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ORIGINAL ARTICLE

Significance of archaeal nitrification in hypoxic waters of the Baltic Sea

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Ammonia-oxidizing archaea (AOA) of the phylum *Thaumarchaeota* are widespread, and their abundance in many terrestrial and aquatic ecosystems suggests a prominent role in nitrification. AOA also occur in high numbers in oxygen-deficient marine environments, such as the pelagic redox gradients of the central Baltic Sea; however, data on archaeal nitrification rates are scarce and little is known about the factors, for example sulfide, that regulate nitrification in this system. In the present work, we assessed the contribution of AOA to ammonia oxidation rates in Baltic deep basins and elucidated the impact of sulfide on this process. Rate measurements with ^{15}N-labeled ammonium, CO_2 dark fixation measurements and quantification of AOA by catalyzed reporter deposition–fluorescence *in situ* hybridization revealed that among the three investigated sites the highest potential nitrification rates (122–884 nmol l^{-1} per day) were measured within gradients of decreasing oxygen, where thaumarchaeotal abundance was maximal (2.5–6.9 $\times$ 10^5 cells per ml) and CO_2 fixation elevated. In the presence of the archaeal-specific inhibitor GC_7, nitrification was reduced by 86–100%, confirming the assumed dominance of AOA in this process. In samples spiked with sulfide at concentrations similar to those of *in situ* conditions, nitrification activity was inhibited but persisted at reduced rates. This result together with the substantial nitrification potential detected in sulfidic waters suggests the tolerance of AOA to periodic mixing of anoxic and sulfidic waters. It begs the question of whether the globally distributed *Thaumarchaeota* respond similarly in other stratified water columns or whether the observed robustness against sulfide is a specific feature of the thaumarchaeotal subcluster present in the Baltic Deeps.
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Introduction

Since the discovery that autotrophic ammonia oxidation is not restricted to the *Bacteria* but is also performed by members of the *Archaea* (Venter *et al.*, 2004; Könneke *et al.*, 2005; Treusch *et al.*, 2005), mesophilic ammonia-oxidizing archaea (AOA) have been recognized as one of the most successful and ubiquitous groups of microorganisms on Earth (Francis *et al.*, 2005; Offre *et al.*, 2013). Phylogenetically affiliated with the novel phylum *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010), AOA occur in high abundances in both terrestrial (Zhang *et al.*, 2010; Pratscher *et al.*, 2011) and aquatic (Yakimov *et al.*, 2011; Biller *et al.*, 2012; Amano-Sato *et al.*, 2013) ecosystems, often outnumbering bacterial ammonia oxidizers (Schleper, 2010). As a consequence, questions arise regarding the ecological niche of AOA, their roles in nitrification and primary production and the environmental factors that regulate their contribution to these processes.

The conversion of ammonia (NH_3) or its protonated form ammonium (NH_4^+) to nitrite (NO_2^-) is the first and generally rate-limiting step in nitrification (except to some extent in the primary nitrite maximum in the oceans; Lomas and Lipschultz, 2006). The relative contributions to ammonia oxidation by autotrophic AOA and ammonia-oxidizing bacteria (AOB) have been inferred using the archaeal and bacterial *amoA* genes that encode subunit A of the key enzyme ammonia monooxygenase (see, for example, Rotthauwe *et al.,* 1997; De Corte *et al.,* 2009; Sauder *et al.,* 2011; Auguet *et al.,* 2012). These studies often revealed the dominance of archaeal over bacterial ammonia oxidizers (Francis *et al.*, 2005; Wuchter *et al.*, 2006; Mincer *et al.*, 2007; Agogué *et al.*, 2008; Newell *et al.*, 2013). Further support for the strong role for AOA in nitrification comes from observations of the co-occurrence of archaeal *amoA* in areas of nitrification activity

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(Caffrey *et al.*, 2007; Beman *et al.*, 2008; Alves *et al.*, 2013) and from metatranscriptomic studies (Baker *et al.*, 2012; Lesniewski *et al.*, 2012). However, to determine the actual contribution and impact of AOA on the nitrogen cycle requires measurements of ammonium oxidation rates, as they cannot be deduced from transcript abundance alone (Mußmann *et al.*, 2011), given that quantification is influenced by, for example, mRNA degradation during sampling (Feike *et al.*, 2012) and, in addition, may not inevitably reflect environmental activity.

In the globally expanding marine oxygen minimum zones and in other oxygen-deficient systems, archaea are prominently embedded in the cycles of carbon (C) and nitrogen (N) (Löscher *et al.*, 2012; Stewart *et al.*, 2012), consistent with the recognition of these systems as hot spots for chemolithoautotrophs (Ulloa *et al.*, 2012). Concerted dissimilatory microbial processes successively transform fixed nitrogen species into dinitrogen gas (Lam and Kuypers, 2011) and autotrophy promotes the fixation of inorganic carbon into biomass (Herndl *et al.*, 2005). Accordingly, AOA have been shown to play an important role within the N (Francis *et al.*, 2007; Stewart *et al.*, 2012) and C cycles (Ingalls *et al.*, 2006). As AOA may contribute strongly to nitrification and primary production, knowledge of their ecology and regulation are crucial for the understanding of ecosystem-relevant N and C cycling in oxygen-deficient zones.

Nitrogen transformations and the responsible microorganisms have also been investigated in the oxic–anoxic transition zones of the water column of the central Baltic Sea (Hannig *et al.*, 2007; Schneider *et al.*, 2010; Hietanen *et al.*, 2012). Oxygen depletion in deep basins is accompanied by gradients of NO_3^-, NO_2^-, H_2S and NH_4^+ that are exploited by different guilds of chemolithoautotrophic prokaryotes (Grote *et al.*, 2008; Labrenz *et al.*, 2010; Glaubitz *et al.*, 2013). Autotrophic ammonia oxidation is an essential ecological function for the N and C cycle in the Baltic Sea that receives high loads of nutrient input from its catchment area (Voss *et al.*, 2011). Nitrification fuels subsequent denitrification that in the water column is mainly carried out by chemolithoautotrophic prokaryotes that link the latter process to the oxidation of reduced sulfur compounds (Brettar and Rheinheimer, 1991; Hannig *et al.,* 2007; Dalsgaard *et al.,* 2013). Anaerobic ammonia oxidation (anammox) has been shown to occur periodically upon major inflow events of oxygen-rich water, causing temporary nonsulfidic conditions (Hannig *et al.*, 2007) in Baltic deep basins. As an extended oxygen- and sulfide-free zone is lacking most of the time (in contrast to the Black Sea; Lam *et al.*, 2007), ammonia oxidation is carried out mostly aerobically because of the sensitivity of anammox bacteria toward oxygen and potentially sulfide (Jin *et al.*, 2012; Carvajal-Arroyo *et al.*, 2013). Where ammonium and oxygen gradients overlap, potential nitrification rates as high as 85–160 nmol l^{-1} per day have been measured (Hietanen *et al.*, 2012). Labrenz *et al.* (2010) reported the dominance of a single thaumarchaeotal subcluster, termed GD2, related to the autotrophic *Candidatus* Nitrosopumilus maritimus (Könneke *et al.*, 2005) that accounted for up to 26% of all prokaryotes in hypoxic waters. The high-level transcription of archaeal *amoA* further indicates a substantial contribution of AOA to ammonia oxidation (Labrenz *et al.,* 2010; Feike *et al.,* 2012). Moreover, the dominance of archaea is in contrast to AOB abundance, as very low cell numbers (<1% of total cell numbers; Bauer, 2003) and *amoA* transcript levels below the detection limit (Labrenz *et al.*, 2010) point to their minor role in ammonia oxidation in Baltic Sea redox gradients. However, cell abundance does not necessarily reflect the respective organism's contribution to a specific process (Musat *et al.*, 2008). AOA and AOB belong to different domains with specific physiologies, and levels of functional gene expression may not be equally extrapolated to the process rates, and hence alternative experimental approaches are required to confirm this hypothesis. In the case of ammonia oxidation, rate measurements can be linked with archaeal vs bacterial contributions by the use of inhibitors specific for either *Bacteria* or *Archaea* (Löscher *et al.*, 2012; Yokokawa *et al.*, 2012).

Sulfide (H_2S) directly impairs metabolism and is therefore toxic to most aerobic microorganisms, including nitrifiers (Joye and Hollibaugh, 1995); thus, its mixing into hypoxic waters (0–10 μmol l^{-1} O_2) such as those of Baltic redox gradients affects microbial activities (Hoppe *et al.*, 1990) and likely also nitrification. The presence of AOA in sulfidic environments (Caffrey *et al.*, 2007; Coolen *et al.*, 2007) suggests that they are able to cope with sulfide, unlike highly H_2S-sensitive AOB (Sears *et al.*, 2004). Among the oxygen-depleted systems worldwide, the Baltic Sea presents an ideal habitat to not only investigate archaeal nitrification but also its response to sulfidic conditions. The high abundance of *Thaumarchaeota* reported by Labrenz *et al.* (2010) in a sulfidic basin of the Baltic Sea suggested that they are the predominating ammonia oxidizers, potentially because they are better adapted than their bacterial counterparts to periodically sulfidic conditions. Erguder *et al.* (2008) reported a shift toward archaeal *amoA* enrichment in a sequential batch reactor when pulsed with sulfide. In the pelagic redox gradient of the Baltic Sea, sulfide is used as an electron donor by chemoautotrophic sulfide-oxidizing bacteria, for example, the autotrophic *Sulfurimonas* sp. subgroup GD17 (Grote *et al.*, 2008) and perhaps by *Gammaproteobacteria* of the SUP05 group (Glaubitz *et al.*, 2013), but short-term mixing events would also expose sulfide-sensitive microbes such as nitrifiers to low and potentially toxic sulfide concentrations. In fact, lateral intrusions and small-scale mixing have been shown to influence microbially mediated

transformations in pelagic redox gradients (Fuchsman *et al*., 2012) and are also frequent in the Baltic Sea (Kuzmina *et al*., 2005; Wieczorek *et al*., 2008; Friedrich *et al*., 2014).

In this study we quantified *Thaumarchaeota* and potential nitrification at different times and locations, assessing the contribution of AOA to this process by using archaea-specific inhibitors. In addition, we sought to elucidate the effect of sulfide on nitrification activity in Baltic Sea redox gradients. Our results provide compelling evidence that *Thaumarchaeota* are indeed responsible for the major portion of ammonia oxidation in hypoxic waters of the Baltic Sea and that they are likely adapted to its periodically occurring anoxic and sulfidic conditions.

Materials and methods

Sampling and physicochemical analyses

Sampling was conducted in the eastern and western Gotland Basin and the Bornholm Basin (locations and coordinates in Figure 1), specifically at Gotland Deep station 271, Landsort Deep station 284 and Bornholm Deep station 213 during cruises with the research vessels *Alkor* (February 2011), *Elisabeth-Mann-Borgese* (July 2011) and *Meteor* (November 2011 and June 2012). During all cruises, water was collected, with emphasis on oxic–anoxic transition zones, in free-flow water bottles attached to a conductivity, temperature, and depth (CTD) probe. For each depth, sampling was conducted with two 5-l free-flow bottles (Hydrobios, Kiel, Germany). Water was taken from one bottle for nitrification and CO_2 fixation rate measurements, followed by sampling for cell counts. At the same time, samples from the second bottle were immediately analyzed on board for O_2, H_2S, NH_4^+, NO_3^- and NO_2^- concentrations (detection limits per liter: 1 μmol for O_2; 0.2 μmol for H_2S; 0.2–0.5 μmol for NH_4^+ and NO_3^-, 0.05 μmol l^{-1} for NO_2^-) according to the method of Grasshoff *et al.* (1983) or Cline (1969) (for H_2S). O_2 was determined via automatic titration (Titrino 702, Metrohm, Herisau, Switzerland); the titer of the thiosulfate solution was determined daily and the solution renewed if deviating >0.1 from the reference value of 1.0. Standards for H_2S were run ranging from 0.1 to 3 mg l^{-1} with an r^2 between 0.9882 and 0.9997. Calibration for NH_4^+ and NO_3^- was done using standards of 10 μmol l^{-1} or 2 μmol l^{-1} for NO_2^-. At Gotland Deep in 2011, water was retrieved in two consecutive CTD casts. Samples for the sulfide-spiking experiment and dissolved inorganic carbon determination were obtained in subsequent separate CTD casts.

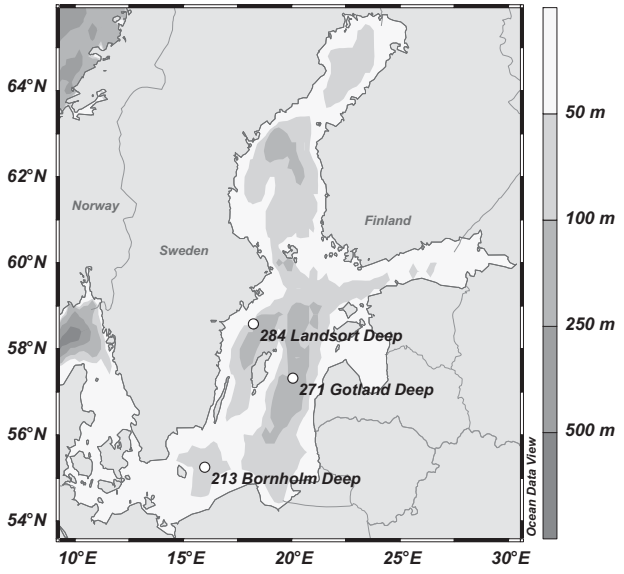

Figure 1 Overview of the locations of the three sampled sites in the Baltic Sea: station 213 at the Bornholm Deep (55° 15.0300 N, 015° 59.1400 E), station 271 at the Gotland Deep (57° 19.2041 N, 020° 02.9291 E) and station 284 at the Landsort Deep (58° 35.0056 N, 018° 14.0344 E).

Quantification of Thaumarchaeota *and* Sulfurimonas *sp. subgroup GD17*

From each depth, samples of 100 ml were fixed for 6–12 h with 0.2 μm filtered formaldehyde (2% final concentration) at 4 °C. From these, volumes of 20–40 ml (profiles) or 4 ml (spiking experiment) were filtered onto 0.2 μm polycarbonate filters (Nuclepore track-etched, Whatman, GE Healthcare, Freiburg, Germany). The filters were dried and stored at −20 or −80 °C until analysis. Catalyzed reporter deposition–fluorescence *in situ* hybridization was performed according to Pernthaler *et al.* (2002) with a few modifications. Briefly, the filters were embedded in 0.1% agarose before digestion with lysozyme and achromopeptidase at 37 °C for 60 and 15 min, respectively. Hybridization with the horseradish peroxidase-labeled Cren679 probe, specifically targeting thaumarchaeotal subcluster GD2 (Labrenz *et al*., 2010), was carried out overnight at 35 °C in the presence of 35% formamide, followed by tyramide signal amplification with Alexa Fluor 488 (Invitrogen, Life Technologies GmbH, Darmstadt, Germany). Hybridization and quantification targeting cells of *Sulfurimonas* sp. subgroup GD17 was conducted with probe SUL90 and 55% formamide according to Grote *et al.* (2007). Dried filter sections were embedded in Vectashield mounting medium (Vector Labs, Burlingame, CA, USA) containing 4′,6-diamidin-2-phenylindol. For enumeration by epifluorescence microscopy, 600–1400 4′,6-diamidin-2-phenylindol-stained cells from randomly selected microscopic fields were inspected using a Zeiss Axioskop 2 mot plus (Zeiss, Oberkochen, Germany).

Potential nitrification rates

Incubations with amended 5 μmol l^{-1} $^{15}NH_4Cl$ (99% ^{15}N, Cambridge Isotope Laboratories, Tewksbury, MA, USA) were conducted as described in Holtappels

et al. (2011) with minor modifications. We consider the measured nitrification rates as potential rates because the addition of $^{15}NH_4Cl$ resulted in ammonium concentrations significantly exceeding the *in situ* concentrations (Supplementary Table S1). The details of the procedure are as follows: sample water was transferred from the CTD into 0.5-l or 1-l glass bottles with a threefold overflow and then closed without headspace with polytetrafluoroethylene-taped butyl rubber stoppers. The bottles were transferred to a cooling room (6–11 °C) where a gas-tight syringe (Hamilton, Bonaduz, Switzerland) was used to inject them with 5 μmol l^{-1} of an anoxic $^{15}NH_4Cl$ solution. Before and after tracer amendment, the total NH_4^+ concentration in the samples was determined in order to calculate the percentage of $^{15}NH_4^+$ labeling that ranged between 21% and 100% (Supplementary Table S1). The water was distributed from the bottle into aliquots of 12 ml in Exetainer glass vials (Labco Ltd, Lampeter, UK). For this, the bottle was placed upside down, gently discharging the sea water by replacement with N_2 through a glass tube into Exetainer vials with threefold overflow one after another. Then, the Exetainers were immediately capped without headspace. Incubations took place in the dark at approximately the *in situ* temperature. Two or three vials were removed approximately every 6 h over a period of 30 h from the beginning of the experiment. Headspace was added to each vial then they were frozen at − 20 °C.

For mass-spectrometric analysis, the frozen samples were thawed, and NO_3^- in a 4.5-ml aliquot reduced to NO_2^-, adding ~0.3 g spongy cadmium per sample, as described by Jones (1984). After horizontal shaking overnight, the tubes were centrifuged and 4 ml of the supernatant was transferred into new 6 ml Exetainer vials. These vials were then closed with caps and flushed with helium (grade 5.0) for 10 min. The NO_2^- pool was reduced to N_2 as described in Füssel *et al.* (2011) by adding 50 μl of 4% sulfamic acid and shaking the samples overnight. With only a small fraction of the bulk NO_2^- labeled, $^{15}NO_2^-$ was converted to $^{15}N^{14}N$. The vials were stored upside down until gas chromatographic isotopic ratio mass spectrometry analysis was carried out within a few days. The accumulation of $^{15}N^{14}N$ was determined by gas chromatographic isotopic ratio mass spectrometry analysis (Delta V plus Isotope Ratio MS, Thermo Finnigan Conflo III, Thermo Fisher Scientific, Waltham, MA, USA) analysis of a 500-μl sample of the headspace N_2. Potential nitrification rates were derived from the excess in the $^{29}N_2/^{28}N_2$ ratio, measured over time during the incubation period, above the initial ratio and from the percentage of ^{15}N labeling of ammonia (see Supplementary Information; Thamdrup and Dalsgaard, 2000).

Sulfide-spiking experiment

At the Gotland Deep site in 2011, water from the zone with the highest potential nitrification rates (122 nmol l^{-1} N per day at a depth of 110 m) was retrieved and transferred to 0.5-l or 1-l bottles as described above but, because of limitations in the sample volume, without overflow. The bottles were randomly assigned to one of four H_2S treatment groups, generated by adding H_2S as spikes from a stock solution of 188 mmol l^{-1} H_2S to final concentrations of 0 (control), 4.1, 8.3 and 16.6 μmol l^{-1}. To detect a potential impact of sulfide addition on nitrification activity after spiking, potential nitrification rates were measured as described above, that is, by subsampling from one bottle of each group into Exetainer vials at 0, 24 and 48 h after spiking, such that nitrification rate determinations covered the intervals 0–24, 24–48 and 48–72 h. The Exetainer incubations were similarly stopped for parallel vials of the control and each sulfide amendment four to nine times during the following 24 h in order to determine potential nitrification rates. In addition, samples from the bottles and Exetainers were fixed with $ZnCl_2$ to measure sulfide concentrations according to Cline (1969).

Dark CO_2 fixation

Rates of dark CO_2 fixation were determined, slightly modified, as described in Jost *et al.* (2010). Sea water was transferred directly from the CTD bottles into 12 ml Exetainer glass vials with threefold overflow. The vials were closed without headspace after which a gas-tight syringe was used to add ~1.85 MBq of $NaH^{14}CO_3$ (Hartmann Analytic GmbH, Braunschweig, Germany) from an anoxic stock solution. Three parallels plus a killed control, in which the sample was fixed with formaldehyde (2% final concentration), were incubated at approximately the *in situ* temperature for 24 h; the exact times were noted. The incubations were stopped by filtering the contents of the vials onto 0.2 μm cellulose acetate (Sartorius, Göttingen, Germany) or polycarbonate (Nuclepore track-etched, Whatman) filters (25 mm diameter). Before filtration, 50 μl of the sample was withdrawn to determine the total radioactivity added to each vial. The filters were exposed to HCl fumes for 0.5–2 h and then transferred into 4 ml of LumaSafe scintillation cocktail (PerkinElmer, Rodgau, Germany). Total- and filter-^{14}C disintegrations per min were analyzed in a PerkinElmer Tri-Carb 2800R liquid scintillation analyzer. CO_2 fixation rates were derived (see Supplementary Information) from the fraction of ^{14}C incorporated in relation to the total activity added and the background concentration of dissolved inorganic carbon ranging between 2003 and 2037 μmol kg^{-1} (Gotland Deep, July 2011). The determination of C_T was performed by coulometry using the SOMMA system designed by Johnson *et al.* (1993). The system was calibrated with certified carbon reference material (Dr A Dickson, University of California, San Diego, La Jolla, CA, USA) and allowed for a precision/accuracy of approximately

$\pm 2\,\mu mol\,kg^{-1}$. Counts of the killed controls were subtracted before the rates were calculated.

Domain-specific inhibition of archaea and bacteria
Taking advantage of the fundamental differences in the translation machinery of *Archaea* and *Bacteria*, we chose inhibitors targeting archaeal or bacterial protein biosynthesis and tested them before *in situ* application. To do so, the chemoautotrophic activity (dark CO_2 fixation rates) of a thaumarchaeotal AOA enrichment culture was used to determine the extent of inhibition. This culture, originally obtained from the redox gradient of the Landsort Deep and enriched over a period of 1.5 years in the presence of streptomycin and NH_4Cl, contained 89–97% archaea (according to catalyzed reporter deposition–fluorescence *in situ* hybridization (probe Arc915); C Berg *et al.*, unpublished data). The archaeal inhibitors, N^1-guanyl-1,7-diaminoheptane (GC_7; Biosearch Technologies, CA, USA) (Jansson *et al.*, 2000) and diphtheria toxin (Sigma-Aldrich, Germany) (Mußmann *et al.,* 2011; Yokokawa *et al.,* 2012), at concentrations of 0.25–2.0 $mmol\,l^{-1}$ and 0.5–10 $\mu g\,ml^{-1}$, respectively, and the bacterial inhibitor erythromycin (VWR, Germany), at concentrations between 10 and 50 $\mu g\,ml^{-1}$ (Yokokawa *et al.,* 2012) were assessed. GC_7 shuts down biosynthesis via cell cycle arrest and specifically targets archaea (Jansson *et al.*, 2000), including *Ca.* N. maritimus SCM1, but it has no effect on AOB (Löscher *et al.*, 2012).

Whereas diphtheria toxin caused no inhibition, GC_7, at a concentration of 1 $mmol\,l^{-1}$, significantly reduced dark CO_2 fixation activity by 81% (one-way analysis of variance $P<0.0004$; Figure 2) within 24 h and was therefore chosen as the archaeal inhibitor for environmental samples. The GC_7 solvent (acetic acid) was likewise tested and had no significant inhibitory effect on AOA (Supplementary Figure S1). For the environmental samples, GC_7 dissolved in 5 $mmol\,l^{-1}$ acetic acid to a concentration of 100 $mmol\,l^{-1}$ was prepared as a stock solution and stored at $-20\,^{\circ}C$ until use. For inhibitor experiments, sample water from the Gotland Deep and the Landsort Deep was amended with $^{15}NH_4Cl$ and then distributed into 6 or 12 ml vials as described above. Immediately afterwards, GC_7 was injected (1 $mmol\,l^{-1}$ final concentration) into the vials that were then incubated alongside the controls (without inhibitor) for 6, 12 or 24 h. Activity was arrested by freezing the samples at $-20\,^{\circ}C$.

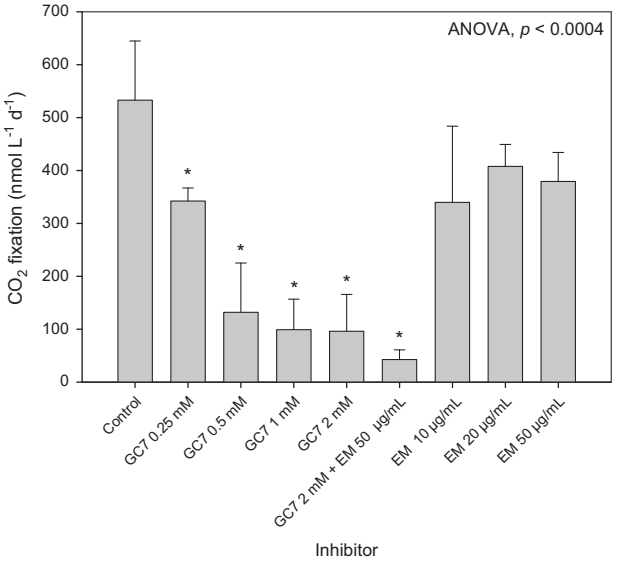

Figure 2 Evaluation of different concentrations of GC_7 and erythromycin (EM) on CO_2 fixation rates in a thaumarchaeotal enrichment culture. Measurements were conducted in triplicate subsamples taken from the culture; error bars show s.d. *Significant ($P<0.0004$) difference of the treatment compared with the control (one-way analysis of variance (ANOVA) and Tukey's pairwise comparison).

Statistical analyses
All statistical analyses were performed with the PAST software package v 3.0 (Hammer *et al.*, 2001). Correlation between potential nitrification rates and the abundance of *Thaumarchaeota* was tested via linear bivariate regression of measured potential nitrification rates and thaumarchaeotal cell counts. Domain-specific inhibition of CO_2 fixation activity in an AOA enrichment culture with inhibitors at several concentrations was compared using one-way analysis of variance followed by Tukey's pairwise comparison. A generalized linear model was used to test for significant difference of regression slopes from zero. Difference in regression slopes of potential nitrification rates with and without GC_7 or erythromycin was tested using one-way analysis of covariance.

Results

Redox zone structure in the Gotland Deep, Landsort Deep and Bornholm Deep
At the Gotland Deep in July 2011, the water column was oxygenated in the upper 119 m, including a hypoxic zone with low oxygen concentrations ($<10\,\mu mol\,l^{-1}$) between 109 and 119 m (Figure 3). An overlap of oxygen with small amounts of sulfide was detected between 114 and 119 m. Below this depth, sulfide concentrations increased. The sampling depths, which spanned from 74 to 119 m, covered a broad peak of nitrate, with a maximum concentration of 8.4 $\mu mol\,l^{-1}$ at 104 m. Nitrite ranged between 0.1 and 0.4 $\mu mol\,l^{-1}$. Ammonium was detectable in the hypoxic zone at concentrations of 0.1–0.2 $\mu mol\,l^{-1}$ and increased progressively with depth. Total prokaryotic cell numbers ranged between 3.2 and 7.5×10^5 cells per ml, with a peak in the hypoxic zone at 110 m. The *Thaumarchaeota* subcluster GD2 accounted for 2–24% of total cell numbers, reaching a maximal abundance of 1.8×10^5 cells per ml at 110 m, that is, within the hypoxic zone.

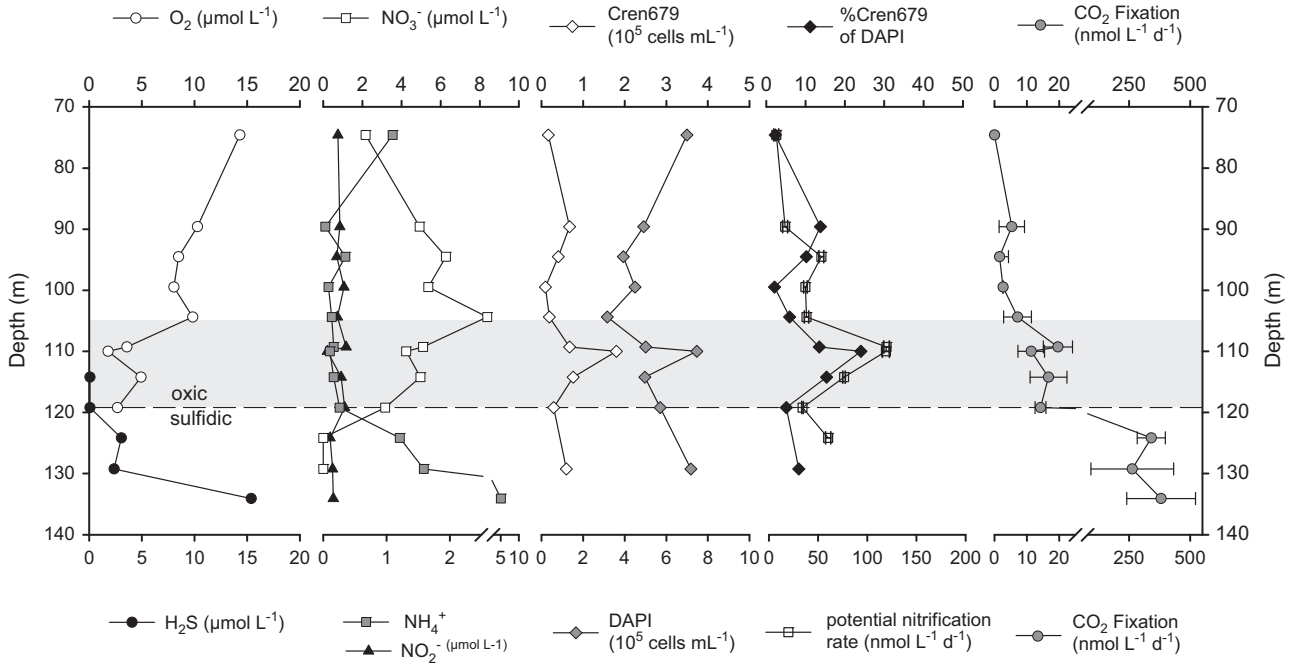

Figure 3 Detailed profile of the Gotland Deep redox zone in July 2011. Concentrations of NO_3^-, NO_2^-, NH_4^+, H_2S and O_2, total prokaryotic cell numbers counted by 4′,6-diamidin-2-phenylindol (DAPI) staining, GD2 thaumarchaeotal cell abundances (catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) probe Cren679), potential nitrification rates and CO_2 fixation rates were determined. The hypoxic zone, where oxygen concentrations were $<10\,\mu mol\,l^{-1}$, is shaded gray. Error bars show s.d. of triplicate samples (CO_2 fixation rates) or the s.e. of the slope of excess ^{29}N (potential nitrification rates).

At the Landsort Deep in June 2012, the hypoxic zone was sampled within a gradient of decreasing nitrate, also covering nitrite maximum (Figure 4). Ammonium concentrations increased pronouncedly, from 0.1 to $1.4\,\mu mol\,l^{-1}$, whereas oxygen concentrations decreased from 22 to $2.7\,\mu mol\,l^{-1}$. Sulfide did not exceed $0.1\,\mu mol\,l^{-1}$. At the Gotland Deep in June 2012, the hypoxic zone was sampled at depths between 70 and 104 m (Figure 5) above the sulfidic/anoxic zone that was not covered by the sampling depths. The physicochemical profile deviated from the typical stratification by showing a narrow band of increased H_2S and NH_4^+ concentrations at 94 m. Above this intrusion, sharp peaks in nitrate and nitrite were located at depths of 85 and 89 m, respectively.

The Bornholm Deep in 2011 was characterized by a declining oxygen gradient, with concentrations as low as $<10\,\mu mol\,l^{-1}$ above the sea floor (Supplementary Table S1), coinciding with NH_4^+ concentrations of 13.6 (November 2011) and $1.8\,\mu mol\,l^{-1}$ (July 2011). The highest potential nitrification rates were detected in November 2011, when *Thaumarchaeota* were highly abundant (4.3×10^5 cells per ml). Also at this site, hydrogen sulfide was detected above the sea floor at a concentration of $23.4\,\mu mol\,l^{-1}$.

Dark CO_2 fixation, as a measure of chemoautotrophic activity in the nonsulfidic, hypoxic zones of the Bornholm Deep, Gotland Deep and Landsort Deep (Figures 3–4, Supplementary Table S1), achieved rates of up to 28, 19, and $58\,nmol\,l^{-1}$ per day, respectively, coinciding with high potential nitrification rates. This level of carbon fixation was at least 20-fold lower than the rates in the upper sulfidic waters of the Gotland Deep that were as high as $380\,nmol\,l^{-1}$ per day (Figure 3).

Potential nitrification rates in Baltic Sea redox zones

Potential nitrification, as evidenced from the increase in ^{15}N label in the NO_2^- and NO_3^- pools with time after $^{15}NH_4^+$ addition, was detectable on all sampling occasions at varying rates and within depth intervals of several meters. Maximal potential nitrification rates in the redox zones of the Gotland Deep (Figure 3) and Landsort Deep (Figure 4a) in 2011 and 2012 were in the range of $133–351\,nmol\,l^{-1}$ per day (Supplementary Table S1). In the Bornholm Deep, maximum rates of $189–884\,nmol\,l^{-1}$ per day were measured within a narrow zone above the sea floor at 78 m depth (Supplementary Table S1). In both the Gotland Deep and Landsort Deep, the zone of maximal nitrification was generally located below the nitrate peak and at oxygen concentrations $<10\,\mu mol\,l^{-1}$, coinciding with the nitrite peak (Figures 3 and 4). Increasing potential rates of nitrification were accompanied by decreasing oxygen concentrations and a high abundance of GD2 *Thaumarchaeota* cells (Supplementary Figures S2 and S3). For the combined data from the Gotland Deep, Landsort Deep and Bornholm Deep, the relationship between potential nitrification rates and thaumarchaeotal abundance varied considerably but the correlation was positive and significant ($P<0.002$, $r^2=0.20$,

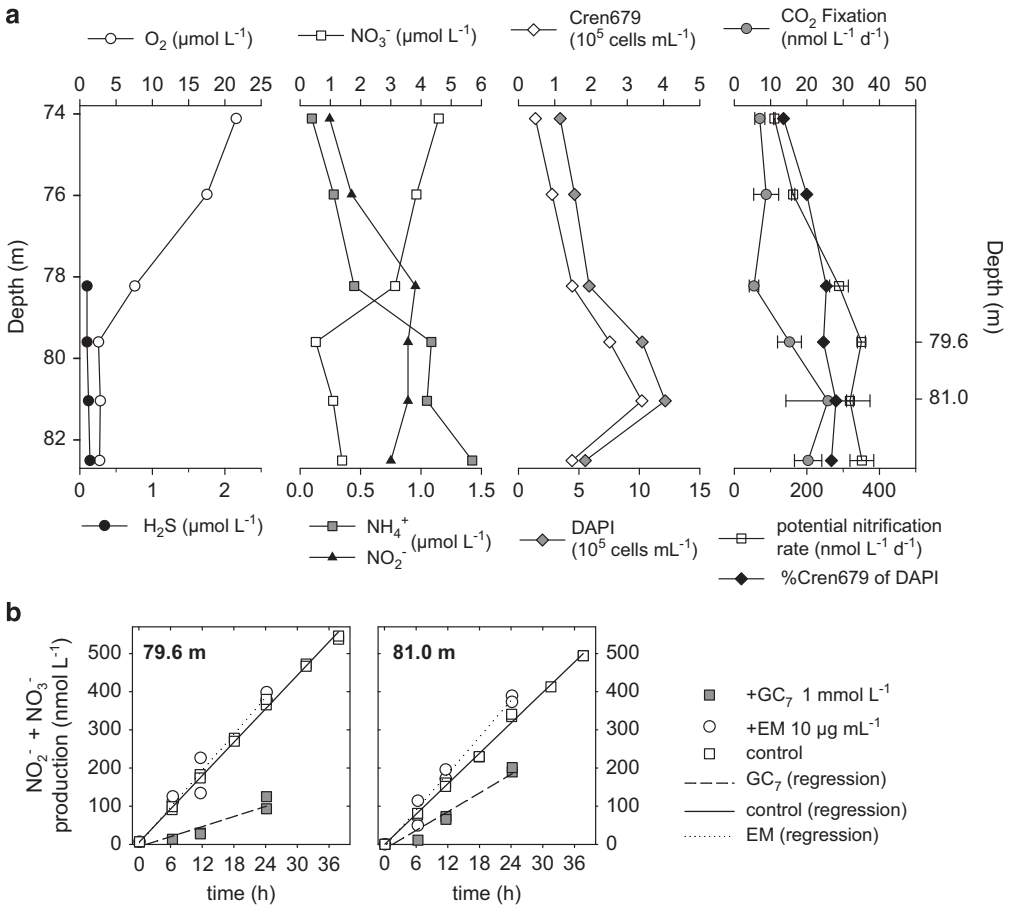

Figure 4 (**a**) Profile within the hypoxic waters of the Landsort Deep redox gradient in June 2012. Error bars show the s.d. of triplicate samples (CO_2 fixation rates) or the s.e. of the slope of excess ^{29}N (potential nitrification rates). Water from 79.6 and 81 m was used to determine nitrification activity in the presence of specific inhibitors. (**b**) Time course of $NO_2^- + NO_3^-$ production from NH_4^+, taking into account both the ambient $^{14}NH_4^+$ and added $^{15}NH_4^+$ concentrations during nitrification. Measurements were made in untreated samples (control) and in the presence of archaeal (GC_7) and bacterial (erythromycin (EM)) inhibitors. Regression slopes of $NO_2^- + NO_3^-$ production of GC_7-treated samples differed significantly from the control at $P<10^{-5}$, whereas those treated with EM were greater than the control at $P>0.1$ (79.6 m) and $P>0.01$ (81 m), respectively (one-way analysis of covariance (ANCOVA)).

$n=46$) (Supplementary Figure S3). At Gotland Deep, July 2011, anammox activity was not detected in incubations from selected depths (114–124 m) as evidenced from the absence of significant $^{29}N_2$ accumulation over time (Supplementary Figure S4).

A potential nitrification rate of 62 nmol l^{-1} per day was also measured in samples taken from a moderately sulfidic depth (3 µmol l^{-1} H_2S, 124 m), at the Gotland Deep in July 2011 (Figure 3). In the hypoxic zone of the Gotland Deep in June 2012 (Figure 5a), potential nitrification rates of 0.2–4.3 nmol l^{-1} per day were measured in depths from 82 to 87 m, close to a narrow band of anoxic water enriched in H_2S and NH_4^+ at 94 m.

Impact of domain-specific inhibitors on nitrification activity

In samples from two depths of the Landsort Deep (79.6 and 81 m) collected in June 2012 and treated with the archaeal inhibitor GC_7, the potential nitrification rate was significantly reduced (one-way analysis of covariance, $P<10^{-5}$) compared with the untreated controls (Figure 4b). Nitrification was most strongly inhibited within the first 6 h (83–86%) at both depths, with the effect tailing off with longer incubation times. In contrast, nitrification activity in samples treated with the bacterial inhibitor erythromycin either did not decrease or increased slightly (one-way analysis of covariance, $P<0.2$ and $P<0.02$, respectively) relative to the untreated controls. In water from the Gotland Deep in June 2012, when a sulfidic intrusion was observed and potential nitrification rates were substantially lower overall, the addition of GC_7 resulted in the complete elimination of nitrification (Figure 5b) as evidenced from negative regression slopes of $NO_2^- + NO_3^-$ production significantly different from the untreated controls (one-way analysis of covariance, 82.1 m: $P<0.4$; 84.5 m: $P<10^{-4}$; 87.0 m: $P<0.05$).

Sulfide-spiking experiment

Sulfide was added to water taken from the nitrification maximum (110 m depth) of the

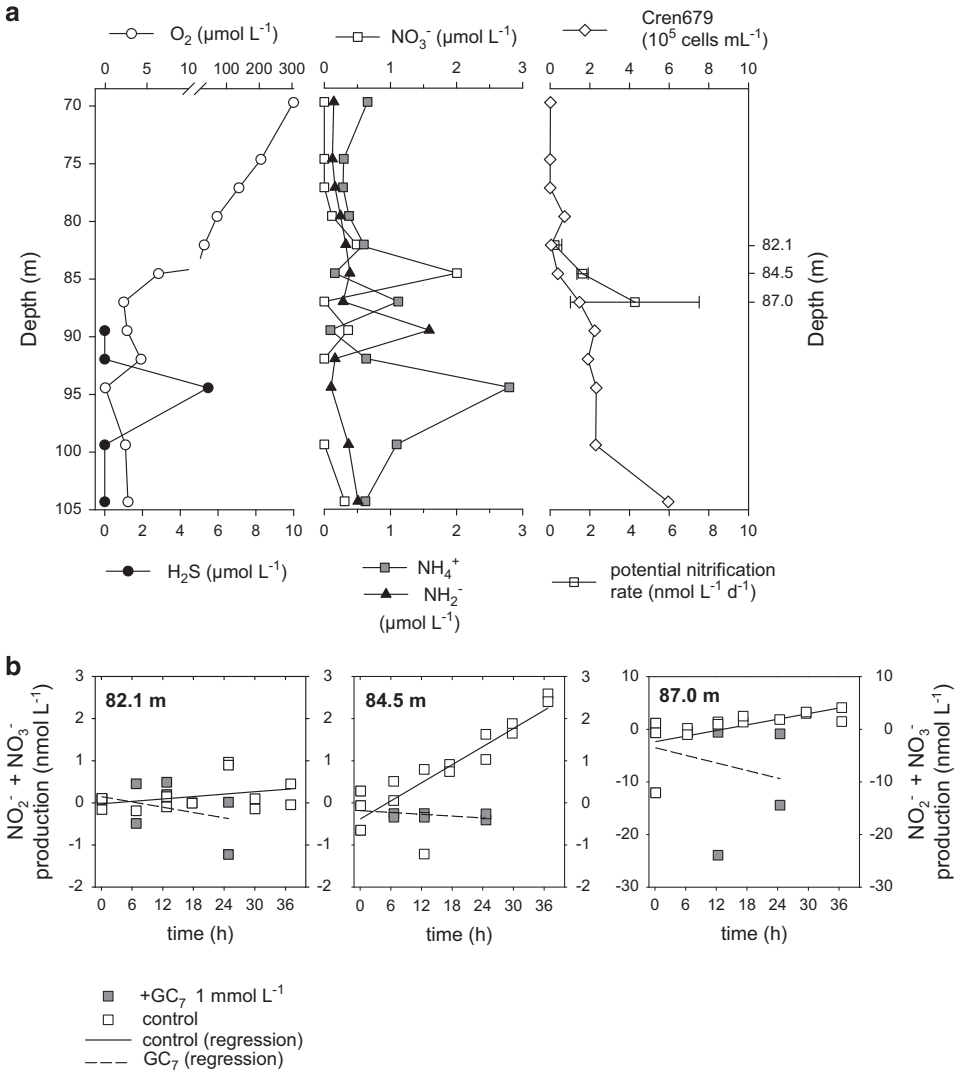

Figure 5 (**a**) Physicochemical profile of the Gotland Deep hypoxic zone in June 2012 during a sulfidic intrusion. Error bars show the s.e. of the slope of excess ^{29}N. Nitrification was measured at three depths above the intrusion and in the presence or absence of the archaeal inhibitor GC_7. (**b**) Time course of NO_2^- and NO_3^- production during nitrification at three depths in the Gotland Deep in 2012.

Gotland Deep in July 2011 (Figure 3) to examine its effect on nitrification. Between sulfide addition and the distribution of samples into the Exetainer vials, sulfide losses were minimal (Supplementary Figure S5); nonetheless, over time sulfide concentrations decreased in both the bottles and the Exetainers (Supplementary Figure S5A vs B). Nitrification was significantly inhibited (one-way analysis of variance, $P<10^{-5}$) by the addition of 4.1 µmol l^{-1} H_2S (Table 1). A higher concentration of H_2S (8.3 µmol l^{-1} H_2S) further decreased potential nitrification rates compared with the untreated control, and 16.6 µmol l^{-1} H_2S completely inactivated nitrification activity. Cell numbers of *Sulfurimonas* sp. subgroup GD17 increased by up to 25-fold during the incubations, especially in those with H_2S addition (Supplementary Figure S6), where CO_2 fixation rates were also higher than in the untreated control (Supplementary Figure S7).

Table 1 Effect of H_2S addition on nitrification activity (nmol l^{-1} per day) in water taken from the Gotland Deep nitrification maximum

	Spikes of H_2S (µmol l^{-1})							
	0 (control)		*4.1*		*8.3*	*16.6*		
0–24 h	132	128	2 (1.5%)	15 (11.5%)	0	0	0	0
24–48 h	190	115	54 (35.4%)	13 (8.5%)	0	0	0	0
48–72 h	246	174	28 (13.3%)	24 (11.4%)	8 (3.8%)	0	0	0

Values in parentheses are the percentage of activity remaining after H_2S spiking relative to mean nitrification in the untreated control samples. Results from each of the duplicate incubations are shown. Nitrification was determined within 0–24, 24–48 and 48–72 h after the addition of sulfide.

Discussion

Our study shows that *Thaumarchaeota* are the main catalyzers of ammonia oxidation in Baltic Sea redox

gradients as revealed by domain-specific inhibition experiments. This habitat, harboring one dominating group of *Thaumarchaeota* (Labrenz *et al.*, 2010), is periodically exposed to sulfidic intrusions from subjacent waters into the nitrification zone. Addressing this ecological feature via a sulfide-spiking experiment, we found that nitrification persisted after sulfide pulses of *in situ*-like concentrations, suggesting a tolerance against sulfide by AOA assemblages in the Baltic Deeps.

Nitrification activity, CO_2 fixation and the distribution of Thaumarchaeota

In hypoxic areas of the Baltic deep basins, nitrification was detected at varying rates during several seasons, covering more than 1 year. Low-oxygen waters above the onset of sulfide consistently hosted maximal thaumarchaeotal abundances (Supplementary Figure S2), showing that AOA are a stable component of Baltic Sea redox gradients as presumed by Labrenz *et al.* (2010). The highest potential nitrification rates (maximum 884 nmol l^{-1} per day) along with abundant *Thaumarchaeota* (4.3×10^5 cells per ml) were detected at Bornholm Deep in November 2011. This site showed an ammonium gradient with concentrations higher than those detected in the other deeps. In general, our data confirm the temporal and spatial variability in potential nitrification rates reported by Hietanen *et al.* (2012) but also underline the persistence of nitrification in the Baltic Sea over time. Anaerobic oxidation of ammonium (anammox), however, was not detected in selected depths of the Gotland Deep (Supplementary Figure S4), and this is in accordance to detectable anammox activity only after the inflow of oxygen-rich water into the Baltic Sea (Hannig *et al.*, 2007). Thus, ammonia oxidation occurred probably mostly aerobically in Baltic Sea redox gradients.

The abundance of marine group I *Thaumarchaeota* in the Black Sea hypoxic zone is one order of magnitude lower, at 4.3×10^4 cells per ml (Lam *et al.*, 2007), than in the Baltic Sea, concurrent with the lower nitrification rates of 5–50 nmol l^{-1} per day (Ward and Kilpatrick, 1991) although potential nitrification rates—as in our study—may be higher than the actual *in situ* nitrification rates (Horak *et al.*, 2013). Similarly, in the Cariaco Basin, *Thaumarchaeota* comprise maximally 9% (Cren537 probe) (Lin *et al.*, 2006) to 13% (Cren679 probe, maximum 1.5×10^4 cells per ml; Gordon Taylor, personal communication) of the total cell counts but also prevail around the redox transition zone. These comparisons show that pelagic Baltic redox gradients offer particularly favorable conditions for both high nitrification activities and high AOA abundances. Apparently, AOA are the best-adapted ammonia oxidizers to the predominant sulfidic conditions in this system, as anammox occurs only during sulfide-free periods (Hannig *et al.*, 2007) and AOB may be more susceptible to sulfide (Joye and Hollibaugh, 1995), making AOA the main utilizers of ammonium. In contrast, in the Black Sea, anammox bacteria obtain nitrite from aerobic ammonia oxidizers and compete with them for ammonium (Lam *et al.*, 2007), whereas in Baltic redox gradients ammonium may exclusively be used by AOA to produce the nitrite that subsequently fuels autotrophic denitrification. The dominance and high cell numbers of AOA in the Baltic Sea basins might be caused by the close proximity of the nitrification zone to sulfidic waters.

The energy provided by aerobic ammonia oxidation serves to carry out CO_2 fixation for growth in autotrophic *Thaumarchaeota*. In our measurements, potential nitrification rates usually were one order of magnitude higher than the corresponding CO_2 fixation activities. This is in line with the 10:1 ratio of N oxidized per C incorporated reported for nitrifiers (Tijhuis *et al.*, 1993; Middelburg, 2011) and suggests that CO_2 fixation in Baltic Sea hypoxic zones is coupled mainly to nitrification by AOA. In general, the rates of CO_2 dark fixation in hypoxic waters were notably lower than those in the upper sulfidic zone, a difference attributed to the activity in the latter of high numbers of chemoautotrophic denitrifiers of the *Sulfurimonas* sp. subgroup GD17 (Grote *et al.*, 2007, 2008; Jost *et al.*, 2008).

Thaumarchaeota *contribute substantially to nitrification*

Our archaea-specific inhibition experiments provide evidence of the major contributions of archaea to nitrification. The Landsort Deep exhibited high potential nitrification rates that were inhibited up to 83–86% by GC_7 (Figure 4); at the Gotland Deep, nitrification rates were comparably low and completely inhibited by the archaea-specific inhibitor (Figure 5). The different inhibition levels at the two sites may be because of their different hydrological histories: Landsort Deep showed a stable stratification, whereas at Gotland Deep, a sulfidic intrusion (Figure 5) may have impaired activities of ammonia oxidizers before sampling, resulting in low potential nitrification rates, and cells being more susceptible to the biosynthesis inhibitor GC_7. In addition, the tailing off of inhibition by GC_7 at Landsort Deep may relate to the degradation of the inhibitor by amine oxidases (Park *et al.*, 1994), such that inhibition was reversible (Jansson *et al.*, 2000), or by the recovery of incompletely inhibited cells.

Taken together, inhibition of archaeal nitrification by GC_7 and the correlation between thaumarchaeotal abundance and nitrification activity in Baltic Sea depth profiles provide complementary evidence that archaea are the main mediators of ammonium oxidation in these waters. Given the low diversity of the archaeal community (Labrenz *et al.*, 2010), the determined ammonia oxidation activities likely rely

on only one well-adapted dominant subcluster, related to *Ca.* N. maritimus.

Impact of sulfide on ammonia-oxidizing Thaumarchaeota

Episodic sulfidic plumes have previously been recognized to occur in oxygen minimum zones, for example, off Peru (Schunck *et al.*, 2013) and the Namibian Shelf (Lavik *et al.*, 2009). In Baltic Sea redox gradients, periodic intrusions of different water layers result in the juxtapositioning of sulfidic, anoxic and low-oxygen waters that undergo temporal changes (Hannig *et al.*, 2007; Bruckner *et al.*, 2013). The sulfidic part of the redox gradient does not permit oxygen-driven ammonia oxidation and, correspondingly, *amoA* expression is minimal here (Labrenz *et al.*, 2010). Instead, at these depths *Thaumarchaeota* may reside inactive. Notably, the detected nitrification potential, likely because of small oxygen additions during sampling (De Brabandere *et al.*, 2012), of 62 nmol l^{-1} per day at a sulfidic depth (Figure 3) suggests that AOA persist during sulfidic conditions and quickly become active as soon as oxygen is again available. Hietanen *et al.* (2012) pointed out that a nitrification potential might extend into deeper layers, even into the sulfidic waters. Physiological adaptations, for example, in cell membrane composition, may enable *Thaumarchaeota* to tolerate the sulfide pulses encountered in the nitrification zone or sulfide accumulation in these waters. This would enable sulfide-tolerant *Thaumarchaeota* to also persist in sulfidic waters and carry out ammonia oxidation after ventilation of sulfidic deep waters caused by major inflow events, thereby sustaining an ecologically important N cycle function under dynamic conditions.

In line with that, our sulfide-spiking experiment showed that nitrification activities in Baltic Sea redox gradients, dominated by *Thaumarchaeota*, are not completely terminated at *in situ*-like concentrations of hydrogen sulfide (Table 1), consistent with the observation from water-column profiles that nitrification is still active at low sulfide concentrations (<4 μmol l^{-1}) (Supplementary Table S1 and Hietanen *et al.*, 2012). Recovery of nitrification after spiking with time was indicated (Table 1) but longer time intervals between sulfide spike and nitrification measurement may show a significant recovery. Added sulfide persisted up to 24 h (Supplementary Figure S5), impairing microbial activities directly at addition, and then declined during the incubation, most likely as a result of biotic oxidation by denitrifiers (Grote *et al.*, 2008) rather than chemical. Sulfide-oxidizing chemolithoautotrophic denitrifiers of the *Sulfurimonas* sp. subgroup GD17 comprise stable (Grote *et al.*, 2007; Labrenz *et al.*, 2007) and active (Grote *et al.*, 2008; Glaubitz *et al.*, 2009) populations in Baltic Sea redox gradients and were stimulated during incubation as indicated by their strongly increased cell numbers (Supplementary Figure S6) and elevated CO_2 fixation activities (Supplementary Figure S7). Thus, potential consumption of $^{15}NO_2^-$ or $^{15}NO_3^-$ produced by nitrification may even have led to underestimated or undetectable nitrification at higher sulfide concentrations. Presuming stoichiometry of chemolithoautotrophic denitrification by *Sulfurimonas gotlandica* str. GD1 as calculated by Bruckner *et al.* (2013), added sulfide would have been sufficient to denitrify the NO_3^- pool. Given the impairment of sulfide pulses on nitrification activity, the supply of oxidized nitrogen in redox gradients by nitrification is also indirectly suppressed by intermittent sulfidic water masses being toxic to nitrifiers.

On the ecosystem level, our findings emphasize the effect of sulfide on nitrifiers in regulating nitrogen budgets in the Baltic Sea and they are in line with those of Joye and Hollibaugh (1995), who showed that the inhibition of nitrification in sediments by sulfide limits nitrogen loss processes. Different histories, that is, lateral intrusions vs small-scale vertical mixing with sulfidic waters, may therefore explain the varying nitrification rates reported in this and previous (Hietanen *et al.*, 2012) studies. For example, at the Bornholm Deep, sulfidic conditions occur less frequently such that nitrification is more stable, proceeding at higher rates and fostering high AOA abundance. Moreover, the portion of *Thaumarchaeota* residing in sulfidic waters provides a high potential for nitrification upon oxygenation and may therefore represent a stable and important component within the N cycle in the Baltic Sea, particularly in light of the dynamic perturbations and lateral intrusions that characterize these waters.

Future studies may investigate how nitrification is potentially initiated in sulfidic water after oxygen spikes or ventilation. Sulfidic pulses may act as a 'switch' by pausing nitrification and stimulating denitrification—after reoxygenation, nitrification may quickly restart again. Such process fluctuations and the coupling between nitrification and denitrification could be a major force that drives N removal in the Baltic and may represent an intriguing mechanism involved in ecosystem functioning. It is furthermore worthwhile to study whether *Thaumarchaeota* in other oxygen-depleted systems respond similarly to sulfidic mixing. The role of *Thaumarchaeota* in sulfidic habitats is not extensively clarified at present and asks for elucidation of the physiological mechanisms that allow for tolerance of these conditions.

Conclusions

Nitrification in Baltic Sea redox gradients was detected as a persistent process together with the presence of *Thaumarchaeota*. The correlation between potential nitrification rates

and thaumarchaeotal abundance and the results of our inhibition experiments support the conclusion that AOA are the main drivers of this process. In a sulfide-spiking experiment with environmental samples, nitrification proved to be robust against lower *in situ*-like sulfide concentrations, as would occur upon mixing with sulfidic waters. Our study emphasizes the role of *Thaumarchaeota* for N cycling in the Baltic Sea and shows that the supply of oxidized N compounds from aerobic ammonium oxidation is mainly dependent on *Thaumarchaeota* that, in the Baltic Sea, are represented by one abundant phylogenetic group. Our results suggest that the dominance and high abundance of AOA in this ecosystem result from a tolerance of the cells to periodic exposures to sulfidic waters and the close proximity of the nitrification zone to sulfidic water layers. It appears worthwhile to examine whether this robustness of archaeal nitrification against sulfide can also be detected among other globally distributed AOA or whether it is an exclusive feature of the Baltic Sea thaumarchaeotal assemblages.

Conflict of Interest

The authors declare no conflict of interest.

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