



Hydrogen isotope fractionation during lipid biosynthesis by *Tetrahymena thermophila*



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ABSTRACT

Hydrogen isotope ratio values of lipids are increasingly used to reconstruct past variation in hydrological conditions. However, apart from recording the hydrogen isotope composition of ambient water, δD values of lipids also depend on specific biosynthetic pathways and growth conditions. We have evaluated the hydrogen isotope fractionation by the ciliated protozoan, *Tetrahymena thermophila*, grown in pure culture at three temperatures (24 °C, 30 °C and 36 °C) and in water with a range of hydrogen isotope composition. *T. thermophila* synthesizes tetrahymanol, a pentacyclic triterpenoid alcohol and the diagenetic precursor of the biomarker gammacerane. We focused our attention on the isotopic controls on tetrahymanol and various fatty acids (FAs).

The δD values of FAs and tetrahymanol correlated linearly with the hydrogen isotope composition of water, but growth temperature was also clearly an important factor controlling lipid D/H composition. Hydrogen isotope fractionation during tetrahymanol biosynthesis changed with higher growth temperatures, resulting in D-depleted signatures relative to water at 24 °C and 30 °C and D-enriched composition at 36 °C. *T. thermophila* grown at 36 °C – a temperature above optimum growth conditions – showed a significant change in lipid composition, with the abundance of tetrahymanol increasing relative to total FAs. We suggest the change is a response to temperature stress and a decrease in the stability of the cell membrane. The temperature effect is also presumed to impact δD_{lipid} by altering the hydrogen isotopic composition of NADPH and potentially intracellular water.

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1. Introduction

Hydrogen isotope ratio values of lipids are determined by the hydrogen isotopic composition of ambient water, the redox-active hydrogen in reducing agents (e.g. NADPH) and, in the case of heterotrophic organisms, carbon substrate and metabolic pathways (Sessions et al., 1999, 2002; Zhang and Sachs, 2007; Zhang et al., 2009a; Dirghangi and Pagani, 2013). Ubiquitous lipids synthesized by a variety of organisms [e.g. palmitic acid ($n\text{-C}_{16}$ fatty acid, FA)] have been used as proxies for water hydrogen isotope composition (Huang et al., 2002; Hou et al., 2006), but the approach is potentially complicated because of differences in biosynthetic D/H fractionation between species responsible for biomarker production, as well as isotopic effects associated with growth conditions. For example, pure cultures of the freshwater green algae *Eudorina unicocca* and *Volvox aureus*, and three species of *Botryococcus braunii* produced $n\text{-C}_{16}$ FA that differed in δD values by ca. 100‰, demonstrating the potential for substantial uncertainty in water D/H reconstruction using lipid compounds (Zhang and Sachs, 2007). On the other hand, lipids synthesized by more specific algal species

or families (e.g. botryococcene from *Botryococcus* algae) can act as more reliable δD_{water} proxies (Zhang and Sachs, 2007).

Tetrahymanol is the source of the biomarker gammacerane, a diagenetic pentacyclic triterpane first observed in the Green River Shale (Hills et al., 1966). Gammacerane is derived from tetrahymanol via sulfurization and consequent carbon–sulfur cleavage (Sinnighe Damsté et al., 1995), as opposed to the initially proposed mechanism involving dehydration and hydrogenation (ten Haven et al., 1989).

The first reported source of tetrahymanol was *Tetrahymena pyriformis* (Mallory et al., 1963), a ciliated Protozoan belonging to the class Oligohymenophorea (subclass Hymenostomatia). However, a land fern and a purple non-sulfur bacterium (*Rhodospseudomonas palustris*) are also known to synthesize it under certain conditions (Kleemann et al., 1990; Rashby et al., 2007). Nonetheless, ciliates are clearly the dominant source of tetrahymanol in sediments (ten Haven et al., 1989), as confirmed by stable carbon isotopic composition (Sinnighe Damsté et al., 1995).

Tetrahymena is predominantly a freshwater organism, although it has been reported from thermal springs and soils (Corliss, 1973). Tetrahymanol was also found to be the dominant neutral lipid in eight strains of marine ciliates, indicating that its synthesis is not restricted to *Tetrahymena* alone, but is common across the class Oligohymenophorea (Harvey and McManus, 1991). Thus, although

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tetrahymanol is not as source-specific as botryococcene, it derives from a more specific source than many sterols and hopanols.

The presence of gammacerane in petroleum is commonly used as a marker for a stratified water column during source-rock deposition under hypersaline conditions (Peters et al., 2005a,b; Rullkötter et al., 1984, 1985; Fowler and McAlpine, 1995; Terken and Frewin, 2000) and tetrahymanol has been reported in ancient and modern hypersaline deposits (ten Haven et al., 1989; Barbe et al., 1990; Romero-Viana et al., 2013). However, tetrahymanol is synthesized predominantly by freshwater and marine bacteriivorous ciliates that are not strictly restricted to hypersaline environments. Tetrahymanol-producing ciliates are bacteriivores, feeding on bacteria at the oxic-anoxic interface and associated mainly with water column stratification (Sinnighe Damsté et al., 1995). Hypersaline lakes are often characterized by stratification and are favorable environments for tetrahymanol-producing ciliates, and account for the association of tetrahymanol and gammacerane with hypersaline deposits.

We have studied D/H fractionation of tetrahymanol and FAs by growing a pure culture of *Tetrahymena thermophila* in water with varying δD values across a range of temperature.

2. Material and methods

2.1. Culture experiments

A *T. thermophila* strain was obtained from the Tetrahymena Stock Center at Cornell University and grown in batch culture (200 ml in 1 l Erlenmeyer flasks) in a growth medium consisting of 5.5 g/l dextrose, 0.625 g/l bacto peptone, 2.5 g/l yeast extract and 33 μ M FeCl₃. It was grown in a shaker bath at 24 °C, 30 °C and 36 °C. For each temperature experiment, five isotopically distinct growth media were used. The hydrogen isotope composition of the medium was altered by adding aliquots of D₂O (99%;

Table 1). All media were sterilized by autoclaving prior to inoculation. A 5–6 ml starter culture was grown in unlabeled medium to early stationary phase and 100 μ l aliquots of the starter culture were used to inoculate all the cultures. Growth curves were obtained with a spectrophotometer (DU 640, Beckman Instruments Inc.) by measuring optical density (i.e. absorbance) at 600 nm (Supplementary material Fig. S1). Cultures were harvested in early stationary phase. Growth rate (divisions day⁻¹) was calculated following Zhang et al. (2009b):

$$k = \log_2(N_1/N_0)/(T_1 - T_0) \quad (1)$$

where k is growth rate, N_0 and N_1 denote respectively the optical density at the beginning and the end of the exponential growth phase, T_0 and T_1 denote respectively the time at the beginning and the end of the exponential growth phase.

2.2. Lipid extraction and analysis

Cultures were filtered using ashed glass microfiber (0.7 μ m) filters and freeze-dried. Lipids were extracted using sonication with 1:1 (v/v) dichloromethane (DCM):MeOH followed by 2:1 (v/v) DCM:MeOH. Base hydrolysis of the total lipid extract (TLE) was performed by adding ca. 5 ml of 2 N KOH in a 9:1 (v/v) water:-MeOH azeotrope, keeping the mixture at 80 °C for 2 h. The mixture was cooled and 5% NaCl aq. (5 ml) was added and extraction with hexane afforded the neutral lipid fraction. The remainder of the TLE was treated with concentrated HCl (to pH < 3) and extracted with hexane to collect the polar fraction containing FAs. The polar fraction was methylated to obtain the FA methyl esters (FAMES) by adding ca. 500 μ l of 10% BF₃/MeOH and holding at 80 °C for 20 min. Ca. 500 μ l of solvent-extracted water was added and extracted with hexane. The hexane extract containing the FAMES was passed through a column of Na₂SO₄ to remove traces of BF₃/MeOH and water and separated into three fractions via silica gel

Table 1
Hydrogen isotope data from *T. thermophila* cultures (St dev, standard deviation).

Name	T (°C)	Tetrahymanol/total FAs	$\delta D_{\text{tetrahymanol}}$ (‰)	St dev	δD_{12C-FA} (‰)	St dev	δD_{14C-FA} (‰)	St dev	
TTh_24C_I	24	2.3	-88.5	0.5	-76.6	3	-67.4	1.3	
TTh_24C_II	24	5.2	-23.7	0.3	-33.2	3.9	-15.5	1	
TTh_24C_III	24	6.8	13.7	2.2	1.7	4.7	27.2	4	
TTh_24C_IV	24	2.3	57.96	0.6	35.2	0.5	88.5	1.4	
TTh_24C_V	24	0.1	72.15	6.4	45.7	2.5	112	2.8	
TTh_30C_I	30	2.1	-56.5	0.2	-	-	-41.1	2.9	
TTh_30C_II	30	1.9	-14	0.4	-	-	24.1	1.7	
TTh_30C_III	30	1.2	18.8	0.1	-	-	82.1	0.1	
TTh_30C_IV	30	3.2	65.88	0.8	-	-	-	-	
TTh_30C_V	30	2.8	118.3	1.5	-	-	173.4	-	
TTh_36C_I	36	34.7	-7.1	3.3	-73.1	0.4	-23.7	1.6	
TTh_36C_II	36	17.8	56.3	0.8	-15.7	1.6	22.2	1	
TTh_36C_III	36	22	87.2	0.5	11.6	2.4	92.4	4	
TTh_36C_IV	36	59.3	155.7	3.2	-	-	136.8	0.7	
TTh_36C_V	36	30.4	164.4	0.4	-	-	167.3	5.9	
Name	δD_{16C-FA} (‰)	St dev	δD_{17C-FA} (‰)	St dev	δD_{18C-FA} (‰)	St dev	$\delta D_{\text{dextrose}}$ (‰)	δD_{water} (‰)	St dev
TTh_24C_I	14.8	1.9	-	-	-	-	30	-40.4	0.3
TTh_24C_II	58.7	-	-	-	92.3	1.7	30	9.96	0.3
TTh_24C_III	89.7	6	-	-	-	-	30	52.3	0.1
TTh_24C_IV	174.9	6.9	-	-	176.5	0.9	30	99.4	0.2
TTh_24C_V	208.4	4.5	-	-	212.7	-	30	128	0.3
TTh_30C_I	-6.4	0.7	3.1	1.6	3.2	1	30	-44.8	0.4
TTh_30C_II	59.8	0.01	-	-	93.5	7	30	12.9	0
TTh_30C_III	88.3	0.8	116.1	8	133.9	13	30	54.7	0.3
TTh_30C_IV	111	0.4	128.2	5.4	155.6	1.5	30	78	0.3
TTh_30C_V	173.6	5.5	207.2	-	210.4	0.2	30	142.5	0
TTh_36C_I	-	-	-	-	29.6	-	30	-42	0.1
TTh_36C_II	-	-	-	-	113.5	-	30	14.4	0.8
TTh_36C_III	-	-	-	-	-	-	30	52.3	0.1
TTh_36C_IV	-	-	-	-	224	-	30	100.3	0.1
TTh_36C_V	-	-	-	-	261.5	0.9	30	129.8	0.4

chromatography using 2 ml hexane, 4 ml DCM and 4 ml MeOH. The DCM fraction was dried under a flow of N₂, dissolved in hexane and analyzed for FAMES.

The neutral lipid fraction was separated into three fractions via silica gel chromatography using 2 ml hexane, 4 ml DCM and 4 ml MeOH representing alkanes, alkenes and alcohols, respectively. The MeOH fraction containing the alcohols was concentrated under N₂ and dissolved in hexane for analysis using a ThermoFinnigan Trace gas chromatography instrument with flame ionization detection (GC-FID). Temperature program for the injector (PTV) was: 80 °C (held 0.30 min) to 320 °C at 14.5 °C/s. The oven temperature program was: 60 °C (1 min) to 320 °C (held 30 min) at 7 °C/min. A Restek Rxi®-1 ms column (60 m × 0.25 mm i.d., 0.25 μm film thickness) was used; tetrahymanol identity was confirmed by analyzing the MeOH fraction with a ThermoFinnigan GC-mass spectrometry (GC-MS) instrument, eluting at ca. 26 min. FAMES were assigned by comparing elution times with standard FAMES analyzed using the same temperature program as above.

2.3. Hydrogen isotope analysis of water, substrate and lipids

The hydrogen isotopic composition of the growth medium was measured in duplicate after autoclaving at the beginning of the culture experiments, using a ThermoFinnigan MAT 253 isotope ratio monitoring mass spectrometer coupled to a Thermo H-Device. Analytical precision for δD measurements of culture media was ± 3‰. In order to determine δD values of the non-exchangeable hydrogen of the substrates used (dextrose, yeast extract and bacto-peptone), each was equilibrated with two different water samples with different δD values following the protocol described by Qi and Coplen (2011). Following equilibration, substrates were analyzed using a Thermo Delta Plus XP stable isotope mass spectrometer coupled to a TC/EA instrument. The δD values of dextrose, yeast extract and peptone were 29.97‰, −95.26‰ and −63.2‰, respectively. However, the uncertainty associated with these estimates can be as much as ± 20‰ (Zhang et al., 2009a).

Lipid δD values were measured using a Finnigan MAT 253 stable isotope mass spectrometer coupled to a Thermo Trace2000 GC instrument with a high temperature conversion system. The H₃⁺ factor averaged 16.52 ± 0.39 and was determined every day prior to sample analysis. We used *n*-alkane isotope standards (Mix A, Indiana University Biogeochemical Laboratory) to monitor instrument accuracy and stability. We anticipated some of the samples would contain lipids more D-enriched than Mix A standard *n*-alkanes. Accordingly, we used a D-enriched eicosanoic acid methyl ester standard (Standard # X, Indiana University Biogeochemical Laboratory) in addition to Mix A. The δD values of both tetrahymanol and FAMES were corrected using Mix A and Standard # X. Average precision of δD measurements of standards was ± 6.8‰. All lipid samples were measured in duplicate. The δD value (−131‰) of the added methyl in the FAMES was determined by methylating a known phthalic acid standard (δD −81.9‰) obtained from Indiana University Biogeochemical Laboratory. All FAME δD values were corrected for the added methyl by mass balance.

Hydrogen isotope data were calculated as follows:

$$\delta D = R_{\text{sample}}/R_{\text{std}} - 1, \quad (2)$$

where $R_{\text{sample}} = (D/H)_{\text{sample}}$. $R_{\text{std}} = (D/H)_{\text{VSMOW}}$, VSMOW represents Vienna Standard Mean Ocean Water.

$$\alpha_{A-B} = (\delta D_A + 1)/(\delta D_B + 1) \quad (3)$$

$$\epsilon_{A-B} = \alpha_{A-B} - 1 \quad (4)$$

where *A* represents lipids and *B* represents water or dextrose (substrate).

3. Results

3.1. Growth rate of *T. thermophila*

T. thermophila growth rate exhibited only small differences with increasing temperature: 1.35 divisions day^{−1} at 24 °C, 1.37 divisions day^{−1} at 30 °C and 1.36 divisions day^{−1} at 36 °C.

3.2. Lipid distribution of *T. thermophila*

Tetrahymanol was the dominant neutral lipid in all cultures, but abundance varied with temperature. At 30 °C, a variety of FAs were observed, including saturated *n*-acids (C₁₄, C₁₆, C₁₇ and C₁₈), with *n*-C_{16:0} the most abundant and *n*-C_{17:0} the least abundant. *T. thermophila* grown at 24 °C contained lower molecular weight FAs dominated by *n*-C_{12:0} and *n*-C_{14:0}, with *n*-C_{16:0} and *n*-C_{18:0} in lower abundance. At 36 °C, *n*-C_{12:0} and *n*-C_{14:0} were dominant, with a very low abundance of *n*-C_{18:0}. The average ratio of tetrahymanol to total FAs (measured as the ratio of tetrahymanol peak area and the sum of the peak areas of all FAs) also showed temperature dependency, with values of 3.4 (± 2.5) at 24 °C, 2.22 (± 0.79) at 30 °C, and, 32.8 (± 16.2) at 36 °C (Table 1, Fig. 2b).

No neutral and polar lipids were detected in either the bacto-peptone or yeast extract used for media preparation.

3.3. Hydrogen isotopic composition of lipids

Results of hydrogen isotope analysis from all culture experiments (Table 1) indicated a temperature effect on lipid hydrogen isotopic composition. For example, with δD_{water} ca. −40‰ and growth temperature 24 °C, 30 °C and 36 °C, δD_{tetrahymanol} values were −88.5‰, −56.5‰ and −7.1‰, respectively (Figs. 1a and 2a). With δD_{water} of ca. 12‰, δD_{FA} values of *n*-C_{12:0} were −33.2‰ and −15.7‰ at 24 °C and 36 °C (Fig. 2a). Isotopic values of *n*-C_{14:0} were −15.5‰, 24.1‰ and 22.2‰ at 24 °C, 30 °C and 36 °C (Figs. 1b and 2a) and *n*-C_{16:0} values were 58.7‰ at 24 °C and 59.8‰ at 30 °C (Fig. 2a). δD_{FA} values of *n*-C_{18:0} were 92.3‰, 93.5‰ and 113.5‰ at 24 °C, 30 °C and 36 °C respectively (Fig. 2a). However, the temperature effect was not uniform for all the lipids, as indicated by the biosynthetic hydrogen isotopic fractionation values calculated between the same lipid at different temperatures (Δε_{T1-T2}, with T1 and T2 representing different growth temperatures, Table 2). Significant temperature effect on biosynthetic fractionation was evident for tetrahymanol and *n*-C_{18:0}, as temperature increased from 30 °C to 36 °C, and for *n*-C_{14:0}, as temperature increased from 24 °C to 30 °C (Table 2). Hydrogen isotope composition of all lipids correlated linearly with δD_{water}, with R² values from 0.97 to 0.99. Slopes of δD_{lipid}−δD_{water} regressions were distinct for different lipids (Fig. 1a and b).

Apart from the temperature effect on δD_{lipid}, FAs were more D-enriched with increasing chain length for all growth temperatures (Fig. 2a), which agrees with observations from higher plants (Sessions et al., 1999; Chikaraishi et al., 2004a), marine brown and red microalgae (Chikaraishi et al., 2004b) and bacteria (Sessions et al., 2002; Campbell et al., 2009). In general, isoprenoids produced by organisms are D-depleted relative to acetogenic lipids (Sessions et al., 1999, 2002). However, for our *T. thermophila* cultures, isoprenoids were generally more D-depleted than acetogenic lipids at 24 °C and 30 °C, but D-enriched at 36 °C (Fig. 2a), due possibly to temperature effects on biosynthetic and metabolic processes.

4. Discussion

4.1. Consideration of carbon substrate

The medium had dextrose as the primary carbon source, but also contained small amounts of peptone and yeast extract, which

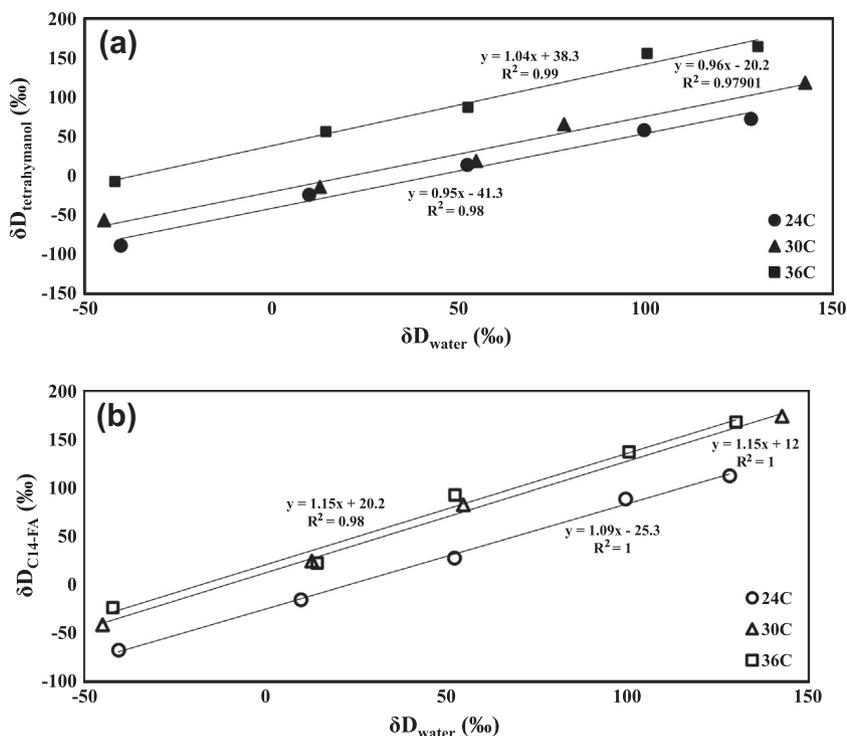


Fig. 1. $\delta D_{\text{lipid}} - \delta D_{\text{water}}$ correlations for tetrahymanol (a) and C₁₄ FA (b) at 24 °C, 30 °C and 36 °C.

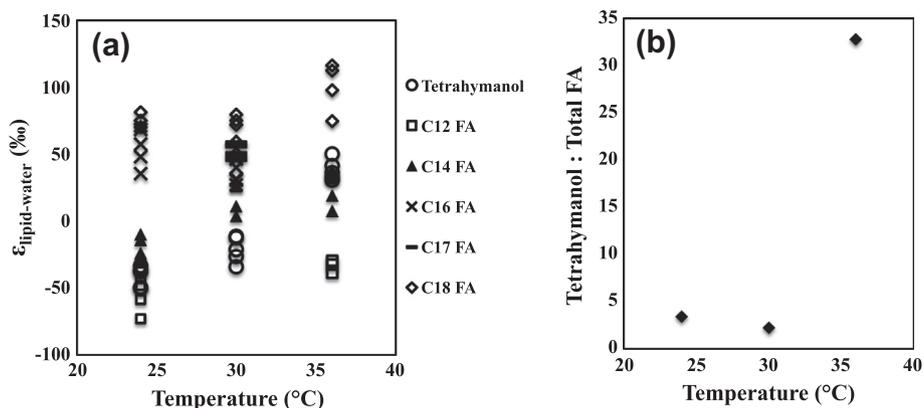


Fig. 2. $\epsilon_{\text{lipid-water}}$ values for different lipids (a) and tetrahymanol/total FAs (b) at 24 °C, 30 °C and 36 °C.

Table 2

$\Delta\epsilon_{T_1-T_2}$ values for *T. thermophila* lipids (St dev, standard deviation).

Lipid	$\Delta\epsilon_{30-24}$ (‰)	St dev	$\Delta\epsilon_{36-30}$ (‰)	St dev	$\Delta\epsilon_{36-24}$ (‰)	St dev
Tetrahymanol	20.1	17.6	63.3	16.8	—	—
C _{12:0} FA	—	—	—	—	10.6	7.2
C _{14:0} FA	44.3	12.6	5.1	10.7	—	—
C _{16:0} FA	-20.9	22.6	—	—	—	—
C _{18:0} FA	-6.2	10.1	36.5	18.1	—	—

are mixtures of various compounds including carbohydrates, proteins and amino acids. Some of them can potentially act as carbon substrates, but δD values of these trace compounds were unknown. Lipids synthesized by *T. thermophila* were significantly D-enriched, indicating that a D-enriched substrate was consumed. This suggests that dextrose ($\delta D_{\text{dextrose}} 29.97\text{‰}$) was the dominant carbon source, compared with yeast extract and peptone, with bulk δD values of -95.26‰ and -63.2‰ , respectively. However, peptone

and yeast extract could have contained certain D-enriched components, which were utilized during growth as carbon source and contributed to the D/H fractionation patterns. Presently, it is not possible to confirm the presence or impact of additional D-enriched components in the yeast extract and peptone.

The δD values of lipids in *T. thermophila* correlated well with ambient water (Fig. 1), demonstrating that δD_{water} exerted a primary influence on δD_{lipid} . This observation is consistent with other

culture experiments showing that water, directly and indirectly via reducing agents such as NADPH or nicotinamide adenine dinucleotide phosphate, contributed 50–80% of all carbon-bound hydrogen in lipids (Zhang et al., 2009a).

4.2. Consideration of temperature and growth effects

Experiments have shown growth temperature impacts on δD_{lipid} in two freshwater algae and two marine coccolithophorid species (Schouten et al., 2006; Zhang et al., 2009b). Higher growth temperature produced more D-depleted lipids, provided that conditions were below optimal growth temperature. A similar effect was observed with a heterotrophic halophilic archaea (Dirghangi and Pagani, 2013). In contrast, our results indicate that *T. thermophila* lipids became more D-enriched with increasing temperature. This was particularly the case for tetrahymanol, which ranged from being D-depleted to D-enriched relative to the hydrogen isotopic composition of water (δD_{water}), depending on growth temperature (Figs. 1a and 2a). Reversal of isotopic trends, as observed for tetrahymanol, has not been reported. Interestingly, increasing temperature did not affect all *T. thermophila* lipids in a similar manner (Fig. 2a). For example *n*-C_{14:0} became increasingly D-enriched with increasing temperature from 24 °C to 30 °C, but did not become more D-enriched at higher temperatures (Figs. 1b and 2a).

Various mechanisms have been proposed which could potentially explain the observed temperature effects on δD_{lipid} , including impact on growth rate, enzyme activity and processes related to NADP⁺ reduction. However, different mechanisms can be adopted by different species in response to change in growth temperature. For example, the freshwater algae *Eudorina uniccocca* and *Volvox aureus* displayed little response in growth rate to temperature, whereas both growth rate and enzymatic activity of the marine coccolithophorids, *Emiliania huxleyi* and *Gephyrocapsa oceanica*, were significantly affected (Schouten et al., 2006; Zhang et al., 2009b).

4.3. Growth rate related processes

Growth rate of organisms is temperature dependent, with the highest rates associated with optimal growth temperatures specific to a particular organism. In previous studies, a negative correlation between $\alpha_{\text{alkenone-water}}$ and growth rate was observed for two coccolithophorid species, *E. huxleyi* and *G. oceanica* (Schouten et al., 2006). Further, lower growth rates were accompanied by a significant D-enrichment in sterols for the marine diatom *Thalassiosira pseudonana*, but no isotopic effect was observed for FAs (Zhang et al., 2009b). Isotopic effects for sterols were possibly related to mixing of isoprenoid compounds generated via both MVA and MEP pathways (each with distinct D/H signatures), given that higher plants and algae maintain the simultaneous presence of both pathways – MVA in the cytoplasm and MEP in the chloroplast (Zhang et al., 2009b). However, this supposition is not applicable to our results because isoprenoid biosynthesis occurs only via the MVA pathway in *T. thermophila*. For halophilic archaea, higher $\alpha_{\text{water-lipid}}$ values are associated with higher growth rate, resulting in more D-depleted lipids (Dirghangi and Pagani, 2013), although in these experiments changes in growth rate occurred as a result of both temperature and salinity variation. In contrast, heterotrophic *E. coli* displayed minimal growth rate effect on water–lipid D/H fractionation (Sessions et al., unpublished results). Therefore, growth rate effects on δD_{lipid} are not systematic and imply that differences in fractionation are likely caused by other mechanisms linked to growth rate. For example, hydrogen isotope exchange between NADPH and water, which results in a partial loss of the D/H signal of NADPH in vitro (Zhang et al., 2009a and references therein) is related to the cellular turnover time of NADPH. However, hydrogen isotope exchange between water and NADPH will only

reduce $\alpha_{\text{water-lipid}}$ and cannot be responsible for the observed isotopic reversal of tetrahymanol at higher temperatures (Fig. 2a).

Alternatively, patterns of $\alpha_{\text{water-lipid}}$ could express changes in the D/H composition of intracellular water. Intracellular water derives from both the surrounding medium and metabolism. During exponential growth of *E. coli*, when growth rate was highest, 53% of intracellular water derived from metabolism, while only 23% derived from metabolism during the stationary growth phase with lower growth rate (Kreuzer-Martin et al., 2006). Further, the δD value of metabolic water became substantially D-depleted during growth, changing from –93‰ during exponential phase to –179‰ during stationary phase – signals that were also recorded in the hydrogen isotopic compositions of FAs (Kreuzer-Martin et al., 2006). These isotopic changes could have been related to the utilization of different carbon substrates in the culture media (e.g. LB medium containing yeast extract and tryptone), or to increased proton pumping, which can make intracellular water more D-enriched during exponential growth (Kreuzer-Martin et al., 2006). A similar growth phase effect was observed for alkenones produced by *E. huxleyi* and *G. oceanica* (Wolhowe et al., 2009), with more D-enriched alkenones in the exponential phase compared with the stationary phase. Wolhowe et al. (2009) attributed this isotopic pattern with growth phase to two possible mechanisms – (i) decreasing biosynthesis of D-depleted isoprenoids and consequent channeling of NADPH to alkenone (acetogenic lipid) synthesis, or (ii) a scenario where intracellular water becomes increasingly isolated from extracellular water. Both mechanisms can be related to the growth phase, but mechanism (i) would cause D-depletion and (ii) would cause D-enrichment in lipids (Wolhowe et al., 2009).

All our *T. thermophila* cultures were extracted in stationary phase, but small differences in growth rate at different temperatures were observed, with highest growth rates at 30 °C and lower growth rates at both 24 °C and 36 °C. If metabolic water effects were at play, lipids would appear more D-enriched at 30 °C and D-depleted at 24 °C and 36 °C. Indeed, D-enrichment was observed for *n*-C_{14:0} at 30 °C relative to 24 °C. In contrast, at 36 °C tetrahymanol shifted from being D-depleted to D-enriched compared with water. Isoprenoids are generally D-depleted relative to FAs (Sessions et al., 1999, 2002) and, hypothetically, a decrease in FA synthesis with increasing temperature, as observed in our experiments (e.g. tetrahymanol/FA 3.42 at 24 °C, 2.22 at 30 °C, and 32.8 at 36 °C; Fig. 2b), could lead to D-enriched tetrahymanol at 36 °C (derived from mechanism (i) proposed by Wolhowe et al., 2009). Unfortunately, absolute lipid abundances for our culture experiments were not quantified and it was not possible to ascertain whether tetrahymanol content increased or FA content decreased at 36 °C. However, tetrahymanol in *Tetrahymena* cell membranes performs the same function as cholesterol in mammals – maintaining membrane fluidity and stability (Nozawa et al., 1974; Wilton, 1983; Nozawa, 2011). Given that optimal growth temperature for *T. thermophila* is 35 °C (Frankel et al., 1980a,b; Frankel, 1999; Frankel and Nelsen, 2001), the anomalously high relative abundance of tetrahymanol at 36 °C indicates increased tetrahymanol demand, and stress on cell membrane integrity. Such a scenario would be accompanied by an increase in tetrahymanol synthesis at higher than optimal temperature, as observed for *T. pyriformis* grown at low (15 °C) and high (39 °C) temperature extremes (Ramesha and Thompson, 1982). If the observed increase in tetrahymanol/FA ratio was due to an increased rate of tetrahymanol synthesis, it would account for our observed D-enrichment in tetrahymanol.

4.4. Temperature effects on metabolism

Temperature exerts a significant control on the metabolism of *Tetrahymena* and other organisms. Studies involving *T. pyriformis*,

with optimal growth temperature of 32.5 °C (Prescott, 1957), revealed considerably higher respiratory rate at 10 °C vs. 30 °C, even though growth rate increased (James and Read, 1957; Levy, 1973). Oxygen consumption experiments for *T. geleii* showed an increase in respiration rate from 10 °C to 25 °C and then a decrease from 25 °C to 35 °C (Pace and Lyman, 1947). Optimal growth temperature for *T. geleii* was 32.5 °C (Prescott, 1958), but the highest O₂ consumption rate was observed at 25 °C (Pace and Lyman, 1947). Above 25 °C, respiration rate decreased with temperature, both at 30 °C and 35 °C (i.e. below and above optimal growth temperature, respectively).

Sensitivity of respiration to temperature in *Tetrahymena* could result from an inhibitory temperature effect on the rate of oxidative phosphorylation (Eichel, 1959a,b; Levy et al., 1969) due to the impact of temperature on respiratory enzymes (Levy, 1973 and references therein). For example, despite the highly unstable nature of *Tetrahymena* enzymes in vitro (Thompson, 1969; Levy, 1973), elevated temperature has been shown to substantially reduce the activity of succinoxidase and DPNH (i.e. NADH) oxidase (Eichel, 1956, 1959a), succinic dehydrogenase (Eichel, 1959b; Levy, 1973), DPNH-cytochrome C reductase (Kamiya and Takahashi, 1961), and lactic oxidase (Eichel and Rem, 1962), on brief exposure. This inhibitory effect could be due to lysophosphatides that originate from the breakdown of particulate phospholipids in *Tetrahymena* cells by the enzyme phospholipase, and cause disruption of membranes (i.e. lysis) and membrane-associated enzymes (Eichel, 1960; Thompson, 1969; Levy, 1973; Mason and Lin, 1979). Thus, lower rates of aerobic respiration in *Tetrahymena* (*T. pyriformis* and *T. geleii*) can occur with increasing temperature even as growth rate increases (Pace and Lyman, 1947; James and Read, 1957). Therefore, we hypothesize that similar reactions with temperature change were associated with *T. thermophila* in our experiments. Aerobic respiration in *Tetrahymena* is a catabolic reaction, which generates NADPH. NADPH acts as a reducing agent during anabolic (biosynthesis) reactions, rates of which are reflected in the growth rate of the organism. A balance is generally maintained between catabolism and anabolism in organisms (Sauer et al., 2004). However, imbalance between them can occur, during which cellular NADPH levels are maintained by transhydrogenase enzymes (Sauer et al., 2004) that drive the interconversion of NADPH and NADH. This process, characterized by substantial D/H fractionation, can produce significantly D-enriched NADPH (Zhang et al., 2009a) and help explain our observation of D-enriched tetrahymanol at temperatures above optimal growth conditions.

Other metabolic effects could be at play as well that impact δD_{lipid} . For example, higher temperature reduces the activity of enzymes (Zhang et al., 2009b) and leads to their replacement by more heat tolerant isoenzymes with different isotopic effects (Zhang et al., 2009b). Further, processes such as hydrogen tunneling that affect the δD value of NADPH are temperature dependent (Zhang et al., 2009b and references therein), and different pathways of NADP⁺ reduction – such as the pentose phosphate pathway – can be adopted under different temperature conditions (Zhang et al., 2009b).

5. Conclusions

T. thermophila was grown in pure culture at three temperatures – 24 °C, 30 °C and 36 °C. Growth rate was highest at 30 °C and lower at 24 °C and 36 °C. Tetrahymanol and FAs of various chain length (*n*-C_{12:0}, *n*-C_{14:0}, *n*-C_{16:0}, *n*-C_{17:0} and *n*-C_{18:0}) were synthesized in varying amounts. The δD values of tetrahymanol and FAs showed near perfect correlation with δD_{water} , although temperature exerted a significant influence on the relationship. Tetrahymanol, in particular, was characterized by a reversal of D/H signal as growth

temperature increased – ranging from being D-depleted (at 24 °C and 30 °C) to D-enriched relative to water (at 36 °C). The relative abundance of tetrahymanol vs. FAs was substantially higher at 36 °C. The observed temperature effects on δD_{lipid} suggest a critical control of temperature on δD values of NADPH hydrogen and possibly intracellular water, due to processes related to growth and metabolism of *T. thermophila*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.orggeochem.2013.09.007>.

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