

Crenarchaeol tracks winter blooms of ammonia-oxidizing Thaumarchaeota in the coastal North Sea

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Abstract

We followed the abundance and distribution of ammonia-oxidizing Archaea (AOA) in the North Sea from April 2003 to February 2005 and from October 2007 to March 2008 by quantification of archaeal genes and core glycerol dibiphytanyl glycerol tetraether (GDGT) membrane lipids in suspended particulate matter, to determine whether their abundance in the North Sea is seasonal. GDGT and gene abundance increased during winters and was low during the summer. Crenarchaeol—a GDGT specific to AOA—was a major fraction of the GDGTs and varied in concert with AOA gene abundance, indicating that AOA are the predominant source of crenarchaeol. The presence of crenarchaeol-based intact polar lipids (IPLs) confirmed that the GDGTs recovered were derived from living AOA, as IPLs are rapidly degraded upon cell senescence and thus their occurrence represents living biomass more robustly than their fossil (i.e., core GDGT) counterparts. Dark incubations of North Sea water sampled during the 2007–2008 seasonal cycle with ^{13}C -labeled bicarbonate revealed incorporation of inorganic carbon into IPL-derived GDGTs, directly showing autotrophic production of Thaumarchaeota biomass during the winter. Inhibition of ^{13}C uptake by nitrification inhibitors confirmed that ammonia oxidation was the main source of energy for carbon fixation. Winter blooms of planktonic AOA in the North Sea were recurrent and predictable, occurring annually between November and February, emphasizing the potential importance of AOA in nitrogen cycling in the North Sea.

The ubiquity of marine Archaea throughout the global seas and oceans has become well-established knowledge over the last two decades (DeLong 2003). From this microbial domain, 16S ribosomal ribonucleic acid (rRNA) gene sequences representative of two major phyla—the Crenarchaeota and Euryarchaeota—are now routinely recovered from temperate marine waters. A particular association has been made between the occurrence of Group I Crenarchaeota, which are now believed to represent the separate phylum Thaumarchaeota (Brochier-Armanet et al. 2008; Spang et al. 2010), and genes coding for the alpha subunit of archaeal ammonia monooxygenase (*amoA*) (the enzyme responsible for the first step of ammonia oxidation), suggesting that these Thaumarchaeota are predominantly ammonia oxidizers (Francis et al. 2005) and may play a significant role in marine nitrification (Wuchter et al. 2006). Thus far, eight thaumarchaeotal (enrichment) cultures have been characterized. These include *Cenarchaeum symbiosum* (Hallam et al. 2006b), *Nitrosopumilus maritimus* (Könneke et al. 2005), “*Candidatus Nitrososphaera gargensis*” (Hatzenpichler et al. 2008), “*Candidatus Nitrosocaldus yellowstonii*” (de la Torre et al. 2008), “*Candidatus Nitrosoarchaeum limnia*” (Blainey et al. 2011), *Nitrososphaera viennensis* (Tourna et al. 2011), and two enrichment cultures from marine sediments (Park et al. 2010), all of which oxidize ammonia and fix bicarbonate. In contrast to the Thaumarchaeota, metabolic functions of marine mesophilic Euryarchaeota remain more enigmatic, and with no currently cultivated representatives to study in physiological detail, their primary biogeochemical functions in the marine environment remain largely unknown.

The cell membrane lipids of Thaumarchaeota consist mainly of glycerol dialkyl glycerol tetraethers (GDGTs) (Fig. 1). Quantification of GDGTs is being increasingly used in addition to conventional molecular techniques in microbial ecology studies of Thaumarchaeota (Sinninghe Damsté et al. 2002a; Pitcher et al. 2009b). Crenarchaeol, a GDGT with a cyclohexane moiety in addition to four cyclopentane moieties, was identified in *Cenarchaeum symbiosum*, a member of the ammonia-oxidizing Archaea (AOA) that lives in symbiosis with the marine sponge *Axinella mexicana* (Preston et al. 1996; Sinninghe Damsté et al. 2002b). Since then, crenarchaeol has been found in nitrifying environments where putative AOA exist (Leininger et al. 2006; Coolen et al. 2007; Pitcher et al. 2009b), and its synthesis by numerous AOA enrichments has been confirmed (Schouten et al. 2008; Pitcher et al. 2010, 2011). This, coupled to the apparent absence of crenarchaeol in (hyper-)thermophilic Crenarchaeota, suggests that crenarchaeol may be a specific biomarker for AOA.

GDGTs as they exist in a viable cell each contain a covalently bound polar headgroup (e.g., Fig. 1). Upon cell senescence, these headgroups are thought to be rapidly lost leaving behind the comparatively recalcitrant core GDGTs, which largely represent the fossilized remains of formerly living cells. Intact polar lipids (IPLs) are labile in comparison and likely represent the presence of (recently) living cells. Cultivated AOA are known to synthesize GDGTs with both sugar and phosphate-based polar headgroups (Schouten et al. 2008; Pitcher et al. 2011), and recently we developed a selected reaction monitoring (SRM) high-performance liquid chromatography–mass spectrometry (HPLC-MS) method to screen for a number of these crenarchaeol-based IPLs directly as a more sensitive method for determining the presence of viable AOA (Pitcher et al. in press).

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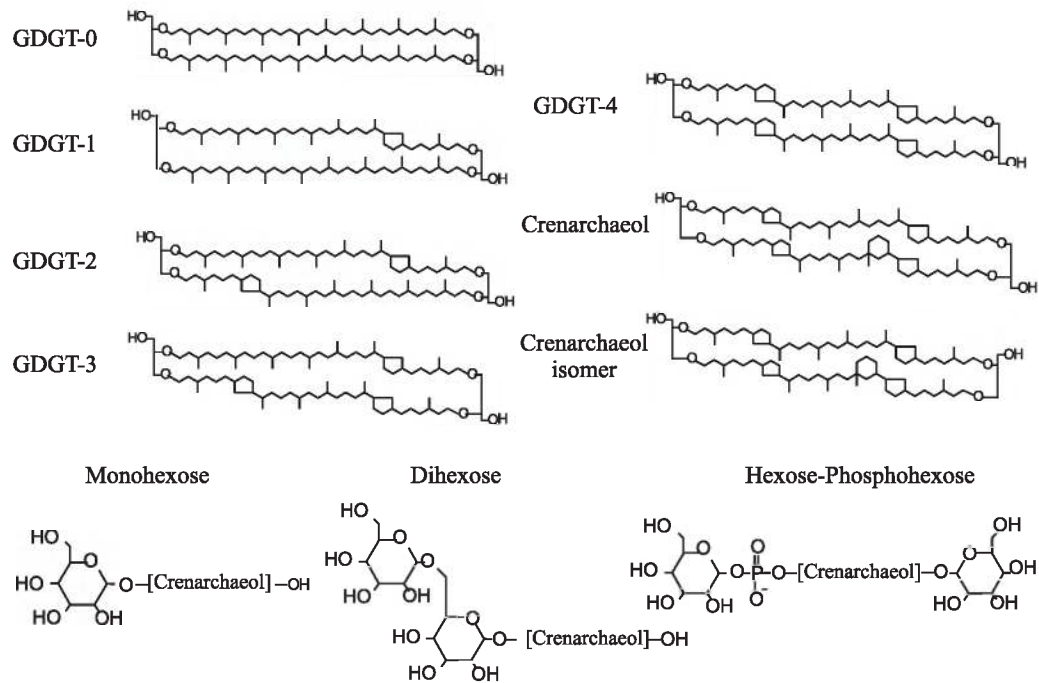


Fig. 1. Structures of GDGTs analyzed in this study. Structures of crenarchaeol-based IPLs analyzed by selected reaction monitoring are shown, with the exception of a monohexose-crenarchaeol + 180-Da headgroup. The structure of the 180-Da moiety remains unknown. Cren' = crenarchaeol regioisomer.

The first direct evidence for autotrophy by marine Thaumarchaeota came from incorporation of ^{13}C -labeled bicarbonate into GDGTs during dark incubation of water sampled from the North Sea (Wuchter et al. 2003). Subsequent analyses of thaumarchaeotal genes (both 16S rRNA and *amoA*) (Wuchter et al. 2006) showed dramatic increases over the winter of 2002–2003, suggesting a particular adaptation for growth during the winter months. Enrichment of a North Sea marine thaumarchaeote confirmed that those Thaumarchaeota were indeed AOA (Wuchter et al. 2006).

Until now it has remained unclear if North Sea AOA exhibit strong seasonal patterns, and how productive they are during times of elevated abundance. Furthermore, since only core GDGTs were analyzed it is unclear if they are all derived from living cells. Therefore, we have expanded upon the data set of Wuchter et al. (2006) by sampling suspended particulate matter (SPM) from the North Sea for an additional 3 yr (2003–2005 and 2007–2008) to determine if and when “blooms” of AOA occur, and to assess the utility of crenarchaeol as a tracer for active AOA in this dynamic coastal system. We have quantified the abundance of Group 1 Thaumarchaeota 16S rRNA genes and *amoA* genes, in addition to archaeal GDGTs including crenarchaeol. Core and IPL-derived GDGTs recovered from SPM sampled during October 2007 to April 2008 were quantified separately and compared with distribution of crenarchaeol-based IPLs analyzed directly via SRM. Finally, water sampled from the North Sea during 2007–2008 was incubated with ^{13}C -labeled bicarbonate, with and without addition of nitrification inhibitors, to determine

the inorganic carbon-fixation activity of Thaumarchaeota and its relation to nitrification.

Methods

Study site and sampling—The sampling site is situated at the western entrance of the North Sea into the Wadden Sea at the island of Texel (53°00'25"N, 4°78'27"E). With each incoming tide, water from the coastal North Sea moves as far as 25 km into the Wadden Sea (Postma 1954). At high tide, water collected at the NIOZ Royal Netherlands Institute for Sea Research (NIOZ) jetty represents Dutch coastal North Sea waters since the estuarine influence is minimal. Strong tidal currents assure that the water is vertically mixed. Therefore, surface water samples taken during high tide are fully oxygenated and representative of the entire water column.

Water samples were taken for desoxyribo nucleic acid (DNA) and lipid analyses from April 2003 to February 2005, and again from October 2007 to April 2008. For DNA analysis, measured volumes (ca. 1 liter) of water were filtered through 0.2- μm -pore-size polycarbonate filters (Schleicher and Schuell) were used during 2003–2005, and Millipore filters (142-mm filter diameter) were used during 2007–2008 and stored at -80°C until extraction. For lipid analyses, a measured volume (ca. 20 liters) of water was filtered sequentially through ashed 3- μm - and 0.7- μm -pore-size glass fiber filters (GFF, Pall, 142-mm filter diameter). GFF filters were stored at -20°C until extraction. Temperature, salinity, and inorganic nutrients were measured weekly as part of a long-term monitoring program of

the NIOZ. It should be noted that frequency and timing of DNA, lipid, and nutrient sampling differed.

Extraction and quantification of total DNA—Six milliliters of extraction buffer (10 mmol L⁻¹ tris(hydroxymethyl)aminomethane HCl, 25 mmol L⁻¹ ethylenediaminetetraacetic acid, 1 vol% sodium dodecyl sulfate, 100 mmol L⁻¹ NaCl) and 0.1 mL of zirconium beads were added to the filters and total DNA was extracted with standard phenol, phenol-chloroform-isoamyl alcohol mixture, and chloroform, and precipitated with ice-cold ethanol (Sambrook et al. 1989). The DNA pellet was redissolved in ultrapure DNA and Dnase-free sterile water (Sigma). From each of the total DNA extracts a subsample was subjected to agarose gel electrophoresis to determine the quality of the extracted DNA.

Real-time quantitative polymerase chain reaction (Q-PCR)—Q-PCR was performed in an iCycler system (BioRad). To quantify the 16S rRNA and *amoA* gene abundance, PCR conditions and primers were used as described in Wuchter et al. (2006). Accumulation of amplified rRNA genes was followed by the increase in fluorescence due to the binding of the fluorescent dye SYBRGreen (Molecular Probes). Reaction mixtures (20 μ L) contained 1 unit of Picomaxx™ High Fidelity DNA polymerase, 2 μ L of 10 \times Picomaxx PCR buffer (both Stratagene), 200 μ mol L⁻¹ of each deoxyribonucleotide triphosphate, 20 μ g of bovine serum albumin, 0.2 μ mol L⁻¹ of primers, 50,000 \times diluted SYBRGreen, 3 mmol L⁻¹ of MgCl₂, and ultrapure sterile water (Sigma). Known amounts of template DNA from each sample were added to each Q-PCR reaction. Quantitative calibration of the samples was achieved using a dilution series containing known gene abundance of purified standards derived from cloned genomic DNA of an AOA previously enriched from the North Sea (Wuchter et al. 2006) to create standard curves ranging from 1 to 10⁸ gene(s) per reaction.

Core GDGT extraction of SPM—The GFF filters were freeze-dried and cut into small pieces with sterile scissors before being ultrasonically extracted four times using dichloromethane (DCM) and methanol (MeOH) (1:1, v:v). The total lipid extracts were eluted over an activated Al₂O₃ column by eluting with MeOH and DCM (1:1, v:v) to obtain a fraction enriched in core GDGTs. Solvent was removed from the eluent under a stream of nitrogen (N₂) and the residue was dissolved by sonication (5 min) in hexane:propanol (99:1, v:v). The resulting suspension was filtered through a 0.45- μ m-pore-size, 4-mm-diameter Teflon filter prior to injection. The GDGTs were analyzed by HPLC-MS with atmospheric pressure chemical ionization (APCI) using conditions modified from Hopmans et al. (2000). Analyses were performed using an HP (Palo-Alto) 1100 series HPLC-MS equipped with an auto-injector and Chemstation chromatography manager software. Separation was achieved on a Prevail Cyano column (2.1 \times 150 mm, 3 μ m; Alltech), maintained at 30°C. GDGTs were eluted isocratically with 99% A and 1% B for 5 min, followed by a linear gradient to 1.8% B in 45 min, where A

= hexane and B = propanol. Flow rate was 0.2 mL min⁻¹. After each analysis, the column was cleaned by back-flushing hexane and propanol (90:10, v:v) at 0.2 mL min⁻¹ for 10 min. Detection was achieved using APCI-MS of the eluent. Conditions for APCI-MS were as follows: nebulizer pressure 4.1 \times 10⁵ Pa, vaporizer temperature 400°C, drying gas (N₂) flow 6 L min⁻¹ and 200°C, capillary voltage -3 kV, corona 5 μ A (~ 3.2 kV). GDGTs were detected by single ion monitoring of their [M + H]⁺ ions and quantified by integration of the peak areas and comparison with a standard curve of a GDGT-0 standard.

Intact polar lipid extraction of SPM—Intact polar lipids (IPLs) were extracted from SPM sampled during 2007–2008 using a modified Bligh and Dyer technique. A known volume of single-phase solvent mixture of MeOH:DCM:phosphate buffer (2:1:0.8, v:v:v) was added to the sample in a glass centrifuge tube and placed in an ultrasonic bath for 10 min. The extract and residue were separated by centrifuging at 1000 \times g for 5 min and the solvent mixture collected in a separate flask (repeated 3 \times). The DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9, v:v:v), and to induce phase separation. The extract was centrifuged at 1000 \times g for 5 min. The DCM phase was collected in a round-bottom flask and the MeOH:phosphate buffer phase was washed two additional times with DCM. The combined DCM phases were reduced under rotary vacuum and evaporated to dryness under a stream of N₂.

An aliquot of each Bligh and Dyer extract was eluted over activated silica gel (60 mesh) with three column volumes of hexane:ethyl acetate (3:1, v:v) and MeOH to obtain fractions enriched in core GDGTs and IPL-GDGTs, respectively (Pitcher et al. 2009a), termed the core lipid (CL) and IPL fractions. To each fraction a known amount of a C₄₆ internal standard was added (Huguet et al. 2006). The CL fractions were evaporated under N₂ to dryness and then analyzed with HPLC-APCI-MS using conditions described above. The IPL fraction was evaporated to dryness and then subject to acid hydrolysis by reflux in 1 mol L⁻¹ HCl for 1 h to cleave any polar headgroups, following methods described in Pitcher et al. (2009b). The resulting IPL-derived core GDGTs were analyzed with HPLC-APCI-MS using conditions described above, and quantification of GDGTs was achieved using the internal standard as described in Huguet et al. (2006).

Selected reaction monitoring of specific IPLs—An aliquot of Bligh and Dyer extract was used for direct analysis of crenarchaeol-based IPLs shown in Fig. 1. Detection was achieved by HPLC-electrospray ionization-MS-MS (Pitcher et al. in press). Separation was achieved on a LiChrospher diol column (250 \times 2.1 mm, 5- μ m particles; Alltech) maintained at 30°C. The following linear gradient was used with a flow rate of 0.2 mL min⁻¹ 100% A:0% B to 35% A:65% B over 45 min, maintained for 20 min, then back to 100% A for 20 min to re-equilibrate the column. Detection of crenarchaeol-based IPLs was achieved by using conditions, and monitoring the transitions, described in Pitcher

et al. (in press). Due to the lack of a purified standard for quantification, crenarchaeol-based IPLs were quantified as the integrated IPL peak area response per liter. This approach reveals the relative distribution of individual IPLs over time, but does not allow the comparison of different IPL absolute abundances.

Stable isotope probing of AOA—Approximately every 2 weeks from October 2007 to April 2008, 4×20 liters of North Sea water was sampled into clean Nalgene containers and incubated with ^{13}C -labeled bicarbonate in the dark at in situ temperatures for approximately 24 h to measure inorganic carbon uptake rates by marine Thaumarchaeota. Four treatments were used: one container was left as a non-treated control, while bicarbonate (300 mg of 99% ^{13}C -labeled, equivalent to an addition of ca. 9% of the total bicarbonate) was added to each of the other three containers. Nitrification inhibitors were added to two of the three ^{13}C -labeled incubations to determine the effects on AOA carbon fixation: 100 mg of nitrapyrin (2-chloro-6-(trichloromethyl)pyridine; 5 mg L^{-1}) was added to one container to inhibit ammonium oxidation by bacteria (Bédard and Knowles 1989; de Bie et al. 2002) and Thaumarchaeota (Park et al. 2010), and 21 g of sodium chlorate (NaClO_3 , 10 mmol L^{-1}) was added to another container to inhibit nitrite oxidation (Belsler and Mays 1980). After each sampling, inhibitors were added immediately and the incubations were gently aerated (bubbled with air) and given ca. 2 h to equilibrate in a dark, temperature-controlled incubation chamber prior to the addition of ^{13}C -labeled substrates. After incubation for approximately 24 h, the water was filtered over $0.7\text{-}\mu\text{m}$ GFF filters and analyzed for GDGTs. The filters were freeze-dried and extracted using a modified Bligh and Dyer technique, and core GDGT and IPL-derived GDGT fractions were generated as described above.

IPL-derived GDGTs were analyzed for ^{13}C -label incorporation by treatment with HI and LiAlH_4 to release the biphytanes (Hoefs et al. 1997). The $\delta^{13}\text{C}$ values of GDGT-derived biphytanes were measured by isotopic-ratio-monitoring (IRM)-gas chromatography-mass spectrometry (GC-MS). Compound-specific ^{13}C analyses were performed with an Agilent 6800 GC coupled to a Thermo Fisher Delta V IRM mass spectrometer. Isotope values were measured against calibrated external reference gas and performance was checked by daily injections of two internal perdeuterated *n*-alkane standards. The $\delta^{13}\text{C}$ values are reported in the standard delta notation against the Vienna Pee Dee Belemnite standard. Samples were run in at least duplicate. Incorporation of ^{13}C into the tricyclic biphytane derived from crenarchaeol (BIP-3) during incubations is expressed as $\Delta\delta^{13}\text{C}$ values (the difference in $\delta^{13}\text{C}$ values between labeled and control incubations).

Results

Seasonal dynamics in environmental parameters—Fig. 2 summarizes chemical and physical data collected from coastal North Sea water sampled at high tides continuously from 2002 to 2008. Concentrations of ammonium (NH_4^+)

were generally highest between November and January, ranging from ca. $10\text{--}13 \mu\text{mol L}^{-1}$ during winter sampling periods (gray vertical bars); however, an anomalous spike in NH_4^+ occurred during mid-May 2004, which was not observed in other seasons. Nitrite (NO_2^-) concentrations were comparatively low throughout the year ($0\text{--}3 \mu\text{mol L}^{-1}$), peaking during the winter as well. Annual declines in NH_4^+ and NO_2^- in the middle of the winter were coinciding with increases in nitrate (NO_3^-), suggesting periods of net nitrification (Wuchter et al. 2006). NO_3^- reached peak concentrations (ca. $60\text{--}90 \mu\text{mol L}^{-1}$) between January and February each year when the coastal North Sea reached its lowest temperatures (ca. $2\text{--}4^\circ\text{C}$). A subsequent steady decline in NO_3^- to ca. $0.2\text{--}0.5 \mu\text{mol L}^{-1}$ was achieved by around August of each year presumably as a result of assimilation by spring- and summer-blooming phytoplankton, while water temperatures rose to maxima of ca. $20\text{--}22^\circ\text{C}$. Annual oscillations in salinity were observed as well, with several sharp dips reaching as low as 20 during the winter, evidencing increased freshwater flux into the North Sea during these times.

Abundances of thaumarchaeotal core GDGT—Fig. 3A–C shows the abundances of core GDGTs (gray circles), crenarchaeol, GDGT-0, and GDGTs 1–3 combined, measured from February 2002 to February 2005 and from September 2007 to April 2008. Overall concentrations of all core GDGTs were low during the spring and summer months, and gradually increased starting around October–November to reach peak abundances during either January or February each year (Table 1). Subsequently, concentrations quickly decreased to near-autumn values by March–April each year. Crenarchaeol and GDGT-0 were dominant among the GDGTs, with maximum winter concentrations reaching $100\text{--}140 \text{ ng L}^{-1}$ and $150\text{--}170 \text{ ng L}^{-1}$, respectively. GDGTs 1–3 represented a minor component of the total GDGT pool, their combined abundance reaching between 20 and 30 ng L^{-1} at winter maxima. In general, the winters in which sampling occurred were typified by analogous peaks of similar concentrations (cf. Fig. 3A–C). Overall, GDGT abundance correlated well with each other ($R^2 = 0.83\text{--}0.97$).

Core and IPL-derived GDGT abundances 2007–2008—Over the winter of 2007–2008, core and IPL-derived GDGT abundances were quantified separately after Bligh and Dyer extraction with GFF filters, as opposed to conventional organic solvent methods used to extract GDGTs from SPM collected during 2002–2005. Total abundances of core and IPL-derived GDGTs over this time period were well correlated with each other ($R^2 = 0.89$), with IPL-derived GDGTs representing an average of $41 \pm 5\%$ of the total GDGT pool (i.e., core and IPL-derived GDGTs combined). Core GDGTs measured during the 2007–2008 winter season were not as abundant as previous years; however, when the abundances of core and IPL-derived GDGTs were combined (black circles; Fig. 3A–C), the GDGT concentrations were comparable from season to season. This suggests that a substantial proportion of the core GDGTs measured during 2002–2005 were derived

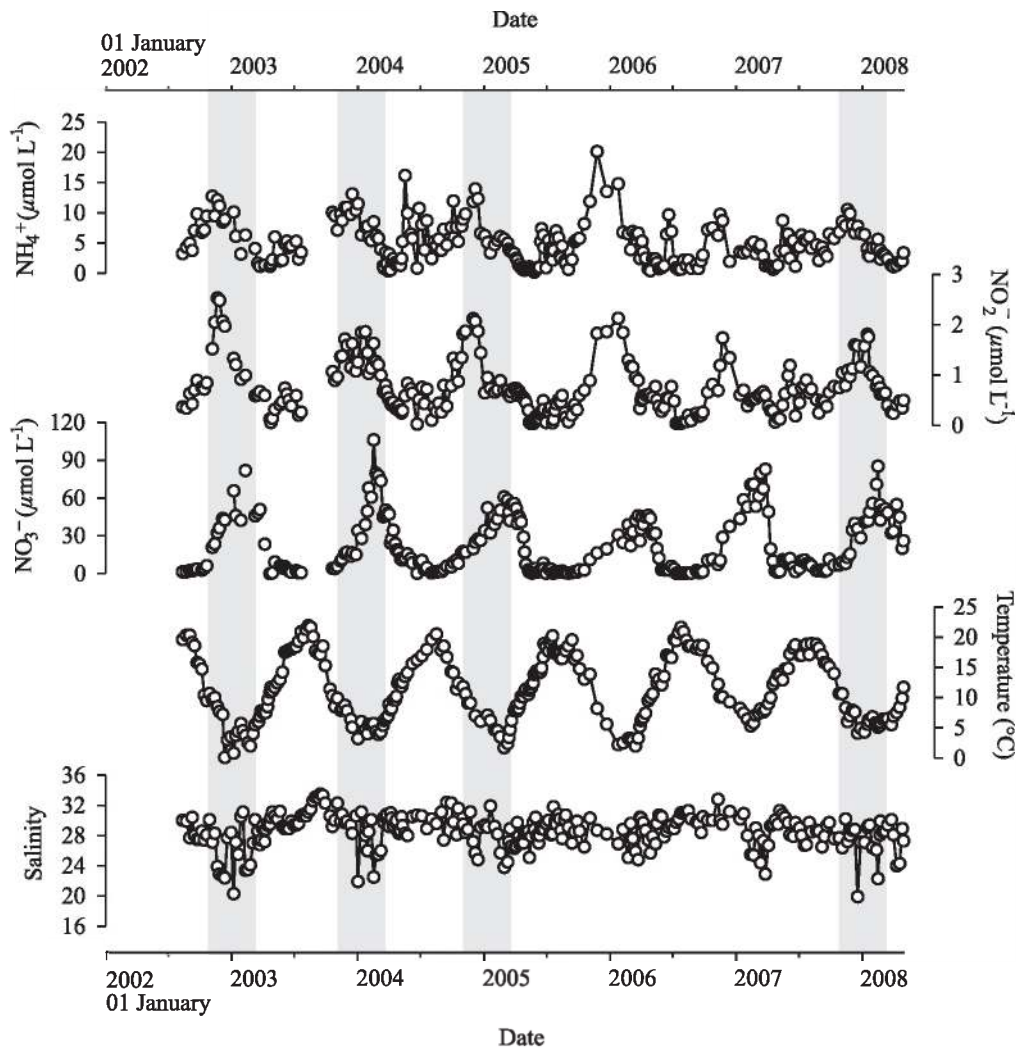


Fig. 2. Inorganic nutrient concentrations ($\mu\text{mol L}^{-1}$), ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-), temperature, and salinity measured from 2002 to 2008. Gray bars indicate winter periods characterized by high abundances of Thaumarchaeota as revealed by crenarchaeol and specific gene abundance (see Fig. 4). Note that these measurements were not performed in 2006 and 2007. Winter periods are characterized by a decline in the ammonium and increase in the nitrate concentrations.

from IPLs that were present in the SPM but lost their polar headgroups during the sample workup. Comparison of the relative GDGT distributions showed that GDGT-0 is present in slightly higher amounts in the core fraction compared to the IPL fraction ($56 \pm 6\%$ as core GDGT of total GDGTs), while for crenarchaeol the major part was present as core lipid ($65 \pm 6\%$ of total GDGTs). Minor GDGTs 1–3 were roughly equally distributed over the core and IPL fractions ($51 \pm 6\%$).

SRM of crenarchaeol-based IPLs—Using a recently developed SRM method (Pitcher et al. in press), we were able to follow the relative abundances of various crenarchaeol-based IPLs (Fig. 1) in the 2007–2008 SPM series (Fig. 4A–D). Crenarchaeol with monohexose (MH) and hexose-phosphohexose (HPH) headgroups were detected at every date sampled, in comparison to crenarchaeol with dihexose and monohexose + 180-Da (“MH + 180”) moieties which were undetectable in some SPM samples

taken near the beginning and end of the sampling period (cf. Fig. 4B,C). IPL-GDGTs with “MH + 180” headgroup have been identified in a number of enriched marine AOA (Schouten et al. 2008; Pitcher et al. 2010); however, the precise structure is still unknown. Individual and total IPL responses correlated well with indirectly quantified IPL-derived crenarchaeol (Fig. 4E) ($R^2 = 0.81\text{--}0.87$), i.e., there was no apparent offset between the two in the North Sea during this sampling period. This suggests that indirectly quantified IPL-derived crenarchaeol is indeed mostly derived from IPLs with crenarchaeol as a core lipid.

Annual variation in archaeal genes—Over the entire sampling period, the abundances of thaumarchaeotal 16S rRNA and *amoA* genes covaried generally well ($R^2 = 0.56$) with an average *amoA* : 16S rRNA ratio of 2.0. This ratio is in the range of those of natural populations of marine AOA which varies from 1 to 3 (Coolen et al. 2007; Hollibaugh et al. 2011; Pitcher et al. in press). Notable increases in gene

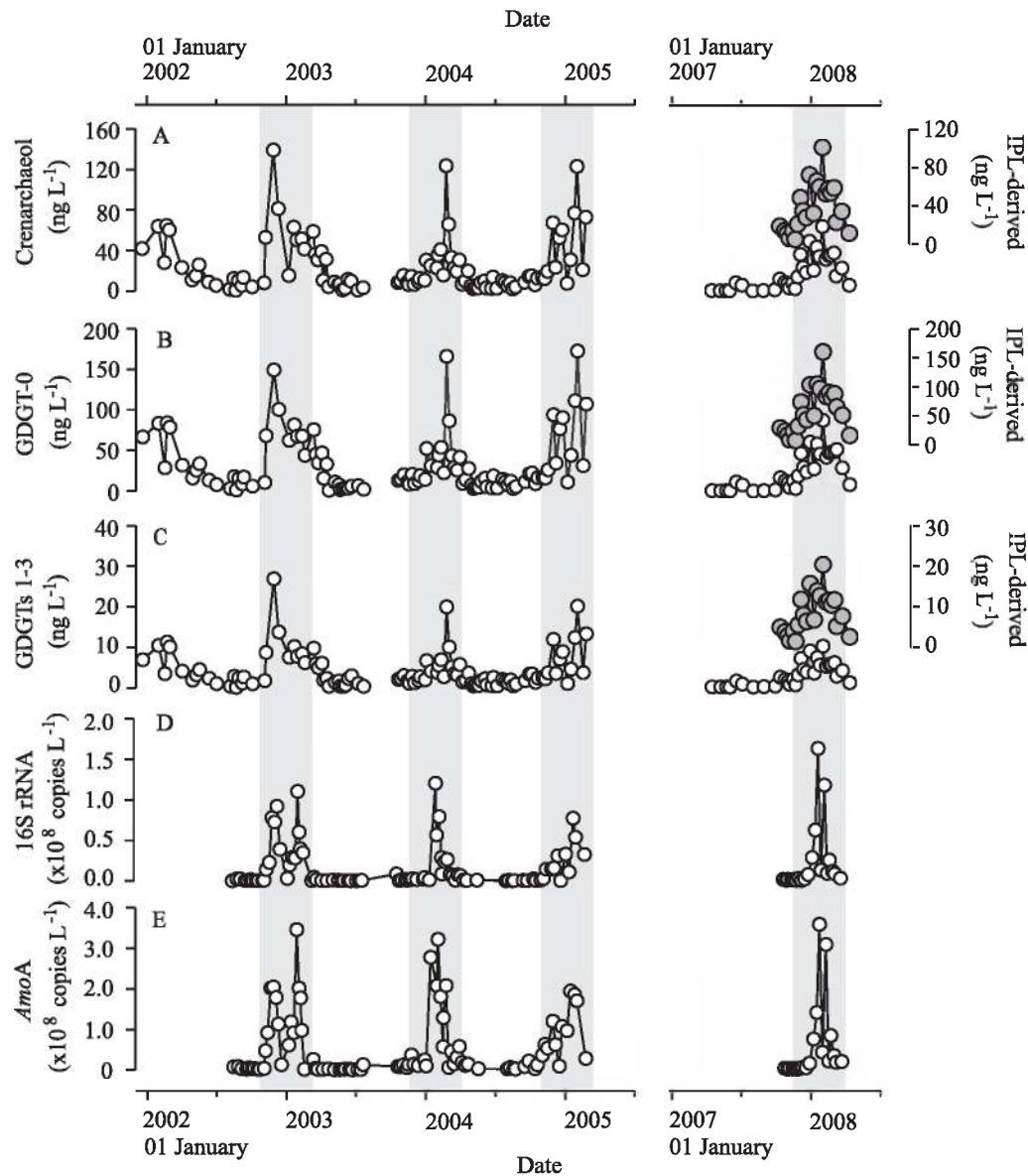


Fig. 3. Concentrations (ng L^{-1}) of core (white circles) and IPL-derived (dark gray circles) (A) crenarchaeol, (B) GDGT-0, and (C) summed GDGTs 1–3 recovered from North Sea suspended particulate matter from 2002–2005 and 2007–2008. Abundances of (D) 16S rRNA genes for Thaumarchaeota and (E) archaeal *amoA* genes peaked during the winter months (gray bars), and matched fairly well to GDGT abundances.

abundance occurred near or just prior to winter each sampling season, with thaumarchaeotal 16S rRNA and *amoA* gene abundances increasing from near detection limit to ca. 10^8 L^{-1} (Fig. 3D,E). Elevated gene abundances ($>10^7 \text{ copies L}^{-1}$) were maintained throughout much of the winter and into the early spring, although the length of this period varied from year to year, ending between the end of March to mid-April (Table 1). In the first sampling season (2002–2003), Wuchter et al. (2006) found two notable peaks in gene abundance of approximately equal magnitude; however, the rest of the sampled winter seasons showed only a single peak (Fig. 3). Despite annual variation in the duration of elevated gene abundances, annual maxima were achieved within a relatively narrow time frame (between 14

January and 2 February, with the exception of Wuchter et al. [2006], who noted an additional “early” peak in November 2002), where Thaumarchaeota and *amoA* abundances increased to 7×10^7 to 2×10^8 and $2\text{--}4 \times 10^8 \text{ genes L}^{-1}$, respectively (Table 1).

¹³C incorporation into crenarchaeol—IRM-GC-MS analysis of HI-LiAlH₄-treated IPLs showed incorporation of ¹³C into GDGT-derived biphytanes. Here we discuss only incorporation into the tricyclic biphytane derived from crenarchaeol (BIP-3), as this GDGT is the most specific to marine AOA (Sinninghe Damsté et al. 2002b). Over the nine incubations performed during the winter of 2007–2008, $\Delta\delta^{13}\text{C}$ values of BIP-3 ranged from 0‰ to 44‰

Table 1. Summary of maximum thaumarchaeotal 16S rRNA and *amoA* gene and core GDGT abundances measured during bloom periods.

Year	Duration of "bloom"		Maximum gene abundances			Maximum GDGT concentrations			
	Start	End	Date	Abundance (copies L ⁻¹)		Date	Concentration (ng L ⁻¹)		
				16S rRNA	<i>amoA</i>		Crenarchaeol	GDGT-0	GDGTs 1–3
2002–2003	07 Nov	12 Mar	21 Nov	7.8×10 ⁷	2.0×10 ⁸	28 Nov	140	150	27
			28 Jan	1.1×10 ⁸	3.5×10 ⁸	01 Jan	63	81	10
2003–2004	03 Nov	21 Apr	02 Feb	7.9×10 ⁷	3.2×10 ⁸	23 Feb	120	170	20
2004–2005	19 Oct	23 Feb	14 Jan	7.7×10 ⁷	1.9×10 ⁸	31 Jan	120	170	20
2007–2008*	27 Dec	21 Mar	22 Jan	1.6×10 ⁸	3.6×10 ⁸	30 Jan	100	160	20

* GDGT abundances are the summed total of quantified core GDGTs and IPL-derived GDGTs.

(Fig. 5), peaking on 30 January, when maximum IPL-GDGT abundances were also observed. In general, the trend in $\Delta\delta^{13}\text{C}$ of BIP-3 followed the seasonal variation in the concentration of HPH-crenarchaeol. The same trend in $\Delta\delta^{13}\text{C}$ values of BIP-3 was visible in the incubations containing the inhibitors nitrapyrin (which inhibits ammonia oxidation) and sodium chlorate (to inhibit nitrite oxidation; Belser and Mays 1980) but the absolute values were much less (Fig. 6), i.e., up to 14‰ for chlorate and up to 5‰ for nitrapyrin.

Discussion

Crenarchaeol as a marker for ammonia-oxidizing Thaumarchaeota in the North Sea—Comparison of annual increases in GDGT and gene abundances show that both occur during similar times in winter periods (Fig. 3, shaded bars). The overall good correspondence between crenarchaeol abundance and *amoA* and thaumarchaeotal 16S rRNA gene abundances showed distinct seasonal increases and decreases, suggesting that crenarchaeol is derived mainly from AOA living in the North Sea. AOA in the North Sea are closely related to *Nitrosopumilus maritimus* (Wuchter et al. 2006; Herfort et al. 2007), a marine AOA known to synthesize crenarchaeol (Schouten et al. 2008). Evidence that crenarchaeol is specific to AOA comes predominantly from culture studies (Sinninghe Damsté et al. 2002b; de la Torre et al. 2008; Schouten et al. 2008). Our field data now provide strong environmental evidence that crenarchaeol recovered from marine environments indeed predominately comes from AOA.

Additional support for the fact that core crenarchaeol (and other GDGTs) measured throughout the time series are derived from living AOA comes from the presence of crenarchaeol-based IPLs (Figs. 3D,E, 5), which are generally indicative of the presence of living cells (Harvey et al. 1986). Correlated absolute abundances of individual GDGTs in the core and IPL-derived fractions suggest they are likely sourced from the same microbial population. If the IPLs and core GDGTs were derived from different sources, i.e., living vs. fossil biomass, one could expect differences in concentration patterns as the controls on delivery of dead material through, e.g., sediment transport is different from the growth of in situ microbial populations. In addition, the good correspondence between HPH-crenarchaeol abundance, gene abundances, and ¹³C incor-

poration into IPL-derived GDGTs indicate that IPL-GDGT concentrations may serve as a proxy to indicate the abundance of Thaumarchaeota.

Comparison of core and IPL-derived GDGT distributions showed that minor GDGTs were present in roughly equal abundance in the CL and IPL fractions. GDGT-0 was present in higher relative abundance in the IPL fraction in contrast to crenarchaeol, which was present in higher relative amounts in the CL fraction. While this could indicate slightly different sources for CL and IPL GDGTs, the comparative GDGT distributions and correlated abundances of core and IPL-derived GDGTs are similar enough to conclude that they are likely derived from similar populations of AOA in the North Sea. A stable thaumarchaeotal population during winter was also evident from denaturing gradient gel electrophoresis analyses of 16S rRNA gene fragments of samples from the 2002–2003 time series (Wuchter 2006). The slight differences in GDGT distributions could be reflective of a number of factors related to the specific headgroups attached to given GDGTs. Differential degradation rates of phospho- and glycolipids (Schouten et al. 2010) could feasibly contribute to slight differences in the core and IPL-derived GDGT pools. Marine AOA synthesize GDGT-based IPLs, with GDGTs 1–4 more closely associated with glycolipids and crenarchaeol more closely associated with phospholipids (Schouten et al. 2008; Pitcher et al. 2010, 2011). Since the latter are more labile once free from the cell membrane (i.e., upon cell senescence), it stands to reason that if the majority of crenarchaeol synthesized by North Sea AOA is bound to a phosphate headgroup, relatively more may be recovered as a core GDGT. The fact that the relative abundance of GDGT-0 is higher in the IPL-derived GDGT pool may reflect that proportionally more GDGT-0 is bound predominantly to sugar-based headgroups, which is less labile.

While AOA recovered from marine water columns are usually attributed to planktonic species, we cannot exclude a contribution by sedimentary AOA in the North Sea, where high concentrations of suspended material persist in the water column (ca. 50 m deep at maximum) throughout the year and the forceful tides combined with rough weather (especially in autumn and winter) undoubtedly result in sediment resuspension from the seafloor. Three sedimentary marine AOA enrichments were recently shown to synthesize crenarchaeol and the crenarchaeol-based IPLs

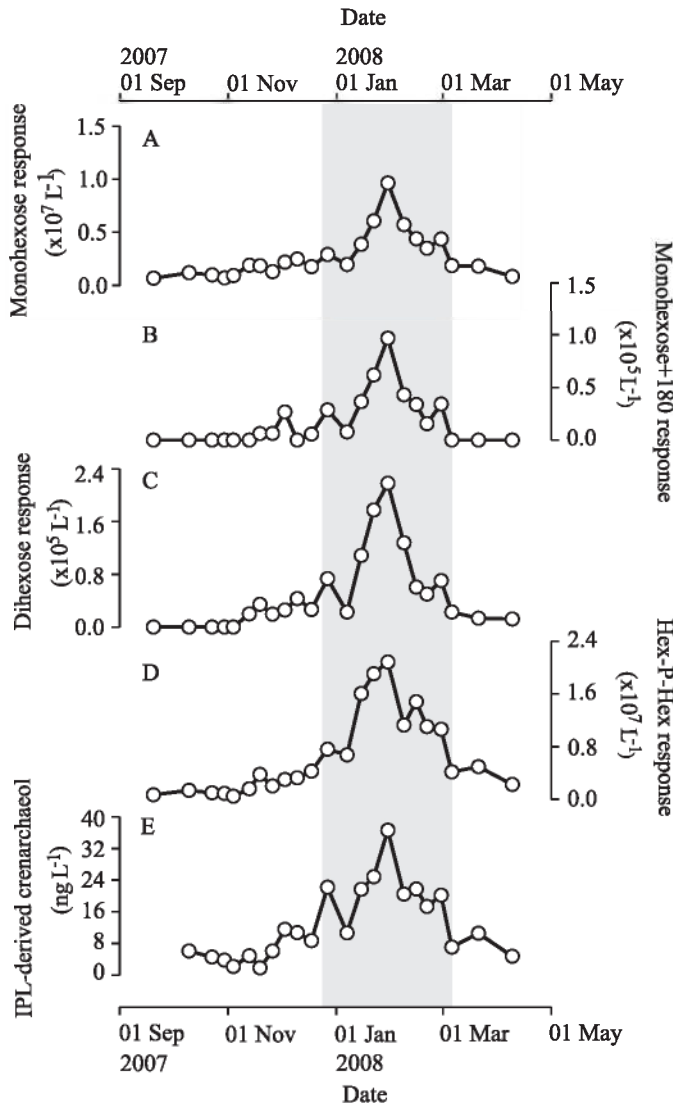


Fig. 4. Seasonal profiles (2007–2008) showing the integrated instrument response for selected reaction monitoring of crenarchaeol-based IPLs with (A) monohexose (MH), (B) monohexose + 180-Da moiety (MH + 180), (C) dihexose (DH), and (D) hexose-phosphohexose (Hex-P-Hex) headgroups, as well as (E) IPL-derived crenarchaeol concentrations.

analyzed in this study (Pitcher et al. 2011), indicating a potential sedimentary origin for the AOA lipids. However, it should be noted that the labeling experiments, performed using water from the North Sea only, showed that incorporation, and thus activity, took place at the same time as increased abundance of crenarchaeol, suggesting that it mostly represents pelagic Thaumarchaeota.

Seasonal recurrence of AOA and their role in the North Sea N cycle—Incorporation of ^{13}C from bicarbonate into IPL-derived crenarchaeol unambiguously shows that during thaumarchaeotal bloom periods at least a portion of the population is autotrophic (Fig. 5). A strong reduction of thaumarchaeal carbon fixation was observed after the addition of nitrapyrin (an inhibitor of ammonium oxida-

tion; de Bie et al. 2002), strongly indicating that the Thaumarchaeota producing crenarchaeol are likely oxidizing ammonium as an energy source for autotrophic fixation of bicarbonate (Fig. 6). Inhibition by nitrapyrin was not complete, suggesting that either (1) the concentration of nitrapyrin was not high enough to completely inhibit ammonia oxidation; (2) that some of the crenarchaeol-synthesizing Thaumarchaeota used alternative substrates for energy, while still fixing inorganic carbon; or (3) that CO_2 was taking up through anaplerotic reactions. Genomic analysis of the thaumarchaeote *Cenarchaeum symbiosum* and environmental samples showed that marine Thaumarchaeota can use other forms of reduced nitrogen as an energy source (Hallam et al. 2006a,b). Significant, albeit less than with nitrapyrin, reduction of carbon fixation by sodium chlorate (which inhibits nitrite oxidation; Belser and Mays 1980) was also observed (Fig. 6). This may suggest that NO_2^- accumulation during the incubation periods likely has affected AOA activity and that nitrite-oxidizing bacteria are essential partners in the natural environment for AOA.

The question remains why the annual peak abundance of AOA always occurs during the winter, with peak abundance occurring in a very narrower time period (Table 1). Similar observations, i.e., Thaumarchaeota peak in abundance during wintertimes, have been made for the greater North Sea (Herfort et al. 2007), waters off the Antarctic Peninsula (Murray et al. 1998), the Santa Barbara Channel in California (Massana et al. 1997), and off the Mediterranean coast of Spain (Galand et al. 2010). One obvious reason why this regular seasonal pattern occurs in these widely different regions would be the competition for ammonium which, with respect to phytoplankton, is more favorable during winter as light and temperature are unsuitable for phytoplankton growth. However, Hollibaugh et al. (2011) reported higher cell numbers of Thaumarchaeota in coastal southeastern U.S. waters in August rather than in April and December, indicating that other factors may also play a role. Experiments with *Nitrosopumilus maritimus* SCM1 suggest a high affinity for ammonium, indicating that marine AOA have a particular adaptation to low ammonium concentrations (Martens-Habbena et al. 2009). However, in situ NH_4^+ concentrations are relatively high (3–20 $\mu\text{mol L}^{-1}$) in the coastal North Sea during the winter, likely due to low uptake by phytoplankton due to light limitation and increased riverine input during periods of high precipitation. A higher contribution of ammonia-oxidizing bacteria (AOB) could be expected during the winter; however, the NH_4^+ concentrations are still low compared to the K_m values reported for some common marine AOB (Martens-Habbena et al. 2009). It is conceivable that AOB do not play an important role in North Sea ammonia oxidation. Indeed, Wuchter et al. (2006) noted much lower gene abundances of bacterial *amoA* than archaeal *amoA* in the North Sea. In any case, the highly predictable seasonality pattern of the Thaumarchaeota in the North Sea, and other regions, suggests that this group is highly optimized for a specific set of environmental conditions, such as light, temperature, and nutrients, and reflecting a low degree of functional redundancy (Furhman et al. 2006).

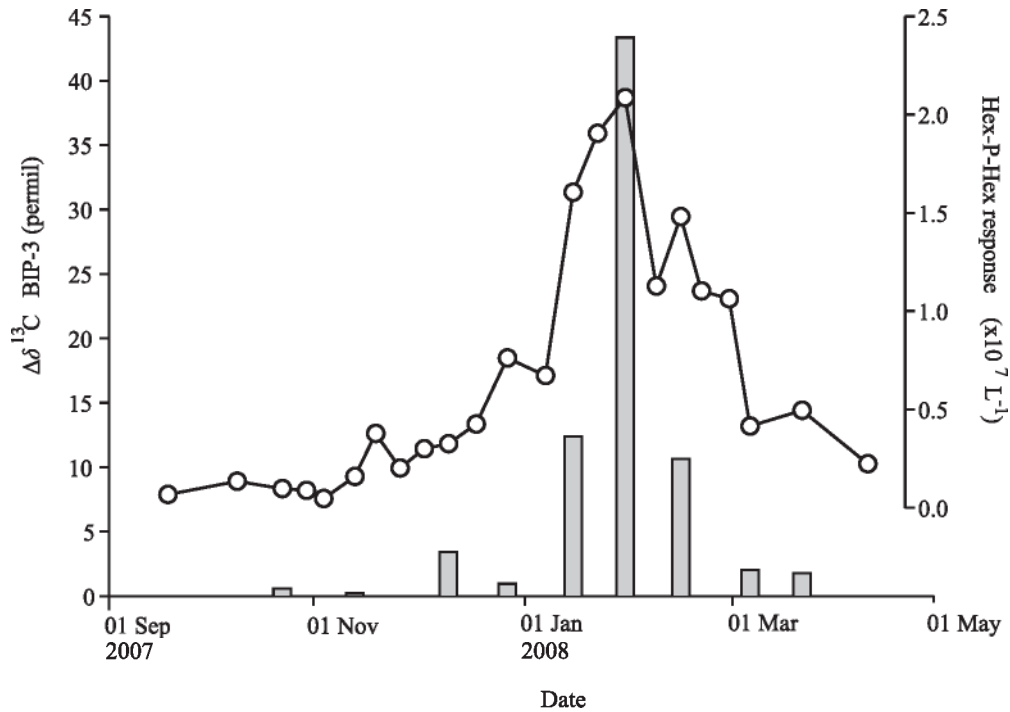


Fig. 5. ^{13}C incorporation into the crenarchaeol-derived biphytane with three cyclic moieties (BIP-3) after incubations performed during the 2007–2008 winter (gray bars). $\Delta\delta^{13}\text{C}$ values represent the difference in ^{13}C content of BIP-3 from non-labeled (control) and ^{13}C -bicarbonate-labeled incubations. Circles indicate the seasonal profile of HPH-crenarchaeol.

Patterns in the abundance of AOA genes and both core and IPL-derived GDGTs in the coastal North Sea demonstrate a pronounced seasonal effect on the ecology of AOA. In doing so, we have noted a direct, long-term

association between crenarchaeol and AOA abundance. While it is still not exactly clear to what extent certain environmental factors influence the AOA ecology in the North Sea, peak abundances during the winter months

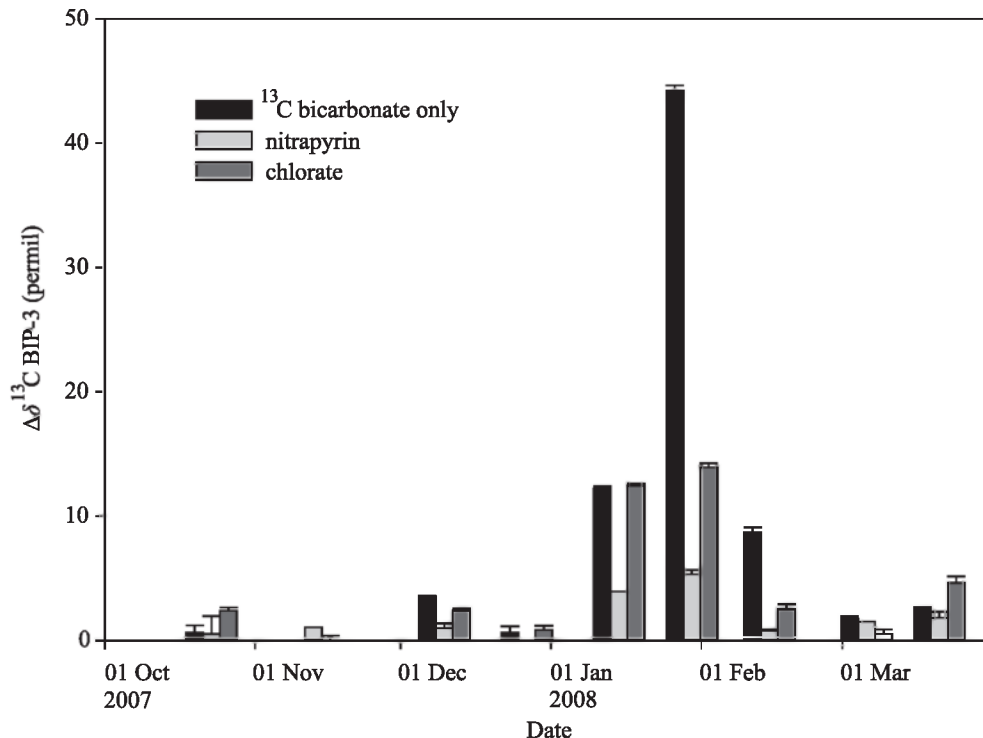


Fig. 6. $\Delta\delta^{13}\text{C}$ values of BIP-3, derived from crenarchaeol, for incubation with ^{13}C bicarbonate only, and with the inhibitors chlorate and nitrapyrin, during the 2007–2008 winter. Error bars indicate standard deviations of replicate measurements.

support previous hypotheses that light regime and competition with phytoplankton and other bacteria are important variables. For example, extensive blooms of the alga *Phaeocystis* occur every year in March or April (Phillipart et al. 2010) and these algae could compete with AOA for available ammonium. ^{13}C incorporation into crenarchaeol, which substantially decreased when ammonia oxidation was inhibited, showed that North Sea AOA are active chemoautotrophs during the winter months, peaking in CO_2 uptake rates at the peak of the bloom. Overall, crenarchaeol appears to be an effective tool to track the occurrence of AOA in the North Sea, and together, the data emphasize the potential importance of AOA in North Sea nitrification and carbon fixation during the winter.

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