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mRNA, rRNA and DNA quantitative stable isotope probing with $H_2^{18}O$ indicates use of old rRNA among soil Thaumarchaeota

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ABSTRACT

RNA is considered to be a short-lived molecule, indicative of cellular metabolic activity, whereas DNA is thought to turn over more slowly because living cells do not always grow and divide. To explore differences in the rates of synthesis of these nucleic acids, we used $H_2^{18}O$ quantitative stable isotope probing (qSIP) to measure the incorporation of ^{18}O into 16S rRNA, the 16S rDNA, *amoA* mRNA and the *amoA* gene of soil Thaumarchaeota.

Incorporation of ^{18}O into the thaumarchaeal *amoA* mRNA pool was faster than into the 16S rRNA pool, suggesting that Thaumarchaea were metabolically active while using rRNA molecules that were likely synthetized prior to $H_2^{18}O$ addition. Assimilation rates of ^{18}O into 16S rDNA and *amoA* genes were similar, which was expected because both genes are present in the same thaumarchaeal genome. The Thaumarchaea had significantly higher rRNA to rDNA ratios than bacteria, though the ^{18}O isotopic signature of thaumarchaeal rRNA was lower than that of bacterial rRNA, further suggesting preservation of old non-labeled rRNA. Through qSIP of soil with $H_2^{18}O$, we showed that ^{18}O incorporation into thaumarchaeal nucleic acids was generally low, indicating slower turnover rates compared to bacteria, and potentially suggesting thaumarchaeal capability for preservation and efficient reuse of biomolecules.

1. Introduction

It is axiomatic in biology that DNA replicates in proportion to cellular division (Marstorp and Witter, 1999). The production of ribosomal RNA (rRNA) is closely coupled to DNA replication because the dividing cells require high rates of protein synthesis (Ruimy et al., 1994), though new rRNA may also be synthesized to maintain a subset of proteins when a cell is not dividing (van Bodegom, 2007). Dormant cells generally have relatively low levels of rRNA because they synthesize fewer new proteins (Lennon and Jones, 2011). In pure cultures, rRNA can be relatively stable during exponential growth (Meselson et al., 1964), during stationary phase (Piir et al., 2011), as intact 70S ribosomes in prokaryotes (Zundel et al., 2009), or as 100S dimers (Wada, 1998). Alternatively, rRNA may be degraded in starved cells or at the transition point into stationary phase, when it can be used as a source of nutrients (Deutscher, 2003). As a cell transitions from dividing to a maintenance state, rRNA declines while the DNA concentration remains constant (Deutscher, 2003; Hsu et al., 1994). Messenger RNA (mRNA) turns over faster than rRNA in a cell (Brenner et al., 1961), and its instability is important for regulation of gene expression and rapid responses to environmental changes (Belasco and Higgins, 1988; Jain, 2002; Steege, 2000). Regulatory RNAs that control persistence of specific mRNAs are ubiquitous in microorganisms. While there are several mechanisms through which mRNA can be temporarily stabilized (Wong and Chang, 1986; Kushner, 2002; Hambraeus et al., 2003; Lee et al., 2003; Baker and Condon, 2004), degradation of mRNA by ribonucleases occurs continuously (Deutscher, 2006). Decay and synthesis of mRNA molecules are carefully controlled to optimize cell survival, growth, performance or metabolic activity.

Some studies (Malik et al., 2015) have examined the turnover of nucleic acids in soil but generally these studies are rare. Characterizing nucleic acids synthesis in soil is challenging because soil has many microhabitats, which can provide highly diverse physico-chemical conditions with notable effects on microbial growth and metabolism. Unlike a cell culture, an environmental sample will contain both older and newly formed nucleic acids. Upon cell death in the environment, the DNA can degrade, be assimilated, or adsorb to surrounding particles (Dlott et al., 2015; Morrissey et al., 2015). Ribosomal RNA is likely less

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persistent than DNA in environmental samples. Rates of mRNA synthesis in environmental samples are likely higher than rates of DNA synthesis, because some protein synthesis is related to cellular maintenance and activity, not growth. For these reasons, turnover of mRNA in environmental samples is presumed to be rapid, more rapid than turnover of DNA, though empirical tests of this hypothesis are rare. Here, we used quantitative stable isotope probing (qSIP) experiments with $H_2{}^{18}O$ (Hungate et al., 2015) to measure synthesis rates of microbial nucleic acids in environmental samples. We quantified the ^{18}O incorporation into nucleic acids of individual thaumarchaeal taxa and used it to estimate nucleic acid synthesis rates.

Ammonia oxidizing Thaumarchaeota (Könneke et al., 2005; Schleper et al., 2005; Venter et al., 2004) have a cosmopolitan distribution, are found in a wide range of habitats including aquatic (Kirchman et al., 2007; Teira et al., 2006; Yakimov et al., 2009) and terrestrial ecosystems (Treusch et al., 2005; Kemnitz et al., 2007), and have a complex chemolithoautotrophic physiology (Hatzenpichler, 2012). They have been found to outnumber ammonia-oxidizing bacteria (AOB) in many ecosystems (Leininger et al., 2006; Adair and Schwartz, 2008; Chen et al., 2008; Shen et al., 2008), suggesting an important role in the nitrogen cycle. They can be detected and quantified through measurements of 16S rRNA or *amoA* gene sequence abundance (Francis et al., 2005). By studying this group of organisms in a quantitative stable isotope probing experiment, it is possible to measure the turnover of DNA, rRNA and mRNA in individual microbial taxa. In the present study, we investigated transcription and replication of the 16S rDNA and the *amoA* gene of soil Thaumarchaeota through $H_2{}^{18}O$ mRNA, rRNA and DNA-qSIP. Specifically, we measured the incorporation of ^{18}O into newly synthetized 16S rRNA and 16S rDNA as well as into the *amoA* gene and its transcripts. For Thaumarchaeota, *amoA* gene transcription indicates cellular activity related to ammonia oxidation, which is central to metabolism for this particular group of organisms. In contrast, 16S rRNA transcription is required for any prokaryotic cell to construct protein synthesis machinery. We hypothesized that replication of both genes would be highly correlated, but that transcription rates would differ.

2. Materials and methods

2.1. Sample collection

Soil samples were collected from three separate locations (N: 34° 57′21.879″ W: 111° 45′14.859", N: 34° 57′21.289″ W: 111° 262 45′14.189″ and N: 34° 57′21.591″ W: 111° 45′14.683") in a semi-arid ecosystem near Sedona, Arizona, USA in April 2014. The soil was characterized as a sandy loam (average pH of 6.95 ± 0.42, soil moisture content of 4.11 ± 0.24% at the time of sampling, organic matter content of 13.7 ± 1.7%, and concentrations of NO_3—N and total P of 13.5 ± 7.0 and 14.3 ± 0.6 ppm respectively (AB-DPTA test, Soltanpour and Schwab, 1977)).

2.2. Incubation and ultracentrifugation

Two grams of overnight air-dried soil were incubated in triplicate with 400 µl of 95 atom % $H_2{}^{18}O$ or with 400 µl of water with natural abundance ^{18}O for 1, 4 or 8 days at room temperature, resulting in a final atom % ^{18}O of approximately 80%. Total RNA and DNA were simultaneously extracted from 1 g of soil using RNA PowerSoil Total RNA Isolation Kit and DNA Elution Accessory Kit (MO BIO Laboratories, Carlsbad, CA). RNA extracts were digested with DNA-*free* DNase Treatment Removal Reagents (Ambion, Life Technologies, Grand Island, NY) to remove any traces of genomic DNA. Nucleic acids were quantified using the Qubit 2.0 Fluorimeter (Life Technologies, Grand Island, NY), separated by ultracentrifugation at 127,000 × *g* in 3.3 ml Optiseal polyallomer tubes (Beckman Coulter, Fullerton, CA) on CsTFA (initial buoyant density of 1.79 g/ml, for RNA) or CsCl (initial buoyant density of 1.73 g/ml, for DNA) density gradients. Samples were separated into approximately 120–150 µl fractions and purified as described in Papp et al. (2018a and 2018b). Briefly, RNA fractions were incubated with 200 µl of isopropanol and 100 ng glycogen at −20 °C overnight, and DNA fractions were incubated with 200–300 µl of water, 300–450 µl of isopropanol and 100 ng of ultra-pure glycogen at room temperature overnight. Fractions were then precipitated with 70% ethanol and resuspended in 20–50 µl 1X TE buffer. RNA from purified fractions and from non-fractionated total RNA extracts was reverse transcribed into complementary DNA (cDNA) using Maxima H-Minus First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA) and random pentadecamer primers (Eurofins MWG Operon, Huntsville, AL). Extracted nucleic acids and corresponding fractions were stored at −80 °C until further processing.

2.3. Sequencing

Sequencing of 16S rRNA gene fragments in cDNA and DNA samples was performed on the Illumina MiSeq platform at the Environmental Genetics and Genomics Facility (EnGGen) at Northern Arizona University using the 2 × 150 paired-end read chemistry and 515F (5' - GTGCCAGCMGCCGCGGTAA - 3′) and 806R (5' – GGACTACVSGGGTATCTAAT – 3') primers. Sequencing data were analyzed using a Quantitative Insights into Microbial Ecology (QIIME) -based (Caporaso et al., 2010) chained workflow (Krohn, 2016). A detailed description of sequencing data processing steps is available at https://github.com/alk224/akutils-v1.2 and in Papp et al. (2018a and 2018b).

2.4. Arch-amoA qPCR

Standards for the archaeal *amoA* qPCR assay were made with DNA purified from qSIP fractions. The archaeal *amoA* gene was amplified in 20 µl reactions containing 2 µl of template and 7.32 µl of RNase-free water, 1X Phusion Mastermix (Water, 10X RedJuice (40% 1M Tris, pH 8.5/phenol red and 60% glycerol), 5X HF Buffer, 10 mM dNTPs, Phusion HSII polymerase), 1.5X $MgCl_2$ and 0.2 µM of a modified archaeal *amoA* primer: Arch-amoAFadapter (5' -caagcagaagacggcatacgagatSTAATGGTCTGGCTTAGACG-3′) and Arch-amoARadapter (5' -caagcagaagacggcatacgagatGCGGCCATCCATCTGTATGT-3′). These primers were modified by attaching the 5′caagcagaagacggcatacgagat 3′ adapter sequence to the 5′end of the archamoA primer to ensure that the *amoA* primer binding sites were not at the ends of the standard templates. Amplification was carried out in a 96-well plate with cycling conditions starting at 95 °C for a 2-min denaturation step, followed by 30 cycles of 30 s at 95 °C, 1 min at 55 °C, and 1 min at 72 °C. After 30 cycles there was a final extension for 3 min at 72 °C followed by an indefinite hold at 4 °C. A subset of 18 PCR products were randomly selected and visualized by gel electrophoresis. All 96 products were subsequently pooled into a 5 ml tube and purified with magnetic beads (0.1% carboxyl-modified Sera-Mag Magnetic Speedbeads, Thermo Fisher Scientific, Freemont, CA) in 18% PEG following a standard bead cleanup protocol with two ethanol washes (DeAngelis et al., 1995), and eluted into 200 µl Tris-Cl (pH 8.0). These PCR products were visualized by gel electrophoresis, and purified using Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Purified products were again visualized by gel-electrophoresis and quantified with Quant-it PicoGreen double-stranded DNA assay kit (Life Technologies, Grand Island, NY) on a BioTek HT Plate reader (BioTek, Vinooski, VT). Serial dilutions, ranging from 10^8 to 10^2 gene copies per µl, were used as the standard curve for assay optimization. They were analyzed in triplicate using a quantitative PCR assay performed on a CFX384 Touch Real-Time PCR Detection System (BIORAD, Hercules, CA, USA). Each 10-µl reaction contained 1 µl of template (i.e. diluted standard), or no template control (NTC), 1X Phusion Mastermix, 1X EvaGreen dye (Biotium), 1.5X $MgCl_2$ and 0.2 µM of each primer: Arch-amoAF (5'

-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCC ATCTGTATGT-3′) (Francis et al., 2005). Cycling conditions started with a denaturation step at 95 °C for 2 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. All runs to quantify the *amoA* gene or its transcripts in fractions were carried out on the CFX384 Detection System, fractions were quantified in triplicate, and standards in quintuplicate (10-μl reactions) with the same components and cycling conditions as describe above.

2.5. Incorporation of ^{18}O into nucleic acids

Fraction density within a sample was corrected based on ^{18}O- IRMS data (Table S1) as described in detail in Papp et al. (2018a). Briefly, nucleic acids were quantified, diluted with a salmon sperm DNA solution to 200 μg oxygen and pipetted into silver capsules (Costech Analytical Technologies Inc., Valencia, CA) in a tray, which was set on a heat block at 50 °C for water to evaporate. Capsules were then closed, weighted, and sent to the Stable Isotope Facility at University of California, Davis, for isotopic analysis. The corrected densities were used for further calculations. Calculations of (1) taxon-specific weighted average densities (WAD_t), (2) weighted averages density shifts (WAD_t shift) and (3) atom percent excess ^{18}O (*APE* ^{18}O) of nucleic acids, including *amoA* mRNA, *amoA* gene, 16S rRNA, and 16S rDNA, were carried out manually. Subsequently, they were also confirmed in R (R Core Team, 2014), using a code available at https://bitbucket.org/QuantitativeSIP/qsip_repo, and described in Hungate et al. (2015).

2.5.1. Weighted average density shifts of nucleic acids

All calculations and equations used for determining weighted average density (*WAD*) shifts of microbial nucleic acids (16S rRNA and rDNA) were previously published in Papp et al. (2018a). The same equations were used for determining the *WAD* shifts of 16S rRNA and rDNA for the thaumarchaeal taxa. We calculated *WAD* of thaumarchaeal *amoA* gene from a given taxon as the weighted average across density fractions. For a given fraction, we multiplied the relative abundance of that thaumarchaeal taxon ($rel.ab._{thaum}$) by the number of *amoA* copies detected via qPCR in that fraction (*amoA copy number*):

$$ABS\ amoA\ copy = rel.\ ab._{thaum} * amoA\ copy\ number \quad (1)$$

yielding an estimate of the absolute abundance of *amoA* copies for a given taxon in a given fraction (*ABS amoA copy*). The WAD for a given thaumarchaeal taxon (WAD_{thaum}) was then calculated as the sum of products of density times the absolute *amoA* copy number abundance in each fraction, divided by the total abundance across all fractions:

$$WAD_{thaum.} = \frac{\sum_{k=1}^{K} (ABS\ amoA\ copy * density)}{\sum_{k=1}^{K} (ABS\ amoA\ copy)} \quad (2)$$

where:

k = fraction of a nucleic acid sample
K = total number of fractions from a nucleic acid sample
density = density of a nucleic acid in each fraction

To obtain a *WAD* shift, we took the difference between *WAD* of nucleic acids from a sample incubated with $H_2{}^{18}O$ and the paired sample incubated with water containing natural abundance levels of ^{18}O. The *WAD* shift reflects the increase in density of nucleic acids following incubation with isotopically labeled water. The procedure for estimating *amoA* mRNA was analogous but the *amoA* copy number was derived from fractions of complementary DNA (cDNA).

2.5.2. Atom percent excess ^{18}O of nucleic acids

All calculations and equations used for determining the atom percent excess ^{18}O (*APE* ^{18}O) values of microbial nucleic acids (16SrRNA and rDNA) were also previously published in Papp et al. (2018a) and in Hungate et al. (2015). Some of these equations are provided in this section as well for clarity. Specifically, we converted *WAD* shifts of thaumarchaeal nucleic acids into atom percent excess ^{18}O (*APE* ^{18}O) values, which show the nucleic acids' isotopic enrichment above natural abundance of the ^{18}O isotope (0.2 atom%). We used equation *(5)* from Papp et al. (2018a) (equation (3) below) to convert *WAD* of thaumarchaeal RNA (16S rRNA or *amoA* mRNA) into atom percent ^{18}O values:

$$WAD = 0.0744 * \text{atom percent } ^{18}O \text{ of RNA} + 1.7803 \quad (3)$$

This equation was obtained by using the known molecular weight of the rRNA molecule at different ^{18}O isotopic contents (natural abundance and 100% ^{18}O content), verified by empirical measurements of the density of RNA at natural abundance ^{18}O. We also used equation (6) from Papp et al. (2018a) (equation (4) below) to convert *WAD* of thaumarchaeal DNA (16S rDNA or *amoA* gene) into atom percent ^{18}O values:

$$WAD = 0.0644 * \text{atom percent } ^{18}O \text{ of DNA} + 1.6946. \dots \dots \quad (4)$$

This equation was generated with results from a culture experiment of growing *E. coli* (strain HB101) at 5 different ^{18}O enrichment levels (natural abundance, 5, 25, 50 and 70% atom fraction ^{18}O). DNA was extracted from these cultures which was centrifuged to determine its density, and analyzed by IRMS to determine its ^{18}O signature. The equation is fully described and explained in Hungate et al. (2015). To calculate the atom percent excess ^{18}O (*APE* ^{18}O), the isotopic values of nucleic acids extracted from non-labeled incubations were subtracted from the isotopic values of nucleic acids extracted from the labeled treatment. We obtained three replicate *APE* ^{18}O values for each thaumarchaeal taxon at each time point.

2.6. rRNA to rDNA ratio calculations

Ratios of rRNA to rDNA were calculated for Thaumarchaea by dividing the relative abundance of RNA in each non-fractionated sample by the relative abundance of rDNA in the same sample. This was done for each replicate at each time point, yielding a total of 9 ratios per treatment. The replicate ratios were then averaged. There were two reasons for averaging the ratios: (1) there was no statistically significant difference over time ($p = 0.404$), and (2) we did not intend to investigate how the ratios changed over time.

2.7. Model of nucleic acid turnover

Details about turnover of microbial rRNA are provided in (Papp et al., 2018b). Briefly, Excel's Solver was used to determine the turnover rate of thaumarchaeal 16S rRNA and *amoA* mRNA that yielded the minimum sum of squared deviations from the observed data. We assumed that 50% of oxygen atoms in the thaumarchaeal nucleic acids originated from water and 50% from organic substrates (Chaney et al., 1972) and used findings from Ostle et al. (2003) and Yuan and Shen (1975) to obtain the starting turnover rates (20% per day, and 25% per hour respectively). After calculating the expected atom percent ^{18}O values of rRNA and mRNA at each of the reported rates (20% per day, and 25% per hour), we compared them to our observed values (on day 1, 4 and 8) and used Excel's Solver function to find the optimal turnover rate for each RNA type.

2.8. Statistical analyses

Statistical analyses were performed in SPSS (IBM SPSS Statistics 24), with statistical significance set to $\alpha = 0.05$. Normality was tested with the Shapiro-Wilk test, homogeneity of variances was tested with Levene's test, and sphericity was tested with Mauchly's test. Additionally, we used a two-way mixed ANOVA to test for a significant interaction between *APE* ^{18}O of the different nucleic acid types (i.e. 16S rRNA, 16S rDNA, *amoA* mRNA, and *amoA* gene) and time. We also used a Spearman's rank order correlation test to assess the strength of correlations between (I) *APE* ^{18}O of 16S rDNA and *amoA* gene, (II) *APE* ^{18}O of *amoA* mRNA and 16S rRNA, (III) *APE* ^{18}O of 16S rRNA and 16S rDNA, and (IV) *APE* ^{18}O of 16S rDNA and 16S rRNA or *amoA* mRNA. Regressions were carried out using a model II regression analysis performed in R to test whether the slope of each correlation was significantly different from 1. We acknowledge that taxa within each replicate are not independent, and therefore, from the standpoint of inference from experimental design, the taxa are pseudoreplicates (taxa within replicate samples). However, the goal of our analyses was not to make inferences about differences between samples, or between groups of samples. The level of pseudoreplication therefore does not affect our conclusions. Combining all taxa within replicates would furthermore result in a significant loss of biological meaning caused by a considerable and particularly interesting variation among the individual taxa. Populations of different taxa are precisely the fundamental groups within fundamental biological and evolutionary units, where we would expect to see with great resolution a decoupling of DNA and mRNA synthesis - if it were to occur. Therefore, regressions in this manuscript show individual taxa within each replicate to illustrate general trends in nucleic acid synthesis with the highest resolution. The regression slopes were bootstrapped, which does not violate assumptions of the approach.

2.9. Accession numbers

All sequences have been deposited in the NCBI Sequence Read Archive (SRA) under accession numbers SAMN07960499 to SAMN07960874, SAMN07965143 to SAMN07965605, and SAMN07968111 to SAMN07968486. Data can directly be accessed at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP123236.

3. Results

3.1. Incorporation of ^{18}O into nucleic acids

The amount of ^{18}O incorporated into thaumarchaeal nucleic acids, expressed as atom percent excess ^{18}O (*APE* ^{18}O), significantly differed over time and between 16S rRNA, the 16S rDNA, *amoA* mRNA, and the *amoA* gene ($p < 0.005$). *amoA* mRNA contained more ^{18}O than thaumarchaeal 16S rRNA ($p < 0.005$) and, with the exception of day 4, the *amoA* gene (Fig. 1, Figure S2, Supplemental material S2). As expected, the 16S rDNA and *amoA* genes had similar ^{18}O enrichment on each day ($p > 0.1$). The ^{18}O isotopic composition of 16S rRNA and *amoA* mRNA increased over time ($p < 0.05$, Fig. 1). The ^{18}O signature of thaumarchaeal 16S rDNA and *amoA* genes also increased over time with a slope significantly greater than zero for both genes (slope = 0.014 for 16S rDNA, and slope = 0.012 for *amoA* gene, Fig. 1, Supplemental material S2).

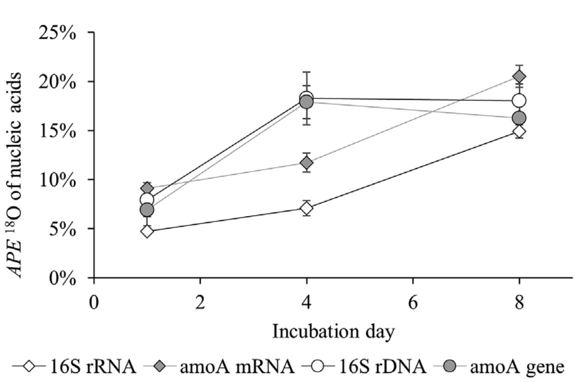

Fig. 1. Atom percent excess (*APE*) ^{18}O of thaumarchaeal 16S rRNA (open diamond), *amoA* mRNA (filled diamond), 16S rDNA (open circle), and *amoA* gene (filled circle) at three incubation time points. Error bars show standard deviation of the mean of taxa (n = 4 taxa for 16S rRNA and *amoA* mRNA, and n = 5 taxa for 16S rDNA and *amoA* gene).

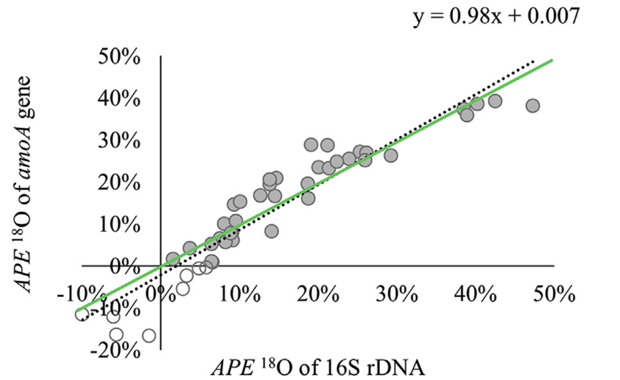

Fig. 2. Correlation between the *APE* ^{18}O of thaumarchaeal *amoA* gene and 16S rDNA (all individual time points are shown). Symbols represent individual thaumarchaeal taxa in replicate soil samples. Filled symbols show positive *APE* ^{18}O values, and open symbols show negative values, which were not included in the analysis. The green line represents a 1:1 line where the *APE* ^{18}O of both genes are equal. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Correlations between isotopic signature of thaumarchaeal nucleic acids

Incorporation of ^{18}O into thaumarchaeal *amoA* genes was strongly, and positively, correlated to incorporation of ^{18}O into 16S rDNA ($\rho(37) = 0.953$, $p < 0.005$, Fig. 2). The equation describing the overall relationship between the two variables showed that the rates of incorporation of ^{18}O into both genes were similar (Fig. 2, Supplemental material S2). Correlation between the isotopic signature of thaumarchaeal *amoA* mRNA and 16S rRNA was also strong and positive ($\rho(34) = 0.860$, $p < 0.005$). The relationship, depicted in Fig. 3, showed that rate of assimilation of ^{18}O isotopes into *amoA* mRNA was greater than into 16S rRNA (Fig. 3, Supplemental material S2). Additionally, the rate of *amoA* mRNA turnover was higher (6.78% per day) compared to the rate of 16S rRNA turnover (4.61% per day). Lastly, incorporation of ^{18}O into 16S rDNA of Thaumarchaeota was correlated with incorporation of ^{18}O into their 16S rRNA (Figure S1A) and *amoA* mRNA (Figure S1B). Model II regression analysis showed that the slopes of both regressions were significantly smaller than one (slope = 0.40 for 16S rRNA vs 16S rDNA, and slope = 0.45 for *amoA* mRNA vs 16S

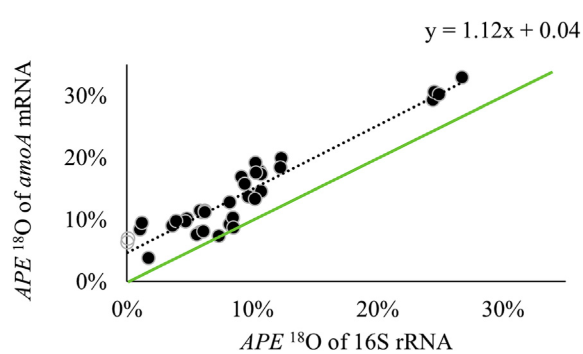

Fig. 3. Correlation between the *APE* ^{18}O of thaumarchaeal *amoA* mRNA and 16S rRNA using all individual incubation time points. Symbols show individual taxa in replicate soil samples. Positive *APE* ^{18}O values are represented by black symbols, and values of ~ zero by open symbols, the latter were not used in the analyses. The green line represents a 1:1 ratio, where the isotopic content of mRNA and rRNA is equal. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

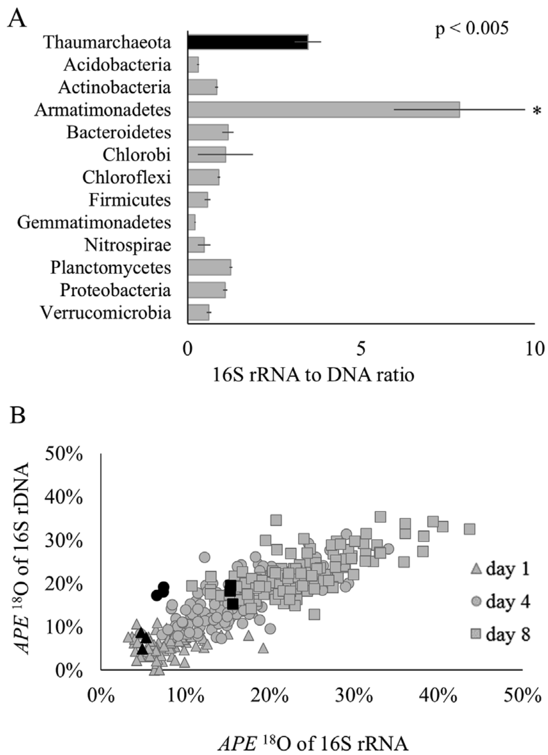

Fig. 4. Ratios of the relative abundance of rRNA over relative abundance of rDNA for major microbial groups in soil (A) and correlation between *APE* ^{18}O of 16S rDNA and 16S rRNA (B). The rRNA to DNA ratios (A) represent means of three replicates and three time points ($n = 9$), error bars show standard deviation of the mean. Significant differences are indicated by *. The correlation (B) shows taxa at deepest taxonomic resolution, day 1 is represented by triangles, day 4 by circles and day 8 by squares; black symbols represent thaumarchaeal taxa, gray symbols represent bacterial taxa. Symbols are bootstrapped means; error bars are not shown for clarity.

rDNA; $p < 0.005$ for both, Supplemental material S2). The isotopic content of nucleic acids was generally lower on day one and increased with incubation time, although there was substantial variation among the replicates at each time point.

3.3. rRNA to rDNA ratios

As a phylum, Thaumarchaeota constituted ~5% of the rDNA and ~16% of the 16S rRNA sequencing libraries (Figure S3), which yielded a relatively high rRNA to rDNA ratio of 3.47. This ratio was significantly higher ($p < 0.005$) than rRNA to rDNA ratios for other bacterial phyla (with the exception of Armatimonadetes, which had an exceptionally high rRNA to rDNA ratio of 7.83). Most bacterial phyla had ratios below 1, with the exception of Proteobacteria (1.08) and Planctomycetes (1.24) (Fig. 4A, Supplemental material S2). Across all bacterial taxa, the correlation between the ^{18}O content of 16S rDNA and 16S rRNA was strong, positive, and significant ($\rho(345) = 0.859$, $p < 0.005$) (Fig. 4B, Supplemental material S2). Relative to bacterial phyla, thaumarchaeal taxa generally incorporated less ^{18}O into their nucleic acids (Fig. 4B, black symbols). The maximum *APE* ^{18}O was ~15% for thaumarchaeal 16S rRNA and ~19% for their 16S rDNA after 8 days of incubation with $H_2{}^{18}O$, while the maximum *APE* ^{18}O for bacterial 16S rRNA was ~43% and ~35% for their 16S rDNA at that time.

4. Discussion

4.1. ^{18}O isotopic content of thaumarchaeal nucleic acids

The natural abundance isotopic composition of newly synthesized nucleic acids within a cell, whether DNA, mRNA, or rRNA, should be similar because they all originate from interchangeable nucleotide pools. Ribonucleotide reductase catalyzes the conversion of ribonucleotides to deoxyribonucleotides (Herrick and Sclavi, 2007; Reichard, 1993), so therefore it is likely that both newly made ribonucleotides and deoxyribonucleotides have a similar ^{18}O content since ^{18}O water was added in very high amounts and fractionation processes could be ignored. Even if fractionation led to differences in natural abundance ^{18}O composition between types of nucleic acids, a large change in natural abundance (e.g., 50‰) corresponds to a very small difference in tracer concentrations (± 0.01 atom % ^{18}O). The observed significant differences in the ^{18}O content of the bulk DNA, mRNA, or rRNA, following the addition of ^{18}O-water can thus only be explained by the fraction of the nucleotide pool composed of older, non-labeled molecules. Additionally, the ^{18}O content of a nucleic acid extract is also affected by the amount of newly synthetized molecules, the turnover rate, the synthesis strategy (*de-novo* vs recycling of nucleotides, discussed further), and the nucleic acid pool size.

^{18}O incorporation into *amoA* mRNA was greater than into the *amoA* gene at the beginning and at the end of our experiment, likely because mRNA synthesis rates were greater than DNA replication rates. This is consistent with the observations of Dumont et al. (2011) throughout a 4-day incubation with ^{13}C-labeled methane, whereby the isotopic signature of *pmoA* mRNA of methanotrophic communities was greater than the ^{13}C isotopic signature of *pmoA* genes. Since growth is not a prerequisite for metabolic activity (Blazewicz et al., 2013), greater ^{18}O content in microbial mRNA relative to the corresponding gene was expected.

Surprisingly, the overall isotopic enrichment of thaumarchaeal *amoA* mRNA was relatively low (~20% after 8 days of incubation with $H_2{}^{18}O$, turnover rate 6.78% per day). This suggested that turnover rate of mRNA may be slower in soil than in pure cultures on media with ideal growth conditions (such as 37°C temperature, Hambraeus et al., 2003) and high cellular activity. In these cultures, studies have measured short half-lives of bacterial mRNAs, ranging anywhere from ~30 s to ~30 min (Belasco and Higgins, 1988; Hambraeus et al., 2003; Rauhut and Klug, 1999; Wong and Chang, 1986). At such a fast turnover rate, we hypothesized that many oxygen atoms in *amoA* transcripts would be replaced with ^{18}O. We did not expect the mRNA to be 100% labeled with ^{18}O because microorganisms assimilate oxygen into ribonucleic acids from both water and other organic or inorganic compounds. In the case of Thaumarchaeota, oxygen is likely to come from water and potentially from fixed carbohydrates as they mostly use inorganic HCO_3^- as a carbon source (Hatzenpichler, 2012; Könneke et al., 2014). Oxygen in the fixed carbohydrates may partially come from water since water is used to produce Acetyl-CoA, a precursor for gluconeogenesis (Berg et al., 2010). Thus, newly synthesized nucleic acids of the Thaumarchaeota could potentially be more labeled with ^{18}O than nucleic acids of heterotrophs, but further research is needed to elucidate the rate of ^{18}O incorporation into newly fixed carbohydrates among autotrophs. Additionally, many ribonucleotides within a cell are likely recycled, and therefore new mRNA sequences will still contain many ^{16}O atoms, which likely contributed to the low ^{18}O content of thaumarchaeal *amoA* mRNA.,

4.2. Chemolithoautotrophic metabolism

The thaumarchaeal *amoA* gene encodes a subunit of the ammonia monooxygenase enzyme (AMO) (Francis et al., 2005), which, in Thaumarchaeota, is responsible for energy generation (Hatzenpichler, 2012). AMO catalyzes the oxidation of ammonia to hydroxylamine, which is the first and rate-limiting step of nitrification (Jia and Conrad, 2009; Leininger et al., 2006; Mertens et al., 2009; Nicol and Schleper, 2006). Preserving *amoA* transcripts for longer periods of time could increase the number of translated AMOs per *amoA* template and directly save energy associated with transcription. Ammonia oxidizing bacteria (AOB) are already known for their high *in vivo* stability of *amo* mRNA, proteins, and ribosomes (Koops et al., 2003; Bock and Wagner, 2006). Our study suggests that soil ammonia oxidizing archaea (AOA) exhibit a similar physiology. Incomplete mRNA labeling was also observed in German agricultural soils (Pratscher et al., 2011). In their study, up to 50% of the *amoA* transcripts had densities below 1.80 g/ml even after 12 weeks of incubation with $^{13}CO_2$ and 100 μg N·g^{-1} dry weight soil. This indicated that the transcripts were not fully labeled with the heavy isotope (Pratscher et al., 2011). The detection of ^{18}O-labeled *amoA* mRNA in our study suggested that soil Thaumarchaea were active in nitrogen cycling (Gubry-Rangin et al., 2010; Huang et al., 2012; Stopnišek et al., 2010; Yao et al., 2011), but relating ^{18}O content of *amoA* mRNAs to ammonia oxidation rates was beyond the scope of this study.

4.3. Presence of old rRNA

We found that Thaumarchaea had higher ^{18}O content in their *amoA* mRNA than 16S rRNA, indicating that old thaumarchaeal rRNA was present in the cells (Deutscher, 2006, 2003; Jain, 2002). We expected the ^{18}O content of newly made mRNA and rRNA to be similar because both are transcribed by the same RNA polymerase (Ebright, 2000; Darst, 2001) and because, within a cell, ribonucleotides used for synthesis of mRNA and rRNA derive from the same nucleotide pool. Thus, if all RNA molecules had very high turnover rates and were recently synthesized, the *APE* ^{18}O of *amoA* and 16S ribosomal RNA should be very similar, which was not what we observed. The difference in *APE* ^{18}O of the two transcripts was most likely due to the presence of a greater amount of older non-labeled rRNA than mRNA. This suggests that mRNA turns over faster than rRNA. The latter, more stable, can thus be reused for maintenance and reduce cell's overall energy expenditure. Ribosome storage has been previously documented by, for example, Wada (1998) who observed formation of 100S dimers, which was proposed to be a mechanism for excess ribosome storage during the stationary phase of growth. Large amounts of stored ribosomes have also been observed in dormant cyanobacteria (Sukenik et al., 2012). Some thaumarchaeal cells may have been in stationary phase and have contained stored ribosomes, thus contributing to overall low isotope content measurements. Yet we observed that the nucleic acids of all taxa were enriched in ^{18}O. Such nucleic acid labeling indicated that at least some individuals within a population were metabolically active and growing.

4.4. Genome replication

As expected, the 16S rDNA and *amoA* genes had very similar isotopic contents. Both genes reside on a single chromosome within a thaumarchaeal cell, and as the cell divided, both became similarly enriched. Our finding of a strong correlation between the *APE* ^{18}O of the 16S rDNA and *amoA* genes with a slope of 1 confirmed that genome replication was uniform, resulting in all genes being labeled with isotopic tracers at the same rate. It also demonstrated that qSIP can measure the rate of genome replication, thus providing a unique opportunity to measure microbial growth rates *in situ*.

Our experiment simulated a rain event. Studies have shown that microbial respiration and growth rates increase after rewetting (Bloem et al., 1992; Iovieno and Bååth, 2008; Pesaro et al., 2004), but the total microbial biomass can decrease (Fierer and Schimel, 2002; Gordon et al., 2008). In $H_2^{18}O$ stable isotope studies, no major changes in the absolute size of bacterial or archaeal populations were found after rewetting because growth was counter-balanced by death (Angel and Conrad, 2013; Blazewicz et al., 2014; Koch et al., 2018). Water addition may have stimulated metabolic activity, including growth, of taxa that might otherwise have stayed dormant in our soil. Water addition increased the soil moisture content from 4% to 20% approximately. Nevertheless, since even desert ecosystems receive precipitation, our experiment remains representative of naturally occurring events and provides valuable information about microbial dynamics following these events.

4.5. Metabolic activity

RNA to DNA ratios are often used as an indicator of microbial metabolic activity. The assumption is that the higher the ratio the higher the activity level because more ribosomes are needed to sustain rapid metabolism. We were thus interested to see how well the ratios compared to qSIP. Through qSIP, we found that Thaumarchaeota incorporated low amounts of ^{18}O into their rRNA, suggesting that synthesis of new rRNA molecules was rather low compared to bacterial taxa. However, we measured high rRNA to rDNA ratios of the soil Thaumarchaeota, which is traditionally interpreted as an indication of high metabolic activity (Roszak and Colwell, 1987; DeAngelis et al., 2010; Baldrian et al., 2012; Dlott et al., 2015). This was in disagreement with result from qSIP but can be explained by the thaumarchaeal capability to preserve and use older ribosomes. The Thaumarchaeota were relatively abundant in the rRNA library but the level of ^{18}O incorporation into their rRNA was low. These two findings suggested that a substantial fraction of their rRNA was synthetized prior to $H_2^{18}O$ addition and was thus at least 8 days old. Our results suggest that ribosomal RNA can be relatively long lived in the environment, whether used by metabolically active bacteria and archaea or perhaps intact and preserved but no longer part of living cells.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.soilbio.2018.12.016.

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