2019

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Citation/Publisher Attribution

Vuillemin, A., Wankel, S. D., Coskun, Ö. K., Magritsch, T., Vargas, S., Estes, E. R.,...Orsi, W. D. (2019). Archaea dominate oxic subseafloor communities over multimillion-year time scales. *Science Advances* *5*(6), eaaw4108. doi: 10.1126/sciadv.aaw4108
Available at: http://dx.doi.org/10.1126/sciadv.aaw4108

ECOLOGY

Archaea dominate oxic subseafloor communities over multimillion-year time scales

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Ammonia-oxidizing archaea (AOA) dominate microbial communities throughout oxic subseafloor sediment deposited over millions of years in the North Atlantic Ocean. Rates of nitrification correlated with the abundance of these dominant AOA populations, whose metabolism is characterized by ammonia oxidation, mixotrophic utilization of organic nitrogen, deamination, and the energetically efficient chemolithoautotrophic hydroxypropionate/hydroxybutyrate carbon fixation cycle. These AOA thus have the potential to couple mixotrophic and chemolithoautotrophic metabolism via mixotrophic deamination of organic nitrogen, followed by oxidation of the regenerated ammonia for additional energy to fuel carbon fixation. This metabolic feature likely reduces energy loss and improves AOA fitness under energy-starved, oxic conditions, thereby allowing them to outcompete other taxa for millions of years.

INTRODUCTION

Marine sediment is estimated to contain >10^{29} microbial cells (*1*), which represent a ubiquitous "deep biosphere," (*2*) extending at least as far as 2500 meters below the seafloor (mbsf) (*3*). Abyssal clay in ultraoligotrophic regions of the ocean is typically oxic through the entire sediment column to the underlying oceanic crust; it is predicted that oxygen (O_2) diffuses through the sediment to the igneous basement in 9 to 37% of the global seafloor (*4*). These aerobic communities have extremely low metabolic activity (*5*) and live near the low-energy limit to life (*4*), surviving at ultraslow average respiration rates (*6*). The diversity, abundance, and metabolic potential of abyssal subseafloor aerobic communities are still poorly understood; genetic characterization of these aerobic communities is limited to the relatively shallow depth of 0.5 mbsf (*7*), compared to communities in anoxic sediment, for which genetic records extend to 2500 mbsf (*3*).

Most studies targeting the deep biosphere of oxic abyssal clay have focused on Pacific sites, where cell abundances decrease from 10^6 cells/cm^3 at the seafloor (surface) to 10^3 cells/cm^3 at ~10 mbsf (*1*, *4*). Isotopic analyses from oxic subseafloor sites in the North Atlantic have suggested that active nitrification takes place, indicating that oxidative nitrogen cycling may be an important feature of microbial communities deep below the abyssal seafloor (*8*, *9*). Consistent with these isotopic studies, the upper 50 cm of the abyssal South Pacific Gyre sediments contain ammonia-oxidizing archaea (AOA) (*7*, *10*), which are a key group contributing to the carbon cycle of the deep ocean in both the water column (*11*–*13*) and benthos (*14*–*17*).

The microbial community of the abyssal seafloor is characterized by diverse assemblages of bacteria and archaea (*18*), with abundances spanning 10^6 to 10^9 cells/cm^3 of sediment, tending toward increased concentrations at higher latitudes (*15*). However, much less is known about the diversity and metabolism of microbial communities in the oxic sediment deep below the seafloor of open-ocean oligotrophic regions (*19*). The dearth of knowledge on aerobic subseafloor microbiomes is especially notable when compared to the relatively higher number of investigations from anoxic subseafloor sites (*20*). Thus, we sought to characterize the diversity, abundance, functional potential, and viability of aerobic microbiomes that reside in oxic subseafloor abyssal red clay from an ultraoligotrophic region of the North Atlantic.

We optimized a DNA extraction protocol for ultraorganic lean sediments that provided increased yields of DNA, enabling sequencing of 16*S* rRNA and ammonia monooxygenase (amoA) genes, as well as metagenomes, from two deep oxic subseafloor sediment cores reaching up to ~15 million years old. In addition, we demonstrate the viability of uncultivated microbial populations via ^{18}O-labeling in long-term (18-month) incubations in the presence of $H_2{}^{18}O$, a method for identifying actively growing microbes (*21*, *22*). The results show that, in these viability experiments, actively growing populations of AOA dominate over all other microbial populations in samples from throughout the cored sediment column. Metagenomic analysis from the same samples shows that the potential coupling of chemolithoautotrophy and mixotrophy, powered by ammonia oxidation, has the potential to fuel multimillion-year subsistence of AOA in this energy-starved habitat.

RESULTS AND DISCUSSION

We obtained sediment via multi-coring (0 to 0.22 mbsf), gravity coring (0.2 to 2.8 mbsf), and deep coring (1 to 28 mbsf) of oxygenated abyssal clay from two sites in the North Atlantic (table S1). These sites are located in the ultraoligotrophic open ocean and characterized by a mean sedimentation rate of 1 m per million years (*4*). Thus, the deepest sample recovered was deposited ca. 28 million years ago. Drawdown of O_2 with increasing depth at both sites (fig. S1) reflects oxidation of organic matter by aerobic microorganisms. The corresponding accumulation of dissolved nitrate (NO_3^-) (Fig. 1) reflects oxidation of released ammonium in C:N Redfield stoichiometry of the organic matter (fig. S2), directly reflective of the activity of aerobic nitrifying microbial communities and consistent with dissolved nitrogen isotopic analyses from similar sites in the North Atlantic (*8*, *9*). These biogeochemical data indicated that nitrification, an important

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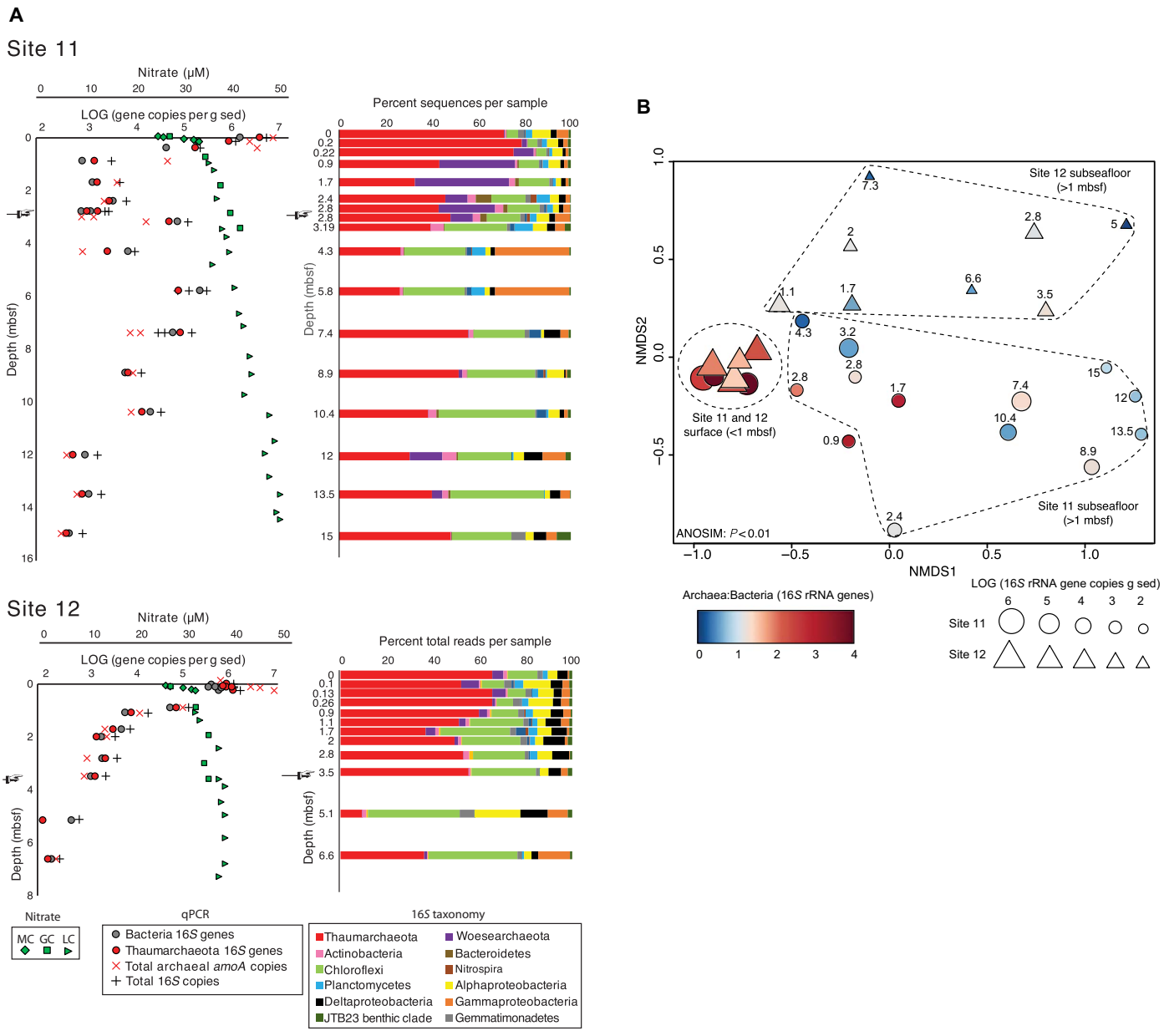

Fig. 1. Microbial diversity, abundance, and nitrification in the North Atlantic abyssal subseafloor. (**A**) Left: Dissolved nitrate, quantitative polymerase chain reaction (qPCR) of 16*S* rRNA and AOA amoA genes, and qPCR-normalized abundance of bacterial and archaeal 16*S* rRNA genes. Right: Diversity of 16*S* rRNA genes. Note that two biological replicates were sequenced at site 11, depth 2.8 mbsf. Arrows show the depths for ^{18}O-labeling incubations. (**B**) Nonmetric multidimensional scaling analysis of 16*S* rRNA genes, the size of the points (samples) are normalized by the number of 16*S* rRNA gene copies per sample (qPCR), the numbers above each point indicate the depth in meters below the seafloor, and the shading indicates the ratio of archaeal to bacterial sequences. The ANOSIM analyses were performed on sample groupings as displayed by the dashed lines. Note that each meter in (A) represents ca. 1 million years of sediment deposition, and the communities in the deepest sample at site 11 have been subsisting for ca. 15 million years.

feature in deep-sea planktonic ecosystems (*11*), is also a major mechanism sustaining these energy-limited aerobic communities deep below the abyssal seafloor over million-year time scales. To better understand the inner workings of microbial survival over such long (geological) time scales, we performed quantitative gene sequencing, metagenomics, and stable isotope probing to characterize the diversity, abundance, metabolism, and activity of specific microbial groups.

Our optimized DNA extraction protocol enabled us to reach a detection limit of 10^2 gene copies per gram of extracted sediment (see Materials and Methods) and allowed us to characterize these subseafloor communities down to 15 mbsf (Fig. 1A). Because the sedimentation rate is 1 m per million years (*4*), microbial communities in the deepest characterized samples (Fig. 1A) have persisted in the subseafloor for ca. 15 million years. To our knowledge, these metagenomic data are the deepest, and geologically oldest, from oxic abyssal subseafloor clay to date.

At both sites, numbers of 16*S* rRNA and amoA gene copies from AOA are up to an order of magnitude greater than numbers of bacterial

16*S* rRNA gene copies at the sediment surface, whereas 16*S* rRNA gene copies affiliated with the Chloroflexi gradually increase in number with increasing depth beneath the seafloor (Fig. 1A). The most abundant Chloroflexi operational taxonomic unit (OTU) at both sites was affiliated with the SAR202 clade, a typical inhabitant of deep-sea water and sediment (*23*). It encodes monooxygenases catalyzing oxygen insertion into relatively unreactive aromatic hydrocarbons, indicating an ability to metabolize older, less reactive, and highly persistent organic matter (*23*). This trait of SAR202 may be selected in subseafloor abyssal clay, where organic matter is relatively unreactive and extremely low in abundance (*4*), but required to sustain long-term survival (*24*). In the South Pacific Gyre oxic subseafloor sediment, O_2 consumption rates are greater than can be explained by heterotrophic particulate organic carbon (POC) oxidation alone (*4*, *25*). O_2 consumption by chemolithoautotrophic AOA accounts for subseafloor respiration that cannot be accounted for by aerobic POC degradation via microbial heterotrophy.

The increase in NO_3^- concentrations with depth together with the high abundance of AOA indicates active nitrification and ammonia oxidation in situ. Higher O_2 drawdown rates and higher nitrification rates occur at site 11 than at site 12, which correlate with higher microbial abundances at site 11 (Fig. 1A and fig. S1). Similar to the O_2 profiles (fig. S1), 16*S* rRNA and amoA gene copies per gram of sediment decrease exponentially with depth at both sites (Fig. 1A). This profile is consistent between samples acquired via multi-coring, gravity coring, and long cores (Fig. 1A and table S1).

Our results show that the extracted sample DNA derives primarily from living cells in the sediment, as opposed to extracellular DNA (eDNA), for several reasons. First, eDNA binds to charged mineral surfaces, making it resistant to DNA extraction (*26*). Our experimental data demonstrate this point, because an artificial eDNA extract containing $>10^9$ 16*S* rRNA genes per gram added to "killed" (autoclaved) controls was only detectable at $<10^3$ 16*S* rRNA genes per gram after reextracting the DNA from the autoclaved sediment (Fig. 2). This low recovery of eDNA with our protocol is presumably due to the majority of eDNA being chemically bound to clay minerals (*26*). In contrast, DNA extracted from "living" (pristine frozen) sediment contained $10^{6.5}$ to 10^2 16*S* rRNA genes per gram of sediment (Fig. 1A). Moreover, the same eDNA extract was used as a nutrient source by growing microbes in "living" (not autoclaved) slurries during a 210-day incubation (Fig. 2). While microbial activity in sediment is increased in slurries compared to intact cores (*27*), the experiment shows that viable cells in the sediment have the potential to use bioavailable eDNA. It also suggests that some microbes can use mineral-bound eDNA as a growth substrate. All of these lines of evidence indicate that extractable DNA from our samples derives primarily from viable cells that were living in the sediment.

To further identify viable microbial populations, we used $H_2{}^{18}O$ in long-term incubations, as ^{18}O incorporation from labeled water can be used to identify actively growing populations within complex microbial communities from environmental samples (*22*). After 7 and 18 months of incubation with $H_2{}^{18}O$, there was measurable respiration, and density gradient centrifugation followed by quantitative polymerase chain reaction (qPCR) revealed that 16*S* rRNA genes were enriched in ^{18}O (fig. S3). High-throughput sequencing of 16*S* rRNA genes in individual density fractions in ^{18}O-labeled and control treatments ("Tag-SIP") identified more than 50 actively growing (^{18}O-labeled) OTUs (table S2), corresponding to 4 to 9% of total OTUs detected in situ (n = 480).

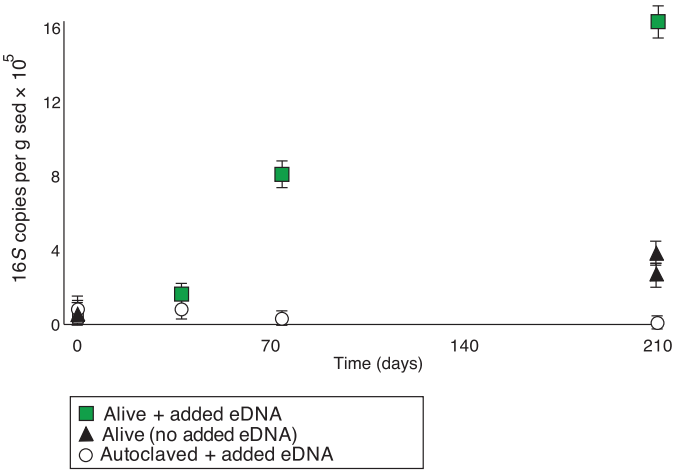

Fig. 2. Utilization of eDNA by living microbes, and a lack of detectable eDNA added to autoclaved sediments. Samples derived from 2.8 mbsf, the depth used for the ^{18}O-SIP experiment from site 11. The lack of DNA detection in autoclaved sediments, where eDNA was added after autoclaving (open circles) indicates that eDNA is tightly bound to sediment particles such as clay minerals and that the DNA extracted with our protocol should target intact living cells, and is unlikely to be biased by eDNA from dead microbes. Error bars are the range across three technical replicates.

Of the 10 most abundant OTUs in situ, three of these were affiliated with AOA (Thaumarchaeota) and were all ^{18}O-labeled, demonstrating their viability and activity (Fig. 3). Most archaeal OTUs that were ^{18}O-labeled exhibited minimal net growth (fig. S4). While the term "net growth" can have different meanings depending on the experimental setup, we use this term in our experiment to refer to the increase in qPCR-normalized abundances of 16*S* rRNA genes per OTU relative to the T0 value (*y* axis in fig. S4, B and C). This minimal net growth by AOA suggests that some ^{18}O-labeling of these archaea may have occurred via growth-independent cell maintenance activities, such as DNA repair. In bacteria, incorporation of ^{18}O from labeled water takes place exclusively during DNA replication and growth (*22*), but it is unknown whether this pattern also applies to archaea. The most abundant OTU in situ at both sites is a viable (^{18}O-labeled) AOA population that persists as the dominant population throughout the entirety of both sampled sediment sequences, dominating the microbial community at multiple depths by over an order of magnitude (Fig. 3). This population of viable AOA has thus dominated both subseafloor ecosystems sampled for millions of years, which is, to our knowledge, the longest timespan that aerobic archaea have ever been reported to dominate a subsurface habitat.

At both sites, the 16*S* rRNA genes and amoA genes from AOA are distinct from planktonic AOA (*28*) and most closely related to benthic AOA, including those detected in abyssal South Pacific Gyre sediment (Fig. 4B and fig. S5) (*7*, *29*). Below 1 mbsf, the microbial communities between sites 11 and 12 are significantly different [Analysis of Similarity (ANOSIM), $P < 0.01$] (Fig. 1B) and have different diversities of protein-encoding genes—with communities at site 11 exhibiting a higher metabolic diversity (Fig. 5A), coinciding with higher rates of nitrification (Fig. 1A). The different rates of nitrification are furthermore consistent with different clades of subseafloor amoA genes at sites 11 and 12 (Fig. 4B). However, a single 16*S* rRNA gene OTU of Thaumarchaeota dominates at both sites (Fig. 4A). This indicates that two different

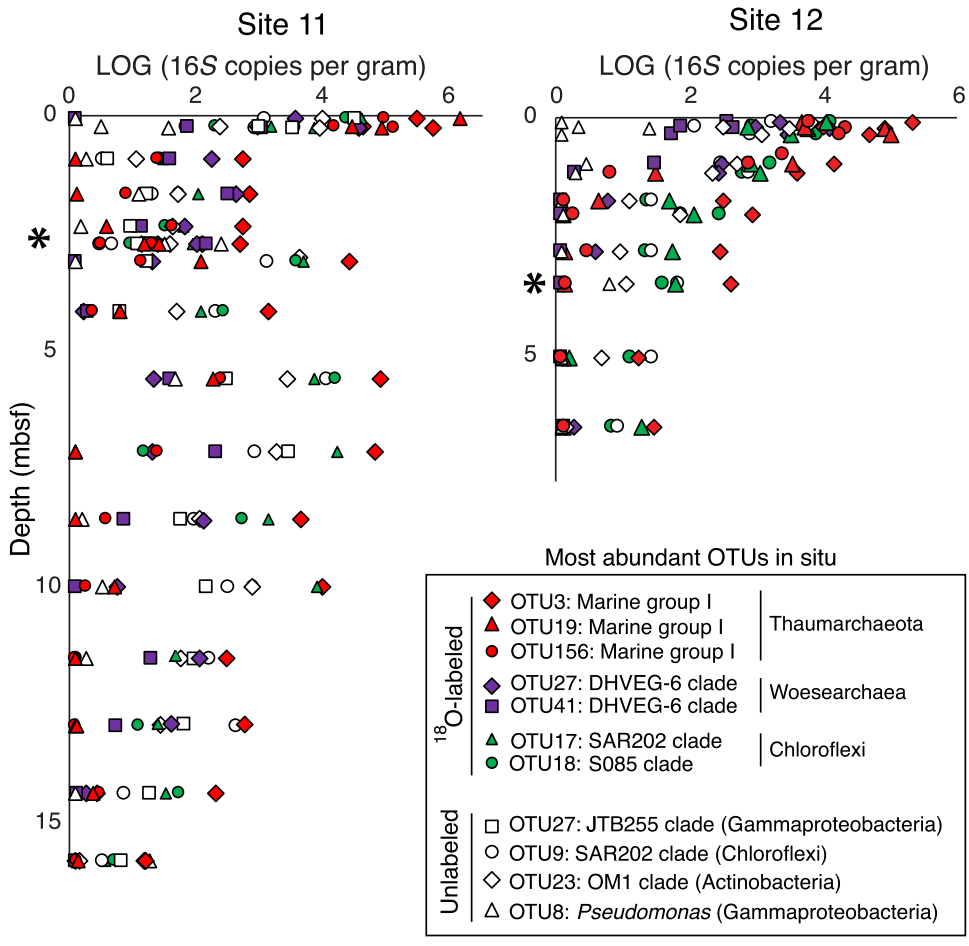

Fig. 3. In situ distributions of the most abundant populations and ^{18}O-labeling. Shown are the most abundant populations in situ, indicating those that were viable (^{18}O-labeled) in $H_2{}^{18}O$ incubations. Asterisks show the depths for ^{18}O-labeling incubations. Note that a single viable population of AOA (Thaumarchaeota), OTU3, dominated the microbial communities at both sites and that each meter represents ca. 1 million years of sediment deposition (thus, the deepest sample is ca. 15 million years old).

ammonia-oxidizing variants within this dominant AOA population are selected at sites 11 and 12, and the AOA variant selected at site 11 possibly underlies the higher nitrification rates compared to site 12.

The in situ dominance of Thaumarchaeota in this energy-starved setting over such long time scales is consistent with the adaptation of marine AOA to oligotrophic settings having low nutrient fluxes (*11*, *30*) such as ammonia (*31*), which is generally below detection in oxic subseafloor clay (*9*). Benthic AOA typically represent separate lineages from planktonic groups (Fig. 4B) (*16*), which may correspond to different physiologies, allowing benthic AOA to exploit subseafloor sediment environments (*32*). Thus, we performed functional metagenomics to gain insights into the potential metabolism underlying the success of these AOA dominating the subseafloor aerobic microbial communities for million-year time scales.

The metagenomes from each sample were sequenced to an average depth of 15 million reads, and de novo assembly resulted in a total of 177,498 contigs across all samples sequenced (table S3). A total of 66 to 95% of the raw reads could be mapped back to the contigs, indicating that the de novo assembled contigs represented the majority of the data obtained (table S3). There is a trend of exponentially decreasing diversity of functional genes with depth at both sites ($r = 0.95$), with the number of protein-encoding genes decreasing faster with time at site 12 compared to site 11 (Fig. 5A). Consistent with the dominance of AOA in the 16*S* rRNA and amoA gene datasets (Fig. 1), metagenomes from both sites are dominated by protein-encoding genes with highest similarity to Thaumarchaeota (Fig. 5B). All amoA genes detected in the metagenomes had highest similarity to Thaumarchaeota. After the Thaumarchaeota, protein-encoding genes throughout the sampled depths at both sites were dominated by the Chloroflexi, Deltaproteobacteria, and Candidate Phyla Radiation (Fig. 5B). The nitrite-oxidizing groups Nitrospirae and Nitrospinae are also represented in the metagenomes, as well as the ANME-2d and NC10 groups in relatively lower abundance (Fig. 5B).

The relative abundances of metabolic pathways and functional gene categories observed in the metagenomes are generally consistent for both sites sampled (Fig. 5C). For example, the hydroxypropionate/hydroxybutyrate (HP/HB) pathway and amoA genes from Thaumarchaeota dominated at each site. Open reading frames (ORFs) that encode proteins with high similarity to those involved in the reductive tricarboxylic acid (rTCA) cycle were detected only at site 11 in relatively low abundance (Fig. 5C), indicating that the organisms encoding this pathway are present at lower abundance than those encoding the HP/HB carbon fixation pathway. Deep-ocean nitrite-oxidizing bacteria (NOB) fix carbon via the rTCA cycle, representing a major component of deep-sea

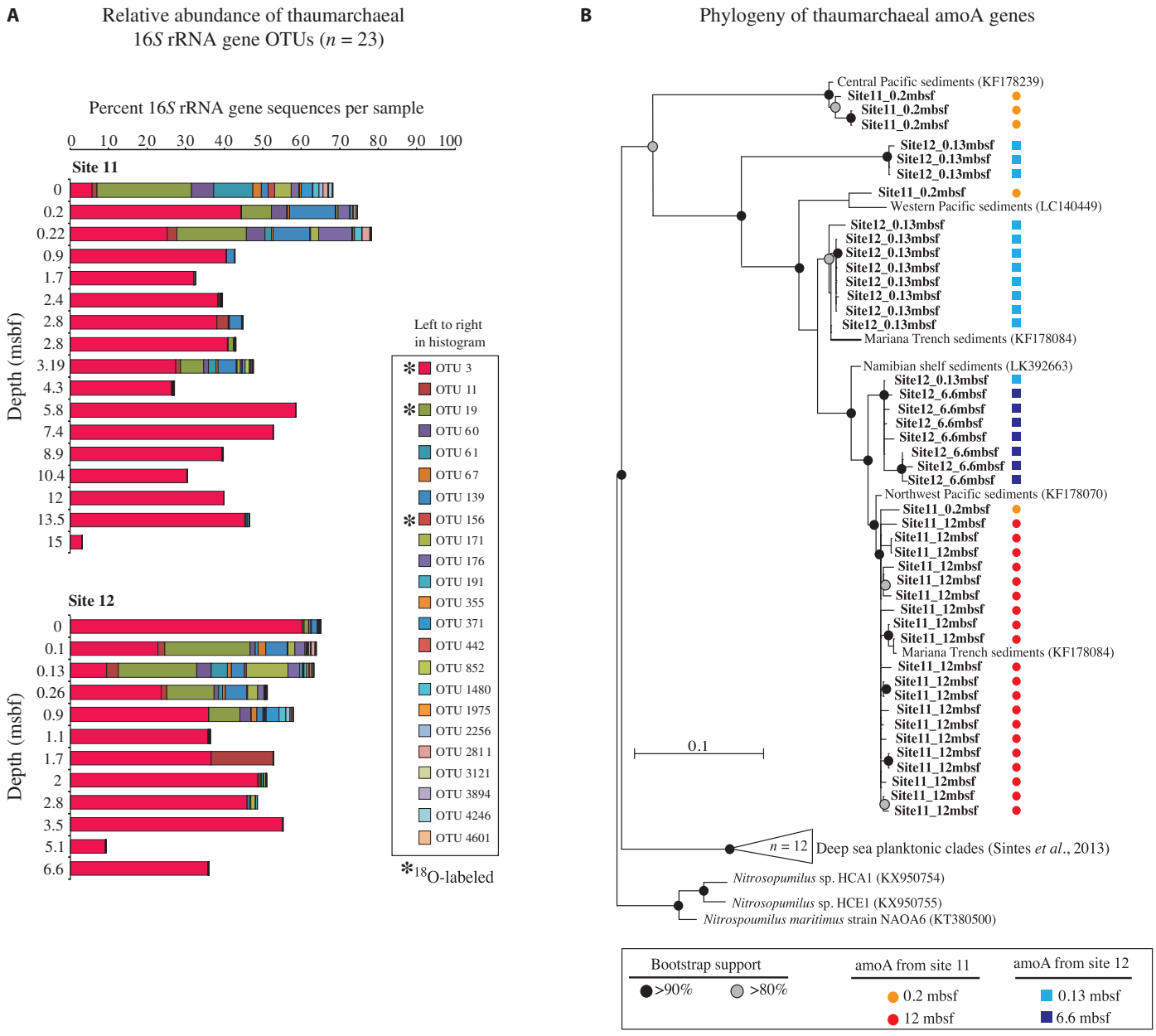

Fig. 4. Diversity of 16*S* rRNA and amoA genes from AOA. (**A**) Histograms showing the relative abundance of AOA OTUs based on 16*S* rRNA gene data. Asterisks mark the OTUs that were ^{18}O-labeled in the long-term incubations. (**B**) Phylogenetic analysis (PhyML) of amoA encoding genes from AOA at sites 11 and 12. Note that amoA genes from sites 11 and 12 form two separate bootstrap-supported clades consisting primarily of deeper (6.6 and 12 mbsf) sequences, which likely derive from the highly abundant OTU3 in these deeper intervals [in (A)]. Tree is based on an alignment of 655 nucleotides under a general time reversible (GTR) model of evolution with four rate categories. Bootstraps were calculated from 100 replications.

bacterioplankton (*33*). Our results indicate that NOB carbon fixation via rTCA also has the potential to proceed in deep subseafloor oxic sediment.

ORFs that encode proteins with similarity to archaeal viruses and bacteriophages at both sites show potential for regeneration of microbial necromass. Viruses have been inferred to be active in subseafloor sediment (*34*), and virus-like particles are abundant in oxic abyssal clay from the South Pacific Gyre (*35*). Viruses may play a key role in regenerating microbial necromass in marine sediment (*36*), possibly enabling subsistence over long time scales (*19*). Increased growth in the presence of the eDNA extract relative to a control (Fig. 2) indicates that microbes in oxic subseafloor oxic sediment have the potential to use necromass (e.g., eDNA) released from viral lysis. Viral lysis of AOA could also explain the minimal net growth of actively growing (^{18}O-labeled) AOA in the long-term incubations (fig. S4, B and C), keeping standing stocks of AOA low. Genes encoding proteins with high similarity to thaumarchaeal viruses (*37*) were relatively abundant in the metagenomes compared to bacteriophages (Fig. 5C).

H_2 generated by radiolysis of water has been proposed as an important energy source in oxic abyssal clay (*5*). The recovered metagenomes

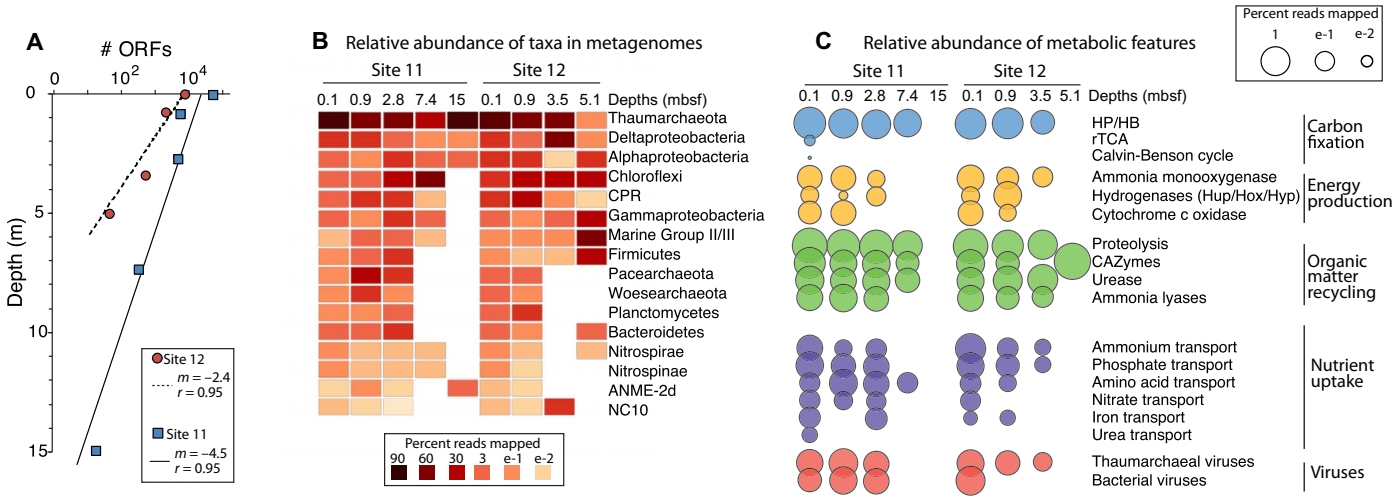

Fig. 5. Taxonomic representation and metabolic potential in metagenomes. (**A**) The number of ORFs as a function of depth at sites 11 and 12. (**B**) Relative abundance of taxonomic groups represented in the metagenomes. (**C**) Relative abundance of metabolic functions in the metagenomes. The amoA and HP/HB cycle genes were only encoded by Thaumarchaeota (AOA). In both (B) and (C), "percent reads mapped" refers to the percentage of raw reads mapped to ORFs encoding contigs.

support this hypothesis as ORFs homologous to Hup/Hox/Hyp hydrogenases involved in H_2 oxidation (*38*) are relatively abundant and detected at both sites (Fig. 5C). The majority have highest similarity to a NiFe-hydrogenase of a "novel Proteobacteria taxon" found in the surface sediment of the South Pacific Gyre (table S4) (*39*). Hydrogenases were also similar to those detected in terrestrial subsurface metagenomes (table S4) from a suboxic/anoxic aquifer (*40*), indicating that multiple groups of H_2-oxidizing microorganisms subsist in the oxic subseafloor sediment. The results indicate that energetically favorable aerobic H_2 oxidation (*41*) is a potentially important form of energy metabolism deep below the abyssal seafloor. Some representatives of the NOB and Woesearchaea, groups that are present in the 16*S* data (Fig. 1A), are also capable of aerobic H_2 oxidation (*38*) (*42*). However, hydrogenases from these groups were not detected in the metagenomes, possibly because of their lower abundance (Figs. 1 and 5B).

Carbon-fixation pathways in the metagenomes are dominated by the HP/HB cycle (Fig. 5C) that was encoded exclusively by Thaumarchaeota (AOA). The dominance of Thaumarchaeota (Figs. 1A and 5B) in this energy-starved setting may be partly explained by their use of a modified HP/HB cycle that is the most energy-efficient aerobic carbon fixation pathway (*43*). An energetically efficient metabolism can be expected to improve fitness in oxic sediment, where subsisting cells must cope with constant energy limitation (*4*). Moreover, the capacity of some AOA to perform mixotrophy (*17*, *28*, *44*, *45*) may allow them to supplement their carbon demand with organic carbon. Not all *amoA*-carrying Thaumarchaeota are obligate chemolithoautotrophs (*46*).

The metagenomes demonstrate a capacity for AOA mixotrophy, as genes encoding thaumarchaeal transmembrane transporters for organic matter including peptides, amino acids, and sugars were all detected (Fig. 6A). The metabolic potential for transport of peptides and amino acids is orders of magnitude greater than that of sugars and urea in these metagenomes, indicating that peptides and amino acids are important carbon sources for AOA mixotrophy (Fig. 5A). This is consistent with other deep-sea planktonic AOA exhibiting a mixotrophic metabolism, including the utilization of organic nitrogen compounds (*45*). Transporters for ammonia lyases, deaminases, and proteases are primarily from AOA in nearly every sample at both sites (Fig. 6A). Ammonia lyases and deaminases are major classes of enzymes responsible for producing ammonia during the degradation of organic nitrogen compounds (amino acids, nucleotides, and proteins), thus indicating that AOA dominated over bacteria in terms of potential ammonia regeneration. These metabolic characteristics of subseafloor AOA are consistent with experiments that showed that planktonic marine AOA use organic nitrogen for mixotrophic growth (*47*, *48*).

In abyssal seafloor sediment, AOA have been proposed to profit mainly from ammonia produced by deaminating heterotrophic bacteria (*39*). However, our deep oxic subseafloor metagenomes illustrate that the metabolic potential for utilization of organic nitrogen and deamination is dominated by mixotrophic AOA that have the potential to oxidize regenerated ammonia via amoA at the source of its creation: inside AOA cells (Fig. 6B). Oxidation of ammonia produced via intracellular deamination represents a fitness advantage in this energy-starved setting, markedly limiting diffusive loss of energy-yielding ammonia from the cell. Such a potential ammonia-concentrating mechanism would provide a fitness advantage for AOA in oxic sediment, where ammonia concentrations are extremely low (*9*). Unique concentrating mechanisms have been proposed as an adaptation of deep-sea planktonic AOA ecotypes to low ammonia concentrations (*28*). Such a mechanism is in line with the "survival of the fewest" model for aerobic subseafloor life (*49*), whereby energetic efficiency provides a selective advantage for long-term microbial subsistence in the oxic seabed underlying oligotrophic regions of the ocean.

Aerobic oxidation of the ammonia produced intracellularly via AOA deamination has the potential to provide additional energy [in the form of adenosine 5′-triphosphate (ATP)] to fuel the HP/HB carbon fixation cycle (Fig. 6B), which is dependent on ATP (*43*). The metabolic potential for coupling of chemolithoautotrophic and mixotrophic metabolism in this manner is correlated with improved fitness, since the genes encoding essential components of such metabolic coupling are dominated by AOA (Fig. 6A) and AOA are the most

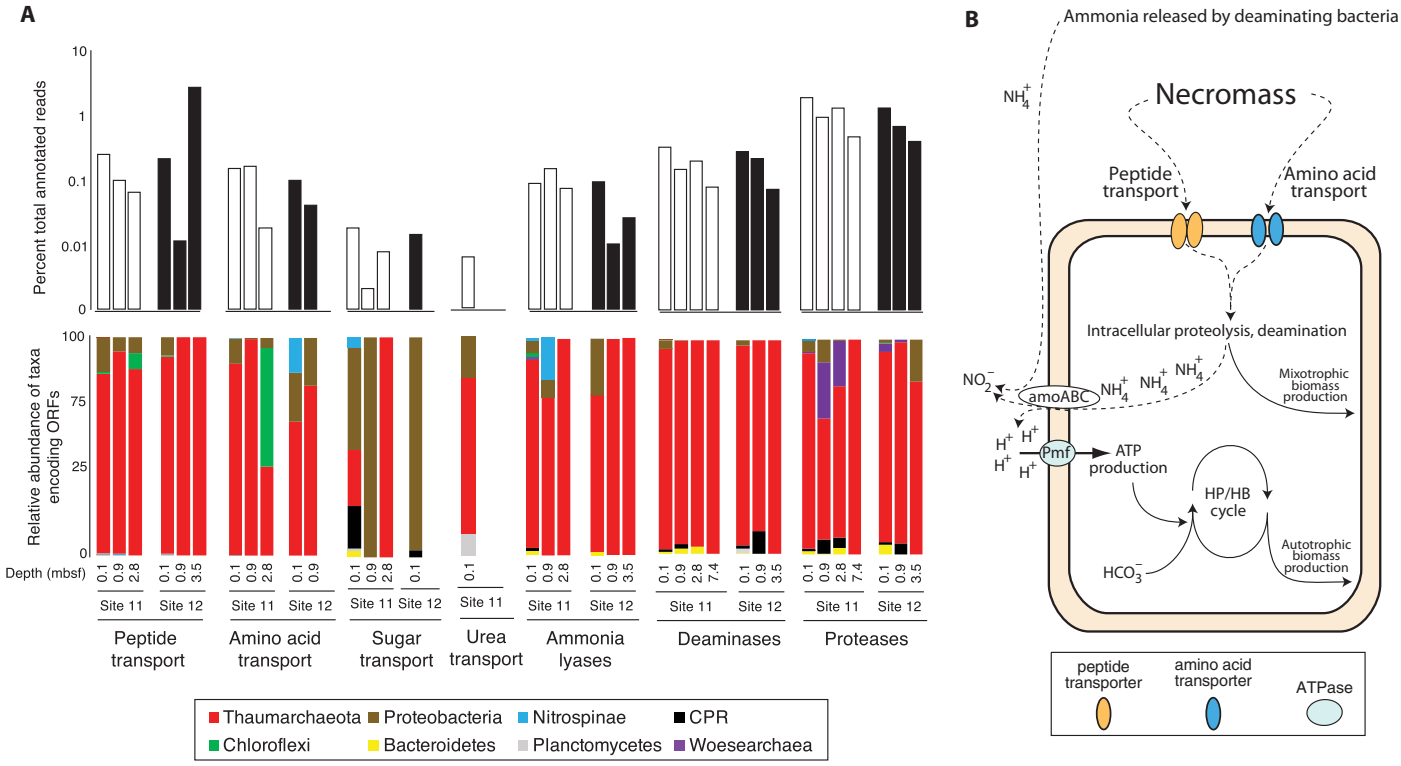

Fig. 6. Metabolic potential for mixotrophic deamination is dominated by Thaumarchaeota. (**A**) Top shows the relative abundance of ORFs in either site 11 (white bars) or site 12 (black bars), corresponding to transporters of organic matter, deaminating (ammonia lyases and deaminases), and proteolytic (proteases) enzymes. Bottom shows the taxonomic affiliation of the same ORFs. (**B**) Conceptual model of how AOA proteolysis and deamination could provide carbon sources for mixotrophic growth and additional energy (ATP) for chemolithoautotrophic growth. The outer lines represent the archaeal cell membrane, which is less permeable and may help to reduce loss of intracellular regenerated ammonia. The "H^+" represents protons derived from oxidation of ammonia that can be used to produce additional energy (ATP) required to fix carbon via the HP/HB cycle. pmf, proton motive force; CPR, Candidate Phyla Radiation.

abundant group throughout the sampled sediment sequence at both sites (Figs. 1, 3, and 4). Obligatory mixotrophic growth of marine AOA occurs using α-ketoglutaric acid as a carbon source (*44*). Since α-ketoglutaric acid is the keto acid produced by the deamination of glutamate, it is plausible that the AOA deaminate amino acids, such as glutamate, oxidize the ammonia for additional energy and then assimilate the resulting deaminated organic carbon (e.g., α-ketoglutaric acid) for building biomass and/or cellular maintenance processes (Fig. 6B).

Anaerobic archaea are ubiquitous and often dominant in anoxic energy-starved marine sediment (*50*–*53*), which has been attributed to several adaptations of anaerobic archaea to energy-stressed anoxic environments (*54*). A major adaptation of archaea for energy stress is often presumed to be the lower permeability of their cell membrane compared to bacteria, which allows them to reduce energy loss (*54*). Our study shows that this general adaptation of archaea may also help populations of aerobic archaea (AOA) to dominate within oxic subseafloor ecosystems by possibly limiting diffusive loss of intracellularly regenerated ammonia. Oxidizing intracellular regenerated ammonia produced via deamination of organic nitrogen would allow these mixotrophic AOA to produce additional ATP that is needed to fix carbon in the HP/HB cycle (*43*). As the ATP requirement for the highly energy efficient carbon fixation cycle in Thaumarchaeota is already relatively low (*43*), the unique mechanism of reducing energy loss shown here would explain how these aerobic archaea can subsist in larger abundance compared to other taxa for millions of years under constant energetic stress in oxic subseafloor sediment.

MATERIALS AND METHODS

Sampling

All samples were taken by Cruise KN223 of the *R/V Knorr*, in the North Atlantic, from 26 October 2014 to 3 December 2014 (Woods Hole, MA to Woods Hole, MA). At both site 11 (22°47.0′ N, 56°31.0′ W, water depth ~5600 m) and site 12 (29°40.6′ N, 58°19.7′ W, water depth ~5400 m), successively deeper subseafloor cores were taken with a multicorer (to ~0.4 mbsf), a gravity corer (to ~3 mbsf), and the 45-m Woods Hole Oceanographic Institution (WHOI) "long corer" piston-coring device (www.whoi.edu/projects/longcore/) (to ~28 mbsf) (table S1). Additional details of sampling at sites 11 and 12 are described by D'Hondt *et al.* (*4*). Subsamples from the core sections for DNA extraction were sampled on board the ship immediately after retrieval with sterile 60-ml syringes with the Luer taper end cut off and frozen immediately at −80°C before DNA extraction. Subsamples for the ^{18}O-labeling experiment were sampled from the core sections in the same manner but were stored at +4°C before incubation setup.

Pore water chemistry

Sedimentary interstitial water samples for nitrate analyses were obtained by standard Rhizon soil moisture samplers (*55*) (Rhizosphere Research Products, Wageningen, The Netherlands). Rhizon sampling extracted pore water from the sediment core by suction filtering through thin tubes of hydrophilic porous polymer with a mean pore diameter of 0.1 μm. Rhizons (5-cm filter in length) were inserted into one end of a whole round core section, and a total volume of up to

10 ml of interstitial water was extracted. Before deployment, Rhizon samplers were soaked in 18 megohms of deionized water for several hours, followed by rinsing with 30 ml of deionized water that was suction-filtered through each Rhizon. After washing, Rhizons were left to dry on filter paper. Nitrate concentrations were measured shipboard using a Metrohm 844 UV/VIS Compact IC with a 150 mm by 4.0 mm Metrosep A Supp 8 150 column. Approximately 0.8 ml of interstitial water was injected manually into a 250-µl sample loop; the sample was then eluted from the column with a 10% NaCl solution. Absorbance at 215 nm was used for quantification. A 50 µM sodium nitrate standard was run after every fifth or sixth sample to correct for instrument drift. Dissolved oxygen concentrations in the equilibrated core sections were measured with needle-shaped optical O_2 sensors (optodes) (PreSens, Regensburg, Germany) as described previously (*56*). The dissolved O_2 data from Expedition KN223 were first reported by D'Hondt *et al.* (*4*). They are archived and available online in the Integrated Earth Data Applications (IEDA) database (http://get.iedadata.org/doi/100519).

DNA extraction

Subcores sampled aseptically with sterile syringes were subsampled aseptically in an ultraviolet (UV)–sterilized DNA/RNA clean HEPA-filtered laminar flow hood. To reduce contamination, the paraffin was removed and the outer 3 cm of sediment was pushed out of the syringe, which was then sliced off with a red-hot, sterile spatula. A second unused, sterile, spatula was used to carefully sample the uncontaminated center of the remaining core sample inside the syringe. DNA extraction was extracted from 10 g of sediment using the method of Orsi *et al.* (*57*), amended with two freeze–thaw steps after homogenization. Briefly, 10 g of sediment was transferred into 50 ml of Lysing Matrix E tubes (MP Biomedicals) containing silica glass beads and homogenized for 40 s at 6 m/s using a FastPrep-24 5G homogenizer (MP Biomedicals) in the presence of 15 ml of preheated (65°C) sterile-filtered extraction buffer [76 volume % 1 M $NaPO_4$ (pH 8), 15 volume % 200 proof ethanol, 8 volume % MoBio's lysis buffer solution C1, and 1 volume % SDS]. The samples were incubated at 99°C for 2 min and frozen overnight at −20°C, thawed, and frozen again at −20°C overnight, followed by additional incubation at 99°C for 2 min and a second homogenization using the settings described above. The additional freeze–thaw steps, particularly freezing overnight, were found to increase DNA yield 2- to 10-fold. After the second homogenization, the samples were centrifuged for 15 min, and the supernatants were concentrated to a volume of 100 µl using 50-kDa Amicon centrifugal filters (Millipore). Coextracted PCR-inhibiting humic acids and other compounds were removed from the concentrated extract using the PowerClean Pro DNA Cleanup Kit (MoBio). Extraction blanks were performed alongside the samples to assess laboratory contamination during the extraction process.

Quantitative polymerase chain reaction

DNA was quantified fluorometrically using a Qubit with a double-stranded DNA high-sensitivity kit (Life Technologies). qPCR was performed using the custom primer dual indexed approach that targets the V4 hypervariable region of the 16*S* rRNA gene (*58*) using updated 16*S* rRNA gene primers 515F/806R (515F, 5′-GTGYCAGCMGCCGCGGTAA-3′; 806R, GGACTACNVGGGTWTCTAAT) that increase coverage of ammonia oxidizing archaea and other marine strains (*59*). To measure the abundance of amoA genes from archaea, the primers Arch amoA-1F (STAATGGTCTGGCTTAGACG) and Arch amoA-2R (GCGGCCATCCATCTGTATGT) were used (*16*). qPCR reactions were prepared using an automated liquid handler (pipetting robot). The epMotion 5070 (Eppendorf) was used to set up all qPCR reactions and standard curves as described previously (*60*). The efficiency values of the qPCR was <90%, and R^2 values were >0.95%. qPCR was performed using white 96-well plates as this was found to increase the signal-to-noise ratio in the SYBR green assay twofold compared to clear plates. The technical variability of 16*S* rRNA gene qPCR measurements was determined to be consistently <5% using the epMotion 5070.

16*S* rRNA and amoA gene sequencing

Barcoded V4 hypervariable regions of amplified 16*S* rRNA genes were sequenced on an Illumina MiniSeq following an established protocol (*58*). This yielded a total of >20,000,000 raw sequencing reads that were then subjected to quality control. To quality control the OTU picking algorithm for the data, we also sequenced a "mock community" alongside our environmental samples. The mock communities contained a defined number of species ($n = 18$) all containing 16*S* rRNA genes >3% difference (*58*). USEARCH version 10.0.240 was used for quality control and OTU picking (*61*), and OTUs were clustered at 97% sequence identity. The taxonomic relationship of OTU representative sequences was identified by BLASTn searches against SILVA database (www.arb-silva.de) version 128. To identify contaminants, 16*S* rRNA genes from extraction blanks and dust samples from the lab were also sequenced. These 16*S* rRNA gene sequences from contaminants were used to identify any contaminating bacteria in our oxic abyssal clay samples. All OTUs containing sequences from these "contaminant" samples were removed before downstream analysis.

qPCR values of 16*S* rRNA genes in DNA extraction blanks were consistently $<10^2$ copies per extraction, and thus, we used 10^2 copies to define our detection limit for the abyssal clay samples. Consistent with this, high-throughput sequencing of amplicons with qPCR values $<10^2$ copies per gram of sediment had up to 50% sequence representation from contaminant taxa, whereas samples with values $>10^2$ copies per gram of sediment had <5% representation from contaminant taxa. This further supported our definition of $<10^2$ as a realistic detection limit. Using samples that had 16*S* rRNA gene copies $>10^2$ copies per gram of sediment, we were able to analyze microbial communities down to ca. 15 mbsf at site 11 and ca. 8 mbsf at site 12 (Fig. 1).

Thaumarchaeal amoA genes amplified via qPCR using the method described above were cloned using the TOPO TA Cloning Kit and Sanger-sequenced at the LMU Munich Sequencing Service at the Faculty of Biology (www.gi.bio.lmu.de/sequencing). Before phylogenetic analysis, the reads were quality trimmed in CLC Genomics using the default settings for quality control.

Experimental setups

DNA-SIP experiments with $H_2{}^{18}O$ were set up at two North Atlantic coring sites: sites 11 and 12 from depths of 2.8 and 3.5 mbsf, respectively. Before setting up the incubations, the subcores were sampled with sterile syringes using the sample aseptic technique used for the DNA extraction. For each sample depth, 7 g of abyssal clay was placed into sterile 20-ml glass flasks and incubated with 4 ml of sterile artificial seawater composed of either $H_2{}^{18}O$ (97% atomic enrichment) or unlabeled artificial seawater. Vials were crimp-sealed, with an oxygenated headspace of approximately 10 ml, and incubated at

8°C. The water content of the clay was measured to be approximately 40% (±5%) of the total weight. This initial water content diluted the final concentration of added $H_2{}^{18}O$ to be ca. 60% of the total water content of the sample. The artificial seawater was different from the pore water at depth because there was no added nitrate, but there was also no added ammonia, which should be similar to the in situ conditions where ammonia is generally below detection (*9*). Oxygen was measured continuously throughout the incubations using noninvasive fiber-optic measurements as described previously (*62*). Small fluctuations in the oxygen measurements in the killed control, and experimental incubations (fig. S3), were likely due to temperature fluctuations of the incubator itself (±1°C), since the noninvasive fiber-optic oxygen sensor spots are temperature sensitive (*62*).

To assess the preservation potential of eDNA, and its ability to bias our study that is based on DNA from living organisms, we monitored microbial growth in the presence and absence of added eDNA over a 210-day incubation experiment. eDNA extracted from a culture of *Rhodococcus erythropolis* was added to sediment slurries from 2.8 mbsf at site 11 at a concentration of 5 ng/g. Microbial growth was measured over time with 16*S* rRNA gene qPCR and also in a control that did not receive the eDNA. As a second control, we added eDNA to autoclaved (dead) sediment. DNA was extracted from each time point and measured with qPCR using the methods described above.

Density gradient fractionation and qPCR

We used Tag-SIP (*63*, *64*) to measure the atom % ^{18}O enrichment of actively growing microbial taxa following the equations described previously for ^{18}O-SIP data (*21*). Briefly, after 7 and 18 months of incubation, DNA was extracted and subjected to cesium chloride (CsCl) density gradient centrifugation as described previously (*60*). The same 16*S* 515F/806R primers (described above) were used in qPCR (described above) to determine density shifts in the peak DNA of buoyant density for each incubation. 16*S* rRNA gene amplicons from each fraction resulting from the density gradient fractionation were Illumina sequenced as described above. To identify contaminants that may have entered during the fractionation process, we also included in the sequencing run extraction blanks from the SIP fractionation. OTUs containing sequences from extraction blanks were removed.

Calculating ^{18}O enrichment

Excess atom % ^{18}O enrichment was calculated for each taxon according to the equations for quantifying per OTU atomic enrichment from Tag-SIP data provided by Coskun *et al.* (*60*). The atom fraction excess of ^{18}O for each OTU ($A_{OXYGENi}$) accounts for the background fractional abundance of ^{18}O (0.002000429) using the following formula

$$A_{OXYGENi} = \frac{M_{LABi} - M_{LIGHTi}}{M_{HEAVYMAXi} - M_{LIGHTi}} \cdot (1 - 0.002000429)$$

where M_{LABi} is the molecular weight of the 16*S* gene for taxon *i* in the labeled treatment, M_{LIGHTi} is the molecular weight of the 16*S* gene for taxon *i* in the unlabeled treatment, and $M_{HEAVYMAXi}$ is the theoretical maximum molecular weight of a fully labeled 16*S* gene with ^{18}O. The lowest detection limit in the density shifts were proposed as 0.0034 to 0.0042 g/ml (*65*), and we selected a conservative lowest limit of 0.005 g/ml for a significant shift. Therefore, throughout this study, we referred to OTUs meeting this criterion as those having incorporated the given substrate.

Metagenome library preparation, sequencing, and bioinformatics analysis

Before library preparation, whole-genome amplifications were performed on DNA extracts through a multiple displacement amplification step of 6 to 7 hours using the REPLI-g Midi Kit (QIAGEN) and following the manufacturer's instructions. We monitored amplification using SYBR green I (Invitrogen) on a CFX Connect qPCR machine, stopping amplifications once the exponential phase was reached. Metagenomic libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina). Quality control and quantification of the libraries were obtained on an Agilent 2100 Bioanalyzer System using the High Sensitivity DNA reagents and DNA chips (Agilent Genomics). Metagenomic libraries were diluted to 1 nM using the Select-a-Size DNA Clean and Concentrator MagBead Kit (Zymo Research) and pooled for further sequencing on the Illumina MiniSeq platform. Contigs were assembled on CLC Genomics Workbench v. 9.5.4 (QIAGEN) using a word size of 20, a bubble size of 50, and a minimum contig length of 300 nucleotides. Reads were then mapped to the contigs using the following parameters (mismatch penalty = 3, insertion penalty = 3, deletion penalty = 3, minimum alignment length = 50% of read length, and minimum percent identity = 95%). We then performed even further stringency controls by removing any contig that had less than 5× coverage, e.g., reads per kilobase mapped (RPKM). The final resulting dataset of contigs was then used for ORF searches and BLAST analysis. Protein-encoding genes and ORFs were extracted using FragGeneScan v. 1.30 and functionally annotated against a large microbial genome database using a bioinformatics pipeline as described previously (*66*). Cutoff values for assigning hits to specific taxa were performed at a minimum bit score of 50, a minimum amino acid similarity of 60, and an alignment length of 50 residues.

For phylogenetic analyses, OTUs of AOAs were aligned with SINA online v.1.2.11 (*67*) and plotted in ARB (*68*) against the SILVA 16*S* rRNA SSU NR99 reference database release 132 (*69*). Closest environmental sequences with nearly full-length sequences (>1400 base pair) were selected as taxonomic references and used to calculate trees using the maximum likelihood algorithm RAxML implemented with the archaeal filter and advanced bootstrap refinement selecting the best tree among 100 replicates (*70*). Partial OTU sequences were added to the tree using the maximum parsimony algorithm without allowing changes of tree topology. Statistical analyses of beta-diversity were performed using R.Studio Version 3.3.0 (*71*) with the vegan package (*72*).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/6/eaaw4108/DC1

Table S1. Coring retrieval for the different samples at both sites.

Table S2. Abundance and atom % ^{18}O enrichment for OTUs that were ^{18}O-labeled at the end of the 18-month experiment.

Table S3. Sequencing and assembly statistics for the subseafloor metagenomes.

Table S4. Hydrogenase encoding ORFs from subseafloor metagenomes.

Fig. S1. Map of sample sites and pore water oxygen profiles from sites 11 and 12.

Fig. S2. Correlations between dissolved nitrate and oxygen at sites 11 and 12.

Fig. S3. Respiration and ^{18}O-labeling of microbial communities in long-term incubations.

Fig. S4. Identification of specific populations that were growing (^{18}O-labeled) in the long-term incubations.

Fig. S5. Phylogenetic analysis of 16*S* rRNA genes (V4 hypervariable region) from AOA OTUs (in red bold font) detected in this study.

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Acknowledgments: We thank N. Fierer and two anonymous reviewers for useful suggestions. **Funding:** This work was supported primarily by the Deutsche Forschungsgemeinschaft (DFG) project OR 417/1-1 granted to W.D.O. Preliminary work was supported by the Center for Dark Energy Biosphere Investigations project OCE-0939564 also granted to W.D.O. Publication of the manuscript was supported by the LMU Mentoring Program. The expedition was funded by the US National Science Foundation through grant NSF-OCE-1433150 to A.J.S, S.D., and R.P. R.W.M. led the expedition. This is a contribution of the Deep Carbon Observatory (DCO). S.D.W. acknowledges partial support from NASA Exobiology (NNX15AM04G). This is Center for Dark Energy Biosphere Investigations (C-DEBI) publication number 463. Portions of this material are based on work supported while R.W.M. was serving at the National Science Foundation. A portion of this work was performed as part of the LMU Masters Program "Geobiology and Paleobiology" (MGAP). **Author contributions:** W.D.O., S.D.W., A.J.S., R.W.M., D.C.S., and S.D. conceived the work and experimental approach. A.V., W.D.O., O.K.C., T.M., S.V., and E.R.E. contributed to the laboratory/bioinformatics analyses and experimental work. R.W.M., D.C.S., E.R.E., and R.P. obtained the samples during the KN223 *R/V Knorr* oceanographic expedition. W.D.O., S.D.W., R.W.M., D.C.S., E.R.E., A.V., S.V., and S.D. discussed and wrote the manuscript and commented on the paper. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** Data are publicly available through NCBI BioProject PRJNA473406. Metagenomes from sites 11 and 12 have Short Read Archive (SRA) BioSample accession numbers SAMN10924458 and SAMN10924459, respectively. The 16*S* data are available in SRA BioSample accessions SAMN10929403 to SAMN10929517. Additional data related to this paper may be requested from the authors.

Submitted 18 December 2018
Accepted 10 May 2019
Published 19 June 2019
10.1126/sciadv.aaw4108

Citation: A. Vuillemin, S. D. Wankel, Ö. K. Coskun, T. Magritsch, S. Vargas, E. R. Estes, A. J. Spivack, D. C. Smith, R. Pockalny, R. W. Murray, S. D'Hondt, W. D. Orsi, Archaea dominate oxic subseafloor communities over multimillion-year time scales. *Sci. Adv.* **5**, eaaw4108 (2019).

Science Advances

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Aurèle Vuillemin, Scott D. Wankel, Ömer K. Coskun, Tobias Magritsch, Sergio Vargas, Emily R. Estes, Arthur J. Spivack, David C. Smith, Robert Pockalny, Richard W. Murray, Steven D'Hondt and William D. Orsi

Sci Adv **5** (6), eaaw4108.
DOI: 10.1126/sciadv.aaw4108

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