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ORIGINAL ARTICLE

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

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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 $^{14}N^{15}N$ 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.
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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 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 could 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 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 the annamox process are ubiquitous and constitute 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 minimum oxygen 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, unstable habitats, characterized by steep chemical and physical gradients because of the mixing of the super heated hydrothermal anoxic fluid with cold

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oxic seawater. Biological communities are distributed along these gradients where the decrease in temperature is more or less correlated with 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 (Takai *et al*., 2001; Alain *et al*., 2002b; Nercessian *et al*., 2003; Schrenk *et al*., 2003).

In the nitrogen cycle, oxidation of ammonium has been demonstrated by 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 fix nitrogen at 92 °C, and this 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 microorganisms 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 of the presence and activity of new anammox bacteria in different hydrothermal areas.

Materials and methods

Samples

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 Victor 6000 and the submersible Nautile. Samples were obtained in such a way that the different biotopes of the ecosystem along the

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	Lucky Strike (depth: 1700 m)	4–8	ND	Cluster A	ND
MO22	Microbial mat on *Bathymodiolus azoricus*				
Mat 2	Lucky Strike (depth: 1700 m)	4–8	ND	Clusters A and B	ND
MO23	Microbial mats				
Mussels 2	Menez Gwen (depth: 850 m)	4–10	ND	Cluster A	ND
MO16 E2	*Bathymodiolus azoricus*				
Shrimp 1	Rainbow (depth: 2300 m)	4–10	ND	Data not shown	ND
EXO6 E1	*Rimicaris exoculata*				
Chimney 1	Lucky Strike (depth: 1700 m)	30	ND	Cluster A	ND
EXO5 E1	Active chimney (iron silica)				
Chimney 4	Lost City (depth: 750 m)	91	20	Cluster B	0.03
EXO17 E1	Carbonate active chimney (pH 10)				
Chimney 3	Lost City (depth: 750 m)	93	ND	Cluster A	ND
EXO16 E1	Carbonate active chimney (pH 10)				
Chimney 6	TAG (depth: 3650 m)	>100	91	Planctomycetes	0.02
EXO13 E1	Active chimney				
Chimney 7	TAG (depth: 3650 m)	>100	40	Planctomycetes	0.01
EXO14	Active chimney				
Chimney 8	Rainbow (depth: 2300 m)	>100	ND	Clusters A and B	0
MO8 E1	Active chimney				
Chimney 10	Rainbow (depth: 2300 m)	153	35	Cluster B	0.03
MOM07	Active chimney				

temperature gradient were covered as good as possible. To this end, active smoker, animals and microbial mats were sampled. Active hydrothermal chimney fragments, where fluid temperature ranged from 30 to 300 °C, were collected by the tele-operated arm of the remote-operated vehicle or Nautile. Samples were transferred to the surface in 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 animal samples (mussels' gills and whole shrimps) were crushed in 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 polymerase chain reaction were carried out as described by Schmid *et al*. (2005), except for DNA extraction from the chimney sample, which was performed using the Fast DNA kit for soil samples (Webster *et al*., 2003). Samples were extracted several times, pooled and concentrated. 16S rRNA partial genes were amplified using the specific anammox primers Pla46F (5′-GGATTAGG CATGCAAGTC-3′), BS820R (5′-TAATTCCTCTATTA GT-3′) and Amx820R (5′-AAACCCCTCTACTTAGTG CCC-3′) (Jetten *et al*., 2005; Schmid *et al*., 2005). Polymerase chain reaction products were subsequently cloned with the TOPO XL cloning kit (Invitrogen, Cergy-Pontoise, France) according to the manual provided by 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 carried out 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 the inferred topologies was tested by 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 phosphocholine-monoalkylether analysis

Lipids were ultrasonically extracted from four chimney samples (4, 6, 7 and 10; approximately 4 g dry weight) according to a modified method of Bligh and Dyer (1959), using three times a mixture of methanol, dichloromethane and phosphate buffer at pH 7.4 (2:1:0.8, vol/vol/vol). The extracts were combined and further dichloromethane and buffer were added to the mixture to achieve a final methanol/dichloromethane/buffer ratio of 1:1:0.9 (vol/vol/vol). The phases were separated and the extraction repeated three more times. An aliquot of the extract was dissolved in a dichloromethane/methanol mixture (9:1, vol/vol) and filtered through a 0.45 µm, 4 mm diameter RC filter.

The C_{20}-[3]-monoalkylether containing a phosphocholine (PC) headgroup (for structure see Figure 1) was analyzed by high-performance liquid chromatography/electrospray ionization-MS/MS according to Boumann *et al*. (2006), with some modifications. Separation was achieved on a LiChrospher diol column (250 mm × 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; and then back to 100% A for 20 min to re-equilibrate the column, where A is hexane/2-propanol/formic acid/14.8 M NH_{3aq} in the ratio 79:20:0.12:0.04 (vol/vol/vol/vol) and B is 2-propanol/water/formic acid/14.8 M NH_{3aq} in the ratio 88:10:0.12:0.04 (vol/vol/vol/vol). Detection of the C_{20}-[3]-monoalkylether-PC was achieved by selec-

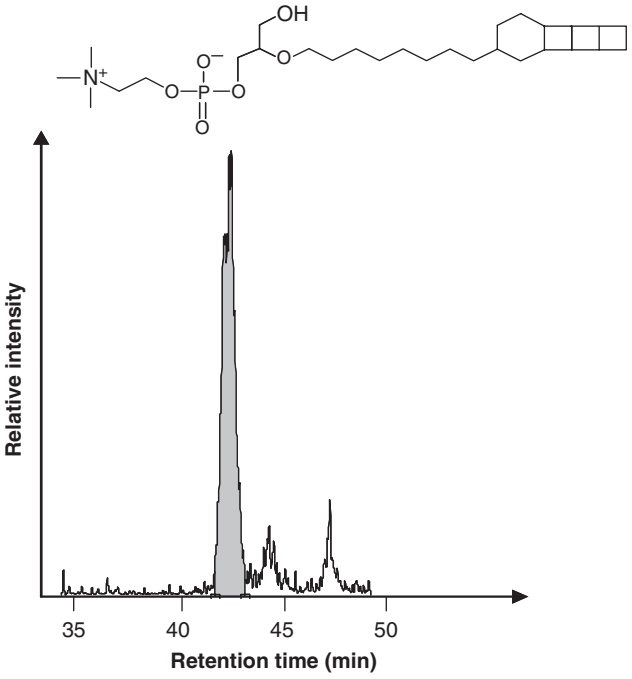

Figure 1 Selective reaction monitoring trace of the intact ladderane monoalkylether lipid with phosphocholine headgroup obtained by high-performance liquid chromatography/electrospray ionization–tandem mass spectrometry analysis of the total-lipid extract of chimney 6 sample and the corresponding structure.

tive reaction monitoring 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 20 V collision energy. Quantification of intact ladderane ether lipids 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 into the column was achieved with this technique.

Activity measurements
Anammox activities were measured for 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 20 μM) and $^{15}NO_2^-$ (final concentration 20 μM) at temperatures of 30, 60 and 85 °C. Three gas analyses were performed after 20, 44 and 68 h incubation. For each measurement, 500 μl gas was injected into a gas chromatogram coupled to an isotope ratio mass spectrometer (Thermo Finnigan delta plus). All gas samples were analyzed 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 carried out at Nijmegen University (The Netherlands).

Nucleotide sequence accession numbers
The EMBL accession numbers of the sequences used in this study are AM941022–AM941038.

Results and discussion

Molecular detection of anammox bacteria in the hydrothermal vent ecosystem
Using anammox-specific primers, 16S rRNA gene sequences were retrieved from different representative samples of the vent ecosystem and from various hydrothermal sites (Figure 2). In the cold part of the ecosystem, several anammox 16S rRNA gene sequences were found in microbial mats and mussel gills (Figure 2). Some of the mussel sequences were related to known anammox bacteria. The similarity of 'mussel 2.2, 2.4' to marine *Candidatus* '*Scalindua* sp' was about 93%, whereas for 'mussel 2.5 and 2.6', a similarity of 97% was observed to *Candidatus* '*Kuenenia stuttgartiensis*'. The other retrieved 16S rRNA gene sequences (mat 1.8, 2.3, 2.4, 2.10 and 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 (8–10 μM), nitrite (0–2 μM), as well as a 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% similarity), one (mussel 2.5) to *Candidatus* '*Scalindua* sp' (97% similarity) and another to uncultivated Planctomycetes (mussel 2.13) from the Black Sea. Temperature, pH and chemical conditions were similar to those measured for 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 P and Sarradin PM, Personal communication). Unfortunately, no activity measurements could be performed to confirm these molecular data because animal samples cannot be preserved at 4 °C without any degradation until analysis.

Hydrothermal vent active chimneys are typically hot and 'anaerobic' 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% similarity) (Kuypers *et al.*, 2003) and from other hydrothermal sites. Interestingly, chimneys 8 and 10 yielded sequences in box B (Figure 2), 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% similarity) was obtained from chimney 8 from the Rainbow vent field where fluid temperatures of up to 300 °C were measured. Owing to high background fluorescence in the chimney samples, fluorescent *in situ* hybridization analysis with specific anammox probes could not be performed.

Ladderane PC-monoalkylether analysis
In addition to 16S rRNA gene sequences, independent specific biomarkers, the 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 a range between 20 and 91 $pg\,g^{-1}$ of sediment.

Activity measurements
To support the molecular and biomarker data, activity measurements were performed on chimney

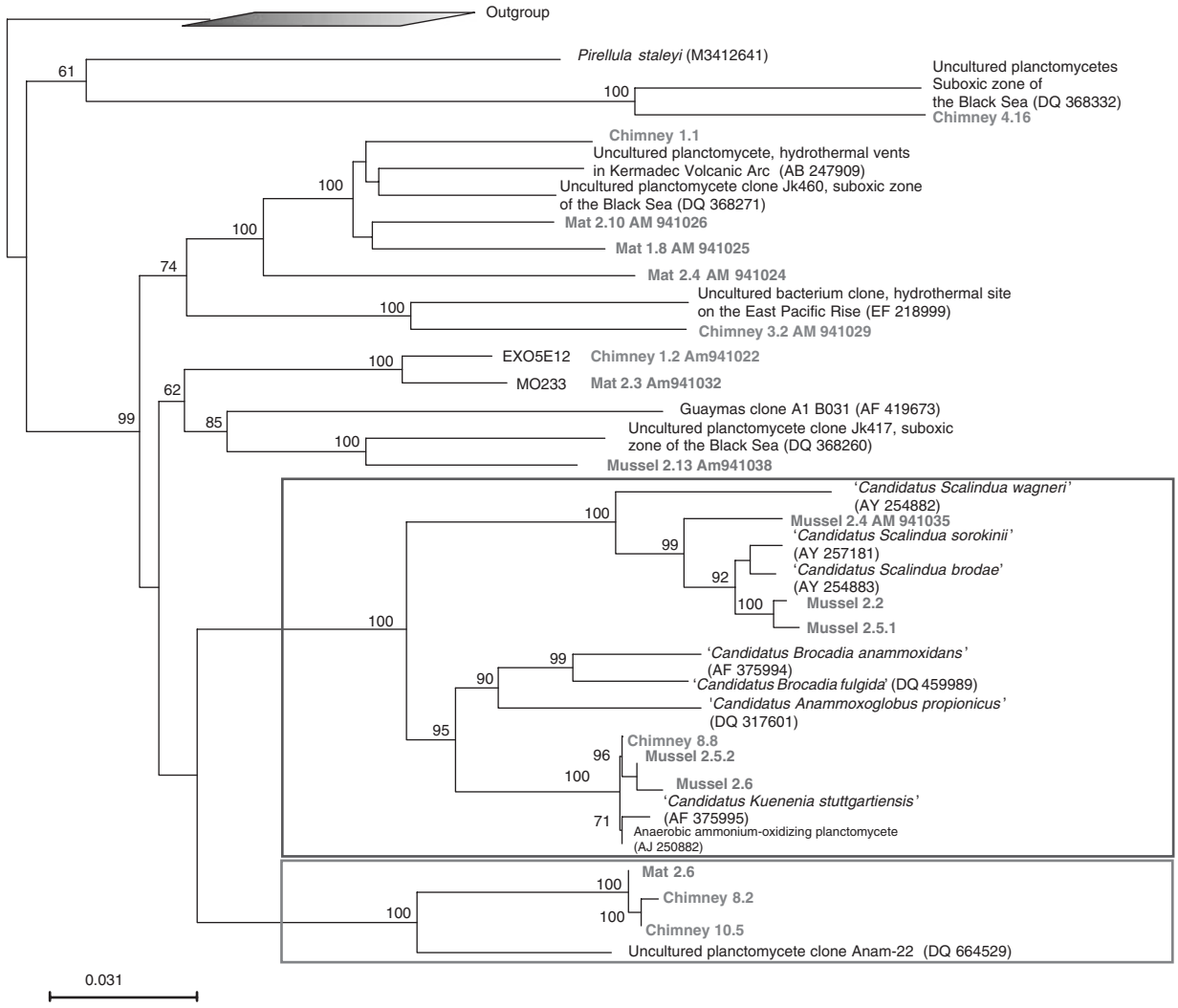

Figure 2 Phylogenetic tree of 16S rRNA gene sequences determined by neighbor-joining analysis. The out group used was Gemmata sp and Isosphaera sp. The numbers at the nodes are the bootstrap values (in percentage). Bootstrap values above 50% are displayed. Scale bar indicates the expected number of changes per sequence position. Cluster 'A' contains all known anammox sequences; cluster B is the potential anammox cluster closest to the anammox cluster, containing DQ 664529.

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

Samples were incubated with 20 μM labeled nitrite and the production of $^{14-15}N_2$ was measured by gas chromatogram coupled to a mass spectrometer. The results are expressed in μM per day per sample. The estimated cellular density corresponding to the measured activity has been calculated from the calibration curve traced with the control *Candidatus* '*Kuenenia stuttgartiensis*'.

samples (Table 2). *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 sample at a rate of 0.02 μM day^{-1}.

At 60 °C, anammox activity was measured in chimney 7 and 10 at a rate of 0.01 μM day^{-1}. Anammox activity could be measured at 85 °C as well in chimney 4 and 10 samples, and the rate was 0.03 μM day^{-1} 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 inferred number of active anammox cells that could be expected from these results was between 4.79×10^3 and 1.44×10^4 cells ml^{-1}.

In conclusion, all our results suggested that anaerobic ammonium-oxidizing bacteria are present and active in hydrothermal vent areas, possibly even at high temperatures. Ladderane lipids, 16S rRNA gene sequences and anammox activity were detected in chimney samples 4, 6, 7 and 10. In addition, for two of them, some sequences retrieved

from chimney samples 4 and 10 clustered in clade B (Figure 2), suggesting that the phylotype might represent a new anammox clade. Our future effort will focus on the enrichment of the 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.

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