Influence of physiological states of *Emiliana huxleyi* cells on their lipids and associated molecular isotopic compositions during microbial degradation

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ABSTRACT

The goal of this study was to examine the potential effect of physiological states of phytoplankton cells on their lipids (phytol, sterols, alkenones, and fatty acids) and associated stable carbon isotopic compositions during microbial degradation. Cells of *Emiliania huxleyi* were cultivated using batch cultures with the same medium but over different growth stages: exponential growth phase (culture-1) and stationary phase (culture-2), respectively. There were remarkable differences in cellular lipid contents and relative compositions between these two cultures: ~ 8 times higher lipid content with higher percentages of poly- and mono-unsaturated fatty acids, and alkenones but lower percentages of saturated fatty acids, phytol, and sterols in culture-2 than in culture-1. Dead phytoplankton cells (killed by freezing) from the two cultures were incubated in the dark with addition of natural seawater (containing natural microbial communities) under aerobic conditions at 17 °C for about one month. Most phytoplankton fatty acids (except for 16:0) from the two cultures were almost completely degraded while most neutral lipids (phytol, sterols and alkenones) (except for 27 Δ^5 from culture-1) were partially degraded with relatively higher percentages of these compounds remaining in culture-2 than in culture-1. Along with degradation of lipids, their stable carbon isotopic compositions varied differently among compound classes: positive shifts (+1.7‰ to +3.9‰) in $\delta^{13}\text{C}$ values for fatty acids; little change for sterols; and for alkenones, either little change in most cases or a negative shift (-3‰) in one case (37:2 alkenone in culture-1). Moreover, relatively larger isotopic shifts (~ +1.5‰ each) for fatty acids 16:0 and 18:1(ω 9) were observed in culture-1 compared to those in culture-2. The results from these experiments imply that the variability in isotopic compositions of phytoplankton lipid compounds during microbial degradation is critically affected by

physiological states of phytoplankton cells due to heterogeneity in isotopic compositions of cellular lipids and differential reactivities of various lipid compounds bound in different intracellular components, which are synthesized through different cell growth phases.

Keywords: phytoplankton cell growth stage; lipid biomarker; compound-specific isotopic composition; microbial degradation; chemical alteration; isotopic alteration.

1. Introduction

As a cosmopolitan coccolithophore, *Emiliania huxleyi* is a significant primary producer in the world's oceans (Westbroek et al., 1993). Together with its related species, *Emiliania huxleyi* synthesize various lipid biomarkers (e.g., fatty acids, sterols, and alkenones) possessing unique compound-specific stable isotopic compositions. These biomarkers have been widely used as important signals to study environmental ecology (Napolitano et al., 1997), organic carbon cycling (Rontani et al., 2009; Volkman et al., 1986; Wakeham and Beier, 1991), to estimate past sea surface temperature (Müller et al., 1998; Prahl and Wakeham, 1987), and to reconstruct ancient atmospheric $p\text{CO}_2$ (e.g., Jasper and Hayes, 1994; Pagani et al., 1999; 2002).

The applications of these lipid biomarkers and their molecular isotopic compositions are based on a fundamental assumption that biogeochemical cycling processes of organic matter in marine systems do not significantly alter original chemical or isotopic signals (Hayes et al., 1990; McCaffrey et al., 1990; Pagani et al., 1999; Prahl et al., 1989). On the one hand, this assumption has been proven to be valid for some organic compounds. For example, decomposition of porphyrins, *n*-alkanes and polycyclic aromatic hydrocarbons did not lead to significant alterations in their isotopic compositions (Freeman et al., 1994; Hayes et al., 1990; Huang et al., 1997; Mazeas et al., 2002). On the other hand, this assumption has been questioned because more and more laboratory and field studies have shown that many organic compounds, from different sources, experience varied changes in their chemical (Gong and Hollander, 1999; Pan and Sun, 2011; Prahl et al., 2010; Rontani et al., 2005) and isotopic (Freeman et al., 1994; Hayes et al., 1990; Macko et al., 1994; Pan et al., 2014; Sun et al., 2004; Teece and Fogel, 2007) signatures during degradation processes. For example, Macko et al. (1994) found that when

~50% of amino acids in the seagrass *Halodule wrightii* were degraded after 4 weeks of incubation, amino acids Glu, Gly, Ala, and Val became depleted in ^{13}C by 5‰, and Asp became enriched in ^{13}C by 2‰ while Leu, Ile, and Pro remained constant. Sun et al. (2004) observed that during degradation of *E. huxleyi* cells in natural oxic and anoxic seawaters, the isotopic compositions of their lipids were altered in diverse ways: ~ 2‰ to 7‰ positive shifts for fatty acids, ~ 4‰ negative shifts for alkenones, and little change for sterols. Teece and Fogel (2007) also observed that during degradation of the salt marsh plant *Spartina alterniflora*, the $\delta^{13}\text{C}$ values of various sugar molecules varied differently. An 85% decrease of glucose concentration resulted in an isotopic enrichment (+5‰) for the remaining compounds. Residual (~ 25%) arabinose was depleted in ^{13}C by 4‰ compared to its original signal. After 79% of the xylose was removed, its $\delta^{13}\text{C}$ value remained constant. It has been, however, unknown what mechanisms drive these diverse isotopic alterations of various organic compounds during biochemical processes.

In the previous study by Pan et al. (2014), the influence of microbial degradation of aged or senescent cells (collected after cell auto-metabolism experiments) on phytoplankton lipids and their molecular stable carbon isotopic compositions has been reported. Subsequently, this study aimed to further elucidate the potential effect of cell physiological states on the variability of algal lipids and their stable carbon isotopic compositions during microbial degradation. Cells of *Emiliania huxleyi* were cultivated in batch cultures through different growth phases: exponential growth phase (culture-1) and stationary growth phase (culture-2), respectively. Intact healthy cells in these two cultures, which had not undergone cell auto-metabolism experiments, were freshly harvested, killed by freezing and then incubated in the dark under aerobic conditions with introduction of natural seawater containing microbial communities. Variations in concentrations

of algal lipids (phytol, sterols, alkenones, and fatty acids) and associated molecular isotopic compositions were followed during a one-month incubation. Possible mechanisms causing these variations are proposed and implications of the study are discussed.

2. Materials and methods

2.1. Materials

Marine haptophyte *E. huxleyi* (CCMP371) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), which is now known as National Center for Marine Algae and Microbiota (NCMA). The strain was initially isolated from the Sargasso Sea (32°N, 62°W; 50 m water depth) in June 1987, and then was made and kept axenic (confirmed by routine tests) in the CCMP. Seawater used for experiments was collected from the Gulf of Mexico (28°10'82"N, 91°36'96"W) in May 2008 by directly pumping surface (2~3 m) water into 25-liter carboys. The seawater used for medium preparation was filtered through Whatman 934-AH glass microfiber filters (pre-combusted at 450 °C for 4 h). The f/50 medium was prepared according to the protocols recommended by CCMP (Guillard, 1962; Guillard, 1975). The seawater for inoculation was stored in 25-liter carboys until incubation began.

2.2. Experimental setup

Cells of *Emiliana huxleyi* were cultivated in batch cultures (in duplicate for both culture-1 and culture-2) in f/50 medium (~ 1 L) under a light:dark (12 h:12 h) regime with a

constant light intensity ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The cultures were manually shaken two times a day, and cell densities were counted every one or two days. Culture-1 was stopped on day 12 (end of exponential phase), and culture-2 on day 35 (late stationary phase). Both cultures were equally divided into a set of small flasks (100 mL each) and then were frozen below -30°C overnight to kill the cells. After thawing, 50 mL of natural seawater was added into each flask to introduce natural microbial communities. These mixtures were then incubated in the dark for 32 days under aerobic conditions by blowing air through the mixed solutions.

2.3. Sampling and cell counting

Transfer and sampling of cultures were conducted in a clean hood, pre-irradiated with a UV lamp for over 30 minutes. The flasks were gently shaken by hand to allow cultures to be homogenous before sampling. One milliliter of culture sample was removed every 1~2 days during cell growth phases, and cells were counted using an Olympus BH-2 microscope with a hemacytometer (Hausser Scientific). Incubation samples (in duplicate for culture-1 and culture-2) were collected at specific time intervals by filtering through Whatman 934-AH glass microfiber filters (pre-combusted at 450°C for 4 h) and then stored at -40°C for future lipid extraction and analysis.

2.4. Extraction and analysis of lipids

Lipid extraction, separation, derivatization and analysis followed the procedure described in previous studies (Sun et al., 1998; Sun and Wakeham, 1999). Lipids, in thawed filters, were

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6 147 extracted using 3×25 mL of mixed methylene chloride and methanol (2:1, v:v) with 6-minute
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8 148 sonication after vortex-shaking. Combined extracts were separated with separation funnels into a
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10 149 methylene chloride phase after addition of 30 mL of 5% NaCl solution. The volume of total lipid
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12 150 extract (TLE) was reduced to near dryness using rotary evaporators. The TLE was then
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14 151 saponified at 92 °C with 6 mL of 0.5 M KOH in CH₃OH / H₂O (95:5) for 2 h. After cooling and
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16 152 addition of 1 mL of 5% NaCl solution, neutral lipids, including phytol, sterols, alkenones, and
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18 153 fatty alcohols, were extracted with 3×6 mL of hexane from the basic solution (pH > 13).
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20 154 Afterward, the solution was acidified (pH < 2) by adding HCl, and fatty acids were extracted
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22 155 with 3×6 mL of hexane. The neutral lipids were reacted in acetonitrile with *N,O*-
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24 156 bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 92 °C for 2 h to form trimethylsilyl (TMS)
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26 157 ethers of sterols and alcohols. The fatty acids were methylated with 5% boron trifluoride (BF₃) in
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28 158 CH₃OH at 92 °C for 2 h to form fatty acid methyl esters (FAMES).
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35 159 Concentrations of neutral lipids and fatty acids were quantified by capillary gas
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37 160 chromatography using a Hewlett-Packard 6890 Gas Chromatograph coupled to a Flame
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39 161 Ionization Detector (GC-FID) with an on-column injector. Lipid compounds were separated with
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41 162 a 30 m $\times$ 0.25 mm i.d. HP-5 column coated with 5% diphenyl : 95% dimethylsiloxane copolymer.
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43 163 The operating temperature programs were: for neutral lipids, 50-170 °C at 10 °C min⁻¹, then 170-
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45 164 310 °C at 3 °C min⁻¹ and finally held for 30 min at 310 °C; and for fatty acids, 50-170 °C at
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47 165 20 °C min⁻¹, then 170-310 °C at 4 °C min⁻¹ and finally held for 10 min at 310 °C. Internal
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49 166 standards of 5 α (H)-cholestane for neutral lipids and nonadecanoic acid methyl ester for fatty
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51 167 acids were added to each corresponding sample prior to quantitative analysis by GC-FID. The
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53 168 relative standard deviation of lipid quantitative analysis was within $\pm 5\%$ based on duplicate
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Identification of lipid compounds was achieved through a Shimadzu QP-5000 Gas Chromatograph-Mass Spectrometer (GC-MS) system using a split injector and a 30 m $\times$ 0.25 mm i.d. XTI-5 column coated with 5% phenyl methyl silicone and helium as carrier gas. The temperature programs for GC were: 50-150 $^{\circ}\text{C}$ at 20 $^{\circ}\text{C min}^{-1}$ followed by 150-310 $^{\circ}\text{C}$ at 4 $^{\circ}\text{C min}^{-1}$, a 30-min hold for neutral lipids and a 10-min hold for fatty acids at 310 $^{\circ}\text{C}$. The operation conditions for MS were: mass range 50-610 Da with a 0.4 s scan interval, 70 eV ionizing energy.

2.5. Isotopic analysis of lipid compounds

Stable carbon isotopic compositions of individual lipid compounds were measured using a Gas Chromatograph-Combustion-Isotope Ratio Mass Spectrometer system (GC-C-IRMS) (Hayes et al., 1990). Lipid compounds were separated by an Agilent 6890 GC using a 30 m $\times$ 0.25 mm i.d. capillary column (DB-1, J&W Scientific) with a split injector installed. The temperature programs for the GC operation were: 50-180 $^{\circ}\text{C}$ at 20 $^{\circ}\text{C min}^{-1}$ followed by 180-310 $^{\circ}\text{C}$ at 3 $^{\circ}\text{C min}^{-1}$ and a 90-min hold at 310 $^{\circ}\text{C}$ for neutral lipids; 50-170 $^{\circ}\text{C}$ at 20 $^{\circ}\text{C min}^{-1}$ followed by 170-300 $^{\circ}\text{C}$ at 4 $^{\circ}\text{C min}^{-1}$ and a 15 min hold at 300 $^{\circ}\text{C}$ for FAMES. Helium, with a flow rate of 1.5 mL min^{-1} , was used as carrier gas. Separated compounds from the GC were sequentially combusted to CO_2 via a Thermo Finnigan Combustion III unit, over previously oxidized Cu/Ni/Pt wires, at 940 $^{\circ}\text{C}$. Water was removed by diffusion through a Nafion selectively permeable membrane (Perma Pure LLC.) flushed with helium. The stable isotopic compositions of CO_2 peaks were measured with a Finnigan Delta XL plus IRMS operated at 3 kV acceleration potential and by magnetic sector mass separation. Stable carbon isotopic

compositions were calculated relative to a standard reference CO₂ and reported in per mil (‰) against Vienna Pee Dee Belemnite (VPDB) standard reference material. Based on measurement of internal standards, the standard deviations of IRMS analyses were $\pm 0.41\text{‰}$ (n = 34) for neutral lipids and $\pm 0.54\text{‰}$ (n = 18) for FAMES. The $\delta^{13}\text{C}$ values of BSTFA ($-38.23 \pm 0.04\text{‰}$) and methanol ($-43.72 \pm 0.15\text{‰}$), which were pre-determined by a Dual-Inlet IRMS, were used to correct stable carbon isotopic compositions of TMS esters and FAMES due to addition of extra carbon atoms during derivatization.

3. Results

3.1. Cell growth of cultures

Cell densities of *Emiliana huxleyi* in culture-1 and culture-2 increased at similar rates from $\sim 1 \times 10^3 \text{ mL}^{-1}$ to maximum levels ($\sim 6 \times 10^5 \text{ mL}^{-1}$) within exponential growth phase (Fig. 1). Culture-1 was stopped on day 12 (close to the end of exponential growth phase) while culture-2 continued for an additional 23 days through stationary phase, in which cell densities remained relatively constant ($6.8 \times 10^5 \pm 1.4 \times 10^5 \text{ mL}^{-1}$) (Fig. 1).

3.2. Total lipid contents and relative lipid compositions in culture-1 and culture-2

Total lipid content ($15.1 \pm 1.6 \text{ pg cell}^{-1}$) of cells in culture-2 (35 days) was ~ 8 times higher than that ($2.0 \pm 0.3 \text{ pg cell}^{-1}$) found in culture-1 (12 days) (Fig. 1), and total neutral lipids increased ~ 23 times while total fatty acids only increased ~ 3 times. In culture-1, cells contained

more fatty acids (~ 75% of total lipids) than neutral lipids while in culture-2, neutral lipids (~ 73% of total lipids) dominated over fatty acids (Fig. 1).

In both cultures, 7 fatty acids and 11 neutral lipids were identified and their relative compositions were different between the two cultures (Fig. 2). In the fatty acid pool, saturated fatty acids (SFA) 14:0 and 16:0, monounsaturated fatty acid (MUFA) 18:1(ω 9), and polyunsaturated fatty acid (PUFA) 22:6 are major constituents (each > 10%). Higher percentages of 18:1(ω 9) and 22:6 fatty acids, but a lower percentage of 14:0 fatty acid, were observed in culture-2 relative to culture-1. Percentages of the other four fatty acids were similar in the two cultures. In the neutral lipid pool, 37:3 alkenone was the dominant (>28%) compound in both cultures, but relative compositions of neutral lipids varied between these two cultures (Fig. 2). Percentages of phytol and two sterols (27 Δ^5 and 28 $\Delta^{5,22}$) were much less in culture-2 relative to culture-1, and percentages of several alkenones (37:2, 38:3-ethyl, 38:2-ethyl, and 39:2-ethyl) were significantly higher in culture-2 than in culture-1. Percentages of other alkenones remained almost same between the two cultures.

3.3. Variations of lipid compounds during incubation experiments

The bacteria-specific fatty acid 18:1(ω 7) was not present in the two initial cultures. This bacterial fatty acid, however, appeared during incubation experiments when natural seawater was introduced. Its concentration increased in the first few days and then gradually declined to relatively stable levels (Fig. 3). The concentration of this bacteria-specific fatty acid in culture-2 was almost double of that in culture-1. In the first 10 days of incubation, phytoplankton lipid concentrations decreased rapidly and then remained at relatively constant but different levels

(Fig. 4, Fig. 5). At the end of incubation, most of fatty acids were degraded more than 90% in both cultures, except for 16:0 (~ 20-30% remained). Various neutral lipid compounds were degraded ~ 8-35% higher in culture-1 relative to culture-2 (Table 1). Degradation rate constants of most compounds, estimated based on initial loss in the first 10 days, were similar in two incubation experiments (Table 2). Variations of concentrations of 37:3 and 37:2 alkenones during microbial degradation have already been published in Pan and Sun (2011). Information on concentrations of 37:3 and 37:2 alkenones can be found in footnotes to Fig. 5, Table 1 and Table 2 in this article.

3.4. Variations of stable carbon isotopic compositions of lipids during incubation experiments

The stable carbon isotopic compositions of different classes of lipid compounds varied in three different ways during incubation experiments (Fig. 4, Fig. 5). The $\delta^{13}\text{C}$ values of fatty acids 14:0, 16:0 and 18:1(ω 9) from both cultures shifted positively (+1.7~3.9‰) within 10 days. Moreover, relatively larger shifts for 16:0 and 18:1(ω 9) fatty acids (~ +1.5‰ more positive shift for both) were observed in culture-1 compared to those in culture-2. There was little change ($\pm 0.4\text{‰}$) in the $\delta^{13}\text{C}$ values of sterols during incubation experiments (Fig. 5). The $\delta^{13}\text{C}$ values of alkenones (37:3 and 37:2) in culture-2 varied little, but that of 37:2 alkenone in culture-1 showed an apparent negative shift (-3‰). The $\delta^{13}\text{C}$ values of phytol and 22:6 fatty acid in this experiment could not be quantitatively determined because of their small IRMS abundance.

4. Discussion

4.1. Biological functions of phytoplankton lipids synthesized at different physiological states

It has been well known that during exponential growth stage, phytoplankton cells synthesize mostly phospholipid as principal lipid components in cell membrane (Brown et al., 1996; Mansour et al., 2003; Volkman et al., 1989), while during stationary phase, the proportion of energy storage lipids such as triacylglycerols (Brown et al., 1996; Dunstan et al., 1993; Mansour et al., 2003) or alkenones (Bell and Pond, 1996; Epstein et al., 2001) increased significantly. In this study, *E. huxleyi* biosynthesized approximately eight times the total lipids in culture-2 (stopped at the late stationary phase) than in culture-1 (stopped at the end of exponential growth) (Fig. 1). The cells in culture-1 contained more fatty acids (~ 75% of total lipids) than neutral lipids. In contrast, due to the much faster increase of neutral lipids (~ 23 times) than that of fatty acids (~ 3 times) during stationary growth, neutral lipids (~ 73% of total lipids) dominated over fatty acids in culture-2 (Fig. 1).

The dominance of fatty acids (~ 75%) in total lipid content in culture-1 indicated the important function of fatty acids as membrane (structure) lipids (Fig. 1). Lower percentage of 14:0 fatty acid but higher percentages of 18:1(ω 9) and 22:6 fatty acids were observed in culture-2 than in culture-1 (Fig. 2). These variations in relative composition suggested the different functions of individual fatty acids in different cellular components (structure vs. storage) between exponential growth (culture-1) and stationary (culture-2) phases. The reduction of fatty acid proportion (~ 27% in total lipid pool) in culture-2 (Fig. 1) indicated that in addition to fatty acids, there were other lipids, such as alkenones, serving as energy storage compounds.

The biosynthesis pathways, distributions in different cellular compartments, and physiological roles of alkenones in *E. huxleyi* cell have not been completely defined. It is known

that *E. huxleyi* produces little triacylglycerols in cellular components (Volkman et al., 1986), but biosynthesizes a large amount of long-chain alkenones through exponential growth (10-20%) (Conte et al., 1998; Prahl et al., 1988) and stationary phase (up to 40%) (Prahl et al., 2003). The results in this study indicated that a higher proportion of neutral lipids (~ 73% of total lipid content) were produced in culture-2 (Fig. 1) and alkenones occurred as major components in the neutral lipid pool (Fig. 2). Alkenones were previously considered only as membrane lipids in regulating membrane fluidity (Brassell, 1993; Prahl and Wakeham, 1987), and therefore the unsaturation degree of alkenones (i.e. the concentration ratio of [37:2]/([37:2] + [37:3])) has been developed to study historic records of paleo-temperature. Later, alkenones have been known as metabolic storage lipids (e.g., Bell and Pond, 1996; Epstein et al., 2001; Pan and Sun, 2011). Additionally, Sawada and Shiraiwa (2004) found that alkenones were produced by *E. huxleyi* predominantly in the endoplasmic reticulum (ER) and coccolith-producing compartment (CPC)-rich fractions over other membrane fractions of the cells. Eltgroth et al. (2005) further demonstrated that alkenones were synthesized in chloroplasts and then exported to cytoplasmic lipid bodies for energy storage and metabolic need. The previous study by Pan et al. (2014) showed that accumulated alkenones in stationary phase were significantly lost during cell respiration, confirming the important function of alkenones as energy storage lipids. In this study, the higher concentrations of neutral lipids in culture-2 relative to those in culture-1 (Fig. 1), along with the dominant proportion of alkenones in the neutral lipid pools (Fig. 2), were consistent with previous findings that the alkenones serve as energy storage lipid compounds and play an important function in cell respiration (or auto-metabolism).

In the neutral lipid pool, phytol (as a degradation product of chlorophyll) and sterols are membrane-associated lipids which play some role in cellular photosynthesis and regulation of

membrane fluidity and permeability, respectively (Cohen et al., 1995; Hartmann, 1998). Thus, in culture-2, their proportions in the neutral lipid pool remained much lower than those in culture-1 because alkenones were largely accumulated as energy storage compounds in culture-2 (Fig. 2). The previous study by Pan et al. (2014) also showed that much less amount of sterols, compared to alkenones and fatty acids, were lost during cell respiration, indicating their non-energy storage function.

4.2. Lipid isotopic compositions in *Emiliania huxleyi* cells along different growth phases

The isotopic compositions of lipids depend on many factors such as carbon sources, effects of carbon assimilation processes, pathways of biosynthesis and metabolism, cell growth rates and physiological states (Benthien et al., 2007; Bidigare et al., 1997). Laws et al. (1995) found that the biological isotopic fractionation (ϵ_p) in a culture of diatom *Phaeodactylum tricornutum* was negatively correlated with the ratio of cell growth rate to aqueous CO_2 concentration ($\mu/[\text{CO}_2]_{\text{aq}}$). The previous study by Pan et al. (2014) showed that cell growth rate in exponential growth phase of batch culture was relatively constant, while it has been known that the dissolved CO_2 concentrations ($[\text{CO}_2]_{\text{aq}}$) apparently decreased during cell growth due to the assimilation by photosynthesis (Benthien et al., 2007). Accordingly, the biological isotopic fractionation factor (ϵ_p) decreased, leading to positive shifts of $\delta^{13}\text{C}$ values of lipid compounds. After reaching stationary phases, cell growth ceased ($\mu = 0$) and $[\text{CO}_2]_{\text{aq}}$ varied much less (Benthien et al., 2007), resulting in less variable or relatively constant $\delta^{13}\text{C}$ values of lipid compounds. In their mesocosm bloom experiments, Benthien et al. (2007) observed that a significant positive shift in compound-specific isotopic compositions of *E. huxleyi*-derived

alkenones occurred during blooms (corresponding to cell exponential growth phases) while little change of isotopic compositions of alkenones was observed after blooms (corresponding to stationary phases).

Based on the experimental results from the previous study (Pan et al., 2014), it has been postulated that (1) during cell exponential growth phase of a batch culture, phytoplankton lipid compounds were biosynthesized primarily as structural components (e.g., phospholipids in cell membranes) while their stable carbon isotopic compositions shifted positively and were thus heterogeneous in the membrane-associated lipid pool; and (2) during cell stationary phase, lipid compounds were produced mainly as energy storage components (e.g., alkenones in lipid vesicles) while their isotopic compositions were relatively constant and were thus homogenous in the energy storage lipid pool. Unlike the aged or senescent cell materials used for degradation experiments in the previous study (Pan et al., 2014), which were collected after cell respiration experiments, the cells harvested from culture-1 and culture-2 of this study were intact healthy cells in exponential growth and stationary phases, representing distinct physiological states of growing cells. The cells in these two cultures had different lipid compositions and isotopic compositions within different intracellular structures (or compartments). Cells from culture-1 contained more membrane-associated lipids with mostly heterogeneous isotope compositions, whereas cells from culture-2 consisted of more storage lipids with largely homogeneous isotope compositions.

4.3. Effect of microbial degradation on isotopic compositions of phytoplankton lipids

There were no bacteria-specific fatty acids such as 18:1(ω 7) (e.g., Kaneda, 1991; Parkes and Taylor, 1983) present in the two initial cultures. By contrast, after natural seawaters were introduced into the incubation samples, 18:1(ω 7) fatty acid appeared and its concentration increased in the first few days (Fig. 3), coinciding with the rapid decrease in concentrations of phytoplankton lipids during the first 10 days (Fig. 4, Fig. 5). When concentrations of phytoplankton fatty acids approached stable levels, the concentration of 18:1(ω 7) also decreased to a relative stable level throughout the rest of incubations, indicating that bacteria played a major role in decomposing phytoplankton lipids. In addition, concentration of this bacteria-specific fatty acid in culture-2 were two times higher than those in culture-1 (Fig. 3), suggesting that bacterial activities were closely related to concentrations of labile substrate materials. Therefore, a more intense response of microbial community to a higher concentration of phytoplankton lipids occurred in culture-2 than in culture-1. Nonetheless, the use of vaccenic acid 18:1(ω 7) to monitor bacterial growth is somewhat limited because vaccenic acid is lacking in several marine bacteria (Zabeti et al., 2010a).

During microbial degradation, most fatty acids, except for 16:0, were significantly decomposed (> 90%), and significant percentages of neutral lipids (~ 20%-90%) were lost (Table 1). The isotopic compositions of remaining lipid compounds varied in three different ways: positive shift (+1.7‰ to +3.9‰) in $\delta^{13}\text{C}$ for various fatty acids; little change for sterols; and for alkenones little change in most cases or negative shift (-3‰) in the case of 37:2 alkenone in culture-1 (Fig. 4, Fig. 5). The diverse variations of compound-specific lipid isotopic compositions were also observed in some other studies (Chikaraishi and Naraoka, 2006; Pan et al., 2014; Sun et al., 2004). In the previous study by Pan et al. (2014), it was observed that the stable carbon

isotopic compositions of *Thalassiosira weissflogii* and *Emiliania huxleyi* lipids shifted positively during cell exponential growth phase while they became relatively constant during stationary phase. When grown cells consumed their intracellular lipids during cell respiration, the isotopic compositions of lipid compounds remained unchanged. By contrast, after the senescent cells were degraded by microbial processes, the isotopic compositions were altered in different ways. Therefore, based on these previous observations, a possible mechanism for diverse isotopic alterations of lipids has been postulated: (1) during cell exponential growth, lipid compounds are largely produced as components of cell membranes and their isotopic compositions are heterogeneous, while during stationary phase, lipid compounds are mainly biosynthesized as energy storage components with relatively homogeneous isotopic compositions; and (2) both cell respiration and microbial degradation result in significant loss of algal lipids but only the latter process causes significant alteration in the isotopic compositions of lipid compounds, which may be due to the difference in utilization of lipid compounds from one pool (respiration case) or two pools (microbial degradation case). Therefore, original heterogeneity in isotopic compositions of lipid compounds bound in different cellular structures, along with their variable reactivities, during microbial degradation are thought to be two major factors causing isotopic alterations. Nevertheless, few studies have tested how the physiological states (or growth phases) of phytoplankton cells affect the isotopic compositions of lipid compounds during microbial degradation.

Indeed, it has been observed that microbial degradation may preferentially decompose lipids in different cellular components (e.g., membrane vs. intracellular storage component) in different environments (Ding and Sun, 2005). Thus, if lipid compounds in cellular structural components (e.g., membranes) with heterogeneous isotopic compositions are selectively

degraded, the isotopic alteration of remaining lipid compounds may be more significant. In contrast, if lipid compounds in storage components (e.g., lipid vesicles) with homogeneous isotopic compositions are preferentially utilized, the isotopic compositions of remnant lipid compounds may change less significantly.

In *E. huxleyi* cells, fatty acids seemed to function mainly as structure (membrane) lipids in culture-1 while their role in storage component was unclear since alkenones were major storage lipids in culture-2 (Fig. 1, Fig. 2). The positive shifts (+1.7‰ to +3.9‰) in $\delta^{13}\text{C}$ values of fatty acids were possibly caused by preferential degradation of fatty acids bound in cell structural component (e.g., membrane) with heterogeneity in isotopic compositions. Although the degradation rate constants of each individual fatty acid between the two cultures were similar (Table 2), relatively higher percentages of fatty acids were degraded (Table 1, Fig. 4) in culture-1 than in culture-2. In other words, more membrane-associated fatty acids with heterogeneous isotopic compositions in culture-1 were preferentially degraded compared to those in culture-2. Therefore, degradation of fatty acids from culture-1 caused relatively larger positive isotopic shifts ($\sim 1.5\text{‰}$ more each) for 16:0 and 18:1(ω 9) compared to those from culture-2 (Fig. 4). This difference in isotopic shifts between the two cultures further implied that the reactivities of lipid compounds in different cellular components might have a potential impact on isotopic alteration of lipid compounds during microbial degradation.

For most alkenones (except for 37:2 alkenone) in culture-1 and culture 2, there was little change in isotopic compositions regardless of the loss of alkenones (Table 1, Fig. 5). Nonetheless, the $\delta^{13}\text{C}$ values of 37:2 alkenone in culture-1 displayed an apparent negative alteration ($\sim -3\text{‰}$) after degradation (Fig. 5). In the previous study (Pan et al., 2014), no apparent isotopic alteration for alkenones was observed, because most alkenones were synthesized during

stationary growth phase as storage lipids, with relatively homogeneous isotopic compositions, and consequent loss of storage alkenones during degradation would not result in obvious isotopic alteration for alkenones. In this study, alkenones were probably produced as membrane component in culture-1 despite their lower contents in the lipid pool (Fig. 1, Fig. 2). If their isotopic compositions in culture-1 were heterogeneous, their degradation might lead to isotopic alterations. But opposite changes between 37:3 and 37:2 alkenones in culture-1 did not support this explanation. This is probably related to differential degradation pathways of different alkenone compounds. For example, a series of experimental studies (Rontani et al., 2005; 2008; 2006; 2009) demonstrated that 37:3 alkenone could be selectively degraded over 37:2 alkenone by bacterial consortia through two different types of decomposing reactions: (1) attacks on double bond (selective for 37:3 over 37:2 alkenone) and (2) attacks on end methyl or keto groups (non-selective). Indeed, one specific microbe (strain *Dietzia maris* sp. S1) was confirmed to selectively degrade alkenones (Zabeti et al., 2010b). More studies are still needed to clarify the contrasting variations in the isotopic compositions of various alkenones.

There was little change in isotopic signals of sterols in spite of their significant degradation (Table 1, Fig. 5). Sterols are mainly membrane-associated lipids (Hartmann, 1998) and degradation of sterols are primarily through the inter-conversion pathways (from sterols to stenones, stanones, stanols, steroiddiols and sterenes) in various environments, which do not involve carbon number changes (e.g., Gagosian et al., 1980). This may be a major reason for little isotopic alterations of sterols during degradation (Sun et al., 2004).

4.4. Implications

Before phytoplankton lipid biomarkers and their isotopic signals can be applied to study biogeochemical cycling of organic matter in natural systems and to reconstruct paleoceanographic records preserved in marine sediments, two critical issues have to be resolved. First, it is necessary to have a clear understanding of the effect of physiological states of phytoplankton cells during growth on the generation of lipids within different cellular structures and on distributions of lipid isotopic compositions. Second, it is important to elucidate the relative reactivities of lipid compounds associated with different cellular compartments and their impact on isotopic alterations during degradation processes.

By comparing field data with culture results, Conte et al. (1998) noticed that the average physiological state of alkenone producers (e.g., *E. huxleyi*) in the open ocean seemed to be more like cells grown in late exponential or stationary growth phases of batch culture. Prahl et al. (2003) further pointed out that: (1) alkenone producers at a continental margin site with a significant nutrient stress appeared to be more like that from cells grown in batch culture; and (2) those at an open ocean site with a sufficient nutrient supply would follow a continuous culture-like growth (Popp et al., 1998). In the previous study by Pan et al. (2014), phytoplankton cells underwent cell respiration (auto-metabolism) in a dark environment before a microbial degradation experiment. Nevertheless, this did not mimic the real physiological states of cells exported from the photosynthetic zone to underlying sediments. Therefore, in order to better represent cell physiological states during bloom and post-bloom, intact cells from both exponential growth and stationary phases were used for incubation experiment through microbial degradation in this study.

During transport of phytoplankton cell-associated lipid biomarkers from surface water to underlying sediments, many biological and biochemical processes (e.g., zooplankton grazing,

cell respiration, and microbial degradation) have varied influences on algal biomarkers and associated isotopic compositions. For instance, Grice et al. (1998) found that the feeding of copepod *Temora longicornis* on the haptophyte *Isochysis galbana* had no influence on the isotopic compositions of alkenones because the copepod seemed not to utilize alkenones for metabolism. Moreover, it is possible that during phytoplankton exponential growth phases, cells mainly biosynthesized structural (membrane) lipids with heterogeneous stable carbon isotopic compositions, while during stationary phase, cells biosynthesized more storage lipids with homogeneous isotopic compositions (Pan et al., 2014). Therefore, during cell respiration, the compound-specific stable carbon isotopic compositions of most lipid biomarkers changed little, although significant amounts of storage lipids (with homogeneous isotopic compositions) were consumed. By contrast, microbial processes might preferentially degrade lipid compounds associated with structural components (with heterogeneous isotopic compositions), resulting in significant isotopic alterations, which have been observed in the previous and current studies. This study further demonstrated that the preferential degradation of membrane-associated lipids (with heterogeneous isotopic compositions) from culture-1 relative to storage compounds (with homogeneous isotopic compositions) from culture-2 could lead to relatively larger isotopic alterations.

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Captions to illustrations

Fig. 1. Variations of *Emiliana huxleyi* cell densities and total lipid contents of cells in culture-1 (12 days) and culture-2 (35 days). Note that filled black dots represent last days of the cultures.

Fig. 2. Relative lipid compositions of cells in culture-1 (12 days) and culture-2 (35 days).

Fig. 3. Variations of concentrations of bacteria-specific fatty acid (BSFA) 18:1(ω 7) during degradation of cells in culture-1 and culture-2.

Fig. 4. Variations of chemical concentrations and stable carbon isotopic compositions of fatty acids during degradation of cells in culture-1 and culture-2.

Fig. 5. Variations of chemical concentrations and stable carbon isotopic compositions of neutral lipids during degradation of cells in culture-1 and culture-2.¹

¹ Please refer to Fig. 3c and Fig. 3d in Pan and Sun (2011) for variations of chemical concentrations (in percentages) of 37:3 and 37:2 alkenones: The Figs. 3c and 3d are corresponding to culture-1 and culture-2 in this article, respectively.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

689 **Table 1**

690 Proportions of degraded vs. remaining lipid compounds. ²

		Neutral lipids					Fatty acids			
		phytol	27Δ ⁵	28Δ ^{5,22}	Et38:3alk	Et38:2alk	14:0	16:0	18:1(ω9)	22:6
Culture 1	Degraded (%)	85	91	79	51	53	96	77	94	~ 100
	Remaining (%)	15	9	21	49	47	4	23	6	~ 0
Culture 2	Degraded (%)	71	63	68	43	26	92	69	91	99
	Remaining (%)	29	37	32	57	74	8	31	9	1

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² Please refer to Fig. 3c and Fig. 3d in Pan and Sun (2011) for proportions of degraded vs. remaining lipid compounds of 37:3 and 37:2 alkenones: The Figs. 3c and 3d are corresponding to culture-1 and culture-2 in this article, respectively.

Table 2

Degradation rate constants (k_d) of lipid compounds.³

		Neutral lipids					Fatty acids			
		phytol	27 Δ^5	28 $\Delta^{5,22}$	Et38:3alk	Et38:2alk	14:0	16:0	18:1(ω 9)	22:6
Culture-1	k_d (d ⁻¹)	0.196	0.164	0.218	0.161	0.172	0.166	0.117	0.141	0.406
	R ²	0.88	0.80	0.86	0.76	0.78	0.98	0.90	0.96	0.95
Culture-2	k_d (d ⁻¹)	0.199	0.166	0.209	0.282	0.124	0.169	0.134	0.249	0.573
	R ²	0.83	0.85	0.82	0.96	0.87	0.94	0.85	0.95	0.91

³ Please refer to Fig. 3c and Fig. 3d in Pan and Sun (2011) for degradation rate constants (k_d) of 37:3 and 37:2 alkenones: The Figs. 3c and 3d are corresponding to culture-1 and culture-2 in this article, respectively.