^- + Moisture + pH	0.536
DOC	0.145	NO_3^- + Moisture + DOC + DON	0.511
NH_4^+	-0.084	NO_3^- + Moisture + DON + pH	0.503
C/N	-0.100	NO_3^- + Moisture + DOC + pH	0.493
pH	-0.192	NO_3^- + Moisture + NH_4^+	0.491

Abbreviations: DOC, dissolved organic carbon; DIN, dissolved inorganic nitrogen; DON, dissolved organic nitrogen; TDN, total dissolved nitrogen; C/N, dissolved organic carbon to total dissolved nitrogen ratio; k, number of possible combined variables in the model.

* $P < 0.05$: ** $P < 0.01$

showed the highest association with community structure, with rank correlation coefficients higher than any of the variables alone (Table 4). Further combinations did not substantially add up to the explanatory power, yielding ρ_w values only slightly higher than that of NO_3^- alone. Comparisons between the environmental data distance matrix and the weighted and unweighted UniFrac distance matrices using BIO-ENV resulted in very low rank correlation coefficients and non-significant association models (data not shown), showing that the association between these AOA communities and their environment could not be explained by their overall taxonomic relations.

The result of the BIO-ENV analysis was clearly illustrated in the NMDS ordination of the AOA communities by the separation between communities under low and high NO_3^-/NH_4^+ ratios and NO_3^- concentrations, with only the Knu-fb community associated with intermediate values (Figure 4). Furthermore, a considerable clustering of the communities in soils with low moisture content was also evident (moisture ≤ 26 % fw soil).

Two sets of variables were subject to forward selection and tested with CCA: the first set included all parameters resulting from direct measurements, while the second set included variables representing potentially relevant relationships between parameters (i.e. ratio values) and all other non-redundant variables (as opposed to C/N vs. DOC and TDN). Both CCA representations showed a consistent separation of AOA populations according to the tundra ecosystem type, with

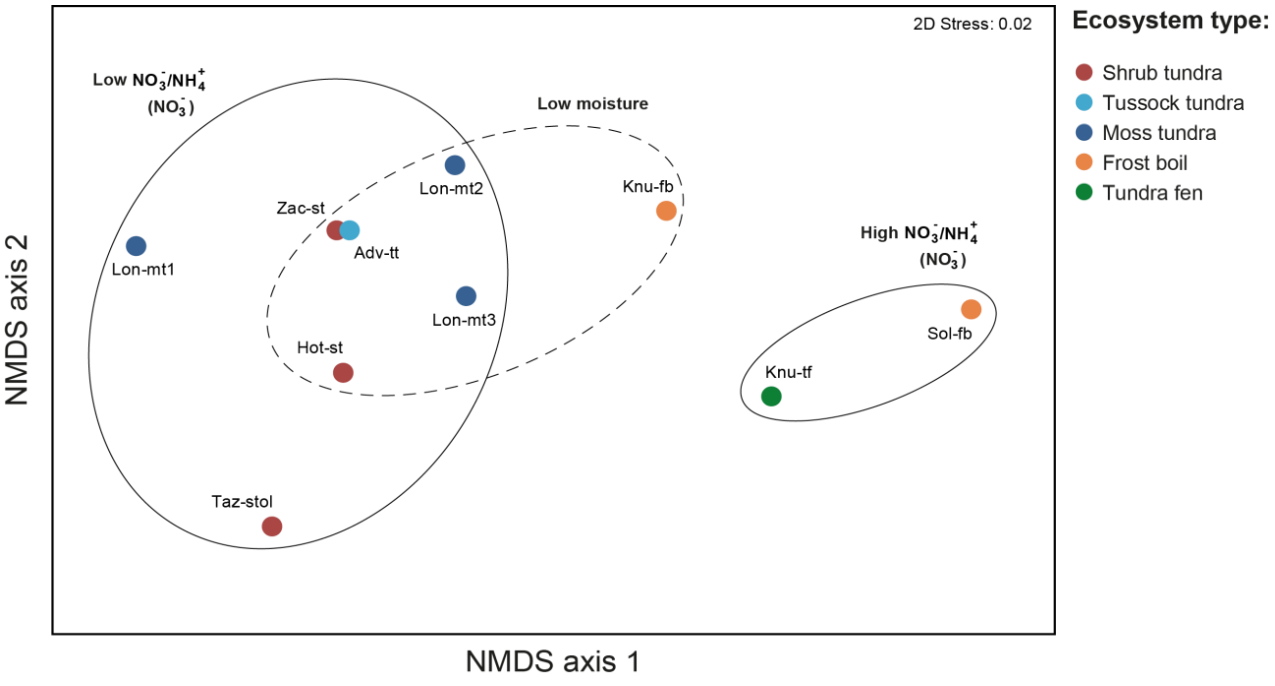


Figure 4 NMDS plot representing relations between AOA communities in the different soils, based on the phylotype relative abundance (1000 re-starts). Different soils are colour-coded according to ecosystem type of origin and the ellipses highlight the clustering according to the environmental variables showing stronger individual association with AOA community structure in the BIO-ENV analysis. Continuous lines depict communities clustered under low NO_3^-/NH_4^+ (≤ 0.19) and NO_3^- (≤ 0.73 µg N g^{-1} dw soil), and high NO_3^-/NH_4^+ (≥ 1.91) and NO_3^- (≥ 19.3 µg N g^{-1} dw soil). The discontinuous line represents the clustering of communities in soils with low moisture content.

the dominant phylotypes in shrub-tussock tundra and in dry moss tundra soils forming separate clusters that were nevertheless closely represented in the ordination (Figure 5). Like in the NMDS ordination (Figure 4), there was a separation of phylotypes dominating close-by sites or similar ecosystems, such as wet and dry moss tundra soils, different frost boils, and the fen peat and frost boil sites from Knudsenheia. These two levels of AOA community partitioning, i.e. between ecosystems and between sites, regardless of the ecosystem, further illustrated the clustering observed in the phylogenetic analysis (Figure 3) and the community dissimilarities obtained with the weighted UniFrac. Among the possible combinations of non-reducible variables, only NO_3^-, moisture content and DOC combined (model 1; Figure 5a) yielded a constrained ordination where the overall canonical axes were statistically significant (P = 0.005; Table 5), explaining 52.2% of the variance in phylotype distribution detected (Table 6). Nitrate explained the highest percentage of variance

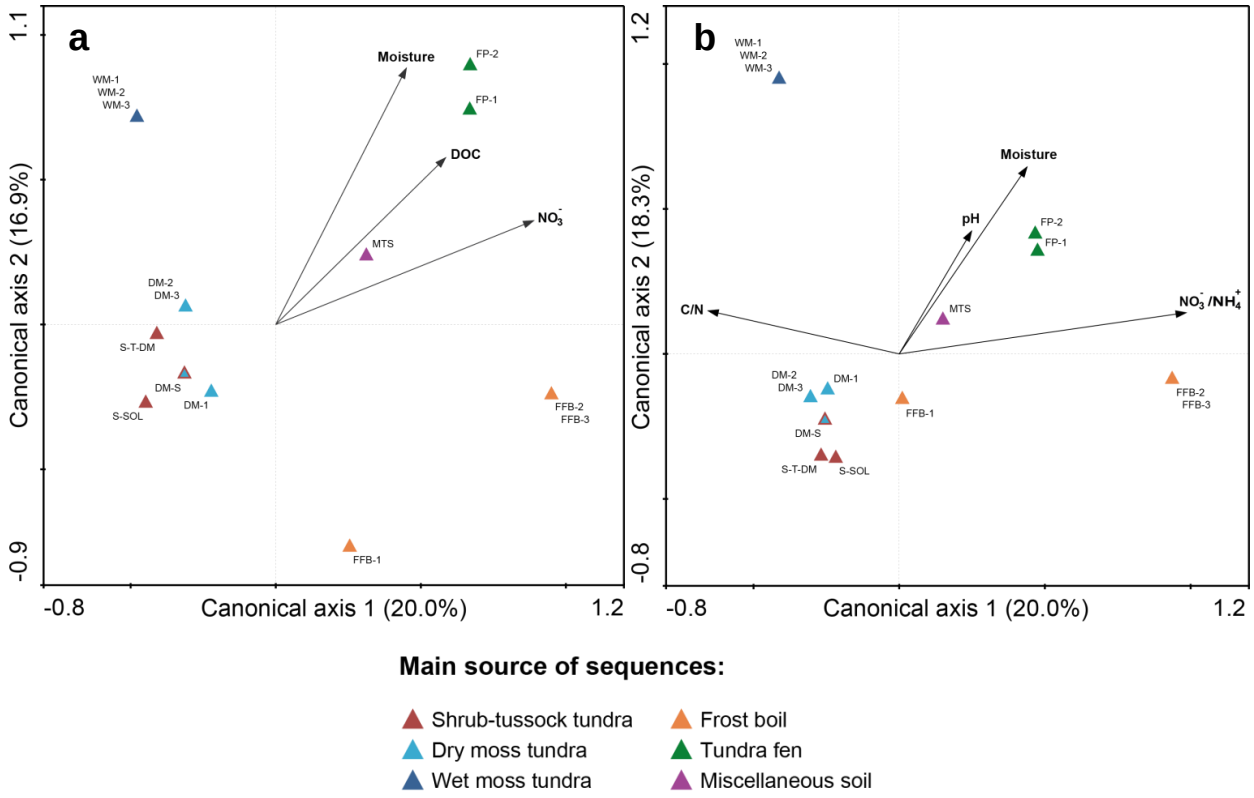


Figure 5 CCA biplots of AOA phylotype distribution in relation to environmental variables. a) model 1, selected non-reducible variables (NO_3^-, moisture and DOC) and b) model 2, selection of reducible variables (NO_3^-/NH_4^+ and C/N), moisture and pH. Phylotypes are represented by the coloured triangles. The colour code indicates the main soil source of sequences in each phylotype and symbols with two colours correspond to phylotypes represented by equivalent proportions of sequences from two distinct ecosystem types. More than one phylotype name close to a single triangle represent phylotypes detected exclusively in that sample. The percentages of phylotype distribution variance explained by the two principal canonical axes are represented close to the axes. Conditional variables are represented by the black arrows. Abbreviations: DOC, dissolved organic carbon; C/N, dissolved organic carbon to total dissolved nitrogen ratio.

(19.3%), with moisture and DOC explaining 17.2% and 15.7%, respectively. However, only the NO_3^- explanatory power was statistically significant ($P < 0.05$), indicating that, individually, moisture and DOC were weakly correlated to AOA distribution (Table 6). The AOA phylotypes mainly present in shrub, tussock and dry moss tundra were generally associated with lower NO_3^-, lower moisture and lower DOC, while the opposite was observed for the fen peat dominating phylotypes (Figure 5a). A clear separation between the communities in shrub-tussock-moss tundra soils and those in the frost boil and tundra fen soils was observed along the first canonical axis ($P < 0.01$) (Figure 5a), which explained 20% of the phylotype distribution and 38.2% of the phylotype-environment relation (Table 5). Additionally, the dominant phylotypes in shrub and tussock tundra appeared to be also slightly segregated from the dominant moss tundra phylotypes along that axis. The high correlation coefficient between the first axis and NO_3^- ($R^2 = 0.89$) indicated that this factor alone might be strongly associated with the separation observed. The dominant phylotypes in the waterlogged soils (Lon-mt1 and Knu-tf) were separated from the low-moisture soils along the second axis, which was correlated with the moisture content ($R^2 = 0.87$) and explained 16.9% of the variance in phylotype distribution. In the second CCA model (Figure 5b), the combination of NO_3^-/NH_4^+, moisture, C/N and pH explained 65.3% of the variance in AOA phylotype distribution ($P < 0.01$; Tables 6). The dominant phylotypes in shrub, tussock and dry moss tundra clustered under lower NO_3^-/NH_4^+ ratios, lower moisture and lower pH, in concomitance with higher C/N ratios (Figure 5b). In turn, the directly opposing situation was observed for the dominant phylotypes in the fen peat soil. Similar to model 1, only moisture appeared to be associated with the differences between dry and wet moss tundra communities, also contributing most of the segregation between frost boil and fen communities, as represented along the second axis (moisture and second axis $R^2 = 0.64$). In model 2, AOA phylotypes were also significantly distributed along the first canonical axis ($P < 0.05$), which explained 20% of the phylotype distribution and 30.7% of the phylotype-environment relation (Table 5). This axis was almost perfectly correlated with the NO_3^-/NH_4^+ ratio ($R^2 = 0.98$), which explained the highest proportion of variance in phylotype distribution (19.9%) with high significance ($P < 0.005$) (Table 6). Comparisons with the first model suggested that NO_3^- was likely to contribute to most of the association observed between NO_3^-/NH_4^+ and phylotype distribution, which was further supported by the positive correlation between the two variables ($R^2 = 0.84$, $P = 0.0005$). Individually, moisture, pH and C/N explained 17%, 15.3% and 13% of phylotype variance in this model, respectively, although without statistical significance (Table 6).

The association between AOA community composition and soil NO_3^-, or NO_3^-/NH_4^+ ratios, obtained with both BIO-ENV and CCA was consistent with previous reports of relations between soil NO_3^- levels and AOA community composition (Wessén *et al*., 2011), as well as changes in community composition after fertilization with either NO_3^- or NH_4^+ (Glaser *et al*., 2010), or with both (Im *et al*., 2011). It would be logical to assume that these correlations might reflect different abilities of the AOA populations to nitrify, possibly as a result of different growth rates and different NH_4^+ affinities and oxidation kinetics, as previously observed for AOB (Horz *et al*., 2004; Webster *et al*., 2005). However, as pointed out above, NO_3^- levels in these soils, and possibly also NH_4^+, seemed to be greatly influenced by the uptake of nitrogen by plants and by inputs through animal faeces deposition. Additionally, NO_3^- assimilatory and dissimilatory microbial processes, as well as possible

differences in NO_2^- to NO_3^- conversion rates, are likely to further preclude a causal association between NO_3^- abundance, or NO_3^-/NH_4^+ ratios, and nitrification activity.

Table 5 Variance in AOA phylotype distribution and phylotype-environment relation explained by the canonical axes of the two selected CCA models

	Environmental variables	*Canonical axes*	*Phylotype distribution % variance explained*	*Phylotype-environment relation % variance explained*
Model 1	NO_3^- Moisture DOC	Axis 1**	20.0	38.2
		Axis 2	16.9	32.4
		Axis 3	15.4	29.4
		Axis 4	18.0	0
		All axes*	70.3	100
Model 2	NO_3^-/NH_4^+ Moisture C/N pH	Axis 1***	20.0	30.7
		Axis 2	18.3	28.0
		Axis 3	15.6	23.7
		Axis 4	11.4	17.6
		All axes**	65.3	100

Abbreviations: DOC, dissolved organic carbon; C/N, dissolved organic carbon to total dissolved nitrogen ratio.

* $P = 0.005$; ** $P < 0.01$; *** $P < 0.05$

Table 6 Total variance in the two CCA models yielding overall significant canonical axes and variance explained by the variables in each model after forward selection

	Total variance in the model[a]	*Total variance explained*[b]	*Environmental variables*	*Variance explained*[c]	*P-value*
Model 1	4.83	2.52 (52.2%)	NO_3^-	0.93 (19.3%)	0.024
			Moisture	0.83 (17.2%)	0.156
			DOC	0.76 (15.7%)	0.289
Model 2		3.15 (65.3%)	NO_3^-/NH_4^+	0.96 (19.9%)	0.003
			Moisture	0.82 (17.0%)	0.162
			C/N	0.74 (15.3%)	0.278
			pH	0.63 (13.0%)	0.465

Abbreviations: DOC, dissolved organic carbon; C/N, dissolved organic carbon to total dissolved nitrogen ratio.

[a] Sum of all non-constrained eigenvalues

[b] Sum of all canonical eigenvalues

[c] Lambda A values

AOA distribution in relation to nitrification rates

Unlike AOB, AOA could be detected in every soil where gross nitrification was measured, with the exception of the Solvatnet fen peat soil (Sol-tf) (Figure 6). Interestingly, previous studies have indeed observed nitrification activity in Arctic soils were no AOB were detected (Jordan *et al*., 1987; Chapin, 1996). Although these observations were based on different methods, they also predated the discovery of AOA, and the current findings suggested that these might have been the main ammonia oxidizers at those sites. In fact, the sole detection of AOA in the Adv-tt, Hot-st and Lon-mt1 sites indicated that archaea might have been the main responsible for the nitrification activity measured in these soils. However, as suggested by Chapin (1996), heterotrophic nitrifiers might contribute greatly to the process, as shown for other soils (Schimel *et al*., 1984; Brierley *et al*., 2001). In the Sol-tf peat soil, where only AOB were detected, gross nitrification rates were 7-fold higher when NH_4^+ was added to a final concentration of approximately 2 mM, in contrast to a 3-fold increase in the Hot-st, no change in the Adv-tt and inhibition in the Lon-mt1 soils. This showed that the AOB present have a potentially higher and/or more prompt response to inputs of NH_4^+,

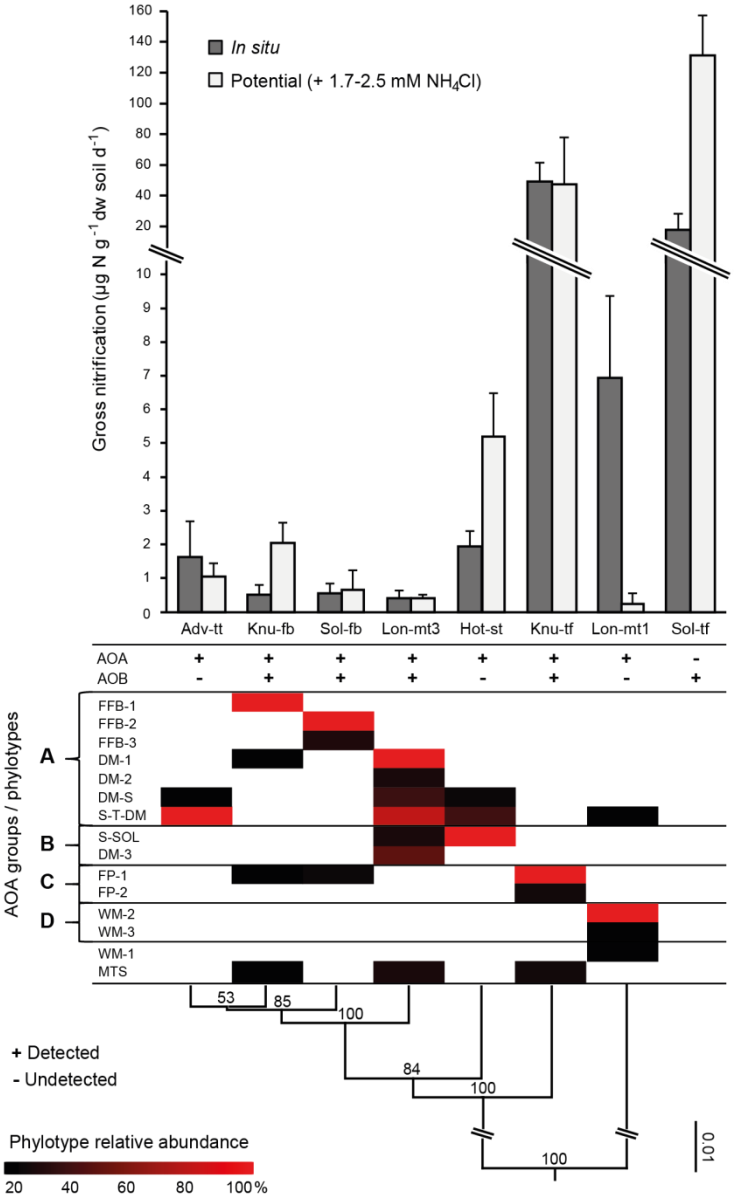


Figure 6 *In situ* and potential gross nitrification rates and presence/absence of detectable AOA and AOB in the corresponding soils. The heatmap depicts the relative abundance of the AOA phylotypes in each soil. The hierarchical clustering dendrogram represents the relation between the overall AOA communities in the soils where nitrification was measured. The clustering was based on weighted UniFrac pairwise distances (with normalized branch weights) and jackknife support values for the clusters were calculated after 1000 permutations. The percentage of permutations that each node was recovered is shown close to the nodes. The scale represents the distance between communities in UniFrac units, where 0 means that two communities are identical and 1 means that they contain mutually exclusive lineages.

indicating a general better adaptation to higher NH_4^+ concentrations than AOA. Moreover, the inhibition of nitrification in the Lon-mt1 when NH_4^+ was added suggested a strict adaptation of the resident AOA population to low concentrations of NH_4^+. Together, these observations are in agreement with the differential AOA and AOB adaptation to distinct inorganic nitrogen conditions that has been reported for other soils (Di *et al*., 2009; Di *et al*., 2010; Verhamme *et al*., 2011). On the other hand, the different gross nitrification rates measured in the AOA-dominated soils (Adv-tt, Hot-st and Long-mt1) suggested that the resident AOA populations (groups A, B and D, respectively) harbor different nitrifying capabilities. The differences in potential gross nitrification particularly suggested that the different populations might nitrify under distinct NH_4^+ conditions, even though these differences might have also resulted from differences in AOA abundance. The amendment with low NH_4^+ amounts (1.7 - 2.5 mM final concentration) nevertheless showed a stimulation of nitrification in soils apparently dominated by AOA affiliated with the soil/sediment lineage (group B; Figure 3 and 6), in contrast to previous studies that observed an increase in nitrification associated only with AOB, probably as a result of the higher NH_4^+ concentrations added (between 4 and 5-fold more than in the present study) (Jia and Conrad, 2009).

The different soils harboured different overall AOA communities dominated by different groups, which, curiously, appeared to generally cluster according to high or low gross nitrification rates, based on the weighted UniFrac distance metric, regardless of the presence of detectable AOB (Figure 6). Given the fact that most communities were dominated by a single AOA group, the clustering greatly reflected the taxonomic relatedness between the dominant phylotypes. The resident AOA populations in the frost boils and in the tussock and dry moss tundra soils formed a tight cluster dominated by members of group A, which was associated with lower *in situ* and potential nitrification rates than those measured in the soils where the AOA communities comprised members of more basal groups (Figure 3 and 6). Exceptionally in the Lon-mt1 soil, nitrification was almost completely abrogated when NH_4^+ was added up to approximately 2 mM, regardless of the relatively high *in situ* rates. In fact, this soil harboured the phylogenetically farthest AOA community among all soils, comprising the only dominating phylotype affiliated with the marine/sediment AOA lineage (Figure 3 and 6). This was in agreement with what has been described for *N. maritimus*, the only isolated AOA in this lineage, which has been shown to be inhibited at NH_4^+ concentrations between 2 and 3 mM (Martens-Habbena *et al*., 2009). Even though the Adv-tt and Hot-st soils showed similar *in situ* nitrification rates and relatively overlapping AOA communities, they differed greatly in their response to additional NH_4^+. In fact, the dominant phylotype in the Hot-st soil (S-SOL) was affiliated with group B, in contrast to all other AOA phylotypes detected in both Adv-tt and Hot-st soils, which were affiliated with group A, as reflected on the clustering analysis (Figure 3). Since the S-SOL phylotype was the only phylotype not shared by both communities, it might have been involved in the higher nitrification response after addition of NH_4^+, possibly representing an AOA group adapted to higher NH_4^+ concentrations. This is further supported by the fact that this phylotype was affiliated with sequences retrieved from soils subject to high NH_4^+ inputs (Figure 3), as well as by the high relative C/N ratio measured in the Hot-st soil, which suggested that this soil was particularly nitrogen-limited (Table 1).

Then again, it should be noted that contemporary factors are likely to influence the different nitrification rates, such as environmental conditions and abundance of nitrifying organisms in the different soils incubated, regardless of their taxonomic affiliation. Although changes in nitrifier community are likely to occur in response to NH_4^+, it is plausible to assume that the incubation period (24 h) was not long enough for these changes to result in drastic community shifts. It has been suggested that temperature and pH might regulate the relative abundance of NH_4^+ and NO_3^- in Arctic soils, by affecting nitrification activity (Atkin, 1996). Yet, the present results showed that nitrification in distinct soils incubated under uniform temperatures exhibited variable rates that sometimes converged into similar values, despite considerable differences in native pH or contemporary NO_3^-/NH_4^+ ratios. Hence, it seems that these factors alone could not explain variations in NH_4^+ and NO_3^- through regulation of nitrification, which in turn might be more directly dependent on the composition of the resident nitrifier community.

AOA identification and nitrifying activity in enrichment cultures

Out of 54 initial enrichment cultures initiated from all Spitsbergen soils, NH_4^+ consumption was observed in 9 of those inoculated with soil from both frost boils (Knu-fb and Sol-fb), dry moss tundra (Lon-mt2 and Lon-mt3) and peat from the Knudsenheia fen (Knu-tf), incubated at 20 ºC for 54 days (data not shown). In the second sub-culturing stage, no NH_4^+ consumption was detected in cultures inoculated with the Sol-fb soil at any of the temperatures in the gradient tested (17, 20, 32 and 37 ºC) and only low amounts of NH_4^+ were consumed in all remaining cultures incubated below 20 ºC. Highest consumption of NH_4^+ was observed in cultures from the Knu-fb and Lon-mt2 soils incubated at 20 ºC temperature for approximately 130 days. During the first and second enrichment stages, there was no obvious difference in NH_4^+ consumption between amendments with either 0.2 or 0.5 mM NH_4Cl (data not shown).

In the third stage of enrichment sub-culturing, archaeal *amo*A genes could be amplified from Knu-fb and Lon-mt2 cultures under all temperature conditions and antibiotic treatments (specific conditions can be found under “Materials and methods”) after 26 days of incubation, while no β-proteobacterial *amo*A was detected. Furthermore, ammonia oxidation activity was confirmed by the almost stoichiometric conversion of NH_4^+ to NO_2^- in several enrichment cultures incubated at either 20 or 28 ºC for a period of 70 days (exemplified in Figure 7a). Together, these results indicated that AOA were probably responsible for the ammonia oxidation observed.

Substantially higher ammonia oxidation was observed at 20 ºC (Figure 7a), although direct comparisons between temperatures could not accurately be made at this stage given the different proportions of inoculum used in some cultures. Yet, subsequent sub-cultivation steps with uniform inoculum proportions confirmed that ammonia oxidation was severely inhibited at temperatures considerably lower or higher than 20 ºC (data not shown). Even though AOA were also detected in cultures at 4 ºC, the small NH_4^+ decrease observed was probably due to assimilatory processes by the microbial community present, since NO_2^- was often not produced. This process might have also been responsible for the slight discrepancy between absolute NH_4^+ consumption and NO_2^- production observed in the cultures with higher ammonia oxidation activity. Nevertheless, small increases in NO_2^- were observed in few Knu-fb cultures incubated at 4 ºC, in which only AOA were

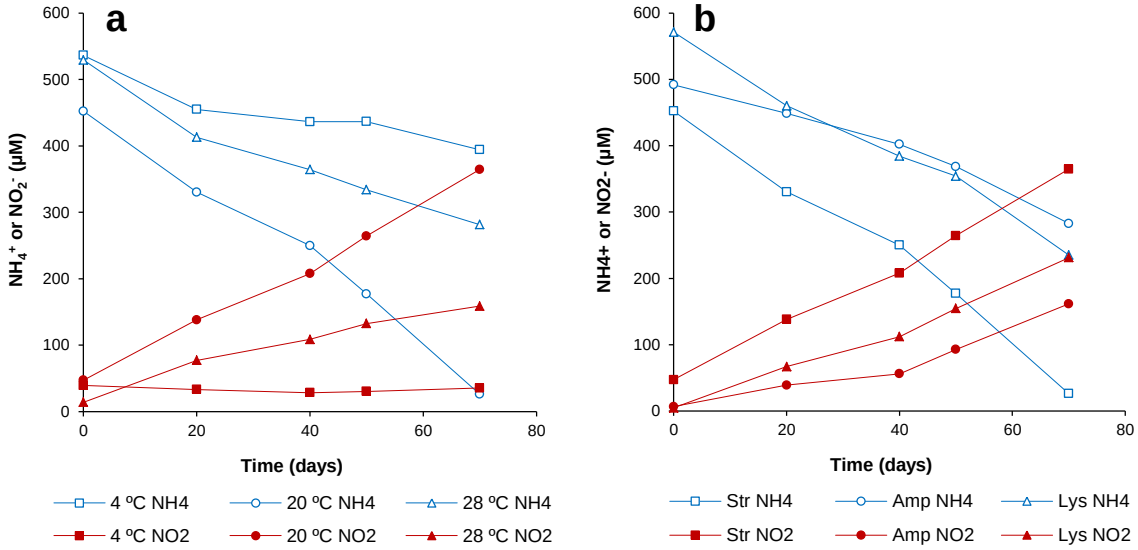


Figure 7 Nitrification activity in AOA enrichments cultures from the Lon-mt2 soil, assessed from NH_4^+ consumption and NO_2^- production over a period of 70 days. a) Nitrification in enrichment cultures treated with streptomycin and incubated at different temperatures; the culture at 20 ºC was inoculated at 20% (v/v) and cultures at 4 ºC and 28 ºC at 7.5% (v/v). b) Nitrification in enrichment cultures incubated at 20 ºC with different antibiotic treatments; the culture with streptomycin was inoculated at 20% (v/v) and cultures with ampicillin or lysozyme at 7.5% (v/v). Ammonium consumption is represented by blue lines and NO_2^- production is represented by red lines. Different symbols indicate cultures under different temperatures (a) or treatments (b).

Abbreviations: Str, streptomycin; Amp, ampicillin; Lys, lysozyme.

detected, suggesting that archaeal-mediated ammonia oxidation might occur at this temperature (data not shown).

Nitrification activity was similar in cultures treated with streptomycin or lysozyme, with slight differences probably resulting from the different proportions of initial inoculum (Figure 7b). In contrast, treatment with ampicillin appeared to have a negative effect on nitrification, by comparison with cultures incubated with the same percentage of inoculum and alternatively treated with lysozyme. Since the level of bacterial contamination was not verified at this stage, it was not possible to assess whether differences in nitrification under different treatments were due to relative bactericidal efficiencies, different tolerance levels of the AOA present or indirect effects resulting from changes in overall microbial community composition.

RFLP fingerprinting of the archaeal *amo*A amplified after 40 days of incubation consistently showed different patterns between Knu-fb and Lon-mt2 enrichment cultures incubated at 20 ºC (Figure 8). Most cultures from both soils incubated at 28 ºC appeared to have an overlapping distinct pattern, which was observed only in one of the cultures at 20 ºC tested. However, almost all cultures where this pattern was observed were also treated with lysozyme, making it difficult to discern whether higher temperature or the lysozyme treatment might have been responsible for the

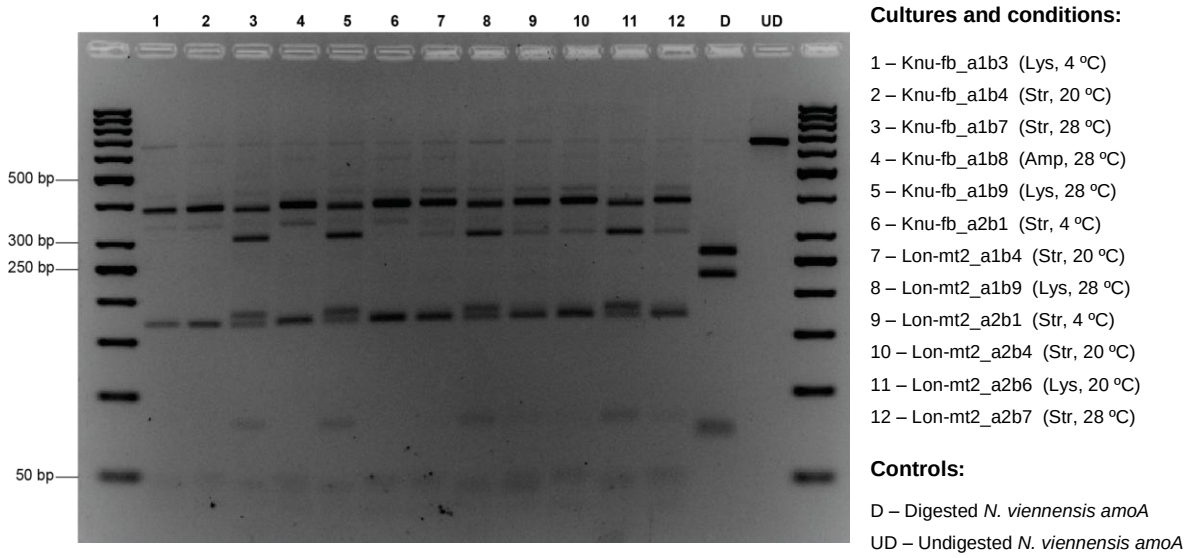


Figure 8 RFLP fingerprinting of archaeal *amo*A genes amplified from selected enrichment cultures incubated for 40 days under different temperatures and antibiotic treatments. Digested and undigested *amo*A genes from *N. viennensis* were used as control reactions. Main DNA ladder band sizes are indicated on the left-hand side of the picture.
Abbreviations: Str, streptomycin; Amp, ampicillin; Lys, lysozyme.

selection observed. In turn, cultures at 4 ºC showed *amo*A patterns identical to those from the same soil source incubated at 20 ºC.

M13 colony PCR products from 3 to 10 clones from each of several enrichment cultures were analysed by RFLP (as described above) and a total of 9 clones were selected for sequencing. The four clones sequenced from a Lon-mt2 enrichment culture with stable nitrification activity at 20 ºC (culture represented in Figure 7a) yielded identical archaeal *amo*A sequences, which clustered into a distinct phylotype closely related to the dominant phylotype in the fen peat (FP-1) within group C (Figure 3). In contrast, the two clones from a Knu-fb culture with similar ammonia oxidation activity under the same conditions were each affiliated with distant phylogenetic lineages: one of them was also associated with group C, clustering within the FP-1 phylotype, while the other clustered within the DM-1 phylotype (group A), which dominated the dry moss tundra soils. Furthermore, the only sequence retrieved from a culture at 4 ºC (from the Knu-fb soil) also clustered within the DM-1 phylotype. In accordance to the RFLP fingerprinting, the two clones from cultures inoculated with different soils (Knu-fb and Lon-mt2) incubated at 28 ºC after lysozyme treatment yielded identical archaeal *amo*A sequences that clustered within the S-T-DM phylotype in group A. This further supported a potential selective effect of either (or both) higher temperature or lysozyme treatment on AOA communities. Given the reduced number of clones sequenced, it was not clear whether the observed diversity actually reflects the enrichment of various AOA phylotypes or might have resulted from the detection of inactive organisms derived from the initial inocula. Nonetheless, the fact that all four clones retrieved from the Lon-mt2 culture yielded the same *amo*A sequence, which was not identified in the clone library from the original soil, strongly suggests that this gene belongs to a low abundant organism that was effectively enriched under the incubation conditions used.

While the present results showed that the AOA present are capable of converting NH_4^+ to NO_2^-, at this stage of enrichment it was not possible to ascertain if this was an autotrophic process. Although bicarbonate was the only carbon source provided in the medium, it is possible that soluble carbon sources originating from the initial soil inocula were still present in the cultures. Additionally, it is also likely that relatively complex microbial communities were present, regardless of the enrichment of AOA, possibly influencing AOA behaviour and growth through diverse interactions, for example by providing organic substances as a result of their metabolism. In fact, the only AOA isolated from soil, *N. viennensis*, appears to grow optimally only after addition of pyruvate or in co-culture with bacteria of the genera *Hyphomicrobium* and *Mesorhizobium*, although the role of these interactions or pyruvate are yet not clear (Tourna *et al*., 2011).

Conclusions

Prevalence of AOA over AOB in cold-soils

The present study showed a widespread presence and remarkable phylogenetic diversity of AOA across high Arctic landscapes, with distinct groups differentially distributed between frost boil, fen peatland, shrub, tussock and moss tundra soils. In contrast, the β-proteobacterial ammonia oxidizers commonly found in soils were surprisingly undetectable in several tundra sites. While in some cases the "niche partitioning" between AOA and AOB appeared to parallel what happens in other environments, particularly in regard to pH, DOC or nitrogen levels, the overall prevalence of AOA in these soils strongly suggested that soils from cold environments might harbour common features that favour the general prevalence of the archaea. This was further supported by similar observations in alpine soils (Nemergut *et al*., 2008). Additionally, the extent of the AOA diversity observed here and the fact that the groups detected are also widely present in other environments preclude a possible high-latitude or "cold-soil" monophyletic clustering. However, the detection of a polyphyletic group of AOA independently related to sequences from cold soils suggested a possible convergence of distinct AOA taxa into putative "cold-adapted" ecotypes at very small phylogenetic scales, as previously observed for some Arctic nitrogen-fixing microorganisms (Deslippe and Egger, 2006).

Multiple environmental factors rather than geography shape AOA communities

The AOA distribution did not show a geographic patterning between Svalbard, Greenland and Siberia sites or between the Svalbard sites, but rather followed a "ecosystem type" (i.e. habitat) distribution. Furthermore, AOA populations also showed considerable local specificity regardless of their taxonomic affiliation, with distinct phylotypes dominating different sites over very small spatial scales and within the same tundra ecosystem. However, while similar tundra ecosystems in different locations generally shared similar AOA populations (i.e. frost boil or shrub tundra soils), different tundra ecosystems with overall similar physicochemical characteristics harboured taxonomically related AOA groups (i.e. shrub, tussock and moss tundra, and to some extent also frost boils). The Arctic tundra is a complex and often patchy environment, to which the AOA populations appeared to be differentially adapted, indicating that this group of organisms might be

more versatile in the ecological niches they occupy than generally assumed. The multivariate analyses of AOA community composition in relation to the soil properties not only supported these distribution patterns but also showed an apparent multifactorial effect of the environment on AOA community composition. The fact that different factors were associated with the differences between distinct populations indeed indicated that different environmental factors were likely to affect the different AOA groups in different degrees. Although stochastic and historical events could not be ruled out, community composition of this monophyletic functional group in Arctic soils appeared to be mostly determined by local environmental conditions rather than by geographic constraints, similar to what has been observed for broader taxonomic groups such as bacteria (Fierer and Jackson, 2006; Van der Gucht *et al*., 2007; Nemergut *et al*., 2011), including those in Arctic soils (Chu *et al*., 2010). Interestingly, the absence of any association between AOA community distances estimated with UniFrac and the environmental parameters suggested that the relation between AOA distribution along environmental gradients might not directly reflect phylogenetic relations but rather exhibit a more "unimodal" type of distribution. This could result from specific phylogenetic groups being adapted to specific ranges of conditions regardless of their taxonomic affiliation, at least on the present scale. Hence, divergent groups of AOA might be adapted to similar conditions in specific environmental factor(s) (e.g. those in the dry and wet moss tundra soils), while close taxonomic groups might in turn be adapted to very different conditions, such as pH and nitrogen conditions (e.g. those in the shrub and dry moss tundra soils).

Cultivation and ammonia oxidation activity of soil AOA from different Arctic sites

The enrichment of AOA in laboratory cultures showed that the resident AOA populations in distinct Arctic soils are indeed capable of oxidizing ammonia, regardless of their abundance in the original soils or their *in situ* activity under the physicochemical conditions measured at the time of sampling. Additionally, the predominance in the cultures of AOA phylotypes affiliated with an uncharacterised basal soil/sediment group (group C) further expanded the number of AOA groups within the soil/sediment lineage that have been directly associated with ammonia oxidation beyond the *Nitrososphaera* clade. Interestingly, the predominance of this AOA group in a nitrogen- and carbon-rich peat soil, potentially under oxygen conditions unfavourable to the aerobic oxidation of ammonia, further emphasized the niche versatility of AOA.

Niche partitioning and low functional redundancy within the AOA

Although the relation between AOA distribution and their environment appeared to be independent on their phylogenetic relatedness, the association between the AOA communities and gross nitrification activity showed a suggestive relation to the taxonomic affiliation of the dominant AOA phylotypes. The variability in *in situ* nitrification rates and as a response to NH_4^+ amendments in soils where either only AOA or AOB were detected, not only supported the previously reported niche partitioning between AOA and AOB in respect to NH_4^+ concentration (Martens-Habbena *et al*., 2009; Di *et al*., 2010; Verhamme *et al*., 2011), but also indicated a niche partitioning within the AOA. These observations suggested that archaea encoding the AMO enzyme differ greatly in their potential to effectively oxidize ammonia and that some groups might rely on alternative primary

energy metabolisms and/or harbor fundamentally different physiologies. The discrepancies in AOA community composition and nitrification activity observed in sites with very similar soil properties (e.g. frost boils) or close geographic location (e.g. moss tundra soils) further suggested that the diversity within the AOA is not functionally redundant and thus categorical assumptions on the whole-phylum level should be avoided. Furthermore, these variations in the nitrifying potential of distinct Arctic soils, regardless of the prevalence of either AOA or AOB, indicated that this process was strongly dependent on the intra-domain identity of the nitrifier groups present and their degree of adaptation to the contemporary soil conditions. Although factors like pH and temperature are known to affect nitrification activity (Booth *et al*., 2005), the native nitrifier populations might indeed constitute a better proxy for nitrification regulation than environmental factors that have very often a wide and unspecific array of effects on ecosystem processes. Together, these observations underlined the importance of better characterizing microbial communities in relation to ecosystem processes and to factors involved in their distribution and activity on different scales. This information is essential for better understanding the regulation of ecosystem processes and should be regarded particularly when defining models of ecosystem response to environmental changes, as emphasized by Singh *et al*. (2010) and Xu *et al*. (2011).

It was not surprising that the association observed between AOA distribution and NO_3^- or NO_3^-/NH_4^+ did not relate to the clustering of the AOA populations according to different gross nitrification rates, given the great dependence of these factors on a number of processes other than nitrification. Although NO_3^- concentration and NO_3^-/NH_4^+ ratios might, to some extent, serve as indicators of ecosystem net nitrification, they can hardly be related to the gross process rates. The strong association between AOA distribution and these factors were rather likely due to: (i) some AOA groups possibly being able to utilise NO_3^- for energy metabolism; (ii) the AOA groups present in the high NO_3^- soils where nitrification rates were low being capable of nitrifying only under very different conditions than those applied during the incubations, or; (iii) the association observed simply representing the indirect effect of an unmeasured environmental factor.

Implications of nitrification potential and AOA distribution in Arctic soils under a changing climate

The fact that the gross nitrification rates measured here were in a similar range than those in warmer soils has also important implications in the context of climate changes, since it constituted evidence that, under similar temperature conditions, microbial communities from Arctic soils have a nitrifying potential comparable to that of the communities in other soils. It is thus plausible to expect that gross nitrification rates in these soils might be enhanced under a warming climate scenario, with potentially critical consequences for the functioning of Arctic ecosystems.

With the recently suggested implication of soil archaea, particularly AOA, in the production of the greenhouse gas N_2O (Rasche *et al*., 2010; Im *et al*., 2011), the diversity and widespread occurrence of AOA in Arctic soils reported here raises yet two more pressing questions regarding the functioning of these ecosystems following environmental changes: first, if AOA are indeed, directly or indirectly, involved in the production of N_2O, how does the increased availability of nitrogen following permafrost thawing influence potential AOA-driven N_2O emissions, and how does this loss of nitrogen to the atmosphere affect the nitrogen availability status of tundra soils? Second,

do all AOA groups hold the same potential to produce (or influence the production of) N_2O, and how does their differential distribution influence the potential for the emissions of this gas across Arctic soils? Elucidation of these and other questions regarding the regulation and responses of the nitrogen cycle to environmental changes in Arctic soils will necessarily require the integration of ecosystem process-based analyses with detailed characterization of microbial communities through carefully designed experiments.

To my knowledge, this is the first description of AOA distribution and diversity in high Arctic soils, as well as the first analysis of the relation between different AOA groups and their potential role in nitrification through a ^{15}N tracing method across different ecological scales of the same biome.

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