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RESEARCH ARTICLE

Discerning autotrophy, mixotrophy and heterotrophy in marine TACK archaea from the North Atlantic

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One sentence summary: Mesothermal TACK archaea in the North Atlantic are predominantly heterotrophic or mixotrophic.

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ABSTRACT

DNA stable isotope probing (SIP) was used to track the uptake of organic and inorganic carbon sources for TACK archaea (Thaumarchaeota/Aigarchaeota/Crenarchaeota/Korarchaeota) on a cruise of opportunity in the North Atlantic. Due to water limitations, duplicate samples from the deep photic (60–115 m), the mesopelagic zones (local oxygen minimum; 215–835 m) and the bathypelagic zone (2085–2835 m) were amended with various combinations of ¹²C- or ¹³C-acetate/urea/bicarbonate to assess cellular carbon acquisition. The SIP results indicated the majority of TACK archaeal operational taxonomic units (OTUs) incorporated ¹³C from acetate and/or urea into newly synthesized DNA within 48 h. A small fraction (16%) of the OTUs, often representing the most dominant members of the archaeal community, were able to incorporate bicarbonate in addition to organic substrates. Only two TACK archaeal OTUs were found to incorporate bicarbonate but not urea or acetate. These results further demonstrate the utility of SIP to elucidate the metabolic capability of mesothermal archaea in distinct oceanic settings and suggest that TACK archaea play a role in organic carbon recycling in the mid-depth to deep ocean.

Keywords: TACK archaea; SIP; heterotrophy; mixotrophy; mesopelagic; North Atlantic

INTRODUCTION

Archaea are ubiquitous and abundant in the deep ocean (DeLong 1992; Fuhrman, McCallum and Davis 1992; DeLong et al. 1994; Stein and Simon 1996; Karner, DeLong and Karl 2001). However, their modes of nutrition remain largely unknown. Much of our current understanding of marine archaeal phyla is derived from DNA sequence data and the few

marine members of the domain that are currently in culture. It is therefore necessary to test TACK archaeal (Thaumarchaeota/Aigarchaeota/Crenarchaeota/Korarchaeota; Guy and Ettema 2011) metabolism using field samples with very little knowledge of their metabolic potential. The abundance of archaea throughout marine ecosystems would imply that their activity greatly influences the modes and efficiency of carbon transfer in

microbial systems. Additionally, the structure of the marine microbial food web plays a major role in oceanic carbon sequestration and the function of the marine ecosystem as a whole (Limardo and Worden 2015). The archaeal contribution to the microbial loop is, however, still poorly understood.

The earliest report of the metabolism of marine thaumarchaea (formerly classified as crenarchaea) from the field was by Ouverney and Fuhrman (2000). Using a microradiographic/fluorescent *in situ* hybridization method (STARFISH, aka MAR-FISH), the researchers demonstrated that archaea represent 14%–43% of the total microbial community at depth in the Mediterranean and Pacific, and ~50% of the archaea at 200 m accumulated ³H-amino acids. Later studies demonstrated the uptake of amino acids by archaea using MAR-FISH in the North Atlantic (Teira et al. 2004, 2006) and Antarctic Circumpolar Current (Herndl et al. 2005); in Arctic waters, the addition of radiolabeled amino acids, whole proteins, glucose and diatom extracellular polymeric substances all produced labeled thaumarchaeal cells (Kirchman et al. 2007). Unfortunately, MAR-FISH approaches have limited phylogenetic resolution and the method does not unambiguously demonstrate that a radiolabeled substrate enters central metabolism. Furthermore, it is unclear whether the positive MAR-FISH signal represents a few operational taxonomic units (OTUs) dominating the community with a specific metabolic capability, or if there are many different community members which share a broader metabolic repertoire.

In contrast to MAR-FISH, metagenomic analyses have shown that many thaumarchaea possess an ammonium monooxygenase (*amoA*) gene (Venter et al. 2004; Treusch et al. 2005), indicating nitrification plays an important role in archaeal metabolism in the water column (Francis et al. 2005; Francis, Beman and Kuypers 2007). Initially, an obligately autotrophic culture of *Nitrosopumilus maritimus*, an ammonia-oxidizing archaeum, was isolated from an aquarium (Könneke et al. 2005). Subsequently, other nitrifying archaeal isolates/co-cultures have been obtained from marine sediments, decaying leaf litter and deep agricultural soils (Park et al. 2010; Jung et al. 2014, 2016). These isolates and other enriched cultures of ammonia-oxidizing archaea (e.g. *Nitrosoarchaeum*) have demonstrated incorporations of ¹³CO₂ into lipids (Park et al. 2010; Jung et al. 2014, 2016; Berg et al. 2015). Several other studies support the chemoautotrophic fixation of carbon by marine archaea *in situ* (e.g. Wuchter et al. 2003; Ingalls et al. 2006). Alternatively, genomic results suggest that marine thaumarchaea may be capable of mixotrophy. Specifically, fosmid sequences from the sponge symbiont *Cenarchaeum symbiosum* revealed the presence of genes associated with autotrophic carbon assimilation, as well as an oxidative tricarboxylic acid cycle (Hallam et al. 2006). Thaumarchaea have also been found to contain genes for 3-hydroxypropionate carbon fixation as well as oligopeptide importers, supporting the concept of mixotrophy (Martin-Cuadrado et al. 2008). Two cultures of *Nitrosopumilus* (strains HCA1 and PS0) were found to require α -ketoglutaric acid to grow in culture (Qin et al. 2014; Kim et al. 2016). However, the role of α -ketoglutarate as a hydrogen peroxide scavenger rather than a metabolic cofactor has been demonstrated for an ammonium-oxidizing archaeal seawater isolate (DDS1; Kim et al. 2016). These metagenomic studies suggest metabolic potential via gene annotation. However, gene annotations can often be tenuous (low similarity) and it is difficult linking genomic information from TACK archaea in marine samples with specific phylogenetic markers, such as 16S rRNA genes. Furthermore, the presence of a gene does not confirm activity, only potential. Therefore, an empirical testing of potential

food sources is essential to definitively prove that a TACK archaeum is capable of metabolizing a particular substrate (Seyler, McGuinness and Kerkhof 2014).

Because most TACK archaea are not in culture, we opted for an SIP approach to determine if the TACK archaeal community is composed of varying levels of heterotrophs, autotrophs or mixotrophs. DNA stable isotope probing (SIP) was used on replicate samples from the North Atlantic Basin to determine the uptake of organic and inorganic ¹³C-carbon for specific OTUs (as defined by terminal restriction fragment length polymorphism (TRFLP) peaks of 16S rRNA genes; Avanniss-Aghajani et al. 1994). SIP methods can elucidate active microorganisms capable of incorporating ¹³C (or ¹⁵N) from specific compounds (Radajewski et al. 2000; Buckley et al. 2007). In SIP, a ¹³C-labeled substrate is used to generate ¹³C-labeled DNA from replicating microbes prior to separation from ¹²C-DNA in cesium gradients, and represents an intermediate approach between metagenomics and MAR-FISH. This method is constrained by the use of specific ¹³C-labeled substrates to generate ¹³C-labeled DNA for separation. Although the approach does not provide a broad representation of potential carbon sources, as does metagenomic analysis of metabolic and carbon assimilation pathways, SIP analysis can demonstrate unambiguous ¹³C incorporation into newly synthesized DNA. ¹³C-substrate concentrations are not similar to *in situ* conditions as with MAR-FISH because SIP requires an isotope atom % enrichment above ambient (generally >25%) to physically separate the newly synthesized ¹³C-DNA obtained during the incubations. SIP has much finer phylogenetic resolution than MAR-FISH, but does not measure *in situ* incorporation rates. This study focused on carbon uptake in marine thaumarchaea and crenarchaea, referred throughout the text as the TACK archaeal superphylum.

MATERIALS AND METHODS

In April 2012, samples were taken onboard the University-National Oceanographic Laboratory System (UNOLS) ship R/V Atlantis, as part of the US CLIVAR/CO₂ Repeat Hydrography program (Wanninkhof et al. 2013). This study was a cruise of opportunity, replicating the World Ocean Circulation Experiment line A20 (longitude 52°W), previously conducted in 2003 (Fig. S1, Supporting Information). As such, water samples were very limited (often <5 L per hydrocast). Therefore, SIP microcosms were established at six stations (indicated by yellow dots) from the epipelagic (60–115 m), the mesopelagic (215–835 m) and the bathypelagic zones (2085–2835 m) at adjacent stations. Duplicate samples (1 L; at stations 30, 45 and 60) were amended with either ¹³C sodium bicarbonate (5 mM)/¹²C sodium acetate (20 μ M) or ¹²C sodium bicarbonate (5 mM)/¹³C sodium acetate (20 μ M) as indicated in Fig. 1. Alternatively, duplicate samples (1 L; at stations 33, 48, 63) were amended with either ¹³C sodium acetate (20 μ M)/¹²C urea (20 μ M) or ¹³C urea (20 μ M)/¹²C sodium acetate (20 μ M). These ¹³C-substrates and concentrations were chosen based on previous findings in salt marsh sediments where the lower concentrations of ¹³C-organic substrates produced greater diversity of active thaumarchaea and bathyarchaea (formerly Miscellaneous Crenarchaeal Group) (Seyler, McGuinness and Kerkhof 2014). A complete list of SIP incubations is provided in Table S1 (Supporting Information).

The intention of this SIP dual amendment scheme was to probe nearly the same populations of TACK archaea in microcosms from similar stations/depths, while tracing the uptake

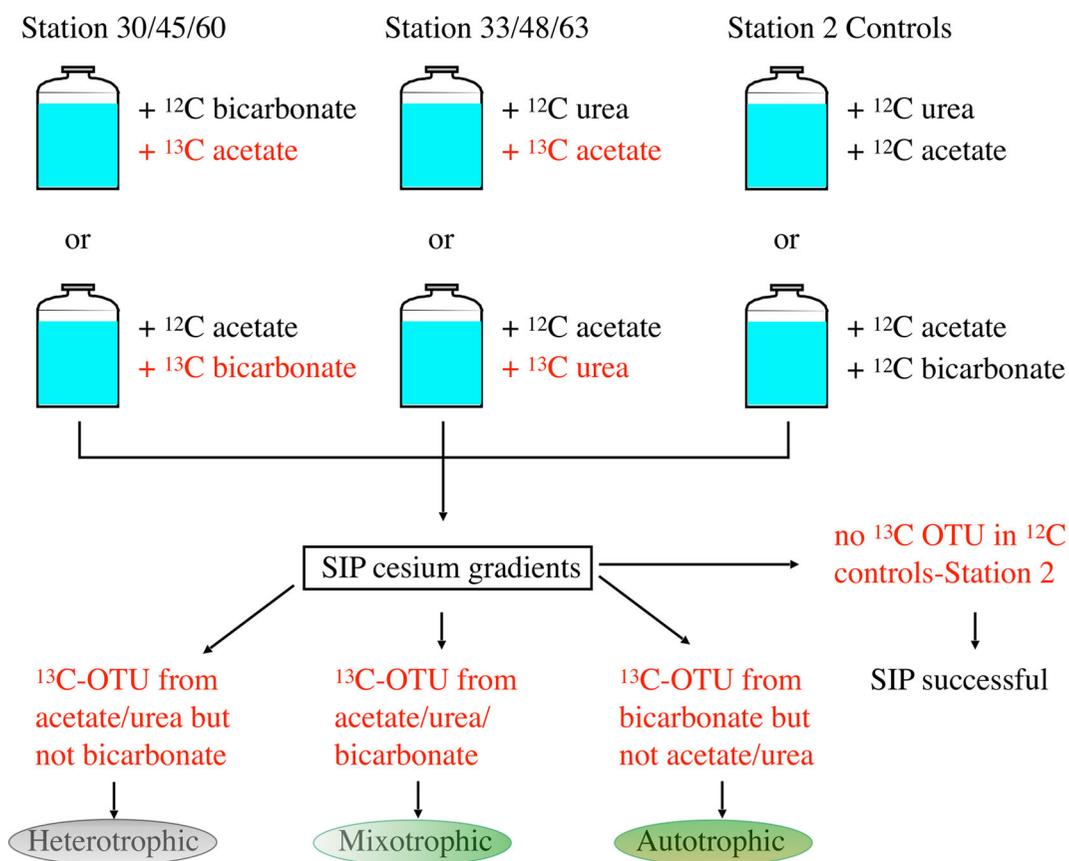


Figure 1. Classification scheme for determining if ^{13}C -labeled OTUs are autotrophic, heterotrophic or mixotrophic. Duplicate samples at each station/depth are supplemented with the ^{12}C or ^{13}C substrates indicated. Unambiguous growth on the ^{13}C is determined by negative amplification in ^{12}C -amended samples for a particular OTU in the ^{13}C carrier band.

of specific ^{13}C -labeled substrates. It was assumed that TACK communities at adjacent stations (e.g. stations 45 and 48) were nearly identical. Furthermore, it was assumed that a 48-h time point would be sufficient to assess ^{13}C -growth, based on previous data from the culturing of ammonia-oxidizing archaea (Könneke et al. 2005) and personal observations in salt marsh SIP incubations. Because of the limited sample volume, the reciprocal $^{12}\text{C}/^{13}\text{C}$ amendment experimental design maximized the possibility of obtaining a 'positive' signal from the SIP incubations while delineating 'negative' signals. For example, if an OTU was observed in the heavy band for ^{13}C -acetate amendments, that OTU should not be observed in the heavy band with ^{12}C -acetate amendments if the TACK archaeum can only utilize acetate (regardless of additional ^{13}C -amendments with different substrates). Likewise, a similar pattern should be observed with ^{13}C -urea or ^{13}C -bicarbonate if that particular substrate is a sole carbon source. However, this assumption will break down if both ^{13}C -acetate and ^{13}C -urea can be utilized or ^{13}C -acetate and ^{13}C -bicarbonate. This dual amendment approach was also implemented because if traditional ^{12}C negative controls were employed for each station/sample, only a single ^{13}C -carbon source could be tested on the cruise, given the limited amount of water available at each hydrocast station. However, it was possible early in the cruise to establish duplicate ^{12}C -controls and ^{13}C -amendments for acetate at station 2. These station 2 ^{12}C and ^{13}C -SIP experiments were incubated for 12, 24, 36, 48 and 72 h to verify the minimum time necessary to obtain a positive ^{13}C -signal for at least a single station. The negative SIP control using

^{12}C sodium acetate was incubated for 72 h. Most negative SIP controls by the criteria outlined above did not amplify under the conditions used in this study.

Finally, bicarbonate was only employed in SIP incubations from the epipelagic and mesopelagic zones based on a prior report that found archaeal ammonia monooxygenase [*amoA*] gene copy numbers were not strongly associated with CO_2 fixation rates (e.g. Agogué et al. 2008). ^{13}C -Bicarbonate was added at ~2.5 times the average concentration in seawater as described above so the ^{13}C -uptake signal would not be obscured by high ambient levels of ^{12}C bicarbonate (Seyler, McGuinness and Kerkhof 2014). ^{12}C -Bicarbonate was also added to the ^{13}C -acetate amendment so any small change in pH did not suppress microbial activity. After the amendments, the SIP microcosms were incubated in covered containers on deck for 48 h to promote ^{13}C -incorporation, with a constant inflow of surface sea water to maintain stable temperature (on average, 22°C).

Biomass was collected from all SIP microcosms on a $0.2\text{-}\mu\text{m}$ filter using vacuum filtration. DNA extractions were performed using modified phenol-chloroform methods as described previously (Sakano and Kerkhof 1998). Frozen filters were suspended in $100\ \mu\text{l}$ buffer (50 mM glucose–10 mM EDTA–25 mM Tris [pH 8.0]) and rapidly frozen with liquid nitrogen and thawed in a 55°C bath four times. Buffer ($125\ \mu\text{l}$), $75\ \mu\text{l}$ EDTA and $100\ \mu\text{l}$ lysozyme solution (10 mg/ml) were then added, and the samples were agitated for 5 min. Fifty microliters of a 50% sodium dodecyl sulfate solution were then added to lyse the cells. The lysate was extracted twice with $800\ \mu\text{l}$ of a phenol-chloroform-isoamyl

alcohol mixture (25:24:1). DNA was precipitated by adding 50 μ l of 3.0 M sodium acetate, 2 μ l of glycogen (20 mg/ml) and 1 ml of 100% ethanol. The DNA was pelleted by centrifugation (16 000 \times g) at 4°C for 20 min, and resuspended in 100 μ l sterile ultrapure water. DNA (300 ng) from each sample was fractionated into 12 C and 13 C bands by isopycnic cesium chloride gradient ultracentrifugation at 80 000 rpm (200 000 \times g) for 36 h, in 600 μ l of 0.9 g/ml CsCl with 300 ng 13 C-labeled *E. coli* DNA as a carrier (modified from Gallagher *et al.* 2005). The use of carrier DNA produces a visible 13 C-lower band in the gradient, increases sensitivity and precludes the need to fractionate the gradient (Gallagher *et al.* 2010; Kerkhof *et al.* 2011; Seyler, McGuinness and Kerkhof 2014; Tuorto *et al.* 2014). Ten microliters were collected by pipette from the 12 C (upper) and 13 C (lower) bands, dialyzed against 10 mM Tris pH 8, and 2 μ l of each sample were amplified by nested PCR with 5'-fluorescently-labeled, 16S rRNA archaea-specific 21F (5'-TCCGGTTGATCCYGCCGG) and 958R (5'-YCCGGCGTTGAMTCCAATT) and thaum/crenarchaeota-specific 7F (5'-TTCCGGTTGATCCYGCCGGACC) and 518R (5'-GCTGGTWTACCGCGGGGCTGA) (Perevalova *et al.* 2003; Ionescu *et al.* 2009; Hong, Kim and Cho 2014; Seyler, McGuinness and Kerkhof 2014) primers. Amplicons (15 ng) were digested with *MnII* in 20 μ l volumes for 6 h at 37°C, and then precipitated using sodium acetate and 95% ethanol (McGuinness *et al.* 2006). Precipitated DNA was dried and resuspended in 19.7 μ l de-ionized formamide with 0.3 μ l ROX 500 size standard (Applied Biosystems (ABI); Foster City, CA). TRFLP fingerprinting was carried out on an ABI 310 genetic analyzer using Genescan software. Peak detection was set at 25 arbitrary fluorescent units. A constant 13 C-amplicon mass for SIP/TRFLP profiling was used for direct comparison of the number of active (i.e. 13 C label-incorporating) thaumarchaeal OTUs within the various samples.

To determine the phylogenetic affiliation of the various 13 C-archaeal peaks, an archaeal 13 C-amplicon clonal library was constructed from microcosms taken from the mesopelagic at station 63, using the Topo TA cloning kit as per the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA), with 16S rRNA archaea-specific 21F/958R primers. These primers were used for cloning because they overlap with the region for next generation 16S tag sequencing (see below), allowing us to draw connections between next-generation sequencing (NGS) and SIP results. Two hundred recombinant clones were screened in a multiplex format as in Babcock *et al.* (2007) to find clones (~900 bp) matching the 13 C-SIP peaks. Fourteen clones were identified and sequenced via Sanger methods using M13 primers (Genewiz Inc., South Plainfield, NJ, USA), yielding 11 unique clone sequences. Seven of these unique clones were matched to OTUs obtained via 16S Illumina sequencing using BLAST, and a maximum likelihood phylogenetic tree was reconstructed using 140 unambiguously aligned bases among 95 taxa (including 23 OTUs identified via 16S amplicon sequencing, see below) with Geneious analysis software (Guindon and Gascuel. 2003; Drummond *et al.* 2009). Genbank accession numbers for the cloned sequences are KT372904–KT372917.

DNA extractions for NGS were performed on a total of 55 filtered samples taken along the Atlantic transect at 16 stations from depths ranging from the surface to 5450 m (Table S2, Supporting Information), using the PowerWater kit from QIAGEN (Germantown, MD), as per the Earth Microbiome Project protocol (<http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/>). 16S rRNA gene amplicon sequencing was performed using the Illumina MiSeq platform on each sample using 515F (5'-AATGATACGGCGACCACCG AGATCTA-

CACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA) and 806R barcoded (5'-CAAGCAGAAGACGG CATACGAGATXXXXXXXXX XXXX AGTCAGTCAGCCGG ACTACHVGGGTWCTAAT) primers (Caporaso *et al.* 2011). Sample processing, sequencing and core amplicon data analysis were performed by the Earth Microbiome Project (www.earthmicrobiome.org; Thompson *et al.* 2017), and all amplicon sequence data and metadata have been made available through the public data portal (qiita.microbio.me/emp). The sequencing data from two samples were discarded via single rarefaction criteria. Data from the remaining 53 samples has been archived at the European Nucleotide Database (Study: ERP016287). Taxa were identified via comparison with the GreenGenes database (<http://greengenes.lbl.gov>), and beta diversity (weighted and unweighted unifrac) was summarized using QIIME version 1.7 (Caporaso *et al.* 2010). The 23 OTUs included in the phylogenetic tree were identified by an analysis of variance (ANOVA) test over depth regime (with a Bonferroni correction value of <0.05).

Quantitative PCR was performed on a subset (22 total) of these amplicon-sequenced samples to verify if the relative abundance of archaea and bacteria was consistent with other reports on archaeal community structure and if similar patterns of increases/decreases in archaeal signals with depth were observed. Agilent Technologies (Santa Clara, CA) Brilliant II SYBR Green qPCR Low ROX Master Mix was used following the manufacturer's protocol, using archaeal 16S A915F (5'-AGGAATTGGCGGGGAGCAG; DeLong 1992)/A1059R (5'-GCCATGCACCCWCCTCT; Yu *et al.* 2005) and bacterial 16S 1114F (5'-CGGCAACGAGCGCAACCC)/1200R (5'-CCATTGTAGCACGTGTGTAGCC; Reysenbach and Pace 1995) primers on a Stratagene MX3500P machine. Standard curves were generated using full-length bacterial or archaeal 16S rRNA genes from estuary surface sediments cloned into a vector and purified using a Zymo Research Zyppy Plasmid Prep Kit (Irvine, CA). Because the bacterial 16S primers amplified both the bacterial and archaeal positive controls, these primers were treated as universal prokaryote primers during analysis of the qPCR data.

RESULTS

The 16S rRNA gene amplicon sequencing results indicated bacteria comprised the majority of the total prokaryotic community, averaging 94% with a range of 83%–99%. Archaea ranged between 0.5% and 16% of the total community, averaging 5% (Fig. S2, Supporting Information). Higher percentages of archaea were most often found in the mesopelagic region (200–1000 m depths). The 16S rRNA gene NGS analysis indicated that archaeal populations were mostly members of phylum Euryarchaeota in the epipelagic zone (60–115 m), and thaumarchaea/crenarchaea between 215 and 5450 m, consistent with previous findings (Karner, DeLong and Karl 2001; Herndl *et al.* 2005). Parvarchaea or 'other' archaea were detected in relatively low numbers (between 0.1% and 8%) in the bathypelagic (2085–2835 m) and benthopelagic (4336–5450 m, 10 m above the seafloor) zones (Fig. S3, Supporting Information). Quantitative PCR suggested that the archaea form a greater percentage of the total prokaryotic population below the mixocline than would be inferred from the NGS data (Fig. S4, Supporting Information). In many cases, the archaeal percentage determined by qPCR was two to three times greater than the percentage estimated from the NGS sequencing data, with one sample more than an order of magnitude higher, possibly due to the differences in priming sets used for the assays or primer mismatch (Parada, Needham and Furhman

Table 1. Active OTUs detected in both duplicate SIP incubations for a particular depth/station/carbon source are indicated. The different ^{13}C -carbon sources are ^{13}C acetate (light gray), ^{13}C urea (medium gray), and ^{13}C bicarbonate (black). Active OTUs detected in one or both SIP incubations are depicted in Supp. Table 3.

TRF (bp)	North Equatorial Current (Sts 30/33)			Sargasso Sea (Sts 45/48)			North Atlantic Drift (Sts 60/63)		
	Epipelagic	Mesopelagic	Bathypelagic	Epipelagic	Mesopelagic	Bathypelagic	Epipelagic	Mesopelagic	Bathypelagic
64			n/a						n/a
66			n/a						n/a
68			n/a						n/a
80			n/a						n/a
82			n/a						n/a
83			n/a						n/a
116			n/a						n/a
117			n/a						n/a
124			n/a						n/a
196			n/a						n/a
223			n/a						n/a
265			n/a						n/a
385			n/a						n/a
452			n/a						n/a
465			n/a						n/a
467			n/a						n/a

 ^{13}C -acetate

 ^{13}C -urea

 ^{13}C -bicarbonate

2015). However, the relative increases and decreases in archaeal community contribution with depth between the two methods were largely in agreement with the exception of the 835 m sample (Fig. S4, Supporting Information).

SIP results indicated that a minimum incubation time of 48 h was required to observe archaeal DNA in the ^{13}C (lower) band with ^{13}C -amendments and no amplification in the ^{13}C (lower) band with ^{12}C -controls (Fig. S5, Supporting Information). Those microcosms at station 2 that were incubated for 12, 24 and 36 h did not produce signal in the ^{13}C (lower) band using thaum/crenarchaeal primers. TACK 16S rRNA gene amplicons from the ^{12}C (upper) and ^{13}C (lower) bands were profiled by TRFLP, and the resulting peaks were considered OTUs. A total of 76 TACK archaeal OTUs were identified in the CLIVAR cruise samples by TRFLP methods (data not shown). Only 16 of these OTUs were found to be active (i.e. capable of synthesizing ^{13}C -labeled DNA from the various ^{13}C -substrates being provided) in replicate SIP incubations (Table 1). However, 31 of the archaeal OTUs detected along the transect were identified as active in at least one of the SIP incubations (Table S3, Supporting Information). Generally, the highest amplification signal from active TACK OTUs (total arbitrary fluorescence in the entire TRFLP profiles) was observed in the ^{13}C -acetate additions from the mesopelagic zone along the entire transect (Fig. 2). The majority of this ^{13}C -acetate amplification signal was detected in both SIP duplicates.

A more detailed breakdown of the active TACK OTUs showed differences with respect to ^{13}C -carbon utilization, depth and station location. No active TACK OTUs were detected in duplicate epipelagic samples (Table 1). The active TACK OTUs in the mesopelagic were 23% autotrophic, 23% heterotrophic and 54%

mixotrophic at station 30/33. Only organic carbon uptake was observed in replicates at stations 45/48/60/63 (Fig. 3); however, bicarbonate uptake was observed in one of the replicates in the epipelagic at station 45 and in one bottle in the mesopelagic at station 60 (Table S3, Supporting Information). For the bathypelagic only heterotrophy was tested and most active TACK OTUs incorporated both ^{13}C -acetate and ^{13}C -urea in the SIP incubations (Table 1). For the OTUs that were detected in both duplicate and single SIP incubations, these patterns remained largely the same. In the epipelagic at stations 45/48, there was one active TACK OTU detected on ^{13}C -acetate, one OTU active on ^{13}C -bicarbonate and five OTUs active on ^{13}C -acetate and ^{13}C -bicarbonate. In the mesopelagic, at stations 30/33, 21% of the 19 active OTUs were heterotrophic, 58% mixotrophic and 21% autotrophic; the 9 active OTUs at stations 45/48 were exclusively heterotrophic; and the 17 active OTUs at stations 60/63 were 41% heterotrophic, 59% mixotrophic and 0% autotrophic. Overall, eight OTUs dominated the active TACK SIP/TRFLP profiles with respect to peak area (TRF 66, 82, 83, 116, 117, 124, 223 and 265). Six of these OTUs were mixotrophic (TRFs 66, 83, 117, 124, 223 and 265). The other dominant OTUs (TRF 82 and 116) were only associated with the uptake of ^{13}C -organic carbon. None of the dominant TACK OTUs appeared to be autotrophic.

In order to assess the phylogenetic affiliation of the various OTUs, a clone library was generated from the ^{13}C amplicons using universal archaeal primers to obtain longer sequence reads. The long read sequences retrieved in this study were distributed throughout the TACK archaea, and Marine Group II and III euryarchaea. Eleven unique clones were identified, with seven clones being members of the phylum Thaumarchaeota (corresponding to the dominant TRFs 82, 83, 116, 223 and 265

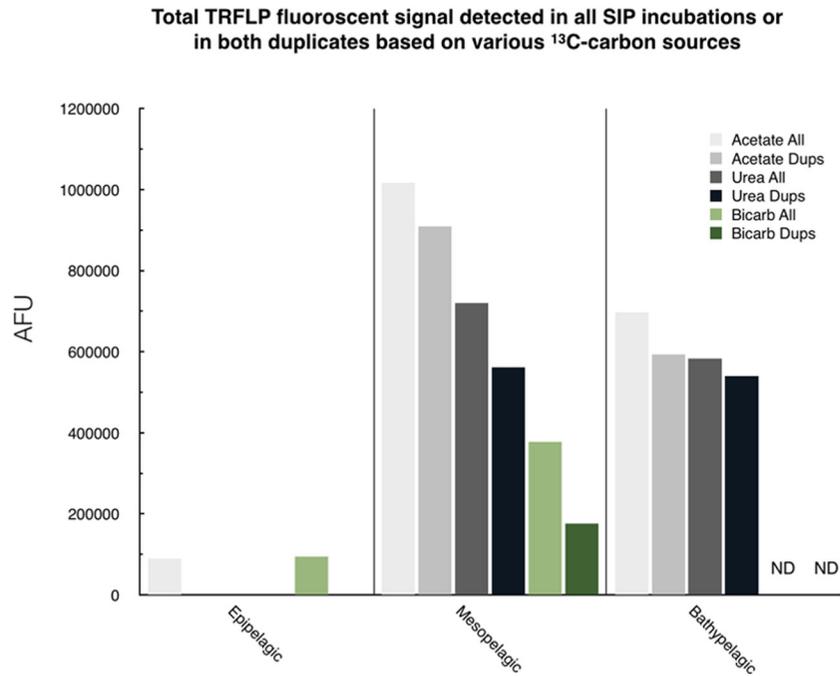


Figure 2. Histograms of arbitrary fluorescence from active OTUs in the total TRFLP profile based on location in the water column or carbon source (¹³C-acetate, ¹³C-urea, ¹³C-bicarbonate). Total AFU signal is presented in profiles where a specific ¹³C-labeled OTU is detected in duplicate incubations (Dups) and in all profiles containing some OTUs only detected in a single incubation (All). The profiles were normalized for the different number of SIP incubations for comparison (as acetate was tested at twice as many stations as urea and bicarbonate). ND = not determined.

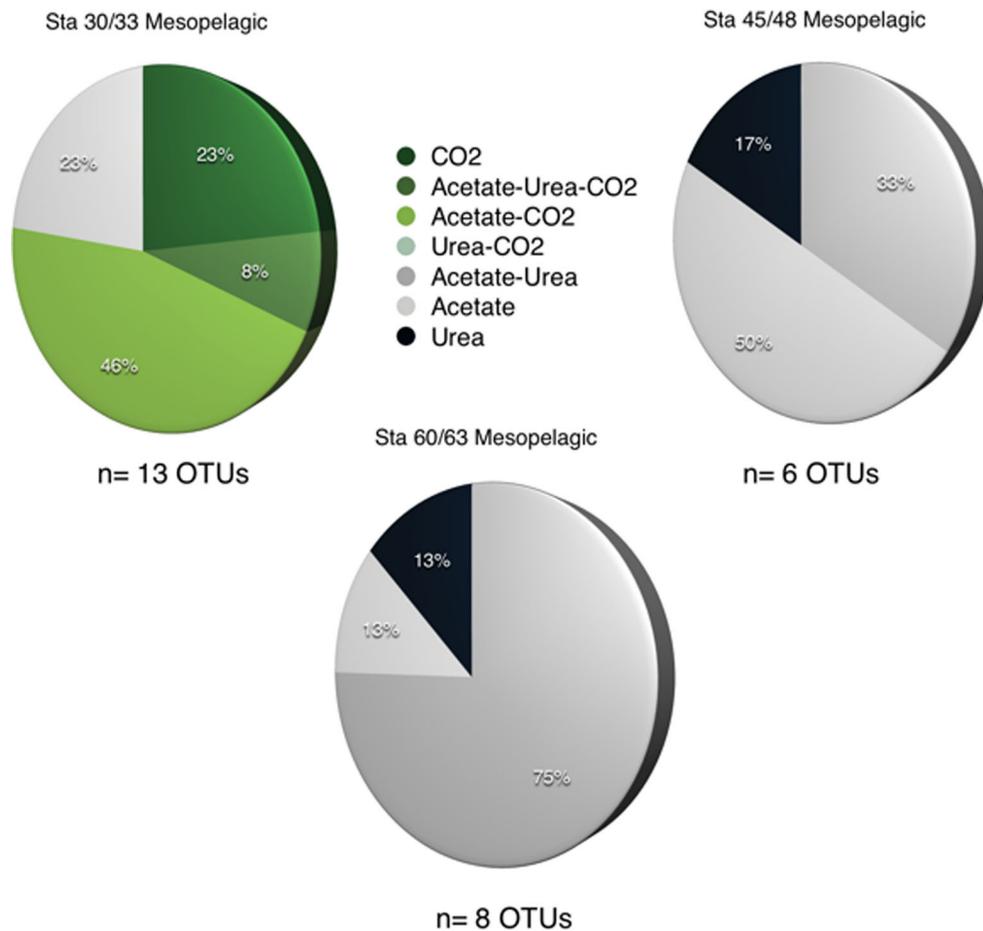


Figure 3. Percentage of active OTUs detected in duplicate SIP incubations from the mesopelagic on the various ¹³C-labeled substrates as indicated.

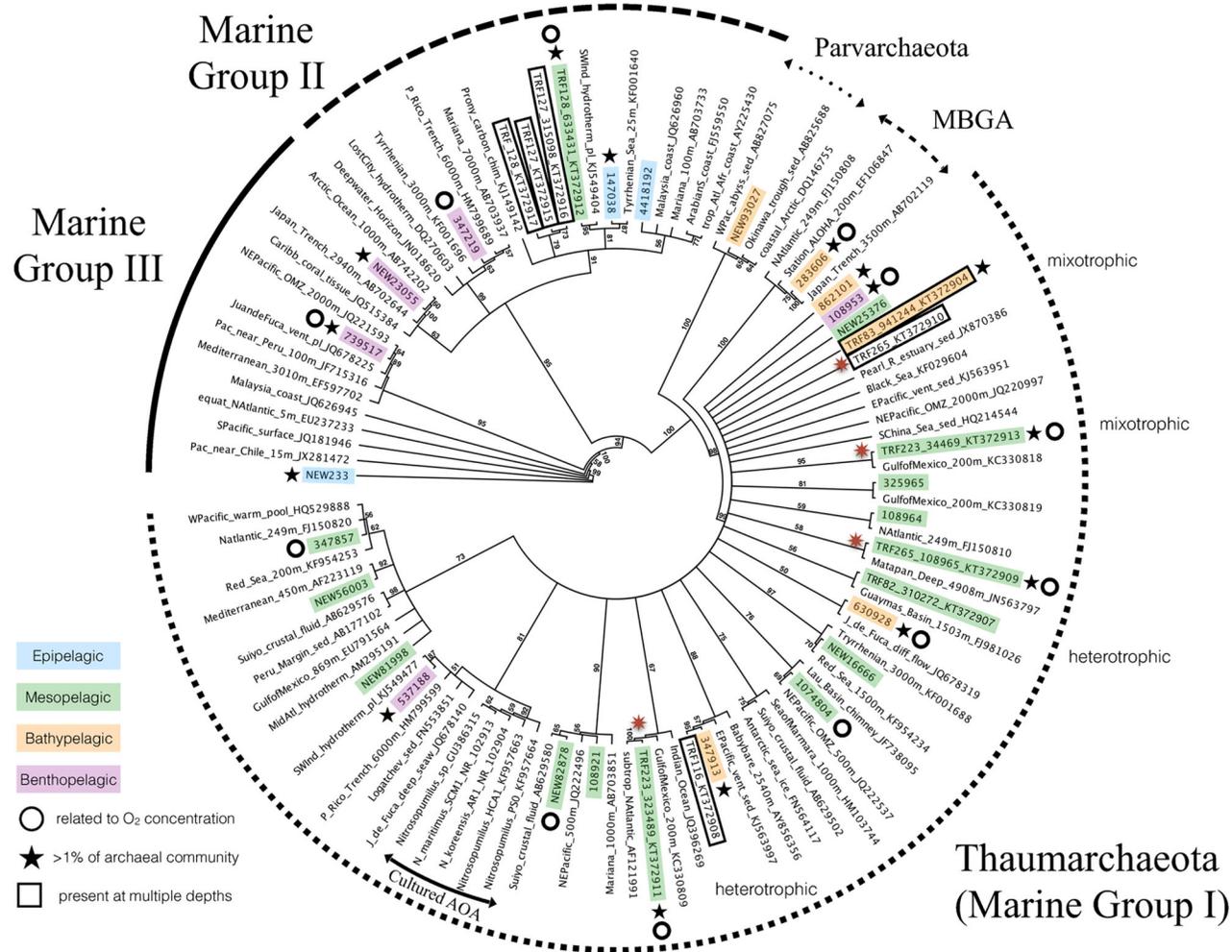


Figure 4. Maximum-likelihood tree of archaeal clones among 95 taxa, including 23 depth-dependent archaeal OTUs from 16S tag sequencing data identified by ANOVA (Bonferroni correction value of <0.05). OTUs that represent $>1\%$ of the total archaeal community are indicated with stars; OTUs correlated with oxygen concentration are indicated with an open circle. The numbers indicate prokMSA ID in the GreenGenes database or NCBI accession numbers. Those OTUs not found in GreenGenes are indicated by the word 'NEW'. OTUs that were identified in the clone library are indicated by TRF[bp length of TRF peak].[prok MSA ID (if a 100% match was available)]. [NCBI accession number].

bp; Fig. 4). In addition to the thaumarchaea, four clones were identified as euryarchaeal members of Marine Group II. All four euryarchaeal clones produced a TRF peak of 127 or 128 bp long, clustering closely together in our phylogenetic tree.

A BLAST search of each clone was also performed against the GreenGenes database using the 140-bp fragment covered by the 515-F/806-R universal prokaryote primers to link with the NGS sequencing data. The results of this search identified all of the thaumarchaeal clones as either *Cenarchaeum* or *Nitrosopumilus* spp. (with a 91–94% match) due to the small size of the region used in the search. Many of the thaumarchaeal OTUs identified by 16S tag sequencing were classified as *Nitrosopumilus* by QIIME using the GreenGenes database (Fig S3). In our phylogenetic tree, the cloned active OTUs clearly fall outside of the cultured *Nitrosopumilus* representatives. The results suggest the apparent dominance of the archaeal community by *Nitrosopumilus* at depth is likely an artifact of the short length of the 16S tag sequencing reads, and the lack of identified representative archaea in the GreenGenes database.

Beta diversity analysis suggested a correlation between the microbial communities and the water mass where the sample was collected (Fig. S6, Supporting Information). Analysis of

similarity of the basin samples (excluding samples taken on the slope or shelf) confirmed that community variance between samples was significantly lower within sampling zones compared to between sampling zones ($R = 0.81$, P -value = 0.01). No significant correlation was detected between microbial beta-diversity and latitude, depth in meters or temperature. An ANOVA test was used to compare OTU frequencies between sampling zones and determine which archaea were differentially represented in one sampling zone over another. These depth-dependent OTUs are included in the phylogenetic tree (Fig. 4). Similarly, a Pearson correlation test was also performed to assess whether archaeal OTUs relative abundance was strongly correlated with oxygen concentration (in Fig. 4, those OTUs with a Bonferroni correction value of <0.05 are indicated with open circles).

DISCUSSION

Much of the recent research focus on marine Thaumarchaeota has concentrated on life strategies revolving around ammonia oxidation and autotrophy. Our SIP results demonstrate that inorganic carbon fixation represents a subset of the full metabolic

capability of the TACK superphylum as a whole. Only two of the observed active TACK OTUs exclusively incorporated bicarbonate, while half of the TACK OTUs readily incorporated both organic and inorganic carbon. These mixotrophic OTUs represented the largest and most frequently observed peaks in TRFLP analysis. However, it should be noted that multiple TACK 16S rRNA genes could be attributed to a single TRFLP peak (e.g. TRF 223 and TRF 265) and our results could represent an autotrophic and a heterotrophic microorganism rather than two mixotrophic TACK archaea.

In a prior study, strict heterotrophy was found to be the prevalent metabolic strategy among the TACK archaea in salt marsh sediments (Seyler, McGuinness and Kerkhof 2014). Likewise, no incorporation of ^{13}C -bicarbonate was detected in the mesopelagic in the Sargasso Sea (stations 45/48; Fig. 3), suggesting that TACK archaea at this location/depth may also adopt a strictly heterotrophic lifestyle, while at other depths/locations these same TRFs were associated with the uptake of both organic and inorganic carbon (Table S3A and B, Supporting Information). However, it should be noted that we did not supplement our ^{13}C -bicarbonate SIP incubations with NH_4^+ and we may detect increased autotrophy with an increase in ammonium concentration. Unfortunately, the use of carrier DNA in our SIP gradients precludes the use of NGS with universal primers, as most of the data retrieved from such methods would be carrier sequence.

The uptake of urea by Thaumarchaeota, particularly in the mesopelagic and bathypelagic, is consistent with the high abundance of urease genes that have been found in this phylum (Alonso-Sáez *et al.* 2012). In our study, incorporation of ^{13}C -urea and ^{13}C -acetate but not ^{13}C -bicarbonate was detected in the mesopelagic at stations 45/48 (Fig. 3). Roughly half of these OTUs did not incorporate ^{13}C -bicarbonate at any station or depth. As with our prior salt marsh study (Seyler, McGuinness and Kerkhof 2014), the ^{13}C -urea is not being incorporated via $^{13}\text{CO}_2$. If that were the case, we should always observe ^{13}C -labeled DNA when the samples are incubated with ^{13}C -bicarbonate. Furthermore, the release of micromolar $^{13}\text{CO}_2$ from ^{13}C -urea would be significantly diluted by the ambient ^{12}C -millimolar bicarbonate in seawater. We should never observe ^{13}C -DNA from ^{13}C -urea if autotrophy were the only pathway to incorporation. Rather this ^{13}C -incorporation from urea in the North Atlantic is consistent with our salt marsh study in which we concluded that TACK archaea run the ornithine cycle in reverse (Seyler, McGuinness and Kerkhof 2014). This incorporation of urea (and acetate) suggests that the role of Thaumarchaeota in organic carbon cycling and remineralization, particularly at depth, may be vital yet underappreciated.

Our SIP results also demonstrated that nearly half of the North Atlantic TACK archaeal community are active (40% of the OTUs detected by SIP/TRFLP) and can double within 24 h (two cellular divisions are required to detect a signal in the 100% labeled ^{13}C carrier band; Seyler, McGuinness and Kerkhof 2014). It is possible that the high percentage of active TACK archaea we observe in our SIP/TRFLP studies is a result of our ^{13}C -incubation conditions, which differed markedly from *in situ* conditions with respect to substrate concentration, temperature and pressure. Yet, changes in incubation temperature, pressure or available substrate cannot bestow a metabolic potential in the TACK archaea where one did not exist in the first place. It is unreasonable to conclude that we are mutating or transforming the TACK archaea and creating a metabolic potential where one did not exist beforehand in our SIP/TRFLP incubations. However, it seems possible that we may be inducing TACK populations that are in-

active *in situ* by incubating at 1 atm and 22°C for 48 h rather than *in situ* conditions. Alternatively, it also is possible that we could not assign activity to members of the *in situ* TACK archaeal community simply because of our SIP incubation conditions, including the requirement of a rapid doubling time. Further SIP-type experiments are needed to definitively show which members of the TACK archaeal community are active under *in situ* conditions.

In conclusion, our SIP results demonstrate that inorganic carbon fixation represents a subset of the full metabolic capability of the TACK superphylum as a whole. Our SIP findings indicate that the mesopelagic zone had the highest diversity of TACK archaea which can utilize the ^{13}C -substrates being tested. Roughly half of these TACK OTUs in the North Atlantic incorporated only ^{13}C -acetate and/or ^{13}C -urea for cellular carbon, while the other half (the most dominant members of the TACK community based on SIP/TRFLP profiles) incorporated both organic (either/both ^{13}C -acetate and ^{13}C -urea) and inorganic (^{13}C -bicarbonate) carbon. Only two TACK archaeal OTUs exclusively incorporated ^{13}C -bicarbonate. Given the diversity of TACK OTUs in the mid- to deep ocean and the metabolic capabilities that can be discerned by these SIP methods, the role of archaea should also be considered in future studies on the recycling of dissolved organic matter and inorganic carbon fixation in the meso- and bathypelagic zones. These findings have direct implications concerning the structure of the marine microbial food web, which is a major determinant in the rate of oceanic carbon sequestration and the type/amount of higher consumers in the ecosystem (Limardo and Worden 2015). Comparable studies to directly measure the uptake of carbon substrates will help predict the fate of dissolved and particulate organic carbon in marine ecosystems.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

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