Supporting Information

Mußmann et al. 10.1073/pnas.1106427108

SI Materials and Methods

Wastewater Treatment Plants Analyzed. In total, 52 wastewater treatment plants (WWTPs) were analyzed in this study. Details regarding these plants including the type of the wastewater and key operational parameters are summarized in Dataset S1. This dataset also provides information on the sampling dates.

Sampling for FISH and DNA Extraction. The oil refineries WWTPs (plants A–E) and the pilot-scale reactor from a tannery waste plant (plant F) from the United Kingdom were sampled as follows: triplicate biofilm and mixed liquor grab samples, respectively, were collected and samples for both DNA extraction and FISH were initially preserved in ethanol [final concentration 50% (vol/vol)]. Samples were kept at 4 °C during transportation to the laboratory and then stored at −20 °C. For FISH, subsamples were taken from the ethanol preserved samples and additionally fixed using paraformaldehyde (PFA). For this purpose, samples were centrifuged and the pellet was washed once with PBS (10 mM potassium phosphate, 150 mM sodium chloride, pH 7.2) and resuspended in PBS before adding three volumes of a PFA solution (3% final concentration, in PBS). Samples from Germany, Switzerland, and Austria ("continental European") were immediately fixed for FISH by adding one to three volumes of a PFA solution (in PBS, final concentration of 2% or 3%, respectively). All these samples were incubated at 4 °C and further processed within 24 h after sampling. Subsequently, all WWTP samples were washed in PBS to remove residual PFA. After a final centrifugation step the pellets were resuspended in PBS: ethanol (1:1) and stored at −20 °C. For DNA analysis samples from continental European WWTPs were collected in 50-mL plastic vials without fixative, cooled, and shipped to the laboratory for further processing and storage at −20 °C.

DNA Extraction. For the United Kingdom sludges, 200 μL of ethanol fixed biofilm sample (refinery plant A) or 250 μL of activated sludge samples (refineries B, C, and E sampling S1-2, and refinery plant D, sampling S1-4) were centrifuged and the pellets were resuspended in 250 μL of double-distilled H_2O to normalize the differences in mixed liquor suspended solids content in the different sludges. DNA was extracted directly from these 250-μL samples. All manipulations were taken into account when calculating gene abundances from qPCR data. For DNA extraction of sludges investigated by Pickering (1) 250 μL of mixed liquor was used. Before extraction, samples were physically disrupted by bead-beating with a Ribolyser (Hybaid Ltd.) for 30 s at a speed of 6.5 m/s. All DNA extracts were recovered using a BIO 101 FastDNA Spin Kit for Soil (Q-Biogene), following the manufacturer's instructions. DNA of the continental European sludges were extracted from pelleted sludge or biofilm using the Power Soil DNA Isolation Kit, (MO BIO Laboratories, Inc.) according to the manufacturer's protocol.

PCR Screening for the Presence of *amoA*-carrying thaumarchaeotes (AEA) and *mcrA* genes. Out of the 52 sampled WWTPs, 49 were surveyed for the presence of the thaumarchaeotal *amoA* gene by PCR using the primers Arch-amoAF and Arch-amoAR (2). The three WWTPs from Vienna (HKA, OMV, VUW) were only screened by CARD-FISH for the presence of thaumarchaeotes. Samples from the United Kingdom were screened using the following PCR conditions: 95 °C for 5 min; 30 cycles consisting of 94 °C for 45 s, 53 °C for 60 s, and 72 °C for 60 s; and 72 °C for 15 min. The continental European WWTP samples were screened according to the following PCR protocol: 94 °C for 5 min, 35 cycles of 94 °C (40 s), 56 °C (40 s), 72 °C (20 s), and a final extension at 72 °C (5 min). The recovered PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen Ltd.).

The archaeal 16S rRNA gene was amplified from DNA extracted from refinery plants A to E with previously published primers 20f (3) and Uni1392 (4), using the following PCR conditions: 95 °C for 3 min; 30 cycles consisting of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 110 s. Final elongation was performed at 72 °C for 7 min and then at 60 °C for 5 min. The recovered PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen Ltd.). The *mcrA* genes, encoding methyl coenzyme M reductase of methanogenic archaea, were amplified using primers mcrA-MLf and mcrA-MLr (5) at an annealing temperature of 55 °C. Thirty PCR-cycles were used for amplification of target DNA. Cloning and sequencing was performed as described by Juretschko et al. (6).

Phylogenetic Analyses and Probe Design. For phylogenetic analyses of the 16S rRNA and AmoA sequences the ARB program package (7) was used. Based on the ARB-SILVA 16S rRNA database SSURef_92_tree_silva_opt.arb (8) maximum parsimony (100 bootstrap resamplings), distance-matrix (ARB Neighbor Joining, Jukes Cantor correction with 1,000 bootstrap resamplings) and maximum-likelihood methods with a 50% conservation filter for archaeal sequences were applied for inferring 16S rRNA trees. Partial sequences were added to the consensus tree using the parsimony criterion without altering the overall topology. For phylogenetic analysis of the ammonia monooxygenase subunit A (AmoA), 188 amino acid positions were considered for maximum Parsimony (100 bootstrap resamplings), distance-matrix (ARB Neighbor Joining with the JTT correction factor), and maximum-likelihood calculations (Phylip-ML). The reference database used for these analyses contained 3,689 archaeal and bacterial sequences. From the different 16S rRNA and AmoA trees, strict consensus trees were constructed for each marker molecule. Furthermore, based on the recovered 16S rRNA sequences of the group I.1b Thaumarchaeota from plant D and plant E sludges, the new oligonucleotide probe Thaum1162 was designed using the PROBE_DESIGN tool of the ARB program package (7) and used for in situ detection of the respective Thaumarchaeota.

Quantitative PCR. Quantification of *amoA* and 16S rRNA genes was conducted on DNA from three independent biological replicate samples from the reactors. Quantitative PCR assays were performed in 96-well plates using a thermocycler IQ5 (Bio-Rad) according to the manufacture's instructions. The abundance of 16S rRNA genes from group I.1b Thaumarchaeota in plant D was quantified as described by Ochsenreiter et al. (9) using 10 pM of primers 771F (5′-ACGGTGAGGGATGAAAGCT-3′) and 957R (5′-CGGCGTTGACTCCAATTG-3′) and the following PCR conditions: initial denaturation at 95 °C for 7 min, followed by 55 cycles of denaturation at 95 °C for 30 s, primer annealing at 54 °C for 45 s, and extension at 72 °C for 45 s. The archaeal *amoA* gene was quantified as described by Treusch et al. (10) using 20 pM each of primers amo196F (5′-GGWGTKCCRGGRACWGCMAC-3′), amo277R (5′-CRATGAAGTCRTAHGGRTA DCC-3′), and 10 pM of the TaqMan probe (5′6-FAM-CAAACCAWGCWCCYT TKGCDACCCA-TAMRA-3′) (Thermo Electron GmbH) under the following PCR conditions: initial denaturation

at 95 °C for 7 min, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 55 °C for 40 s, extension at 72 °C for 20 s. Some of the archaeal *amoA* sequences recovered from the refinery wastewater treatment reactors contained mismatches with the oligonucleotides used in the qPCR assay. The assay was empirically tested using cloned sequences containing the mismatches to ensure that these would be detected. Running the assay under these conditions with DNA from the refinery WWTPs never resulted in amplification of nonspecific products and the estimates of thaumarchaeal abundance based on qPCR, FISH, and lipid analysis were all consistent.

The bacterial *amoA* gene was quantified as described by Rotthauwe et al. (11) using 6 pM of primers AmoA 1-F (5′-GGGGTTTCTACTGGTGGT-3′), and AmoA 2-R (5′-CCCCTCKGSAAAGCCTTCTTC-3′), and the following PCR conditions: initial denaturation at 95 °C for 7 min, followed by 40 cycles of denaturation at 95 °C for 20 s, primer annealing at 60 °C for 1.5 min, and extension at 72 °C for 1.5 min. For calibration of the 16S rRNA gene and *amoA* gene assays standards were generated from cloned sequences recovered from plant D by amplification from plasmids using vector based primers. DNA concentrations of standards were determined using a DNA spectrophotometer NanoDropND-1000. Standards were serially diluted to concentrations ranging from 10^8 to 10^3 copies/μL. Standards were run in duplicate and the environmental samples were run in triplicate. Besides primers each PCR (20 μL) contained 3 μL DNA template and either a Taqman-probe (see above) or 1% SYBR Green I (Sigma; 10,000 × concentration in DMSO) in 10 μL iQ-Supermix PCR reagent (Bio-Rad) and 6 μL of molecular biology-grade water. Two negative controls without template were included in each assay, as well as two samples spiked with standard DNA to check for PCR inhibition. After SYBR Green assays, melting curves of sample- and clone-derived PCR products were compared and confirmed the presence of only a single peak to demonstrate the specificity of the PCR. After calibration with the standards, the abundance of *amoA* and 16S rRNA genes per milliliter of sludge was calculated. Values of PCR efficiency/slope/R^2 of the standard curve were (69%/−4.395/0.988) for the 16S rRNA gene of Thaumarchaeota group I.1b, (100% /−3.317/0.958) for ammonia-oxidizing bacteria (AOB) *amoA*, and (83%/−3.593/0.992) for AEA *amoA*.

FISH and CARD-FISH. For quantification of total cell numbers and AEA numbers, PFA-fixed samples of plant D were sonicated for 30 s on ice (Bandelin; Sonopuls, cycle 2, amplitude 20%) and were subsequently filtered on polycarbonate membranes (0.2-μm pore size, 47 mm; Millipore). For quantification of AEA, polycarbonate filters were sectioned directly after filtration. Untreated sections were used for counts of the total cell number (see below), whereas the remaining sections were used for catalyzed reporter deposition (CARD)-FISH according to Ishii et al. (12), with slight modifications. Specifically, filter membranes were mounted with 0.1% agarose. Endogenous peroxidases were inactivated by a treatment with 0.15% H_2O_2 in methanol for 30 min. Afterward, membranes were washed in water and ethanol (1 min each). Cells were permeabilized by proteinase K [15 μg/mL (Sigma) in 0.1 M Tris, 0.01 EDTA, pH 8.0, 5–8 min at room temperature] with subsequent washing in water (1 min) and inactivation of proteinase K by 0.01 M HCl for 20 min. Buffers used in hybridization, washing and amplification were prepared as described previously (13). Samples and peroxidase-labeled probes were hybridized for 3 h at 46 °C. Probe Thaum1162 was hybridized for 16 to 20 h to ensure hybridization in 16S rRNA regions of decreased accessibility (14). The signal was amplified using carboxy-fluorescein–labeled tyramides for 45 to 60 min at 46 °C. Subsequently, samples were washed first in water and then in ethanol (1 min each). Finally, DNA was stained by DAPI (1 μg/mL). AEA cell numbers were determined by calculating the ratio of the total cell number of untreated filter sections (thereby avoiding a potential bias because of lysis of some microbial cells because of the application of the CARD-FISH protocol) and AEA number of proteinase K-treated filter sections. Standard FISH for AOB and archaea using Cy3- and FITC-labeled probes was performed on PFA-fixed samples according to Daims et al. (15). AOB probes Nso1225, NEU, NmV, Nso192 cluster 6a, and the respective competitors were mixed in equimolar concentrations and hybridized at 35% formamide (AOB mix).

Modeling Autotrophic Ammonia Oxidizer Abundance. The abundance of ammonia oxidizers in the plant D was estimated from levels of ammonia removal using the model developed by Rittman and colleagues (16, 17). Ammonia oxidizer biomass (X_{AO}) was estimated using the following equation:

$$X_{AO} = \frac{\theta x}{\theta}\left[\frac{Y_{AO}}{1 + b_{AO} * \theta x} * \Delta Ammonia\right],$$

where X_{AO} is the biomass of ammonia oxidizers in milligrams per liter, θ_x is the mean cell residence time in days, θ is the hydraulic retention time in days, Y_{AO} is the growth yield of ammonia oxidizers (0.34 kg VSS/kg NH_4^+ − N), b_{AO} is the endogenous respiration constant of ammonia oxidizers (0.15 d^{-1}), and $\Delta_{Ammonia}$ is the difference in influent and effluent ammonia concentrations in milligrams per liter.

The yield value used was decided based on calculation of the growth yield of *Nitrosopumilus maritimus* based on the data presented in Könneke et al. (18). The yield was 1.15 g dw/mol N, which compared favorably with the range of growth yields reported for AOB in the literature [0.1–1.4 gdw/mol N (19)]. We therefore used the same growth yield and other physiological parameters that have previously been used to estimate the abundance of AOB in WWTP based on ammonia removal (16, 17). Hydraulic parameters, such as mean cell residence time and hydraulic retention time, and ammonia removal data required by the model were calculated from operational data provided by the plant operators. The AEA biomass values obtained from the model were converted to biovolume using a conversion factor of 310 fg·C·μm^3 (19). Cell numbers were calculated from biovolume data on the basis of the mean cell size of thaumarchaeotes (d = 1.47 ± 0.24 μm diameter, n = 50 cells) (Fig. 1) and the AOB (d = 0.75 ± 0.08 μm diameter, n = 25 cells) (Fig. 2) using CARD-FISH confocal laser scanning microscopy from sludge samples from refinery plant D.

Archaea *amoA* mRNA Analysis. Sludge from plant D (S5) was aliquoted (4 mL in 50-mL tubes) in three replicates and 2.0 mM NH_4Cl was added. In addition, three replicates without NH_4Cl amendment served as control treatments. The sludge was incubated overnight at 30 °C without shaking. Subsequently, the sludge samples were stored at −80 °C until RNA extraction. RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and finally dissolved in 100 μL water. Five microliters of community RNA served as template for reverse transcription and subsequent PCR amplification using the Super Script III One Step RT-PCR kit (Promega) according to the manufacturer's protocol. To check for possible DNA contamination, parallel reactions were set up without the initial reverse transcription. For reverse transcription and PCR amplification, primers Arch-amoAF and Arch-amoAR (2) were used with the following conditions: reverse transcription was performed at 45 °C for 45 min and was followed by enzyme inactivation of 2 min at 94 °C. Subsequent PCR was conducted as follows: denaturation at 94 °C for 30 s, primer annealing at 56 °C for 1 min, elongation at 68 °C for 2 min, and a final elongation at 68 °C for 7 min. The PCR product was cloned and sequenced as described above.

Combined FISH and Microautoradiography. Activated sludge from plant D, reactor A was sampled on 7.5.2008 (S5), chilled and transported to the laboratory within 48 h. In addition, activated sludge from this plant (reactor A) was sampled at 9.10.2008 (S6), transported to the laboratory without chilling (to test whether chilling negatively affects the metabolic activity of the members of the Thaumarchaeota), and was processed within 30 h. For sampling S5 the sludge was diluted 10-fold with filtered (0.2-μm pore size) supernatant and was preincubated for 3 h each at 26 °C with 0.05 mM NH_4Cl. The preincubated sludge was subsequently aliquoted into 10-mL vials with a total activated sludge volume of 2 mL. These vials were amended (duplicate incubations) with 0.15, 0.5, and 5 mM NH_4Cl, respectively. After addition of 7.5 μCi [^{14}C]-bicarbonate (Hanke Laboratory Products), the samples were incubated for 6 h at 26 °C (in situ T) without shaking. For sampling S6, the sludge was diluted fourfold and directly amended with 2 μCi [^{14}C]-bicarbonate and 0.5 mM of ammonium, and incubated for 4 h at 31 °C (in situ T) without shaking.

In both experiments controls were included by using sludge without addition of ammonium as well as with sludge treated with 1.8% formaldehyde before the incubation to check for physiological activity of the thaumarchaeotes without added substrate and for chemography, respectively. After incubation, biomass was fixed with 1.8% formaldehyde, as previously described (15). FISH staining was performed as described above. Microautoradiography (MAR) was performed as described earlier with modifications (20). The hybridized samples were dipped in preheated (48 °C) LM-1 emulsion (Amersham), exposed for 7 to 28 d at 4 °C in the dark and developed in Kodak D19 (40 g/L of distilled water) before microscopy. For sampling S6, the FISH-MAR procedure was altered by using slightly different chemicals and by application of membrane filters according to the protocol of Alonso et al. (21).

Diphtheria Toxin-Inhibition Experiment and Ammonium Measurements. Three microliters of undiluted plant D sludge (S5, 7.5.2008) was amended with 0.5 mM of NH_4Cl and with diphtheria toxin (1 μg/mL; Sigma) and was incubated in 10-mL vials in triplicates for 5 h at 27 °C without shaking. Controls were performed without amendment of diphtheria toxin. Finally, the remaining ammonium concentration was measured in the supernatant according to (22). Nitrate formation was tested semiquantitatively by nitrate test strips (Merck).

Nitrogen Fixation Assay. Cooled (4 °) and uncooled sludge samples were sent and stored at 4 °C and room temperature (~25 °C), respectively, before analyses were performed. Nitrogen fixation was assessed by following the incorporation of the stable isotope ^{15}N of $^{15}N_2$ into the microbial biomass as described previously (23). Briefly, 3-mL sludge samples were incubated at 27 °C in an artificial $^{15}N_2$:O_2 atmosphere (80:20%, vol/vol; $^{15}N_2$ at 98 at% ^{15}N; Cambridge Isotope Laboratories) in headspace vials (18 mL, butyl rubber septa) for 14.5 h under constant horizontal shaking (100 rpm). Untreated controls were incubated under the same conditions but with ambient N_2 to determine the natural abundance of ^{15}N in the sludge. After incubation, sludge samples were centrifuged at 12,000 × *g*, the supernatant decanted, and the pelleted biomass dried at 60 °C overnight. Samples were finely ground in a ball mill (MM2000; Retsch GmbH & Co. KG) and aliquots of 0.5-mg dry material were weighed in tin capsules. The abundances of ^{15}N (at% ^{15}N) were determined with a continuous-flow isotope ratio mass spectrometer (Delta Advantage; Thermo), linked to an elemental analyzer. All experiments were done in triplicate.

Measurements of Crenarchaeol Concentrations in Two Activated Sludges. For lipid analysis, 2 × 50 mL of refinery D sludge (S5, 7.5.2008) reactor A, 2 × 50 mL of refinery D, reactor B sludge, and and 1 × 50 mL from Ingolstadt sludge (May 2008) were centrifuged and lyophilized. Preweighed, freeze-dried reactor material was ultrasonically extracted three times with an organic solvent mixture of dichloromethane (DCM):methanol (MeOH) (2:1, vol/vol). Total lipid extracts were collected in a round-bottom flask, evaporated to dryness under rotary vacuum, redissolved in DCM, and dried again over Na_2SO_4. To each extract, 0.1 μg of a C_{46} internal standard was added before it was chromatographed over activated Al_2O_3. The glycerol dibiphytanyl glycerol tetraether (GDGT)-containing fraction was eluted with DCM:MeOH (1:1, vol/vol), collected, and dried under a stream of N_2, redissolved in hexane:isopropanol (99:1, vol/vol) and filtered through a 0.45-μm pore size, 4-mm diameter, Teflon filter.

Archaeal GDGTs were analyzed using HPLC atmospheric pressure chemical ionization mass spectrometry (APCI-MS) by applying conditions slightly modified, as previously reported (24, 25). Analyses were performed using an HP 1100 series LC/MSD equipped with an autoinjector and Chemstation chromatography manager software. For the first 5 min, elution was isocratic with 99% hexane and 1% isopropanol, followed by a gradient to 1.8% isopropanol in 45 min. The flow rate was 0.2 mL/min. Separation was achieved on a Prevail Cyano column (2.1 × 150 mm, 3 μm; Alltech) maintained at 30 °C. After each analysis the column was cleaned by back flushing hexane/propanol (9:1, vol/vol) at 0.2 mL/min for 10 min. Detection was achieved by positive ion APCI with the following conditions: nebulizer pressure (N_2) 60 psi, vaporizer temperature 400 °C, drying gas (N_2) flow 6 L/min and temperature 200 °C, corona current 5 μA, capillary voltage −3kV. Archaeal GDGTs were detected with single ion monitoring of their protonated molecules $[M + H]^+$. SIM parameters were set to detect protonated molecules of common isoprenoid-tetraethers (*m/z* 1,304, 1,302, 1,300, 1,298, 1,296, 1,294, 1,292, 1,290, and 1,288) as well as the internal standard (*m/z* 744), with a dwell time of 237 ms per ion. Archaeal tetraethers were quantified according to Huguet et al. (26). Because of coelution with crenarchaeol, concentrations of GDGT-4 (*m/z* 1,294) corrected for the $[M+H+2]^+$ isotope peak of crenarchaeol (27).

Compound-Specific ^{13}C Analysis of GDGT-Derived Biphytanes. Activated sludge from plant D (reactor A) was sampled at 9.10.2009 (S6), transported to the laboratory without chilling, and was processed within 30 h. The sludge was amended with 0.5 mM [^{13}C]-labeled sodium bicarbonate (99% atom ^{13}C; Sigma) and 1.0 mM of NH_4Cl and aliquoted into triplicate flasks (50 mL), with a total sludge volume of 15 mL. Sludge incubated without addition of ammonia served as control. As an additional control, sludge amended with ammonia and bicarbonate was inhibited by mercury chloride to reveal unspecific bicarbonate adsorption. The sludge was then incubated in the dark for 18 h at 31 °C without shaking. Nitrate formation was tested semiquantitatively by nitrate test strips. After centrifugation the solid sludge fraction was processed further and GDGTs were extracted. GDGTs were subjected to ether bond cleavage as described by Hoefs et al. (28) and analyzed by gas chromatography coupled to mass spectrometry (GC/MS) for biphytanes on a ThermoFinnigan-TRACE gas chromatograph coupled with a ThermoFinnigan DSQ quadrupole mass spectrometer. Compound-specific δ^{13}C analyses were performed on the aliphatic fraction using an Agilent 6800 GC coupled to a ThermoFisher Delta V isotope ratio monitoring mass spectrometer. Isotope values were measured against calibrated external reference gas. The δ^{13}C values for individual compounds are reported in the standard δ notation against the Vienna Pee Dee Belenite standard.

1. Pickering RL (2008) How hard is the biomass working? Can cell specific uptake rates be used to optimise the performance of bacterial biomass in wastewater treatment plants? PhD thesis (Newcastle University, Newcastle upon Tyne, UK).
2. Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* 102:14683–14688.
3. Massana R, Murray AE, Preston CM, DeLong EF (1997) Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara Channel. *Appl Environ Microbiol* 63:50–56.
4. Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR, Stahl DA (1986) Microbial ecology and evolution: A ribosomal RNA approach. *Annu Rev Microbiol* 40:337–365.
5. Juottonen H, Galand PE, Yrjälä K (2006) Detection of methanogenic Archaea in peat: Comparison of PCR primers targeting the *mcrA* gene. *Res Microbiol* 157: 914–921.
6. Juretschko S, et al. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl Environ Microbiol* 64:3042–3051.
7. Ludwig W, et al. (2004) ARB: A software environment for sequence data. *Nucleic Acids Res* 32:1363–1371.
8. Pruesse E, et al. (2007) SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 35: 7188–7196.
9. Ochsenreiter T, Selezi D, Quaiser A, Bonch-Osmolovskaya L, Schleper C (2003) Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ Microbiol* 5:787–797.
10. Treusch AH, et al. (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* 7:1985–1995.
11. Rotthauwe J-H, Witzel K-P, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* 63:4704–4712.
12. Ishii K, Mussmann M, MacGregor BJ, Amann R (2004) An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments. *FEMS Microbiol Ecol* 50:203–213.
13. Pernthaler A, Pernthaler J, Amann R (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microbiol* 68:3094–3101.
14. Yilmaz LS, Noguera DR (2004) Mechanistic approach to the problem of hybridization efficiency in fluorescent in situ hybridization. *Appl Environ Microbiol* 70:7126–7139.
15. Daims H, Stoecker K, Wagner M (2005) Fluorescence in situ hybridization for the detection of prokaryotes. *Advanced Methods in Molecular Microbial Ecology*, eds Osborn AM, Smith CJ (Bios Advanced Methods, Abingdon), pp 213–239.
16. Rittmann BE, et al. (1999) Molecular and modeling analyses of the structure and function of nitrifying activated sludge. *Water Sci Technol* 39(1):51–59.
17. Furumai H, Rittmann BE (1994) Evaluation of multiple-species biofilm and floc processes using a simplified aggregate model. *Water Sci Technol* 29(10-11):439–446.
18. Könneke M, et al. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437:543–546.
19. Fry JC (1990) Direct methods and biomass estimation. *Methods Microbiol* 22:41–85.
20. Lee N, et al. (1999) Combination of fluorescent in situ hybridization and microautoradiography—A new tool for structure-function analyses in microbial ecology. *Appl Environ Microbiol* 65:1289–1297.
21. Alonso C, Pernthaler J (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. *Appl Environ Microbiol* 71: 1709–1716.
22. Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol Fertil Soils* 6:68–72.
23. Fürnkranz M, et al. (2008) Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. *ISME J* 2:561–570.
24. Hopmans EC, Schouten S, Pancost RD, van der Meer MTJ, Sinninghe Damsté JS (2000) Analysis of intact tetraether lipids in archaeal cell material and sediments by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun Mass Spectrom* 14:585–589.
25. Schouten S, Huguet C, Hopmans EC, Kienhuis MVM, Damsté JSS (2007) Analytical methodology for TEX86 paleothermometry by high-performance liquid chromatography/ atmospheric pressure chemical ionization-mass spectrometry. *Anal Chem* 79:2940–2944.
26. Huguet C, et al. (2006) An improved method to determine the absolute abundance of glycerol dibiphytanyl glycerol tetraether lipids. *Org Geochem* 37:1036–1041.
27. Weijers JWH, Schouten S, van der Linden M, van Geel B, Damsté JSS (2004) Water table related variations in the abundance of intact archaeal membrane lipids in a Swedish peat bog. *FEMS Microbiol Lett* 239:51–56.
28. Hoefs MJL, et al. (1997) Ether lipids of planktonic archaea in the marine water column. *Appl Environ Microbiol* 63:3090–3095.

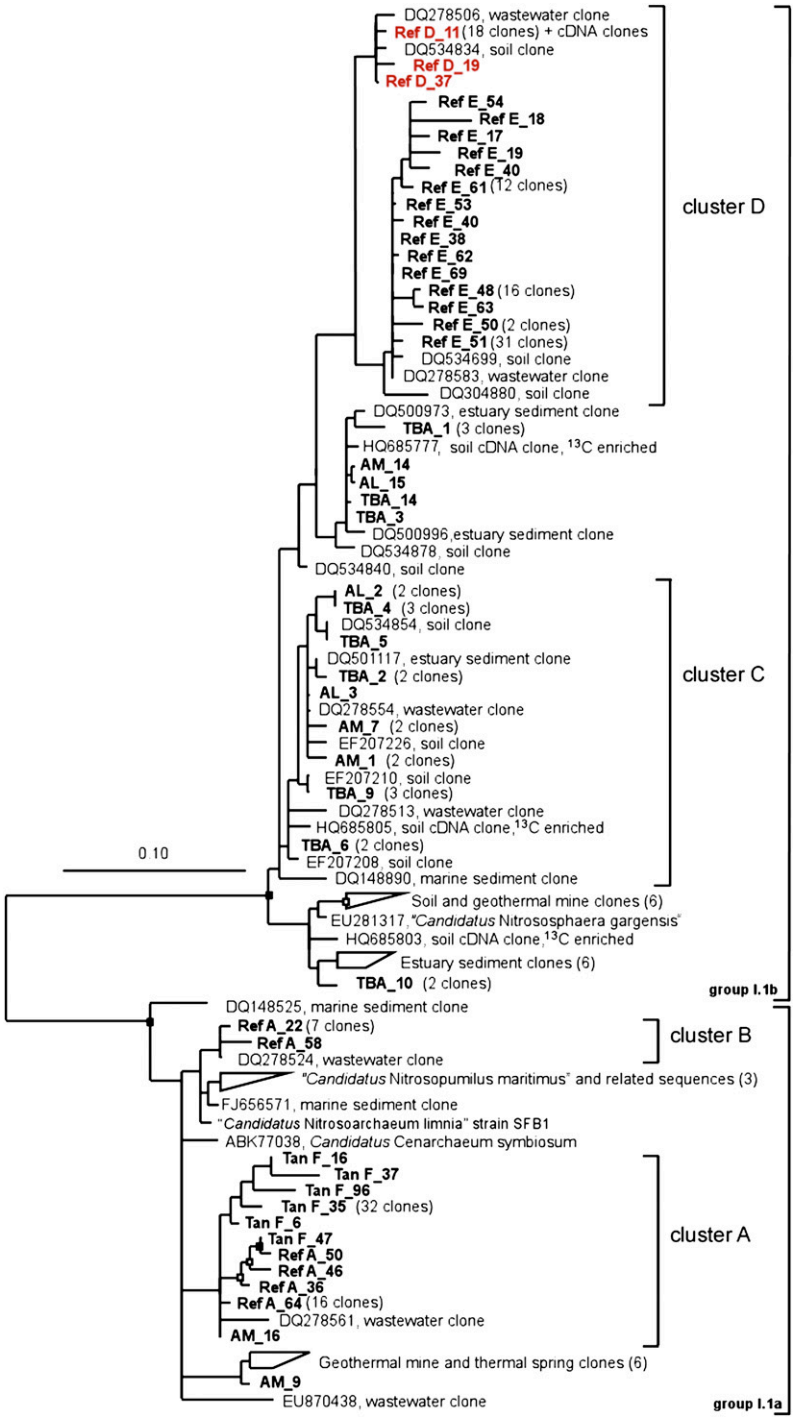

Fig. S1. Phylogeny of the 193 thaumarchaeotal AmoA sequences recovered from seven activated sludge samples. Based on different treeing methods, a consensus tree was constructed considering 188 amino acid positions. Sequences were grouped based on a nucleotide sequence identity cut-off >99%. Bootstrap support >70% (□), bootstrap support >90% (■) using 100 iterations (Maximum Parsimony). Cluster designations were made according to Park et al. (1). Clones were retrieved from six industrial WWTPs [animal rendering and dairy plant Ampfing (AM); animal rendering plant Lyss (TBA); oil refinery plants A, D, E, and tannery plant F (Ref A, D, E, and TanF)] and the municipal plant Altmannstein (AL). Clones from refinery plant D are labeled in red. (Scale bar, 10% estimated sequence divergence.)

1. Park HD, Wells GF, Bae H, Criddle CS, Francis CA (2006) Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. *Appl Environ Microbiol* 72:5643–5647.

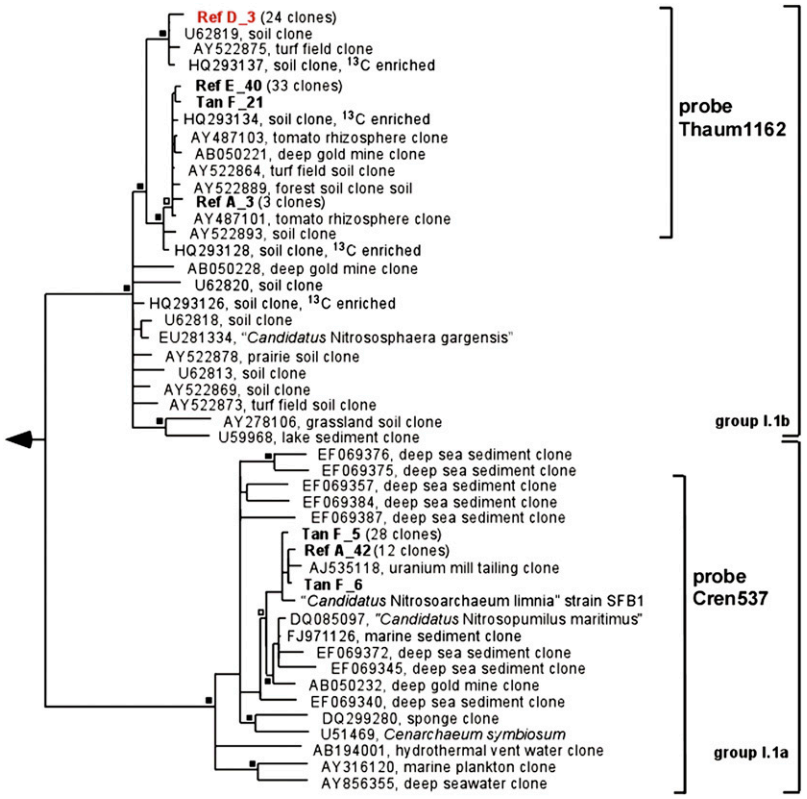

Fig. S2. Phylogeny of the 102 thaumarchaeotal 16S rRNA sequences recovered from four activated sludge samples: Refinery plants A, D, and E (Ref A, D, E) and tannery plant F (Tan F). Based on different treeing methods, a consensus tree was constructed. Sequences were grouped based on a sequence identity cut-off >99%. Bootstrap support >70% (□); bootstrap support >90% (■) using 100 iterations (Maximum Parsimony). Brackets indicate specificities of probes Cren537 and Thaum1162. Clones from refinery plant D are labeled in red. (Scale bar, 10% estimated sequence divergence.)

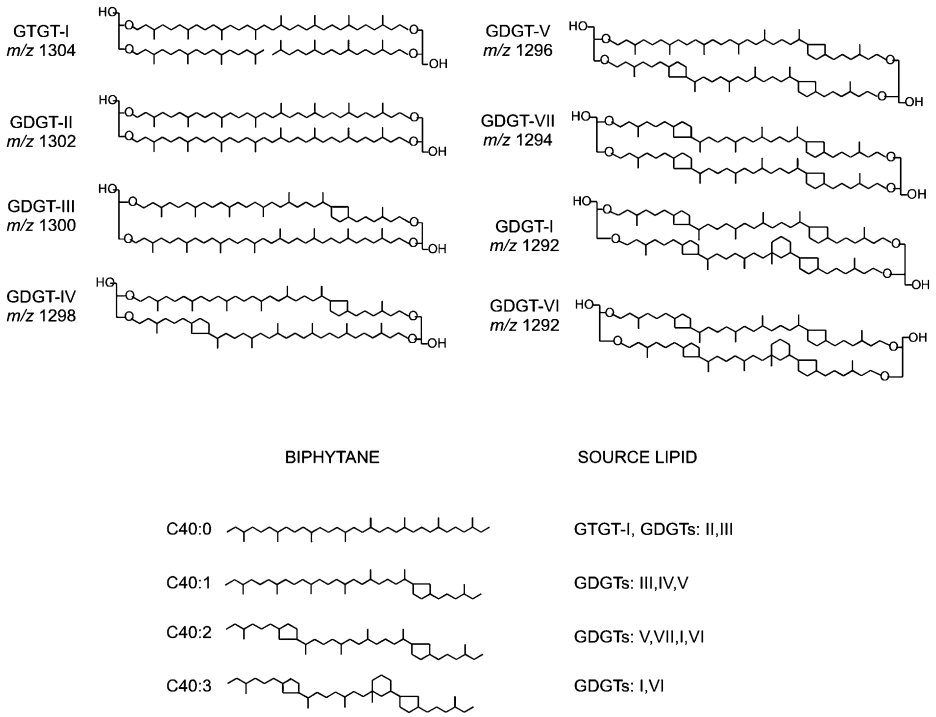

Fig. S3. Structures of archaeal glycerol trialkyl glycerol tetraethers (GTGT) and GDGTs referred to in Table S2.

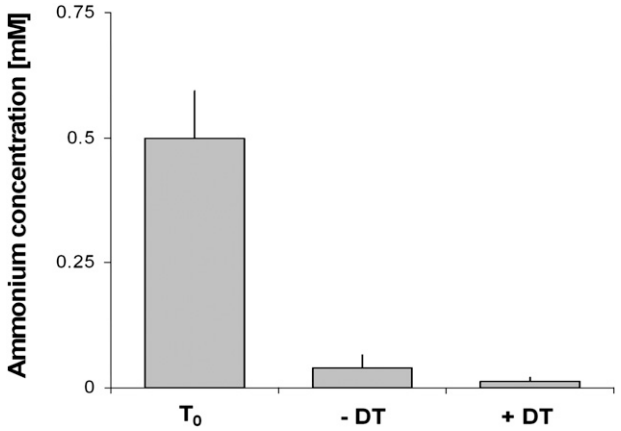

Fig. S4. Ammonium concentration in plant D sludge (07.05.2008) amended with 0.5 mM ammonium at the beginning of the experiment (T0), and after 2.5 h of incubation at 27 °C in the absence (–DT) or presence of 1 μg/mL diphtheria toxin (+DT). After 2.5 h in both samples, nitrate formation could be detected with test strips (not shown).

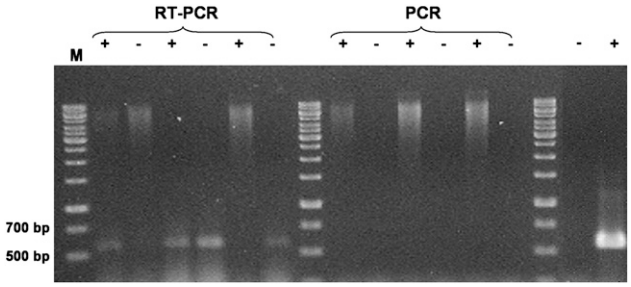

Fig. S5. Detection of mRNA of thaumarchaeotal *amoA* in ammonia-amended (+) and unamended (–) plant D sludge (07.05.2008). (*Left*) mRNA detection by RT-PCR. (*Center*) PCR control for DNA contamination in the same samples. Each experiment was performed with three replicates. On the right side of the gel an *amoA* PCR-negative and -positive control are shown, respectively. M, size marker. It should be noted that *amoA* expression was detected in two of the three replicates independent of the addition of ammonium.

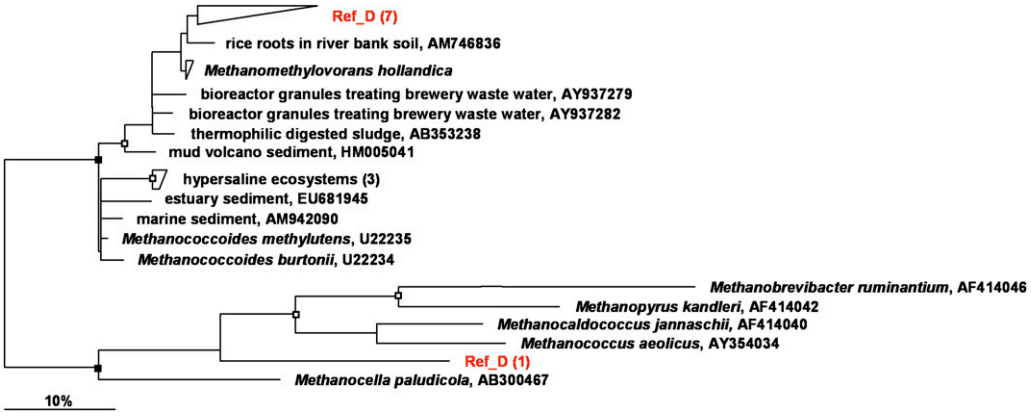

Fig. S6. Phylogenetic analysis of McrA sequences (152-aa positions considered) inferred from cloned *mcrA* gene fragments from plant D. Based on different treeing methods, a consensus tree was constructed using a strict consensus rule. Two short sequences from the plant D sludge were added to the tree without changing the overall tree topology. Bootstrap support >70% (□); bootstrap support >90% (■) using 100 iterations (Maximum Parsimony). (Scale bar, 10% estimated sequence divergence.)

Table S1. Oligonucleotide probes used for FISH or CARD-FISH

Probe	Specificity	Sequence (5′- 3′)	Label	Formamide %	Source
EUB_I	Most bacteria	GCT GCC TCC CGT AGG AGT	HRP	10–35%	(1)
NONsense	Control probe	AGA GAG AGA GAG AGA GAG	HRP	10–35%	(2)
Arch915	Most archaea	GTG CTC CCC CGC CAA TTC CT	HRP/Cy3	10–35%	(3)
Cren512	Thaumarchaeota, most crenarchaeota except Thermoprotei	CGG CGG CTG ACA CCA G	HRP	5%	(4)
Comp_Cren512a	Competitor for Cren512	CGG CGG CTG GCA CCA G		5%	Present study
Comp_Cren512b	Competitor for Cren512	CGG CGG CTG GCA CCC G		5%	Present study
Cren537	Marine group I.1a thaumarchaeota	TGA CCA CTT GAG GTG CTG	HRP	20%	(5)
Thaum1162	Subcluster of the thaumarchaeotal cluster I.1b	TTC CTC CGT CTC AGC GAC	HRP	20%	Present study
Comp_Thaum1162	Competitor for Thaum1162	TTC CTC CGC CTC AGC GGC		20%	Present study
Eury499	Most Euryarchaeota	CGG TCT TGC CCG GCC CT	HRP	20%	(4)
NEU	*Nitrosomonas eutropha*, *Nitrosomonas europaea,*	CCC CTC TGC TGC ACT CTA	Cy3	35%	(6)
CTE	Competitor for NEU	TTC CAT CCC CCT CTG CCG		35%	(6)
Nso192 cluster 6a	*Nitrosomonas oligotropha* lineage (cluster 6a)	CTT TCG ATC CCC TAC TTT CC	Cy3	35%	(7)
Comp_Nso192 cluster 6a	Competitor for Nso192	CTT TCG ATC CCC TGC TTC C	Cy3	35%	(7)
NmV (Ncmob)	*Nitrosococcus mobilis*	TCC TCA GAG ACT ACG CGG	Cy3	35%	(8)
Nso1225	Most β-proteobacterial ammonia oxidizing bacteria	CGC CAT TGT ATT ACG TGT GA	Cy3	35%	(9)
Nso190	Some β-proteobacterial ammonia oxidizing bacteria	CGA TCC CCT GCT TTT CTC C	Cy3	40%	(9)

1. Amann RI, et al. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 56: 1919–1925.
2. Hatzenpichler R, et al. (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci USA* 105:2134–2139.
3. Stahl DA, Amann R (1991) *Nucleic Acid Techniques in Bacterial Systematics*, eds Stackebrandt E, Goodfellow M (John Wiley & Sons Ltd., Chichester, United Kingdom), pp 205–248.
4. Jurgens G, et al. (2000) Identification of novel Archaea in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent in situ hybridization(1). *FEMS Microbiol Ecol* 34 145–56.
5. Teira E, Reinthaler T, Pernthaler A, Pernthaler J, Herndl GJ (2004) Combining catalyzed reporter deposition fluorescence in situ hybridization and microautoradiography to detect substrate utilization by bacteria and Archaea in the deep ocean. *Appl Environ Microbiol* 70:4411–4414.
6. Wagner M, Rath G, Amann R, Koops HP, Schleifer KH (1995) In situ identification of ammonia-oxidizing bacteria. *Syst Appl Microbiol* 18:251–264.
7. Adamczyk J, et al. (2003) The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. *Appl Environ Microbiol* 69:6875–6887.
8. Juretschko S, et al. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl Environ Microbiol* 64:3042–3051.
9. Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl Environ Microbiol* 62:2156–2162.

Table S2. Concentrations of GDGTs and glycerol trialkyl glycerol tetraethers (GTGT) from refinery plant D (7.5.2008) and the Ingolstadt WWTP, which served as a control plant without AEA detectable by molecular methods

	Concentration (μg/g dry sludge)							
	GTGT-I	GDGT-II	GDGT-III	GDGT-IV	GDGT-V	GDGT-VII	GDGT-I	GDGT-VI
Plant D Reactor A	0.1	3.8	0.9	0.9	0.5	1.9	8.8	0.4
Plant D Reactor B	0.1	3.8	1.1	1.0	0.6	2.4	10.9	0.4
Ingolstadt (control)	0.5	92.9	0.3	0.3	0.1	0.1	0.1	0.01

Table S3. Incorporation of ^{15}N from $^{15}N_2$ into biomass in refinery D sludge and δ ^{13}C of sludge organic matter

Identifier	at% C	δ $^{13}C/^{12}C$	at% N	δ $^{15}N/^{14}N$	δ $^{15}N/^{14}N$ mean	SD
October 2010						
4 °C-I	32.27	−28.761	4.35	15.842		
4 °C-II	32.51	−28.708	4.40	15.857		
4 °C-III	33.80	−28.792	4.59	15.895	**15.865**	0.027
4 °C+^{15}N-I	31.40	−28.765	4.28	15.856		
4 °C+^{15}N-II	32.40	−28.839	4.45	15.807		
4 °C+^{15}N-III	32.33	−28.852	4.46	15.752	**15.805**	0.052
RT-I	33.69	−28.968	4.50	15.606		
RT-II	33.46	−28.758	4.45	15.806		
RT-III	34.21	−28.769	4.58	15.869	**15.760**	0.137
RT+^{15}N-I	32.48	−28.869	4.44	15.761		
RT+^{15}N-II	32.79	−28.785	4.47	15.758		
RT+^{15}N-III	32.65	−28.855	4.49	15.677	**15.732**	0.048
November 2010						
RT-I	35.66	−27.627	4.36	21.393		
RT-II	34.87	−27.626	4.26	21.450		
RT-III	33.94	−27.661	4.17	21.321	**21.388**	0.065
RT+^{15}N-I	35.37	−27.618	4.27	21.563		
RT+^{15}N-II	35.28	−27.711	4.29	21.429		
RT+^{15}N-III	34.83	−27.776	4.30	21.380	**21.457**	0.095

4 °C, RT, sludge samples were sent and stored at 4 °C and room temperature (~25 °C), respectively; at%: atom percent; I, II, III: replicate number. All incubations were conducted at 27 °C.

Table S4. δ ^{13}C analysis of GDGT-derived biphytanes from ^{13}C labeling experiments

	C40:0	C40:1	C40:2	C40:3*	Phytane
T_o	−56.0 ± 1.7	−43.1 ± 0.7	−44.3 ± 1.5	−46.2 ± 1.9	−60.4 ± 0.3
$T_{(end)}$ †	−48.1 ± 1.0	−37.8 ± 1.1	−38.2 ± 0.5	−38.9 ± 0.8	−52.8 ± 0.3
$T_{(end)}$ ‡	−41.7 ± 0.6	−35.5 ± 1.5	−37.4 ± 0.6	−39.5 ± 1.1	ND

Incorporation of ^{13}C into crenarchaeol-derived biphytanes in refinery plant D (sampling S6, 9.10.2009) amended with 0.5 mM ^{13}C-labeled bicarbonate with or without addition of 1 mM NH_4^+. Errors indicate the SD from three to four measurements. ND, not determined.

*C40:0, C40:1, C40:2 and C40:3 refer to biphytanes with 0–3 cycloalkyl rings, respectively (Fig. S3). C40:3 biphytane contains two cyclopentane and a cyclohexane ring, which is thought to be exclusively derived from crenarchaeol and thus to be highly specific to thaumarchaeotes.

†Experiment with addition of ^{13}C labeled bicarbonate.

‡Experiment with addition of ^{13}C labeled bicarbonate and NH_4^+.

Other Supporting Information Files

Dataset S1 (XLSX)