Exploring lipid $^2\text{H}/^1\text{H}$ fractionation mechanisms in response to salinity with continuous cultures of the diatom *Thalassiosira pseudonana*

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ABSTRACT

The hydrogen isotopic ($^2\text{H}/^1\text{H}$) composition of lipids in microalgae is significantly depleted relative to extracellular water. While a variety of growth conditions influence the magnitude of ^2H -depletion, the effect of salinity is of particular interest due to the paleohydrological applications of lipid $^2\text{H}/^1\text{H}$. In previous studies, lipid-water $^2\text{H}/^1\text{H}$ fractionation was shown to decrease as salinity increased, a response largely independent of lipid type, species, or setting. The mechanism responsible for this response remains uncertain, primarily because salinity is rarely isolated as the sole variable in laboratory cultivation experiments investigating hydrogen isotope systematics in microalgae. Here we report the lipid–water $^2\text{H}/^1\text{H}$ fractionation response to salinity in nutrient-replete continuous cultures of the centric diatom *Thalassiosira pseudonana*. In six cultures with the same growth rate at salinities between 14–40 ppt, lipid–water $^2\text{H}/^1\text{H}$ fractionation decreased linearly as salinity increased by 1.3‰/ppt in fatty acids ($\text{C}_{14:0}$, $\text{C}_{16:0}$, $\text{C}_{16:1}$) and by 1.0‰/ppt in the sterol 24-methylcholesta-5,24(28)-dien-3 β -ol. A constant growth rate between cultures reveals that the fractionation response to salinity is independent of growth rate. Sensitivity tests using a simple hydrogen flux model indicated that at high salinity a greater proportion of metabolic NAD(P)H in lipids at the expense of photosynthetic NADPH can cause ^2H -enrichment. Additionally, increased exudate release or decreased hydrogen transport can enrich both lipids and cell–water in ^2H . The 1.0–1.3‰/ppt increase in lipid–water $^2\text{H}/^1\text{H}$ fractionation observed in *T. pseudonana* is within the 0.8–2‰/ppt range observed in field studies and culture studies, supporting the application of algal lipid $^2\text{H}/^1\text{H}$ as a paleosalinity proxy.

1. Introduction

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25 The $^2\text{H}/^1\text{H}$ ratio of individual phytoplankton lipids preserved in sediments (Sachse et
26 al., 2012), is a promising tool for reconstructing past hydrologic variability (Pahnke et al.,
27 2007; van der Meer et al., 2007, 2008; Sachs et al., 2009; Smittenberg et al., 2011; Leduc et
28 al., 2013; Vasiliev et al., 2013; Atwood and Sachs, 2014; Kasper et al., 2014; Zhang et al.,
29 2014; Petrick et al., 2015; Simon et al., 2015; Nelson and Sachs, 2016; Richey and Sachs,
30 2016). Hydroclimate reconstructions rely on the link between the hydrogen isotopic
31 composition of environmental waters and fluxes of water through the hydrologic cycle
32 (Craig, 1961; Dansgaard, 1964; Craig and Gordon, 1965; Gat, 1996). Deuterium/protium
33 ($^2\text{H}/^1\text{H}$) ratios of environmental samples are expressed as $\delta^2\text{H}_{\text{sample}}$ ($\delta^2\text{H}_{\text{sample}} =$
34 $[(^2\text{H}/^1\text{H})_{\text{sample}}/(^2\text{H}/^1\text{H})_{\text{VSMOW}}] - 1$) where VSMOW is Vienna Standard Mean Ocean Water.
35 Molecular paleohydrology relies on the relationship between the $\delta^2\text{H}$ of photosynthetically
36 produced lipids and that of their environmental source water. Culture experiments with a
37 variety of microalgae species have shown that $\delta^2\text{H}_{\text{lipid}}$ is almost perfectly correlated with
38 $\delta^2\text{H}_{\text{water}}$ with R^2 values in excess of 0.99 (Paul, 2002; Englebrecht and Sachs, 2005; Zhang
39 and Sachs, 2007).

40 Phytoplankton lipids do not directly record the actual isotopic composition of source
41 water. The offset between $\delta^2\text{H}_{\text{lipid}}$ and $\delta^2\text{H}_{\text{water}}$, the lipid–water $^2\text{H}/^1\text{H}$ fractionation,
42 represents the net isotopic effect of cellular processes associated with lipid biosynthesis.
43 Fractionation is characterized by an apparent fractionation factor, $\alpha_{\text{lipid–water}}$ where $\alpha_{\text{lipid–water}}$
44 $= (^2\text{H}/^1\text{H})_{\text{lipid}}/(^2\text{H}/^1\text{H})_{\text{water}} = (\delta^2\text{H}_{\text{lipid}} + 1000)/(\delta^2\text{H}_{\text{water}} + 1000)$. Most biosynthetic and all
45 photosynthetic products appear to be ^2H -depleted compared to ambient water resulting in
46 $\alpha_{\text{lipid–water}} < 1$. Lipid–water $^2\text{H}/^1\text{H}$ fractionation factors can vary widely for different species

of phytoplankton and different compounds (Sessions et al., 1999; Zhang and Sachs, 2007; Sachse et al., 2012), requiring the use of taxon-specific biomarkers.

The magnitude of $\alpha_{\text{lipid-water}}$ for any particular phytoplankton lipid can also be significantly influenced by several environmental and physiological parameters. This has important implications for proper interpretations of paleoclimate from molecular archives, prompting recent efforts to understand and characterize factors that impact microorganism $\alpha_{\text{lipid-water}}$ including growth rate (Schouten et al., 2006; Zhang et al., 2009b; Sachs and Kawka, 2015), growth stage (Wolhowe et al., 2009, 2015; Chivall et al., 2014; Heinzelmann et al., 2015a, 2015b), irradiance (van der Meer et al., 2015), temperature (Estep and Hoering, 1980; Stiller and Nissenbaum, 1980; Wolhowe et al., 2009; Zhang et al., 2009b), metabolism (Zhang et al., 2009b; Osburn et al., 2011; Dirghangi and Pagani, 2013a, 2013b; Dawson et al., 2015; Heinzelmann et al., 2015a, 2015b), and salinity (Schouten et al., 2006; Sachse and Sachs, 2008; Sachs and Schwab, 2011; Chivall et al., 2014; M'Boule et al., 2014; Nelson and Sachs, 2014; Heinzelmann et al., 2015a; Kasper, 2015; Sachs et al., 2016)

Of particular concern for the application of molecular paleohydrology is the fact that increasing salinity can have a significant influence on $\alpha_{\text{lipid-water}}$ in eukaryotic microalgae and cyanobacteria. Apparent lipid–water $^2\text{H}/^1\text{H}$ fractionation in alkenones decreased ($\alpha_{\text{lipid-water}}$ increased) $\sim 3\text{‰/ppt}$ in *Emiliana huxleyi* and *Gephyrocapsa oceanica* grown in batch cultures at different salinities (Schouten et al., 2006) (but also at different temperatures and growth rates as discussed in Zhang et al. (2009b)). Apparent lipid–water $^2\text{H}/^1\text{H}$ fractionation decreased $\sim 0.7\text{--}1\text{‰/ppt}$ in cyanobacteria lipids from several hypersaline ponds on Christmas Island (Sachse and Sachs, 2008), in the dinoflagellate lipid dinosterol along the Chesapeake Bay estuary (Sachs and Schwab, 2011), and in dinosterol and brassicasterol

from a global survey of saline and hypersaline lakes (Nelson and Sachs, 2014). In alkenones from batch-cultured open-ocean haptophytes $^2\text{H}/^1\text{H}$ fractionation decreased $\sim 1\text{--}2\text{‰}$ /ppt in exponential phase (Chivall et al., 2014; M'Boule et al., 2014), and $\sim 0.8\text{--}1\text{‰}$ /ppt in stationary and decline phases of the coastal species (Chivall et al., 2014). $\text{C}_{16:0}$ fatty acid from batch cultures in exponential, stationary, and decline phases all displayed decreasing $^2\text{H}/^1\text{H}$ fractionation of $\sim 1.5\text{‰}$ /ppt (Heinzelmann et al., 2015a). $^2\text{H}/^1\text{H}$ fractionation decreased $\sim 1.2\text{--}2.2\text{‰}$ /ppt in nitrogen-limited chemostats of *E. huxleyi* maintained at the same growth rate (Sachs et al., 2016). It has been proposed that this nearly universal trend may be due to lower growth rates at sub-optimal salinity (Sachse and Sachs, 2008; Sachs and Schwab, 2011), decreased water transport across the outer cell membranes (Sachse and Sachs, 2008; Sachs and Schwab, 2011; Heinzelmann et al., 2015a; Sachs et al., 2016), increased production of small, compatible solutes (osmolytes) resulting in increased recycling of intracellular water and a progressively ^2H -enriched cell water pool (Sachse and Sachs, 2008; Sachs and Schwab, 2011), or an increase in the exudation of ^2H -depleted organic matter from cells at high salinity resulting in a ^2H -enriched cell water pool (Sachs et al., 2016).

In order to clarify the biochemical mechanisms responsible for the “salinity effect” we conducted nutrient-replete steady state continuous culture experiments that had the same growth rate across a 26 ppt range of salinities with the diatom *T. pseudonana* to isolate the effect of salinity on $^2\text{H}/^1\text{H}$ fractionation in phytoplankton lipids. The diatom *T. pseudonana* was chosen because it is an extensively studied model organism (Armbrust et al., 2004) that can survive in a wide range of salinities (0–45 ppt). Continuous cultures are constant volume cultures that are maintained indefinitely in exponential growth at a growth rate set by the

media supply rate. They provide an advantage over batch cultures (Bull, 2010) by allowing for reproducible, steady state growth rates at several different salinities while maintaining constant temperature, light, nutrient, and chemical conditions. We present lipid–water $^2\text{H}/^1\text{H}$ fractionation values in three fatty acids and 24-methyl-cholesta-5,24(28)-dien-3 β -ol (referred to as “sterol” for the remainder of the paper). This work complements a recent study using nitrogen-limited chemostats (Sachs et al., 2016) by investigating the effect of salinity on $\alpha_{\text{lipid-water}}$ in nutrient-replete continuously cultured microalgae; both studies support the universality of a 1–2‰/ppt sensitivity of $\alpha_{\text{lipid-water}}$ to salinity. A simple model of hydrogen fluxes indicated that lipid–water $^2\text{H}/^1\text{H}$ fractionation is sensitive to: (i) proportionally more lipid hydrogen derived from the ^2H -enriched pool of metabolically reduced nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) vs the ^2H -depleted pool of photosynthetically reduced NADPH, (ii) increased incorporation of cell-water-hydrogen into organic material, (iii) decreased transport of water in and out of cells, or (iv) and increased extracellular release of organic hydrogen.

2. Methods

2.1. Continuous cultures

The diatom *T. pseudonana* (CCMP 1335) was grown in continuous cultures at a range of salinities chosen to ensure that approximately identical growth rates could be maintained across treatments: 14, 18, 27, 31, 36, and 40 ppt. An additional culture was attempted at 9 ppt which had a lower growth rate. Continuous cultures were grown in 5 l of

f/2 media made from mixing MilliQ water with Instant Ocean aquarium salts (Spectrum Brands, Middleton, WI) and adding trace metals, vitamins, and macronutrients to achieve concentrations of 882 μM NO_3^- , 36 μM PO_4^{3-} , and 106 μM $\text{Si}(\text{OH})_4$ (Guillard and Ryther, 1962; Guillard, 1975). Cultures were grown under 24 h light ($224 \pm 54 \mu\text{mol}/\text{m}^2\text{s}$) at 20 °C with an inflow/outflow rate 4.7–5.0 ml/min. Detailed culture parameters are provided in Supplementary Table S1. Cultures were monitored via *in vivo* fluorescence (IVF) twice daily. Steady state conditions (taken as IVF varying less than 15% of the mean of all measurements) were maintained for 2–4 days before twice-daily subsampling for chlorophyll *a* (Chl *a*) and dissolved nutrients for a further 2–3 days. Chl *a* and nutrient subsamples were analyzed according to standard methods (UNESCO 1994). Additional 1 ml subsamples were preserved with formalin for cell density, cell diameter, and cell volume analyses on a Beckman Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter, Fullerton CA, USA). For details of the cell enumeration, see the Supplementary material (Section 1). Growth rates in divisions per day (div/d) were estimated using dilution rate (flow-rate/volume) divided by $\ln(2)$ (Wood et al., 2005) and averaged over the 2–3 day steady state sampling period. After 5–6 total days of steady state growth, each entire culture was harvested onto a pre-combusted Whatman 142 mm diameter 0.7 μm pore size GF/F filter and immediately frozen at –20 °C.

2.2. Water and lipid $\delta^2\text{H}$

Samples for isotopic analysis of the culture media were taken at the start and end of each continuous culture. $\delta^2\text{H}_{\text{water}}$ was measured six times per sample (the first three measurements were discarded to avoid memory effects) on a Picarro L2130-i Isotopic

Liquid Water Analyzer with A0211 High-Precision Vaporizer (Picarro, Inc., Santa Clara, CA) and normalized to VSMOW using three in-house water standards with $\delta^2\text{H}$ of $-107.3 \pm 0.5\text{‰}$, $-76.1 \pm 0.3\text{‰}$, and $14.8 \pm 1.0\text{‰}$. The average precision of sample measurements was 0.19‰.

Subsections of each frozen harvest filter were freeze-dried and cut into small pieces. Lipids were extracted by an Accelerated Solvent Extractor (ASE-200, Dionex Corp., Sunnyvale, CA, USA) using dichloromethane:methanol (DCM:MeOH; 9:1, v:v) at 1500 psi and 100 °C for three 5 min cycles. Total lipid extracts (TLEs) were saponified with 1N KOH at 70 °C for 12 h followed by liquid–liquid extractions with hexane. Aliquots of the saponified TLEs were silylated by dissolving them in 20 µl of pyridine and 20 µl of bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich, St. Louis, MO, USA) and heating at 60 °C for 1 h. Lipid abundances were determined via gas chromatography–flame ionization (GC-FID) and recovery corrected using *n*-C₃₆-alkane added to each sample prior to ASE extraction. Lipid identifications were made by comparing mass spectra generated by gas chromatography–mass spectrometry (GC–MS) to previously published mass spectra and laboratory standards (Zhang et al., 2009b). GCFID and GC–MS parameters were the same as in Sachs et al. (2016) and are provided in SupplementaryTable S5.

The remaining saponified TLEs were then purified using one-step column chromatography with a solid phase of 0.5 g aminopropyl gel (Supelco, Lot #2511301, Part # 5-7205). The sterol-containing fraction was eluted first in 8 ml of DCM:isopropyl alcohol (3:1, v:v), followed by the fatty acids in 6 ml 4% acetic acid in diethyl ether. The fatty acid containing fractions were methylated by dissolving samples in 1 ml hexane with 2 ml MeOH:acetyl chloride (10:1, v:v) and heating for 12 h at 60 °C, followed by recovery via

liquid-liquid extraction into hexane. Phthalic acid of known isotopic composition ($-95.5 \pm 2.2\text{‰}$, Dr. Arndt Schimmelmann, Indiana University) was methylated via the same method to determine the isotopic composition of the methylating agent. Sterol-containing fractions were acetylated by dissolving in 20 μl of pyridine plus 20 μl of acetic anhydride of known hydrogen isotopic composition ($-123.8 \pm 8.2\text{‰}$) and heating at 70 °C for 0.5 h.

Lipid $\delta^2\text{H}$ values were measured via gas-chromatography isotope-ratio mass spectrometry (GC-IRMS) according to the settings in Sachs et al. (2016) and provided in Supplementary Table S5. Injections of external isotopic standards spanning a range of retention times and isotopic compositions of known hydrogen isotopic composition (Supplementary Table S5) were injected separately after every 3–6 samples. Fatty acid and sterol data were evaluated and corrected using external standards as in Nelson and Sachs (2014b). Fatty acids were corrected for methylation and sterol for acetylation by mass balance calculations. The average $\delta^2\text{H}_{\text{standard}}$ precision of multiple injections for external and internal standards was 3.5‰.

3. Results

3.1. Continuous cultures

None of the monitored culture parameters changed systematically with salinity except the siliceous acid remaining in the media after filtration (residual silica) (Table 1). Further details can be found in the Supplementary material (Sections 1 and 2). Continuous cultures at salinities 14, 18, 27, 31, 36, and 40 ppt could be maintained within a narrow

range of growth rates between 1.90 and 1.99 div/d with a 1.97 ± 0.03 div/d mean while the fresher culture at salinity 9 ppt had a lower growth rate at 1.62 ± 0.03 div/d (Table 2).

3.2. δ^2H values of water and lipids

Isotope measurements and lipid concentrations are reported in Table 3. Concentrations of myristic ($C_{14:0}$), palmitic ($C_{16:0}$), and palmitoleic ($C_{16:1}$) fatty acids and the sterol were not linearly correlated with salinity (Table 1). The culture media that was sampled when the cells were harvested and had nearly constant δ^2H_{media} across all cultures averaging $-74.4 \pm 2.3\text{‰}$ (Fig. 1). Two media samples taken at the start of steady state conditions reveal that the isotopic composition of media during culture growth was constant with δ^2H_{media} values of $-75.6 \pm 0.12\text{‰}$ (31 ppt) and $-73.6 \pm 0.42\text{‰}$ (36 ppt). All lipids were 2H -depleted compared to culture water, δ^2H_{lipid} values increased as salinity increased, and were well correlated with salinity with R^2 values between 0.84 and 0.98 (Fig. 1). Fatty acids were the least depleted lipids. $\delta^2H_{C_{14:0}}$ was between -248‰ and -218‰ , a range of 30‰ with a mean of $-234 \pm 12\text{‰}$. $\delta^2H_{C_{16:1}}$ was between -234‰ and -206‰ , a range of 29‰ with a mean of $-221 \pm 13\text{‰}$. $\delta^2H_{C_{16:0}}$ was between -247‰ and -218‰ , a range of 28‰ with a mean of $-232 \pm 1\text{‰}$. $\delta^2H_{\text{sterol}}$ values were more depleted than the fatty acids with a mean of $-381 \pm 11\text{‰}$ and had a similar range (30‰) across the salinity treatments between -396‰ and -366‰ . δ^2H_{lipid} values for all compounds from the low growth-rate 9 ppt culture were the most depleted samples and are included in Table 3 and Fig. 1.

Fractionation factors increased, implying less $^2H/^1H$ fractionation, as salinity increased for all lipids (Table 3) and were highly correlated with salinity (R^2 was between 0.85 and 0.99) (Fig. 2 and Table 1). $\alpha_{C_{14:0}\text{--media}}$ was between 0.813 and 0.844 with a $0.828 \pm$

0.013 mean, $\alpha_{C16:1-media}$ was between 0.826 and 0.858 with a 0.842 ± 0.013 mean, $\alpha_{C16:0-media}$ was between 0.813 and 0.844 with a 0.830 ± 0.014 mean, and $\alpha_{sterol-media}$ was between 0.653 and 0.684 with a 0.669 ± 0.011 mean. Since the hydrogen isotope fractionation response to salinity was the primary objective of these experiments, the freshest continuous culture (9 ppt) with a lower growth rate was not included in the linear regression analyses. If the low growth-rate data were included the α -salinity relationship became more sensitive to salinity for $\alpha_{C14:0-media}$ (0.0016 ± 0.00012 instead of $0.0013 \pm 0.00007 \text{ ppt}^{-1}$) while the other lipids remained within the error of the estimated slopes (Supplementary material Fig. S3).

4. Model design and results

A coarse hydrogen flux model was used as a hypothesis development tool to test the sensitivity of $\alpha_{Lipids-Media}$ (capitalized to refer to model pools) to changes in hydrogen flux proportionality. The stationary model (Fig. 3) design included 6 boxes, 19 fluxes, the fractionation factor 0.4 associated with photosynthetic reduction of $NADP^+$ to NADPH via ferredoxin- $NADP^+$ reductase in photosystem 1 (PS1) (Luo et al., 1991; Schmidt et al., 2003), and an estimated fractionation factor 0.75 for the metabolic reduction of $NAD(P)^+$ to NAD(P)H using carbon-bound hydrogen (Schmidt et al., 2003; Sachs and Kawka, 2015). Fractionations associated with other processes were ignored ($\alpha = 1$) for most experiments both for simplicity and the fact that they are largely unknown (although some ranges of values are explored in the Supplementary Fig. S6). The Cell Water pool, two organic hydrogen pools (Lipids and Other Organics), and two reductant pools (Photosynthetic NADPH and Metabolic NAD(P)H) had isotopic compositions determined by isotope mass

balance equations (Supplementary material Section 3). The Media pool represented an infinite source of non-cellular hydrogen with constant $\delta^2\text{H}_{\text{Media}} = 0\text{‰}$. This model incorporates the key principals from a previously published 3-endmember model (Sachs and Kawka, 2015; Sachs et al., 2016). However, rather than prescribing endmember isotopic compositions, each hydrogen pool (except Media) was free to have variable isotopic compositions, which is important for testing the sensitivity of both Lipids and Cell Water.

Each flux represented an important biosynthetic, catabolic, or non-biosynthetic process responsible for moving hydrogen ($^2\text{H} + ^1\text{H}$) between reservoirs. The sum of the fluxes into and out of each box was zero for the assumed steady state conditions. Photosynthetic reduction of NADP^+ to NADPH was set to an arbitrary constant value ($J_{\text{Wat_PSI}} = 1$) and a range of values was evaluated for six testable metabolic parameters that characterize the fluxes (Supplementary Table S6). Assumed relationships between these metabolic parameters, $J_{\text{Wat_PSI}}$, and other fluxes (Supplementary Table S7) make it possible to explore the sensitivity of $\alpha_{\text{Lipids-Media}}$ without the need to constrain or measure any individual flux.

Fig. 4 illustrates how variations in each metabolic parameter influence $\alpha_{\text{Lipids-Media}}$. For individual metabolic parameter tests, all other metabolic parameters are set to default values specified in Supplementary Table S6 and Fig. 3. Changes in the PSI vs MET Parameter (x) (Sachs and Kawka, 2015) altered the proportion of Photosynthetic NADPH vs Metabolic NAD(P)H fluxes available for synthesis of Lipids and Other Organics. To achieve a 0.03 increase in $\alpha_{\text{Lipids-Media}}$ (the approximate range observed in our cultures), $(1-x)$ must increase from 0.5 (default) to 0.7 (Fig. 4a), meaning Lipids received a larger flux of Metabolic NAD(P)H vs Photosynthetic NADPH while Other Organics received a smaller

flux of Metabolic NAD(P)H vs Photosynthetic NADPH (Supplementary Fig. S4). The Metabolic Reductant Parameter (M) determined the size of several fluxes (essentially the rate of catabolism) and also altered the proportion of Photosynthetic NADPH vs Metabolic NAD(P)H fluxes for synthesis of Lipids and Other Organics. To achieve a 0.03 increase in $\alpha_{\text{Lipids-Media}}$, M must increase from 1 (default) to 1.7 (Fig. 4b), meaning a larger flux of Metabolic NAD(P)H vs Photosynthetic NADPH to Lipids and Other Organics (Supplementary Fig. S4). The Biosynthetic Cell Water Parameter (B) determines the proportion of Cell Water-hydrogen used for synthesis of Other Organics (vs NAD(P)H) and the Equilibrium Exchange Parameter (E) sets the amount of non-enzymatic equilibrium exchange between Cell Water and Other Organics. Increasing the flux of Cell Water-hydrogen to Other Organics by increasing B or E from 1 (default) to 4 for ^2H -enriched Lipids is enough to result in a 0.03 increase in $\alpha_{\text{Lipids-Media}}$ (Fig. 4c). The Hydrogen Transfer Parameter (HT) determines the flux sizes between Media and Cell Water. Decreasing HT from 1 (default) to 0.1, which restricted the fluxes between Media and Cell Water, resulted in a 0.03 increase in $\alpha_{\text{Lipids-Media}}$ (Fig. 4d). Percent Extracellular Release (PER) prescribed how much organic hydrogen was expelled from the cells. Increasing PER by 25% resulted in a 0.03 increase in $\alpha_{\text{Lipids-Media}}$. Decreasing HT or increasing PER were the only ways to ^2H -enrich Cell Water in this model (Fig. 5), whereas changes in x , M , B , and E resulted in Lipids that were ^2H -enriched without appreciably changing the isotopic composition of Cell Water (Supplementary Fig. S5).

5. Discussion

Continuous cultures of *T. pseudonana* provide unequivocal evidence that $^2\text{H}/^1\text{H}$ fractionation decreases in lipids as salinity increases (Fig. 2). The 1.0–1.3‰/ppt salinity dependence observed in fatty acids and the predominant sterol in this species is similar to the sensitivity reported in field and batch culture studies with other species and lipids (Sachse and Sachs, 2008; Sachs and Schwab, 2011; M'Boule et al., 2014; Heinzelmann et al., 2015a; Sachs et al., 2016). Since the cultures were grown under steady state conditions and at the same rate of growth across salinity treatments we can rule out the hypothesis put forth by Sachs and Schwab (2011) that the $^2\text{H}/^1\text{H}$ fractionation response in microalgal lipids is indirectly caused by salinity-induced changes in growth rate. This is further supported by a recent study of the coccolithophorid *E. huxleyi* maintained at the same growth rate using nitrogen-limited chemostats at a range of salinities (Sachs et al., 2016).

A six-box model was constructed to investigate the sensitivity of $\alpha_{\text{Lipids-Media}}$ to changes in basic cell physiology and metabolism. Several mechanisms emerge to explain salinity-induced variations in $\alpha_{\text{Lipids-Media}}$ as discussed below.

5.1. Varying proportions of reductant from Metabolic NAD(P)H vs Photosynthetic NADPH (x and M)

Photosynthetically produced NADPH is estimated to be ~600‰ depleted in ^2H relative to intracellular water (Luo et al., 1991; Schmidt et al., 2003) owing to the fact that electrolysis of water is associated with a large isotope effect. Reductant from other (metabolic) sources is expected to be less ^2H -depleted (Schmidt et al., 2003). Metabolic NAD(P)H can be generated in microalgae by NADP^+ -reducing enzymes that participate in the oxidative pentose phosphate (OPP) pathway including glucose-6-phosphate

dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase. In diatoms the OPP pathway is restricted to the cytosol (Wilhelm et al., 2006; Kroth et al., 2008; Gruber et al., 2009; Michels et al., 2014), but NAD(P)H can be generated by glycolytic enzymes in all cellular compartments (Smith et al., 2012) and the Entner-Doudoroff pathway in the mitochondria (Fabris et al., 2012). Additionally, NAD(P)H can be generated by isocitrate dehydrogenase and malate dehydrogenase in the TCA cycle, aldehyde dehydrogenase, pyruvate dehydrogenase, NAD(P)H-malic enzyme (Granum et al., 2005; Kroth et al., 2008), the membrane bound NADH-NADPH converting enzyme transhydrogenase, and other NAD⁺ and NADP⁺ reducing catabolic reactions (reviewed in Pollak et al., 2007; Spaans et al., 2015). Folate metabolism (Fan et al., 2014) may also contribute to metabolic NAD(P)H in diatoms (Hyde et al., 2008)

Separate pools of NAD(P)H with different isotopic compositions have been invoked to explain variable lipid–water ²H/¹H fractionation in: (i) microorganisms with heterotrophic, photoautotrophic, and chemoautotrophic metabolisms (Zhang et al., 2009b; Osburn et al., 2011; Dirghangi and Pagani, 2013a, 2013b; Dawson et al., 2015; Heinzemann et al., 2015b), (ii) carbon-autonomous plant organs such as leaves (Gamarra and Kahmen, 2015), (iii) phytoplankton cells growing at different rates (Sachs and Kawka, 2015), and recently in (iv) the haptophyte *Emiliania huxleyi* grown at the same growth rate across a wide range of salinities (Sachs et al., 2016). We build on this line of reasoning and propose that similar mechanisms may play a role at high salinity in diatoms and other microalgae by: (i) increasing the flux of Metabolic NAD(P)H (vs Photosynthetic NADPH) to Lipids while increasing the flux of Photosynthetic NADPH (vs Metabolic NAD(P)H) to Other Organics (increasing (1–x) in the model), or (ii) increasing the flux of hydrogen from

Other Organics to Metabolic NAD(P)H and thereby increasing the flux of Metabolic NAD(P)H (vs Photosynthetic NADPH) to Lipids and Other Organics (increasing M in the model) (Supplementary Fig. S4).

In microalgae adjusting to salinity, changes in M or x might follow from physiological responses required to maintain osmotic and/or energy balances. Osmotic balance in microalgae is achieved by the synthesis and accumulation of small organic molecules (osmolytes) which behave as non-toxic compatible solutes to help maintain vital cellular functions (Brown, 1978; Kirst, 1989; Galinski, 1995). Non-lipid biochemical constituents were not measured in these cultures so the hypothesis that increased production of osmolytes (and therefore a potential shift in biochemical composition) may “shuttle” light hydrogen to the osmolyte pool leaving intracellular water, NADPH, and lipids more enriched cannot be ruled out (Sachs and Schwab, 2011). This process was mimicked in the 6-box model by increasing the parameter $(1-x)$ which caused the proportion of Lipid-hydrogen derived from Metabolic NAD(P)H and Other-Organic-hydrogen derived from Photosynthetic NADPH to increase. Increasing $(1-x)$ in the 6-box model is analogous to increasing $(1-x)$ in the 3-endmember model (Sachs and Kawka, 2015; Sachs et al., 2016) and has the same result, except that the Other Organics pool adds demand for NAD(P)H flux and all cellular pools of hydrogen are free to have variable isotopic compositions in the former. This analysis does not increase the total flux of reductant used in cells; it merely switches the relative proportion of ^2H -depleted reductant flux to Lipids vs Other Organics. The result is a decrease in Lipid–Media $^2\text{H}/^1\text{H}$ fractionation (increase in $\alpha_{\text{Lipid–Media}}$) (Fig. 3a) with a concomitant increase in Organic–Media $^2\text{H}/^1\text{H}$ fractionation (decrease in $\alpha_{\text{Organics–Media}}$) (Supplementary Fig. S5a) and could happen if osmolytes use a greater proportion of

²H-depleted photosynthetic NADPH compared to lipids. Under high salt conditions osmolyte production required for cell survival might occur at the expense of lipid production, leaving lipids more ²H-enriched. Increasing (1-*x*) caused $\alpha_{\text{Lipid-Media}}$ to approach unity (Fig. 3a), but did not appreciably enrich NAD(P)H or intracellular water in ²H as Sachs and Schwab (2011) hypothesized (Supplementary Fig. S5). Therefore, while our model supports their mechanism, it is perhaps more nuanced than originally proposed.

Alternatively, a similar result was accomplished by increasing *M* in the 6-box model. This sensitivity test is somewhat analogous to increasing (1-*x*) in the 3-endmember model (Sachs and Kawka, 2015; Sachs et al., 2016) except the 6-box model more realistically incorporates a source for metabolic NAD(P)H. When the flux of hydrogen from Other Organics to Metabolic NAD(P)H increased, the flux of Metabolic NAD(P)H to both Lipids and Other Organics also increased and $\alpha_{\text{Lipid-Media}}$ approached unity (Fig. 3b). This model analysis increased the total flux of reductant derived from metabolic NAD(P)H while the photosynthetic flux remained unchanged. Physically this might occur if cells have a greater demand for reducing power at high salinities. Importantly, previous investigations of protein activity and transcript abundance suggest that reduction of Metabolic NAD(P)H increases with salinity. For instance, increased expression levels of OPP enzymes have been observed in diatoms grown at high salinity (Cheng et al., 2014). Bacterial cells (Danevčič and Stopar, 2011), macroalgae (Huan et al., 2014), and cyanobacteria (Rai et al., 2013, 2014) grown at high salinity showed increased OPP enzyme activity. Several higher plant studies imply that increased OPP activity or protein abundance occur in response to salinity changes (Nemoto and Sasakuma, 2000; Huang et al., 2003; Wang et al., 2008; Yu et al., 2011; Yang et al., 2014; Gao et al., 2016). In high salt conditions, NAD(P)⁺ reducing proteins such as pyruvate

dehydrogenase, malate dehydrogenase, and OPP enzymes were up-regulated in the halotolerant green alga *Dunaliella* (Liska, 2004). All of these studies indicate that phototrophs may require more reducing power at high salinities. Since growth performance was either different or not reported in salt treatments, it is not immediately clear if the observed enzyme activities and transcript abundances are a direct response to salt or an indirect response to growth, highlighting the importance of continuous culture experiments.

If high salinity conditions result in either: (i) ^2H -enriched NAD(P)H available for lipid synthesis when photosynthetically produced NADPH is preferentially shuttled to osmolytes, or (ii) overall more metabolically produced NAD(P)H flooding the reductant pool, then lipids should become ^2H -enriched. Indeed, heterotrophic bacteria and archaea don't have access to pools of photosynthetic NADPH and don't display $\delta^2\text{H}$ -enrichment at high salinity (Dirghangi and Pagani, 2013a; Heinzemann et al., 2015a). If more metabolic NAD(P)H is used by photoautotrophs during lipid synthesis at high salinities, this could explain why salinity-dependent $^2\text{H}/^1\text{H}$ fractionation is observed in photoautotrophs but not heterotrophs. Increasing the flux of Metabolic NAD(P)H to just Lipids ($1-x$), or increasing the flux of metabolic NAD(P)H to both Lipids and Other Organics (M) are mechanisms that are likely to be directly linked to increased production of compatible solutes and/or energy balance, and as such are the most likely explanations for decreased $^2\text{H}/^1\text{H}$ fractionation at increased salinity.

5.2. Varying proportions of hydrogen from intracellular water (*B* and *E*)

NMR studies indicate acetogenic lipids derive about 50% of their hydrogen from NAD(P)H, 25% from intracellular water, and 25% from acetyl-CoA (Saito et al., 1980;

Robins et al., 2003; Schmidt et al., 2003; Zhang et al., 2009b). Isoprenoid lipids derive ~20–40% of their hydrogen from NADPH and the remainder from either acetyl-CoA when synthesized via the cytosolic MVA pathway, or pyruvate and glyceraldehyde-3-phosphate (GAP) when synthesized via the plastidic DOXP/MEP pathway (Sessions et al., 2002). Pyruvate, acetyl-CoA, and GAP are central metabolites under high demand by several competing cellular functions such as carbohydrate, lipid, protein, and nucleic acid synthesis and catabolism (Oliver et al., 2009; Hemmerlin et al., 2012). One way in which salinity might influence the proportion of cell-water-derived hydrogen in biochemicals is by increasing the likelihood that carbon-bound hydrogen on the primary metabolite precursors pyruvate, acetyl-CoA, and GAP is derived from cell-water. For example, shifting demands on the primary metabolite pools could change the relative importance of different pyruvate synthesis pathways that impart different proportions of hydrogen derived from water vs metabolites on the lipid-destined methyl group (see Fig. 6 in Sachs et al., 2016), effectively changing the metabolic parameter B .

Additionally, hydrogen in pyruvate precursors can exchange with intracellular water through the presence or activity of isomerases at the glucose level (Katz and Crespi, 1970). The enzyme glucose-6 phosphate isomerase (which converts glucose-6-phosphate to fructose-6-phosphate and back) can facilitate both intramolecular and intermolecular exchange of hydrogen between the sugar and the aqueous solvent. Investigation of this enzyme in different cell types has revealed heterogeneity in the amount of back and forth conversion of sugars and suggests that environmental parameters can influence this exchange (Malaisse-Lagae et al., 1989). At high salinity, glucose-6 phosphate isomerase from *Dunaliella salina* was strongly induced by salinity at both the protein and gene level

(Cui et al., 2010). Any associated fractionation effects aside (Supplementary Fig. S6), it is plausible that salinity could increase the proportion of ^2H -enriched cell-water-derived hydrogen in lipid precursors, and by extension, lipids (akin to increasing the model metabolic parameter B) by altering the relative or absolute presence or activity of these enzymes.

Relatedly, preferences for specific lipid biosynthesis isoenzymes may modify the amount of cell-water-hydrogen in lipids (akin to increasing the model metabolic parameter E). There are numerous fatty acid synthesis isoenzymes responsible for incorporating hydrogen during lipid synthesis. For instance, Zhang et al. (2009b) noted that there are many variants of enoyl-ACP reductase enzymes involved in the second reductive step of fatty acid biosynthesis (Massengo-Tiassé and Cronan, 2009). The enzymes FabI and FabL directly transfer hydrogen from NAD(P)H to the growing fatty acid chain, but FabK involves the indirect transfer of H^- via a nitrogen-bound hydrogen on the flavin cofactor. Since N-bound hydrogen undergoes rapid exchange with water, flavoproteins that transfer hydrogen to organic material can indirectly enrich products in ^2H (Simon and Kraus, 1976; Chisla and Massey, 1989; Schmidt et al., 2003). For fatty acids at least, enhancement of FabK over FabI or FabL could incorporate more cell-water-hydrogen into lipids. While it is not yet known if FabK occurs in eukaryotic microalgae, or if salinity can induce a shift in Fab isoenzymes, the presence of the gene encoding for FabK was inferred from the transcriptome of the diatom *Nitzschia* sp. (see Table 1 in Cheng et al., 2014)). Since these enzymes are not involved in isoprenoid synthesis, an analogous process would need to be invoked to explain the similar salinity response of sterol $\delta^2\text{H}$ values to salinity in diatoms.

Finally, osmolytes in phytoplankton are typically small organic molecules with a large proportion of exchangeable hydrogen, such as amino acids and derivatives, polyols and sugars, methylamines, methylsulfonium compounds, and urea (Yancey, 2005). Sachs and Schwab (2011) proposed that osmolyte production might result in ^2H -enriched pools of cell water and NAD(P)H. If a normal fractionation factor (i.e., one that results in a product that is isotopically depleted compared to the reactant) characterizes the enzymatic or equilibrium exchange of hydrogen from Cell Water to Other Organics, the model yields ^2H -enriched lipids (Supplementary Fig. S6). These mechanisms for incorporating additional cell-water-hydrogen into lipids (*B* and *E*) are less direct (and therefore more difficult to test) than changes in NAD(P)H related to *x* or *M* but cannot be ruled out.

5.3. Variations in Hydrogen Transport (*HT*):

Both $\delta^2\text{H}_{\text{OtherOrganics}}$ and $\delta^2\text{H}_{\text{CellWater}}$ become more ^2H -enriched as *HT* approaches zero, likely the result of recycling of intracellular water without full replenishment from the media (Fig. 4). One widely proposed mechanism for decreased fractionation at elevated salinity is “increased recycling” of cell water resulting from diminished transport of environmental water into cells (Sachse and Sachs, 2008; Sachs and Schwab, 2011; Heinzelmann et al., 2015a; Sachs et al., 2016). Investigations of intracellular water from both prokaryotic and mammalian cells (Kreuzer-Martin et al., 2005, 2006; Kreuzer et al., 2012) indicate that the isotopic composition of cell-water-hydrogen and oxygen can differ from that of growth media. Since the permeability of the lipid bilayer is high and the residence time of water inside a cell is short (García-Martín et al., 2001) decreasing transport of environmental water across the cell membrane might be achieved by

transcriptional down-regulation of aquaporins or adjustment of the probability that aquaporins are open (Hasegawa et al., 2000; Boursiac et al., 2005; Horie et al., 2011). The model indicates that a hydrogen flux across the cell membrane that is smaller than the photosynthetic flux of hydrogen increases $\alpha_{\text{Lipid-Media}}$ and causes intracellular water to become ^2H -enriched relative to external water (Fig. 4a). Reduced transport of environmental water (to fluxes smaller than the total flux of hydrogen required for biosynthesis) therefore remains a viable mechanism for explaining decreased fractionation at increased salinity, but a hypothetical one pending demonstration of changes in $\delta^2\text{H}_{\text{CellWater}}$ in photoautotrophs.

5.4. Variations in the release of organic hydrogen (*PER*)

Depending on growth conditions and species, phytoplankton cells can exude 2–76% of their photosynthetically fixed carbon (Van Oostende et al., 2013; Thornton, 2014). Phytoplankton *PER* is considered to be a normal physiological function of healthy cells and likely plays a protective role in stressful environments (Thornton, 2014). Several studies indicate that both phytoplankton and bacteria increase *PER* and alter its chemical composition (Abdullahi et al., 2006) in response to suboptimal growth conditions (Zlotnik and Dubinsky, 1989; Alcoverro et al., 2000) such as elevated salinity (Maršálek and Rojíčková, 1996; Liu and Buskey, 2000; Abdullahi et al., 2006; Zhang et al., 2011; Aslam et al., 2012; Steele et al., 2014). Experiments with diatom cultures at high salinity showed increased frustule-bound exopolysaccharides but decreased transparent exopolysaccharides (Bussard et al., 2016). The model sensitivity analysis indicated that increasing *PER* causes $\alpha_{\text{Lipid-Media}}$ to approach unity (Fig. 3e) when more ^2H -depleted organic material is expelled from the cell. This was the only model process besides restricted uptake of extracellular

water (low *HT*) that caused significant changes in modeled $\delta^2\text{H}_{\text{CellWater}}$ (Fig. 4b), supporting the hypothesis recently put forward in Sachs et al. (2016) that increased exudation of ^2H -depleted organic matter may lead to ^2H -enriched cell-water. Investigations of the isotopic composition of intracellular water and exudate fluxes could help elucidate whether changes in *HT* or *PER* can explain decreased fractionation with increased salinity.

5.5. Model limitations

Interestingly, the data for the 9 ppt experiment fell below the α -salinity trends for the fatty acids (Supplementary Fig. S3a–c), but on the α -salinity trend for the sterol (Fig. S3d). In previous studies comparing *T. pseudonana* nutrient-replete (higher growth rate) continuous cultures to nutrient-limited (lower growth rate) chemostats, $\alpha_{\text{fattyacids-media}}$ was not influenced by growth rate while $\alpha_{\text{sterol-media}}$ displayed increased fractionation at increased growth rate (Zhang et al., 2009a; Sachs and Kawka, 2015). Thus the 9 ppt treatment in this study behaved as the previous studies would suggest: presumably the decreased growth rate pushed the sample onto the trend for the sterol α -salinity relationship while there was no “bolstering effect” for the fatty acid α -salinity relationships since acetogenic lipids do not seem to be influenced by growth rate in *T. pseudonana* (Zhang et al., 2009a; Sachs and Kawka, 2015).

The stationary model presented is inadequate for evaluating the effect of changes in growth rate or growth phase on $\alpha_{\text{Lipids-Media}}$. Because catabolism equaled anabolism in a single population of cells, the model is more representative of a culture in “stationary phase”. The 6-box model is not directly comparable to the continuous cultures that were held in steady state by dilution with a constantly overturning population of cells. However,

because a nearly identical salinity effect was observed in batch cultures that were in exponential, stationary, and death phases (Heinzelmann et al., 2015a), the 6-box model represents a first step in determining the mechanisms underlying the sensitivity of $\alpha_{\text{Lipids-Media}}$ to salinity.

Adding other hydrogen reservoirs to the model and additional isotopically distinct pool of metabolic NAD(P)H might reveal nuances governing the H isotopic composition of lipids. For instance, measured fatty acid $\delta^2\text{H}$ was more sensitive to salinity than the sterol in this study, and recent culture studies with haptophytes revealed different $\alpha_{\text{lipid-water}}$ sensitivities in isoprenoid and acetogenic lipids (Kasper, 2015; Sachs et al., 2016). Since it has been suggested that *n*-alkyl ^2H -enrichment relative to isoprenoid lipids might be due to isotopically different pools of NAD(P)H (Sessions et al., 1999; Sachse and Sachs, 2008), a more complex model might inform unexplained patterns of hydrogen isotope fractionation in microorganisms. While the *n*-alkyl lipid precursor acetyl-CoA is synthesized in the chloroplast (e.g., Shtaida et al., 2015), the precursor to isoprenoid compounds (isopentenyl pyrophosphate, IPP) is synthesized either in the cytoplasm via the “eukaryotic” mevalonic acid (MVA) pathway or in the plastid via the “prokaryotic” 1-deoxy-D-xylulose-5-phosphate/2-methyl-erythroyl-4-phosphate pathway (DOXP/MEP) (Lohr et al., 2012). In diatoms, sterol synthesis has characteristics of bacterial, higher plant, and fungal biosynthetic pathways, possibly a result of their unique evolutionary origin (Desmond and Grihaldo, 2009; Fabris et al., 2014) likely resulting in steroidal precursors from both the MVA and DOXP/MEP pathways (Lohr et al., 2012 and references therein). Therefore, incorporating compartmentalization into a model could further illuminate differences in $^2\text{H}/^1\text{H}$ fractionation between fatty acids and isoprenoids and differences between species.

From the sensitivity analyses of the 6-box hydrogen flux model we hypothesize that:

(i) variations in the source of NAD(P)H and/or increased production of Metabolic NAD(P)H vs Photosynthetic NADPH is the most likely mechanism responsible for the salinity sensitivity of $\alpha_{\text{Lipids-Media}}$, with (ii) increased incorporation of Cell Water-hydrogen into Other Organics a viable second mechanism. Two additional mechanisms caused a significant ^2H -enrichment of both lipids and intracellular water, including: (iii) restricted hydrogen transport from media into cells, and (iv) increased extracellular release of organic material, insight that could not be obtained from the previous 3-endmember model (Sachs and Kawka, 2015; Sachs et al., 2016). These might contribute to ^2H -enrichment of lipids at high salinities if it can be shown that intracellular water can be isotopically distinct from media in photoautotrophs. Considering these mechanisms separately is useful for the purposes of sensitivity analysis, but it is important to note that a combination of these mechanisms may well be responsible for the observed salinity effect on lipid fractionation.

6. Conclusions

The diatom *T. pseudonana* was grown at steady state in nutrient-replete continuous cultures at six salinities between 14–40 ppt and at a lower growth rate at 9 ppt. Over the 26 ppt salinity range in which the cultures had equivalent growth rates, $\delta^2\text{H}$ values increased by 1.3‰/ppt in $\text{C}_{14:0}$, $\text{C}_{16:0}$, and $\text{C}_{16:1}$ fatty acids, and by 1.0‰/ppt in 24-methyl-cholesta-5,24(28)-dien-3 β -ol. Growth rate differences, previously hypothesized to contribute to the observed sensitivity of lipid $\delta^2\text{H}$ to salinity, can be ruled out as a causal mechanism in both *T. pseudonana* and *E. huxleyi* (Sachs et al., 2016). A 6-box model revealed four mechanisms

that might contribute to ^2H -enrichment in lipids as salinity increases: (i) increases in the relative contribution of metabolic NAD(P)H (vs photosynthetic NADPH), (ii) increased incorporation of cell-water-hydrogen into organic material, (iii) restricted exchange of water between cells and the environment, and (iv) increased release of organic exudates. Although each mechanism could potentially impact $\alpha_{\text{lipid-water}}$, shifts in the relative supply of metabolic NAD(P)H, at the expense of photosynthetic NADPH, is the most parsimonious mechanism.

Irrespective of the underlying cause of diminished $^2\text{H}/^1\text{H}$ fractionation in microalgal lipids under increased salinity, there is strong empirical support from field, batch cultures, nitrogen limited chemostats, and now nutrient-replete continuous cultures that this response is robust and likely universal. In virtually all lipids and species $^2\text{H}/^1\text{H}$ fractionation decreases with salinity by 1–2‰/ppt. The consistency of these observations implies that biomarker lipid $\delta^2\text{H}$ values may be used to reconstruct salinity in paleoclimate and paleoenvironmental studies.

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Appendix A. Supplementary data

Supplementary Tables 1–7 and Supplementary Figures 1–6 are in the Supplementary material document.

The code used for the phytoplankton hydrogen and deuterium flux model can be found online at <http://dx.doi.org/10.5281/zenodo.XXXXX>

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Table captions

Table 1. Linear regression results of culture parameters on salinity. Slopes with P values less than 0.05 are in bold.

Table 2. Average growth rate, in vivo fluorescence (IVF), cell density, cell diameter, and cell volume during the last 2–3 days of steady state conditions.

Table 3. Lipid concentrations and $\delta^2\text{H}$ values of media and lipids from continuous cultures of *T. pseudonana*. $\delta^2\text{H}_{\text{media}}$ is the average of 3 measurements. $\alpha_{\text{lipid-media}} = (1000 + \delta^2\text{H}_{\text{lipid}})/(1000 + \delta^2\text{H}_{\text{media}})$ is reported with propagated errors from $\delta^2\text{H}_{\text{lipid}}$ and $\delta^2\text{H}_{\text{media}}$.

Figure captions

Fig. 1. Isotopic composition ($\delta^2\text{H}_{\text{sample}}$) as a function of salinity for culture media, fatty acids, and a sterol in *T. pseudonana*. Fatty acids included myristic ($\text{C}_{14:0}$), palmitic ($\text{C}_{16:0}$) and palmitoleic ($\text{C}_{16:1}$) acids. The sterol was 24-methyl-cholesta-5,24(28)-dien-3 β -ol. Lines are the linear regressions of $\delta^2\text{H}_{\text{lipid}}$ on salinity for the 14–40 ppt cultures and do not include the 9 ppt culture which had a lower growth rate. The 9 ppt data fall below the fatty acid regression lines but on the sterol regression line. Error bars represent standard deviations of three $\delta^2\text{H}_{\text{media}}$ measurements and 3–6 $\delta^2\text{H}_{\text{lipid}}$ measurements.

Fig. 2. Apparent fractionation factors, $\alpha_{\text{lipid-media}}$, as a function of salinity for three fatty acids and one sterol from nutrient-replete continuous cultures of *T. pseudonana*. Shown for comparison are fractionation factors for lipids from nitrogen-limited continuous cultures (chemostats) from the marine coccolithophorid *E. huxleyi* from Sachs et al. (2016) including eight alkenones, three fatty acids, one sterol, and phytol. Three separate chemostat cultures were conducted at 32 ppt and two at 22 ppt salinity for *E. huxleyi*. All values are averages of at least three measurements. Error bars represent propagated error for lipid and water measurements and are smaller than the symbols in some cases. Solid lines are linear regressions of data, dotted lines indicate linear regressions that were not significant below the $P = 0.05$ level.

Fig. 3. Schematic for 6-box model of hydrogen mass and isotope fluxes in a population of phytoplankton cells. Fractionation factors associated with all fluxes are set to unity except for $J_{\text{Wat_PSI}}$ ($\alpha_{\text{PSI}} = 0.4$) and $J_{\text{Org_MET}}$ ($\alpha_{\text{MET}} = 0.75$).

Fig. 4. Sensitivity of $\alpha_{\text{Lipids-Media}}$ to changes in metabolic parameters using default values where $\delta^2\text{H}_{\text{Media}} = 0\text{‰}$. Lipid-Media $^2\text{H}/^1\text{H}$ fractionation decreases (i.e., $\alpha_{\text{Lipids-Media}}$ approaches unity) as (a) $(1-x)$ (the amount of hydrogen from metabolic NAD(P)H vs photosynthetic NADPH) increases, (b) M (size of the metabolic NAD(P)H flux relative to PS1) increases, (c) B and E increase (where B is the amount of organic-hydrogen source from cell-water-hydrogen relative to total NAD(P)H, and E is the equilibrium exchange between organic and cell-water-hydrogen), (d) HT (Hydrogen Transport) is less than photosynthesis and decreases, and (e) PER (Percent Extracellular Release) increases.

Fig. 5. Sensitivity of $\delta^2\text{H}_{\text{Reservoir}}$ to changes in metabolic parameters using default values and $\delta^2\text{H}_{\text{Media}} = 0\text{‰}$. All non-media boxes become ^2H -enriched as (a) HT (Hydrogen Transport) decreases and (b) PER (Percent Extracellular Release) increases.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1

Table 1.

Parameter	R ²	p level	Slope	Slope SE	Intcpt	Intcpt SE
Cell Density ($\times 10^5$ cells/ml)	0.01	0.86	0.00	0.02	1.2	0.5
Cell Diameter (μm)	0.54	0.09	0.02	0.01	3.0	0.2
Cell Volume (μm^3)	0.50	0.12	0.23	0.10	14	2
in vivo fluorescence (fsu)	0.60	0.07	5	2	117	54
Chl <i>a</i> (pg/cell)	0.44	0.15	0.04	0.02	-0.1	0.5
Fatty acid C _{16:0} ($\mu\text{g}/\text{cell}$)	0.21	0.37	0.2	0.2	0	6
Fatty acid C _{16:1} ($\mu\text{g}/\text{cell}$)	0.27	0.29	0.3	0.2	-3	6
Fatty acid C _{14:0} ($\mu\text{g}/\text{cell}$)	0.21	0.36	0.10	0.09	1	3
Sterol ($\mu\text{g}/\text{cell}$)	0.47	0.13	0.02	0.01	-0.1	0.3
Residual NO ₃ ⁻ ($\mu\text{M}/\text{cell}$)	0.11	0.52	8	11	359	1
Residual PO ₄ ³⁻ ($\mu\text{M}/\text{cell}$)	0.05	0.67	0.1	0.3	13	2
Residual NO ₂ ⁻ ($\mu\text{M}/\text{cell}$)	0.02	0.80	0.02	0.09	3	1
Residual NH ₄ ⁺ ($\mu\text{M}/\text{cell}$)	0.00	0.97	0.00	0.04	0.2	0.2
Residual Si(OH) ₄ ($\mu\text{M}/\text{cell}$)	0.82	0.01	-1.0	0.2	46	7
$\alpha_{\text{C16:0-media}}$	0.95	< 0.001	0.0013	0.0001	0.794	0.004
$\alpha_{\text{C16:1-media}}$	0.86	< 0.001	0.0012	0.0002	0.809	0.007
$\alpha_{\text{C14:0-media}}$	0.99	< 0.001	0.00125	0.00007	0.794	0.002
$\alpha_{\text{sterol-media}}$	0.85	< 0.001	0.001	0.0002	0.641	0.006

Table 2

Table 2.

Salinity	Growth rate (div/d)	SD	IVF (fsu)	SD	<i>n</i>	Cell density (×10 ⁵ cells/ml)	SD	Cell diameter (μm)	SD	Cell volume (μm ³)	SD	<i>n</i>
9*	1.62	0.03	121	9	5	0.8	0.2	3.2	0.1	18	2	4
14	1.97	0.02	210	5	6	1.1	0.1	3.3	0.1	19	2	6
18	1.99	0.02	195	13	7	1.0	0.1	3.3	0.1	18	2	6
27	1.99	0.05	221	13	6	1.2	0.1	3.3	0.1	16	1	6
31	1.97	0.01	255	10	7	1.6	0.3	3.4	0.1	22	2	7
36	1.97	0.04	232	15	7	1.4	0.3	3.4	0.1	20	2	6
40	1.90	0.05	352	24	5	0.6	0.2	3.9	0.2	25	5	5

*Lower growth rate culture

Table 3

Table 3.

Salinity (ppt)		9*	14	18	27	31	36	40
Media	$\delta^2\text{H}$	-75.5	-74.5	-75.1	-74.7	-75.7	-74.0	-73.2
	SD	0.1	0.5	0.2	0.2	0.1	0.1	0.3
C14:0	[pg/cell]	2.4	1.9	2.6	5.1	1.8	1.7	6.8
	$\delta^2\text{H}$ (‰)	-272	-248	-247	-235	-232	-223	-218
	<i>n</i>	3	3	3	3	5	3	4
	SD (‰)	2	4	4	2	4	3	2
	α	0.788	0.813	0.814	0.827	0.831	0.839	0.844
	SD	0.002	0.005	0.004	0.002	0.005	0.003	0.003
C16:0	[pg/cell]	2.5	3	3.9	8.5	2.9	1	15.1
	$\delta^2\text{H}$ (‰)	-269	-247	-245	-237	-225	-218	-218
	<i>n</i>	3	3	3	3	6	3	4
	SD (‰)	3	4	3	3	4	2	1
	α	0.791	0.813	0.817	0.825	0.838	0.844	0.844
	SD	0.003	0.004	0.003	0.003	0.004	0.002	0.001
C16:1	[pg/cell]	1.7	1.9	2.9	5.5	1.9	0.9	14.3
	$\delta^2\text{H}$ (‰)	-255	-234	-236	-224	-213	-206	-211
	<i>n</i>	3	3	3	3	3	3	4
	SD (‰)	2	3	2	3	5	3	2
	α	0.806	0.828	0.826	0.839	0.851	0.858	0.851
	SD	0.002	0.003	0.002	0.003	0.006	0.003	0.002
Sterol	[pg/cell]	0.6	0.2	0.4	0.4	0.3	0.4	1
	$\delta^2\text{H}$ (‰)	-398	-396	-388	-384	-379	-366	-374
	<i>n</i>	1	6	3	4	5	3	4
	SD (‰)	-	4	7	14	1	8	4
	α	0.652	0.653	0.662	0.666	0.672	0.684	0.676
	SD	-	0.004	0.007	0.015	0.001	0.009	0.004

*Lower growth rate culture