LETTERS

Archaea predominate among ammonia-oxidizing prokaryotes in soils

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Ammonia oxidation is the first step in nitrification, a key process in the global nitrogen cycle that results in the formation of nitrate through microbial activity[1,2]. The increase in nitrate availability in soils is important for plant nutrition, but it also has considerable impact on groundwater pollution owing to leaching. Here we show that archaeal ammonia oxidizers are more abundant in soils than their well-known bacterial counterparts. We investigated the abundance of the gene encoding a subunit of the key enzyme ammonia monooxygenase (*amoA*) in 12 pristine and agricultural soils of three climatic zones. *amoA* gene copies of Crenarchaeota (Archaea) were up to 3,000-fold more abundant than bacterial *amoA* genes. High amounts of crenarchaeota-specific lipids, including crenarchaeol, correlated with the abundance of archaeal *amoA* gene copies. Furthermore, reverse transcription quantitative PCR studies and complementary DNA analysis using novel cloning-independent pyrosequencing technology demonstrated the activity of the archaea *in situ* and supported the numerical dominance of archaeal over bacterial ammonia oxidizers. Our results indicate that crenarchaeota may be the most abundant ammonia-oxidizing organisms in soil ecosystems on Earth.

Autotrophic ammonia-oxidizing bacteria (AOB) of the β- and γ-subgroups of proteobacteria have so far been considered the most important contributors to aerobic ammonia oxidation[3,4]. These organisms usually comprise only a small fraction of the microbiota[5–7]. Recently, we found genes encoding subunits of a potential ammonia monooxygenase (AMO), the key enzyme of AOB, on a metagenomic soil clone alongside a ribosomal RNA operon of Archaea, affiliated with the phylum Crenarchaeota[8]. After addition of ammonia to bulk soil samples their transcription was induced[8]. Highly similar *amoA* and *amoB* sequences and a linked *amoC* gene were also identified in a metagenomic study of the Sargasso Sea indicating the presence of all three enzyme subunits in crenarchaeota[9,10]. The predicted existence of ammonia-oxidizing archaea (AOA) was ultimately confirmed by cultivation of a chemolithoauto-

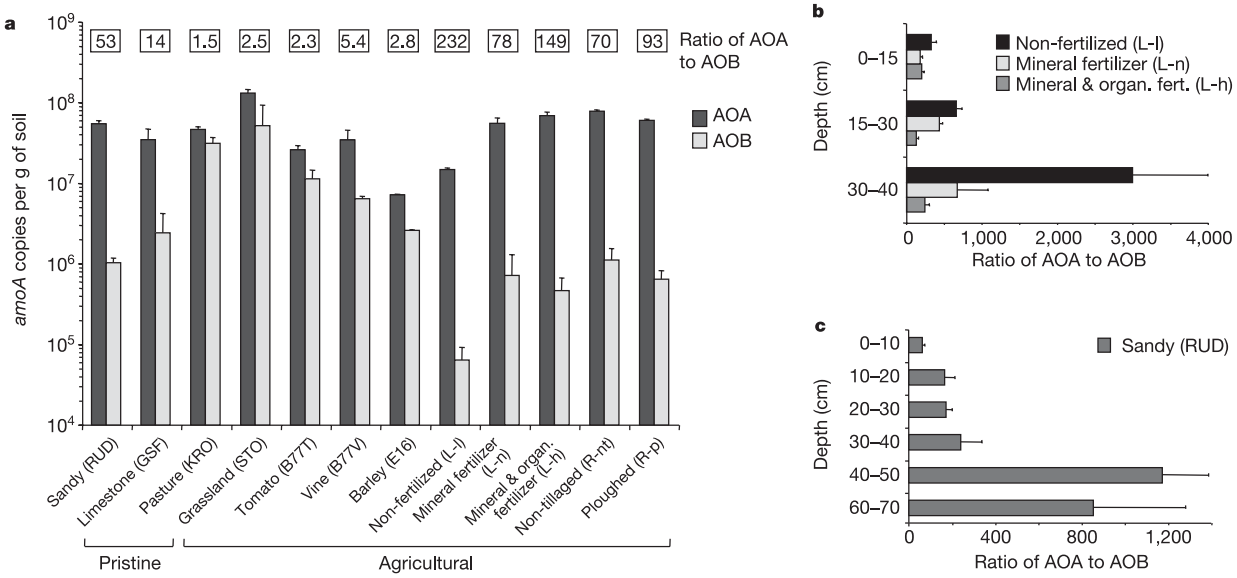

Figure 1 | Archaeal *amoA* genes outnumber bacterial *amoA* genes in topsoils and in deeper layers. a, Abundance of AOA and AOB in 12 different topsoils (0–10 cm) expressed as *amoA* copy numbers per g of dried soil, analysed from three independent replicates per site. Ratios of AOA to AOB *amoA* copies are shown in boxes above the chart. **b**, Ratios of AOA to AOB *amoA* gene copies in soil depth profiles of agricultural soils L-l, L-n and L-h (see Table 1). **c**, Ratios of AOA to AOB *amoA* gene copies in a sandy ecosystem (RUD). The ratios increased with depth in all but one soil (L-h) owing to a dramatic decrease in AOB while AOA stayed constantly high (for absolute numbers see Supplementary Table S2). Error bars indicate standard deviation.

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Table 1 | Soil sample characteristics and locations

Name	Soil type	pH	WEON	WEOC	Location
RUD[18]	Sandy grassland	7.1	0.9	7.6	Darmstadt, Germany
GSF	Limestone grassland	6.9	1.08	10.2	Munich, Germany
KRO[24]	Pasture land	6.1	3.76	25.85	Bergen, Norway
STO[24]	Arable grassland	5.5	1.16	5.5	Bergen, Norway
B77T	Sandy soil, growing tomato	6.0	0.76	5.4	Santorini, Greece
B77V	Sandy soil, growing grapes	6.2	0.87	5.6	Bergen, Norway[24]
E16	Sandy soil, growing barley	6.0	0.56	3	Bergen, Norway[24]
L-l[17]	Sandy loam, non-fertilized	6.4	1.22	35.2	Bad Lauchstädt, Germany
L-n[17]	Sandy loam, mineral fertilizer	6.3	2.34	57.1	Bad Lauchstädt, Germany
L-h[17]	Sandy loam, mineral and organic fertilizer	6.7	4.76	85.1	Bad Lauchstädt, Germany
R-nt	Silty clay loam, non-tillaged	7.3	2.3	16.3	Rommersheim, Germany
R-p	Silty clay loam, ploughed	7.3	2.4	7.7	Rommersheim, Germany

Typical soil types from the region of sampling were used, providing a spectrum from sandy soils (Greece) to silty clay loam soils (Rommersheim, Germany). RUD was sampled from 0 to 70 cm depth in 10-cm increments. Other depth profiles were taken from three plots of agricultural soil under different management regimes at Bad Lauchstädt (0–15 cm, 15–30 cm and 30–40 cm). Triplicate soil samples were taken at each site. All soil samples were snap-frozen and stored at −80 °C. WEON, water extractable organic nitrogen (mg per kg of dry weight soil); WEOC, water extractable organic carbon (mg per kg of dry weight soil). For more details of soil characteristics, see Supplementary Table S1.

trophic, marine strain which used ammonia as sole energy source and produced nitrite in nearly stoichiometric conversion[11]. It contained *amoA*, *amoB* and *amoC* genes highly similar to those from the metagenomic soil clone and the marine sequences.

The goal of this study was to quantify ammonia-oxidizing crenarchaeota in terrestrial ecosystems to evaluate their potential ecological impact. For this purpose, we have focused on two biomarkers, the *amoA* gene, which has frequently been used to quantify bacterial ammonia oxidizers[5–7], and tetraether lipids diagnostic for crenarchaeota[12]. Because the archaeal and bacterial *amoA* genes are sufficiently divergent from each other[8,13,14], two specific primer sets were used in real-time (quantitative) polymerase chain reaction (qPCR) to determine the absolute and relative abundance of archaeal and bacterial ammonia oxidizers. Twelve different soils following a sequence from northern to southern Europe were investigated, including natural and managed ecosystems exhibiting different soil types with a wide range of pH, water extractable carbon and nitrogen (for details on soils see Table 1 and Supplementary Fig. S1 and Supplementary Table S1).

In all samples, the archaeal *amoA* copy numbers were high, ranging from 7×10^6 to 1×10^8 per g of dry soil (Fig. 1a, Supplementary Table S1). In contrast, bacterial *amoA* genes varied considerably over three orders of magnitude, similar to earlier findings of AOB abundance in agricultural soils[5–7,15,16]. Archaeal *amoA* genes dominated over bacterial *amoA* genes in all soils, with ratios ranging from 1.5 to over 230 in topsoils. To analyse the distribution of AOA and AOB at different depths, we investigated an agricultural soil (from Bad Lauchstädt), which has been treated with different amounts and qualities of fertilizers for more than 100 years (ref. 17), and a natural, pristine calcareous grassland soil site (Am Rotböll[18], Fig. 1b and c). Bacterial *amoA* genes in the former declined significantly with depth in the unfertilized and inorganically fertilized sites while archaeal *amoA* genes stayed high, resulting in a maximal AOA to AOB ratio of 3,000. In contrast, both archaeal and bacterial *amoA* copy numbers varied little with depth at the site treated additionally with manure (L-h) and these higher levels of AOA and AOB *amoA* were associated with the highest bioavailability of nitrogen and carbon (Table 1). Interestingly, archaeal *amoA* copy numbers also varied little with depth in the sandy ecosystem (RUD), while the abundance of bacterial *amoA* gene copies again decreased significantly, resulting in an AOA to AOB *amoA* ratio of >1,000 at 40 cm (Fig. 1c, Supplementary Table S2). The relative abundance of AOA with respect to the total microbiota varied mostly between 1% and 5% (Supplementary Table S2, assuming arbitrarily an average microbial genome size of 4 megabases, Mb), whereas the highest fraction of AOB in soil microbiota was only 0.23% (STO). To analyse possible differences in community structure of archaea in topsoil and deeper soil horizons, we cloned and sequenced AOA *amoA* from the sandy ecosystem at depths of 0–10 cm and 60–70 cm. A comparison of 36 sequences from each depth shows highly similar *amoA* genes (with 24% maximal sequence divergence at the DNA level), but also clustering, indicating differences in population structure of AOA with depth that might reflect the occurrence of different 'ecotypes' (see tree in Supplementary Fig. S3).

To exclude the possibility that quantification was biased by differences in the sensitivity of the primer sets or the efficiency of the qPCR methods, five different primer combinations for archaeal and bacterial *amoA* and 16S rRNA genes, respectively, were used in a most-probable-number (MPN) PCR experiment (Supplementary Information, Supplementary Table S3 and Supplementary Fig. S4). The results obtained on serial dilutions of two soil DNAs confirmed, with independent primer sets the dominance of AOA over AOB.

Isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) are unique membrane lipids of archaea and are often used as biomarkers to study their presence and distribution[19,20]. Among these lipids, crenarchaeol has been identified exclusively in crenarchaeota, particularly from marine environments but also in freshwater sediments and peat bogs[12,21]. We investigated the amount of archaea-derived isoprenoidal GDGT in ten of the soils. Values ranged from 0.04 to 3.24 μg per g of soil, of which a significant fraction was crenarchaeol, showing that it occurs in considerable amounts in the soil ecosystems

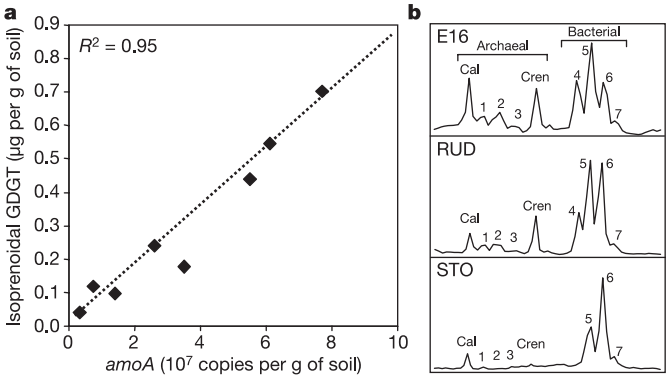

Figure 2 | Isoprenoidal tetraether lipids in soils and correlation to *amoA* gene copies. a, bivariance plot of GDGT abundance (μg per g of dry soil) versus AOA *amoA* copies (10^7 per g of dry soil). The high coefficient of correlation ($R^2 = 0.95$; $n = 8$) is consistent with ammonia oxidizers constituting a major proportion of crenarchaeota (values from managed pastures KRO and STO excluded). **b**, Chromatographic traces of archaeal and bacterial membrane lipids in three different surface soils (E16, RUD and STO; see Table 1), determined by high-pressure LC-APCI-MS. Isoprenoidal GDGTs are attributed to archaea, whereas non-isoprenoidal GDGT have been proposed to derive from bacteria[26]. Cal, caldarchaeol; Cren, crenarchaeol; numbers refer to GDGT structures as displayed in Supplementary Fig. S6.

studied (0.02–0.33 μg per g of soil; Supplementary Tables S1 and S2). A good correlation was found between the amount of GDGT (or crenarchaeol alone) and the abundance of archaeal *amoA* gene copies in eight out of ten soil samples tested, which is consistent with ammonia-oxidizing archaea constituting a significant proportion of crenarchaeota (Fig. 2, and Supplementary Table S1 and Supplementary Fig. S5 for crenarchaeol:*amoA*).

To determine whether *amoA* genes from AOA and AOB populations are actively transcribed in soil and whether transcription correlates with gene abundance, we isolated total RNA from three soils and quantified relative cDNA copies of *amoA* genes after reverse transcription. Remarkably, *amoA* cDNA of both populations was detected in all soils and their ratio correlated well with quantitative DNA measurements; that is, archaeal transcripts dominated in RUD (sandy ecosystem) and KRO (pasture), while almost equal amounts of cDNA copies were detected in STO (grassland) (Fig. 3).

To analyse the activity of AOA and AOB at the community level and to verify their relative abundances by an independent technique, we performed large-scale analysis of a cDNA library using pyrosequencing technology. The cDNA library was obtained from RUD soil in which archaeal 16S rRNA and *amoA* genes were found in approximately equal numbers and archaeal 16S rRNA gene sequences belonged exclusively to crenarchaeota subgroup 1.1b in which the *amo* genes have been detected (ref. 18 and T.U. and C.S., unpublished data). After second-strand synthesis on randomly reverse-transcribed total RNA the resulting double-stranded cDNA was used directly for sequencing without a cloning procedure or PCR amplification[22]. Out of 210,523 assignable cDNA reads (of a total of 314,041 reads) 1.37% were affiliated with archaea (Supplementary Table S4), a similar proportion to that estimated by *amoA* qPCR of the same sample (1.0% of the microbial cells contained an archaeal *amoA* gene, Supplementary Table S2, RUD 0–10 cm). This is consistent with the assumption that most if not all of the archaea in this soil are capable of ammonia oxidation. While *amoA* cDNA of archaea and bacteria was readily quantified from the same RNA preparations by qPCR (Fig. 3), we found only two reads in the 30 Mb 'pyro-library' that could be assigned to archaeal *amoA* and none was associated with bacterial *amoA* (Supplementary Table S5). However, this is not unexpected given that the vast amount of cDNA appears to be derived from stable ribosomal RNA transcripts (at least 30% of the reads represent 16S rRNA genes, Supplementary Table S4).

To compare further the relative proportions of non-thermophilic crenarchaeota and AOB, we used full-length 16S rRNA genes of both groups to query this database. We identified 570 crenarchaeota- and 37 AOB-assigned sequence reads (Fig. 4a); the ratio of 15:1 is similar to the AOA:AOB ratio determined by *amoA* cDNA copies in the same sample (a ratio of 16:1, see RUD Fig. 3). Similarly, more sequences of crenarchaeota than of AOB were identified when querying the database with a universal probe or with various group-specific probes in different regions of the 16S rRNA gene (Fig. 4b, c and Supplementary Table S5), which is again consistent with dominance of active AOA over AOB in the sample.

In conclusion, our data provide evidence for high abundance of AOA in soils, and extrapolation suggests that they represent the most abundant ammonia-oxidizing organisms in soil ecosystems on Earth. Their high numbers in various ecosystems and at greater soil depths indicate that these organisms are adapted to a broad range of growth conditions and might therefore have a more versatile metabolism than AOB, perhaps being able to grow mixotrophically. Genes predicted to encode components of a modified-3-hydroxypropionate cycle known in carbon-fixing hyperthermophilic crenarchaeota as well as an oxidative tricarboxylic acid cycle have been found in the marine relative, the sponge symbiont *Cenarchaeum symbiosum*[23]. This is consistent with both an autotrophic and an organotrophic lifestyle. Furthermore, the genome from *Cenarchaeum* revealed genes encoding homologues of urease and urea transporters beside the *amoA*, *amoB* and *amoC* genes, indicating the potential of AOA to oxidize various reduced nitrogen compounds[23].

Although numerically abundant and transcriptionally active, it remains to be shown whether archaea in soil also dominate with respect to their nitrification activities. It will be important to identify

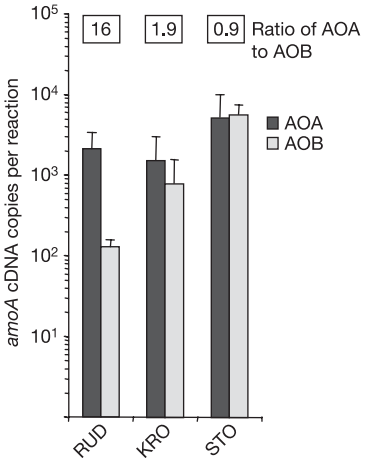

Figure 3 | *amoA* cDNA copies and their AOA:AOB ratios in three different soils. Average of three independent qPCR experiments performed on reverse-transcribed total RNA from fresh soil samples taken in January 2006. Copy numbers of AOA *amoA* transcripts are comparable to those published earlier from the same soil but from a different season[8]. Error bars indicate standard deviation.

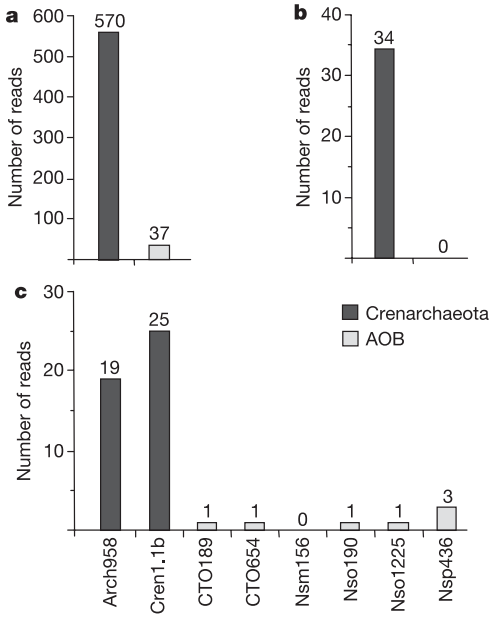

Figure 4 | Identification of rRNA transcripts from crenarchaeota and AOB in 30 Mb of sequence determined from a RUD soil cDNA library (314,000 reads with an average length of 96.4 bp). **a**, Search performed by aligning all reads against a set of four full-length crenarchaeal and six full-length AOB 16S rRNA genes, covering the major phylogenetic clusters of these groups, similarity cut-off 97%. **b**, 1,134 sequences that aligned to the universal phylogenetic 16S rRNA probe Uni1392 (ref. 27) were screened for affiliation with AOB or (cren)archaea by keyword searches conducted on individual Blast output files using classification names of Bergey's manual. **c**, (Cren)archaea- and AOB-specific 16S rRNA probes were used in an '*in silico*' hybridization experiment, allowing up to one mismatch. All reads identified in **a**, **b** and **c** were verified manually by individual Blast searches against the non-redundant database (see Supplementary Information for more details).

the parameters influencing AOA and AOB populations in soils and to quantify and compare their specific activities under varying environmental conditions. If archaea contribute significantly to nitrification, as their abundance now suggests, estimates of the ecological impact of ammonia oxidation (including greenhouse gas emissions) based on bacterial ammonia-oxidizing activity will need to be re-assessed.

METHODS

Extraction and preparation of nucleic acids. Nucleic acid (RNA and DNA) extractions from all soils were performed using a modification of the method described in ref. 25 after optimization of bead beating times, which led to maximal yield of DNA or of quantified copies of *amoA* in qPCR (see Supplementary Information for details). cDNA was prepared as described previously[8]. DNA concentration was measured by incubating different dilutions of extracted DNA with the fluorescent dye SYBRGreenI (Molecular Probes, see Supplementary Information).

Real-time PCR. For each soil sample, 5 ng of DNA pooled from three replicate samples or 2 μl of cDNA, respectively, was used to quantify the copy numbers of archaeal *amoA* genes and transcripts as described before[8]. The same procedure was applied for *amoA* from bacteria using bacterial *amoA*-specific primers and SYBRGreenI. Both real-time techniques yielded highly reproducible standard curves with fosmid 54d9 (ref. 8) (for archaeal *amoA*) and the cloned *amoA* genes from *Nitrosomonas europaea* ATCC 19718, *Nitrosospira multiformis* ATCC25196, *Nitrosospira* NpAV and *Nitrosovibrio tenuis* NV-12, respectively. All qPCR reactions had high efficiencies and detection limits were as low as ten copies of AOB *amoA* and 30 copies of AOA *amoA* (see Supplementary Information for details). All sample and standard reactions were performed in triplicate and an average value was calculated. Possible inhibitory effects on PCR performance from co-extracted polyphenolic compounds in soil extracts were estimated by three different techniques as described in Supplementary Information. Inhibitory effects were negligible in all samples.

Extraction and quantification of GDGTs. GDGTs were extracted from soil with a polar solvent and extracts were cleaned by Al_2O_3-solid phase extraction (SPE) and filtration through 0.45 μm polytetrafluoroethylene (PTFE) filters. GDGT fractions were analysed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) on a cyanopropyl (CN) -column and protonated molecular ions were recorded in selected ion monitoring (SIM) as described previously[21,26] (for details see Supplementary Information).

High-throughput sequencing of cDNA. Total RNA isolated from RUD soil was subjected to reverse transcription with random hexanucleotides and second-strand synthesis (for details see Supplementary Information). The resulting cDNA was sheared into fragments of 100 to 500 base pairs (bp) in length and subsequently used for library construction as described previously[22]. One run on a 70 × 75 mm PicoTiterPlate format was performed on a GS20 Genome Sequencer (Roche Applied Sciences/454 Life Sciences), yielding 314,041 successful sequencing reads. Sequence data were processed as FastA files for all further data analysis, including megablast or primer searches as described in the Supplementary Information.

Received 11 April; accepted 19 June 2006.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank V. Torsvik for use of the qPCR machine, S. L. Jørgensen and V. Torsvik for discussions and L. Knudsen and L. Tomsho for technical assistance. K. Zink is acknowledged for running LC-APCI-MS analyses. We thank for help or support in soil sampling: V. Torsvik (for KRO and STO), E. Schulz (for L-l, L-n and L-h from a long-term field trial in Bad Lauchstädt, Germany), C. Emmerling (for R-nt and R-p), B. Winkler (for GSF), E. Vavoulidou (for B77V, B77T and E16 from the NAGREF Soil Science Institute of Athens). Most of this project was financed through an initial funding of the University of Bergen given to C.S. Part of the pyrosequencing project was paid through the Department of Health using Tobacco Settlement Funds to S.C.S.

Author Contributions The project was conceived and the manuscript was written by C.S., assisted by co-authors. Soil samples were collected and characterized for general parameters by M.S., T.U. and S.L. DNA and RNA extractions were performed by M.S. and S.L. and real-time PCR by S.L.; MPN-PCR and clone libraries were performed by T.U.; GDGT analyses was carried out by L.S.; ds cDNA synthesis and high-throughput sequencing including data analyses was performed by T.U., J.Q. and S.C.S.; and *amoA* phylogeny was performed by G.W.N. and J.I.P.

Author Information Sequences obtained in this study were deposited at GenBank (NCBI) with accession numbers DQ534808–DQ534888. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to C.S. (christa.schleper@bio.uib.no).