The ISME Journal
January 2009 ; Volume 3 (1) : Pages 117-123
http://dx.doi.org/10.1038/ismej.2008.72
Copyright © 2009 Nature Publishing Group

The original publication is available at http://www.nature.com

Archimer http://www.ifremer.fr/docelec/
Archive Institutionnelle de l'Ifremer

Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal vents

Nathalie Byrne[1, *], Marc Strous[2], Valentin Crépeau[1], Boran Kartal[2], Jean-Louis Birrien[1,] Markus Schmid[2], Françoise Lesongeur[1], Stefan Schouten[3], Andrea Jaeschke[3], Mike Jetten[2], Daniel Prieur[1] and Anne Godfroy[1]

[1] Laboratoire de Microbiologie des Environnements Extrêmes UMR6197 IFREMER, Centre de Brest–BP70/IUEM, Plouzané, France
[2] Department of Microbiology, Institute for Water and Wetland Research, Radboud University Nijmegen, Nijmegen, The Netherlands
[3] Department of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, Den Burg, The Netherlands

*: Corresponding author : N. Byrne, Phone : 00 33 (0)2 98 22 46 74, email address : nathalie.byrne@univ-brest.fr

Abstract:

Recent studies indicate that ammonia is an important electron donor for the oxidation of fixed nitrogen, both in the marine water column and sediments. This process, known as anammox, has so far only been observed in a large range of temperature habitats. The present study investigated the role of anammox in hydrothermal settings. During three oceanographic expeditions to the Mid-Atlantic Ridge, hydrothermal samples were collected from five vent sites, at depths ranging from 750 to 3650 m from cold to hot habitats. Evidence for the occurrence of anammox in these particular habitats was demonstrated by concurrent surveys, including the amplification of 16S rRNA gene sequences related to known anammox bacteria, ladderanes lipids analysis and measurement of a 14N15N dinitrogen production in isotope-pairing experiments at 60 and 85 °C. Together these results indicate that new deep-branching anammox bacteria may be active in these hot habitats.

Keywords: anammox, micro-organisms, activity, 16S rRNA, ladderanes, hydrothermal vent

1. Introduction

Research on anammox − the anaerobic oxidation of ammonium − has a long history. Since 1932, anomalous nitrogen losses were noticed in water sediments (Allgeier et al., 1932) and anoxic fjords (Richards, 1965). In the last decade, anammox bacteria have been actively investigated, leading to a basic understanding of the metabolism and biodiversity of these unique prokaryotes (Strous et al., 1999a)

In oceanic ecosystems and in anoxic basins and fjords, denitrification (the microbial conversion of nitrate to N_2) was previously considered as the main process converting fixed nitrogen to gaseous N_2. It was recently discovered that the anaerobic oxidation of ammonium coupled to nitrite reduction can be responsible for a significant fraction of N_2 production in marine sediments (Thamdrup and Dalsgaard, 2002). Nutrient profiles, activity measurements, ladderane lipids analysis, 16S rRNA gene sequences and FISH (Fluorescent In Situ Hybridization), showed that *Candidatus* "Scalindua sorokinii" was present and active in the anoxic basin of the Black Sea (Kuypers et al., 2003). In the meantime, many studies have shown that the anammox bacteria and process are ubiquitous and constitutes a substantial sink of fixed nitrogen in the oceans (Dalsgaard et al., 2003; Kuypers et al., 2003; Penton et al., 2006; Schmid et al., 2007). The process is also significant in oxygen minimum zones (Kuypers et al., 2005; Hamersley et al., 2007; Jaeschke et al., 2007), sediments (Engstrom et al., 2005; Penton et al., 2006) and estuaries (Trimmer et al., 2003; Tal et al., 2005). These are all mesophilic to cold environments and it is presently unknown whether anammox bacteria are also active at higher temperatures in marine ecosystems.

Hot environments are significant in past and present oceans and deep sea hydrothermal vents are well known examples of such environments. Deep-sea hydrothermal vents are small, patchy and unstable habitats, characterised by steep chemical and physical gradients because of the mixing of the super heated hydrothermal anoxic fluid with cold oxic sea water. Biological communities are distributed along these gradients where the decrease in temperature is more or less correlated to the transition from anoxic to oxic conditions. Reduced compounds are available along the gradient and can be used as energy sources by the prokaryotes.

Since the discovery of hydrothermal vents in 1977, microbiological studies were primarily devoted to the high temperature part of this ecosystem and resulted in the isolation of numerous prokaryotes (Miroshnichenko, 2004). New species belonging to both the *Archaea* and *Bacteria* were isolated and described. In addition, molecular approaches have revealed astonishing microbial diversity which includes numerous as-yet-uncultivated organisms that likely reflect the unusual environmental setting of the deep sea hydrothermal vent environment (Takai et al., 2001; Alain et al., 2002b; Nercessian et al., 2003; Schrenk et al., 2003).

In the nitrogen cycle, the oxidation of ammonium has been demonstrated *via* the isolation of thermophilic heterotrophic nitrifiers growing aerobically at 65°C (Mével and Prieur, 1998). Thermophilic nitrate-reducers (denitrifiers) belonging to the archaeal and bacterial domains have also been isolated and described (Alain et al., 2002a). A methanoarchaeon was recently found to fixed nitrogen at 92°C and by the way completes the current understanding of the nitrogen cycle in high temperature environments (Mehta and Baross, 2006). Even more recently, thermophilic autotrophic nitrifiers were enriched from terrestrial hot springs (de la Torre et al., 2008) On the other hand, numerous unsuccessful attempts have been made to enrich or isolate autotrophic nitrifiers from hot environments. The present study is the first to address the presence and activity of anammox microorganism at high temperatures. So far, the highest temperature at which anammox activity has been observed was 43°C, namely for a laboratory culture enrichment of *Candidatus* "Brocadia anammoxidans" (Strous et al., 1999b).

In this study, various samples, collected along the temperature gradient of several Mid Atlantic Ridge vent fields, were processed through molecular, chemical and microbiological methods for detection of anammox bacteria and/or anammox activity. The result of this investigation yielded strong indication for the presence and activity of new anammox bacteria in different hydrothermal areas.

2. Materials and methods

Samples (Table 1):

Hydrothermal samples were retrieved from five hydrothermal sites; Rainbow (36,2°N ; 33,9°W), Lucky Strike (37,29°N ; 32,28°W), Lost City (30,07°N ; 42,07°W), TAG (26°08'N, 44°49'W) and Menez Gwen (37,85°N ; 31,51°W) on the Mid Atlantic Ridge (Table 1), during the scientific cruises EXOMAR in 2005, MoMARETO in 2006 and MoMARDREAM in 2007 on the R/V Atalante and R/V Pourquoi Pas? using the remote operated vehicle (ROV) Victor 6000 and the submersible Nautile. Samples were obtained in such a way that the different biotopes of the ecosystem along the temperature gradient were covered as good as possible. To this end active smoker, animals and microbial mats were sampled. Active hydrothermal chimneys fragments where fluid temperature was ranging from 30 to 300°C were collected by the teleoperated arm of the ROV or Nautile. Samples were transferred to the surface into a previously decontaminated insulated box. Mussels and shrimps were collected in insulated boxes and using a slurp gun device respectively. Microbial mats were sampled using the PEPITO water sampler (Sarrazin and Sarradin, 2006) and concentrated on 0.22 µm pore-size polycarbonate filters while on board. Chimney and animals samples (mussels' gills and whole shrimps) were crushed into sterile sea water. One aliquot was immediately transferred to a flask or bottle, flushed with 100% helium and stored at 4°C, for further anammox activity measurements. A second aliquot was frozen at -80°C for DNA extraction. Microbial mat samples were stored at -80°C for DNA extraction.

Molecular techniques and phylogenetic analysis

DNA isolation and PCR were done as described by (Schmid et al., 2005), except for DNA extraction from chimney sample; that was performed using the Fast DNA kit for soil samples (Webster et al., 2003). Samples were extracted several times, pooled and concentrated. 16S Ribosomal RNA partial genes were amplified using the specific anammox primers Pla46F (5'-GGATTAGGCATGCAAGTC-3'), BS820R(5'-TAATTCCTCTATTAGT-3') and Amx820R (5'-AAACCCCTCTACTTAGTGCCC-3') (Jetten et al., 2005; Schmid et al., 2005). PCR products were subsequently cloned with the TOPO XL cloning kit (Invitrogen) according to the manual of the manufacturer and sequenced at OUEST-Genopole. Roscoff France. The molecular work was carried out in the laboratory in Brest where no culture of anammox planctomycetes was ever present.
BLAST homology searches were done to determine phylogenetic affiliations. Sequences were aligned using the BioEdit software version *7.0.5* (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and ClustalW. Trees were constructed using the PHYLO_WIN program on the basis of evolutionary distance and maximum likelihood methods (Galtier et al., 1996). The robustness of inferred topologies was tested by the bootstrap resampling of trees calculated on the basis of the evolutionary distance, neighbor-joining algorithm with Jukes–Cantor correction. The overall tree topology was confirmed by further analysis with distance matrix and maximum parsimony methods.

Ladderane PC-monoalkylether analysis:

Lipids were ultrasonically extracted from four chimney samples (4, 6, 7 and 10; ca. 4 g dry weight) according to a modified method of Bligh and Dyer (Bligh and Dyer, 1959), using three times a mixture of methanol, dichloromethane (DCM) and phosphate buffer at pH 7.4 (2:1:0.8 v/v). The extracts were combined and further DCM and buffer were added to the mixture to achieve a final methanol/DCM/buffer ratio of 1:1:0.9 (v/v). The phases were separated and the extraction repeated three more times. An aliquot of the extract was dissolved in a DCM/methanol mixture (9:1 v/v) and filtered through a 0.45 µm, 4 mm diameter RC filter.
The C_{20}-[3]-monoalkylether containing a phosphocholine (PC)-headgroup (see structure in Figure 2) was analyzed by HPLC/electrospray ionization (ESI)-MS/MS according to (Boumann et al., 2006) with some modifications. Separation was achieved on a LiChrospher diol column (250 mm x

2.1 mm, 5 µm particles) maintained at 30°C. The following linear gradient was used with a flow rate of 0.2 mL min^{-1}: 90% A:10% B to 70% A: 30% B over 10 min, maintained for 20 min, then to 35% A:65% B in 15 min, maintained for 15 min, then back to 100% A for 20 min to re-equilibrate the column, where A = hexane/2-propanol/formic acid/14.8 M NH_{3aq} ratios 79:20:0.12:0.04 (v/v/v/v) and B = 2-propanol/water/formic acid/14.8 M NH_{3aq} ratios 88:10:0.12:0.04 (v/v/v/v). Detection of the C_{20}-[3]-monoalkylether-PC was achieved by Selective Reaction Monitoring (SRM) of the transition from *m/z* 530, the $[M+H]^+$ ion, to *m/z* 184 (corresponding to the PC-headgroup), with 1.5 mTorr Argon as collision gas and 20V collision energy. Quantification of intact ladderane etherlipids was done by an external calibration curve of an isolated C_{20}-[3]-monoalkylether-PC standard (43% purity). A detection limit of 10 pg injected on column was achieved with this technique.

Activity measurements

Anammox activities were measured on chimney samples 4, 6, 7, 8 and 10 stored under a helium atmosphere. Each sample was incubated with a mixture of $^{14}NH_4^+$ (final concentration 20µM) and $^{15}NO_2^-$ (final concentration 20µM) at three different temperatures 30, 60 and 85°C. Three gas analyses were done after 20, 44, and 68 hours incubation. For each measurement, 500 µl gas were injected into a GC coupled to an isotope ratio mass spectrometer (Thermo Finnigan delta plus). Gas samples were all analysed for their content of $^{14}N^{15}N$ dinitrogen gas, a direct evidence for anammox activity (Strous et al., 1999a; Kartal et al., 2007). Activity measurements were done at Nijmegen University (Netherlands).

Nucleotide sequence accession numbers:

The EMBL accession numbers of the sequences used in this study are AM941022 to AM941038.

3. Results and discussion

Molecular detection of anammox Bacteria in the hydrothermal vent ecosystem

Using anammox specific primers, 16S ribosomal RNA gene sequences were retrieved from different representative samples of the vent ecosystem and from various hydrothermal sites (Figure 1). In the cold part of the ecosystem, several anammox 16S rRNA gene sequences were found in microbial mats and mussel gills (Figure 1). Some of the mussel sequences were related to known anammox bacteria. The similarity to marine *Candidatus* “Scalindua species” was about 93% of similarity for “Mussel 2.2, 2.4”, while for “Mussel 2.5 and 2.6” a similarity of 97% was obtained to *Candidatus* “Kuenenia stuttgartiensis”. The other retrieved 16S rRNA gene sequences (Mat 1.8, 2.3, 2.4, 2.10, Mussel 2.13) were related to uncultivated bacteria outside the known anammox clade.
All these sequences branched close to the root of the anammox line of descent. Sequences with highest similarity to these were previously detected in geothermal areas and in the deep, sulfidic water column of the Black Sea. Presently, it is not possible to assign these 16S rRNA gene sequences to bacteria with verified anammox metabolism. In mat samples, the concentrations of ammonium (8-10µM), nitrite (0-2µM), as well as the pH range between 6.2 and 8, and temperature between 4 and 10°C (Sarradin et al., 1999) are compatible with the physiology of the known anammox bacteria (Strous et al., 1999a; Jetten et al., 2005).
At the Menez Gwen vent field, three anammox sequences were retrieved from mussel gills (Mussel 2.2, 2.4, 2.5). One sequence (Mussel 2.6) was distantly related to the genus *Candidatus* “Kuenenia.sp” (93% of similarity), one to *Candidatus* “Scalindua species” (Mussel 2.5) (97% of similarity) and the other to uncultivated Planctomycetes (Mussel 2.13) from the Black Sea. Temperature, pH and chemical conditions were similar to those measured on the microbial mats from Lucky Strike (Sarradin et al., 1999). Nevertheless, as oxygen is present in the mussel gills, the anammox reaction should theoretically be inhibited. But, high concentrations of sulfide

measured in the inner shell water could induce temporary or local anoxic conditions (Dando & Sarradin pers.com). Unfortunately no activity measurements could be performed to confirm these molecular data because animal samples can not be preserved at 4°C without any degradation until analysis.

Hydrothermal vent active chimneys are typically hot and "anaerobics" habitats where suitable amounts of nitrites and ammonium are present. Chimney 1 yielded sequences distantly related to Planctomycetes from the sulfidic basin of the Black Sea (up to 82 % of similarity) (Kuypers et al., 2003) and from other hydrothermal sites. Interestingly, chimneys 8 and 10 yielded sequences in box B (Figure 1), forming a clade with sequence "mat 2.6" and a sequence from a biofilter treating pig manure. Finally, a sequence closely related to the genus *Candidatus* "Kuenenia. sp" (98% of similarity) was obtained from chimney 8 from the Rainbow vent field where fluid temperatures up to 300°C were measured. Due to high background fluorescence in the chimney samples, FISH (Fluorescent In Situ Hybridization) analysis with specific anammox probes could not be performed.

Ladderane PC-monoalkylether analysis:

In addition to 16S ribosomal RNA gene sequences, independent specific biomarkers, so-called ladderane lipids, were used to trace anammox bacteria in hydrothermal chimney samples (Table 1). We specifically targeted the intact ladderane monoether lipid with a PC headgroup as phospholipids are derived from living biomass rather than dead cell material (White et al., 1979). Furthermore, this lipid is present in nearly all presently known anammox genera (Rattray et al., 2008). We detected this PC ladderane lipid in all four chimney samples 4, 6, 7 and 10 at range between 20 and 91 pg/g of sediment.

Activity measurements: Table 1

In order to support the molecular and biomarker data, activity measurements were performed on chimney samples (Table 1). *Kuenenia stuttgartiensis* cells, used as positive control were active at 30°C and showed no activity at 60 and 85°C. Anammox activity was also detected at 30°C for the chimney 6 at a rate of 0.02 µM/day.

At 60°C, anammox activity was measured in chimney 7 and 10 at a rate of 0.01µM/day. Anammox activity could even be measured at 85°C in chimneys 4 and 10 and the rate was 0.03 µM/day at 85°C. These rates are in the range of anaerobic ammonium oxidation rates measured in the Black Sea (Kuypers et al., 2003) and in the Benguela upwelling system (Kuypers et al., 2005). The number of active anammox cells inferred that could be expected from these results was comprised between 4.79×10^3 and 1.44×10^4 cells/ml.

In conclusion, all our results suggested that anaerobic ammonium oxidizing bacteria are present and active in hydrothermal vent areas, possibly even at high temperatures. Ladderanes lipids, 16S rRNA gene sequences and anammox activity were detected in Chimney 4, 6, 7 and 10. Additionally, for two of them some sequences retrieved from chimney 4 and 10 clustered in clade B (figure 1) suggesting that the the phylotype might represent a new anammox clade. Our future effort will focus on the enrichment of members of this cluster in a laboratory scale bioreactor.

Anammox metabolism in marine ecosystems was an important discovery for the oceanic nitrogen cycle (Dalsgaard et al., 2003; Kuypers et al., 2003; Meyer et al., 2005), anammox bacteria highlighted in hydrothermal ecosystems could allow a better comprehension of the nitrogen cycle in the deep ocean

Acknowledgements

The authors want to thank Anne Godfroy, Pierre-Marie Sarradin and Josée Sarrazin, Françoise Gaill, respectively, chief scientist of the EXOMAR, MoMARETO and MoMARDREAM cruises, as well as the captain and crew of the research vessels *Atalante* and *Pourquoi pas?* and the Victor and Nautile team. Jelle Eygenstein (RU Nijmegen) is thanked for his help on the isotope ratio mass spectometer. Katharina Ettwig is thanked for solving the gas contamination problems. All people working in the department of Microbiology (RU Nijmegen) and in the LMEE laboratory in Brest are thanked for the hospitality and help during various part of the work. We thank Karine Alain for helpfull reading of the manuscript. This work was supported by ANR DEEP OASES and Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (grant for NB).

References

Alain, K., Querellou, J., Lesongeur, F., Pignet, P., Crassous, P., Raguenes, G. et al. (2002a) *Caminibacter hydrogeniphilus* gen. nov., sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium isolated from an East Pacific Rise hydrothermal vent. *Int J Syst Evol Microbiol* **52**: 1317-1323.

Alain, K., Olagnon, M., Desbruyeres, D., Page, A., Barbier, G., Juniper, S.K. et al. (2002b) Phylogenetic characterization of the bacterial assemblage associated with mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis. FEMS Microbiol. Ecol.* **42**: 463-476.

Allgeier, R.J., Peterson, W.H., Juday, C., and BirgeE, A. (1932) The anaerobic fermentation of lake deposits. *Int. Rev. Hydrobiol.*

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**: 911-917.

Boumann, H.A., Hopmans, E.C., van de Leemput, I., Op den Camp, H.J.M., van de Vossenberg, J., Strous, M. et al. (2006) Ladderane phospholipids in anammox bacteria comprise phosphocholine and phosphoethanolamine headgroups. *FEMS Microbiology Letters* **258**: 297-304.

Dalsgaard, T., Canfield, D., Petersen, J., Thamdrup, B., and Acuna-Gonzalez, J. (2003) N2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature* **422**: 606-608.

de la Torre, J.R., Walker, C.B., Ingalls, A.E., Konneke, M., and Stahl, D.A. (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environmental Microbiology* **10**: 810-818.

Engstrom, P., Dalsgaard, T., Hulth, S., and Aller, R.C. (2005) Anaerobic ammonium oxidation by nitrite (anammox): Implications for N2 production in coastal marine sediments. *Geochimica et Cosmochimica Acta* **69**: 2057-2065.

Galtier, N., Gouy, M., and Gautier, C. (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. . *Computer Applicattions in the Biosciences* **12**: 543-548.

Hamersley, M., Lavik, G., and Woebken, D. (2007) Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. *Limnol oceanogr* **71**: 1066-1071.

Jaeschke, A., Hopmans, E., Wakeham, S., Schouten, S., and Damste, J. (2007) The presence of ladderanes lipids in the oxygen minimum zone of the Arabian Sea indicates nitrogen loss through anammox. *Limnol oceanogr* **52**: 780-786.

Jetten, M.S., Schmid, M., Van de Pas-Schoonen, K.T., damste, J.S.S., and Strous, M. (2005) Anammox Organisms: enrichment, cultivation and environmental analysis. *Methods in Enzymology* **397**: 34-57.

Kartal, B., Kuypers, M.M.M., Lavik, G., Schalk, J., Op den Camp, H.J.M., Jetten, M.S.M., and Strous, M. (2007) Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. *Environmental Microbiology* **9**: 635-642.

Kuypers, M.M., Lavik, G., Woebken, D., Schmid, M., Fuchs, B.M., Amann, R. et al. (2005) Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc Natl Acad Sci U S A* **102**: 6478-6483.

Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jorgensen, B.B., Kuenen, J.G. et al. (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422**: 608-611.

Mehta, M., and Baross, J. (2006) Nitrogen fixation at 92°C by a hydrothermal vent archaeon. *Science* **314**: 1783-1785.

Mével, G., and Prieur, D. (1998) Thermophilic heterotrophic nitrifiers isolated from Mid-Atlantic Ridge deep-sea hydrothermal vents. *Canadian Journal of Microbiology* **44**: 723-733.

Meyer, R.L., Risgaard-Petersen, N., and Allen, D.E. (2005) Correlation between anammox activity and microscale distribution of nitrite in a subtropical mangrove sediment. *Appl Environ Microbiol* **71**: 6142-6149.

Miroshnichenko, M.L. (2004) Thermophilic microbial communities of deep-sea hydropthermal vents. *Microbiology* **73**: 1-13.

Nercessian, O., Reysenbach, A.-L., Prieur, D., and Jeanthon, C. (2003) Archaeal diversity associated with in situ samplers deployed on hydrothermal vents on the East Pacific Rise (13°N). *Environmental Microbiology* **5**: 492-502.

Penton, C.R., Devol, A.H., and Tiedje, J.M. (2006) Molecular Evidence for the Broad Distribution of Anaerobic Ammonium-Oxidizing Bacteria in Freshwater and Marine Sediments. *Appl. Environ. Microbiol.* **72**: 6829-6832.

Rattray, J.E., Jack. van de Vossenberg, Ellen C. Hopmans, Boran Kartal, Laura van Niftrik, W. Irene C. Rijpstra et al. (2008) Ladderane lipid distribution in four genera of anammox bacteria. *Arch Microbiol* **DOI 10.1007/s00203-008-0364-8**.

Richards, F.A. (1965) Anoxin basins and fjords. *Chemical Oceanography. Academic Press, London*: 611-645.

Sarradin, P.-M., Caprais, J.-C., Riso, R., Kerouel, R., and Aminot, A. (1999) Chemical environment of the hydrothermal mussel communities in the Lucky Strike and Menez Gwen vent fields, Mid Atlantic Ridge. *Cahier de Biologie Marine* **40**: 93-104.

Sarrazin, J., and Sarradin, P.M. (2006) MoMARETO: a cruise dedicated to the spatiao temporal dynamic and the adaptations of hydrothermal vent fauna on the mid-Atlantic Ridge *International Research INTERRIDGE NEWS* **15**: 24-33.

Schmid, M.C., Risgaard-Petersen, N., van de Vossenberg, J., Kuypers, M.M.M., Lavik, G., Petersen, J. et al. (2007) Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. *Environmental Microbiology* **9**: 1476-1484.

Schmid, M.C., Maas, B., Dapena, A., van de Pas-Schoonen, K., van de Vossenberg, J., Kartal, B. et al. (2005) Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. *Appl Environ Microbiol* **71**: 1677-1684.

Schrenk, M.O., Kelley, D.S., Delaney, J.R., and Baross, J.A. (2003) Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. In *Appl Environ Microbiol*, pp. 3580-3592.

Strous, M., Kuenen, J.G., and Jetten, M.S. (1999a) Key physiology of anaerobic ammonium oxidation. *Appl Environ Microbiol* **65**: 3248-3250.

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T. et al. (1999b) Missing lithotroph identified as new planctomycete. *Nature* **400**: 446-449.

Takai, K., Komatsu, T., Inagaki, F., and Horikoshi, K. (2001) Distribution of Archaea in a black smoker chimney structure. *Appl Environ Microbiol* **67**: 3618-3629.

Tal, Y., Watts, J.E., and Schreier, H.J. (2005) Anaerobic ammonia-oxidizing bacteria and related activity in Baltimore inner harbor sediment. *Appl Environ Microbiol* **71**: 1816-1821.

Thamdrup, B., and Dalsgaard, T. (2002) Production of N2 through Anaerobic Ammonium Oxidation Coupled to Nitrate Reduction in Marine Sediments. *Appl. Environ. Microbiol.* **68**: 1312-1318.
Trimmer, M., Nicholls, J., and Deflandre, B. (2003) Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. *Appl. Environ. Microbiol.* **69**: 6447-6454.
Webster, G., C. J. Newberry, J. C. Fry, and Weightman., A.J. (2003) Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16s rDNA-based techniques: a cautionary tale. *Journal of Microbiological Methods* **55**: 155-164.
White, D.C., Davis, W.M., Nickels, J.S., King, J.D., and Bobbie, R.J. (1979) Determination of the Sedimentary Microbial Biomass by Extractable Lipid Phosphate. *Oecologia* **40**: 51-62.

Figures

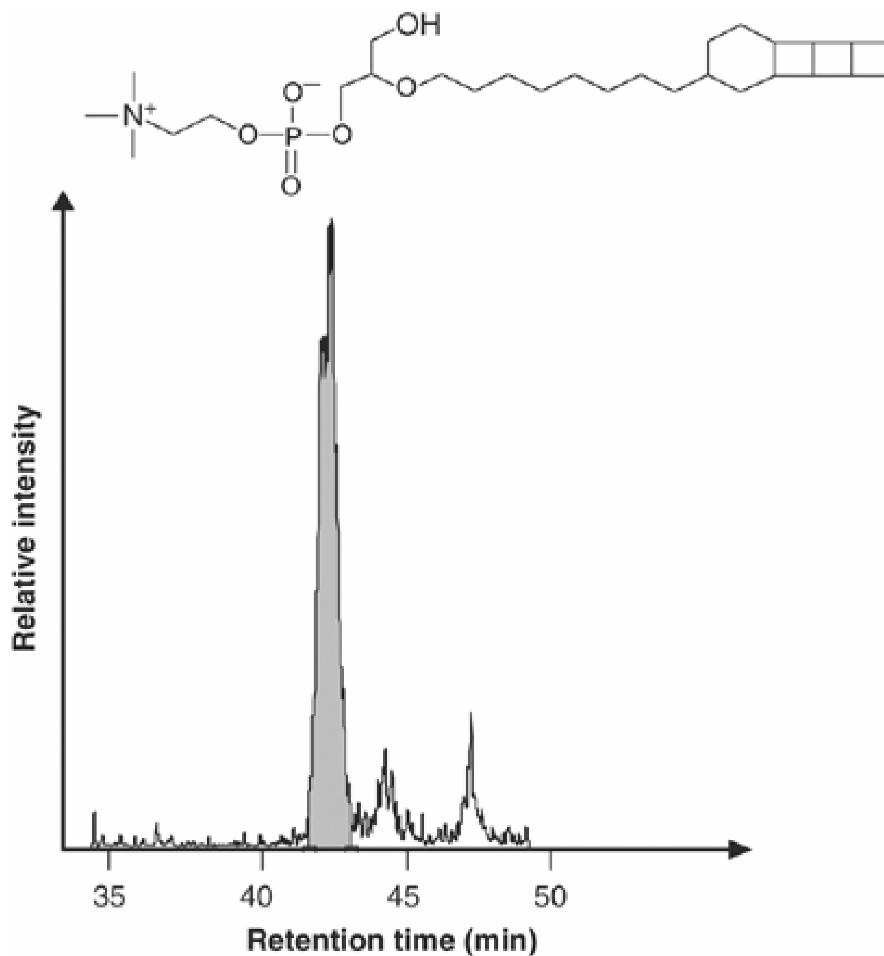

Figure 1 :
Phylogenetic tree of 16S rRNA gene sequences determined by neighbour-joining analysis. The outgroup used was *Gemmata. sp and Isosphaera. sp*. The numbers at the nodes are the bootstrap values (as percentage). Bootstrap values above 50% are displayed. Scale bar indicates the expected number of changes per sequence position.
Cluster “A”: containing all known anammox sequences, cluster “B”: the potential-anammox cluster closest to the anammox cluster, containing DQ664529

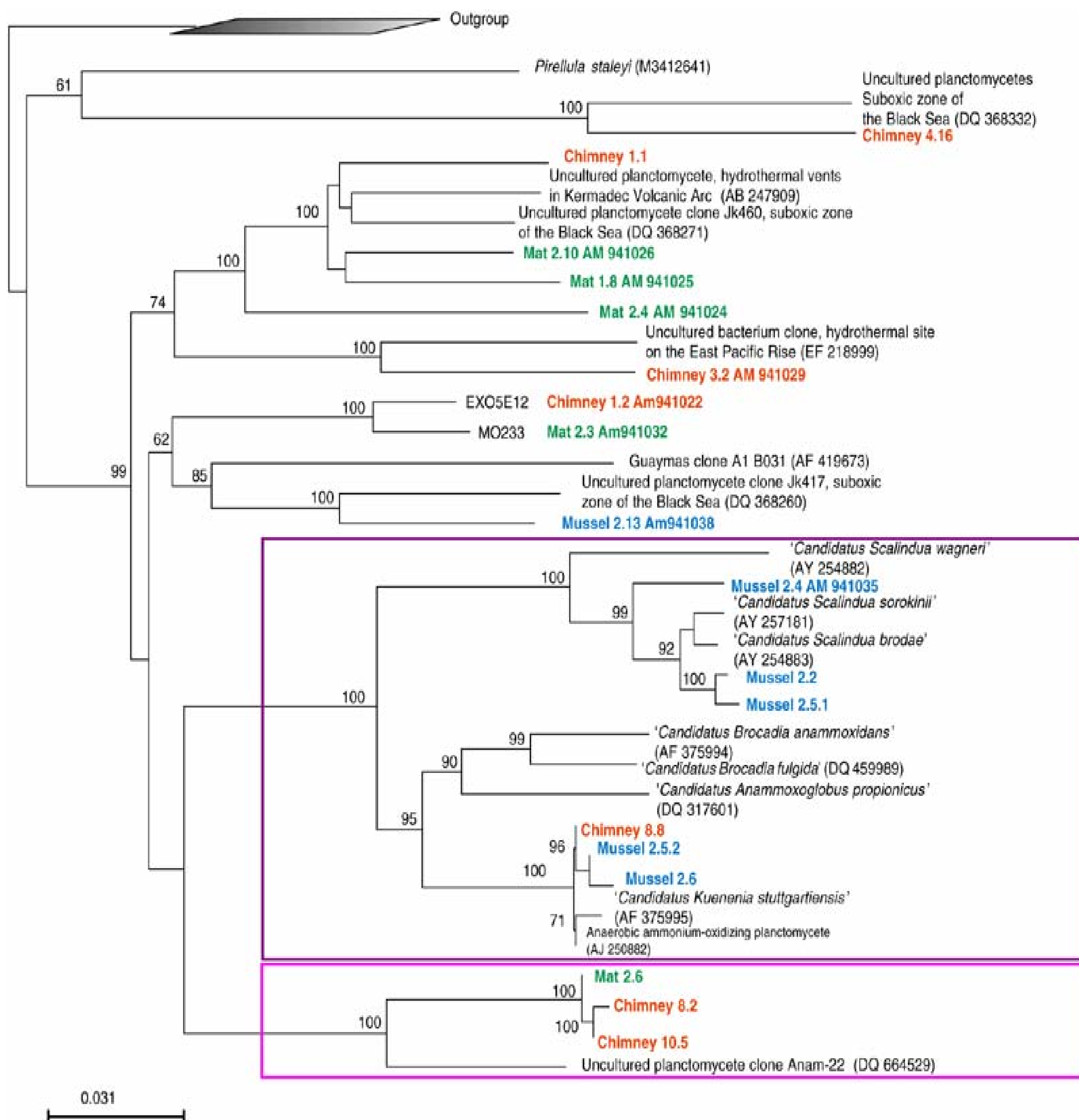

Figure 2: Selective reaction monitoring (SRM) trace of the intact ladderane monoalkylether lipid with phosphocholine (PC) headgroup obtained by HPLC/electrospray ionization (ESI)-tandem mass spectrometry analysis of the total lipid extract of chimney 6 sample, and corresponding structure.

Tables

Table 1 Main characteristics of the hydrothermal samples: sampling sites, temperature, sample types, molecular biology results and rates of anammox

	Samples	*Temperature (°C)*	*Lipids analysis—ladderanes PC-mono ether (V) pg/g 'sediment'*	*Molecular biology*	*Anammox activity (nmol ml^{-1} sample per day = μM day^{-1})*
Mat 1 MO22	Lucky Strike (depth: 1700 m) Microbial mat on *Bathymodiolus azoricus*	4–8	ND	Cluster A	ND
Mat 2 MO23	Lucky Strike (depth: 1700 m) Microbial mats	4–8	ND	Clusters A and B	ND
Mussels 2 MO16 E2	Menez Gwen (depth: 850 m) *Bathymodiolus azoricus*	4–10	ND	Cluster A	ND
Shrimp 1 EXO6 E1	Rainbow (depth: 2300 m) *Rimicaris exoculata*	4–10	ND	Data not shown	ND
Chimney 1 EXO5 E1	Lucky Strike (depth: 1700 m) Active chimney (iron silica)	30	ND	Cluster A	ND
Chimney 4 EXO17 E1	Lost City (depth: 750 m) Carbonate active chimney (pH 10)	91	20	Cluster B	0.03
Chimney 3 EXO16 E1	Lost City (depth: 750 m) Carbonate active chimney (pH 10)	93	ND	Cluster A	ND
Chimney 6 EXO13 E1	TAG (depth: 3650 m) Active chimney	>100	91	Planctomycetes	0.02
Chimney 7 EXO14	TAG (depth: 3650 m) Active chimney	>100	40	Planctomycetes	0.01
Chimney 8 MO8 E1	Rainbow (depth: 2300 m) Active chimney	>100	ND	Clusters A and B	0
Chimney 10 MOM07	Rainbow (depth: 2300 m) Active chimney	153	35	Cluster B	0.03

Table 1: Main characteristics of the hydrothermal samples: sampling sites, temperature, sample types, molecular biology results, and rates of anammox.

Table 2 Measurements of activity rates for the hydrothermal vent samples

Chimney	*Temperature (°C)*	*N_2 production (nmol ml^{-1} sample per day)*	*Cell density (cells ml^{-1})*
6	30	0.02	8.9×10^3
7	60	0.01	4.79×10^3
10	60	0.01	4.79×10^3
4	85	0.03	1.44×10^4
10	85	0.03	1.44×10^4

Table 2: Measurements of activity rates for the hydrothermal vent samples. Samples were incubated with 20 µM of labelled nitrite and the production of $^{14-15}N2$ was measured by GC coupled to a mass spectrometer. The results are expressed in µM/day/sample. The estimated cellular density corresponding to the measured activity has been calculated from the calibration curve traced with the control *Candidatus* “Kuenenia stuttgartiensis”.