# IMPACTS OF BIOGEOCHEMICAL PROCESSES ON PHYTOPLANKTON-PRODUCED LIPID BIOMARKERS AND THEIR STABLE CARBON ISOTOPIC COMPOSITIONS

by

### HAI PAN

(Under the Direction of Ming-Yi Sun)

### ABSTRACT

Phytoplankton-produced lipid biomarkers and their compound-specific stable carbon isotopic compositions have been widely used to study organic carbon cycling and paleoceanography. One big uncertainty in their applications is the impact of biogeochemical cycling of organic matter on their stabilities. Although it has been assumed that the changes of these signals during biogeochemical processes are insignificant, accumulating evidence has indicated that these signals underwent diversified variations from their generation to preservation. This dissertation aims to examine (1) how physiological states of phytoplankton affect generation of cellular lipids and their stable carbon isotopic compositions, and (2) how cell respiration and microbial degradation processes alter these signals.

Three series of laboratory experiments were conducted to reach my goals: (1) to grow two phytoplankton species (*Thalassiosira weissflogii* and *Emiliania huxleyi*) through exponential growth and stationary phases in batch culture, followed by dark respiration, and then microbial degradation; (2) to conduct microbial degradation of *E. huxleyi* cells collected from different growth phases; and (3) to incubate <sup>13</sup>C-labeled tripalmitin in natural oxic and anoxic sediments. Bulk parameters (TOC, TN, C/N, and isotopes), lipids (alkenones, fatty acids, sterols and phytol) and associated molecular isotopic compositions were monitored for samples from these experiments.

The results showed: (1) cell growth phases had an important influence on generation of chemical and isotopic signals; (2) cell respiration had little impact on chemical and isotopic signals although significant fractions of lipids were utilized; (3) microbial degradation could cause diversified alterations for chemical and isotopic signals but these alterations were more or less dependent on physiological states of phytoplankton cells; and (4) chemical reactivity of lipid compounds played one major role in altering compound-specific isotopic compositions when the compounds existed in several different pools in natural systems.

Therefore, the general conclusion is that heterogeneous production of chemical and isotopic signals over different cell growth phases and selective degradation of lipids bound in different cellular components are major causes for alterations of chemical and isotopic signals during biogeochemical cycling. More studies are needed to elucidate the relations among cell physiological states, intracellular structures, and distributions of chemical and isotopic signals within these structures.

INDEX WORDS: Phytoplankton, Lipid biomarkers, Isotopic composition, Paleotemperature index  $U_{37}^{K'}$ , Physiological state, Cell growth phase, Cell respiration, Microbial degradation, *Thalassiosira weissflogii*, *Emiliania huxleyi*, Isotopic alteration, Chemical reactivity, Sediment, Redox conditions

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### DEDICATION

This dissertation is dedicated to my dear father and mother, Yeyao Pan and Yingxin Du, whose love, patience and support have helped me to become who I am today; to my beloved grandmother, Xueyun Lu, who passed away in Shanghai when I was pursuing my PhD study in Athens; and to my cherished younger sister, Yan Pan, who has always been my supporter and helped me with my responsibility for the family when I am far away from home.

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### CHAPTER 1

#### GENERAL INTRODUCTION AND LITERATURE REVIEW

#### 1. Literature review

We are now living in a rapidly changing world and the changes in climate and environments greatly affect our lives (Haines et al., 2006; McMichael et al., 2006). On the other hand, human's activities also significantly impact the Earth. For example, fossil fuel burning has rapidly increased CO<sub>2</sub> concentration in atmosphere (Falkowski et al., 2000), which has led to dramatic changes of environmental conditions such as temperature increase and global warming, ice coverage decline in polar areas, sea level rising, and ocean acidification (Caldeira and Wickett, 2003; Feely et al., 2004; Semiletov, 2004; Ridley et al., 2005; Kennedy, 2012). All these changes are closely related to global carbon cycling among atmosphere, ocean, and land (Fig.1.1). Each year, fossil fuel burning contributes about  $5.3 \times 10^{15}$  g of CO<sub>2</sub> to atmosphere (Prentice et al., 2001), of which approximately 36% enters into ocean as a net sink (Millero, 1995; Feely et al., 2004; Sabine et al., 2004).

Carbon is cycled in the oceans through biological pump and solubility pump (Chisholm, 2000). As shown in Fig. 1.2, approximately  $5 \times 10^{16}$  g of organic carbon is produced by marine phytoplankton each year in oceans (Martin et al., 1987), which is the second largest organic carbon production in earth biosphere (Harvey et al., 1995). Most phytoplankton-produced organic compounds in surface waters are recycled in water column through biological and

biochemical processes (Berner, 1989), which provides food and energy for higher trophic level organisms (Park et al., 2002; Kainz et al., 2004). Although only a very small portion (<0.1%) of the phytoplankton-produced organic carbon is eventually buried in marine sediments (Berner, 1989), important records of historic biogeochemical cycling, past climatic and environmental conditions are to some extent preserved (Dean et al., 1986; Hollander and McKenzie, 1991; Summons, 1993). For example, Meyers (1994) suggested that elemental C/N ratios and  $\delta^{13}$ C values of total organic matter seemed to particularly reserve paleoenvironmental information for multi-million-year time periods, although most initial aquatic organic matter was destructed and altered during sinking, sedimentation, and after sedimentation.

However, there are some uncertainties limiting the application of total organic matter and associated stable carbon isotopic composition. First, mixing of organic matters with distinct isotopic compositions from various sources would make it difficult to differentiate the sources of organic matters. For example, a contribution of 50 % of terrestrial C<sub>3</sub> plants with a  $\delta^{13}$ C value of -26‰ and 50% of estuarine C<sub>4</sub> plants with a  $\delta^{13}$ C value of -14 ‰ will result in a misleading 100% input of marine phytoplankton with a  $\delta^{13}$ C value of -20‰. Second, chemical and biochemical decomposition and degradation processes could also lead to either depletion or enrichment of <sup>13</sup>C in total organic matter due to the selective loss of isotopically enriched (i.e., amino acids and carbohydrates) or depleted (i.e., lipids and lignins) components during export of organic matter from surface water to underlying sediments (Benner et al., 1987; Lehmann et al., 2002). Third, input of organic matter with distinct isotopic composition from microbial biomass through early diagenesis could also change the  $\delta^{13}$ C value of total organic matter (e.g. Canuel et al., 1997a; Boschker et al., 1999b; Teece et al., 1999).

In order to avoid the above limitations in applications of total organic matter and associated isotopic composition, phytoplankton-produced lipid biomarkers have been widely applied as biomarkers to study organic carbon cycling and paleoceanographic records. Although phytoplankton lipids account for a relatively smaller fraction (~5-20%) than carbohydrates and proteins of total organic matter in phytoplankton biomass (Parsons, 1961), their structures are more source-specific and less reactive than proteins and carbohydrates (Harvey et al., 1995). For instance, the relative abundances of specific lipid biomarkers (e.g., sterols, alkenones, and fatty acids) in natural environments have been applied to measure organic carbon fluxes from surface seawater to underlying sediments (Wakeham et al., 1997; Bac et al., 2003), assess the sources of organic matter (Harvey et al., 1988; Harvey, 1994; Volkman et al., 1998; Shin et al., 2008), characterize microbial community (Wakeham et al., 2003), reconstruct past records of sea surface temperature (Prahl and Wakeham, 1987; Müller et al., 1998). Moreover, the  $\delta^{13}$ C values of lipid biomarkers have been utilized to characterize microbial community (Boschker and Middelburg, 2002) and estimate ancient atmospheric *p*CO<sub>2</sub> records (Jasper and Hayes, 1994; Pagani et al., 1999; Pagani et al., 2002).

There are many factors that may have influences on the chemical signals of algal lipid biomarkers during biosynthesis progresses. Lipid contents and compositions of growing cells can be affected by environmental conditions such as temperature and salinity (Xu and Beardall, 1997; Zhu et al., 1997), light and UV-B irradiation (Brown et al., 1996; Skerratt et al., 1998), and concentrations of nutrient and CO<sub>2</sub> (Dempster and Sommerfield, 1998; Riebesell et al., 2000b). Algal lipids are also dependent on cell physiological states or cell growth phases (Dunstan et al., 1993; Brown et al., 1996; Zhu et al., 1997; Mansour et al., 2003; Lv et al., 2010). For example, the alkenone unsaturation ratio ( $U_{37}^{K'} = [37:2]/([37:2] + [37:3])$ , representing the relative concentrations of C<sub>37</sub> unsaturated alkenones produced by Prymnesiophyeae such as *Emiliania huxleyi*, has been known to be well correlated with the temperature of cell growth (Prahl et al., 1988; Müller et al., 1998) and been widely applied to assess the past sea surface temperatures. Nonetheless, the alkenone-based temperature index  $(U_{37}^{K'})$  can also be affected by other factors such as cell growth rate or growth phase (Herbert, 2001).

Marine microalgae biosynthesize diverse lipid compounds for various biological functions in distinct cellular structures during different growth phases (Sukenik and Carmeli, 1990; Bell and Pond, 1996; Brown et al., 1996; Mansour et al., 2003). Although most algal species biosynthesize polar phospholipids in their cell membrane as structural components, different algal species produce different lipid compounds as energy storage compounds in their cytoplasm (Bell and Pond, 1996; Brown et al., 1996). During exponential growth phase, more structural lipids are biosynthesized (Volkman et al., 1989; Sukenik and Carmeli, 1990; Brown et al., 1996; Mansour et al., 2003) whereas during stationary phase, more storage lipids are produced by cells (Dunstan et al., 1993; Bell and Pond, 1996; Brown et al., 1996; Epstein et al., 2001; Mansour et al., 2003; Lv et al., 2010). In various lipid classes, fatty acids in various ester complexes (Suen et al., 1987; Sukenik and Wahnon, 1991; Zhu et al., 1997; Lv et al., 2010) and long chain alkenones (Prahl and Wakeham, 1987; Brassell, 1993; Bell and Pond, 1996; Epstein et al., 2001; Sawada and Shiraiwa, 2004; Eltgroth et al., 2005a; Pan and Sun, 2011) are associated with either cell membrane components or energy storage compounds. Phytol, as a constituent of chlorophyll, and sterols are generally bound with algal membrane (Cohen et al., 1995; Hartmann, 1998).

Furthermore, there are also many factors that could control the isotopic compositions of algal lipids during biosynthesis. These effects include carbon acquisition mechanism (Laws et

al., 1998; Rost et al., 2002), compound synthesis pathways (Laws et al., 2001), algal growth rate and growth phases (Laws et al., 1995; Bidigare et al., 1997; Popp et al., 1998a; Riebesell et al., 2000a; Benthien et al., 2007), and cell size and geometry (Popp et al., 1998b; Burkhardt et al., 1999). For instance, Laws et al. (1995) observed that isotopic fractionation of carbon by phytoplankton were controlled by both  $CO_2$  concentration and cell growth rate. Bidigare et al. (1997) further suggested that changes in cell growth rate had a significant effect on the isotopic compositions of alkenones. Benthien et al. (2007) conducted a series of mesocosm bloom experiments dominated by *Emiliania huxleyi* under three distinct  $CO_2$  partial pressures, and found that during cell exponential growth phase there were large positive alterations in alkenone isotopic composition (4-5‰) in all treatments whereas during stationary phase there were little changes. Nevertheless, it has been unclear about the biosynthetic pathways and intracellular distributions of alkenones during cell growth under variable conditions (Laws et al., 2001). It is also unknown if isotopic compositions of lipid compounds are homogeneously distributed in different cellular structures.

The fundamental assumption of applications of biomarkers and associated molecular isotopic compositions is that the alteration of these phytoplankton-generated chemical and isotopic signals is small enough to be neglected during biogeochemical cycling processes such as degradation of organic matter (Prahl et al., 1989; Hayes et al., 1990; McCaffrey et al., 1990; Pagani et al., 1999) (Fig. 1.2). However, more and more field and laboratory studies have shown that different organic compounds from various sources experience diversified changes in their chemical signatures during biogeochemical cycling processes. For example, the  $U_{37}^{K'}$  index increased significantly with depth in the open-ocean water column, possibly caused by selective degradation of  $C_{37}$  alkenones with different double bonds (Freeman and Wakeham, 1992;

Christodoulou et al., 2009; Rontani et al., 2009). Gong and Hollander (1999) measured the concentration profiles of alkenones in surface sediments from the Santa Monica Basin and noticed a biased temperature record (up to  $2.5^{\circ}$ C) resulting from a preferential degradation of C<sub>37:3</sub> over C<sub>37:2</sub> alkenones under oxic conditions, compared to that in a nearby anoxic sediment. Nonetheless, Prahl et al. (1989) observed that in the Madeira Abyssal Plain (MAP) sediments, even when up to 85% of alkenones degraded under both oxic and anoxic conditions, the index appeared to be stable over ~8 kyr. By contrast, Hoefs et al. (1998) argued that oxidation actually resulted in significant changes in the  $U_{37}^{K'}$  index in the same MAP sediments (but at different sections). Contrasting results of the effect of alkenone degradation on the index were also observed by many experimental studies (Teece et al., 1998; Sun et al., 2004; Rontani et al., 2005; 2008; 2009; Prahl et al., 2010; Zabeti et al., 2010).

Moreover, controversial results have also been reported for the variations of isotopic compositions of biomarkers during biogeochemical processes. For example, degradation of *n*-alkanes and polycyclic aromatic hydrocarbons did not cause apparent alteration in their isotopic compositions (Hayes et al., 1990; Freeman et al., 1994; Huang et al., 1997; Mazeas et al., 2002). On the contrary, Macko et al. (1994) observed that after 4 weeks of incubation, ~50% of amino acids in the seagrass *Halodule wrightii* were degraded, and some amino acids were depleted in <sup>13</sup>C (~5‰ decrease) while others either became enriched (~2‰ increase) or remained constant. Sun et al. (2004) also illustrated the diversified isotopic variations of different *Emiliania huxleyi* produced lipids after microbial degradation in natural oxic and anoxic seawaters: negative shift in  $\delta^{13}$ C values for alkenones, positive shift for fatty acids, and no change for sterols. In addition, differential isotopic alterations of sugar molecules during degradation of salt marsh plant *Spartina alterniflora* was observed Teece and Fogel (2007): residual (~25%) arabinose was

depleted in <sup>13</sup>C by -4‰ compared to the original signal; 85% decrease in glucose resulted in an isotopic enrichment (+5‰) for remaining compounds; and after 79% of xylose was removed, the  $\delta^{13}$ C value remained constant. Nevertheless, it has been unclear what mechanisms cause these diversified isotopic alterations for different biomarkers during biogeochemical cycling processes.

### 2. Hypotheses

Based on previous studies, three hypotheses were proposed to elucidate potential internal and external factors affecting chemical and isotopic signals during cell growth, respiration, and microbial degradation:

(1) Algal lipid compounds are biosynthesized as structure (membrane) and intracellular energy storage components during different growth phases, which may result in heterogeneity in chemical/isotopic signals within different cellular components;

(2) Lipid compounds associated with different cellular components are utilized differently during cell respiration (auto-metabolism) and microbial degradation, which may lead to significant alteration in chemical/isotopic signals of remaining compounds;

(3) Chemical reactivity may play the more important role than kinetic isotopic fractionation in altering isotopic compositions of lipids during microbial degradation.

### 3. Objectives

The major purpose of this dissertation is to elucidate the potential factors affecting phytoplankton-produced lipid biomarkers (phytol, sterols, alkenones, and fatty acids) and their stable carbon isotopic compositions during cell growth, respiration, and microbial degradation. One focus will be on internal factors, which are related to cell growth phases, cellular structures, and production of lipid compounds with varying isotopic compositions. The other focus will be on external factors such as consumption of lipid compounds by cell respiration (autometabolism) or degradation of lipid compounds by microbial processes. This research is trying to figure out how these internal and external factors collaborate to alter the chemical and isotopic signals of lipid biomarkers during biogeochemical cycling processes. The specific objectives are: (1) To determine variations in lipid compositions of two algal species (diatom *Thalassiosira weissflogii* and haptophyte *Emiliania huxleyi*) during cell growth (exponential and stationary phases), respiration (auto-metabolism), and subsequent microbial degradation;

(2) To determine variations in stable carbon isotopic signals ( $\delta^{13}$ C) of lipid compounds produced by two algal species during cell growth, respiration, and microbial degradation;

(3) To examine effect of microbial degradation on chemical and isotopic signals of intact algal cell derived lipid biomarkers harvested at cell exponential and stationary growth stages, respectively;

(4) To determine variations of the alkenone-based  $U_{37}^{K'}$  index derived from *E. huxleyi* during cell growth, respiration, and microbial degradation;

(5) To examine relative roles of kinetic isotope fractionation vs. chemical reactivity (using <sup>13</sup>C-labeled tripalmitins at different carbon atom sites) in altering compound-specific isotopic signals during microbial degradation in oxic and anoxic sediments.

### 4. Structure of the dissertation

This dissertation consists of six chapters. Chapter 1 (this chapter) is a general introduction and literature review, and Chapter 6 is a general summary. Chapter 2 to Chapter 5 are four main chapters, which are inherently linked to each other.

Chapter 2 aimed to elucidate potential internal and external factors and their collaborative effects on the  $U_{37}^{K'}$  index during cell growth, respiration, and microbial degradation. Two series of laboratory experiments were conducted under a constant temperature. In the first series, *E*.

*huxleyi* cells were first cultured under a light:dark regime throughout exponential growth and stationary phases, followed by cell respiration under continuous dark, and subsequent microbial degradation of the senescent cells. The second series was related to the incubation of killed *E*. *huxleyi* cells harvested respectively in the exponential growth phase and in the late stationary phase in natural seawater. Variations of cell density and alkenone concentrations over experiments were followed and the effects of various processes and intracellular associations of alkenones on the  $U_{37}^{K'}$  index were examined.

Chapter 3 was designed to examine (1) generation of various lipid compounds and their stable carbon isotopic compositions during different growth phases; and (2) relative roles of cell respiration (auto-metabolism) and microbial degradation in altering chemical and isotopic signals. Diatom *Thalassiosira weissflogii* and haptophyte *Emilliania huxleyi* were batch cultured under a light:dark regime through cell exponential growth and stationary phase, followed by cell respiration under dark, and then microbial degradation with the introduction of natural seawater. Bulk parameters (POC, TN, C/N,  $\delta^{13}$ C-POC, and  $\delta^{15}$ N-TN) of algal cells and chemical concentrations and isotopic compositions of algal lipids (phytol, sterols, alkenones, and fatty acids) were determined. The relationships between the variations of chemical and isotopic compositions of algal lipids and cellular physiological states and environmental conditions were explored. Implications of the results were also addressed.

Chapter 4 followed the findings of Chapter 3 and aimed to further elucidate the potential effect of cell physiological states (or growth phases) on the variability of algal lipids and their stable carbon isotopic compositions during microbial degradation. Killed *Emiliania huxleyi* cells, harvested in the exponential growth phase and the stationary growth phase respectively, were dark incubated under aerobic conditions with introduction of natural seawater (containing

microbial communities). Variations in algal lipid concentrations (phytol, sterols, alkeones, and fatty acids) and associated stable carbon isotopic compositions were determined during onemonth incubation. Possible mechanisms leading to these variations were proposed and implications of the study were discussed.

Based on results in Chapter 4, Chapter 5 was particularly designed to further examine the relative roles of kinetic isotopic fractionation vs. chemical reactivity in changing stable carbon isotopic composition of lipid compounds during microbial degradation. <sup>13</sup>C-labeled (labeling at different carbon sites in fatty acid chains) and unlabeled tripalmitins were spiked in different proportions and then incubated in natural oxic (top 1 cm) and anoxic (>10 cm) sediments with primitively differential lipid contents and compositions. The concentrations of naturally-occurred (e.g., 14:0, 16:1, 18:1, and 20:5/20:4) fatty acids were determined and their degradation rate constants were estimated. The variations of tripalmitin-derived 16:0 fatty acid concentrations and associated isotopic compositions were also measured. The relationship between isotopic alteration and the relative proportion of <sup>13</sup>C-labeled compound in the total pool was investigated and the implications of the findings were discussed.

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Fig. 1.1. Schematic net carbon flows (inputs and exports) among atmosphere, ocean, and land influenced by human activities such as fossil fuel burning and cement production.



Fig. 1.2. Schematic organic carbon cycling in ocean system<sup>1</sup>

<sup>1)</sup> based on data from Berner (1989).

## CHAPTER 2

# VARIATIONS OF ALKENONE-BASED PALEOTEMPERATURE INDEX $(U_{37}^{K'})$ DURING *EMILIANIA HUXLEYI* CELL GROWTH, RESPIRATION (AUTO-METABOLISM), AND MICROBIAL DEGRADATION <sup>1</sup>

<sup>&</sup>lt;sup>1</sup> Pan, H., Sun, M.-Y., 2011. Organic Geochemistry 42, 678-687.

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## Abstract

To clarify effects of biological and biochemical processes on the alkenone-based paleotemperature index ( $U_{37}^{K'}$ ), we conducted two series of experiments under a constant temperature: (1) culturing *Emiliania huxleyi* (CCMP371, axenic strain) throughout exponential growth and stationary phases under a light:dark regime, followed by cell respiration (autometabolism) under continuous dark, and subsequently by microbial degradation under continuous dark by killing cells and inoculating with natural seawaters; and (2) microbial degradation of killed *E. huxleyi* cells, which were harvested respectively in exponential growth and stationary phases, in natural seawaters.

Our results showed that alkenone compositions (represented by K37/K38 and K38e/K38m ratios) of *E. huxleyi cells* differed significantly in different growth phases or in different physiological states. These ratios decreased slightly during cell respiration but changed little during microbial degradation. The  $U_{37}^{\kappa'}$  index varied in different modes: (1) shifting positively (+0.13 unit) in the exponential growth phase but negatively (-0.1 unit) in stationary phase; (2) remaining constant during cell respiration even when ~50% of cellular alkenones were lost; (3) changing insignificantly during microbial degradation of the senescent cells (survived after cell respiration) and the cells harvested in exponential growth phase but increasing by 0.05 unit during microbial degradation of the cells harvested in late stationary phase. Collective observations in this study imply that the physiological state of cells, production rates of di- and tri-unsaturated alkenones during different growth phases, and susceptibilities of alkenones bound with different cellular structures (membrane vs. intracellular energy storage component) during microbial degradation have potential influences on the  $U_{37}^{\kappa'}$  index and thus on paleotemperature records preserved in sediments.

## 1. Introduction

Chemical signals preserved in marine sediments retain substantial information on the history of past biological productivity, paleoclimate change, and paleoenvironmental conditions (Dean et al., 1986; Volkman, 1986; Hollander and McKenzie, 1991; Summons, 1993). One group of specific organic compounds, long chain unsaturated alkenones, have been considered to be a useful paleotemperature indicator (Marlowe, 1984; Brassell et al., 1986a). These compounds are exclusively biosynthesized in surface water by Prymnesiophyceae including *Emiliania huxleyi, Isochrysis galbana, Chrysotila lamellosa* and *Gephyrocapsa oceanica*, which are distributed from coastal areas to the open ocean (Volkman et al., 1980a; Marlowe et al., 1984; Patterson et al., 1994; Conte et al., 1995; Volkman et al., 1995). The relative concentrations of  $C_{37}$  unsaturated alkenones were defined as an index ( $U_{37}^{K^*} = [37:2]/([37:2] + [37:3])$ , which was well correlated with the temperature of cell growth. The correlation has been confirmed by laboratory culture experiments (Prahl et al., 1988) and field measurements for core-top sediments from the global ocean (Müller et al., 1998) and widely accepted as a robust tool for assessment of historic records of the sea surface temperature.

Besides the temperature dependence, other internal and external factors have been recognized to affect the  $U_{37}^{K'}$  index (Schneider, 2001). The first internal factor is related to interspecies differences (e.g., *E. huxleyi* vs. *G. oceanica*), which produce alkenones with different temperature dependence (Prahl and Wakeham, 1987; Conte et al., 1995; Volkman et al., 1995). Second, even for the same species (e.g., *E. huxleyi*), different strains or clones produce  $C_{37:3}$  vs.  $C_{37:2}$  alkenones disproportionally, resulting in variable  $U_{37}^{K'}$  indexes (Popp et al., 1998a; Riebesell et al., 2000b; Benthien et al., 2007). Third, cell growth rate or growth phase plays an important role in controlling the index (Herbert, 2001). Alkenones are thought to be intracellular

energy compounds though they are also suggested to occur in membranes (Prahl et al., 1988; Brassell, 1993; Bell and Pond, 1996; Pond and Harris, 1996; Eltgroth et al., 2005b).

External factors affecting the  $U_{37}^{K'}$  index in natural environments involve physical and biochemical processes such as lateral transport of remote alkenones (produced in water with different temperature), bioturbational mixing, and degradation of alkenones (Schneider, 2001). Although alkenones are susceptible to degradation in the water column and surface sediments, contrasting evidence has continuously emerged and stimulated a debate on the influence of alkenone degradation on the  $U_{37}^{K'}$  index. In the open-ocean water column, the  $U_{37}^{K'}$  index increased significantly with depth, likely caused by selective degradation processes (Freeman and Wakeham, 1992; Christodoulou et al., 2009; Rontani et al., 2009). The concentration profiles of alkenones measured in surface sediments from the Santa Monica Basin also indicated a preferential degradation of C37:3 over C37:2 alkenone under oxic conditions, causing a biased temperature record (up to 2.5°C) compared to that in a nearby anoxic sediment (Gong and Hollander, 1999). Conversely, in the Madeira Abyssal Plain (MAP) sediments, the index appeared to be stable even when up to 85% of alkenones degraded under both oxic and anoxic conditions over ~8 kyr (Prahl et al., 1989). However, Hoefs et al. (1998) argued that oxidation indeed caused significant changes in the  $U_{37}^{K'}$  index in the same MAP sediments (but at different sections). Many experimental studies (Teece et al., 1998; Sun et al., 2004; Rontani et al., 2005; 2008; 2009; Prahl et al., 2010; Zabeti et al., 2010) also showed contrasting results on the effect of alkenone degradation on the index.

This study aimed to elucidate potential internal and external factors and their collaborative effects on the  $U_{37}^{K'}$  index during cell growth, respiration, and microbial degradation. We conducted two series of laboratory experiments under a constant temperature. The first series

included culturing *E. huxleyi* cells under a light:dark regime throughout exponential growth (12 days) and stationary phases (additional 27 days), followed by respiration of grown-up cells under continuous dark over 21 days, and subsequently by microbial degradation of the senescent cells with inoculation of natural seawaters over 40 days. The second series was to incubate killed *E. huxleyi* cells, which were harvested respectively in the exponential growth phase (likely dominated by membrane compounds) and in the late stationary phase (abundant in intracellular storage compounds), in natural seawater. We monitored variations of cell density and alkenone concentrations over experimental courses and examined the effects of various processes and intracellular associations of alkenones on the  $U_{37}^{K'}$  index.

## 2. Experimental

#### 2.1. Materials

*E. huxleyi* (calcifying strain 12-1, CCMP371) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The strain was originally isolated from the Sargasso Sea (32°N, 62°W; 50 m water depth) in June 1987. The initial axenic culture was made in February 2002 and remained axenic (confirmed by routine tests). Seawaters for preparation of culture medium and inoculation of natural microbes were collected from Georgia coast (31°05'N, 81°16'W, September 2005 and 31°25N, 77°21W, May 2006) and the Gulf of Mexico (28°16'N, 91°59'W, September 2006 and 28°10'N, 91°36'W, May 2008) by direct pumping of surface (1 m) waters into large carboys. The seawater used for medium preparation was filtered through Whatman 934-AH glass microfiber filters (pre-combusted at 450°C for 4 hours) and then sterilized. The f/50 medium was prepared according to the protocol recommended by CCMP (Guillard, 1962; Guillard, 1975). The seawater used for inoculation were stored in carboys until incubation experiments.

## 2.2. Experimental set-up

The first series of experiments (Fig. 2.1a) consisted of cell growth, respiration (autometabolism), and microbial degradation. First, axenic *E. huxleyi* cells were batch cultured in f/50 medium (1 - 2.3 l) under a light:dark (12h:12h) regime with a constant light intensity (50  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>) through phase I (12 days, exponential growth) and phase II (following 27 days, stationary phase). Second, the grown-up cells were kept in the same medium in continuous dark over 21 days (phase III, cell respiration). Transfer and sampling of cultures were carried out in a clean hood, which was pre-irradiated with a UV lamp over 30 min. Third, the cultures were divided equally into a set of small flasks (100 ml each) and the cells were killed by freezing below -30°C overnight. After thawing the cultures, 50 ml of natural seawater was added to the flasks and incubated in the dark for 40 days with aerobic conditions by continuously purging the solution with air (phase IV, microbial degradation). The second series of experiments (Fig. 2.1b) was to incubate killed *E. huxleyi* cells, which were harvested respectively from the exponential growth phase (12 days) and during late stationary phase (35 days), by inoculating with natural seawater in the same way as the first series of experiments. All experiments were conducted under a constant temperature (17°C).

## 2.3. Sampling and cell counting

Before sampling, the flasks were gently shaken by hand to create uniform cell cultures. One milliliter of culture was taken for cell counting every 1-2 day during phases I, II, and III using an Olympus BH-2 microscope with a hemacytometer (Hausser Scientific). Subsamples (25 - 300 ml, depending on cell density) in all phases were collected by filtering the cultures through glass microfiber filters (Whatman 934-AH) and then stored at -40°C for future lipid extraction.

## 2.4. Lipid analysis

The method for lipid extraction, separation, and derivatization were adopted from the previous studies (Sun et al., 1998; Sun and Wakeham, 1999). Lipids on thawed filters were extracted by  $3 \times 25$  ml methylene chloride-methanol (2:1, v/v) with 6 min sonication each time. Combined extracts were partitioned into a methylene chloride phase with addition of 30 ml 5% NaCl solution. The volume of total lipids extract (TLE) was reduced to near dryness by rotary evaporation. The TLE was then saponified at 92°C with 6 ml 0.5 M KOH in MeOH/H<sub>2</sub>O (95:5) for 2 hours. After cooling and addition of 1 ml 5% NaCl solution, the neutral lipids, including alkenones, sterols, phytol and fatty alcohols, were extracted from the basic solution (pH > 13) with  $3 \times 6$  ml hexane. After drying, the neutral lipids were reacted with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in acetonitrile to form TMS-ethers of sterols and alcohols.

Concentrations of neutral lipids were quantified by capillary gas chromatography using a Hewlett-Packard 6890 GC with an on-column injector and a flame ionization detector (Sun et al., 1998; Sun and Wakeham, 1999). The lipid compounds were separated with a 30 m × 0.25 mm i.d. HP-5 column coated with 5%-diphenyl-95%-dimethylsiloxane copolymer operated with a temperature program of 50-170°C at 10°C min<sup>-1</sup>, then 170-310°C at 3°C min<sup>-1</sup> and finally held for 30 min at 310°C. Internal standard [5 $\alpha$ (H)-cholestane] was added to each samples before the GC analysis to aid in quantification. The relative standard deviation of lipid analysis was within ±5% based on duplicate measurements. Identification of lipid compounds was achieved with a Shimadzu QP-5000 GC-MS system using a split injector and a 30 m × 0.25 mm i.d. XTI-5 column coated with 5% phenyl methyl silicone and helium as carrier gas. Operating conditions were: mass range 50-610 Da with a 0.4 s scan interval; 70 eV ionizing energy; GC temperature

program 50-150°C at 20°C min<sup>-1</sup> followed by 150-310°C at 4°C min<sup>-1</sup> and a 30 min hold at 310°C.

## 3. Results

## 3.1. Variations of cell density during experiment series 1

The culture started at an initial cell density of  $\sim 3 \times 10^3$  cell ml<sup>-1</sup> and reached a maximum density ( $\sim 7 \times 10^5$  cells ml<sup>-1</sup>) in 12 days (Table 2.1, Fig. 2.2). During exponential growth phase, cell growth rate was 0.67 divisions d<sup>-1</sup> (Fig. 2.2a). During stationary phase, cell density remained at a nearly constant level of  $7.2\pm0.9\times10^5$  cell ml<sup>-1</sup> (Fig. 2.2a). When grown-up cells in the culture were placed in continuous dark (auto-metabolism, phase III), cell density remained almost constant ( $4.7\pm0.5\times10^5$  cells ml<sup>-1</sup>) over 21 days (Table 2.1, Fig. 2.2b).

## 3.2. Production and degradation of alkenones in experiment series 1

Concentrations of total alkenones ( $\Sigma$ Alk) in *E. huxleyi* cells varied between cell growth phases (Table 2.1). In exponential growth phase (I), alkenone concentrations were low and slowly increased: from 1.3 pg cell<sup>-1</sup> at day 6 to 2.8 pg cell<sup>-1</sup> at day 12 (end of exponential phase). Through the stationary phase (II), the concentrations of total alkenones continuously increased to 19.4 pg cell<sup>-1</sup> (~7× of that in exponential phase). Alkenone compositions were dominated by C<sub>37</sub> (37:2 and 37:3) and C<sub>38</sub> (38:2 ethyl, 38:2 methyl, 38:3 ethyl, and 38:3 methyl) compounds while C<sub>39</sub> (39:2 ethyl and 39:3 ethyl) compounds accounted for less than 9% of the total (Table 2.1). To better demonstrate variations in alkenone compositions, we adopted two widely used ratios: (1) K37/K38, defined as C<sub>37</sub>/C<sub>38</sub> ketones; and (2) K38e/K38m, defined as C<sub>38</sub> ethyl/C<sub>38</sub> methyl ketones. These ratios represent the relative proportions of alkenones with different structural features (chain length and location of keto functional group). They were significantly different between different phases of cell culture (Fig. 2.2a). In the exponential phase, K37/K38 ratios

were greater than one (C<sub>37</sub> alkenones more abundant) while in the stationary phase, they were lower than one (C<sub>38</sub> alkenones more abundant). The K38e/K38m ratios in the exponential phase were relatively lower than those in the stationary phase. Concentrations of individual 37:2 and 37:3 alkenones followed similar variations as total alkenones (Fig. 2.2d). The production rates of C<sub>37:3</sub> and C<sub>37:2</sub> alkenones in phase I were similarly low (~0.06 pg cell<sup>-1</sup> d<sup>-1</sup> for each compound) while in phase II, the production rate of C<sub>37:3</sub> was ~3× faster than that of C<sub>37:2</sub> (Fig. 2.2d).

During cell respiration (phase III), concentrations of total alkenones gradually decreased (Table 2.1) and the relative compositions (K37/K38 and K38e/K38m ratios) slightly declined (Fig. 2.2b). Meanwhile, 47-48% of  $C_{37:2}$  and  $C_{37:3}$  alkenones were lost regardless of a constant cell density over 21 days (Fig. 2.2e). Degradation rate constants of  $C_{37:3}$  and  $C_{37:2}$  alkenones, estimated based on the 1st-order kinetics, were almost the same ( $k_{d-37:3}/k_{d-37:2} = 1.03$ ) (Fig. 2.2e).

When the senescent cells (survived after respiration phase) were killed and incubated in natural seawater, concentrations of total alkenones continuously decreased (Table 2.1) but the K37/K38 and K38e/K38m ratios showed little variation (Fig. 2.2c). Approximately 55-59% of C<sub>37:3</sub> and C<sub>37:2</sub> alkenones were lost over 40 days of incubation (phase IV). Degradation rate constants (following the 1st-order kinetics) of two alkenones were similar ( $k_{d-37:3}/k_{d-37:2} = 1.06$ ) although they were slightly higher than those in phase III (Fig. 2.2f).

#### 3.3. Degradation of alkenones in experiment series 2

*E. huxleyi* cells for the experiment series 2 were harvested from the cultures respectively at the 12th day (exponential growth phase) and the 35th day (late stationary phase). Although the cell densities in two cultures were similar  $(5.0\pm0.6\times10^5 \text{ cells ml}^{-1} \text{ for } 12 \text{ days and } 6.4\pm0.6\times10^5 \text{ cells ml}^{-1}$  for 35 days), the cellular alkenone concentration was ~36× higher in the culture of 35 days than that of 12 days (Table 2.1). The alkenone compositions were also different between

two cultures: relatively higher K37/K38 and lower K38e/K38m ratios in the cells harvested from culture of 12 days than those from culture of 35 days (Figs. 2.3a and 2.3b). When the cells from the 12 d culture were incubated in natural seawaters after they were killed, the concentrations of total alkenones decreased (Table 2.1) while the K37/K38 and K38e/K38m ratios remained constant over the course of incubation (Fig. 2.3a).  $C_{37:3}$  and  $C_{37:2}$  alkenones were degraded at similar rates ( $k_{d-37:3}/k_{d-37:2} = 0.95$ ) and approximately 60% of each alkenone was lost over 30 days (Fig. 2.3c). For cells harvested from culture of 36 days, total alkenone concentrations also decreased in the same way as the cells harvested from culture of 12 days (Table 2.1) and the K37/K38 and K38e/K38m ratios remained constant during incubation (Fig. 2.3b). However,  $C_{37:3}$  alkenone degraded apparently faster than  $C_{37:2}$  alkenone ( $k_{d-37:3}/k_{d-37:2} = 3.49$ ) and ~36% of  $C_{37:3}$  and ~20% of  $C_{37:2}$  were lost respectively during incubation (Fig. 2.3d).

## 3.4. Variations of the $U_{37}^{K'}$ index in all experiments

During the exponential growth phase (I) of experiment series 1, the  $U_{37}^{K'}$  index increased by 0.13 unit (Fig. 2.4a). In contrast, through the stationary phase (II) of experiment series 1, the  $U_{37}^{K'}$  index decreased by 0.10 unit (Fig. 2.4a). During the cell respiration phase (III) of experiment series 1, the  $U_{37}^{K'}$  index remained almost constant (Fig. 2.4b, standard deviation (SD)  $< \pm 0.004$ ). During the subsequent microbial degradation phase (IV) of experiment series 1, the  $U_{37}^{K'}$  index scattered in a narrow range (Fig. 2.4c, SD  $< \pm 0.01$ ). When cells with low-alkenone content (harvested in the exponential growth phase) were incubated in natural seawater (experiment series 2), variations of the  $U_{37}^{K'}$  index were insignificant (Fig. 2.4d, SD  $< \pm 0.006$ ). However, when cells with high-alkenone content (harvested in the late stationary phase) were incubated in natural seawater (experiment series 2), the index increased apparently (~0.05 unit) in the first a few days and then remained at a constant level (Fig. 2.4e).

## 4. Discussion

# 4.1. Factors affecting the cellular alkenone composition and $U_{37}^{K'}$ index during cell growth

Alkenone compositions (represented by K37/K38 and K38e/K38m ratios) varied significantly throughout the exponential and stationary phases of cell culture, with a continuous increase in total cellular alkenone concentration. From a field study, (Rosell-Melé et al., 1994) showed that the K37/K38 ratio distributions seemed to be correlated with biogeographic areas of alkenone producers in the Atlantic Ocean, implying that these ratios may be species-dependent. On the other hand, based on laboratory culturing study, Conte et al. (1998) found that the chain length distribution (K37/K38 ratio) of alkenones within E. huxleyi and G. oceanica cells changed with cell growth phase. Our results (Fig. 2.2a) are consistent with those by Conte et al. (1998): higher ratios (>1) in exponential growth phase and lower ratios (<1) in stationary phase. Prahl et al. (2003; 2006) further pointed out that nutrient stress during cell culturing (in stationary phase) could reduce the K37/K38 ratio. In contrast, the K38e/K38m ratios progressively increased from the cell exponential growth phase to stationary phase. This is caused by a rapid increase in ethyl alkenone compared to methyl alkenone in the stationary phase. Conte and Eglinton (1993) suggested that ethyl alkenone might be biosynthesized via an oxidation-reduction pathway, different from that of methyl alkenone. It is evident that the physiological state of cells (growth phases and nutrient conditions) has a strong impact on cellular alkenone composition.

Although the *E. huxleyi* (strain 12-1, CCMP371) was cultured under a constant temperature (17°C) in this study, the cellular  $U_{37}^{K'}$  indexes varied over a range of 0.16 - 0.38 during cell growth. According to the generally accepted  $U_{37}^{K'}$ -cell growth temperature calibration

 $(T = (U_{37}^{K'} - 0.043)/0.033)$  for the *E. huxleyi* strain NEPCC 55a culture (Prahl and Wakeham, 1987) or derived from core-top sediment samples and the surface seawater temperatures (SSTs) of the global ocean (Müller et al., 1998), the values correspond to a temperature range from 3.6°C to 10.2°C, far lower than the actual culturing temperature. However, when another calibration ( $T = (U_{37}^{K'} + 0.762)/0.063$ ) derived from *E. huxleyi* strain EH2 culture (Sawada et al., 1996) was applied, the inferred temperature range was between 14.7°C and 18.1°C, quite close to the actual culturing temperature. Deviation from the generally accepted calibration has been observed by many culturing studies and is attributed to the genetic difference between strains of *E. huxleyi* (Conte et al., 1998; Popp et al., 1998a; Riebesell et al., 2000b).

It is interesting that the cellular  $U_{37}^{K'}$  index varied in different modes between different growth phases in our experiments (Fig. 2.4a). Several factors may be responsible for different variations of the index during different growth phases. First, growth rate of *E. huxleyi* cells was found to have different impacts on the cellular  $U_{37}^{K'}$  indexes of calcifying and noncalcifying species (Popp et al., 1998a). For noncalcifying species, the index was inversely correlated with the cell growth rate while a positive relationship between the index and the cell growth rate existed for calcifying species. The *E. huxleyi* strain used in our culture experiment is a calcifying species, so the positive shift of the index during exponential growth phase is expected. Prahl et al. (2003) observed that fast growth of *E. huxleyi* (strain NEPCC 55a) during exponential phase did not result in significant variations in the  $U_{37}^{K'}$  index but the index indeed declined during stationary phase of the culture.

Second, variability of the cellular  $U_{37}^{K'}$  index depends on the relative production rates of  $C_{37:3}$  and  $C_{37:2}$  alkenones in each growth phase, which directly affect the relative proportions of

two alkenones. Based on the definition, the  $U_{37}^{K'}$  index can be expressed as  $1/[1 + (C_{37:3}/C_{37:2})]$ . When 37:3 alkenone was produced much faster than 37:2 alkenone in stationary phase, the ratio of  $C_{37:3}/C_{37:2}$  increased, which led to a decrease in the index. On the other hand, the  $(C_{37:3}/C_{37:2})$ ratios in exponential growth phase tended to decrease from the initial ratio when  $C_{37:3}$  and  $C_{37:2}$ alkenones were produced at similar rates. Decrease in the  $(C_{37:3}/C_{37:2})$  ratios resulted in an increase in the  $U_{37}^{K'}$  index.

Third, associations of alkenones within different cellular structures may affect the index although physiological roles of alkenone molecules have not been completely understood (Eglinton and Eglinton, 2008). Early studies presumed that alkenones are biosynthesized mainly as membrane-bound compounds, which regulate membrane fluidity (Brassell et al., 1986b; Prahl et al., 1988). Fernández et al. (1994) suggested that alkenones in *E. huxleyi* cells served to control buoyancy when coccoliths were produced on the cell surface. Many marine algae produce a high content of triglycerides as common metabolic energy storage compounds, but their content in *E. huxleyi* cells was very low (Volkman, 1986; Pond and Harris, 1996). Instead, *E. huxleyi* cells produce ample alkenones as substitute compounds for their metabolism needs. Epstein et al. (2001) reported that the content of alkenones within the *E. huxleyi* cells continued to increase throughout the exponential and stationary phases of cell growth but decreased when the grown-up cells were placed in complete dark over a few weeks. Our study also showed similar variations of alkenone concentrations in cell growth and dark respiration, further supporting that alkenones could be produced as energy storage compounds and used by autometabolism of living cells during respiration.

Sawada and Shiraiwa (2004) examined the distributions of alkenones and related compounds (alkyl alkenoates and alkenoic acids) in isolated membrane and organelle fractions of

*E. huxleyi* cells and found that predominantly high percentage of these compounds was in the endoplasmic reticulum (ER) and coccolith-producing compartment (CPC)-rich membrane fraction. Eltgroth et al. (2005b) further pointed out that polyunsaturated long-chain alkenones were synthesized in chloroplasts and then exported to cytoplasmic lipid bodies where they were stored for future metabolism. In general, membrane-bound lipids are mainly produced in the exponential growth phase when new cells are building up their membrane structures while intracellular storage lipids are largely made during stationary phase when the buildup of cellular structural components is completed (Bell and Pond, 1996). Production of alkenones in different cellular structures may follow different temperature-dependence. For example, synthesis of membrane compounds is generally dependent on temperature due to homeoviscous adaption to maintain membrane fluidity (Hazel, 1988) while production of metabolic storage compounds is controlled by temperature due to kinetic effects such as enzyme optimization (Epstein et al., 2001).

Other environmental conditions (e.g., light intensity, nutrient availability, and CO<sub>2</sub> level) also affect cell growth and synthesis of alkenones, which in turn affect the cellular  $U_{37}^{K'}$  index (Conte et al., 1995; Popp et al., 2006; Benthien et al., 2007). As shown by many studies (Epstein et al., 2001; Prahl et al., 2003; Benthien et al., 2007), the concentrations of nutrients and CO<sub>2</sub> in batch culture medium greatly reduced from exponential to stationary phases while biosynthesis of alkenones within *E. huxleyi* cells continued or even accelerated when nutrients became depleted. This is particularly obvious for the nitrogen-starved situation because storage lipids do not need nitrogen. However, influence of nutrients may result in a difference in relative production rates of C<sub>37:3</sub> vs. C<sub>37:2</sub> alkenones (Epstein et al., 2001). On the other hand, Riebesell et al. (2000b) demonstrated that increases of CO<sub>2</sub> concentration led to dramatic increases of

alkenone content in *E. huxleyi* cells and as a proportion of organic carbon but there was no report showing a systematic effect of CO<sub>2</sub> concentration on the  $U_{37}^{K'}$  index.

# 4.2. Degradation of alkenones during cell respiration and its impact on the $U_{37}^{K'}$ index

During the phase III of experiment series 1, cell density remained almost constant but the concentrations of total alkenones declined markedly (Table 2.1), clearly indicating that alkenones were used as energy source compounds for cell metabolism. Both K37/K38 and K38e/K38m ratios decreased slightly during this phase (Fig. 2.2b), suggesting that shorter chain and ethyl alkenones might be selectively degraded by cell respiration. However, the cellular  $U_{37}^{K'}$  indexes remained nearly invariable (SD <±0.004) during this phase (Fig. 2.4b). It seems that the invariability in the index is likely controlled by loss rates of C<sub>37:3</sub> and C<sub>37:2</sub> alkenones during auto-metabolism of living cells. Indeed, the degradation rate constants of C<sub>37:3</sub> and C<sub>37:2</sub> alkenones bound in initial concentration (Fig. 2.2b). This suggests that C<sub>37:3</sub> and C<sub>37:2</sub> alkenones bound in intracellular storage components are non-selectively degraded by cell auto-metabolism.

However, other studies observed different variation patterns of the index during cell respiration (in complete darkness). For example, Epstein et al. (2001) conducted isothermal batch culturing and respiration experiments using two *E. huxleyi* strains (BT6, from Sargasso Sea and 55a, from the subarctic Pacific) and their results showed that the index of BT6 cells declined (from 0.1 to 0.07, corresponding to a temperature decrease of  $-1.0^{\circ}$ C) over 15 days of dark respiration while that of 55a cells increased (from 0.51 to 0.55, corresponding to a temperature increase of  $+1.2^{\circ}$ C) during 18 days of dark respiration. Moreover, Prahl et al. (2003) observed that when exponentially growing cells of *E. huxleyi* (strain 55a) were placed in dark for 5 days, the index shifted positively from 0.55 to 0.66, corresponding to a calculated temperature change

of +3.3°C. But a 5-day dark incubation of the grown-up cells (already in stationary phase) in their study did not result in a significant change of the index.

There are several possible explanations for different variations of the index during respiration of E. huxleyi cells. First, the genetic difference between strains is likely responsible for their variable functions utilizing cellular alkenones during cell respiration. For example, strain 55a cells in two studies (Epstein et al., 2001; Prahl et al., 2003) showed the same changes in the index while two different strains (BT6 and 55a) in one study (Epstein et al., 2001) exhibited opposite changes in the index. In this study, the strain (CCMP371) did not show a change in the index during cell respiration. Second, relative roles of cell respiration vs. bacterial degradation among studies may be different. In our study, the original E. huxleyi strain was confirmed to be axenic by the CCMP. Although we could not rule out the possibility of bacterial presence during carefully-controlled culturing and respiration experiments, evidence indicates an insignificant role of lipid-degrading bacteria in our experiments. For example, no bacteriaspecific biomarkers (e.g., iso- and anteiso-15:0, 18:1(n-7) fatty acids) were detected in our culture samples but they appeared in the samples with inoculation of natural seawater. In addition, concentrations of cellular sterols during our respiration experiment did not significantly decline but they decreased significantly when the culture was inoculated with natural seawater. In contrast, the cultures in other studies were not axenic and bacteria might play an important role in degrading cellular alkenones during dark incubation, which could not be distinguished from loss of storage alkenones due to cell auto-metabolism. Third, relative proportions of alkenones bound in membrane and intracellular storage components may have an impact on variation of the index during cell respiration. As shown by Prahl et al.'s study (2003), the index increased during dark respiration of exponentially growing cells, which might have a lower

proportion of storage alkenones. Respiration of grown-up cells in stationary phase, which might have a high proportion of storage alkenones, resulted in much less (Epstein et al., 2001) or no change in the index (Prahl et al., 2003; this study). However, it is still unknown how differently the alkenones bound in different cellular structures are utilized by cell respiration.

## 4.3. Effect of microbial degradation of alkenones on the $U_{37}^{K'}$ index

The effect of diagentic processes on stability of the  $U_{37}^{K'}$  index was ignored in early development of the alkenone proxy for paleothermometry (Prahl et al., 1989; McCaffrey et al., 1990). However, accumulated evidence from field observations (Hoefs et al., 1998; Gong and Hollander, 1999; Kim et al., 2009) and laboratory experiments (Rontani et al., 2005; 2006; 2008; Prahl et al., 2010; Zabeti et al., 2010) has demonstrated that the index can be significantly altered during degradation of alkenones. Unlike most lipid compounds, degradation of alkenones in natural environments is largely driven by microbial processes and not by animal's activities. For example, some studies (Volkman et al., 1980b; Grice et al., 1998) revealed that zooplankton's grazing neither digested alkenones nor altered the  $U_{37}^{K'}$  index. Benthic macrofauna in sediments were unable to assimilate alkenones during grazing (Ding and Sun, 2006). However, the effects of microbial degradation of alkenones on the  $U_{37}^{K'}$  index vary with environmental conditions and microbial communities (Teece et al., 1998; Rontani et al., 2005; 2008; Zabeti et al., 2010).

In this study, we conducted microbial degradation experiments using *E. huxleyi* cells with three different treatments (senescent cells survived from respiration experiment, intact cells collected from exponential growth phase and from late stationary phase respectively). During incubation, concentrations of total alkenones continuously decreased (Table 2.1) but the K37/K38 and K38e/K38m ratios remained almost constant (Figs. 2.2c, 2.3a and 2.3b), indicating that microbial degradation did not result in fundamental changes in alkenone compositions.

Although the initial concentrations of alkenones in these cell materials were different, they varied by the same pattern over the incubation course: a rapid decrease in the first 5-10 days and followed by a slow decrease or remained at a relatively constant level.

Similar to respiration experiment, we can compare the relative degradation rate constants of  $C_{37:3}$  vs.  $C_{37:2}$  alkenones to examine the effect of alkenone degradation on the index. For the senescent cells and cells collected in the exponential growth phase, the ratios of degradation rate constants of  $C_{37:3}$  and  $C_{37:2}$  alkenones were close to 1 (Figs. 2.2f and 2.3c). Thus, no significant variations of the index were observed in these two cases (Figs. 2.4c and 2.4d). However, for the cells collected in late stationary phase,  $C_{37:3}$  alkenone was preferentially degraded over  $C_{37:2}$ alkenone (Fig. 2.3d) and the ratio of degradation rate constants was greater than 3 (Fig. 2.3d), resulting in an apparent increase in the index (Fig. 2.4e).

There has been a debate on the selective degradation of alkenones. Teece et al. (1998) showed that >80% of initial alkenones in slurry systems under oxic, sulphate-reducing, and methanogenic conditions were non-selectively degraded and would not cause significant change in the  $U_{37}^{K^*}$  index. Kim et al. (2009) reported that alkenones in surface sediments were selectively degraded only under oxic conditions and resulted in a small but significant increase (up to 0.06 units) of the index. A series of experimental studies (Rontani et al., 2005; 2006; 2008; 2009) demonstrated that  $C_{37:3}$  alkenone could be selectively degraded over  $C_{37:2}$  alkenone by bacterial consortia, resulting in a moderate increase in the  $U_{37}^{K^*}$  index. Two types of decomposing reactions for alkenones were characterized: one with attacks on end methyl or keto groups, which were non-selective (Rontani et al., 2008). Recently, one specific microbe (strain *Dietzia maris* sp. S1) was confirmed to selectively degrade alkenones (Zabeti et al., 2010).

In this study, we observed selective degradation only for one case, where the cells were collected in late stationary phase. Although alkenones in cell membrane and intracellular storage component were not differentiated, we may speculate that alkenones in senescent cells and intact cells collected in the exponential growth phase are dominantly associated with cell membrane while alkenones in intact cells collected in the stationary phase are largely bound in intracellular storage component. Alkenone compositions (also relative proportions of  $C_{37:3}$  vs.  $C_{37:2}$ ) in different structural components might differ, as shown by varying K37/K38 and K38e/K38m ratios in different treatments. Bacteria might non-selectively degrade alkenones bound with one cellular structure (membrane) but they may selectively degrade alkenones in two cellular structures (membrane and intracellular storage component). Therefore, selective degradation of alkenones in natural environments can be caused by both external forces (different bacterial species) and internal causes (alkenones associated with different cellular structures). It has been shown that intracellular structural associations have important influences on the degradation of algal fatty acids (Ding and Sun, 2005a) and chloropigments (Ding and Sun, 2005b).

## 4.4. Implications for applications of the $U_{37}^{K'}$ index

The results from this experimental study support the previous finding that the alkenonebased  $U_{37}^{\kappa}$  index depends not only on temperature but also on other factors during cell growth (Conte et al., 1998; Epstein et al., 1998; Prahl et al., 2003). Variability in the cellular index between different growth phases of isothermally cultured is most likely related to physiological states of alkenone producers. As indicated by Prahl et al. (2003), physiological states of alkenone producers in natural marine systems may be determined by nutrient supply. Nutrient availability may control chemical composition and relative production rates of di- and tri-unsaturated alkenones within cellular structures (Versteegh et al., 2001; Benthien et al., 2007). Under different physiological states, cells produce different proportions of membrane and storage alkenones with different temperature-dependences (Hazel, 1988; Epstein et al., 2001). Thus, the first question that needs to be clarified is: what is physiological state of cells that deliver alkenones to underlying sediments in nature? Based on comparison between culture results and field data, Conte et al. (1998) indicated that the average physiological state of alkenoneproducers in the open ocean seemed to be more like cells in late log or stationary phase of batch culturing. Prahl et al. (2003) further pointed out that alkenone-producers at an open ocean site with a sufficient nutrient supply would follow a continuous culture-like growth (Popp et al., 1998a) and export a  $U_{37}^{K'}$  signal, which is consistent with the water temperature at the depth of production, to the seafloor. In contrast, the  $U_{37}^{K'}$  signal exported to the seafloor at a continental margin site with a significant nutrient stress seemed to be more like that from cells grown in batch-culture (Prahl et al., 2003). However, diversified variations of the cellular index during cell growth among different strains imply that changes in physiological state may also depend on genetic factor, which reserves more investigations.

The next important question is: how can the  $U_{37}^{K'}$  index be altered by biological and biochemical processes during transport of alkenone-carrying cells from surface water to underlying sediment. In natural systems, alkenone degradation by cell respiration (autometabolism) cannot be distinguished from alkenone degradation by microbial processes. Thus, it is impossible to clarify the individual effect of these different processes on the  $U_{37}^{K'}$  index by field measurements. This experimental study allowed us to exclusively examine the effect of cell auto-metabolism on the index and our results indicated that although storage alkenones were significantly lost during cell respiration, there was no significant effect on the index. Increase of the index with water depth in oceans are attributed to selective degradation of alkenones by microbial processes (Freeman and Wakeham, 1992; Christodoulou et al., 2009; Rontani et al., 2009), and the roles of specific bacteria in selective degradation of alkenones have been clarified (Rontani et al., 2005; 2008; Zabeti et al., 2010). This study further suggests that microbial processes may selectively degrade alkenones only when cellular storage alkenones are abundant (e.g., cells in stationary phase or in physiological state with nutrient-deplete conditions). Therefore, collaboration of physiological control (internal) and microbial role (external) is likely a driving force to alter the index in natural systems, but further researches are needed.

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Table 2.1. Summary of results of experiment series 1 and series 2: Cell density, total alkenone concentration ( $\Sigma$ Alk), and relative proportions (%) of group alkenones.

Phase	Time	Density $(10^5 \text{ colls } \text{m}^{1-1})$	$(ng \text{ coll}^{-1})$	$\frac{lk}{(ug ml^{-1})}$	%K37	%K38m	%K38e	%K38	%K39
Experiment 1: cell culturing through exponential (I) and stationary (II) phases									
I	3	0.1	/	/	/	/	/	/	/
Ι	6	0.6	1.3	0.1	49	18	26	44	6.5
Ι	9	4.6	0.8	0.4	52	19	24	43	4.8
I/II	12	6.4	2.8	1.8	48	14	32	46	6.6
II	15	7.3	4.4	3.2	46	13	34	47	7.2
II	18	7.6	5.8	4.4	46	11	36	47	7.4
II	21	8.5	6.9	5.9	45	12	35	47	7.4
II	24	6.5	10.0	6.5	44	13	36	49	7.6
II	27	7.5	11.5	8.6	45	12	36	48	7.5
II	30	7.1	12.9	9.1	45	12	36	48	7.5
II	33	6.2	16.4	10.2	44	11	37	48	7.5
II	36	5.8	17.6	10.2	44	12	37	49	7.8
II	39	5.3	19.4	10.3	44	12	36	48	7.5
Experiment 1: cell respiration (phase III)									
ĪĦ	0	5.4±0.2	$14.2 \pm 1.3$	7.9±0.7	42±2	$11\pm0$	39±2	50±1	7.9±0.3
III	3	4.1±0.1	19.4±1.7	8.1±0.7	41±3	11±0	40±2	51±2	8.1±0.6
III	6	5.5±0.1	12.6±0.6	6.8±0.3	$44\pm0$	13±0	36±0	$49 \pm 0$	7.6±0.1
III	9	4.5±0.8	17.7±1.1	6.7±0.4	43±1	$12\pm0$	37±0	50±0	7.8±0.1
III	12	4.7±0.4	11.3±0.1	5.7±0.1	42±0	13±0	37±0	50±0	7.9±0.0
III	15	4.5±0.3	$10.7 \pm 0.5$	5.1±0.2	41±1	13±0	38±1	51±1	8.1±0.1
III	18	4.9±0.2	9.2±0.3	4.7±0.2	39±2	$14\pm0$	39±2	52±2	8.2±0.1
III	21	4.0±0.5	$12.9\pm0.0$	4.5±0.0	39±1	13±0	39±1	53±1	8.2±0.1
Experiment 1: degradation of senescent cells (phase IV)									
IV	0			3.5±0.3	$40\pm0$	$14\pm0$	37±0	51±0	$8.8 \pm 0.0$
IV	2			3.1±0.3	39±0	13±0	39±0	52±0	9.0±0.0
IV	5			$2.4{\pm}0.5$	39±0	$14\pm0$	38±0	52±0	9.1±0.0
IV	10			2.6±0.5	$40\pm0$	$14\pm0$	$37\pm0$	51±0	$8.8 \pm 0.0$
IV	18			$2.2\pm0.2$	39±0	$14\pm0$	38±0	52±0	9.0±0.0
IV	25			$1.7\pm0.1$	$40\pm0$	15±1	37±1	51±0	$8.8 \pm 0.0$
IV	40			1.6±0.3	$40\pm0$	15±1	37±1	51±0	$8.7 \pm 0.0$
Experiment 2: degradation of cells collected at the end of exponential growth phase									
	0			$0.2 \pm 0.1$	48±2	19±0	27±1	46±1	6.5±1.5
	2			$0.2 \pm 0.1$	49±2	$18\pm0$	27±1	45±1	6.1±1.1
	4			$0.1 \pm 0.1$	48±2	$18\pm0$	26±1	45±1	$7.0\pm0.8$
	7			$0.1 \pm 0.0$	$48 \pm 1$	19±0	27±1	$46 \pm 0$	6.1±0.6
	11			$0.1 \pm 0.0$	49±2	$18\pm0$	27±1	46±1	5.8±1.1
	18			$0.1 \pm 0.0$	52±1	20±0	28±1	$48 \pm 1$	/
	25			$0.1 \pm 0.0$	52±2	20±0	28±1	$48 \pm 2$	/
	32			$0.1 \pm 0.0$	52±2	20±1	28±1	48±2	/
Experiment 2: degradation of cells collected in late stationary phase									
	0			$6.5\pm0.1$	$44\pm0$	11±1	38±1	$49 \pm 0$	7.1±0.1
	2			$4.9\pm0.1$	$45 \pm 0$	$11\pm0$	36±0	$48\pm0$	$7.2\pm0.1$
	4			4.6±0.1	45±0	$11\pm0$	37±0	$48 \pm 0$	$7.2\pm0.1$
	7			$4.2 \pm 0.1$	45±0	11±0	37±1	$48 \pm 0$	7.3±0.1
	11			4.2±0.2	45±0	11±0	37±0	48±0	7.2±0.0
	18			4.5±0.1	45±0	11±0	36±0	48±0	7.1±0.0
	25			4.8±0.4	45±0	11±1	37±1	48±0	7.2±0.0
	32			$4.4 \pm 0.7$	45±0	11±1	37±1	$48 \pm 0$	7.3±0.0



Fig. 2.1. Experimental setup: (a) the first series of experiment (cell growth, respiration, and microbial degradation); (b) the second series of experiment (microbial degradation of cells collected from exponential and stationary phases respectively).



Fig. 2.2. Variations of cell density, K37/K38 and K38e/K38m ratios, and 37:3 and 37:2 alkenone concentrations during experimentseries 1: cell growth (a and d), cell respiration (b and e), and microbial degradation (c and f).


Fig. 2.3. Variations of K37/K38 and K38e/K38m ratios, and 37:3 and 37:2 alkenone concentrations during experiment-series 2: microbial degradation of *E. huxleyi* cells harvested respectively from the exponential phase (a and c) and the stationary phase (b and d).



Fig. 2.4. Variations of the  $U_{37}^{K'}$  index during experiment-series 1 (a: cell growth phases; b: cell respiration; and c: microbial degradation) and experiment-series 2 (d: microbial degradation of cells harvested from the 12 d culture; e: cells from the 36 d culture).

## CHAPTER 3

# EFFECTS OF CELL GROWTH, RESPIRATION, AND MICROBIAL DEGRADATION ON LIPIDS AND ASSOCIATED MOLECULAR ISOTOPIC COMPOSITIONS OF *THALASSIOSIRA WEISSFLOGII* AND *EMILIANIA HUXLEYI*<sup>1</sup>

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#### Abstract

This study examines the effects of biochemical processes on algal lipids (phytol, sterols, alkenones, and fatty acids) and their stable carbon isotopic compositions by conducting a series of laboratory experiments. Two marine algal species, Thalassiosira weissflogii and Emiliania huxleyi, were batch cultured through cell exponential growth (phase I) and stationary period (phase II), followed by dark respiration (phase III) and subsequent microbial degradation (phase IV). During culturing, T. weissflogii continuously produced a variety of neutral lipids and fatty acids with similar rates between phase I and phase II. By contrast, E. huxleyi produced neutral lipids at faster rates but fatty acids at slower rates in phase II than in phase I. However, the isotopic compositions of lipid compounds between the two species varied in a similar pattern during cell culturing: significant positive shifts (5%-10%) in  $\delta^{13}$ C occurred through phase I while during phase II, the isotopic compositions of most lipids remained relatively constant or small negative shifts (2‰-3‰) occurred. During dark respiration, the two species lost their cellular lipids by varying percentages: >50% of alkenones from E. huxleyi, >50% of all fatty acid compounds from T. weissflogii while 20-60% of individual fatty acids from E. huxleyi, and <20% of phytol and sterols from both. No matter how much lipid compound was lost, the  $\delta^{13}$ C values of most compounds (except phytol of T. weissflogii, with a ~-3‰ shift) remained relatively constant (in a range of  $\leq \pm 1\%$ ) in phase III. During microbial degradation, most fatty acids (except 16:0 fatty acid from E. huxleyi) were lost, but significant amounts of sterols (>30%) from both species and alkenones (~20%) from E. huxleyi survived in phase IV. In phase IV, the isotopic compositions of individual lipids varied in three different ways: positive shift  $(>+2\infty)$  for phytol and most fatty acids; little change or small fluctuations (within  $\pm 1\infty$ ) for sterols and alkenones; and a negative shift (>-2‰) for 20:5 FA from T. weissflogii. The results of this study imply: (1) isotopic compositions of phytoplankton lipid compounds are heterogeneously generated through different cell growth phases, which is likely dependent on cell physiological states, medium conditions, and relative proportions of intracellular compartments; and (2) microbial degradation plays a more important role in altering isotopic compositions of lipid compounds compared to cell respiration.

#### 1. Introduction

Marine phytoplankton produces approximately  $5 \times 10^{16}$  g organic carbon each year in oceans (Martin et al., 1987), which is the second largest organic carbon production in earth biosphere (Harvey et al., 1995). Phytoplankton production in surface waters provides food and energy for higher trophic level organisms (Park et al., 2002; Kainz et al., 2004) and most phytoplankton-produced organic compounds are recycled in the water column through biological and biochemical processes (Berner, 1989). Only a very small fraction (<0.1%) of the phytoplankton-produced organic carbon is ultimately buried in marine sediments (Berner, 1989), but important signals for past histories of climatic and environmental conditions as well as biogeochemical cycling are somewhat preserved (Dean et al., 1986; Hollander and McKenzie, 1991; Summons, 1993). Although phytoplankton lipids account for a relatively smaller fraction (~5-20%) of total organic matter in phytoplankton biomass (Parsons, 1961), they have been widely applied as biomarkers to study organic carbon cycling and paleoceanographic records because their structures are more source-specific and less reactive than proteins and carbohydrates (Harvey et al., 1995).

The relative abundances of specific lipid biomarkers (e.g., sterols, alkenones, and fatty acids) in natural environments have been used to detect the sources of organic matter (Harvey et al., 1988; Harvey, 1994; Volkman et al., 1998; Shin et al., 2008), characterize microbial community (Wakeham et al., 2003), measure organic carbon fluxes from surface seawater to underlying sediments (Wakeham et al., 1997; Bac et al., 2003), and reconstruct past sea surface temperature (Prahl and Wakeham, 1987; Müller et al., 1998). Furthermore, the compound-specific stable isotopic compositions of lipid biomarkers have been used as powerful tools to

characterize the microbial community (Boschker and Middelburg, 2002) and reconstruct ancient atmospheric  $pCO_2$  records (Jasper and Hayes, 1994; Pagani et al., 1999; Pagani et al., 2002).

Many studies have suggested that the chemical and isotopic compositions of algal lipid biomarkers are largely impacted by environmental conditions during photosynthesis. For example, light and UV-B irradiation (Brown et al., 1996; Skerratt et al., 1998), temperature and salinity (Xu and Beardall, 1997; Zhu et al., 1997), and concentrations of nutrient and CO<sub>2</sub> (Dempster and Sommerfield, 1998; Riebesell et al., 2000b) can affect lipid compositions of growing cells. Cellular lipids are also dependent on cell physiological states and cell growth phase (Dunstan et al., 1993; Brown et al., 1996; Zhu et al., 1997; Mansour et al., 2003; Lv et al., 2010). Moreover, the isotopic compositions of algal lipids are controlled by carbon acquisition mechanisms (Laws et al., 1998; Rost et al., 2002), compound synthesis pathways (Laws et al., 2001), algal growth rate and growth phases (Laws et al., 1995; Bidigare et al., 1997; Popp et al., 1998a; Riebesell et al., 2000a; Benthien et al., 2007), and cell size and geometry (Popp et al., 1998b; Burkhardt et al., 1999).

Marine microalgae biosynthesize various lipids for different functions in different intracellular structures over different growth stages (Sukenik and Carmeli, 1990; Bell and Pond, 1996; Brown et al., 1996; Mansour et al., 2003). Most species produce polar phospholipids in their cell membrane as structural components. However, different algal species make different lipid compounds as energy storage compounds in their cytoplasm (Bell and Pond, 1996; Brown et al., 1996). It has been observed that during exponential growth phase, more structural lipids are produced (Volkman et al., 1989; Sukenik and Carmeli, 1990; Brown et al., 1996; Mansour et al., 2003) while during stationary phase, more storage lipids are made by cells (Dunstan et al., 1993; Bell and Pond, 1996; Brown et al., 2003; Lv et

al., 2010). Among lipid classes, phytol, as a constituent of chlorophyll, and sterols are generally associated with algal membranes (Cohen et al., 1995; Hartmann, 1998). Fatty acids in various ester complexes (Suen et al., 1987; Sukenik and Wahnon, 1991; Zhu et al., 1997; Lv et al., 2010) and long chain alkenones (Prahl and Wakeham, 1987; Brassell, 1993; Bell and Pond, 1996; Epstein et al., 2001; Sawada and Shiraiwa, 2004; Eltgroth et al., 2005a; Pan and Sun, 2011) are bound either as cell membrane components or as energy storage compounds.

It was previously thought that the carbon isotopic compositions of phytoplankton and individual compounds are mainly controlled by the availability of aqueous CO<sub>2</sub> (Arthur et al., 1985; Hayes et al., 1989; Rau et al., 1989). Laws et al. (1995) pointed out that both CO<sub>2</sub> concentration and cell growth rate are major factors controlling the isotopic fractionation of carbon by phytoplankton. More evidence (e.g., Bidigare et al., 1997) suggested that variations in cell growth rate had a critical impact on the isotopic compositions of alkenones. By conducting a mesocosm bloom experiment dominated by *Emiliania huxleyi* under three distinct CO<sub>2</sub> partial pressures, Benthien et al. (2007) documented that there were large positive shifts in alkenone isotopic composition (4-5‰) during cell exponential growth phase in all treatments while there was little change during stationary phase. However, the biosynthetic pathways and intracellular distributions of alkenones during cell growth under variable conditions are largely unknown (Laws et al., 2001). It is also unclear whether isotopic compositions of lipid compounds are homogeneous in different cellular structures.

Cell respiration and microbial degradation are two important processes recycling algal lipids in natural environments (Voet and Voet, 1995; Brown et al., 1996; Manoharan et al., 1999; Eltgroth et al., 2005a; Ding and Sun, 2005a). While cell respiration mainly consumes lipids in intracellular storage compartments (Voet and Voet, 1995; Brown et al., 1996; Manoharan et al., 1999; Eltgroth et al., 2005a), microbial degradation may decompose lipids in both membrane and intracellular storage compartments (Ding and Sun, 2005a). It was observed that under variable environmental conditions (e.g., oxic and anoxic), lipids bound in different intracellular compartments may be selectively degraded (Ding and Sun, 2005a). So far, there have been few studies to directly distinguish the relative roles of respiration and microbial process in degrading algal lipids. In addition, little is known about the effects of respiration and microbial degradation on the stability of isotopic composition of phytoplankton-produced lipid compounds.

Applications of biomarkers and their molecular isotopic compositions are based on a controversial assumption that biogeochemical cycling processes such as the degradation of organic matter have an insignificant influence on the stability of these signals after they are generated in phytoplankton cells (Hayes et al., 1990; Pagani et al., 1999). However, accumulated evidence has showed that different organic compounds from various sources experience diversified changes in their isotopic composition during degradation. For example, degradation of *n*-alkanes and polycyclic aromatic hydrocarbons did not result in significant changes in their isotopic compositions (Hayes et al., 1990; Freeman et al., 1994; Huang et al., 1997; Mazeas et al., 2002). By contrast, Macko et al. (1994) observed that when ~50% of the amino acids in the seagrass Halodule wrightii were degraded after 4 weeks of incubation, some amino acids were depleted in <sup>13</sup>C (~5‰ decrease) while others either became enriched (~2‰ increase) or remained constant. Teece and Fogel (2007) also observed different  $\delta^{13}$ C alterations of sugar molecules during degradation of the salt marsh plant (Spartina alterniflora): 85% decrease in glucose concentration resulted in an isotopic enrichment (+5‰) for remaining compounds; after 79% of xylose was removed, the  $\delta^{13}$ C value remained constant; and the remaining (~25%) arabinose was depleted in <sup>13</sup>C by -4‰ compared to the original signal. Sun et al. (2004) demonstrated the

diversified isotopic alterations of various lipids from *Emiliania huxleyi* cells during degradation in natural oxic and anoxic seawaters: negative shift in <sup>13</sup>C for alkenones, positive shift for fatty acids, and no change for sterols. Nevertheless, it has been unclear what mechanisms cause these diversified isotopic alterations for different biomarkers during biogeochemical cycling processes.

This experimental study was designed to examine the effects of biological and biochemical processes on algal lipids and associated molecular isotopic signals. The main focuses were on: (1) generation of lipids and their stable carbon isotopic compositions during different growth phases; and (2) relative roles of cell respiration (auto-metabolism) and microbial degradation in altering chemical and isotopic signals. *Thalassiosira weissflogii* (diatom) and *Emilliania huxleyi* (haptophyte) were batch cultured in a light:dark regime through cell exponential growth and stationary phase, followed by cell respiration under dark, and then microbial degradation with the introduction of natural seawater. Bulk parameters (POC, TN, C/N,  $\delta^{13}$ C-POC, and  $\delta^{15}$ N-TN) and chemical and isotopic compositions of algal lipids (phytol, sterols, alkenones, and fatty acids) were analyzed to explore the relationships between the variations of chemical and isotopic compositions of algal lipids and cellular physiological state and environmental conditions. Implications of the results from this study were also discussed.

#### 2. Experimental

#### 2.1. Materials

Marine diatom *T. weissflogii* (CCMP1010) and marine haptophyte *E. huxleyi* (CCMP371) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The strain of *T. weissflogii* was originally isolated from the Gulf Stream (37°N, 65°W) in December 1969 and the strain of *E. huxleyi* from the Sargasso Sea (32°N, 62°W; 50 m water depth) in June 1987. Two strains were made and remained to be axenic

(confirmed by routine tests) in the CCMP. Seawater was collected from the Georgia coast (31°05'N, 81°16'W; in September 2005 and 31°25'N, 77°21'W; in May 2006) and from the Gulf of Mexico (28°16'N, 91°59'W; in September 2006 and 28°10'N, 91°36'W; in May 2008) by directly pumping surface (1 m) water into large carboys (50 liters). The seawater used for medium preparation was filtered through the Whatman 934-AH glass microfiber filters (precombusted at 450 °C for 4 h) and then sterilized by autoclaving. The f/2 (for *T. weissflogii*) and f/50 (*E. huxleyi*) media were prepared according to the protocols recommended by the CCMP (Guillard, 1962; Guillard, 1975). The seawater used for inoculation was stored in carboys until incubation experiments.

#### 2.2. Experimental set up

First, *T. weissflogii* and *E. huxleyi* cells were batch cultured (duplicate for each species) in f/2 and f/50 media (~ 2.3 L) respectively under a light:dark (12 h:12 h) regime with a constant light intensity (50 µmol photons  $m^{-2} s^{-1}$ ). The cultures were hand shaken twice and cell densities in each culture were counted every day. Algal cells were cultured through exponential growth phase (phase I: 12 d for *T. weissflogii* and 10 d for *E. huxleyi*) and stationary phase (phase II: 14 d for *T. weissflogii* and 32 d for *E. huxleyi*). Then, the grown cells were left in the same media but the light was turned off (phase III, dark respiration). The *T. weissflogii* cells were kept under continuous dark conditions over 14 d and *E. huxleyi* over 21 d. After phase III, the cultures were equally divided into a set of small flasks (100 mL each) and frozen below -30 °C overnight to kill the cells. After thawing, 50 mL of natural seawater was added into each flask to introduce natural bacterial assembly. They were then incubated under dark and aerobic conditions for 40 days by purging the solution with air (phase IV, microbial degradation).

#### 2.3. Sampling and cell counting

Transfer and sampling of cultures were carried out in a clean hood, which was preirradiated with a UV lamp for 30 min. Before sampling, the flasks were gently shaken by hand for a while to make uniform cell cultures. One milliliter of culture was taken every 1–2 days during phases I, II and III and the cells were counted using an Olympus BH-2 microscope with a hemacytometer (Hausser Scientific). Subsamples (25–300 mL, depending on cell density) in all phases were collected at certain time points by filtering the cultures through glass microfiber filters (Whatman 934-AH) and then stored at –40 °C for future lipid extraction and analysis.

#### 2.4. Analysis of bulk parameters

To monitor variations of bulk parameters (POC, TN, C/N,  $\delta^{13}$ C-POC, and  $\delta^{15}$ N-TN) of each algal species, parallel culture experiments (phases I, II, and III) were conducted. Subsamples (15-200 mL, based on cell density) at certain time points were collected by filtering the cultures through glass microfiber filters (Whatman 934-AH, precombusted at 450 °C for 4 hours) and kept frozen below -40°C until analysis. Thawed samples were treated with 10% HCl to remove inorganic carbon, air-dried in a fume hood and then analyzed for the bulk parameters using a Carlo Erba CHN element analyzer (EA) coupled with a Finnigan Delta C Stable Isotope Mass Spectrometer.

#### 2.5. Extraction and analysis of lipids

Lipid extraction, separation, and derivatization followed the procedure described in previous studies (Sun et al., 1998; Sun and Wakeham, 1999). Lipids in experimental samples were extracted with  $3 \times 25$  mL methylene chloride:methanol (2:1, v:v) with 6 min sonication each time. Combined extracts were partitioned into a methylene chloride phase with the addition of 30 mL 5% NaCl solution. The volume of total lipid extract (TLE) was reduced to nearly dry

by rotary evaporation. The TLE was then saponified at 92 °C with 6 mL 0.5 M KOH in MeOH:H<sub>2</sub>O (95:5) for 2 h. After cooling and the addition of 1 mL 5% NaCl solution, the neutral lipids, including alkenones, sterols, phytol and fatty alcohols, were first extracted from the basic solution (pH > 13) with 3 × 6 mL hexane. Subsequently, the solution was acidified (pH < 2) by adding HCl and the fatty acids were then extracted with  $3 \times 6$  mL hexane. The neutral lipids were reacted with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in acetonitrile at 92 °C for 2 h to form TMS ethers of sterols and alcohols. The fatty acids were methylated with 5% BF<sub>3</sub>-MeOH at 92 °C for 2 h to form FAMEs (fatty acid methyl esters).

Concentrations of neutral lipids and fatty acids were quantified by capillary gas chromatography using a Hewlett–Packard 6890 GC with an on-column injector and a flame ionization detector (Sun et al., 1998; Sun and Wakeham, 1999). The lipid compounds were separated with a 30 m × 0.25 mm i.d. HP-5 column coated with 5% diphenyl:95% dimethylsiloxane copolymer operated with the following temperature programs: for neutral lipids, 50-170 °C at 10 °C min<sup>-1</sup>, then 170-310 °C at 3 °C min<sup>-1</sup> and finally held for 30 min at 310 °C; for fatty acids, 50-170 °C at 20 °C min<sup>-1</sup>, then 170-310 °C at 4 °C min<sup>-1</sup> and finally held for 10 min at 310 °C; for fatty acids, 50-170 °C at 20 °C min<sup>-1</sup>, then 170-310 °C at 4 °C min<sup>-1</sup> and finally held for 10 min at 310 °C. Internal standards  $5\alpha$ (H)-cholestane for neutral lipids and nonadecanoic acid methyl ester for fatty acids were added to each corresponding sample before the GC analysis to aid in quantification. The relative standard deviation of lipid analysis was within  $\pm 5\%$  based on duplicate measurements. Identification of lipid compounds was achieved with a Shimadzu QP-5000 GC–MS system using a split injector and a  $30 \text{ m} \times 0.25 \text{ mm}$  i.d. XTI-5 column coated with 5% phenyl methyl silicone and helium as carrier gas. Operating conditions were: mass range 50-610 Da with a 0.4 s scan interval; 70 eV ionizing energy; GC temperature program 50-150 °C at

20 °C min<sup>-1</sup> followed by 150–310 °C at 4 °C min<sup>-1</sup> and a 30 min hold for neutral lipids and a 10 min hold for fatty acids at 310 °C.

#### 2.6. Isotopic analysis of lipid compounds

Stable carbon isotopic compositions of individual lipids were determined by using a Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometer system (GC-C-IRMS) (Hayes et al., 1990). Lipid compounds were separated through an Agilent (HP) gas chromatograph with a  $30 \text{ m} \times 0.25 \text{ mm}$  i.d. capillary column (DB-1, J and W Scientific) and a split injector installed. The temperature program for the GC operation was 50-180 °C at 20 °C min<sup>-1</sup> followed by 180-310 °C at 3 °C min<sup>-1</sup> and a 90 min hold at 310 °C for neutral lipids, and 50-170 °C at 20 °C min<sup>-1</sup> followed by 170-300 °C at 4 °C min<sup>-1</sup> and a 15 min hold at 300 °C for FAMEs. Helium with a flow rate of 1.5 mL min<sup>-1</sup> was used as carrier gas. Eluted peaks from the GC were combusted (via a Thermoquest Finnigan GC Combustion III unit) to CO2 over CuO/Ni/Pt wires at 850 °C and water was removed by diffusion through a Nafion selectively permeable membrane (Perma Pure LLC.) flushed with helium. The stable isotopic compositions of CO<sub>2</sub> peaks were measured with a Finnigan Delta XL plus IRMS operated at 3 kV acceleration potential and by magnetic sector mass separation. Stable carbon isotope compositions were expressed relative to a standard reference CO<sub>2</sub> and reported in per mil (‰) against VPDB (Vienna Pee Dee Belemnite). Based on internal standard measurements, the standard deviations of IRMS analyses were  $\pm 0.40\%$  (n=21) for neutral lipids and  $\pm 0.35\%$  (n=23) for FAMEs. The  $\delta^{13}$ C values of BSTFA (38.23  $\pm 0.04\%$ ) and methanol ( $43.72 \pm 0.15\%$ ), which were pre-determined by 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. Bulk organic parameters through cell growth and dark respiration phases

During culture experiments for bulk parameter analyses, cell densities of *T. weissflogii* and *E. huxleyi* varied in a similar way (Fig. 3.1). They increased rapidly from  $\sim 1 \times 10^3$  mL<sup>-1</sup> to  $2.2 \times 10^5$  mL<sup>-1</sup> in 13 days (*T. weissflogii*) and to  $5.3 \times 10^5$  mL<sup>-1</sup> in 11 days (*E. huxleyi*) in phase I (exponential growth phase) respectively. The densities remained at relatively constant levels ( $\sim 4.2 \pm 1.4 \times 10^5$  mL<sup>-1</sup> for *T. weissflogii* and  $\sim 5.3 \pm 1.1 \times 10^5$  mL<sup>-1</sup> for *E. huxleyi*) within phase II (stationary) and phase III (dark respiration).

Algal POC contents in two cultures continuously increased through cell growth phases I and II (Fig. 3.1): from 3.5 mg C L<sup>-1</sup> to 82.8 mg C L<sup>-1</sup> (*T. weissflogii*) and from 0.6 mg C L<sup>-1</sup> to 22.9 mg C L<sup>-1</sup> (*E. huxleyi*). During dark respiration phase III, the POC contents gradually decreased to 56.9 mg C L<sup>-1</sup> (*T. weissflogii*) and to 13.1 mg C L<sup>-1</sup> (*E. huxleyi*) (Fig. 3.1). By contrast, TN contents in two cultures varied differently (Fig. 3.1). In *T. weissflogii* culture, the TN continuously increased from 0.5 mg N L<sup>-1</sup> to 8.2 mg N L<sup>-1</sup> in phases I and II and then remained constant within phase III. In *E. huxleyi* culture, the TN increased only in phase I (0.1 mg N L<sup>-1</sup> to 0.5 mg N L<sup>-1</sup>) and then remained relatively constant within phases II and III. As a result, the C/N ratios in two cultures varied differently: fluctuated in a small range of 8-12 within three phases in *T. weissflogii* culture while in *E. huxleyi* culture, dramatically increased from ~10 (phase I) to ~60 (end of phase II) and dropped to 34 in phase III (Fig. 3.1).

The stable carbon isotopic composition of POC ( $\delta^{13}$ C-POC) in two cultures varied in a similar trend but at different levels (Fig. 3.1). The  $\delta^{13}$ C values of *T. weissflogii* POC increased from -21.4‰ to -15.9‰ in phase I and then remained relatively constant throughout phases II and III. The  $\delta^{13}$ C values of *E. huxleyi* POC increased from -24.1‰ to -20.6‰ in phase I and

early stationary phase II and then remained relatively constant throughout late stationary phase II and phase III. There were some differences in the stable nitrogen isotopic composition of TN between two cultures (Fig. 3.1). In *T. weissflogii* culture, the  $\delta^{15}$ N values of TN became enriched from -3‰ to +5.5‰ through phases I and II and then remained unchanged in phase III. In *E. huxleyi* culture, enrichment in nitrogen isotope occurred only in phase I ( $\delta^{15}$ N values from -5.3‰ to 6.2‰) and then slightly fluctuated in phases II and III.

#### 3.2. Algal lipid compositions in all phases

In the experiment of *T. weissflogii*, 14 fatty acids and 5 neutral lipids were identified (Fig. 3.2). For fatty acids, saturated fatty acids 14:0 and 16:0, monounsaturated fatty acid 16:1( $\omega$ 7), and polyunsaturated fatty acids 16:3 and 20:5 were major components (>10% for each). There were different variation patterns for different fatty acids throughout four phases (Fig. 3.2). For example, the relative compositions of polyunsaturated fatty acids (16:3, 20:5 and 22:6) continuously increased (15% to 28%, 11% to 21% and 2% to 7%, respectively) from phase I to phases II and III but dropped dramatically (to 6%, 6% and 2%, respectively) in phase IV. The relative compositions of 16:1 and 16:0 fatty acids decreased continuously from 28% and 24% to 17% and 11% through phases I, II and III), but increased to 22% and 19% in phase IV. The relative composition of 14:0 fatty acid remained relatively constant at about 10% throughout all phases. In phase IV, bacteria-specific fatty acids such as iso-15:0, anteiso-15:0 and 18:1( $\omega$ 7) appeared and even became a major component (>15%). For neutral lipids, phytol and 28 $\Delta$ <sup>5, 24</sup> were dominant compounds and their relative compositions remained almost constant (~ 62% and ~ 25%) throughout the experiment (phases I to IV).

In the experiment of *E. huxleyi*, 8 fatty acids and 9 neutral lipids were identified (Fig. 3.2). For fatty acids, saturated fatty acids 14:0 and 16:0, monounsaturated fatty acid 18:1(ω9)

and polyunsaturated fatty acid 22:6 are major components. Unlike the patterns of polyunsaturated fatty acids in T. weissflogii culture, the relative composition (18~19%) of 22:6 fatty acid did not change through phases I and II but decreased to 11% in phase III and further to 3% in phase IV. The relative composition of  $18:1(\omega 9)$  in E. huxleyi culture had a similar variation pattern as that of polyunsaturated fatty acids in T. weissflogii culture: increasing continuously from 29% to 47% through phases I. II and III but decreased to 15% in phase IV. The relative compositions of two major saturated fatty acids showed contrasting variations: continuous decrease for 14:0 while continuous increase for 16:0 from phase I to phase IV. In addition, bacteria-specific fatty acid  $18:1(\omega 7)$  appeared and became a dominant component (>30%) in phase IV of E. huxleyi experiment. For the neutral lipids, long chain alkenones dominated the pool while phytol and sterols only contributed less than 10%. In spite of the low relative compositions, phytol and sterols had larger variations compared to those of alkenones in the experiment (all phases). For example, the relative compositions of phytol decreased from 6% to less than 1% after exponential growth (phase I) and the relative composition of  $28\Delta^{5, 22}$ decreased from 8% in phase I to 3% through phases II to IV. By contrast, the relative compositions of 37:3, 37:2, Me38:3 and Me38:2 were relatively constant (~30%, 12%, 10% and 4%, respectively) throughout the experiment.

# 3.3. Variations of lipid compounds and their $\delta^{13}$ C in phases I and II

Two cultures (*T. weissflogii* and *E. huxleyi*) for lipid/isotope analyses started at a similar density ( $\sim 3 \times 10^3 \text{ mL}^{-1}$ ) but reached different maximum levels in different times (Fig. 3.3). The density of *T. weissflogii* reached the maximum level ( $\sim 2 \times 10^5 \text{ mL}^{-1}$ ) in 11 d and slightly increased afterwards. *E. huxleyi* reached the maximum density ( $\sim 7 \times 10^5 \text{ mL}^{-1}$ ) in 10 d and remained relatively constant in following 30 d.

Concentrations of neutral lipids and fatty acids varied differently among compounds, between growth phases and between cultures (Figs. 3.3 and 3.4). For *T. weissflogii*, the concentrations of two major neutral lipids (phytol and  $28\Delta^{5, 24(28)}$  sterol) continuously increased from phase I to phase II. By contrast, concentrations of *E. huxleyi* neutral lipids ( $27\Delta^5$  and  $28\Delta^{5,22}$  sterols, 37:3 and 37:2 alkenones) increased much less in phase I than in phase II. Calculations of production rates in two cultures (Table 3.1) indicated that *T. weissflogii* cells biosynthesized neutral lipids at similar rates between phase I and phase II while *E. huxleyi* cells produced neutral lipids at much faster rates (2-10×) in phase II than in phase I. Variations in concentrations of fatty acids in two cultures were more diversified. For example, concentrations of saturated (14:0 and 16:0) and monounsaturated (16:1) fatty acids in *T. weissflogii* culture increased at similar rates in phase I and phase I but polyunsaturated (16:3 and 20:5) fatty acids were produced at faster rates in phase II than in phase I (Table 3.1). For *E. huxleyi* culture, all fatty acids (14:0, 16:0, 16:1, and 22:6) varied similarly: they were produced at faster rates in phase I than in phase II than in phase II than in phase II (Table 3.1).

Compound-specific stable carbon isotopic compositions ( $\delta^{13}$ C) of lipids varied similarly between two cultures but differently between phases and among compounds (Figs. 3.3 and 3.4). In phase I, most compounds in the two cultures (except 18:1 and 22:6 fatty acid of *E. huxleyi*) showed the same enrichment trend with a large range of +5‰ to +10‰. However, in phase II, their isotopic compositions in different cultures varied in different ways. For example, the isotopic compositions of most neutral lipids (except  $28\Delta^{5, 24(28)}$  sterol of *T. weissflogii*) and all polyunsaturated fatty acids (16:3, 20:5 and 22:6) in two cultures remained relatively constant while those of saturated fatty acids (14:0 and 16:0) became depletion with a range of -2‰ to -3‰. For monounsaturated fatty acids, 16:1 in *T. weissflogii* culture had a depletion while 18:1 in *E. huxleyi* culture remained a relatively constant isotopic composition in phase II.

# 3.4. Variations of lipid compounds and their $\delta^{13}$ C in phase III

During dark respiration (phase III), cell densities in two cultures remained relatively constant within two weeks (for *T. weissflogii*) and three weeks (for *E. huxleyi*) (Fig. 3.5). Concentrations of different neutral lipids and fatty acids in two cultures decreased with time but at varying rates (Figs. 3.5 and 3.6). In general, less than 21% of neutral sterols and phytol were lost in two cultures but much greater percentages (51-61%) of alkenones were lost in *E. huxleyi* culture during phase III (Table 3.2). Fatty acids in two cultures varied differently: all fatty acids in *T. weissflogii* culture were lost remarkably (43-69%) while in *E. huxleyi* culture, much greater percentage (63%) of 22:6 was lost compared to 18:1 (17% loss) (Table 3.2). No matter how much lipids were lost during phase III, the isotopic compositions of most compounds (except phytol in *T. weissflogii* culture) fluctuated in a small (< $\pm$ 1‰) range in phase III. Isotopic composition of phytol became depletion by ~-3‰ during phase III.

# 3.5. Variations of lipid compounds and their $\delta^{13}$ C in phase IV

When cells in cultures (after phase III) were physically killed and natural seawater was introduced, concentrations of neutral lipids and fatty acids decreased further (Figs. 3.7 and 3.8). In phase IV, approximately 38-46% of phytol and sterols, 22-29% of alkenones, and 27-71% of fatty acids were lost (Table 3.2). After a 40-day incubation, 33-53% of phytol and sterols as well as 18-20% of alkenones remained in the samples (Table 3.2). However, the remaining percentages of fatty acids varied over a much larger range (1-33%), dependent on culture and compound structures (Table 3.2). In *T. weissflogii* culture, concentrations of all fatty acids dropped below 6.5% (compared to initial concentration in growing culture) but in *E. huxleyi* 

culture, only 1.5% of polyunsaturated 22:6 concentration remained while 33% of saturated 16:0 remained (Table 3.2). The isotopic compositions of these lipid compounds in phase IV varied in three different ways: (1) positive shift (over +2‰); (2) little change or small fluctuation (within  $\pm$ 1‰); and (3) negative shift (over -2‰). Phytol and most fatty acids followed the first way and sterols/alkenones followed the second way (Figs. 3.7 and 3.8). Only one fatty acid (20:5 in *T. weissflogii*) followed the third way (Fig. 3.8).

#### 4. Discussion

# 4.1. Generation of chemical and isotopic signals through different growth phases of algal cells

Our results showed that the bulk parameters (POC, TN, C/N ratio,  $\delta^{13}$ C, and  $\delta^{15}$ N) in two cultures varied in different ways through exponential growth and stationary phases (Fig. 3.1). Both *T. weissflogii* and *E. huxleyi* continuously produced organic carbon in their cells from the beginning of culturing to the end of stationary phase even when cell densities reached constant maximum levels. However, TN production in the two cultures displayed different patterns. The TN in *T. weissflogii* culture continuously increased through phases I and II while the TN in *E. huxleyi* culture increased only in phase I and remained to be constant C/N ratios were observed in *T. weissflogii* culture and remarkably variable C/N ratios occurred in *E. huxleyi* culture, which indicated that two algal species biosynthesized organic matter with different compositions and their productions varied with cell growth phases. It has been well known that cell growth phase is a critical factor affecting intracellular fractions of proteins, carbohydrates, and lipids of phytoplankton (Dunstan et al., 1993; Brown et al., 1996; Zhu et al., 1997; Hatate et al., 1998; Mansour et al., 2003). The extremely high C/N ratio (>50) in phase II of *E. huxleyi* culture is

likely caused by a larger proportion (40-60%) of carbon incorporation into lipids than that ( $\sim$ 20%) of carbon incorporation into proteins (Fernández et al., 1994).

Similar to bulk parameters, intracellular lipids in algal cells were produced differently between two cultures through different phases (Figs. 3.3 and 3.4). For example, concentrations of neutral lipids and fatty acids in *T. weissflogii* culture increased at similar rate through phases I and II (Table 3.1). By contrast, concentrations of neutral lipids in the *E. huxleyi* culture increased at 2-10× faster rates in phase II than in phase I while concentrations of fatty acids in the culture varied oppositely: faster rates in phase I than in phase II (Table 3.1). Moreover, relative compositions of lipids in two cultures also varied between phases I and II (Fig. 3.2). For example, percentages of two major polyunsaturated fatty acids (16:3 and 20:5) in *T. weissflogii* were higher in phase II than in phase I while those of saturated fatty acids (14:0 and 16:0) varied oppositely between two phases. In *E. huxleyi* culture, variations of monounsaturated 18:1( $\omega$ 9) fatty acid percentage between phase I and phase II seemed to be more significant than polyunsaturated 22:6 and saturated 16:0 fatty acids. All these variations in lipid abundance and relative compositions through different cell growth phases indicate that algal cells synthesize different lipid compounds in different intracellular structures for different functions.

It is known that marine microalgae produce polar lipids and nonpolar lipids within their cells. Polar lipids consist of mostly phospholipids and glycolipids, which are main components of cell membrane and chloroplast membrane, respectively (Sukenik and Carmeli, 1990; Brown et al., 1996; Mansour et al., 2003). Non-polar lipids are primarily used as metabolic storage components (Sukenik and Carmeli, 1990; Mansour et al., 2003). Many marine algae, including most diatom species, generally produce triacylglycerols as energy storage compounds within their cell cytoplasm (Brown et al., 1996). However, some species such as *E. huxleyi* have little

triacylglycerols in their cells (Volkman et al., 1986). Instead, these species produce a greater amount of alkenones as substitute compounds for energy storage (Bell and Pond, 1996) although alkenones also serve as membrane lipids to regulate membrane fluidity (Prahl and Wakeham, 1987; Brassell, 1993). Thus, in this study, fatty acids and alkenones extracted from the culture samples are considered to be associated either with cell membrane or with intracellular storage components. Other lipids such as phytol and sterols are known to be associated mostly with chloroplast thylakoid membrane (Cohen et al., 1995) and algal cell membrane (Hartmann, 1998), respectively.

It has been documented that proportions of lipids bound in different intracellular structures are dependent on cell physiological states or growth phases (Sukenik and Carmeli, 1990; Brown et al., 1996; Mansour et al., 2003). For example, phospholipids are a principal lipid component in algal cells during cell exponential growth (Volkman et al., 1989; Brown et al., 1996; Mansour et al., 2003) while the proportion of storage lipids such as triacylglycerols (Dunstan et al., 1993; Brown et al., 1996; Mansour et al., 2003) or alkenones (methyl and ethyl ketones) (Bell and Pond, 1996; Epstein et al., 2001) increased significantly during stationary phase when nutrients (nitrogen or phosphorus) in the cultures become depleted. Recently, Lv et al. (2010) separated membrane and intracellular storage fatty acids from two algal species (*Isochrysis* sp. and *Gymnodinium* sp.) and their results showed that most (84-100%) fatty acids were membrane-associated compounds in exponential growth phase. Although our study did not directly separate membrane vs. storage lipids (fatty acids and alkenones), it is expected that the relative proportions of these compounds would vary between phases I and II, according to a similar pattern observed by other previous studies.

From our results, it is obvious that the isotopic compositions of phytoplankton POC and lipids also varied differently between different growth phases (Figs. 3.1, 3.3 and 3.4). During cell exponential growth phase I, the  $\delta^{13}$ C values of POC in both *T. weissflogii* and *E. huxleyi* cultures shifted positively (+5.5‰ and +3.5‰, respectively), whereas during stationary phase II, these values remained relatively constant. The compound-specific isotopic compositions of lipids in both *T. weissflogii* and *E. huxleyi* cultures followed the similar pattern: significant positive shift (+5‰ to +10‰) of  $\delta^{13}$ C values in phase I, and relatively constant or small negative alteration (-2‰ to -3‰) of  $\delta^{13}$ C values in phase II. These variations in stable carbon isotopic compositions through different growth phases are likely related to cell physiological states and culture conditions.

Many experimental and field observations (Popp et al., 1989; Rau et al., 1989; Freeman and Hayes, 1992) have suggested that the availability of aqueous CO<sub>2</sub> is a major factor controlling isotopic composition of organic compounds made by phytoplankton. However, other factors such as nutrient availability, cell growth rate, carbon acquisition mechanisms, irradiance, and cell size and geometry have been recognized to affect the isotopic compositions of organic compounds (Laws et al., 1995; Rau et al., 1996; Bidigare et al., 1997; Popp et al., 1998a; Popp et al., 1998b; Burkhardt et al., 1999; Keller and Morel, 1999; Riebesell et al., 2000a; Gervais and Riebesell, 2001; Rost et al., 2002). Based on a continuous culture of diatom *Phaeodactylum tricornutum*, Laws et al. (1995) found that the biological isotopic fractionation ( $\epsilon_p$ ) was negatively related to a combined factor of cell growth rate to aqueous CO<sub>2</sub> concentration ( $\mu/[CO_2]_{aq}$ ) rather than [CO<sub>2</sub>]<sub>aq</sub> alone. In our batch cultures, the cells in phase I grew at a relatively constant rate (0.67±0.19 d<sup>-1</sup> for *E. huxleyi* and 0.64±0.32 d<sup>-1</sup> for *T. weissflogii*) but the dissolved CO<sub>2</sub> concentration ([CO<sub>2</sub>]<sub>aq</sub>) in the medium rapidly decreased due to uptake by phytoplankton photosynthesis. Thus, the  $\varepsilon_p$  (biological fractionation associated with carbon fixation) would decrease, resulting in positive shifts of  $\delta^{13}$ C for POC and lipid compounds in phase I. However, both *T. weissflogii* and *E. huxleyi* cells in two cultures stopped growing ( $\mu =$ 0) and [CO<sub>2</sub>]<sub>aq</sub> varied slowly during stationary phase II (Benthien et al., 2007). Therefore, the  $\delta^{13}$ C values of phytoplankton-POC and lipid compounds remained less variable or relatively constant in phase II.

By conducting a series of mesocosm experiments dominated by *E. huxleyi* at three distinct initial CO<sub>2</sub> levels, Benthien et al. (2007) found that isotopic compositions of alkenones varied differently during bloom and post-bloom periods regardless of the initial concentrations of CO<sub>2</sub>. Their results are very similar to those observed in our study: the large positive shift (4-5‰) in alkenone isotopic composition appeared during bloom (equivalent to the exponential growth phase) with presence of replete nutrients while the isotopic compositions of alkenones remained relatively unchanged during post-bloom (equivalent to stationary phase) when nutrients became depleted. According to Benthien et al.'s explanation, the positive shift of isotopic composition of alkenones during bloom was probably caused by either the reduction in CO<sub>2</sub> leakage out of cell or the increase in relative proportion of HCO<sub>3</sub><sup>-</sup> uptake. Benthien et al. (2007) further indicated that after nutrients were depleted during the post-bloom period, the interaction of carbon source and cellular uptake dynamics by *E. huxleyi* reached a steady state, leading to relatively unchanged isotopic compositions of alkenones although these compounds were still biosynthesized.

Based on above discussion, we can speculate: (1) during cell exponential growth phase, two algal species produce lipid compounds mainly as membrane components (e.g., phospholipids) but the stable carbon isotopic compositions of these compounds are variable with remarkable changes in the concentrations of dissolved  $CO_2$  and nutrients; and (2) during stationary phase, two species produce different lipid compounds (e.g., triacylglycerols for *T. weissflogii* and alkenonesas for *E. huxleyi*) as energy storage components but their isotopic compositions are much less variable or relatively constant due to stable dissolved  $CO_2$ concentration and depletion of nutrients. If these speculations are proven to be true, we can further expect that the different intracellular pools for lipid compounds within phytoplankton cells and heterogeneous distributions in their isotopic compositions may be potential factors causing variations in compound-specific isotopic compositions during biogeochemical cycling of phytoplankton-derived organic matter.

#### 4.2. Effect of cell respiration on chemical and stable isotopic signals

Our results showed that during dark respiration phase III, ~30-40% of POC was lost while TN remained to be constant in two cultures, resulting in significant decreases in C/N ratios (Fig. 3.1). These changes of the bulk parameters suggested that living algal cells preferentially used lipids (non-nitrogen-containing compounds) than proteins (nitrogen-containing compounds) for their auto-metabolisms (Voet and Voet, 1995; Manoharan et al., 1999; Eltgroth et al., 2005a). Indeed, we observed that 51-61% of alkenones in *E. huxleyi* culture and 43-69% of fatty acids in *T. weissflogii* culture were consumed while the cell densities in two cultures remained to be constant during phase III. These results indicated that *E. huxleyi*. used alkenones and *T. weissflogii* used fatty acids-associated compounds (e.g., triacylglycerols) as main energy sources, respectively. Concentrations of phytol and sterols in two cultures decreased much less (<21%) than fatty acids or alkenones, since they were largely associated with membrane components and did not function as energy storage compounds.

The role of intracellular lipids for cell auto-metabolism during dark respiration has been widely recognized. For example, Manoharan et al. (1999) found that dinoflagellate *Prorocentrum minimum* survived after 10 days of dark incubation by using triacyglycerols and galactolipids as an energy source while its cellular phospholipid content showed little change. Many other studies (Epstein et al., 2001; Eltgroth et al., 2005a) observed that lipid components associated with PULCA (polyunsaturated long-chain alkenones, alkenoates, and alkenes) in the haptophyte algae *Emiliania huxleyi* and *Isochrysis galbana* increased in abundance under nutrient depletion (stationary phase) and disappeared under extended darkness, indicating that PULCA were produced as energy storage compounds and preferentially consumed during cell auto-metabolism.

Although POC and storage lipids were largely consumed during dark respiration of the two cultures, their stable carbon isotopic compositions (except phytol in *T. weissflogii*) fluctuated in a small range ( $\leq$ ±1‰) and no clear variation patterns were seen. A possible reason for unchanged isotopic signals of lipids during dark respiration is related to the original generation of cellular lipids and associated isotopic signals through different phases of cell growth. As discussed above (see 4.1.), storage lipid compounds were largely produced during stationary phase and the isotopic compositions of these compounds were more homogeneous compared to those produced during exponential growth phase as membrane components. When lipids in intracellular storage component were selectively utilized for auto-metabolism during dark respiration, no isotopic fractionation would occur for isotopically-uniform compounds in this pool.

There has been a debate on the effect of respiration on isotopic composition of organic matter (or compounds). Some evidence (e.g., Laws et al., 1995; Lin and Ehleringer, 1997)

indicated that the effects of both dark respiration and photo-respiration of plant cells were so small that they could be neglected in estimating isotopic fractionation during biological processes. For example, Laws et al. (1995) observed that the  $\delta^{13}$ C values of marine diatom *Phaeodactylum tricornutum* ( $\delta^{13}C_p$ ) did not show any temporal variation during the 12h dark period. On the contrary, Ghashghaie et al. (2003) pointed out that both dark respiration and photo-respiration of plants could result in significant isotopic fractionation in organic matter <sup>13</sup>C but the fractionation factors were variable among species and with environmental conditions. For instance, Degens et al. (1968) found that the  $\delta^{13}$ C values of green flagellate *Dunaliella tertiolecta* (clone 'Dun') decreased about -5‰ in the first 5 days of a prolonged (19 days) respiration in darkness.

#### 4.3. Effect of microbial degradation on chemical and stable isotopic signals

In phase IV of our experiments, senescent cells (survived from phase III) in two cultures were physically killed and natural seawater was introduced. Remarkable changes occurred immediately in the relative fatty acid compositions (Fig. 3.2): some bacteria-specific fatty acids, which were absent in phases I, II, and III, appeared in phase IV. For example, iso-15:0 and anteiso-15:0 occurred in *T. weissflogii* samples while  $18:1(\omega7)$  occurred in both *T. weissflogii* and *E. huxleyi* samples. Moreover,  $18:1(\omega7)$  became a dominant compound (20-30%) in the fatty acid pools of both samples. Concentrations of all algal lipid compounds decreased rapidly in the first 10 days of incubation and then remained at different levels (Figs. 3.7 and 3.8). All these variations indicated that bacteria entered the samples due to addition of natural seawater and played a primary role in degrading phytoplankton-produced lipid compounds. After 40 days of incubation, small percentages ( $\leq 7\%$ ) of various fatty acids (except  $18:1(\omega9)$  and 16:0 in *E. huxleyi* samples with 12% and 33% respectively) relative to the initial amounts in the culture (at

the end of phase II) remained while relatively larger percentages of neutral compounds (33-53% of phytol and sterols and 18-20% of alkenones) remained.

To examine the relative importance of cell respiration vs. microbial degradation as well as relative reactivities of various lipid compounds during both processes, we estimated the consumption and degradation rate constants (k<sub>c</sub> for consumption due to respiration and k<sub>d</sub> for loss due to microbial degradation) based on the first-order kinetic model (Westrich and Berner, 1984; Ding and Sun, 2005b). Comparisons between rate constants (Table 3.3) reveal several interesting implications: (1) It is obvious that microbial degradation rates of all lipid compounds were faster than consumption rates by cell respiration (2.3-8.5× for T. weissflogii case and 3.5-25.2× for E. huxleyi case). (2) During dark respiration, consumption rates of fatty acids/alkenones were faster than those of neutral phytol/sterols (2-6× for T. weissflogii case and 2-14× for E. huxleyiuxleyi case). (3) During microbial degradation, rates of fatty acids were much faster (5-22×) than those of neutral lipids in T. weissflogii case but in E. huxleyiuxleyi case, rates of neutral lipids were comparable ( $<2\times$ ) with those of saturated fatty acids although those of polyunsaturated fatty acids were still 2.6-6.3× faster. (4) Unsaturation (number of double bond) of fatty acids seemed to have no significant effect during cell respiration but it was important for fatty acid degradation (at least  $2 \times$  faster than those of saturated compounds) in both T. weissflogii and E. huxleyi cases. (5) The degradation rate constant values of various lipid compounds were comparable with those reported by other studies (Sun and Wakeham, 1994; Canuel and Martens, 1996; Harvey and Macko, 1997; Sun et al., 1997; Grossi et al., 2001; Sun et al., 2002; Grossi et al., 2003) although the experimental systems, substrate types, and environmental conditions were remarkably different from each other.

Unlike cell dark respiration (phase III), microbial degradation of lipid compounds in phase IV did cause isotopic alterations for remaining compounds. However, different compounds experienced different alterations (Figs. 3.7 and 3.8): most fatty acids (except 20:5 in *T. weissflogii* case) and phytol shifted positively; sterols and alkenones had little variations; 20:5 fatty acid of *T. weissflogii* shifted negatively. Diversified changes in isotopic compositions of lipids during degradation were observed previously in different systems. For example, Sun et al. (2004) found that in natural oxic and anoxic seawaters (taken from surface 30 m and deep 900 m layers in the Cariaco Basin), degradation of *E. huxleyi*-derived lipids resulted in positive (for fatty acids) and negative (for alkenones, especially in anoxic seawater) shifts in their isotopic compositions of plant lipids during transport/degradation processes were also observed in a plant-soil system (Chikaraishi and Naraoka, 2006). Their results showed that some compounds such as long-chain *n*-alkanes, *n*-alkanoic acids and alkanols enriched in <sup>13</sup>C and depleted in <sup>2</sup>H from raw plant leaves to soils but other compounds such as anteiso-alkanes, phytol, and sterols had little variations in their carbon and hydrogen isotopic compositions.

For a long time, the effect of degradation on isotopic composition of organic compounds has been assumed to be too small to be counted (Sackett, 1964; Myers, 1974; Sweeney et al., 1978; Arthur et al., 1985; Dean et al., 1986). Indeed, some studies have demonstrated clear evidence to support this assumption. Examples include porphyrins (Hayes et al., 1990), *n*-alkanes (Huang et al., 1997), and aromatic hydrocarbons (Freeman et al., 1994). However, contrasting evidence has emerged to question this assumption. Good examples covered wide range of natural organic compounds: amino acids (Macko et al., 1994), carbohydrates (Teece and Fogel, 2007), and lipids (Sun et al., 2004; Dai et al., 2005). However, it has been unclear what mechanisms are

responsible for isotopic alterations of different organic compounds during degradation. Actually, there are many uncertainties in generation of isotopic compositions of organic compounds by organisms and the impact of generation processes on the stability of isotopic signals during biogeochemical cycling in natural environments.

From the literature and this study, we now know that phytoplankton cells produce membrane and intracellular lipids respectively in exponential growth and stationary phases and isotopic compositions of lipids are more variable in growth phase than in stationary phase. Thus, we further infer that isotopic compositions of lipids associated with cell membrane may be more heterogeneous while those in intracellular storage components may be more homogeneous. So, if the lipid compounds bound in membrane and storage components have different reactivities during degradation, one consequence would be isotopic alteration for the remaining compounds. In fact, Ding and Sun (2005a) found that algal fatty acids bound in membrane and intracellular storage component were degraded differentially but the relative selectivity would be affected by environmental conditions. Therefore, both heterogeneity in isotopic compositions of cellular lipids and their differential reactivities during degradation are potential causes for isotopic alteration of organic compounds. Indeed, many studies have observed heterogeneous isotopic compositions between compound classes (e.g., Degens, 1969; Deines, 1980; Benner et al., 1987; Lehmann et al., 2002), between individual compounds within the same class (e.g., Schouten et al., 1998; Riebesell et al., 2000b), and even within the single molecule (Schouten et al., 2008). Different precursors and synthesis pathways are two major causes for heterogeneous isotopic compositions of organic compounds (Hayes, 1993; 2001). However, more studies are needed to clarify the impact of the heterogeneity on isotopic alteration of organic compounds during degradation.

The above speculation seems to be valid for interpreting the isotopic variations of fatty acids, especially for T. weissflogii case, in which fatty acids are produced either as phospholipids in membrane or as triacylglycerols in storage component and they carry different isotopic compositions. Meanwhile, production rates of fatty acids are similar in phases I and II of T. weissflogii culture, resulting in comparable pool sizes for membrane and storage component. Therefore, selective consumption of storage fatty acids (with more homogeneous isotopic composition) in phase III (dark respiration) did not cause isotopic alteration. But selective degradation in phase IV between two fatty acid pools (with heterogeneous isotopic composition) could result in isotopic alteration. However, the cases of alkenones (little isotopic alteration) could not be explained by the same speculation. One possible reason is too small pool size of alkenone in membrane although with heterogeneous isotopic composition. Most alkenones are produced in phase II and have relatively constant isotopic compositions. Degradation of alkenones was likely dominated by loss of compounds in the large storage pool. Sun et al. (2004) observed significant isotopic alteration of E. huxleyi-derived alkenones in Cariaco seawater but the cells were harvested from the culture in phase I. That means that alkenones in that study might be largely bound in a membrane pool with heterogeneous isotopic composition. For sterols in two algal samples, no isotopic alteration was observed during degradation, similar to observations by previous studies in different systems (Sun et al., 2004; Chikaraishi and Naraoka, 2006). One possible reason is that sterols are associated only with membranes (Hartmann, 1998) and another reason is that degradation of sterols is primarily through inter-conversion pathways (to stenones, stanones, stanols, steroiddiols and sterenes) in various environments, which do not involve carbon number changes (Gagosian et al., 1980). Although phytol is also a membrane compound, its isotopic composition was altered during respiration (phase III) and degradation

(phase IV) but in different directions. The mechanisms to cause positive and negative isotopic alteration of phytol in two processes are unclear. However, there is clear evidence showing that phytoplankton and other plants can biosynthesize phytol by using different pathways from different precursors, resulting in substantial intramolecular heterogeneity in its isotopic composition (Schouten et al., 2008).

#### 4.4. Implications

To apply chemical/isotopic signals buried in marine sediments to study paleoceanograpic records, it is important to understand how these signals are generated by phytoplankton in surface seawater and how they are modified during biogeochemical cycling processes in water and sediments. Interpretation of these signals buried in sediments needs to clarify the following questions: (1) what is the physiological state (or growth phase) of sinking cells that deliver chemical/isotopic signals to sediments? (2) whether or not the chemical/isotopic signals (associated with different cellular components or compartments) are homogeneous? and (3) which degradation process of organic compounds (e.g., by cell respiration, animal grazing, or microbial decomposition) has a significant impact on the isotopic signals?

From our results, it appears that different lipid compounds are generated at different rates during different growth stages (or at different physiological states), probably associated with different intracellular components and with varying proportions. It is also clear that the compound-specific stable carbon isotopic compositions of lipids are differently generated during different growth stages or in different cellular components. Moreover, it seems that microbial degradation (with selectivity between two intracellular pools) plays a more important role in altering isotopic signals of lipid compounds compared to cell respiration (or auto-metabolism, with only one storage pool involved) although both processes degrade algal lipids significantly. Therefore, the heterogeneity in isotopic signals and the relative reactivities of lipid compounds bound in different intracellular pools are two potential causes for alteration of chemical/isotopic signals in natural environments.

To extrapolate laboratory results to field study is always challenging because no laboratory simulation can exactly mimic the natural systems (Bidigare et al., 1997; 1999; Laws et al., 2001). Phytoplankton grow in oceans with varying nutrient and trace metal conditions (Martin et al., 1991; Hutchins and Bruland, 1998; Hutchins et al., 2002) but the effects of cell physiological state related to nutrient- or Fe-limitation cannot be distinguished from other factors by field measurements. Therefore, laboratory culturing has been used as an effective approach to study the relation between cell physiology and chemical/isotopic signals under well-controlled conditions. Both batch and continuous culturing methods have been widely used by many researchers. Advantages and disadvantages of these two culturing methods were thoroughly discussed by Laws et al. (2001). Each laboratory culturing system seems to be roughly analogous to distinct regimes in the real world. For example, the average physiological state of alkenoneproducing algae in the open ocean is more like cells in the late log or stationary phase of batch culturing (Conte et al., 1998). Prahl et al. (2003) pointed out that growth of alkenone producers at an open ocean site with a sufficient nutrient supply would follow a continuous culture-like pattern while at a continental margin site with a significant nutrient stress, cell growth seemed to be more like that in batch culture. By conducting a mesocosm bloom experiment of E. huxleyi, Benthien et al. (2007) demonstrated that exponential growth of cells in the experimental system was similar to bloom in natural environments while stationary phase is similar to post-bloom situation.

Some field studies have observed variations of isotopic compositions of lipids in the water column and sediments with depth but the impact of degradation has not been seriously considered. For example, Freeman and Wakeham (1992) found that when alkenone concentrations decreased rapidly with water depth in the Black Sea, which reflects a loss of alkenones due to degradation, the  $\delta^{13}$ C values of alkenones became depleted by ~-3‰. However, the variations were not attributed to degradation but rather than to (1) alkenone sources from several prymnesiophyte populations (with different isotope compositions); and (2) debris from a single population that grew under variable CO<sub>2</sub> concentrations. Recently, Coolen et al. (2006) analyzed fossil DNA in the Black Sea sediments and pointed out that *E. huxleyi* was the sole source of alkenones in that area, thus ruling out the possibility of multiple alkenone-producers. On the other hand, Riebesell et al. (2000b) confirmed that *E. huxleyi* could produce alkenones with  $\delta^{13}$ C values from -22‰ to -32‰ over a wide range of CO<sub>2</sub> concentrations (1.1–53.5 µmol/L). But there is no direct evidence showing whether the depth-dependent changes in  $\delta^{13}$ C of alkenones in the Black Sea water column are related to changes in CO<sub>2</sub> concentration with time.

In summary, our findings from this study reveal additional possibilities for isotopic alteration of lipid biomarkers during biogeochemical cycling of organic matter in natural environments. Indeed, the explanation on isotopic alteration of organic compounds in natural systems is very difficult because many internal (cellular structures) and external (environmental conditions) factors are involved. We have little knowledge on the mechanism of how these factors work together or separately to make changes on chemical/isotopic signals. More studies are needed to further elucidate the heterogeneity of isotopic compositions of lipid compounds bound in membrane and intracellular storage components as well as their relative reactivities under different environmental conditions.

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Table 3.1. Production rates ( $\mu$ g mL<sup>-1</sup> d<sup>-1</sup>) of lipid compounds during cell growth (phases I and II). R<sup>2</sup> is the correlation coefficient of data fitting and II/I is the ratio of rates between phase II and phase I.

Phase	Neutral lipids of <i>T.w.</i>		Fatty acids of <i>T.w.</i>						
	phytol	$28\Delta^{5, 24(28)}$	14:0	16:3	16:1(ω7)	16:0	20:5		
Ι	0.038	0.011	0.031	0.073	0.114	0.071	0.058		
$(R^2)$	(0.99)	(0.99)	(0.99)	(0.97)	(0.99)	(1.00)	(0.97)		
II	0.022	0.010	0.033	0.126	0.106	0.054	0.089		
$(R^2)$	(0.98)	(1.00)	(0.95)	(0.87)	(0.89)	(0.90)	(0.94)		
II/I	0.58	0.91	1.06	1.73	0.93	0.76	1.53		

Phase		Neutral lip	pids of <i>E.h</i> .		Fatty acids of <i>E.h.</i>				
	$27\Delta^5$	$28\Delta^{5, 22}$	37:3 alk	37:2 alk	14:0	16:0	18:1( <b>ω</b> 9)	22:6	
Ι	0.003	0.008	0.030	0.011	0.061	0.011	0.044	0.034	
$(R^2)$	(0.73)	(0.88)	(0.75)	(0.73)	(0.86)	(0.93)	(0.85)	(0.92)	
II	0.014	0.077	0.067	0.025	0.012	0.007	0.026	0.013	
$(R^2)$	(0.99)	(0.94)	(0.97)	(0.88)	(0.76)	(0.81)	(0.85)	(0.92)	
II/I	4.67	9.63	2.23	2.27	0.33	0.64	0.59	0.38	

%	Neutral lipids of T.w.		Fatty acids of <i>T.w.</i>						
	phytol	$28\Delta^{5, 24(28)}$	14:0	16:3	16:1(ω7)	16:0	20:5		
Respired	21.1	15.3	43.3	52.1	69.0	66.5	49.6		
Degraded	46.1	43.1	50.3	46.8	26.6	27.1	48.6		
Remaining	32.8	41.5	6.4	1.1	4.3	6.4	1.9		

Table 3.2. Percentages of respired (auto-metabolized), degraded and remaining lipid compounds of *T. weissflogii* and *E. huxleyi* relative to their initial concentrations.

%		Neutral li	pids of <i>E</i> . <i>h</i>	•	Fatty acids of <i>E</i> . <i>h</i> .			
	$27\Delta^5$	$28\Delta^{5, 22}$	37:3 alk	37:2 alk	14:0	16:0	18:1(ω9)	22:6
Respired	8.9	20.4	60.6	51.1	49.4	34.0	16.6	62.9
Degraded	38.1	43.8	21.6	28.6	44.0	33.0	71.3	35.6
Remaining	53.1	35.8	17.8	20.2	6.6	33.0	12.1	1.5

Phase	Neutral lipids of <i>T.w.</i>		Fatty acids of <i>T.w.</i>						
	phytol	$28\Delta^{5, 24(28)}$	14:0	16:3	16:1(ω7)	16:0	20:5		
$k_{c} \left( d^{-1} \right)$	0.021	0.016	0.042	0.062	0.098	0.087	0.060		
$(R^2)$	(0.86)	(0.71)	(0.96)	(0.98)	(0.99)	(0.95)	(0.94)		
$k_{d} \left( d^{-1} \right)$	0.024	0.038	0.273	0.527	0.299	0.197	0.488		
$(R^2)$	(0.88)	(0.83)	(0.90)	(0.94)	(0.96)	(0.98)	(0.94)		
$k_d/k_c$	1.14	2.38	6.5	8.5	3.05	2.26	8.13		

Table 3.3. Respiration (auto-metabolism, phase III) rate constants (k<sub>c</sub>) and microbial degradation (phase IV) rate constants (k<sub>d</sub>) of lipid compounds of *T. weissflogii* and *E. huxleyi*.

Phase		Neutral li	pids of <i>E.h</i> .		Fatty acids of <i>E.h.</i>				
	$27\Delta^5$	$28\Delta^{5, 22}$	37:3 alk	37:2 alk	14:0	16:0	18:1( <b>ω</b> 9)	22:6	
$k_{c} (d^{-1})$	0.005	0.014	0.035	0.034	0.049	0.031	0.024	0.070	
$(R^2)$	(0.32)	(0.85)	(0.96)	(0.97)	(0.98)	(0.94)	(0.63)	(0.78)	
$k_d (d^{-1})$	0.126	0.224	0.149	0.122	0.173	0.220	0.576	0.789	
$(R^2)$	(0.90)	(0.94)	(0.93)	(0.96)	(0.93)	(0.89)	(0.94)	(0.97)	
$k_d/k_c$	25.2	16	4.26	3.59	3.53	7.1	24	11.27	



Fig. 3.1. Variations of cell density, POC, TN, C/N,  $\delta^{13}$ C-POC and  $\delta^{15}$ N-TN of *T. weissflogii* (first six plots) and *E. huxleyi* (last six plots) during phase I (exponential growth stage); phase II (stationary phase); and phase III (dark respiration).



Fig. 3.2. Lipid compositions (fatty acids and neutral lipids separately) of *T. weissflogii* (upper two plots) and *E. huxleyi* (lower two plots) during cell growth (phases I and II), dark respiration (phase III) and microbial degradation (phase IV).



Fig. 3.3 Variations in cell density, concentrations of neutral lipid compounds, and their stable carbon isotopic compositions during cell growth (phases I and II) of *T. weissflogii* (top row of plots) and *E. huxleyi* (lower row of plots).



Fig. 3.4. Variations in concentrations of fatty acids, and their stable carbon isotopic compositions during cell growth (phases I and II) of *T. weissflogii* (top row of plots) and *E. huxleyi* (lower row of plots).



Fig. 3.5. Variations in cell density, concentrations of neutral lipid compounds, and their stable carbon isotopic compositions during dark respiration (phase III) of *T. weissflogii* (top row of plots) and *E. huxleyi* (lower row of plots).



Fig. 3.6. Variations in concentrations of fatty acids, and their stable carbon isotopic compositions during dark respiration (phase III) of *T. weissflogii* (top row of plots) and *E. huxleyi* (lower row of plots).



Fig. 3.7. Variations in concentrations of neutral lipid compounds, and their stable carbon isotopic compositions during microbial degradation (phase IV) of *T. weissflogii* (top row of plots) and *E. huxleyi* (lower row of plots).



Fig. 3.8. Variations in concentrations of fatty acids, and their stable carbon isotopic compositions during microbial degradation (phase IV) of *T. weissflogii* (top row of plots) and *E. huxleyi* (lower row of plots).

# CHAPTER 4

# DEGRADATION OF *EMILIANIA HUXLEYI* CELLS: INFLUENCE OF DIFFERENT PHYSIOLOGICAL STATES ON LIPIDS AND THEIR ISOTOPIC COMPOSITIONS<sup>1</sup>

<sup>&</sup>lt;sup>1</sup> Pan, H., Sun, M.-Y., Culp, R. A. To be submitted to Organic Geochemistry.

# Abstract

The goal of this study is to clarify the potential effect of cell physiological states on variabilities of algal lipids (phytol, sterols, alkeones, and fatty acids) and their stable carbon isotopic compositions during microbial degradation. Emiliania huxleyi cells were batch cultured in the same medium but over different growth stages: exponential growth phase (culture 1) and the stationary phase (culture 2), respectively. There were apparent differences in cellular lipid contents and relative compositions between the two cultures:  $\sim 8 \times$  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 algal cells (killed by freezing) from two cultures were dark incubated with introduction of natural seawater (containing natural microbial community) under aerobic conditions at 17°C over one month. Most fatty acids (except for 16:0) from two cultures were almost completely degraded while most neutral lipids (phytol, sterols and alkenones) (except for  $27\Delta^5$  from culture 1) were partially degraded with relatively higher percentages of these compounds remaining in culture 2 than in culture 1. The stable carbon isotopic compositions of lipids varied differently among compound classes: positive shift (+1.7% to +3.9%) in  $\delta^{13}$ C for various fatty acids; little change for sterols; and for alkenones either little change in most cases or negative shift (-3‰) in one case (37:2 alkenone in culture 1). Nonetheless, relatively larger shifts (~1.5‰ each) for fatty acids (16:0 and 18:1) were observed in culture 1 compared to those in culture 2. The results imply that variability in isotopic compositions of lipid compounds during microbial degradation may be largely affected by heterogeneous isotopic compositions of cellular lipids synthesized through different cell growth phases (or physiological states) and differential reactivities of various lipid compounds bound in different intracellular structures.

#### 1. Introduction

*Emiliania huxleyi* is a cosmopolitan coccolithophore existing in nearly all marine environments except in the Arctic Ocean and high-latitude Southern Ocean, and is a significant primary producer in the world's oceans (Westbroek et al., 1993). The lipid biomarkers (e.g., fatty acids, sterols, and alkenones) and their isotopic compositions derived from *E. huxleyi* (and certain of its related species) have been widely used to study organic carbon cycling (Volkman et al., 1986; Wakeham and Beier, 1991; Rontani et al., 2009), to estimate past sea surface temperature (Prahl and Wakeham, 1987; Müller et al., 1998), and to reconstruct ancient atmospheric  $pCO_2$  (e.g., Jasper and Hayes, 1994; Pagani et al., 1999; 2002).

The controversial assumption underlying the applications of biomarkers and their molecular isotopic compositions is that during biogeochemical processes the changes of these signals are too small to be counted (Prahl et al., 1989; Hayes et al., 1990; McCaffrey et al., 1990; Pagani et al., 1999). Nevertheless, more and more laboratory and field studies have shown that various organic compounds from different sources experience diversified changes in their chemical (Gong and Hollander, 1999; Rontani et al., 2005; Prahl et al., 2010; Pan and Sun, 2011) and isotopic (Hayes et al., 1990; Freeman et al., 1994; Macko et al., 1994; Sun et al., 2004; Teece and Fogel, 2007) signatures during transport/cycling processes. For example, decomposition of *n*-alkanes and polycyclic aromatic hydrocarbons did not lead to significant alterations in their isotopic compositions (Hayes et al., 1990; Freeman et al., 1994; Fueaga et al., 1994; Huang et al., 1997; Mazeas et al., 2002). By contrast, Macko et al. (1994) found that after 4 weeks of incubation when ~50% of amino acids in the seagrass *Halodule wrightii* were degraded, some amino acids were enriched (~2‰ increase) in  $^{13}$ C while others either became depleted (~5‰ decrease) or remained constant. Sun et al. (2004) demonstrated that during degradation of

various lipids from *Emiliania huxleyi* cells in natural oxic and anoxic seawaters the alterations in isotopic compositions of those lipids diversified: ~ 2‰ - 7‰ positive shift for fatty acids, ~ 4‰ - 6‰ negative shift for alkenones, and no change for sterols. Teece and Fogel (2007) also observed that during degradation of the salt marsh plant *Spartina alterniflora*, changes in  $\delta^{13}$ C of sugar molecules varied: 85% decrease of glucose concentration resulted in an isotopic enrichment (+5‰) for the remaining compounds; residual (~25%) arabinose was depleted in <sup>13</sup>C by -4‰ compared to the original signal; and after 79% of the xylose was removed, the  $\delta^{13}$ C value remained constant. In Chapter 3 of this dissertation, the diversified alterations of isotopic compositions of algal lipids (derived from diatom *Thalassiosira weissflogii* and haptophyte *E. huxleyi*) were also observed during microbial degradation. Therefore, possible effect of biogeochemical cycling on biomarker applications needs to be carefully considered (Hedges and Prahl, 1993).

Up to now, it has been unclear what mechanisms drive these diversified isotopic alterations for different biomarkers during biochemical processes. Sun et al. (2004) postulated that: (1) because of the differences in original precursors and synthesis pathways for various lipid compound classes with distinct chemical structures (i.e., sterols, alkenones, and fatty acids), carbon atoms at specific functional groups may have distinct isotopic ratios from other carbon atoms in the molecules; (2) degradation reactions at these specific functional groups would lead to the structure-dependent isotopic fractionations for various lipid compound classes. These speculations (Sun et al., 2004) could explain the same trend in isotopic alteration for each individual lipid class, but it could not explain the diversified variations in the same lipid class (e.g., fatty acids) observed in our current study.

In Chapter 3 of this dissertation, we also postulated an alternative way for diversified isotopic alterations of lipids based on our new results that: (1) during cell exponential growth, lipid compounds are largely produced as components of cell membranes and their isotopic compositions are heterogeneously distributed, while during stationary phase, lipid compounds are mainly biosynthesized as storage components with relatively homogeneous distribution of isotopic compositions; (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) and two pools (microbial degradation case). Therefore, original heterogeneity in isotopic compositions of lipid compounds bound in different cellular structures and their variable reactivities during microbial degradation are thought to be two major factors causing isotopic alterations. However, cell materials used for previous degradation experiment (phase IV, Chapter 3) were senescent algal cells after dark respiration experiment (phase III). It is unknown how differently the lipids bound in intact cells, grown in different growth phases, are degraded by microbial processes. Moreover, it is also unclear what is the effect of cell physiological states (or growth phases) on the variability of lipid isotopic compositions during microbial degradation.

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. *Emiliania huxleyi* cells were batch cultured through different growth phases: the exponential growth phase (culture 1) and the stationary growth phase (culture 2), respectively. Dead cells (killed by freezing) in two cultures were dark incubated under aerobic conditions with introduction of natural seawater containing microbial communities. Variations in concentrations of algal lipids (phytol, sterols, alkeones, and fatty acids) and associated isotopic compositions were followed during a one-month incubation. Possible mechanisms causing these variations are proposed and implications of the study are addressed.

# 2. Experimental

#### 2.1. Materials

Marine haptophyte *E. huxleyi* (CCMP371) was provided by the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). 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 for experiment was collected from the Gulf of Mexico (28°10'82"N, 91°36'96"W) in May 2008 by direct 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 the CCMP (Guillard, 1962; Guillard, 1975). The seawater for inoculation was stored in 25-liter carboys until incubation experiments.

# 2.2. Experimental setup

*E. huxleyi* cells were batch cultured (in duplicate for both cultures 1 and 2) in f/50 medium (~1 L) under a light:dark (12 h:12 h) regime with a constant light intensity (50  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>). The cultures were hand shaken twice and cell densities in each culture were counted every one or two day. 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 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. They

were then incubated for 32 days under dark and aerobic conditions which was manipulated by purging the solution with air.

# 2.3. Sampling and cell counting

Transfer and sampling of cultures were conducted in a clean hood, which was preirradiated with a UV lamp over 30 min. The flasks were gently shaken by hand to allow cultures to be uniform before sampling. One milliliter of culture sample was taken every 1–3 days during cell growth phases and the cells were counted using an Olympus BH-2 microscope with a hemacytometer (Hausser Scientific). Incubation samples (in duplicate for cultures 1 and 2) were collected at certain time points by filtering the cultures through glass microfiber filters (Whatman 934-AH) and then stored at –40°C for future lipid extraction and analysis.

# 2.4. Extraction and analysis of lipids

Lipid extraction, separation, and derivatization followed the procedure described in previous studies (Sun et al., 1998; Sun and Wakeham, 1999). Lipids in thawed filters were extracted with  $3 \times 25$  mL methylene chloride:methanol (2:1, v:v) with 6 min sonication each time after vortex-shaking. Combined extracts were separated into a methylene chloride phase with addition of 30 mL 5% NaCl solution. The volume of total lipid extract (TLE) was reduced to nearly dry by rotary evaporators. The TLE was then saponified at 92°C with 6 mL 0.5 M KOH in MeOH:H<sub>2</sub>O (95:5) for 2 h. After cooling and the addition of 1 mL 5% NaCl solution, the neutral lipids, including alkenones, sterols, phytol and fatty alcohols, were first extracted with  $3 \times 6$  mL hexane from the basic solution (pH > 13). Afterward, the solution was acidified (pH < 2) by adding HCl and then the fatty acids were extracted with  $3 \times 6$  mL hexane. The neutral lipids were reacted in acetonitrile with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)

at 92°C for 2 h to form TMS ethers of sterols and alcohols. The fatty acids were methylated with 5% BF<sub>3</sub> in MeOH at 92°C for 2 h to form FAMEs (fatty acid methyl esters).

Concentrations of neutral lipids and fatty acids were quantified by capillary gas chromatography using a Hewlett-Packard 6890 GC with an on-column injector and a flame ionization detector (Sun et al., 1998; Sun and Wakeham, 1999). The lipid compounds were separated with a  $30 \text{ m} \times 0.25 \text{ mm}$  i.d. HP-5 column coated with 5% diphenyl:95% dimethylsiloxane copolymer, and the operating temperature programs were: for neutral lipids, 50–170°C at 10°C min<sup>-1</sup>, then 170–310°C at 3°C min<sup>-1</sup> and finally held for 30 min at 310°C; for fatty acids, 50-170°C at 20°C min<sup>-1</sup>, then 170-310°C at 4°C min<sup>-1</sup> and finally held for 10 min at 310°C. Internal standards such as 5a(H)-cholestane for neutral lipids and nonadecanoic acid methyl ester for fatty acids were added to each corresponding sample before the GC analysis for quantification. The relative standard deviation of lipid analysis was within  $\pm 5\%$  based on duplicate measurements. Identification of lipid compounds was achieved through a Shimadzu QP-5000 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 operation conditions for MS were: mass range 50–610 Da with a 0.4 s scan interval; 70 eV ionizing energy; and the temperature program for GC was 50–150°C at 20°C min<sup>-1</sup> followed by 150–310°C at 4°C min<sup>-1</sup> and a 30 min hold for neutral lipids and a 10 min hold for fatty acids at 310°C.

# 2.5. Isotopic analysis of lipid compounds

Stable carbon isotopic compositions of individual lipid compounds were measured using a Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometer system (GC-C-IRMS) (Hayes et al., 1990). Lipid compounds were separated by an Agilent 6890 gas chromatograph with a 30 m  $\times$  0.25 mm i.d. capillary column (DB-1, J & W Scientific) and a split injector installed. The temperature programs for the GC operation were: 50-180°C at 20°C min<sup>-1</sup> followed by 180-310°C at 3°C min<sup>-1</sup> and a 90 min hold at 310°C for neutral lipids; 50-170°C at 20°C min<sup>-1</sup> followed by 170-300°C at 4°C min<sup>-1</sup> and a 15 min hold at 300°C for FAMEs. Helium with a flow rate of 1.5 mL min<sup>-1</sup> was used as carrier gas. Separated compounds from the GC were combusted (via a Thermoquest Finnigan GC Combustion III unit) to CO<sub>2</sub> over CuO/Ni/Pt wires at 850°C and water was removed by diffusion through a Nafion selectively permeable membrane (Perma Pure LLC.) flushed with helium. The stable isotopic compositions of CO<sub>2</sub> 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 CO<sub>2</sub> and reported in per mil (‰) against VPDB (Vienna Pee Dee Belemnite). Based on internal standard measurements, the standard deviations of IRMS analyses were ±0.41‰ (n = 34) for neutral lipids and ±0.54‰ (n = 18) for FAMEs. The  $\delta^{13}$ C values of BSTFA (38.23 ± 0.04‰) and methanol (43.72 ± 0.15‰), which were predetermined by 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 *E. huxleyi* in cultures 1 and 2 increased at similar rates from  $\sim 1 \times 10^3$  mL<sup>-1</sup> to maximum levels ( $\sim 6.0 \times 10^5$  mL<sup>-1</sup>) within exponential growth phase (Fig. 4.1). Culture 1 was stopped on day 12 (end of exponential growth phase) while culture 2 continued for an additional 23 days through stationary phase, in which cell densities remained relatively constant ( $6.8 \pm 1.4 \times 10^5$  mL<sup>-1</sup>) (Fig. 4.1).

#### 3.2. Total lipid contents and relative lipid compositions in cultures 1 and 2

Total lipid content (15.1±1.6 pg cell<sup>-1</sup>) of cells in culture 2 (35 days) was ~8× higher than that (2.0±0.3 pg cell<sup>-1</sup>) in culture 1 (12 days) (Fig. 4.1), in which total neutral lipids increased ~23× while total fatty acids only increased ~3×. 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. 4.1).

In both cultures, 7 fatty acids and 11 neutral lipids were identified and their relative compositions were different between two cultures (Fig. 4.2). In the fatty acid pool, saturated fatty acids (SFA) 14:0 and 16:0, monounsaturated fatty acid (MUFA) 18:1( $\omega$ 9), and polyunsaturated fatty acid (PUFA) 22:6 are major constituents (each > 10%). Higher percentages of 18:1( $\omega$ 9) and 22:6 fatty acids but a lower percentage of 14:0 fatty acid were observed in culture 2 than in culture 1. Percentages of the other four fatty acids were similar in the two cultures. In the neutral lipid pool, 37:3 alkenone was a dominating (>28%) compound in both cultures, but relative compositions of neutral lipids varied between the two cultures (Fig. 4.2). Percentages of phytol and two sterols (27 $\Delta$ <sup>5</sup> and 28 $\Delta$ <sup>5, 22</sup>) were much less, and percentages of several alkenones (37:2, Et38:3, Et38:2, and Et39:2) were significantly higher in culture 2 than in culture 1 while other alkenones remained almost same percentages between two cultures.

# 3.3. Variations of lipid compounds during incubation experiments

There was no  $18:1(\omega7)$ , a bacteria-specific fatty acid, present in two initial cultures. However, this bacterial fatty acid appeared during incubation experiments and the concentrations increased in the first few days and then gradually declined to relatively stable levels (Fig. 4.3). The concentrations of this bacteria-specific fatty acid in culture 2 almost double those in culture 1. In the first 10 days of incubation, concentrations of algal lipids decreased rapidly and then remained at relatively stable but different levels (Figs. 4.4 and 4.5). For most cases, over 90% of fatty acids (except 16:0), were lost in both cultures while approximately 20-30% of 16:0 fatty acid remained in both cultures after incubation. For neutral lipids, higher (8-35% more) percentages of various compounds were lost in culture 1 than in culture 2 (Table 4.1). However, degradation rate constants for most compounds (mostly based on initial loss in the first 10 days) were somewhat similar in the two incubation experiments (Table 4.2).

# 3.4. Variations of stable carbon isotopic compositions of lipid compounds during incubation experiments

The stable carbon isotopic compositions of compounds varied in three different ways among different lipid classes during incubation experiments (Figs. 4.4 and 4.5). The  $\delta^{13}$ C values of fatty acids (14:0, 16:0 and 18:1( $\omega$ 9)) from both cultures shifted positively (+ 1.7-3.9‰) within 10 days. Moreover, relatively larger shifts for 16:0 and 18:1( $\omega$ 9) fatty acids (~1.5‰ more positive shift for each) were observed in culture 1 compared to those in culture 2. There was little change (within ±0.4‰) in the  $\delta^{13}$ C values of sterols during incubation experiments. The  $\delta^{13}$ C values of most alkenones (37:3 and 37:2) varied little, but that of 37:2 alkenone from culture 1 showed apparent negative alteration (~ -3‰). The  $\delta^{13}$ C values of phytol and 22:6 fatty acid in this experiment could not be quantitatively determined because of their too small IRMS peaks.

# 4. Discussion

# 4.1. Biological functions of algal lipids synthesized at different physiological states

It has been observed that during cell exponential growth, algal cells synthesize mostly membrane lipids (e.g., phospholipid) as principal lipid components (Volkman et al., 1989; Brown et al., 1996; Mansour et al., 2003), while during cell stationary phase, the proportion of storage lipids such as triacylglycerols (Dunstan et al., 1993; Brown et al., 1996; Mansour et al.,

2003) or alkenones (methyl and ethyl ketones) (Bell and Pond, 1996; Epstein et al., 2001) increased significantly. In this study, *E. huxleyi* biosynthesized much more ( $\sim 8 \times$ ) total lipids in culture 2 (stopped at the end of stationary phase) than in culture 1 (stopped at the end of exponential growth) (Fig. 4.1). Algal cells contained more fatty acids ( $\sim 75\%$  of total lipids) than neutral lipids in culture 1. But due to the much faster increase of neutral lipids ( $\sim 23 \times$ ) than that of fatty acids ( $\sim 3 \times$ ) during stationary growth, neutral lipids ( $\sim 73\%$  of total lipids) dominated over fatty acids in culture 2 (Fig. 4.1).

The dominance of fatty acids (~75%) in total lipid content in culture 1 indicated the important function of fatty acids as membrane (structure) lipids (e.g., phospholipids) (Fig. 4.1). Lower percentage of 14:0 fatty acid but higher percentages of  $18:1(\omega 9)$  and 22:6 fatty acids were observed in culture 2 than in culture 1. These variations suggested the different functions of individual fatty acids in different cellular (structure vs. storage) components between exponential growth (culture 1) and stationary (culture 2) phases. But the reduction of the fatty acid proportion (~27% in total lipid pool) in culture 2 implied that there were probably some other lipids functioning as energy storage lipids besides fatty acids.

The biosynthesis pathways, distributions in different cellular compartments, and physiological role of alkenones in *E. huxleyii* cell have not been well understood. It is known that *E. huxleyi* produces little triacyglycerols in cellular carbon (Volkman et al., 1986), but biosynthesizes a large amount of long-chain alkenones instead through exponential growth (10-20%) (Prahl et al., 1988; Conte et al., 1998) and during stationary phase (up to 40%) (Prahl et al., 2003). Our results also indicated that a higher proportion of neutral lipids (~73% of total lipid content) were produced in culture 2 (Fig. 4.1) and alkenones occurred as major components in the neutral lipid pool (Fig. 4.2). Alkenones were previously considered only as membrane lipids
in regulating membrane fluidity (Prahl and Wakeham, 1987; Brassell, 1993) and therefore the unsaturation degree of alkenones, that is the concentration ratio of [37:2]/([37:2] + [37:3]), has been developed to study history of paleo-temperature. But recently, alkenones have been observed as metabolic storage lipids (e.g., Bell and Pond, 1996; Epstein et al., 2001; Pan and Sun, 2011). 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. Eltgroth et al. (2005a) further suggested that alkenones might be synthesized in chloroplasts and then exported to cytoplasmic lipid bodies for energy storage and metabolism need. From Chapter 3 of this dissertation, we know that accumulated alkenones in stationary phase were significantly lost during dark respiration, confirming again the important function of alkenones as storage lipids. The higher concentrations of alkenones in culture 2 relative to those in culture 1 (Fig. 4.5) and dominant proportion of alkenones in the neutral lipid pools (Fig. 4.2) observed in this study were consistent with previous finding that the alkenones serve as storage lipid compounds and play an important function in cell respiration (or auto-metabolism).

In the neutral lipid pool, both phytol (as a degradation product of chlorophyll) and sterols are membrane-associated lipids which play roles 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 storage compounds in culture 2 (Fig. 4.2).

# 4.2. Distributions of lipid isotopic compositions in *E. huxleyi* cells along different growth phases

The isotopic compositions of lipids depend on many factors such as carbon sources, the effects of carbon assimilation processes, the pathways of metabolism and biosynthesis (Hayes, 1993; Schouten et al., 1998; Riebesell et al., 2000b; Hayes, 2001; Schouten et al., 2008), and cell growth rate and physiological states (or growth phases) (Laws et al., 1995; Bidigare et al., 1997; Benthien et al., 2007). Laws et al. (1995) found that the biological isotopic fractionation ( $\varepsilon_p$ ) in a culture of diatom Phaeodactylum tricornutum was negatively correlated with the ratio of cell growth rate to aqueous CO<sub>2</sub> concentration ( $\mu/[CO_2]_{aq}$ ). In Chapter 3 of this dissertation, we have shown that the cell growth rates in the exponential growth phase of batch culture were relatively constant while dissolved  $CO_2$  concentrations ( $[CO_2]_{aq}$ ) dropped down quickly due to the assimilation by photosynthesis (Benthien et al., 2007). Accordingly, the biological isotopic fractionation factor ( $\varepsilon_n$ ) decreased, leading to positive shifts of  $\delta^{13}$ C values of lipid compounds. After reaching stationary phases, cell growth ceased ( $\mu = 0$ ) and  $[CO_2]_{aq}$  varied little (Benthien et al., 2007), resulting in less variable or relatively constant  $\delta^{13}$ C values of lipid compounds. In their mesocosm bloom experiments, Benthien et al. (2007) also 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 experimental results, some speculations have been put forward in Chapter 3 of this dissertation: (1) during algal cell exponential growth phase of a batch culture, lipid compounds were biosynthesized primarily as structural components (e.g., phospholipids in cell

membranes) while their stable carbon isotopic compositions varied (with positive shifts) and were thus heterogeneously distributed in the membrane-associated lipid pool; (2) during cell stationary growth 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 homogenously distributed. Unlike the senescent cell materials used for degradation experiments in Chapter 3, which were collected after cell respiration phase III, the cells collected in culture 1 and culture 2 of this study were intact cells, representing distinct physiological states of growing cells. The cells in these two cultures have different lipid and isotopic distributions within different intracellular structures (or compartments). Cells from culture 1 contained more membrane-associated lipids with heterogeneous distribution of isotope compositions, whereas cells from culture 2 consisted of more storage lipids with homogeneous distribution of isotope compositions.

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

There was no bacteria-specific fatty acids such as  $18:1(\omega 7)$  (e.g., Parkes and Taylor, 1983; Kaneda, 1991) present in two initial cultures. However, when natural seawaters were introduced into the incubation samples,  $18:1(\omega 7)$  fatty acid appeared and its concentrations increased in first a few days, coinciding with the rapid decrease of concentrations of algal lipids during first 10 days. When concentrations of algal fatty acids approached stable levels, the concentrations of  $18:1(\omega 7)$  also decreased to a relative stable level throughout the incubations (Fig. 4.3), indicating that bacteria played a major role in decomposing algal lipids. In addition, the concentrations of this bacteria-specific fatty acid in culture 2 were 2× higher than those in culture 1 (Fig. 4.3), suggesting that bacterial activities were closely related to concentrations of

labile substrate materials. That means that a more intensive response of microbial community to a higher concentration of algal lipids in culture 2 than in culture 1.

During microbial degradation, most fatty acids (except 16:0) were significantly decomposed (>90%) and significant percentages of neutral lipids (~ 20% - 90%) were lost (Table 4.1). The isotopic compositions of remaining compounds among different lipid classes varied in three different ways: positive shift (+1.7% to +3.9%) in  $\delta^{13}$ C for various fatty acids; little change for sterols; and for alkenones either little change in most cases or negative shift (-3‰) in one case (37:2 alkenone in culture 1). The diversified variations of compound-specific lipid isotopic compositions were also observed in some previous studies (Sun et al., 2004; Chikaraishi and Naraoka, 2006; Chapter 3 of this dissertation). In Chapter 3, we speculated that both heterogeneous distribution of isotopic compositions of cellular lipids and their differential reactivities during microbial degradation are primary factors controlling the isotopic alterations of lipid compounds. 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, 2005a). Thus, if lipid compounds in cellular structural components (e.g., membranes) with heterogeneous distribution of isotopic compositions are selectively degraded, the isotopic alteration of remaining lipid compounds may be more significant. By contrast, if lipid compounds in storage components (e.g., lipid vesicles) with homogeneous distribution of isotopic compositions are preferentially utilized, the isotopic compositions of remnant lipid compounds may change less significant.

In *E. huxleyi* cells, fatty acids seemed to function mainly as structure (membrane) lipids in culture 1 (Fig. 4.1) while their role in storage component were unclear since alkenones were major storage lipids in culture 2 (Figs. 4.1 and 4.2). The positive shifts (+1.7% to +3.9%) in  $\delta^{13}$ C values of fatty acids were possibly caused by preferential degradation of fatty acids in cell structural component (e.g., membrane) with heterogeneity in isotopic compositions. Although the degradation rate constants of each individual fatty acid between two cultures were similar (Table 4.2), relatively higher percentages of fatty acids were degraded (Table 4.1, Fig. 4.4) in culture 1 than in culture 2. In other words, more membrane-associated fatty acids with heterogeneous distribution of isotopic compositions in culture 1 were preferentially degraded compared to culture 2. Therefore, degradation of fatty acids from culture 1 caused a relatively larger positive shift (~ 1.5‰ more each) for 16:0 and 18:1( $\omega$ 9) compared to that from culture 2 (Fig. 4.4). This difference in isotopic shifts between 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 one  $C_{37}$  alkenone) in cultures 1 and 2, there is little change in isotopic compositions no matter how much alkenones were lost (Table 4.1, Fig. 4.5). However, the  $\delta^{13}$ C values of 37:2 alkenone in culture 1 displayed an apparent negative alteration (~-3‰) after degradation (Fig. 4.5). In Chapter 3 of this dissertation, we did not observe the apparent isotopic alteration for any alkenone. It was because most alkenones (unlike fatty acids) were synthesized as storage lipids with homogeneous isotopic compositions during stationary growth phase 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. If their isotopic distributions in the 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 speculation. This is probably related to differential degradation pathways of different alkenone compounds. For example, a series of experimental studies (Rontani et al., 2005; 2006; 2008; 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., 2010). More studies are still needed to clarify the contrasting variations in alkenone isotopic compositions.

There is little change in isotopic signals of sterols in spite of their significant degradation (Table 4.1, Fig. 4.5). 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 we can apply algal lipid biomarkers and their isotopic signals to study biogeochemical cycling of organic matter in natural systems and to reconstruct paleoceanographic records preserved in marine sediments, we should have a clear understanding on the effects of physiological states of phytoplankton cells during growth on the generation of lipids within different cellular structures and on distributions of lipid isotopic compositions. On the other hand, it is also important to elucidate the relative reactivities of lipid compounds associated with different cellular compartments and their impact on isotopic alterations.

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; (2) those at an open ocean site with a sufficient nutrient supply would follow a continuous culture-like growth (Popp et al., 1998a). In Chapter 3 of this dissertation, algal cells underwent cell respiration (auto-metabolism) in a dark environment before a microbial degradation experiment, but 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 sediment, many biological and biochemical processes (e.g., zooplankton grazing, cell respiration, and microbial degradation) have different influences on algal biomarkers and associated isotopic compositions. For instance, Grice et al. (1998) found that the result of the copepod *Temora longicornis* feeding 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 algal cell exponential growth phases, cells biosynthesized mainly structural (membrane) lipids with heterogeneous stable carbon isotopic compositions, while during stationary phase, cells biosynthesized more storage lipids with homogeneous isotopic compositions. Therefore, in Chapter 3 of this dissertation, our results showed that 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. On the contrary, microbial processes may preferentially degrade lipid compounds associated with structural components (with heterogeneous isotopic compositions), resulting in isotopic alterations (Chapter 3 and this chapter). Especially, this study demonstrated that the preferential degradation of membrane-associated fatty acids (with heterogeneous isotopic compositions) from culture 1 relative to storage fatty acids (with homogeneous isotopic compositions) from culture 2 could lead to relatively larger isotopic shifts.

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		Neutral lipids								Fatty acids			
		phytol	27Δ5	28Δ5,22	37:3alk	37:2alk	Et38:3alk	Et38:2alk	14:0	16:0	18:1( <b>ω</b> 9)	22:6	
Culture 1	Degraded (%)	85	91	79	50	54	51	53	96	77	94	~ 100	
	Remaining (%)	15	9	21	50	46	49	47	4	23	6	~ 0	
Culture 2	Degraded (%)	71	63	68	35	19	43	26	92	69	91	99	
	Remaining (%)	29	37	32	65	81	57	74	8	31	9	1	

Table 4.1. Proportions of degraded vs. remaining lipid compounds.

		Neutral lipids								Fatty acids			
		phytol	27Δ5	28Δ5,22	37:3alk	37:2alk	Et38:3lk	Et38:2alk	14:0	16:0	18:1(ω9)	22:6	
Culture 1	$k_{d}\left(d^{-1}\right)$	0.196	0.164	0.218	0.147	0.155	0.161	0.172	0.166	0.117	0.141	0.406	
	$R^2$	0.88	0.80	0.86	0.76	0.80	0.76	0.78	0.98	0.90	0.96	0.95	
Culture 2	$k_{d} (d^{-1})$	0.199	0.166	0.209	0.272	0.078	0.282	0.124	0.169	0.134	0.249	0.573	
	$R^2$	0.83	0.85	0.82	0.95	0.80	0.96	0.87	0.94	0.85	0.95	0.91	

Table 4.2.	Degradation rate constants $(k_d)$ of lipid con	mpounds.
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Fig. 4.1. Variations of *E. huxleyi* cell densities and total lipid contents of cells in culture 1 (12 days) and culture 2 (35 days).



Fig. 4.2. Relative lipid compositions of cells in culture 1 (12 days) and culture 2 (35 days).



Fig. 4.3. Variations of concentrations of bacteria-specific fatty acid (BSFA) 18:1( $\omega$ 7) during degradation of cells in cultures 1 and 2.



Fig. 4.4. Variations of chemical concentrations and stable carbon isotopic compositions of fatty acids during degradation of cells in cultures 1 and 2.



Fig. 4.5. Variations of chemical concentrations and stable carbon isotopic compositions of neutral lipids during degradation of cells in cultures 1 and 2.

# CHAPTER 5

# TEST OF RELATIVE ROLES OF ISOTOPIC FRACTIONATION VS. CHEMICAL REACTIVITY OF LIPIDS IN ALTERING MOLECULAR ISOTOPIC SIGNALS DURING MICROBIAL DEGRADATION IN MARINE SEDIMENTS <sup>1</sup>

<sup>&</sup>lt;sup>1</sup> Pan, H., Sun, M.-Y., Culp, R. A. To be submitted to Chemical Geology.

#### Abstract

This experimental study was designed to examine the relative roles of kinetic isotopic fractionation vs. chemical reactivity in altering stable carbon isotopic composition of lipid compounds during microbial degradation. <sup>13</sup>C-labeled (labeling at different carbon positions of fatty acid chains) and unlabeled tripalmitins were spiked in varying proportions and then incubated in natural oxic (top 1 cm) and anoxic (> 10 cm) marine sediments. In anoxic sediments, neither natural fatty acids nor tripalmitin-derived 16:0 fatty acid were apparently degraded and hence no significant variation in stable carbon isotopic composition of 16:0 fatty acid was observed. However, in oxic sediments, both naturally occurred fatty acids and spiked tripalmitin-derived 16:0 fatty acid were obviously degraded by 26% - 95%. For natural fatty acids (e.g., 14:0, 16:1, 18:1, 20:5/20:4, and  $>C_{20:0}$ ), degradation rates varied according to the following order: polyunsaturated > monounsaturated > short chain saturated > long chain saturated fatty acids, reflecting variable reactivities of natural lipid compounds from different sources. Tripalmitin-derived 16:0 fatty acid degraded at an at least 2-3× rate compared to that in natural sediments. Meanwhile, isotopic compositions of 16:0 fatty acid in the oxic sediments shifted negatively during incubation. It appears that the magnitudes in isotopic shift are dependent on the amount of spiked <sup>13</sup>C-labeled compound in the sediments but not related to the labeling position of <sup>13</sup>C in the molecular structure. These results provide a direct evidence that the relative reactivities of lipid compounds from different sources (or different pools), which originally have heterogeneous isotopic signatures, may cause alterations in molecular isotopic composition during microbial degradation in natural environments.

#### 1. Introduction

Lipid biomarkers (e.g., sterols, alkenones, and fatty acids) and associated compound specific stable carbon isotope compositions have been widely applied to study biogeochemical cycling of organic matter (Hayes et al., 1990; Boschker et al., 2005; Dai and Sun, 2007), characterize microbial community (Boschker and Middelburg, 2002) and reconstruct historic records of climatic and environmental conditions (Jasper and Hayes, 1994; Pagani et al., 1999; Pagani et al., 2002). The fundamental assumption underlying the applications of lipid biomarkers and their molecular isotopic compositions is that these signals remain intact since their formation by phytoplankton in surface water until preservation in marine sediments (Hayes et al., 1990; Pagani et al., 1999). That implies that biogeochemical cycling of organic matter in water column and surface sediments have no significant impacts on these signals although most (>99%) phytoplankton-produced organic compounds are destructed. However, many field and laboratory studies have shown that the stable carbon isotope compositions of various organic compounds can be altered in various ways through biochemical degradation (Freeman and Wakeham, 1992; Macko et al., 1994; Sun et al., 2004; Dai et al., 2005; Chikaraishi and Naraoka, 2006; Teece and Fogel, 2007).

Theoretically, incomplete and unidirectional processes such as biologically mediated reactions may cause kinetic isotope fractionations, that is, when the rate of chemical reaction is affected by atomic mass at a certain position in a reacting molecule, a kinetic isotope fractionation occurs (Hoefs, 2009). The isotopic composition of a new product should be depleted in <sup>13</sup>C (isotopically lighter) due to kinetic fractionation during unidirectional chemical reactions (Hoefs, 2009). However, many previous studies have observed that isotopic compositions of organic compounds from natural sources can be altered in diverse ways during

degradation (Macko et al., 1994; Sun et al., 2004; Chikaraishi and Naraoka, 2006; Teece and Fogel, 2007; Chapters 3 and 4 of this dissertation). For example, Macko et al. (1994) observed that when ~50% of amino acids in the seagrass *Halodule wrightii* were degraded after 4 weeks of incubation, some amino acids were enriched (~2‰ increase) in <sup>13</sup>C while others either became depleted (~5‰ decrease) or remained constant. Teece and Fogel (2007) also found that during degradation of salt marsh plant *Spartina alterniflora*, the  $\delta^{13}$ C values of its sugar molecules changed differentially: an isotopic enrichment (+5‰) for remaining compound after 85% glucose was decomposed; an isotopic depletion (-4‰) for residual (~25%) arabinose; and constant  $\delta^{13}$ C values for xylose after 79% of the compound was removed. Sun et al. (2004) showed that after microbial degradation in natural oxic and anoxic seawaters the isotopic compositions of *Emiliania huxleyi* derived lipids underwent diversified alteration: ~2‰ - 7‰ positive shift for fatty acids, ~4‰ - 6‰ negative shift for alkenones, and little change for sterols. But up to now, there has been no convincing explanation for the mechanisms that drive diversified isotopic alterations of organic compounds during biogeochemical cycling.

Kinetic isotopic fractionation during biochemical decomposition of organic matter could be one of the driving forces. For instance, it has been postulated by Sun et al. (2004) that since fatty acids were degraded primarily through decarboxylation (Sun et al., 1997) and the carbon at the carboxyl group of fatty acids is isotopically lighter than overall carbons bound in the chain (DeNiro and Epstein, 1977), the degradation of fatty acids would result in positive shifts of  $\delta^{13}$ C values for surviving compounds. Furthermore, different susceptibility to degradation of various organic matter pools with distinct isotopic composition could also cause isotopic alteration of remaining organic matter. For example, during degradation of vascular plants such as *Spartina alterniflora*, selectively more loss of polysaccharide (relatively enriched in <sup>13</sup>C) than lignin components (relatively depleted in <sup>13</sup>C) resulted in the remnant organic matter depleted in <sup>13</sup>C (Benner et al., 1987). Additionally, contribution of bacteria produced lipids (e.g., fatty acids) with different  $\delta^{13}$ C values from those of plant lipids (substrates) may also alter the  $\delta^{13}$ C values of residual lipids after microbial degradation (Canuel et al., 1997b; Gong and Hollander, 1997; Boschker et al., 1999a; Teece et al., 1999).

Based on our experimental results (Chapter 3 of this dissertation), some new perspectives were proposed: (1) isotopic compositions of phytoplankton lipid compounds are heterogeneously generated through different cell growth phases and distributed with varying proportions in different intracellular compartments, which are dependent on cell physiological states and environmental conditions; and (2) microbial degradation can selectively decompose these lipid compounds with distinct isotopic compositions located in different intracellular compartments, leading to diversified alterations of isotopic compositions of remaining lipid compounds. In other words, other than the kinetic isotope effect, a combination of isotopic heterogeneity of lipid compounds in different intracellular compartments and variable reactivities of these compounds during microbial degradation might lead to the diversified isotopic alterations.

In Chapter 4 of this dissertation, our experimental results also showed that after microbial degradation of the haptophyte *Emiliania huxleyi* cells,  $\delta^{13}$ C values of remaining fatty acids in culture 1, which was collected at the end of exponential growth (probably dominated by membrane lipids), shifted more positively than those collected from culture 2 (in late stationary phase with higher proportions of storage lipids). Meanwhile, larger fractions of membrane lipids (with heterogeneous isotopic distribution) from culture 1 were degraded than those of storage lipids from culture 2 (with homogeneous isotopic distribution), further suggesting the important

role of reactivity of organic compounds in altering isotope compositions of remnant lipids during microbial degradation.

This study was specifically designed to further examine the relative roles of kinetic isotopic fractionation vs. chemical reactivity in altering stable carbon isotopic composition of lipid compounds during microbial degradation. <sup>13</sup>C-labeled (labeling at different carbon positions of fatty acid chains) and unlabeled tripalmitins were spiked in varying proportions and then incubated in natural oxic (top 1 cm) and anoxic (>10 cm) sediments with originally distinct lipid compositions and contents. The variations of chemical concentrations of naturally-occurring (e.g., 14:0, 16:1, 18:1, and 20:5/20:4) fatty acids were measured and their degradation rate constants were estimated. The variations of tripalmitin-derived 16:0 fatty acid concentration and its isotopic composition during incubation were also followed. The relationship between isotopic alteration and the pool size of reactive substrate (<sup>13</sup>C-labeled compound) was explored and the implications of the results are discussed.

#### 2. Experimental

#### 2.1. Sediment and seawater sampling

Sediments and water used in this study were collected from Doboy Sound ( $31^{\circ}24'17''$  N,  $81^{\circ}17'67''$  W) near Sapelo Island, Georgia, USA on January 5, 2007. Surface seawater (0-2 m, temperature 16.4 °C, and salinity 31.4) was pumped through a set of filters ( $25 \mu m$ ) into 50 L carboys. Sediments were first collected using a box core sampler. The top 1 cm surface sediments (oxic) and >10 cm deep sediments (anoxic) were scraped from the sediment cores and passed through a 0.5 mm mesh size sieve separately to remove macrobenthos, large shells and detritus. Operations related to anoxic sediments were conducted in a polyethylene glove bag inflated with a continuous flow of nitrogen gas.

#### 2.2. Labeled tracers

Tripalmitin, as an important metabolic storage compound (triacylglycerol) of many algae, was used for this study. It contains three 16:0 fatty acids esterified with a glycerol. Two different <sup>13</sup>C-labeled tripalmitins were purchased from the Cambridge Isotope Laboratories, Inc. One was 1,1,1-<sup>13</sup>C-tripalmitin labeled at the carboxyl carbon (1st carbon) of three fatty acid chains. Another was 2,2,2-<sup>13</sup>C-tripalmitin labeled at the second carbon site (middle chain) of three fatty acid chains. In addition, an unlabeled bulk tripalmitin was bought from Sigma Chemical Co. Three substrate solutions (S1, S2, and S3) were prepared for incubation experiments with the same concentrations (~1.25×10<sup>-3</sup> mmol mL<sup>-1</sup> each) but with different fractions of <sup>13</sup>C-labeled tripalmitin (Table 5.1). Substrate S1 was the mixture of 1,1,1-<sup>13</sup>C-tripalmitin and bulk tripalmitin (1:99, v:v); substrate S2 was the mixture of 2,2,2-<sup>13</sup>C-tripalmitin and bulk tripalmitin (15:85, v:v); and substrate S3 was the mixture of S1 and S2 (1:1, v:v).

#### 2.3. Experiment setup and treatments

Oxic and anoxic sediment–seawater systems were simulated by setting a thin layer (~ 1.5 mm) of sediment in large water reservoirs with distinct redox conditions (Aller and Mackin, 1989; Sun et al., 1993; Sun and Wakeham, 1998). First, ~2 mL of each substrate solution (S1, S2, and S3 in hexane) was spiked onto ~2 g of sieved, ground, and combusted (at 450 °C for 4 h) fine sediment as a carrier. After solvent was evaporated in a fume hood, each spiked carrier was mixed with ~150 g of collected oxic or anoxic sediments by hand stirring for 30 min. The well-mixed spiked sediments were then used to fill a set of small plug rings (1.5 mm depth and 5.7 cm i.d.) to create thin sediment layers (S1O, S2O, and S3O for oxic incubation; S1A, S2A, and S3A for anoxic incubation). These small rings were placed on the bottom of two seawater reservoirs (~10 L), where oxic and anoxic environments had already been set up by purging with air (for

oxic) or with  $N_2/CO_2$  mixture (for anoxic) for 3 days. Both oxic and anoxic incubations were conducted in the dark at 17°C (close to the in-situ collection temperature of 16.4 °C). The pH of the seawater in the anoxic reservoir was maintained according to the previous study (Ding and Sun, 2005a) with a flow ratio  $N_2:CO_2$  of about 10:1. For both oxic and anoxic environments, one or two small rings were taken out at certain time points over a two-month incubation period. The sediments in each small ring were transferred to a 50 mL polypropylene tube and frozen at -40°C for future lipid analysis and water content measurement.

#### 2.4. Extraction and analysis of fatty acids

The method of lipid extraction, separation, and derivatization was adopted from previous studies (Sun et al., 1998; Sun and Wakeham, 1999). Lipids in thawed sediments were extracted first with 15 mL methanol and then with  $3 \times 15$  mL methylene chloride: methanol (2:1, v:v). During each extraction, samples were sonicated for 10 min right after vortex-shaking. Combined extracts were partitioned into a methylene chloride phase by addition of 20 mL 5% NaCl solution. The volume of total lipid extract (TLE) was reduced to almost dry with a rotary evaporator. The TLE was then saponified at 92°C with 6 mL 0.5 M KOH in MeOH:H<sub>2</sub>O (95:5) for 2 h. After cooling and addition of 1 mL 5% NaCl solution, the neutral lipids were extracted with  $3 \times 6$  mL hexane from the basic solution (pH > 13). After the solution was subsequently acidified (pH < 2) by adding HCl, the fatty acids were extracted with  $3 \times 6$  mL hexane. The fatty acids in the extracts were methylated with 5% BF<sub>3</sub> in MeOH at 92°C for 2 h to form fatty acid methyl esters (FAMEs).

Concentrations of fatty acids (or FAMEs) were quantified by capillary gas chromatography using a Hewlett–Packard 6890 GC with an on-column injector and a flame ionization detector (Sun et al., 1998; Sun and Wakeham, 1999). Compound separation was accomplished with a 30 m × 0.25 mm i.d. HP-5 column (Hewlett-Packard) coated with 5% diphenyl: 95% dimethylsiloxane copolymer, and the operating temperature program was: 50-170°C at 20°C min<sup>-1</sup>, then 170-310°C at 4°C min<sup>-1</sup> and finally held for 10 min at 310°C. Internal standard (nonadecanoic acid methyl ester) for quantification was added to each sample right before the GC analysis. Identification of fatty acid compounds was achieved by a Shimadzu QP-5000 GC–MS system using a split injector and a 30 m × 0.25 mm i.d. XTI-5 column (Resteck) coated with 5% phenyl methyl silicone and helium as carrier gas. The operation conditions for MS were: mass range 50–610 Da with a 0.4 s scan interval; 70 eV ionizing energy; and the GC temperature program was: 50–150°C at 20°C min<sup>-1</sup> followed by 150–310°C at 4°C min<sup>-1</sup> and a 10 min hold for fatty acids at 310°C.

### 2.5. Isotopic analysis of fatty acids

The measurement of stable carbon isotopic compositions of fatty acids was achieved by a Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometer system (GC-C-IRMS) (Hayes et al., 1990). Fatty acids were separated by an Agilent 6890 gas chromatograph with a 30 m  $\times$  0.25 mm i.d. capillary column (DB-1, J & W Scientific) and a split injector installed. The temperature program for the GC was: 50-170°C at 20°C min<sup>-1</sup>, followed by 170-300°C at 4°C min<sup>-1</sup>, and a 15 min hold at 300°C. Helium with a flow rate of 1.5 mL min<sup>-1</sup> was used as carrier gas. Separated fatty acids from the GC were combusted (via a Thermoquest Finnigan GC Combustion III unit) to CO<sub>2</sub> over CuO/Ni/Pt wires at 850°C and water was removed by diffusion through a Nafion selectively permeable membrane (Perma Pure LLC.) flushed with helium. The stable isotopic compositions of CO<sub>2</sub> peaks were measured with a Finnigan Delta V 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 CO<sub>2</sub> and reported in per

mil (‰) against VPDB (Vienna Pee Dee Belemnite). Based on internal standard measurements, the standard deviations of IRMS analyses was  $\pm 0.35\%$  (n = 30) for FAMEs. The  $\delta^{13}$ C values of methanol (43.72  $\pm$  0.15‰) were pre-determined by IRMS to correct stable carbon isotopic compositions of FAMEs due to addition of an extra carbon atom during methylation.

#### 3. Results

#### 3.1. Fatty acid abundances and compositions in natural background sediments

In the natural background sediments, 17 fatty acids (FA) in oxic sediments and 14 fatty acids in anoxic sediments were identified (Fig. 5.1). The total concentration of fatty acids in oxic sediments (~109 µg/g dry sed.) was about 5× higher than that in anoxic sediments (~22 µg/g dry sed.). In oxic sediments, dominant fatty acids were saturated fatty acid (SFA) 16:0 (~18%), monounsaturated fatty acid (MUFA) 16:1( $\omega$ 7) (~19%), polyunsaturated fatty acid (PUFA) 20:5/20:4 (~14%) and bacteria-specific fatty acid (BSFA) 18:1( $\omega$ 7) (~ 12%). In anoxic sediments, major fatty acids were SFA 16:0 (~ 24%) and BSFA 18:1( $\omega$ 7) (~ 10%) while 18:1( $\omega$ 9) and 20:5/20:4 fatty acids were absent. In both oxic and anoxic sediments, concentrations of long chain 20:0, 22:0, 24:0 and 26:0 fatty acids (LCFA) were at similar level (0.7-1.7 µg/g dry sed.). By contrast, the concentrations of SFA, MUFA, PUFA and BSFA in oxic sediments were ~3-33× higher than those in anoxic sediment. More specifically, the concentration of 16:0 fatty acid in oxic sediments was ~ 4× higher than that in anoxic sediments.

## 3.2. Relative compositions of fatty acids in experimental oxic and anoxic sediments

After addition of <sup>13</sup>C-labeled and unlabeled tripalmitins, the relative percentage of 16:0 in three experimental oxic sediments (S1O, S2O and S3O) increased to ~38% while MUFA 16:1( $\omega$ 7), PUFA 20:5/20:4, and BSFA 18:1( $\omega$ 7) remained as major components (~10% for each)

(Fig. 5.2). In the three experimental anoxic sediments (S1A, S2A and S3A), 16:0 fatty acid was the dominate compound (~75%) while all other fatty acids were below 5% (Fig. 5.2).

#### 3.3. Variations in concentrations of natural fatty acids in oxic and anoxic sediments

In experimental anoxic sediments (S1A, S2A, and S3A), concentrations of all naturally occurred fatty acids were low and showed little changes during incubation (data not shown). However, in oxic sediments (S1O, S2O, and S3O), concentrations of major natural fatty acids varied in different patterns (or at different rates) during incubation (Table 5.2, Fig. 5.3). For example, concentration of 14:0 fatty acid decreased slowly but continuously over incubation. Concentrations of 16:1( $\omega$ 7), 18:1( $\omega$ 7), and 20:5/20:4 decreased rapidly in the first 10 days of incubation and then remained at relatively constant levels (~40% for 16:1 and 18:1 and ~10% for 20:5/20:4). Concentrations of LCFA (e.g., 24:0) fluctuated in a small range during incubation.

# 3.4. Variations of 16:0 fatty acid (naturally-occurred and tripalmitin-derived) in oxic and anoxic sediments

Just like other natural fatty acids in the three experimental anoxic sediments (S1A, S2A, and S3A), concentrations of 16:0 fatty acid, including naturally-occurring and tripalmitin-derived, fluctuated in a range of  $\pm 5 \ \mu g/g$  dry sediment and no apparent decreasing trend was observed during incubations (Fig. 5.4). By contrast, in the three experimental oxic sediments (S1O, S2O, and S3O), concentrations of 16:0 fatty acid continuously decreased during incubation (Fig. 5.4). Approximately 50% of total 16:0 fatty acid was lost over two months, which were similar in three cases.

# 3.5. Variations of $\delta^{13}$ C values of 16:0 fatty acid during incubations

In experimental anoxic sediments (S1A, S2A, and S3A), stable carbon isotopic compositions of 16:0 fatty acid fluctuated and no clear variation trend was observed during

incubation (Fig. 5.4). However, in experimental oxic sediments (S1O, S2O, and S3O), when the concentrations of 16:0 fatty acid decreased rapidly in the first 10 days, the  $\delta^{13}$ C values of 16:0 fatty acid declined remarkably (Fig. 5.4). The negative shifts were -10‰, -94‰, and -61‰ relative to the initial values for S1O, S2O, and S3O treatments respectively.

### 4. Discussion

#### 4.1. Effect of redox conditions on lipid degradation

In this study, ~ 26% - 95% of both naturally occurring fatty acids (e.g., 14:0, 16:1, 18:1, and 20:5/20:4) and spiked tripalmitin-derived 16:0 fatty acid were degraded in all treated oxic sediments (S1O, S2O, and S3O) during the two months of incubation (Figs. 5.3 and 5.4). Nonetheless, there was no apparent degradation of either natural fatty acids or tripalmitin-derived 16:0 fatty acid in all treated anoxic sediments (S1A, S2A, and S3A) over two months of experiment (Fig. 5.4).

Some field studies also showed that degradation of lipids was less significant in anoxic sediments than in oxic sediments. For example, Canuel and Martens (1996) observed lipid degradation in deep anaerobic sediments was remarkably slower than that at surface sediment exposed to oxygenated water in Cape Lookout Bight (North Carolina, USA), an area with high annual sedimentation rates (Canuel et al., 1990). Sun and Wakeham (1994) found that the degradation rate constants for most lipid compounds in anoxic Black Sea sediments were an order of magnitude lower than those in coastal sediments (with a top oxic layer).

However, many laboratory studies (Harvey et al., 1995; Sun et al., 1997; Ding and Sun, 2005a) showed that lipid compounds were significantly and comparably degraded in both oxic and anoxic seawaters and sediments. In Harvey et al.'s (1995) experiments, anoxic settings were made by purging previously oxic culture medium with argon, and anaerobic microbial consortia

were introduced to degrade algal derived organic matter in anoxic seawater. In sediment-water incubation systems by Sun et al. (1997) and Ding and Sun (2005a), anoxic sediments were converted from surface oxic sediments (top 1-2 cm). In this study, anoxic sediments were directly collected from deep anoxic layer of sediments (>10 cm). Therefore, the differences in experimental setups and actual anaerobic microbial communities involved in various experimental systems may be a major reason for different effects of redox conditions on lipid degradation observed by various studies.

#### 4.2. Reactivities of fatty acids

In this study, degradation rate constants of naturally occurred fatty acids (e.g., 14:0, 16:1, 18:1, and 20:5/20:4) (Table 5.2) varied with the following order: polyunsaturated > monounsaturated > short-chain (<C<sub>20</sub>) saturated > long-chain (>C<sub>20</sub>) saturated fatty acids, implying that variable reactivities of naturally occurred lipid compounds are likely dependent on the sources where they come from. It was found that phytoplankton-derived fatty acids (e.g., 14:0, 16:1, and 20:5/20:4) had higher loss rates than those vascular plants derived long chain fatty acids (LCFA, e.g., 24:0) (Canuel and Martens, 1996). The Concentrations of LCFA (e.g., 24:0) varied little between the top oxic and deep anoxic sediments and also during incubations in this study, indicating that these lipid compounds from terrestrial sources had been reworked before into marine environments and only the refractory compounds remained by the time of deposition in coastal marine sediments (Canuel and Martens, 1996; Wakeham et al., 1997).

In addition, structures of lipid compounds (if from the same source) also affect their degradation. For example, unsaturation (number of double bonds) in lipid structure has a great influence on degradation of many lipid compounds (Harvey and Macko, 1997). The preferential degradation of polyunsaturated fatty acids relative to other fatty acids was observed through
water column into sediments (Wakeham et al., 1997). In both oxic and anoxic seawater, higher degradation rates of mono- and polyunsaturated fatty acids than those of saturated fatty acids were also observed (Farrington et al., 1977; Wakeham and Beier, 1991; Harvey and Macko, 1997; Sun et al., 1997; Sun et al., 2004).

### 4.3. Alteration of compound-specific isotopic composition of 16:0 fatty acid

Since the microbial degradation is a unidirectional reaction, the isotope fractionation factor ( $\alpha$ ) of unreacted substrate (total 16:0 FA in here) for each treatment (S1O, S2O, and S3O) could be calculated by the Rayleigh equation (e.g., Mariotti et al., 1981; Fogel and Cifuentes, 1993):

$$\mathbf{R}_{s}/\mathbf{R}_{s0} = \mathbf{f}^{(\alpha-1)} \tag{1}$$

or 
$$\ln (R_s/R_{s0}) = (\alpha - 1) \times \ln (f)$$
 (2)

where  $R_{s0}$  is the isotope ratio of the substrate at time zero,  $R_s$  is the isotope ratio of the substrate with time, and  $f = (C_s/C_{s0})$ , is the fraction of substrate that is unreacted. By fitting experimental data using the Rayleigh equation (Fig. 5.5), the fractionation factors ( $\alpha$ ) of 16:0 FA were calculated to be 1.012, 1.140, and 1.069 for S1O, S2O, and S3O treatments, respectively. No matter how the tripalmitins were labeled (either at 1,1,1-<sup>13</sup>C or 2,2,2-<sup>13</sup>C position), the isotopic compositions of remaining 16:0 fatty acid shifted in all cases when significant amounts of compound were degraded. This implies that isotopic alteration of compounds during degradation is probably not caused by kinetic fractionation at specific reaction sites within molecular structure. Instead, the isotopic alteration of compound is likely related to relative reactivities of the compounds in different pools, which carry variable isotopic signals.

Moreover, it was unusual that the  $\delta^{13}$ C values of residual total 16:0 FA shifted negatively ( $\Delta \delta = -10\%$ , -94‰, and -61‰ for S1O, S2O, and S3O treatments, respectively). The isotopic

shifts should be positive if the isotope alteration was primarily induced by kinetic fractionation associated with incomplete and unidirectional processes such as biologically mediated reactions (Hoefs, 2009). Sun et al. (2004) observed positive shifts (up to +4.5‰) of remnant 16:0 fatty acid derived from the haptophyte Emiliania huxleyi after microbial degradation in both oxic and anoxic seawaters, which was attributed to (1) the carbon at the carboxyl group of fatty acids is isotopically lighter than overall carbons bound in the chain (DeNiro and Epstein, 1977); and (2) fatty acids are degraded primarily through decarboxylation (Sun et al., 1997). In the Chapters 3 and 4 of this dissertation, positive isotopic shifts of most fatty acids in two phytoplankton cultures were also observed when these compounds were largely degraded. These positive variations in isotopic compositions are postulated to be a consequence from heterogeneous isotopic distributions of compounds within different cellular structures (formed during different cell growth phases) and selective degradation of compounds between two pools. This study manipulated an experimental system containing a specific compound (16:0 fatty acid) in two pools (spiked and natural sediments) while the spiked compounds had remarkably higher isotopic compositions. If the compounds in the spiked pool (with highly labeled carbon) are more labile than those in natural sediments (with lower isotopic compositions), then the negative shifts in isotopic compositions of the compounds during degradation can be expected.

To clarify the relative reactivities of 16:0 fatty acid in spiked and natural pools, we distinguished <sup>13</sup>C-labeled compounds from the total fatty acid pool and estimated the degradation rate constants of labeled compounds in three oxic incubations. The actual concentrations of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA ( $C_1$ ) in each treatment during microbial degradation can be calculated from the following isotopic mass-balance equations:

$$\delta_{t} = f_{n} \times \delta_{n} + f_{u} \times \delta_{u} + f_{l} \times \delta_{l}$$
(3)

$$\mathbf{f}_{n} + \mathbf{f}_{u} + \mathbf{f}_{l} = 1 \tag{4}$$

where  $\delta_t$ ,  $\delta_n$ ,  $\delta_u$ , and  $\delta_l$  are  $\delta^{13}$ C values of total, natural, unlabeled tripalmitin-derived, and  $^{13}$ C-labeled tripalmitin-derived 16:0 FA, respectively; and  $f_n$ ,  $f_u$ , and  $f_l$  are the fractions of natural, unlabeled tripalmitin-derived, and  $^{13}$ C-labeled tripalmitin-derived 16:0 FA in total 16:0 FA pool, respectively. The  $\delta^{13}$ C values of total 16:0 FA ( $\delta_t$ ) were calculated from the measured  $\delta^{13}$ C values of total 16:0 FAME in this study (after correction of the  $\delta^{13}$ C value of methanol due to methylation). The  $\delta^{13}$ C values of natural and unlabeled tripalmitin-derived 16:0 FA were assumed to be at natural background level (i.e.,  $\delta_n \approx \delta_u \approx -26\%$ ). The  $\delta^{13}$ C values of  $^{13}$ C-labeled tripalmitin-derived 16:0 FA ( $\delta_l$ ) can be calculated by the equation below:

$$\delta_{\rm l} = [(R_{\rm l} - R_{\rm std})/R_{\rm std}] \times 1000\% = 4963\%$$
(5)

in which  $R_1$  is the isotope ratio of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA ( $R_1 = 1/15 = 0.06667$ ), and  $R_{std}$  is the isotope ratio of standard (VPDB: Vienna Pee Dee Belemnite;  $R_{std} = 0.01118$ ). By combining equations (3) to (5), the fraction of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA in total 16:0 FA pool ( $f_1$ ) can be calculated from the following equation:

$$f_{l} = (\delta_{t} - \delta_{n})/(\delta_{l} - \delta_{n}) = (\delta_{t} + 26\%)/(4963\% + 26\%)$$
(6)

Subsequently, the concentrations of  ${}^{13}$ C-labeled tripalmitin-derived 16:0 FA (C<sub>l</sub>) were calculated with the equation below:

$$C_l = f_l \times C_t \tag{7}$$

where  $C_t$  is the measured concentration of total 16:0 FA.

Based on the resolved concentrations of <sup>13</sup>C-labeled 16:0 fatty acids, the degradation rate constants ( $k_d$ ) of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA in oxic sediments (S1O, S2O, and S3O) were estimated by using the first-order multi-G model (Westrich and Berner, 1984; Sun et al., 2004) (Fig. 5.6). The resulting degradation rate constants of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA were 2-3× larger than those of total 16:0 FA in all treatments (S1O, S2O, and S3O) (Table

5.3). This indicated that <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA degraded at an apparently faster rate than naturally occurring 16:0 FA. Consequently, the preferential loss of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA (<sup>13</sup>C enriched 16:0 FA) in total 16:0 FA pool during microbial degradation led to the significant negative shifts of  $\delta^{13}$ C values of remaining total 16:0 FA in all three oxic sediments (S1O, S2O, and S3O).

In this study, we defined the 'apparent enrichment factors ( $\epsilon$ )' for each treatment (S1O, S2O, and S3O) since the isotopic shifts might not be caused by kinetic isotopic fractionation rather than due to selective degradation of compounds in two pools with distinct isotopic compositions. The 'apparent enrichment factors' were calculated by the following equation (e.g., Mariotti et al., 1981; Fogel and Cifuentes, 1993):

$$\varepsilon = (\alpha - 1) \times 1000 \tag{8}$$

It was noted that there was a strong linear relationship between the 'apparent enrichment factors ( $\epsilon$ )' (12.1, 139.7, and 68.5 for S1O, S2O, and S3O, respectively) and the initial proportions of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA in total FA: the higher proportion of <sup>13</sup>C-labeled tripalmitin-derived 16:0 FA in total FA, the larger the 'apparent enrichment factor' (Fig. 5.7). Thus, it further confirmed that isotopic alteration of 16:0 fatty acid during degradation was caused by the relative reactivities of the compounds from two pools with distinct isotopic compositions.

### 4.4. Geochemical implications

Phytoplankton produced lipid compounds in surface water (during either bloom or nonbloom period) can be partially transported to underlying surface sediments in various forms (dead cells, fecal pellets, and adsorbed on mineral particles) while they have different biolabilities (or reactivities) for microbial degradation processes (Keil et al., 1994; Mayer, 1994; Hedges and Keil, 1995). From previous chapters of this dissertation, we also know that lipids are produced in different cellular structures with different isotopic distributions and selective degradation of lipids between different structural pools can result in diversified isotopic alterations of compounds. Therefore, kinetic isotopic fractionation may not be responsible for isotopic alteration of lipid compounds during degradation. In this study, we further prove that if the lipid compounds carry heavier isotopic compositions and are more reactive than those with lighter isotopic compositions and more refractory during degradation processes, the isotopic alteration will be negative for remaining compounds. Therefore, the relative reactivities of lipid compounds between different pools are one major cause for isotopic alteration during degradation processes. In natural environments, of course, there are many other factors affecting isotopic signals of biomarkers but they are hard to distinguish from the effect of degradation. More studies are needed to compare laboratory results with field data to eventually understand the mechanisms which drive isotopic alteration of organic compounds during biogeochemical cycling.

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Treatments	Oxic			Anoxic			
	S10	S2O	S3O	S1A	SA	S3A	
f <sub>1,1,1-TP</sub> (%)	0.66	0	0.31	0.90	0	0.445	
f <sub>2,2,2-TP</sub> (%)	0	8.85	4.65	0	13.35	6.675	
$\mathrm{f}_{\mathrm{bulk-TP}}(\%)$	65.34	50.15	57.04	89.10	75.65	81.88	
$f_{natural}$ (%)	34	41	38	10	11	11	
$\delta^{13}C_{\text{ calculated }}(\%)$	13	424	229	25	649	337	
$\delta^{13}C_{\text{measured}}$ (‰)	11.7±0.2	476.1±0.3	250.2±0.1	23.3±4.3	630.7±18.1	314.0±5.3	

Table 5.1. Parameters of stable carbon isotopic treatments in initial oxic (S1O, S2O, and S3O) and anoxic (S1A, S2A, and S3A) incubation experiments.<sup>1</sup>

<sup>1)</sup> f <sub>1,1,1-TP</sub> and f <sub>2,2,2-TP</sub> are the fractions of 1,1,1-<sup>13</sup>C-tripalmitin and 2,2,2-<sup>13</sup>C-tripalmitin derived C<sub>16:0</sub> fatty acid in total C<sub>16:0</sub> fatty acid in total C<sub>16:0</sub> fatty acid pool; f <sub>bulk-TP</sub> is the fraction of unlabeled tripalmitin derived C<sub>16:0</sub> fatty acid in total C<sub>16:0</sub> fatty acid pool; and f <sub>natural</sub> is the fraction of natural sedimentary C<sub>16:0</sub> fatty acid in total C<sub>16:0</sub> fatty acid pool.  $\delta^{13}$ C <sub>calculated</sub> and  $\delta^{13}$ C <sub>measured</sub> are calculated and measured stable carbon isotopic compositions of initial total 16:0 FA in sediments.

Oxic treatment	14:0 FA		16:1(ω7) FA		18:1(ω7) FA		20:5/20:4 FA	
	$k_{d}\left(d^{\text{-}1}\right)$	$\mathbb{R}^2$	$k_d (d^{-1})$	$\mathbb{R}^2$	$k_d (d^{-1})$	$R^2$	$k_d (d^{-1})$	$\mathbb{R}^2$
S10	-0.009	0.67	-0.068	0.90	-0.135	0.82	-0.365	0.96
S2O	-0.007	0.82	-0.073	0.69	-0.146	0.97	-0.361	0.96
S3O	-0.007	0.46	-0.078	0.77	-0.161	0.80	-0.360	0.97

Table 5.2. Degradation rate constants  $(k_d)$  of naturally occurred fatty acids in oxic sediments (S1O, S2O, and S3O).

Treatments		Total 16	5:0 FA	<sup>13</sup> C-labeled 16:0 FA	
		$k_d (d^{-1})$	$R^2$	$k_d (d^{-1})$	$R^2$
Oxic	S10	0.010	0.73	0.034	0.85
	S2O	0.010	0.82	0.027	0.85
	S2O	0.013	0.87	0.029	0.90
	S1A	< 0.001	0.05	/	/
Anoxic	$S2A^*$	0.001	0.05	/	/
	S3A <sup>*</sup>	< 0.001	0.04	/	/

Table 5.3. Degradation rate constants ( $k_d$ ) of total and <sup>13</sup>C-labeled tripalmitin-derived16:0 fatty acids in oxic (S1O, S2O, and S3O) and anoxic (S1A, S2A, and S3A) sediments.

\* Without day 64.



Fig. 5.1. Concentrations of fatty acids in natural background oxic and anoxic sediments.



Fig. 5.2. Relative fatty acid compositions in treated (with addition of <sup>13</sup>C-labeled and non-labeled tripalmitins) oxic and anoxic sediments.



Fig. 5.3. Variations in concentrations of naturally occurred fatty acids in oxic sediment during incubations.



Fig. 5.4. Variations in concentrations and isotopic compositions of total 16:0 fatty acid (including naturally-occurred and added tripalmintin-derived) in oxic and anoxic sediments during incubation.



Fig. 5.5. Estimation of isotopic fractionation factor ( $\alpha$ ) of total 16:0 fatty acid by curve fit with Rayleigh equation.



Fig. 5.6. Variations in concentrations of <sup>13</sup>C-labeled tripalmitin-derived 16:0 fatty acids in oxic sediments (S1O, S2O, and S3O) during incubation.



Fig. 5.7. The relationship between 'apparent enrichment factor' ( $\epsilon$ ) and the initial fractions of <sup>13</sup>C-labeled tripalmitin-derived 16:0 fatty acid in the total 16:0 fatty acid pools of three treatments (S1O, S2O, and S3O) in oxic sediments.

### **CHAPTER 6**

#### GENERAL SUMMARY

The impacts of biogeochemical processes on phytoplankton-produced lipid biomarkers (phytol, sterols, alkenones, and fatty acids) and their stable carbon isotopic compositions were examined through a series of laboratory studies. The variations of chemical and isotopic signals of lipid compounds were followed during algal cell exponential growth and stationary phases, cell respiration (auto-metabolism), and microbial degradation of senescent cells (after respiration) and intact cells (from exponential growth and stationary phases, respectively). Furthermore, the relative roles of kinetic isotopic fractionation vs. chemical reactivity of lipids in altering compound-specific isotopic compositions during microbial degradation in marine oxic and anoxic sediments were clarified.

# 1. Effects of *E. huxleyi* cell growth, respiration, and microbial degradation on alkenone compositions and alkenone-based paleotemperature index $U_{37}^{K'}$

The  $U_{37}^{K'}$  index shifted positively (+0.13 unit) in cell exponential growth phase but negatively (-0.1 unit) in stationary phase, which can be caused by nutrient conditions, the physiological state of cells, and relative production rates of di- and tri-unsaturated alkenones between different intracellular structures. During cell respiration, ~50% of cellular alkenones were lost, clearly indicating the role of alkenones as intracellular storage components. The  $U_{37}^{K'}$ index remained constant during cell respiration, resulting from the non-selective degradation of intracellular storage alkenones by cell auto-metabolism. It is possible that alkenones were mainly bound with membrane components of intact cells from exponential growth phase, while for intact cells from stationary phase alkenones were largely associated with intracellular storage component. Alkenones in senescent cells (after respiration) were likely associated with membrane component. During microbial incubation, bacteria might non-selectively degrade alkenones bound in one cellular structure (membrane) but selectively degrade alkenones in two cellular structures (membrane and intracellular storage component). Therefore, the variations in  $U_{37}^{K'}$  index were dependent on proportions of alkenones distributed between different intracellular structures, which was controlled by the physiological state (growth phase) of phytoplankton cells.

## 2. Effects of *T. weissflogii* and *E. huxleyi* cell growth, respiration, and microbial degradation on lipid biomarkers and associated stable carbon isotopic compositions

Relative compositions of lipid compounds varied through different cell growth phases. During cell growth, *T. weissflogii* produced a variety of neutral lipids and fatty acids at similar rates between phase I and phase II, whereas *E. huxleyi* produced neutral lipids at faster rates but fatty acids at slower rates in phase II than in phase I. Fatty acids and alkenones were associated either with cell membrane or with intracellular storage components, while phytol and sterols were associated mostly with chloroplast thylakoid membrane and algal cell membrane, respectively. The compound-specific isotopic compositions of lipids in both *T. weissflogii* and *E. huxleyi* cultures followed a similar pattern: significant positive shift (+5‰ to +10‰) of  $\delta^{13}$ C values in phase I, and relatively constant or small negative alteration (-2‰ to -3‰) of  $\delta^{13}$ C values in phase II. Accordingly, speculation has been put forward based on observations from previous and my studies: (1) during cell exponential growth phase, lipid compounds were biosynthesized mainly as structural components (e.g., phospholipids in cell membranes) while their stable carbon isotopic compositions shifted positively and were therefore heterogeneously distributed in the membrane-associated components; (2) during cell stationary growth phase, lipid compounds were produced mainly as energy storage components (e.g., alkenones in lipid vesicles of *E. huxleyi* cells) while their isotopic compositions were relatively constant and were thus homogenously distributed.

During dark respiration (auto-metabolism), *E. huxleyi* used alkenones and *T. weissflogii* used fatty acid-associated compounds (e.g., triacylglycerols) as main energy sources, respectively. Since phytol and sterols were largely associated with membrane components and did not function as energy storage compounds, concentrations of these compounds in the two cultures decreased much less than fatty acids or alkenones. No matter how much lipid compound was lost during cell respiration, the  $\delta^{13}$ C values of most compounds (except phytol of *T. weissflogii*, with a ~-3‰ shift) remained relatively constant (in a range of <±1‰) because only lipids in intracellular storage component with homogeneous distribution of isotopic compositions were selectively utilized for auto-metabolism.

During microbial degradation of senescent cell (after respiration), most fatty acids (except 16:0 fatty acid from *E. huxleyi*) were lost, but significant amounts of sterols (>30%) from both species and alkenones (~20%) from *E. huxleyi* survived. The isotopic compositions of individual lipids varied in three different ways: positive shift (>+2‰) for phytol and most fatty acids; little change or small fluctuation (within  $\pm$ 1‰) for sterols and alkenones; and negative shift (>-2‰) for 20:5 FA from *T. weissflogii*. Selective degradation of fatty acids bound in different cellular components (membrane and storage) with possible heterogeneous distribution of isotopic composition could result in isotopic alteration of remaining fatty acids. Degradation

of alkenones was probably dominated by loss of compounds in the large storage pool (compared to the very small membrane pool), resulting in little isotopic alteration. Because sterols are associated only with membrane and degradation of sterols is primarily through inter-conversion pathways without a change in carbon number, no isotopic alteration was observed for remnant sterols. The mechanisms causing positive and negative isotopic alteration of phytol in both cell respiration and microbial degradation are unclear, although there is clear evidence that substantial intramolecular isotopic heterogeneity of phytol exists due to the different biosynthesis pathways from different precursors by phytoplankton.

## 3. Effects of microbial degradation of intact *E. huxleyi* cells on lipid biomarkers and associated stable carbon isotopic compositions

Intact *E. huxleyi* cells were harvested in cell exponential growth phase (culture 1) and stationary phase (culture 2), respectively. There were significant differences in cellular lipid contents and relative compositions between two cultures: ~8× 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. After microbial degradation, most fatty acids (except for 16:0) from two cultures were nearly totally degraded while most neutral lipids (phytol, sterols and alkenones) (except for  $27\Delta^5$  from culture 1) were partially degraded with relatively higher percentages of these compounds remaining in culture 2 than in culture 1. The stable carbon isotopic compositions of lipids varied diversely among compound classes: positive shift (+1.7‰ to +3.9‰) in  $\delta^{13}$ C for various fatty acids; little change for sterols; and for alkenones either little change in most cases or negative shift (-3‰) in one case (37:2 alkenone in culture 1).

Moreover, degradation of fatty acids from culture 1 caused a relatively larger positive shift (~ 1.5‰ more each) for 16:0 and  $18:1(\omega 9)$  compared to that from culture 2, and it is because more membrane-associated fatty acids with heterogeneous distribution of isotopic compositions in culture 1 were preferentially degraded compared to culture 2. The difference in isotopic shifts between two cultures after microbial degradation 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.

The differential isotopic variations between 37:3 and 37:2 alkenones could be caused by distinct degradation pathways of different alkenone compounds, but more studies are still necessary to elucidate the contrasting variations in alkenone isotopic compositions.

## 4. Relative roles of kinetic isotopic fractionation vs. chemical reactivity in altering molecular isotopic signals during microbial degradation in sediments

Natural oxic and anoxic marine sediments were spiked with <sup>13</sup>C-labeled (labeling at carbonyl and non-carbonyl carbon positions of fatty acid chains) and unlabeled tripalmitins and then incubated in oxic and anoxic sediment-water systems, respectively. In anoxic sediments, neither natural fatty acids nor tripalmitin-derived 16:0 fatty acid were apparently degraded and therefore no significant variation in stable carbon isotopic composition of 16:0 fatty acid was observed. By contrast, in oxic sediments, both spiked tripalmitin-derived 16:0 fatty acid and naturally occurred fatty acids were obviously degraded. Tripalmitin-derived 16:0 fatty acid degraded at an at least 2-3× higher rate compared to that in natural sediments, while isotopic compositions of 16:0 fatty acid shifted negatively during oxic incubation. It appears that the extents of isotopic shift are dependent on the relative proportions of spiked <sup>13</sup>C-labeled compound in the sediments but not related to the labeling position of <sup>13</sup>C in the molecular

structure. These results provided direct evidence that the relative chemical reactivities of lipid compounds from different sources (or different pools), which originally have heterogeneous isotopic signatures, may lead to alterations in molecular isotopic composition during microbial degradation in natural environments.

### 5. Future directions

In this dissertation, the stable carbon isotopic compositions of lipid compounds (phytol, sterols, alkenones, and fatty acids) in different cellular structures (membrane vs. intracellular storage component) during different growth phase (exponential vs. stationary growth) have not been directly measured. Therefore, further studies are necessary to clarify the heterogeneous distribution of isotopic composition in lipid compounds associated with various cellular components in different cell growth phases. Batch cultures and continuous chemostatic cultures should be conducted to simulate different cell physiological states (or growth phases). Collected algal cells can be fractionated with suitable methods into certain cellular components (e.g., chloroplast fraction, lipid body fraction, and cell membrane fraction). Lipid compounds and their isotopic compositions associated with these components should be analyzed to further elucidate the status of isotopic distribution of lipid compounds in various cellular components during cell different physiological states.