Distribution of Cold-Adapted Ammonia-Oxidizing Microorganisms in the Deep-Ocean of the Northeastern Japan Sea

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We investigated the quantities and phylogenies of *amoA* genes of ammonia-oxidizing archaea (AOA) belonging to *Crenarchaeota* and ammonia-oxidizing bacteria (AOB) belonging to *Betaproteobacteria* in water columns and deep-ocean cold seep sediment in the northeastern Japan Sea with a competitive PCR (cPCR) assay. Water samples were collected at depths of 2000 m and 2956 m. Sediment samples were collected where white bacterial mats had developed. The cPCR analysis revealed five to ten times more AOA than betaproteobacterial AOB in both the water columns and sediment. The abundance of the crenarchaeotal *amoA* gene was estimated at 6×10^1 and 3×10^2 copies ml^{-1} in the water columns at depths of 2000 m and 2956 m, and 1×10^8 and 1×10^7 copies g^{-1} in pelagic brown sediment and black sediment, respectively. Most archaeal *amoA* clones from water column at 2000 m fell into the Deep Marine Group. Most archaeal *amoA* clones from pelagic brown sediment were less closely related to known environmental clones. Moreover, incubation experiments revealed nitrite production at 4°C and 10°C. The results indicate that psychrophilic AOA and AOB may be responsible for nitrification in the deep-ocean region of the northeastern Japan Sea.

Key words: ammonia-oxidizing archaea, ammonia-oxidizing bacteria, *amoA*, *Crenarchaeota*, psychrophilic

Ammonia-oxidizing microorganisms (AOM) utilize ammonia as their major source of energy and carbon dioxide as their main source of carbon. They catalyze the conversion of ammonia (NH_3) to nitrite (NO_2^-) and are widespread in biospheres such as the sea, freshwater, and soil. Hence, AOM play an important role in the global nitrogen cycle.

Ammonia monooxygenase (AMO) is a key enzyme that catalyzes the reaction producing hydroxylamine during the bacterial oxidation of ammonia. The widespread distribution and phylogenetic diversity of ammonia-oxidizing bacteria (AOB) belonging to the *Betaproteobacteria* in natural environments such as the sea, freshwater, and soil have

been demonstrated by using a molecular ecological approach based on the α subunit of the AMO (*amoA*) gene^{4,5,19,24,26,30}. The *amoA* genes affiliated with the *Gammaproteobacteria* have been found in the ocean upper depth of 1000 m³². More recently, an environmental genomic study has suggested that ammonia-oxidizing archaea (AOA) exist in soil because a novel *amoA* gene was found in the same metagenomic fragment accompanied with 16S rRNA gene of *Crenarchaeota*²⁸. Isolation of the ammonia-oxidizing archaeon ‘*Candidatus Nitrosopumilus maritimus*’ belonging to the marine group I *Crenarchaeota* has indicated a definitive link between the novel *amoA* gene and archaeal ammonia oxidation¹⁴. Several studies with different sets of PCR primers for the archaeal *amoA* gene have demonstrated the worldwide distribution and phylogenetic diversity of AOA in natural environments^{3,7,9,16,20,31}.

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The ocean is cold (less than 5°C) at depths of more than 1000 m. The ratio of nonthermophilic archaea to all planktonic prokaryotes increases gradually in deeper water columns of the ocean¹³. Marine Group I *Crenarchaeota* are relatively abundant among all archaea in deep-water columns of the meso- and bathypelagic oceans at more than 1000 m^{10,13}. Positive correlations between the abundance of *Crenarchaeota* and nitrite were observed in the Arabian Sea²⁷. A lipid analysis with a compound-specific isotope-based approach demonstrated that CO₂ was a major carbon source for *Crenarchaeota* in the mesopelagic ocean at 670 m¹². An enrichment culture of coastal water of the North Sea suggested that mesophilic *Crenarchaeota* contributed mainly to the oxidization of ammonia³³. Furthermore, the abundance of archaeal *amoA* genes was greater than that of bacterial *amoA* in the upper (less than 1000 m) open ocean of the Atlantic³³. These results suggested a significant role for AOA in nitrification in the ocean. However, relatively little is known about the ratio of AOA to AOB or the rate of nitrification in the water column and sediment of the cold deep ocean at more than 1000 m.

In this study, we quantified the *amoA* genes of crenarchaeotal AOA and betaproteobacterial AOB in water columns and cold seep sediments in the deep ocean (more than 1000 m) of the northeastern Japan Sea with competitive PCR (cPCR). We developed two competitors for AOA and AOB with environmental clones obtained from sediment samples used in this study. In addition, phylogenies of *amoA* genes were also investigated with a PCR-cloning analysis. In order to clarify the potential of ammonia-oxidation by AOA and/or AOB under low temperature conditions, nitrite and nitrate concentrations in culture media for AOM were investigated at 4°C, 10°C, and 20°C with samples of water columns and sediment as an inoculum.

Materials and Methods

Sample collection

Water column samples were collected from two depths, 2000 m (designated NR) and 2956 m (designated NY), in the northeastern Japan Sea (43°20'N, 139°39'E) using Niskin bottles deployed on the manned research submersible *Shinkai 6500* (dive no. 6K960) on cruise YK06-07 in June 2006. Temperatures at both depths were 0.2°C. NY was collected just above cold seep sediments where bacterial white mats had developed (43°20.21'N, 139°39.85'E). Water samples were stored at 4°C and transferred to the laboratory within three days after sampling. A 1300 ml volume of sea-water was filtered with Millex®-GP (pore size, 0.2

µm, Millipore, Billerica, MA, USA). One-third of the filter for molecular analysis was kept in a 2 ml centrifuge tube for DNA extraction and then frozen at -20°C until DNA extraction. One-third of the filter for the incubation experiment was kept in a sterile plastic tube (50 ml) with filtered sea-water.

Sediment samples were collected from site M1 (43°20'N, 139°39'E), described previously by Arakawa *et al.*², at the southern foot of the Shiribeshi seamount by using MBARI cores deployed on the *Shinkai 6500* (dive no. 6K960). A core sample was collected from an area where small white microbial mats were observed (2965 m; 43°19.99'N, 139°39.91'E). Ten grams of pelagic brown sediment (designated CR) was obtained from the core at 0–5 cm below the seafloor (bsf). Another core sample was collected from an area where white microbial mats were observed (2961 m; 43°20.22'N, 139°39.86'E). Ten grams of black sediment (designated CBK) was obtained from the core at 5–10 cm bsf. It is presumed that sea water just above cold seep sediments contains molecular oxygen compared to the black sediment. Sediment samples were stored at 4°C and transferred to the laboratory within three days. A part of each sediment sample was kept in a 1.5 ml centrifuge tube and then frozen at -20°C until DNA extraction.

Amplification of the *amoA* gene

Nucleic acids were extracted from 0.5 g of sediment ($n=3$) and filter ($n=1$) with an ISOIL for Beads Beating Kit (Nippon Gene, Toyama, Japan) following the manufacturer's instructions, after vortexing vigorously the plastic tube containing lysis solutions and beads in the kit for 45 sec. Nucleic acids extracted in 20 µl of Tris-EDTA (TE) buffer were stored at -20°C until PCR. Partial *amoA* gene fragments were amplified with TaKaRa EX Taq DNA polymerase (Takara Bio, Otsu, Japan). Nucleotide primers for the PCR were crenAMO_F and crenAMO_R for the crenarchaeotal *amoA* gene⁹, and *amoA*-1F and *amoA*-2R for the betaproteobacterial *amoA* gene²⁶. The amplification was performed with a MyCycler thermal cycler (BIO-RAD, Hercules, CA). The protocol for PCR cycling is described in Table 1. PCR products were visualized on a 1.5% (w/v) agarose gel stained with ethidium bromide. Two reaction mixtures were pooled, excised from gels, and extracted with a QIAGEN II Gel Extraction Kit (QIAGEN, Valencia, CA). Amplified gene fragments of *amoA* were cloned with a TOPO TA PCR cloning kit (Invitrogen, Carlsbad, CA). White colonies were transferred to new LB broth agar plates with kanamycin, grown overnight at 37°C, and amplified with the vector-specific primers M13F and M13R. After

Table 1. Protocol for PCR cycling

Primer set	Target gene	PCR cycle	Reference
crenAMO_F and crenAMO_R	crenarchaeotal <i>amoA</i> gene	94°C for 5 min, 25 to 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, and final extension at 72°C for 3 min.	9
<i>amoA</i> -1F and <i>amoA</i> -2R	betaproteobacterial <i>amoA</i> gene	94°C for 5 min and then 25 to 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 45 sec, and final extension at 72°C for 3 min.	26
NOC1-45f and NOC2-1168r	16S rRNA gene of gammaproteobacterial ammonia-oxidizing bacteria	94°C for 5 min, 25 to 35 cycles of 94°C for 30 sec, 61°C for 30 sec, and 72°C for 45 sec, and final extension at 72°C for 3 min.	8

PCR products were electrophoresed again in a 1.5% agarose gel to check the length of the bands, amplicons were purified with a QIAquick PCR purification kit (QIAGEN) prior to sequencing.

Sequencing and phylogenetic analysis

M13F/M13R PCR products were sequenced with a Big-Dye v.3.1 sequencing kit (PE Applied Biosystems, Foster City, CA) on a Model 3100 DNA sequencer (PE Applied Biosystems). M13F/M13R PCR products (archaeal and bacterial *amoA* genes) were sequenced using vector primers M13F and M13R. Similarities of the *amoA* gene sequences were investigated in the databases of the National Center for Biotechnology Information and the DNA Data Bank of Japan using BLAST¹⁾. Clones having $\geq 97\%$ nucleotide sequence similarity in each sample (NR, NY, CR, and CBK) were assigned to the same representative type. Deduced amino acid sequences for environmental *amoA* genes were determined from 590 bp of AOA and 444 bp of AOB nucleotide sequences. The nucleotide and amino acid sequences were aligned by using the CLUSTAL W program within MEGA3 software¹⁵⁾. Phylogenetic trees for *amoA* of AOA and AOB were based on 184 and 138 amino acid sequences, respectively. Phylogenetic trees were inferred by the neighbor-joining and maximum parsimony methods implemented in MEGA3. The p-distance (amino acids) model within the neighbor-joining method was used for *amoA*. Bootstrap values were determined from 1000 iterations.

Construction of competitors

The competitor DNA fragments were constructed according to a previously described method^{6,34)}. The partial archaeal *amoA* gene fragment (540 bp) was amplified directly from *Escherichia coli* containing the vector designated pAOA with a partial *amoA* gene (CR-G3N006) using primers crenAMO_F and AamoA-TN1 (5'-ACTGGACT-GTTATAGAACT-3'). The partial betaproteobacterial

amoA gene fragment (391 bp) was amplified directly from *Escherichia coli* containing the vector designated pAOB with a partial *amoA* gene (CR-G3N026) using BamoA-TN1 (5'-AACTGGATGATCACAGCA-3') and *amoA*-2R-TC (5'-CCCCTCTGCAAAGCCTTCTTC-3')²³⁾. The amplified archaeal and betaproteobacterial *amoA* gene fragments were reamplified with primers crenAMO_F and AamoA-TN2 (5'-CCCACTTTGACCAAGCGGCCATACTGGACTGT-TATAGAACT-3' [primer crenAMO_R underlined]) for AOA, and BamoA-TN2 (5'-GGGGTTTCTACTGGTGG-TAACTGGATGATCACAGCA-3' [primer *amoA*-1F underlined]) and *amoA*-2R-TC for AOB, respectively, and then cloned into the pCR2.1-TOPO vector with a TOPO TA PCR cloning kit. After incubation of *E. coli* at 37°C, the plasmids were extracted with a Quantum Prep Plasmid Miniprep Kit (BIO-RAD), and then used for cPCR as the archaeal *amoA* competitor (designated cAOA) and bacterial *amoA* competitor (designated cAOB).

cPCR

To estimate the abundance of archaeal and bacterial *amoA* genes in the water column and sediment samples, amplifications were performed as described above with 25- μ l volumes containing 0.5 μ l of extracted nucleic acids and 0.5 μ l of competitor cAOA or cAOB using primers crenAMO_F and crenAMO_R, and *amoA*-1F and *amoA*-2R. PCR products were analyzed by electrophoresis in 2% (w/v) Nusieve 3:1 agarose (FMC, Rockland, ME) gels, and then photographed with UV transillumination and a Polaroid MP4 Instant Camera System. The images were analyzed with Photoshop CS2 (Adobe Systems Inc., San Jose, CA).

Incubation experiments

To investigate the potential oxidation of ammonia by AOA and/or AOB obtained from the deep ocean under low-temperature conditions, incubation experiments were conducted with sea-water (NR and NY) and sediment (CR and

CBK) samples using two types of media. Modified Artificial Sea Water (ASW-Cl) medium²¹⁾ contained (NH₄)₂SO₄ 1.0 g, NaCl 20 g, MgCl₂·6H₂O 10 g, Na₂SO₄ 3.91 g, CaCl₂ 1.1 g, KCl 0.66 g, NaHCO₃ 0.19 g, K₂HPO₄ 0.01 g, Fe-EDTA (III) 0.01 g, MOPS [3-(*N*-Morpholino) propane-sulfonic acid] 10 g, and 1 ml of bromothymol blue solution (bromothymol blue 0.01 g l⁻¹, Na₂HPO₄·2H₂O 0.123 g l⁻¹, and KH₂PO₄ 0.005 g l⁻¹ in distilled water) in 1000 ml of distilled water. The pH of the ASW and ASW-Cl media was adjusted to 8.0. Synthetic Crenarchaeota medium (SCM)¹⁴⁾ contained NaCl 26 g, MgCl₂·6H₂O 5 g, MgSO₄·7H₂O 5 g, CaCl₂ 1.5 g, KBr 0.1 g, 1 ml of nonchelated trace element mixture, 1 ml of vitamin mixture (4-aminobenzoic acid 40 mg l⁻¹, D-biotin 10 mg l⁻¹, nicotinic acid 100 mg l⁻¹, calcium D-pantothenate 50 mg l⁻¹, and pyridoxine dihydrochloride 150 mg l⁻¹ dissolved in 10 mM sodium phosphate buffer [pH 7.1]), 1 ml of thiamin solution (thiamine chloride dihydrochloride 100 mg l⁻¹ dissolved in 25 mM sodium phosphate buffer [pH 3.4]) and 1 ml of vitamin B₁₂ solution (cyanocobalamin 50 mg l⁻¹ dissolved in distilled water), 1 ml of selenite-tungstate solution (NaOH 0.4 g l⁻¹, Na₂SeO₃·5H₂O 6 mg l⁻¹, and Na₂WO₄·2H₂O 8 mg l⁻¹), 10 ml of KH₂PO₄ (4 g l⁻¹), 1 ml of bicarbonate solution (NaHCO₃ 84 g l⁻¹), and 1 ml of NH₄Cl (1 M) in 1000 ml of distilled water. The pH of SCM was adjusted to 7.0. A plastic tube (50 ml) containing filtered seawater with filtered cells was mixed well to use as an inoculum. Two spoonfuls of sediment were transferred into 50 ml of filtered sea-water and then mixed well to make slurry samples. Three milliliters of the water and slurry sample was used to inoculate a hard cotton-wool-plugged 100-ml Erlenmeyer flask containing 30 ml of ASW-Cl, and SCM, respectively, and incubated at 10°C and 20°C in the dark for 84 days. After the incubation, the flask was incubated at 4°C in the dark. The concentration of nitrite in the medium was determined colorimetrically with Griess-Ilosvey reagent¹¹⁾ (quantitative limit, 5 µM). Nitrate was measured with an ion chromatography system-1000 attached to an UltiMate 3000 Variable Wavelength Detector (DIONEX, Sunnyvale, CA) (quantitative limit, 5 µM).

Nucleotide sequence accession numbers

The *amoA* gene sequences were submitted to DDBJ/EMBL/GenBank and have been assigned the following accession numbers: AB289348 to AB289401.

Results and Discussion

Quantitative analysis of the *amoA* gene in water columns

To study the distribution of microorganisms responsible for the oxidation of ammonia at low temperatures in water columns of the deep ocean, a quantitative analysis of the *amoA* gene was performed with cPCR. The abundance of the *amoA* gene of AOA in the water column NR at a depth of 2000 m was estimated at 6×10¹ copies ml⁻¹ (*n*=1) (Fig. 1A). In this study, the quantitative limit was 1×10¹ copies ml⁻¹. On the other hand, the *amoA* gene of AOB was not detected by the 35-cycle PCR in the water column NR. The detection limit of cPCR for the *amoA* gene of AOB at a cycle number of 35 was 1×10² copies µl⁻¹ in the extracted nucleic acid solution. Moreover, the abundance of the *amoA* gene of AOA was 1×10³ copies µl⁻¹ in the extracted nucleic

Fig. 1. Comparative abundance of *amoA* genes between crenarchaeotal ammonia-oxidizing archaea (AOA) and betaproteobacterial ammonia-oxidizing bacteria (AOB). (A) Abundance of *amoA* genes in water columns. (B) Abundance of *amoA* genes in cold seep sediment samples. NR, water column at a depth of 2000 m; NY, water column at a depth of 2956 m; CR, brown sediment (0–5 cm bsf); CBK, black sediment (5–10 cm bsf). n.d. shows that the *amoA* gene was not detected. In this study, quantitative limits were 1×10¹ copies ml⁻¹.

acid solution. Thus, the *amoA* gene of AOA can be estimated to be at least ten times more abundant than that of AOB in the water column NR (0.2°C) at 2000 m. Although these values are likely underestimated due to loss of nucleic acids during the extraction procedure, the ratio of gene abundance in AOA to AOB is considered accurate, if the PCR efficiency for AOA and AOB amplification is similar. Similarly, it has been reported that the abundance of the *amoA* gene of AOA was higher than that of AOB in the upper 1000 m of water columns of the North Atlantic Ocean³³.

The abundance of *amoA* in the water column NY at a depth of 2956 m was estimated at 3×10^2 copies ml⁻¹ for AOA ($n=1$), and 1×10^1 copies ml⁻¹ for AOB ($n=1$) (Fig. 1A). In this study, the quantitative limit was 1×10^1 copies ml⁻¹. Therefore, it is concluded that the abundance of the *amoA* gene of AOA was probably higher than that of AOB in water column NY at 2956 m just above cold seep sediments as well as in water column NR at a depth of 2000 m. In addition, the abundance of the crenarchaeotal *amoA* gene was greater at 2956 m than at 2000 m. Abundance of marine *Crenarchaeota* group I reportedly increased in ambient cold seawater around deep-sea hydrothermal environments compared to normal seawaters²⁹. It is known that hydrothermal fluids contain CH₄ and CO₂, and NH₄⁺, depending on the sampling site¹⁷. Methane was detected previously at this water column just above cold seep sediments². It thus seemed that the nutrient flux from seafloor induces an increase in the abundances of AOA and AOB in water columns.

Quantitative analysis of the *amoA* gene in cold seep sediments

The abundance of the *amoA* gene of AOA in sediment CR was estimated at $(1 \pm 2) \times 10^8$ copies g⁻¹ [wet weight] ($n=3$), while that of the *amoA* gene of AOB was estimated at $(2 \pm 2) \times 10^7$ copies g⁻¹ ($n=3$) (Fig. 1B). This result demonstrated that the abundance of the *amoA* gene of AOA was probably higher than that of AOB in brown sediment CR (0–5 cm bsf).

The abundance of the *amoA* gene of AOA in sediment CBK was estimated to be $(1 \pm 0.5) \times 10^7$ copies g⁻¹ [wet weight] ($n=3$), while that of the *amoA* gene of AOB was estimated at $(5 \pm 5) \times 10^6$ copies g⁻¹ ($n=3$) (Fig. 1B). Therefore, it is concluded that the abundance of the *amoA* gene of AOA was probably greater than that of AOB in black sediment CBK (5–10 cm bsf). Additionally, the *amoA* genes of AOA and AOB were less abundant in black sediment CBK than in brown sediment CR (Fig. 1B). The smell of hydro-

gen sulfide was detected in black sediment CBK. It is presumed that the amount of molecular oxygen is decreased in black sediment CBK compared to the layers above, because remarkable anaerobic oxidation of methane occurred with the detection of sulfate-reducing bacteria belonging to *Deltaproteobacteria* in black sediment².

Phylogenetic analysis of the *amoA* gene of AOA

Sixteen representative clone types were obtained from 40 archaeal *amoA* gene sequences of samples. Clones obtained from the water column NR fell mostly (90%) into the phylogenetic cluster of the deep marine group containing the clone ETNP 4 from a water column of the Eastern Tropical North Pacific, the clone BS15.8.24 from a water column of the Black Sea⁷, and HF500 and HF4000 from water columns of Monterey Bay and the Central Pacific, respectively⁹ (Fig. 2). It has been reported that the clusters of the water columns A and B mostly consisted of *amoA* genes, suggesting that a significant portion of the archaeal community may be responsible for the first step of chemolithoautotrophic nitrification in water columns of the ocean⁷. Similarly, it is likely that AOA corresponding to the *amoA* genes within the cluster of the Deep Marine Group (water column B) are important microbial populations for nitrification in the water column NR at a depth of 2000 m.

Clones obtained from sediment sample CR mostly (91%) fell into the cluster of Sediment A including the *amoA* gene of the chemolithoautotrophic ammonia-oxidizing archaeon ‘*Candidatus Nitrosopumilus maritimus*’¹⁴) (Fig. 2). The similarity of *amoA* gene sequences between CR clones within the cluster of Sediment A and those of ‘*Candidatus Nitrosopumilus maritimus*’ was 89% to 91%. In addition, CR clones, that fell into the cluster of Sediment A-1, were less closely related to environmental clones retrieved from several environments (91%–92% nucleotide similarity). This clustering suggests that *amoA* genes within the Sediment A cluster represent a unique and psychrophilic AOA type in the deep-ocean cold seep sediments. Clones CR-G3N128 and CBK-G3N131 represented a separate lineage.

Phylogenetic analysis of the *amoA* gene of AOB

Nine representative clone types were obtained from 14 betaproteobacterial *amoA* gene sequences of NY, CR, and CBK samples. All clones fell into the *Nitrosospira* cluster²⁵) containing NEamoA-5 (96% to 98% nucleotide similarity) and NFamoA-1 (96% to 99% nucleotide similarity) detected in deep-ocean sediment in the Nankai Trough, Japan³⁰.

Fig. 2. Phylogenetic tree based on the deduced, partial amino acid sequences (184 length) of the archaeal *amoA* gene as determined by neighbor-joining analysis. Boldface type indicates *amoA* clones obtained in this study. The scale bar represents an estimated sequence divergence of 2%. Numbers at nodes are bootstrap values >50% that support the branching pattern appearing to the right of the value. Right sides of bootstrap values were determined by the maximum-parsimony method. German soil 54d9 (AJ627422) and OKR-C-22 (DQ148882) were used as the outgroup. Classification of the phylogenetic groups was described previously^{7,9}.

Gammaproteobacterial AOB

To investigate the distribution of gammaproteobacterial AOB in water columns and sediment, we attempted to amplify the PCR product with a primer set for the 16S rRNA gene of gammaproteobacterial AOB⁸⁾. However, no PCR product was detected in any sample.

Nitrite production at less than 10°C

To clarify the potential for ammonia-oxidation by AOA and/or AOB under low temperature conditions, nitrite concentrations in three types of media for AOM were determined after an 84-day incubation at 10°C and 20°C in the dark. A remarkable difference in nitrite production was found at between 10°C and 20°C (Fig. 3). Remarkable nitrite production was observed in seven media (ASW-Cl-CR, -CBK, -NY, -NR, and SCM-CBK, -NY, -NR) for all cultures at 10°C. The concentrations of nitrate in ASW-Cl-CR and SCM-CR at 10°C were 5.6 μ M and 11.3 μ M, respectively. Nitrate was not detected from other samples (detection limit, 5 μ M). Furthermore, the nitrite concentrations in ASW-Cl-CR, -CBK, -NY, -NR, and SCM-NR kept increasing after the incubation temperature was changed to 4°C (Fig. 4). Therefore, these results demonstrated that nitrite production occurred at 4°C, implying the potential for nitrite production in the deep ocean of the northeastern Japan Sea. It is, however, still unclear to what extent AOA and AOB might contribute to nitrite production. In addition, it is presumed that denitrification occurs during the incubation, because genes for denitrification have been detected in deep-sea sediment³⁰⁾.

Fig. 3. Comparison of nitrite concentrations in culture media (ASW-Cl, and SCM) at 10°C and at 20°C after 84-day incubations. NR and NY were water columns at depths of 2000 m and 2956 m, respectively. CR, brown sediment CR (0–5 cm bsf); CBK, black sediment CBK (5–10 cm bsf). The asterisk shows that the concentration was less than the quantitative limit (5 μ M).

Fig. 4. Changes in nitrite concentrations in culture media at 4°C after 84-day incubations at 10°C. NR and NY were water columns at depths of 2000 m and 2956 m, respectively. CR, brown sediment CR (0–5 cm bsf); CBK, black sediment CBK (5–10 cm bsf).

Conclusion

This study demonstrated that crenarchaeotal AOA were likely to be more abundant than beta- and gammaproteobacterial AOB in water columns and cold seep sediment of the deep ocean at a depth of more than 1000 m. Furthermore, this study suggests that potential nitrite production takes place at 4°C in the water column at a depth of 2000 m. Approximately 71% of the surface of the Earth is covered with oceans²²⁾. In addition, the deep ocean (1–5°C; more than 1000 m) is in area estimated to account for approximately 88% of the world's oceans¹⁸⁾. Hence, it is important to clarify whether the psychrophilic AOM in the deep ocean play an important role in the global nitrogen and carbon cycles.

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