Putative ammonia-oxidizing Crenarchaeota in suboxic waters of the Black Sea: A basin-wide ecological study using 16S ribosomal and functional genes and membrane lipids

Running title: Putative nitrifying Archaea in the Black Sea

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ABSTRACT

Within the upper 400 m at western, central, and eastern stations in the world's largest stratified basin, the Black Sea, we studied the qualitative and quantitative distribution of putative nitrifying Archaea based on their genetic markers (16S rDNA, *amoA* encoding for the alfa-subunit of archaeal ammonia monooxygenase), and crenarchaeol, the specific glycerol diphytanyl glycerol tetraether (GDGT) of pelagic Crenarchaeota within the Group I.1a. Marine Crenarchaeota were the most abundant Archaea (up to 98% of the total archaeal 16S rDNA copies) in the suboxic layers with oxygen levels as low as 1 μM including layers where previously anammox bacteria were described (Kuypers et al., 2003). Different marine crenarchaeotal phylotypes (both 16S rDNA and *amoA*) were found at the upper part of the suboxic zone as compared to the base of the suboxic zone and the upper 15-30 m of the anoxic waters with prevailing sulfide concentrations of up to 30 μM. Crenarchaeol concentrations were higher in the sulfidic chemocline as compared to the suboxic zone. These results indicate an abundance of putative nitrifying Archaea at very low oxygen levels within the Black Sea and might form an important source of nitrite for the anammox reaction.

INTRODUCTION

The Black Sea is the largest permanently stratified basin in the world, being devoid of oxygen and containing abundant sulfide from about 100 m depth to the seafloor at 2200 m. A 20-to 30-m-deep suboxic layer depleted in both O_2 and sulfide overlies the sulfide zone (Jørgensen et al., 1991). This permanent gradual redoxcline offers great opportunities to study the distribution of prokaryotes (Vetriani et al., 2003) involved in the cycling of nitrogen (N). Within the suboxic zone (with <10 μM of oxygen), bacteria falling in the order Planctomycetales and performing anaerobic ammonia oxidation (anammox) thrive (Kuypers et al., 2003). In the anammox reaction, ammonium (NH_4^+) is anaerobically oxidized with nitrite (NO_2^-) to dinitrogen (N_2). Anammox is an important anaerobic process responsible for the removal of fixed inorganic nitrogen from the Black Sea (Kuypers et al., 2003). The nitrite required for oxidation of ammonium in the anammox process may be produced during the bacterial reduction of nitrate to nitrite (i.e. the first step in denitrification) or during the microbial oxidation of ammonium to nitrite (i.e. nitrification).

Until recently, it was thought that only a few members of Betaproteobacteria (e.g. species of the genera *Nitrosomonas* and *Nitrospira*) (Beaumont et al., 2004; Taylor and Bottomley, 2006) and Gammaproteobacteria (*Nitrosococcus oceani*) (Ward and O'Mullan, 2002) were involved in either of the two steps of nitrification. However, despite their critical role in the biogeochemical cycling of nitrogen in both pelagic and benthic oceanic environments, aerobic ammonia-oxidizing bacteria (AOB) often comprise only 0.1% of bacterial assemblages (Ward et al., 2000). In contrast, pelagic non-thermophilic marine Crenarchaeota within the Group I.1a (Schleper et al., 2005) are ubiquitous and abundant in the ocean (DeLong et al., 1994; Herndl et al., 2005; Ingalls et al., 2006; Karner et al., 2001; Stein and Simon, 1996), and there is growing evidence that at least some of these marine picoplankton are nitrifiers as well (Könneke et al., 2005; Treusch et al., 2005; Wuchter et al.,

2006). Although some pelagic Crenarchaeota may utilize amino acids as a carbon source (Herndl et al., 2005; Ouverney and Fuhrman, 2000), it has been shown that some pelagic Crenarchaeota may be autotrophs (Hoefs et al., 1997; Pearson et al., 2001) capable of light-independent bicarbonate fixation (Herndl et al., 2005; Wuchter et al., 2003). Recent analyses of the natural distribution of radiocarbon in archaeal membrane lipids from mesopelagic waters of the North Pacific gyre suggested that chemoautotrophy is the predominant archaeal metabolism at depth (Ingalls et al., 2006). Further insight into the potential energy source of pelagic Crenarchaeota comes from whole genome shotgun analysis of DNA sequences derived from the Sargasso Sea in which potential ammonia monooxygenase genes (e.g. *amoA*) associated with presumptive archaeal contigs were identified (Venter et al., 2004), suggesting that some pelagic Crenarchaeota may be capable of performing chemoautotrophic nitrification. Another study of fosmids derived from complex soil libraries identified *amoA* sequences related to those of the Sargasso Sea and were linked to a crenarchaeotal ribosomal RNA operon (Schleper et al., 2005) and genes encoding for ammonia monooxygenase subunits including *amoA* have recently also been identified in the marine sponge symbiont *Cenarchaeum symbiosum* (Hallam et al., 2006). Only recently, the first cultivated representative from this archaeal lineage (Könneke et al., 2005) was brought into culture (*Candidatus* '*Nitrosopumilus maritimus*') and was shown to grow solely on bicarbonate and ammonia as carbon and energy sources and this nitrifying species carried all genes encoding for the subunits of archaeal ammonia monoxygenase (Könneke et al., 2005). A recent *amoA* clone library study showed that ammonia-oxidizing Archaea (AOA) are widespread in oxic to suboxic water bodies of marine waters as well as in soils and sediments (Francis et al., 2005).

The glycerol diphytanyl glycerol tetraether (GDGT) membrane lipid "crenarchaeol", thought to be a unique core membrane lipid of pelagic Crenarchaeota (Sinninghe Damsté et al., 2002b), was previously found in the oxygen minimum zone (150-1200 m) of the Arabian

Sea where oxygen levels are less than 5 μM and it was suggested that pelagic Crenarchaeota are facultative anaerobes (Sinninghe Damsté et al., 2002a). This assumption is supported by the observation that crenarchaeol concentrations maximize at the chemocline of the Black Sea (Wakeham et al., 2003). Despite the predominance of crenarchaeol in the suboxic zone of the Black Sea, a previous clone library with the most predominant prokaryotic 16S rDNA revealed only two phylotypes of marine Crenarchaeota, whereas the remaining 90% of the archaeal clones were affiliated with pelagic Group II marine Euryarchaeota (Vetriani et al., 2003). Instead, in open ocean settings marine Crenarchaeota are much more abundant than the marine Group II Euryarchaeota (Herndl et al., 2005; Karner et al., 2001). Recently, 12 phylotypes of archaeal *amoA* were also recovered from a narrow interval of the suboxic waters from the Black Sea (at densities of 15.7, 15.8 and 15.9) (Francis et al., 2005).

In order to shed further light on the ecology of putative nitrifying Archaea in the Black Sea, we performed a high resolution basin-wide qualitative and quantitative survey of archaeal signatures (crenarchaeol, 16S rDNA and *amoA*) in relation to the abundance of N-species (NH_4^+, NO_2^- and NO_3^-), oxygen and sulfide. Our survey covered the entire water column at the stations of western, central, and eastern Black Sea, but since genetic and lipid markers of marine Crenarchaeota were low in abundance in the deeper sulfidic water layers, we focus here on the upper 400 m, spanning the oxic, suboxic and upper sulfidic zones. Our study revealed that the diversity of marine Crenarchaeota (based on the phylogeny of archaeal 16S rDNA and *amoA*) and their abundances as revealed by quantitative PCR (copy numbers of 16S rDNA and *amoA* of marine Crenarchaeota) were far greater than reported previously (Vetriani et al., 2003). Based on the distribution of the crenarchaeotal phylotypes (*amoA* and 16S rDNA) in relation to nutrients and oxygen concentration, we conclude that most of the marine Crenarchaeota located within the suboxic layer are putative nitrifiers and live at oxygen levels ≤1 μM.

Results and discussion

Location of the suboxic zone, N-species, and onset of sulfide

Particulate organic matter (POM) was collected by in-situ filtration from the central (R/V *Knorr* 172-8 station 5; May 2003), western (R/V *Meteor* 51-4 stations 7605 and 7620; December 2001) and eastern (R/V *Knorr* 172-8 station 7; May 2003) regions of the Black Sea (Fig. 1) from various depth intervals between 10 and 2000 m; for the purposes of this study, we focused on the 10-400 m depth range (Table 1). The suboxic zone at approximately 50-120 m depth occurs where the concentrations of both O_2 and sulfide are extremely low and do not exhibit perceptible vertical or horizontal gradients (Codispoti et al., 1991; Murray et al., 1989); typically O_2 concentrations of <10 μM are used to define the suboxic zone. Recent measurements of O_2 and sulfide provided higher precision and lower detection limits of ca. 3 μmol for both O_2 and sulfide (Konovalov et al., 2003) and show that the depth and breadth of the suboxic zone varies seasonally and on at least decadal scales (Konovalov et al., 2005; Murray et al., 1995; Murray and Yala-del-Rio, 2006; Yakushev et al., 2005). For example, during the R/V Knorr cruise in 2003, the depth of the suboxic zone deepened from the western basin (55-80 m) to the central basin (70-90 m) to the eastern basin (90-120 m) (see O_2 and sulfide data of G. W. Luther on the Knorr-2003 Black Sea web site http://www.ocean.washington.edu/cruises/Knorr2003). The suboxic zone in May 2003 was shallower than that observed in December of 2001, reflecting deeper and more intense physical mixing of the water column in winter vs. spring.

At the central station of the Black Sea (Fig. 1) sampled in May, 2003, O_2 was not detectable at 68 m, and sulfide was detected (detection limit 0.04 μM) at a depth of 85 m (σ_τ = 16.07) (Fig. 2E; http://www.ocean.washington.edu/cruises/Knorr2003). Ammonia concentrations dropped from >20 μM at ~130 m to below detection limit at the base of the suboxic zone [(Fig. 2F; data of Murray and Fuchsman on the Knorr-2003 Black Sea web site

(http://www.ocean.washington.edu/cruises/Knorr2003)]. A small nitrite peak of up to 0.05 µM was found at the base of the photic zone, where ammonia concentrations reached undetectable level (Fig. 2F). At depths between ~40 and 45 m where oxygen concentrations reached up to 300 µM, a second, (upper), nitrite maxima occurred, which agrees well with previous findings (Murray and Yala-del-Rio, 2006; Ward and Kilpatrick, 1990). A nitrate peak with up to 5 µM of nitrate was observed between the two nitrite maxima (Fig. 2F).

Oxygen was not detectable below ~80 m (σ_τ = 15.66) at the western stations (Kuypers et al., 2003) (Fig. 2A) sampled in December 2001 and detectable sulfide concentrations were measured below 100 m (σ_τ = 16.04) (Manske et al., 2005) (Fig. 2A). A distinct nitrite peak was found between 88 and 92 m in the lower part of the sub-oxic zone (Kuypers et al., 2003) (Fig. 2B). The nitrate peak (up to 5 µM) was located between the lower nitrite peak and 43 m.

For the eastern site, also sampled in May 2003, oxygen was <10 µM below 75 m (σ_τ = 15.39) became undetectable at 85 m (σ_τ = 15.54) and sulfide was first detected at 117 m (σ_τ = 16.10; http://www.ocean.washington.edu/cruises/Knorr2003). (Fig. 2I). Similar to the central site, two nitrite peaks (both 0.03 µM) were observed. The lower peak was found at 102 m, 15 m above the first detectable levels of sulfide and the upper peak was found where oxygen levels reached a maximum of 300 µM (Figs. 2I, J).

These results showed that the depth and width of the suboxic zone differed from station to station as evident from other studies (Codispoti et al., 1991; Jørgensen et al., 1991; Manske et al., 2005; Murray et al., 1995), with a more shallow chemocline in the central Black Sea. The boundary of upward diffusing sulfide was found at almost identical densities of ~16.07 (central station 5, 85 m), 16.04 (western station 7605, 95 m), and 16.10 (eastern station 7, 117 m). Note that *Knorr* stations 5 and 7 were sampled in spring of 2003, whereas *Meteor* stations 7605 and 7620 were sampled in winter of 2001.

Qualitative distribution of archaeal 16S rDNA and *amoA* in the upper 400 m.

Basin-wide, we determined whether the oxygenated, suboxic, and sulfidic part of the photic zone as well as the upper sulfidic zone in the water column that does not receive light would harbor different communities of Archaea. We performed a PCR with primers for the 16S rDNA of the domain Archaea and separated the amplicons by denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) followed by subsequent phylogenetic analysis of excised and sequenced DGGE fragments. In addition we developed a PCR/DGGE based method to determine to phylogeny of archaeal *amoA*.

Central site, Station 5

Eighteen DGGE fragments with unique or similar melting positions in the gel were excised for which 12 represented unique phylotypes (Fig. 3B). Eight out of twelve phylotypes clustered with the marine Crenarchaeota (Group I.1a; Fig. 4) and were detected in the suboxic as well as in the slightly sulfidic waters down to 115 m but were below the detection limit in the fully oxygenated waters at 10 and 30 m at station 5 (Fig. 3B). In general, the fully oxygenated waters at 10 m and 30 m contained very little template DNA of Archaea which resulted in the non-specific amplification of bacterial 16S rDNA (DGGE bands denoted with a "b" in Fig. 3). DGGE fragments that resulted in poor quality sequences of Archaea were indicated with 'a' and were not used for phylogenetic analysis either. The yield of PCR-amplified archaeal 16S rDNA was low between 130 and 300 m and as such, no phylotypes of marine Crenarchaeota could be identified from sulfidic waters below 130 m (Fig. 3B). All sequenced DGGE fragments from the suboxic zone were found to be marine Crenarchaeota, whereas three phylotypes related to uncultured members of the Miscellaneous Crenarchaeota Group (characterized after Inagaki et al. [2003]); orange marked bands in Fig. 3B) and a Euryarchaeotal sequence (green marked band) were also identified from the sulfidic zone between 92 and 100 m but will be discussed elsewhere (Coolen et al., in preparation). Some

of the sequenced DGGE bands (Figs. 3B, 4) within the top of the suboxic zone at 75 m at the eastern and western sampling sites showed up to 100% sequence similarity to the 16S rDNA of the recently cultured nitrifying marine Crenarchaeote *Candidatus* "*Nitrosopumilus maritimus*" (Könneke et al., 2005) and the recently obtained crenarchaeotal putative nitrifying enrichment culture from the North Sea (Wuchter et al., 2006). The sponge symbiont *Cenarchaeum symbiosum* (Preston et al., 1996) (denoted in bold in Fig 4) was the closest relative of additional sequenced DGGE bands (Fig 3) found at this depth. The sequences of the predominant bands 30 and 31 (Fig. 3B) at 62 m were closely related to sequences found in hydrothermal vent habitats (Nercessian et al., 2003; Huber et al., 2002) (Fig. 4). All those sequences were unique for the top of the suboxic zone (Figs. 3B and 4). It is obvious that, although DGGE bands 30 and 31 melted at different positions in the gel (Fig. 3B), their sequences were identical (Fig. 4), which is probably due to the degeneracy (Kowalchuk et al., 1997) in the forward primer used during PCR. The same holds for bands 33 and 34, and 36 and 37 (Fig. 3B) which were unique for the deeper part of the suboxic zone (70 m and 77 m) and prevailed even in anoxic waters down to 115 m with sulfide concentrations of up to 30 µM (Fig. 2E). The sequence of the latter DGGE bands was identical to the sequence of clone BSA14-89 which was previously recovered at a depth of 89 m within the Black Sea (Vertriani et al., 2003). Our high resolution phylogenetic analysis of archaeal 16S rDNA thus clearly revealed archaeal community shifts even between marine Crenarchaeota at various depths in the suboxic zone as well as the top of the sulfidic zone.

In the same suboxic and sulfidic waters between 62 and 100 m as where the eight unique 16S rDNA sequences of marine Crenarchaeota were found, PCR/DGGE of archaeal *amoA* (Fig. 5B) and subsequent sequencing of the recovered DGGE fragments revealed nine unique phylotypes of archaeal *amoA* (Fig. 6). Most of the sequences (Figs. 5B, 6) appeared to be present in all of these layers but a shift in the intensity of the DGGE bands (Fig. 5B),

which indicates a shift in the relative distribution of the different *amoA* phylotypes (Fig 6), might result from an adaptation of *amoA*-carrying putative nitrifying Archaea to thrive under different levels of oxygen or even in the presence of sulfide.

Five out of nine sequences clustered with the BS15.7 to BS 15.9 phylotypes (denoted in bold, Fig. 6) that were previously also identified at densities of 15.7, 15.8, and 15.9 kg m^{-3} within suboxic waters of the Black Sea (Francis et al., 2005), which, according to these authors, bracketed the lower nitrite peak. In addition, we recovered four new phylotypes BS_AOA-6, 7, 10, 11. The latter four sequences affiliated with the recently obtained crenarchaeotal enrichment culture from the North Sea (Wuchter et al., 2006) and the recently isolated *Candidatus* '*Nitrosopumilus maritimus*' (Könneke et al., 2005). On the other hand, sequences related to BS 15.7_19 (DQ148696) and BS15.8_24 (DQ148723) from the previous clone library (Francis et al., 2005) were not recovered with our PCR/DGGE method. It is clear that with both our novel slightly degenerate primers as well as the slightly degenerate primers of Francis and coworkers (2005), certain archaeal *amoA* phylotypes escaped PCR amplification.

Western Black Sea, Stations 7605/7620

In the western Black Sea, eight unique 16S rDNA phylotypes were recovered and 100% of the recovered DGGE fragments in the suboxic layer at 75 m were attributed to marine Crenarchaeota. As for the central sampling site, the DGGE band pattern of the marine Crenarchaeota (Fig. 3A) showed clear differences between the upper part (75 m) and the base of the suboxic zone (95-100 m) and the sulfidic waters down to 130 m. For example, DGGE bands 4 to 6 were unique for the top of the suboxic layer at 75 m (Fig. 3A). The sequenced DGGE fragments 4 and 5 (phylotypes BS 7620_75m MGI-4 and 5 in Fig. 4) appeared for the first time whereas the phylotypes 1,2,3, and 6 at this depth were also recovered from top of the suboxic zone at the central site (Figs 3B and 4). Band 9 (Fig. 3A) was only recovered from

the lower part of the suboxic zone including the upper sulfidic waters down to 130 m with sulfide concentrations of up to ~10 μM (Fig. 2A) and showed a 100% sequence similarity with the Black Sea clone previously detected by Vetriani et al. (2003) (Fig. 4). This sequence was therefore also identical to the sequence of the predominant DGGE bands found in the deeper part of the suboxic and and upper part of the sulfidic zone of the central site (Fig. 3B). Note that due to the degeneracy of the forward primer used during PCR (Table 2), also here, the predominant DGGE band 9 carried the same sequence as bands 10 and 16. Two of the sequenced DGGE fragments found at 130 m (in orange; Fig. 3A) grouped with the Miscellaneous Crenarchaeota Group and the DGGE fragments in the green and blue boxes (Fig. 3A) represented unclassified lineages of Euryarchaeota, which will be discussed elsewhere (Coolen et al., in preparation).

The same eight unique phylotypes of archaeal *amoA* as described from the central site were found in the suboxic and sulfidic waters of the western station (Fig. 5A). Interestingly, the archaeal *amoA* DGGE band 11 was unique for the sulfidic water at 130 m depth well below the sulfide chemocline and was not detected from the suboxic zone (Fig. 3A).

Eastern site, Station 7

One crenarchaeotal phylotype (DGGE band 60) was found in the fully oxygenated water layer at 30 m at the eastern station (Figs. 3C, 4) and also here, the highest diversity of marine Crenarchaeota (six phylotypes) was found in the suboxic water layers between 75 and 95 m. At this station, the sulfidic waters were found below 117 m and no other Archaea besides marine Crenarchaeota were detected in the suboxic layers down to 115 m. Within the upper part of the sulfidic zone, the same Miscellaneous Crenarchaeota and unclassified Euryarchaeota as found at the other two stations were recovered only from the sulfidic waters below 117 m (Figs. 3C, 4). No crenarchaeotal sequences were recovered from below 130 m. The vertical distribution of the crenarchaeotal phylotypes was comparable to the vertical

distribution found within the suboxic to sulfidic waters at the central and western stations (Figs. 3C and 4). All sequences of DGGE bands 61-77 were unique for the suboxic zone, whereas bands 80 and 82 were unique for the layer just above the first appearance of sulfide (115 m) and the sulfidic water at 130 m (Figs. 3C, 4).

The archael *amoA* band pattern was quite similar to those found at the two other stations (Fig. 5C), and again band 11- but this time also band 10- were unique for the sulfidic waters at 130 m.

Quantitative distribution of archaeal biomarkers

Recently, CARD-FISH analysis revealed that Crenarchaeota dominated in the suboxic zone of the central station, where they comprised up to 95% of the total number of archaeal cells (max. 7.10^7 cells L^{-1}) and outnumbered the euryarchaeotal cells by two orders of magnitude (Lin et al., 2006). We expected to find a similar total number of archaeal 16S rDNA copies since previous findings have shown that Archaea contain only one or two 16S rDNA copies per genome (Klappenbach et al., 2001; Fogel et al., 1999; Wuchter et al., 2006). Instead, the absolute number of archaeal 16S rDNA and crenarchaeotal 16S rDNA copies per sample was more than one order of magnitude lower than the archaeal and crenarchaeotal cell counts by CARD-FISH. This discrepancy was probably caused by differences in the filtration methods used. For CARD-FISH, a small volume (~20 mL) was filtered over 0.2 μm pore-size polycarbonate membranes (Lin et al., 2006) and few cells should have escaped during filtration. In order to collect enough material for the analysis of the lipids, we filtered a large volume (~100 to 600 L) through a pair of glass fiber filters, each with a nominal pore 0.7 μm pore-size. Small cells may have passed the filters at the start of the filtration. One-third of each filter was used for the quantitative PCR as well as the phylogenetic analysis and the remaining part of each filter for the GDGT-analysis. Therefore, in order to compensate for (a) possible loss of small cells in the beginning of the filtration, (b) a possible unequal

distribution of the cells on the GFF filter and (c) loss of DNA due to adsorption of DNA to glass fiber filters as likely reasons for the unexpectedly lower copy numbers, we here only present the vertical relative quantitative distribution of archaeal *amoA* or 16S rDNA copies in comparison to the total archaeal 16S rDNA copies (Figs. 2C, G, K) instead of reporting the absolute copy numbers.

Central Black Sea (Station 5)

Quantitative PCR analysis using primers selective for marine Crenarchaeota and primers for the archaeal Domain (Table 2) revealed that 16S rDNA copies of marine Crenarchaeota were most predominant in the suboxic layer at 62 m in the presence of ~10 μM of oxygen (93 ± 13% of the total archaeal 16S rDNA copies) (Fig. 2H). This is in agreement with the DGGE results (Fig. 3B) which showed that all sequenced DGGE fragments in this suboxic layers were marine Crenarchaeota. Based on the DGGE results, one would at first sight also expect that the two layers directly below 62 m (at 70 m or at 77 m) would harbor ~100% marine Crenarchaeota. The lower abundance of marine Crenarchaeota compared to the total number of archaeal copies present in these samples (Fig. 2H) can, however, be explained by the fact that part of the archaeal DNA resulted in smeary bands (indicated with a X in Fig. 3B) which were not isolated and sequenced and most likely did not represent marine Crenarchaeota.

amoA was most abundant at 70 m and still comprised up to 28% of total archaeal gene copies in the sulfidic part of the water column down to 100 m (Figs. 2F, G). Whereas the genetic markers *amoA* and 16S rDNA of marine Crenarchaeota were most abundant within the suboxic part of the water column, crenarchaeol, the GDGT specific for pelagic Crenarchaeota (Sinninghe Damsté et al., 2002b) was even more abundant at 100 m in the presence of 15 μM of H_2S (Figs. 2F, G, H). In agreement with our results, crenarchaeol and GDGT-0, both assigned to pelagic Crenarchaeota, were previously also found to be the

predominant GDGTs within the slightly sulfidic waters at 100 and 130 m depth at station BSK-2 which is located close to the central site 5 (Wakeham et al., 2003).

The western Black Sea (stations 7605/7620)

The maximum abundance (up to 100 ± 20% of the total archaeal copies) of the genetic markers of marine Crenarchaeota (Fig. 2C) in the western basin was found just above (75 m) and below (95 m) the nitrite peak (Fig. 2B) in the suboxic zone. Unfortunately, no POM was available from the nitrite maximum at 88-92 m for the analysis of archaeal lipid and genetic markers (Table 1 and Figs. 2b, c). Whereas the maxima of genetic markers of marine Crenarchaeota (both 16S rDNA and *amoA*) was found in the suboxic layer (Fig. 2C), crenarchaeotal *amoA* still represented ~50% of the total archaeal copies (Fig. 2C) at 115 m in the presence of 5 μm of sulfide. At this depth, 15 m below the base of the suboxic zone, a maximum in the concentration of crenarchaeol was detected (Fig. 2E).

The eastern Station 7

As for the eastern basin, the highest relative abundance of the genetic markers of marine Crenarchaeota compared to the total archaeal copy numbers was found at the top of the suboxic zone (Fig. 2K). As for the central station 5, the relative abundance of crenarchaetoal *amoA* (Fig. 2K) was still high at 130 m (~40% of the total archaeal copies) in the presence of ~ 6 μM of sulfide (Fig. 2I). At this depth, the crenarchaeol concentration was found to be highest for this station (Fig. 2L) which is in agreement with the results from the western sites 7605/7620.

Vertical distribution of marine Crenarchaeota **in relation to oxygen and sulfide concentrations**

The most striking observation of our study is that at all Black Sea stations the crenarchaetoal genetic markers were most predominant in the suboxic layer whereas maximum concentrations for crenarchaeol were even found slightly deeper. Crenarchaeotal

gene sequences were barely detected in the oxic waters of the Black Sea, even though other studies have shown that this group of Archaea is relatively abundant in oxic open ocean waters from a wide variety of locations (e.g. Bano et al., 2004; Francis et al., 2005; Herndl et al., 2005; Hershberger et al., 1996; Ingalls et al., 2006; Karner et al., 2001; Massana et al., 2000). The presence of crenarchaeol in the oxygen minimum zone of the Arabian Sea, where oxygen levels were less than 5 μM, provided indirect evidence that marine Crenarchaeota are capable of thriving at low oxygen levels (Sinninghe Damsté et al., 2002a), which is in agreement with our findings. At all studied sites in the Black Sea, we found that crenarchaeol concentrations were even highest (40-45 ng L^{-1}) just below the suboxic zone with sulfide concentrations of up to several tens of μM (Figs. 2D, H, L) and where the relative abundance of crenarchaeotal *amoA* still comprised up to 50% of the total archaeal copies (Figs. 2C, G, K).

Crenarchaeol is more refractory than the DNA of marine Crenarchaeota (Wuchter et al., 2005) and, therefore, is not useful in discriminating dead from living cells. Thus a substantial fraction of the GDGTs encountered in the sulfidic waters just below the suboxic zone may derive from suspended or sinking dead cell debris. Indeed, sediment trap studies have shown that crenarchaeol-containing particles are efficiently transported to the sediment (Wakeham et al., 2003), indicating that there is a mechanism for packaging small archaeal cells into larger sinking particles. Our genetic markers (16S rDNA and *amoA*) are a much stronger indicator of living microorganisms and it is unlikely that dead cell material would accumulate within the sulfidic zone because the largest density shift where cells are more likely to accumulate is found above the crenarchaeotal DNA maximum, at the top of the suboxic zone (Figs. 2A, E, I).

The observed dominance of the marine Crenarchaeota in suboxic waters and the upper part of the sulfidic zone is in good agreement with the FISH data recently reported for the

central Station 5 (Lin et al., 2006), which also showed an abundance of Crenarchaeota in suboxic and anoxic waters. In addition, at all investigated locations, different phylotypes of marine Crenarchaeota (both 16S rDNA and *amoA*) were found in the top of the sulfidic zone, and those phylotypes were not detected in the suboxic zone (Figs. 3-6). This contradicts the assumption that the genetic and lipid markers in the sulfidic waters were derived from dead material and our results would indicate that living marine Crenarchaeota were present even in the sulfidic waters with up to a few tens of μM sulfide (Fig. 2). The presence of marine Crenarchaeota in "cold" sulfidic waters of the Black Sea is in agreement with the recent findings of Koch and coworkers (2006) who reported sequences of marine Crenarchoaeta in German sulfidic marsh waters.

The observed increase in crenarchaeol below the suboxic zone (Figs 2D, H, L) may also point towards species-specific variability in the level of cellular crenarchaeol biosynthesis. For example, below the onset of sulfide at 130 m at the eastern site, the crenarchaeol concentration was highest (Fig. 2L) and this coincided with the presence of the marine crenarchaeotal 16S rDNA sequence BS 7_130m-80 which was identical to a clone that was previously identified within the oxic/anoxic chemocline of the Black Sea at 89 m (clone BSA 14-89; Vetriani et al., 2003) (Figs. 3C, 4). These sequences were not found in shallower suboxic waters, whereas sequences BS 7_95m 74 to 77, found in the suboxic layer and which affiliated with Group I.1a sequences recovered from hydrothermal vents, were not identified in the sulfidic waters (Figs. 3C, 4). In addition, the occurrence of crenarchaeotal *amoA* sequence BS_AOA-11 (Figs. 5, 6) was restricted to the sulfidic zone at the western and eastern stations. The relative abundance based on the intensity of the DGGE band of sequence BS_AOA-11 was also greater in the sulfidic waters at 92 m at the central station as compared to the suboxic layers (Fig. 5).

Possible role of marine Crenarchaeota **in nitrification within the suboxic zone of the Black Sea**

Both *Candidatus* '*Nitrosopumilus maritimus*' (Könneke et al., 2005) and the crenarchaeotal enrichment culture from the North Sea (Wuchter et al., 2006) were found to accumulate nitrite upon oxidation of ammonium as they apparently lack the physiology to oxidize nitrite to nitrate. Our results indicate that the lower nitrite peak (Figs 2B, F, J) found at all three of our Black Sea stations could at least partly result from archaeal ammonia oxidation to nitrite. Indications that marine Crenarchaeota could be involved in nitrification in our study is provided by the abundance of crenarchaeotal *amoA* mainly within the suboxic water layers with oxygen levels as low as 1 μM and that both *amoA* and 16S rDNA were below the detection limit and, therefore, at least 2 orders of magnitude lower within the fully oxygenated water layers where ammonia reaches undetectable levels.

When *amoA* was normalized to its maximum abundance in the waters of the central Black Sea (data not shown), *amoA* appeared to be most abundant at 77 m where the lower nitrite maximum was also found but when *amoA* was normalized as a percentage of total archaeal 16S rDNA copies, its abundance was highest at 70 m (Fig. 2G). Unfortunately, we did not have POM material from the exact depth of the lower nitrite peak for the western and eastern sites (compare Table 1 with Figs. 2b,c,j,k). However, crenarchaeotal *amoA* sequences (sequences starting with BS_15.7, BS_15.8 and BS_15.9 in Fig. 6) were recently also identified (but not quantified) from a narrow region of the Black Sea's suboxic zone, with densities indicative of the presence of the lower nitrite optimum (densities of 15.7, 15.8, and 15.9) (Francis et al., 2005). All this suggests that marine Crenarchaeota within the suboxic zone of the Black Sea could be involved in archaeal nitrification.

Our filter samples from western stations 7605 and 7620 had previously been analyzed for the presence of biomarkers indicative of aerobic methanotrophic bacteria (Schubert et al.,

2006). According to these authors, the concomitant presence and abundance of phylotypes of type I aerobic methanotrophs (Methylococcaceae) and lipid biomarkers with depleted isotopic signatures indicative for a methanotrophic metabolism all indicates that methanotrophic bacteria are responsible for aerobic methane oxidation at the chemocline at a depth down to 115 m at stations 7605 and 7620 (Schubert et al., 2006) despite the presence of up to 5 µM of sulfide. Concentrations of total soluble sulfide of ~15 µM has been shown to completely inhibit the oxidation of ammonia by enrichment cultures of aerobic ammonia oxidizing bacteria (Sears et al., 2004). Whether the presence of 5 µM of sulfide as found at 115 m of the western station (Fig. 2A, C) could still support archaeal nitrification in the Black Sea should be studied in detail with enrichment cultures or by the analysis of *amoA* gene expression such as described by Treusch et al. (2005). Unfortunately, the POM collected by filtration used in the present study were not suitable for the analysis of the extremely labile messenger RNA (mRNA) of *amoA*.

Co-occurrence of anammox and marine Crenarchaeota **within the suboxic zone of the Black Sea**

Kuypers et al. (2003) found anammox bacteria between 62 and 100 m at the western stations 7605/7620, with the highest concentration of ladderanes, the specific lipid biomarkers of anammox, at 90 m, coincident with the nitrite concentration maximum. Unfortunately no filtered POM from the Kuypers et al. (2003) survey from this exact depth was available for the parallel analyses of archaeal lipid- and genetic markers. Nonetheless, using subsamples of the same filters, we now report that the highest relative abundance of archaeal *amoA* occurs at 95 m, within 5 m of the nitrite maximum where Kuypers et al (2003) described the second highest concentration of anammox biomarkers.

Based on nutrient profiles, FISH with specific probes, ^{15}N tracer experiments and the distribution of specific ladderane membrane lipids, Kuypers et al (2003) showed that

ammonia diffusing upwards from the anoxic deep water is consumed by anammox bacteria in the suboxic layer. It was concluded that the anammox bacteria oxidize ammonium with nitrite formed via bacterial nitrification and denitrification. Part of the lower nitrite peak (Fig. 2B) may result from bacterial denitrification (Ward and Kilpatrick, 1990) but the archaeal *amoA* optimum at 95 m (Fig. 2D) indicates that part of the nitrite needed for the anammox reaction could derive from nitrification by marine Crenarchaetoa. At the same time both groups of ammonia oxidizers could compete for the presence of the available ammonia.

Conclusions

Our results show that basin-wide, genetic markers (16S rDNA and archaeal *amoA*) of pelagic marine Crenarchaeota are predominantly present in the suboxic water layer with oxygen concentrations as low as 1 μM, but they are also present in sulfidic waters. Crenarchaeol was detected in the same water layers as where the genetic markers were found but its abundance was found to be highest at the top of the sulfidic zone. Phylogenetic analysis of PCR-amplified and sequenced 16S rDNA containing DGGE fragments revealed that different species of marine Crenarchaeota occurred in the suboxic layers compared to the low-sulfide which suggests that marine Crenarchaeota living under low-sulfide conditions biosynthesize higher levels of crenarchaeol than marine Crenarchaeota living in suboxic layers. The concomitant presence with anammox bacteria could imply that the AOA are involved in nitrification under very low oxygen levels and that AOA provide nitrite which is needed for the anammox reaction. Future analyses that involve $^{15}N\ NH_4^+$ labeling experiments and qualitative plus quantitative reverse transcriptase PCR of archaeal *amoA* transcripts would reveal the absolute link between the diversity and activity of these putative archaeal nitrifiers and provide more details about their oxygen demand as well as their tolerance for soluble sulfide concentrations.

Experimental procedures

Sampling

Particulate organic matter (POM) for the analysis of archaeal GDGTs and genetic markers was collected by in situ filtration of 400 to 900 L of water on glass fiber filters (GFF for tetraethers) from discrete water depths in the upper 300 to 400 m at stations located in the western [stations 7605 (42˚40.7'N, 30˚14.7'E) and 7620 (42˚56.2'N, 30˚01.9'E)], central (station 5; 43˚06'33"N, 34˚00'61"E), and eastern (station 7; 42˚44'93"N, 37˚30'00"E) regions of the Black Sea. The western stations were sampled during the R/V *Meteor* cruise M51/4 in December 2001 and the central and eastern stations were sampled during the R/V *Knorr* cruise K172 leg 8 in May 2003 (http://www.ocean.washington.edu/cruises/Knorr2003)). All filters were kept frozen at -40˚C until further analysis. Details about sampling depths and densities (σ_τ) can be found in Table 1.

Geochemical analyses

Water samples for nutrient analyses were obtained by a pumpcast conductivity–temperature–depth (CTD) system equipped with an oxygen sensor. Before analyses, $ZnCl_2$ was added to the samples from the anoxic part of the water column to precipitate sulfide. Nitrate, nitrite and ammonium concentrations (detection limits 0.1, 0.01 and 0.5 µM, respectively) were determined on board with an autoanalyzer, immediately after sampling. Oxygen and sulfide on the *Knorr* cruise were determined according to Konovalov et al (2003) with detection limits of ~3 µmol L^{-1} for O_2 and 3 nmol L^{-1} for H_2S. The geochemical analyses at the western stations 7605 and 7620 have been described previously (Kuypers et al., 2003).

Lipid extraction for the analysis of tetraether membrane lipids

The GFF filters were extracted with organic solvents and part of the total extract was fractionated into apolar and polar fractions (*Meteor* samples) or neutral lipids, glycolipids and phospholipids (*Knorr* samples) using silica gel. Intact GDGTs were analyzed by dissolving an

aliquot of the polar or glycolipid fractions in hexane/n-propanol (99:1 v/v) to achieve a concentration of 2 mg/mL, filtering through a 0.45 μm polytetrafluoroethylene (PTFE) filter and subsequent high performance liquid chromatography (HPLC) coupled to atmospheric pressure chemical ionization mass spectrometry (APCI-MS) as described by (Hopmans et al., 2000) with minor modifications. Samples were analyzed on a Prevail CN column (150 x 2.1 mm, 3 μm; Alltech Associates Inc., USA) using a 0.2 ml/min flow-rate. The mass range scanned was m/z 1225-1325. GDGTs were quantified by integration of peaks in summed chromatograms of $[M+H]^+$ and $[M+H]^+$+1 and compared with a standard curve obtained using a GDGT-0 standard.

Total DNA extraction

For the QPCR and phylogenetic analysis outlined below, a third part of the GFF filters was extracted using the UltraClean™ Soil DNA Kit Mega Prep following the directions of the manufacturer (Mobio, Carlsbad, CA, USA). Prior to extraction, the filters were sliced with a sterile scalpel in order to enhance the extraction. The concentration of each DNA extract was quantified with the fluorescent dye PicoGreen (MoBiTec, Göttingen, Germany). The quality of the DNA was checked by agarose gel electrophoresis. Undiluted, as well as 2, 5, 10, 20, and 50 times diluted DNA extracts were subjected to quantitative PCR (QPCR) reactions in order to determine, based on the expected distance of the threshold cycles for each diluted sample, whether PCR-inhibiting co-extracted impurities within the DNA extracts were present. The extraction efficiency of this method was determined by performing DAPI cell counts of filtered POM as well as from resuspended pellets after the cell lyses step during DNA extraction.

Quantitative real-time PCR (QPCR)

The copy numbers of 16S rDNA (Archaea and marine Crenarchaeota) and archaeal *amoA* in all samples were determined using an iCycler system (Biorad). A total of 40 cycles were run

with PCR conditions and reagents as described previously (Coolen et al., 2006) but with annealing temperatures and primer combinations as listed in Tables 2 and 3. One microliter of template DNA with known concentrations (10 ng) of template DNA (fluorescently measured [Picogreen, Molecular Probes]), was added to the reaction mixtures. Accumulation of newly amplified double stranded gene products was followed online as the increase in fluorescence due to the binding of the fluorescent dye SYBRgreen (Molecular Probes). Calibration of the samples was performed with known copies (between 10^{-2} and 10^{7}) of *Sulfolobus acidocaldarius* DSM 639 (QPCR for archaeal 16S rDNA) or the enriched marine Crenarchaeote from the North Sea (QPCR for both 16S rDNA of marine Crenarchaeota and archaeal *amoA*) which were generated during PCR with the same primers as used for the amplification of the environmental genes (Table 3). As a control of the specificity of the QPCR, the runs were repeated with only 32 cycles so that most amplicons reached the threshold cycle. In addition, one microliter of the first reaction with 32 cycles was added to a fresh mixture of PCR ingredients and run for 15 cycles but this time with primers including the 40-bp-long GC clamp to allow subsequent DGGE analysis (Muyzer et al., 1993). Aliquots of these QPCR products were run on an agarose gel in order to identify unspecific PCR products such as primer dimers or fragments with unexpected fragment lengths. See Table 3 for the expected fragment lengths. Sequence analysis of the excised DGGE fragments (see methods below) revealed the diversity of the amplicons generated by QPCR and therefore was the ultimate proof that the QPCR reactions were in fact specific.

Primer design

PCR primers for the quantitative and qualitative analysis of archaeal *amo*A (Tables 2 and 3) were designed based on alignments of archaeal *amoA* from the Sargasso Sea (NCBI accession numbers AACY01007942, AACY01075168, AACY01575171, AACY0101435967) and German soil (NCBI accession no. AJ627422). Known copy numbers

of PCR-amplified *amoA* from an enriched marine Crenarchaeote from the North Sea (Wuchter et al., 2006) was used as a positive control during the reactions. DNA of *amoA* containing AOB, ß-Proteobacteria (*Nitrosomonas europeae* and *Nitrospira briensis*) and γ-Proteobacteria (*Nitrosococcus oceanus*), served as a control for the specificity of the archaeal *amoA* PCR reactions. Initially, a gradient QPCR (ICycler, Biorad) with template DNA of the crenarchaeotal enrichment culture and *Nitrosomonas europeae* was performed to determine the optimal annealing temperature (Table 3) for maximum specificity of the PCR reactions.

Phylogeny of sequenced DGGE fragments

The PCR-amplified partial 16S rDNA of Archaea (Tables 2 and 3) was separated by DGGE (Muyzer et al., 1993) using the conditions as described previously (Coolen et al., 2004). For the DGGE analysis of archaeal *amo*A, the gel was run only for 3.5 hours at 200V instead of 5 hours. All processes after electrophoresis including sequence analysis of excised DGGE fragments have been described previously (Coolen et al., 2006). Sequence data were compiled using ARB software (Ludwig et al., 2004) and aligned with complete length sequences of closest relatives obtained from the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using the ARB FastAligner utility. The 16S rRNA gene sequences were analysed using the ARB phylogeny software package (Ludwig *et al*., 2004). The Fast Aligner V1.03 tool implemented in ARB was used for automatic alignment of the recovered Black Sea sequences with their closest relatives from the NCBI database. Then, the phylogenetic bootstrap tree of Fig. 4 (1000 replications) was first reconstructed based on 820-bp-long available sequences of the closest relatives employing the Neighbour-Joining method (Saitou and Nei, 1987). The shorter aligned environmental 16S rDNA sequences from this study were inserted afterwards without changing overall tree topology employing the Parsimony Interactive tool implemented in the ARB software package.

The bootstrap tree (1000 replications) of Fig. 6, was first constructed based on 606-bp-long available archaeal *amoA*-like sequences using the Felsenstein correction .The shorter aligned environmental *amoA* sequences from this study were inserted afterwards without changing overall tree topology employing the Parsimony Interactive tool.

Sequences obtained in this study have been deposited in the NCBI sequence database under accession numbers (*submitted*).

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Figure legends

Figure 1. Sampling sites within the Black Sea.

POM from closely located stations in the western part (station 7605 [42˚30'99"N:30˚14'27"E] and 7620 [42˚55'56"N:30˚03'65"E]) was collected during the R/V Meteor cruise M51/4 in December 2001. POM from stations 5 (central site [43˚06'33"N:34˚00'61"E]) and 7 (eastern site [42˚44'93"N:37˚30'00"E]) was collected during the R/V Knorr cruise K172/8 in May 2003.

Figure 2. Nutrient, and the abundance of biomarkers for marine Crenarchaeota in the upper 400 m of the western (left panel), central (middle panel) and eastern (right panel) stations in the Black Sea.

(*A,E,I*): Oxygen (μM), density (σ_τ), and, for the central and eastern station, H_2S (μM) The H_2S profile at the western stations (Fig. A) was obtained from (Manske et al., 2005). (*B, F, J*): The nutrients NH_4^+, NO_2^- and NO_3^- (μM). The nutrients at the western sampling sites (Fig. A) were obtained from (Kuypers et al., 2003). O_2, H_2S, and nutrient data for central station 5 and eastern station 7 are from the Knorr 2003 Black Sea web site http://oceanweb.ocean.washington.edu/cruises/Knorr2003. (*C, G, K*): *amoA* and 16S rDNA of marine Crenarchaeota as quantified by QPCR (% of total archaeal copies). (*D, H, L*): crenarchaeol concentration (ng L^{-1}). The grey area represents the suboxic zone as defined by oxygen concentrations less than 10 μm and the base of the sulfidic zone. The grey bar in Fig. 2A-D indicates the suboxic zone where previously biomarkers of anammox bacteria were found (Kuypers et al., 2003).

Figure 3. DGGE analysis of the predominant PCR-amplified partial 16S rDNA of the archaeal domain of POM obtained from (*A*) the water column of the western site (stations 7605/7620), (*B*) central site (station 5), and (*C*) eastern site (station 7) in the Black Sea.

Numbers above the gels represent water depths (m) of POM. Depths indicated in light blue, dark blue, and black represent respectively oxygenated, suboxic, and sulfidic waters. DGGE bands (in total 79) that were sliced from the gels and subsequently sequenced are indicated with numbers. Crenarchaeotal groups: DGGE fragments which represented sequences of marine Crenarchaeota carry red numbers and their phylogeny with closest relatives is displayed in Fig. 4. Bands in orange rectangles grouped with uncultured members of the Miscellaneous Crenarchaeota Group. DGGE bands in blue and green rectangles grouped with unclassified Euryarchaeota and their phylogeny will be discussed elsewhere (Coolen et al., in preparation). Two archaeal DGGE bands that resulted in poor sequence quality are indicated with 'a' in the figure. The samples above the suboxic layers apparently contained low template DNA of archaeal 16S rDNA and resulted in the non-specific amplification of bacterial 16S rDNA. The DGGE bands that were found to represent bacterial 16S rDNA are indicated with 'b' in the figure. DGGE bands indicated with 'a' or 'b' were not used for phylogenetic analysis. Smeary, unsequenced bands are indicated with "X".

Figure 4. Phylogenetic tree showing the affiliation of predominant 16S rDNA sequences of Crenarchaeaota retrieved from the water column (POM) (white text in black boxes) with selected reference sequences of Crenarchaeota from the NCBI database. Crenarchaeotal 16S rDNA sequences that were previously found in the suboxic and anoxic waters of the Black Sea (Vetriani et al., 2003) as well as the North Sea enrichment culture (Wuchter et al., 2006) and Candidatus *"Nitrosopumilus maritimus"* (Könneke et al., 2006) are denoted in bold. Bar indicates 0.1 fixed point mutations per nucleotide. Numbers at nodes indicate bootstrap values out of 1000 replications for phylogenetic trees calculated by Neighbour-Joining and Parsimony methods. The sequences from the Black Sea were determined from the DGGE analysis shown in Fig. 3. For example, the crenarchaeotal (Marine Group I; MGI) phylotype

of DGGE band 30 from a depth of 62 m at station 5 within the Black Sea (BS) is named in the tree as BS 5_30m_MGI-30.

Figure 5. DGGE analysis of the predominant PCR-amplified partial *amoA* of AOA obtained from the vertical water column (POM) of the western site (station 7605/7620), central site (station 5), and eastern site (station 7). Eleven major DGGE bands were observed that showed unique positions in the gel but a total of 41 bands were excised to generate replicates.

Figure 6. Phylogenetic tree showing the affiliation of predominant archaeal *amo*A sequences retrieved from the DGGE of Fig. 5 (white text in black boxes) with reference archaeal *amoA* sequences from the NCBI database. The number of replicate DGGE bands that have been sequenced for each unique phylotype is indicated in brackets. *AmoA* of the soil fosmid clone 54d9 (Soil Crenarchaeota group I.1b) was used as outgroup. Sequences with accession numbers starting with DQ are described by Francis et al. (2005). The *amoA* sequences of the clone library (Francis et al., 2005) that were previously recovered from a narrow part of the suboxic zone of the Black Sea with densities of 15.7, 15.8, and 15.9 as well as *amoA* found in the North Sea enrichment culture (Wuchter et al., 2006) and Candidatus *"Nitrosopumilus maritimus"* (Könneke et al., 2006) are denoted in bold (e.g. BS15.8_11). Sequences starting with "T" were recovered from deep waters of the Atlantic Ocean and described by Wuchter et al. (2006). The Sargasso Sea sequences were found on large genome fragments from which AACY01075167 also carried the 16S rDNA which could be assigned to marine Crenarchaeota. Bar indicates 0.1 fixed point mutations per nucleotide. Numbers at nodes indicate bootstrap values out of 1000 replications for phylogenetic trees calculated by Felsenstein correction and Parsimony methods.

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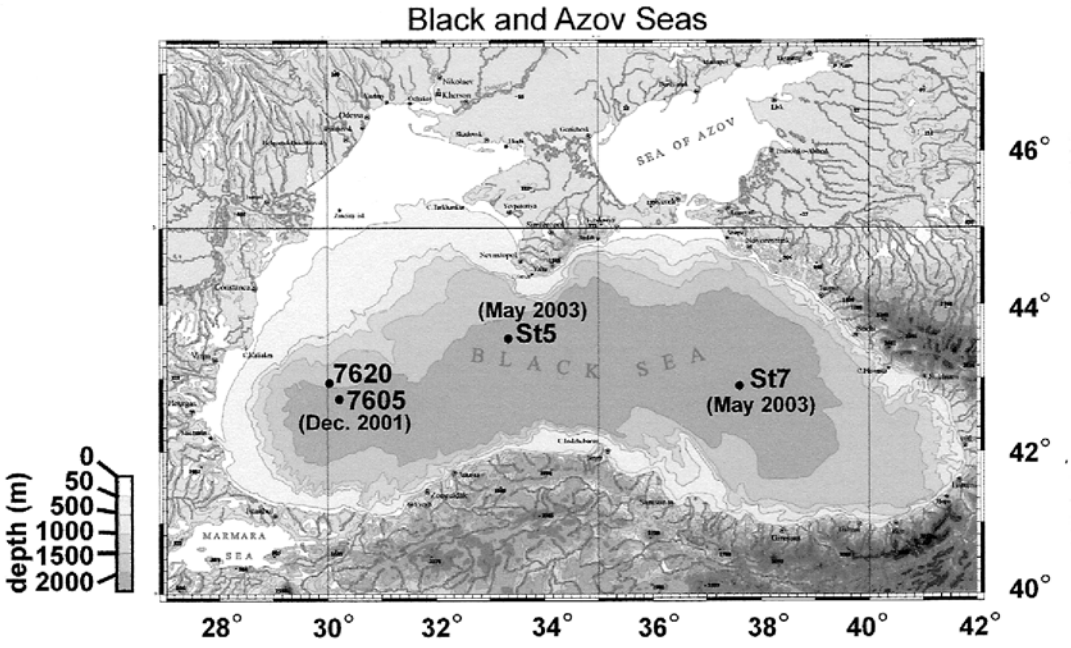


Fig. 1. Coolen et al 2006

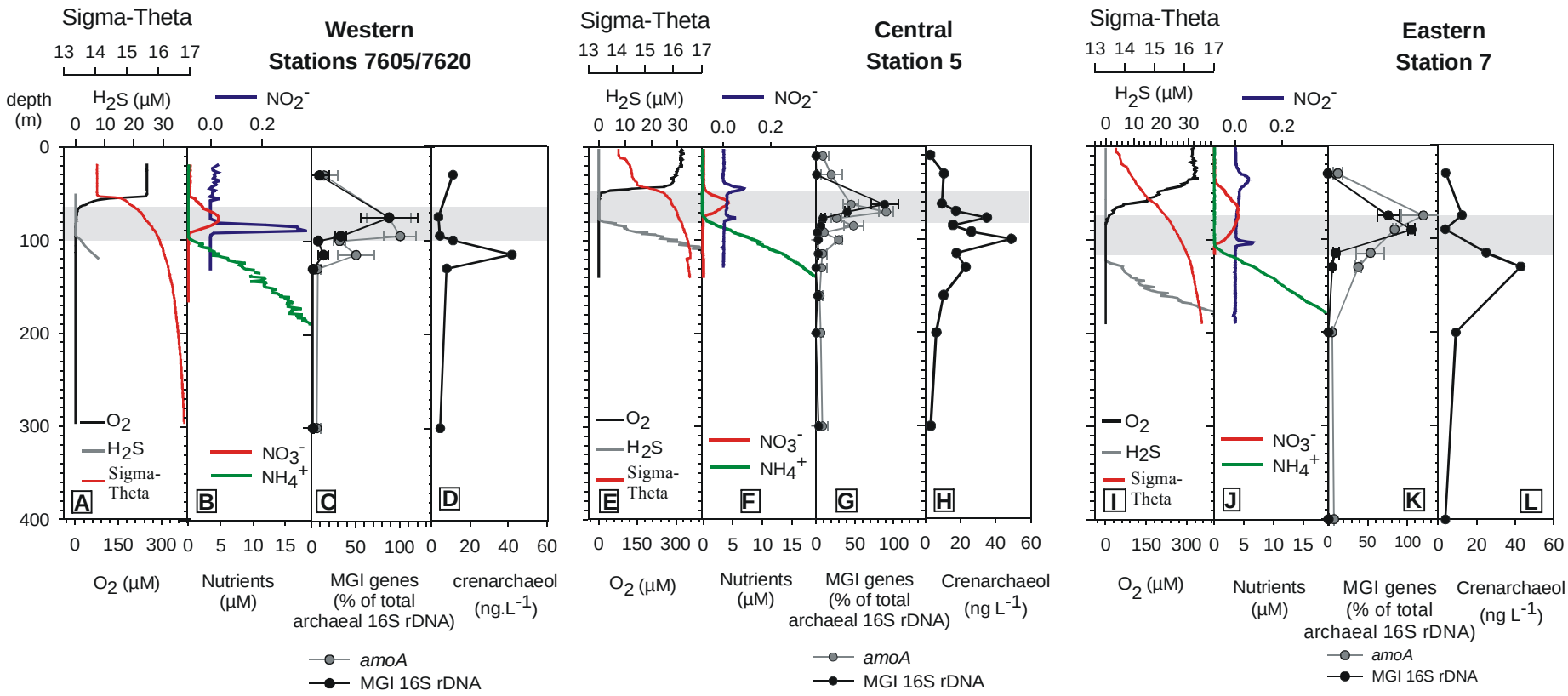
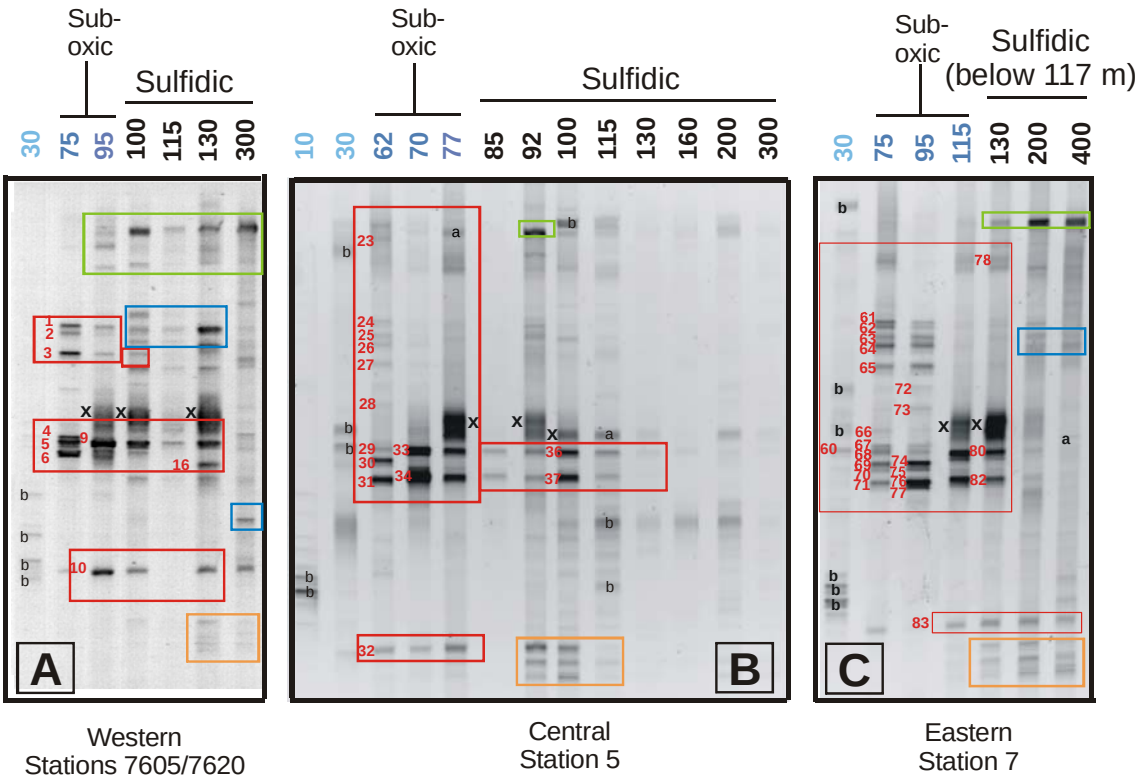
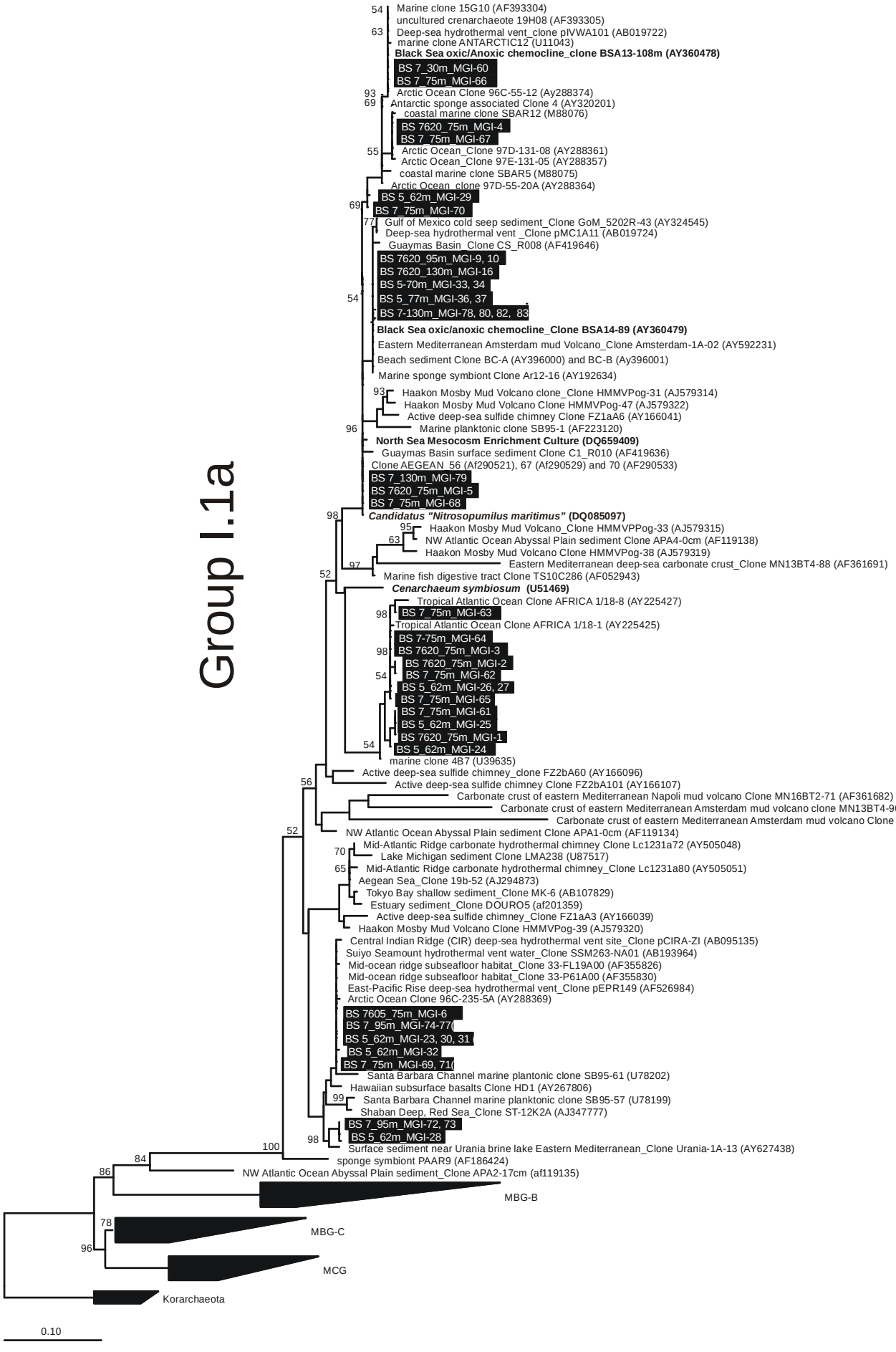


Fig. 2 Coolen et al., 2006



Coolen et al., Fig. 3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Marine clone 15G10 (AF393304)
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marine clone ANTARCTIC12 (U11043)
Black Sea oxic/Anoxic chemocline_clone BSA13-108m (AY360478)
BS 7_30m_MGI-60
BS 7_75m_MGI-66
Arctic Ocean Clone 96C-55-12 (Ay288374)
Antarctic sponge associated Clone 4 (AY320201)
coastal marine clone SBAR12 (M88076)
BS 7620_75m_MGI-4
BS 7_75m_MGI-67
Arctic Ocean_Clone 97D-131-08 (AY288361)
Arctic Ocean_Clone 97E-131-05 (AY288357)
coastal marine clone SBAR5 (M88075)
Arctic Ocean_clone 97D-55-20A (AY288364)
BS 5_62m_MGI-29
BS 7_75m_MGI-70
Gulf of Mexico cold seep sediment_Clone GoM_5202R-43 (AY324545)
Deep-sea hydrothermal vent _Clone pMC1A11 (AB019724)
Guaymas Basin_Clone CS_R008 (AF419646)
BS 7620_95m_MGI-9, 10
BS 7620_130m_MGI-16
BS 5-70m_MGI-33, 34
BS 5_77m_MGI-36, 37
BS 7-130m_MGI-78, 80, 82, 83
Black Sea oxic/anoxic chemocline_Clone BSA14-89 (AY360479)
Eastern Mediterranean Amsterdam mud Volcano_Clone Amsterdam-1A-02 (AY592231)
Beach sediment Clone BC-A (AY396000) and BC-B (Ay396001)
Marine sponge symbiont Clone Ar12-16 (AY192634)
Haakon Mosby Mud Volcano clone_Clone HMMVPog-31 (AJ579314)
Haakon Mosby Mud Volcano Clone HMMVPog-47 (AJ579322)
Active deep-sea sulfide chimney Clone FZ1aA6 (AY166041)
Marine planktonic clone SB95-1 (AF223120)
North Sea Mesocosm Enrichment Culture (DQ659409)
Guaymas Basin surface sediment Clone C1_R010 (AF419636)
Clone AEGEAN_56 (Af290521), 67 (Af290529) and 70 (AF290533)
BS 7_130m_MGI-79
BS 7620_75m_MGI-5
BS 7_75m_MGI-68
Candidatus "Nitrosopumilus maritimus" (DQ085097)
Haakon Mosby Mud Volcano_Clone HMMVPPog-33 (AJ579315)
NW Atlantic Ocean Abyssal Plain sediment Clone APA4-0cm (AF119138)
Haakon Mosby Mud Volcano Clone HMMVPog-38 (AJ579319)
Eastern Mediterranean deep-sea carbonate crust_Clone MN13BT4-88 (AF361691)
Marine fish digestive tract Clone TS10C286 (AF052943)
Cenarchaeum symbiosum (U51469)
Tropical Atlantic Ocean Clone AFRICA 1/18-8 (AY225427)
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Tropical Atlantic Ocean Clone AFRICA 1/18-1 (AY225425)
BS 7-75m_MGI-64
BS 7620_75m_MGI-3
BS 7620_75m_MGI-2
BS 7_75m_MGI-62
BS 5_62m_MGI-26, 27
BS 7_75m_MGI-65
BS 7_75m_MGI-61
BS 5_62m_MGI-25
BS 7620_75m_MGI-1
BS 5_62m_MGI-24
marine clone 4B7 (U39635)
Active deep-sea sulfide chimney_clone FZ2bA60 (AY166096)
Active deep-sea sulfide chimney Clone FZ2bA101 (AY166107)
Carbonate crust of eastern Mediterranean Napoli mud volcano Clone MN16BT2-71 (AF361682)
Carbonate crust of eastern Mediterranean Amsterdam mud volcano clone MN13BT4-90 (AF361692)
Carbonate crust of eastern Mediterranean Amsterdam mud volcano Clone MN13BT4-97 (AF3616
NW Atlantic Ocean Abyssal Plain sediment Clone APA1-0cm (AF119134)
Mid-Atlantic Ridge carbonate hydrothermal chimney Clone Lc1231a72 (AY505048)
Lake Michigan sediment Clone LMA238 (U87517)
Mid-Atlantic Ridge carbonate hydrothermal chimney_Clone Lc1231a80 (AY505051)
Aegean Sea_Clone 19b-52 (AJ294873)
Tokyo Bay shallow sediment_Clone MK-6 (AB107829)
Estuary sediment_Clone DOURO5 (af201359)
Active deep-sea sulfide chimney_Clone FZ1aA3 (AY166039)
Haakon Mosby Mud Volcano Clone HMMVPog-39 (AJ579320)
Central Indian Ridge (CIR) deep-sea hydrothermal vent site_Clone pCIRA-ZI (AB095135)
Suiyo Seamount hydrothermal vent water_Clone SSM263-NA01 (AB193964)
Mid-ocean ridge subseafloor habitat_Clone 33-FL19A00 (AF355826)
Mid-ocean ridge subseafloor habitat_Clone 33-P61A00 (AF355830)
East-Pacific Rise deep-sea hydrothermal vent_Clone pEPR149 (AF526984)
Arctic Ocean Clone 96C-235-5A (AY288369)
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BS 7_95m_MGI-74-77(
BS 5_62m_MGI-23, 30, 31
BS 5_62m_MGI-32
BS 7_75m_MGI-69, 71(
Santa Barbara Channel marine plantonic clone SB95-61 (U78202)
Hawaiian subsurface basalts Clone HD1 (AY267806)
Santa Barbara Channel marine planktonic clone SB95-57 (U78199)
Shaban Deep, Red Sea_Clone ST-12K2A (AJ347777)
BS 7_95m_MGI-72, 73
BS 5_62m_MGI-28
Surface sediment near Urania brine lake Eastern Mediterranean_Clone Urania-1A-13 (AY627438)
sponge symbiont PAAR9 (AF186424)
NW Atlantic Ocean Abyssal Plain sediment_Clone APA2-17cm (af119135)
MBG-B
MBG-C
MCG
Korarchaeota
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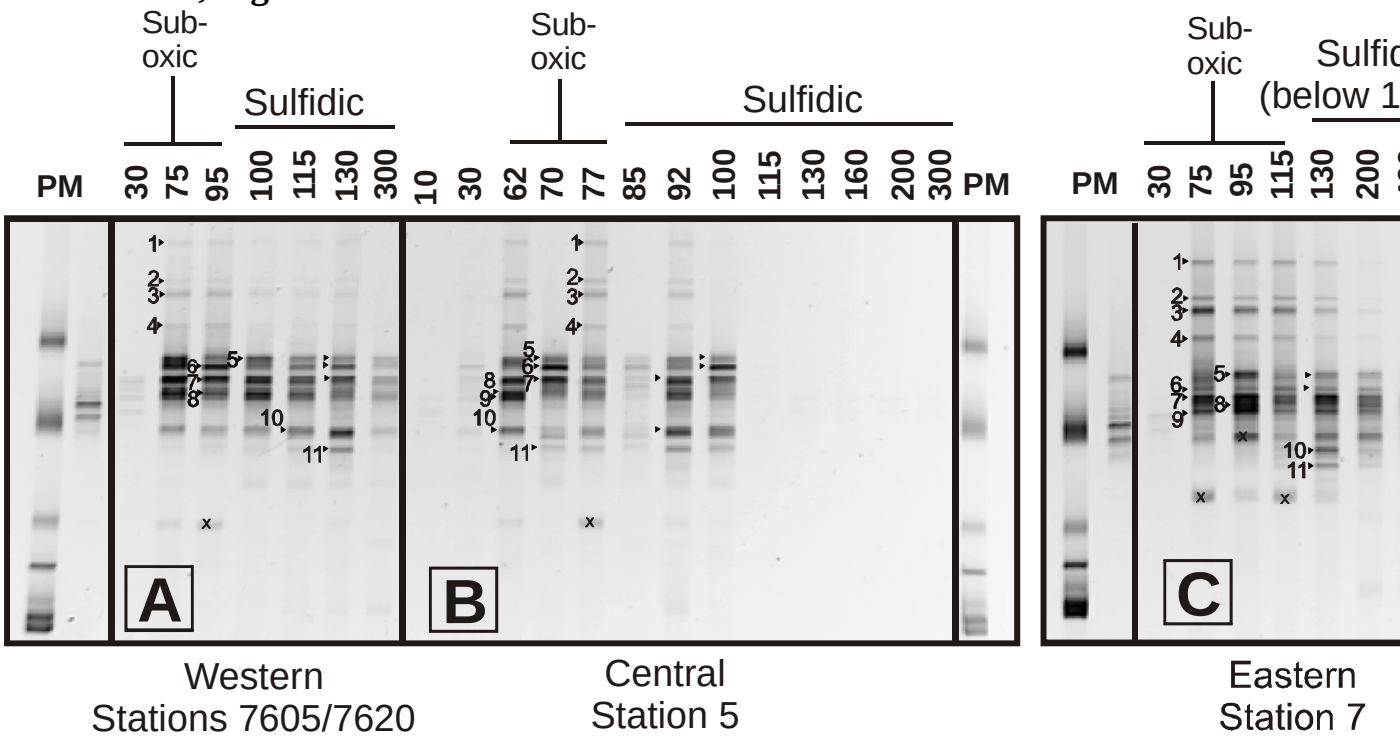
Coolen et al., Fig. 4



Coolen et al., Fig. 5

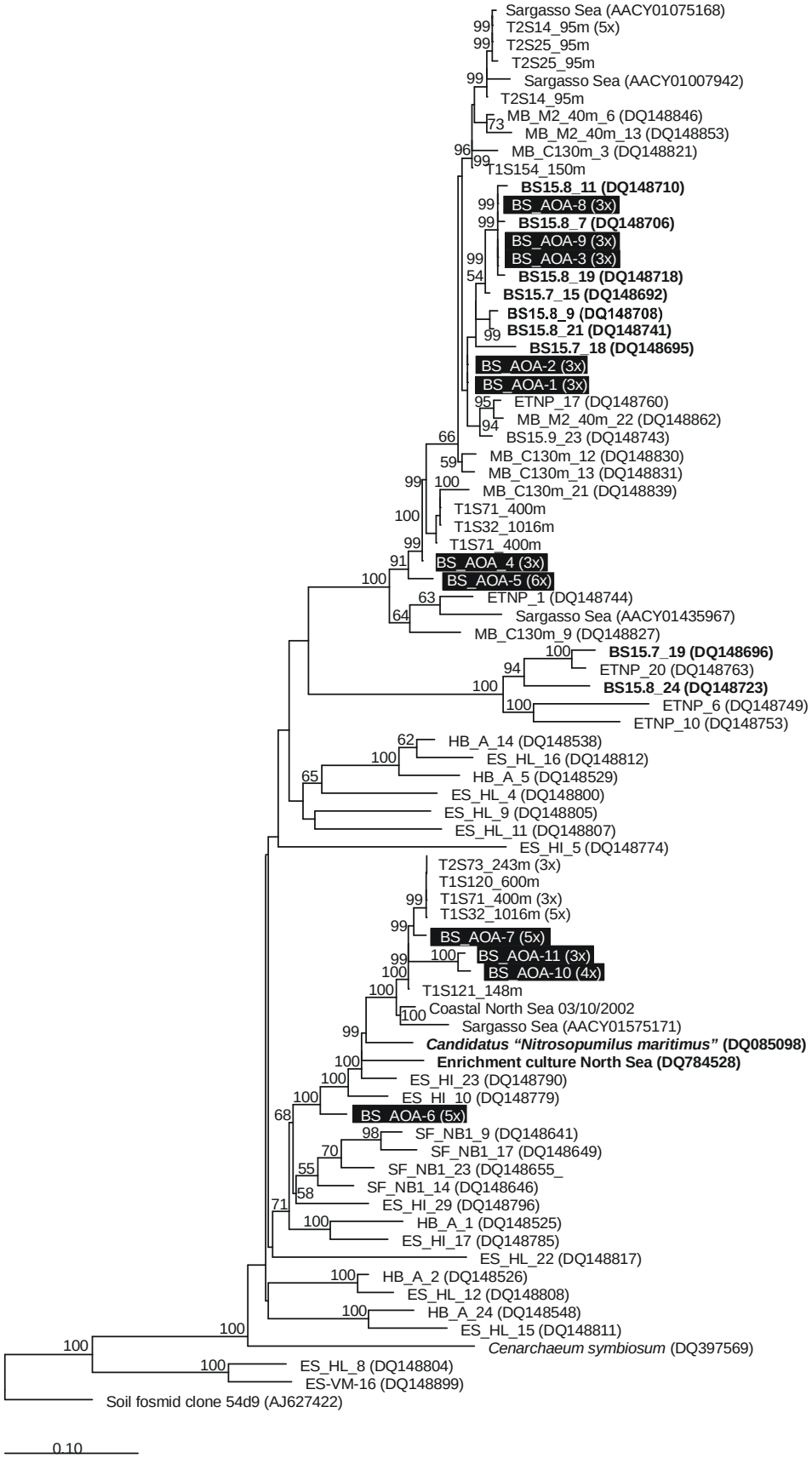


Fig. 6 Coolen et al., 2006

Table 1. Densities at depths where water was filtered in order to collect POM

Western		Central		Eastern	
Stations 7605 and 7620		Station 5		Station 7	
mbsl	density (σ_θ)	mbsl	density (σ_θ)	mbsl	density (σ_θ)
30	14.06	10	14.07	30	14.20
75	15.55	30	14.47	75	15.39
95	15.96	62	15.60	95	15.70
100	16.04	70	15.80	115	16.10
115	16.19	77	15.93	130	16.26
130	16.32	85	16.07	200	16.61
300	n.d.	92	16.16	400	16.95
		100	16.21		
		115	16.31		
		130	16.55		
		160	16.80		
		200	16.88		
		300	16.99		

The filters with POM collected from station 7605 are underlined. mbsl (meter below sea level). n.d. (not determined).

Table 2. Information about the PCR primers used during this study.

Primer #	Primers	*E. coli* positions	Primer sequence	Complementary sequence found in 16S rDNA of:	Reference
I	Parch519f	518-534	5'-CAG CMG CCG CGG TAA-3'	Domain Archaea	(Øvreås et al., 1
II	Arch915r	548-563	5'-[GC-clamp][a]GTG CTC CCC CGC CAA TTC CT-3'	Domain Archaea	(Stahl and Amann
III	MCGI-391f	391-413	5'-AAG GTT ART CCG AGT GRT TTC-3'	MGI	(Takai et al., 20
IV	MCGI-554r	537-554	5'-TGA CCA CTT GAG GTG CTG-3'	MGI	(Teira et al., 20
Primer #	**Primers**		**Primer sequence**	**Complementary sequence found in *amoA* of:**	**Reference**
V	AOA-*amo*A-f		5'-CTG AYT GGG CYT GGA CAT C-3'	MGI	This work
VI	AOA-*amo*A-r		5'-[GC clamp][a] TTC TTC TTT GTT GCC CAG TA-3'	MGI	This work

Additional information about primer combinations for the group-selective PCR amplification of 16S rDNA or archaeal *amo*A can be found in Table 3. [a] For DGGE purposes only, a 40 bp-long GC-rich clamp (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3') (Muyzer et al., 1993) was attached to the 5'-end of the primer. [b] Complementary reverse primer of probe MGI 391-413 described previously (Takai et al., 2004). [c] Primer sequence is identical to the sequence of probe Cren537 (Teira et al., 2004).

Table 3. Primer combinations used in this study.

Primer set	**primers**	**Select for**	**Fragments (bp) excl. GC-clamp**	**Annealing 40 sec. at:**	**Controls: 10^5 gene copies of**
A	I + II	Domain Archaea	420	63ºC	Complete 16S rDNA of *Sulfolobus* sp.
B	III + IV	MGI	122	61 ºC	Complete 16S rDNA of North Sea enrichment culture
C	V + VI	AOA	256	58.5ºC	Fragment generated with primers VII and VIII from North Sea enrichment culture (Wuchter et al., 2006)

In addition, this table shows the selectivity of the primers, fragment lengths of the amplicons, annealing step conditions during PCR, and the type of template DNA of species that served as controls during PCR and to calibrate the QPCRs. Additional information about the primers can be found in Table 2.