INFLUENCES OF CELL GROWTH RATE AND CELLULAR STRUCTURES ON MOLECULAR STABLE CARBON ISOTOPIC COMPOSITION OF PLANKTONIC FATTY ACIDS

by

QIANHUI QIN

(Under the Direction of Ming-Yi Sun)

ABSTRACT

In order to explore the influence of intracellular structures and cell growth rates on the stable carbon isotopic compositions of cellular fatty acids in marine phytoplankton, two series of culturing experiments of a typical diatom, *Thalassiosira pseudonana*, were conducted: (1) batch culturing through exponential and stationary growth stages to study the influence of cellular structures on molecular carbon isotopic compositions; and (2) culturing in continuous culture environments with four growth rates to examine the influence of cell growth rate on molecular carbon isotopic compositions of fatty acids bound in cellular components. The results from all experiments showed that $\delta^{13}C$ values of unsaturated fatty acids in intracellular energy storage component were enriched (1-2‰) than those in membrane associated component. With the decrease in growth rate, the $\delta^{13}C$ values of unsaturated fatty acids were generally enriched (1-5‰). The result of this research can potentially influence the applications of fatty acid biomarkers to study sources of organic matters and food webs.
INDEX WORDS: cellular fatty acids of *Thalassiosira pseudonana*, molecular stable carbon isotopic composition, batch and continuous cultures, cell growth stages and rates
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DEDICATION

This thesis is dedicated to my dear parents, Zhijie Qin and Lihua Xie. Their support, love and encouragement make me able to pursue my dream; to my maternal grandfather, Guilin Xie, who passed away while I was studying in the U.S.; and to my fiancé, Zuopin Qin, who makes my life delightful and colorful.
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1.1 LITERATURE REVIEW

Accumulating evidence suggests that the global climate is now changing as a result of human activities—most importantly, the release of greenhouse gases from fossil fuels (McMichael et al., 2004). The increases of greenhouse gases, especially CO$_2$, has led to significant changes of environmental conditions, such as sea level rising, ice coverage decline in polar areas and ocean acidification. (Caldeira and Wickett, 2003; Feely et al., 2004; Semiletov, 2004; Ridley et al., 2005; Kennedy, 2012). Increasing concerns about global climate changes require a comprehensive understanding of both the carbon cycle and the paleo-history record (Sun et al. 2004). Each year, fossil fuel burning contributes about $5.3 \times 10^{15}$ g of CO$_2$ 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).

Solubility pump and biological pump are the two main processes of carbon cycling in the ocean. Biological pump is essential to the carbon cycling in the ocean. The atmospheric CO$_2$ would be around 480 ppm instead of 400 ppm without the biological pump. Marine phytoplankton produces approximately $5 \times 10^{16}$ g organic carbon per year in the ocean (Siegenthaler and Sarmiento, 1993; Hedges et al., 1997), which is the second largest organic carbon production in earth biosphere (Harvey et al., 1995). Roughly 70
percent of the CO$_2$ taken up by phytoplankton is recycled near the surface, and the remaining 30 percent sinks into the deeper waters before being converted back into CO$_2$ by marine bacteria (Falkowski et al. 2000). Only about 0.1 percent of the organic carbon fixed at the surface reaches the seafloor to be buried in the sediments. (Sabine et al., 2004). Despite the small portion of carbon preserved in the sediments, organic matter preserved in marine sediments retains substantial information regarding the history of past productivity, paleoclimate change, and paleoenvironmental conditions (Dean et al., 1986; Hollander and McKenzie, 1991; Summons, 1993).

An important method for inferring the biological source and the ecological setting in which organisms existed is the use of isotopic tracers of carbon in organic matter (Fogel and Cifuentes, 1993). Stable isotope compositions of bulk organic matter have integrative signals from processes that have occurred over the life of the organism, whereas those of individual compounds can record specific events in life (Fogel and Cifuentes, 1993). Isotope techniques, especially compound-specific isotopic composition measurements, have stimulated extensive studies of carbon cycling and showed great promise for paleoceanography (Hayes et al., 1990; Jasper and Hayes, 1990; Freeman et al., 1992; Macko et al., 1994; Pagani et al., 1999, Pagani et al., 2002; Sun et al., 2004). The isotopic composition of the molecule can indicate the isotopic composition of the parent organism and that, in turn, can reveal the carbon source utilized by the producer and thus its position within the ancient ecosystem. Because these factors are in turn dependent on environmental conditions, the distribution of $^{13}$C among natural products is a sensitive paleoenvironmental indicator and can provide a great deal of information about ancient biogeochemical processes (Hayes, 1993).
Lipids, as one of major fractions of phytoplankton biomass, play an important role in controlling growth and reproduction of many marine animals (Volkman et al., 1989; Mansour et al., 2003). Molecular structures of lipids are more specific and source related compared to proteins and carbohydrates, so they have been widely used as biomarkers to study carbon cycle and paleoceanography (Gagosian et al., 1980; Volkman et al., 1986). The applications of the isotopic signals of lipids include the reconstruction of sea surface temperature (SST) using alkenones and their $\delta^{13}C$ signals (e.g., Brassell et al., 1986; Prahl and Wakeham, 1987); the estimation of partial pressure of CO$_2$ ($p$CO$_2$) using alkenones and their $\delta^{13}C$ signals (Jasper and Hayes, 1994; Pagani et al., 1999; Pagani et al., 2002); the proof of the enhancement of the biomass of archaea through anaerobic oxidation of methane at the gas hydrate deposits in the Gulf of Mexico using GDGT and their $\delta^{13}C$ signals (Zhang et al., 2002) etc.

In spite of the wide applications of biomarkers and associated molecular isotopic compositions, the fundamental assumption of the applications 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; Pagani et al., 1999). However, there are many uncertainties about the linkage between surface water parameters and sedimentary signals (Sun et al., 2004). There are also more and more evidence indicating diversified changes in isotopic signals owing to biogeochemical cycling processes in the ocean. For example, 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 $^{13}C$ (~5‰ decrease); Teece and Fogel (2007) reported that during the degradation of salt
marsh plant *Spartina alterniflora*, arabinose was depleted in $^{13}$C by -4‰ while glucose was enriched by 5‰; Sun et al. (2004) observed 4‰-6‰ negative shifts of alkenones during the oxic and anoxic microbial degradation of *Emiliania huxleyi*, while for the fatty acids, there were 2.5‰-7‰ positive shifts during the same degradation process. Pan (2012) reported significant positive isotopic shifts of various lipids (5‰-10‰) through the exponential growth stage of *Thalassiosira weissflogii* and *Emiliania huxleyi*.

It is now believed that cellular lipid yield and composition of microalgae cells are greatly affected by the biosynthesis factors such as different cell growth stages, cellular structures and different growth rates (Dunstan et al., 1993; Brown et al., 1996; Zhu et al., 1997; Hatate et al., 1998; Mansour et al., 2003). In general, microalgae biosynthesize lipid compounds are mainly phospholipids and triacylglycerols in cell membrane and intracellular energy storage component (Dunstan et al., 1993; Brown et al., 1996; Zhu et al., 1997; Mansour et al., 2003; Lv et al., 2010). And the relative proportions of membrane and intracellular storage fatty acids varied along different growth phases (Lv et al., 2010).

Besides the yield and composition of lipids, the isotopic compositions of algal lipids are also affected by the biosynthesis factors. Algal growth stages and rates, carbon acquisition mechanism, compound synthesis pathways (Laws et al., 1995; Bidigare et al., 1997; Popp et al., 1998a; Riebesell et al., 2000; Benthien et al., 2007), and cell size and geometry (Popp et al., 1998b; Burkhardt et al., 1999) are examples of the biosynthesis factors. However, there is little understanding of how these biosynthesis factors affect the yield of cellular lipids and the stable carbon isotopic composition of lipids quantitatively.
1.2 HYPOTHESE

Based on literatures and previous researches, two hypotheses were proposed to illuminate the influences of two important biosynthesis factors—cellular structures and growth rates on molecular stable carbon isotopic composition of planktonic fatty acids:

(1) Phytoplankton biosynthesizes lipid compounds as membrane and intracellular energy storage components over different growth stages, which may result in heterogeneity in carbon isotopic compositions of fatty acid compounds bound in different cellular structures;

(2) Different growth rates of phytoplankton at the exponential stage may result in heterogeneity in stable carbon isotopic compositions of fatty acid compounds bound in different cellular components.

1.3 OBJECTIVES

The major objective of this research is to explore the influences of biosynthesis factors, including cellular structures, cell growth stages and growth rates on the isotopic compositions of phytoplankton-produced fatty acids. The specific objectives are:

(1) To characterize cellular fatty acid compositions of the testing diatom.

(2) To differentiate fatty acids bound in cell membrane and storage component.

(3) To determine molecular stable carbon isotopic compositions of fatty acids bound in different cellular structures.

(4) To study the effects of different cell growth rates on cellular fatty acid compositions and their isotopic compositions.
CHAPTER 2

METHODS

2.1 MATERIALS

Marine diatom *Thalassiosira pseudonana* (NCMA1335) was used in this research. *Thalassiosira pseudonana* is a common centric diatom. *Thalassiosira pseudonana* strain 3H was isolated in September 1958 from a shallow embayment on the south shore of Long Island, New York. This species has a worldwide distribution, including Canada, Great Lakes, Pacific Ocean, Australia, Britain, Spain, East China Sea etc. It has had a large impact on algal research as a model organism. In 2004, its complete genome was sequenced, making this strain a valuable laboratory tool for a wide range of scientific investigation (Armbrust et al., 2004). It is also commonly utilized by aquaculture and algal biomass industries. Besides, the main fatty acids of *Thalassiosira pseudonana* have been well studied. Volkman et al. (1989) extracted and measured that the main fatty acids of *Thalassiosira pseudonana* are 14:0, 16:0, 16:1, 16:2, 16:3, 16:4, 18:4, 20:5/4. These fatty acids account for over 95% of the total fatty acids of *Thalassiosira pseudonana*.

Marine diatom *Thalassiosira pseudonana* (NCMA1335) was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA). The strain of *Thalassiosira pseudonana* was originally isolated from Moriches Bay, Forge River, Long Island, New York, USA (40.756° N, 72.82° W). The strain was made and remained to be axenic (confirmed by routine tests) in the NCMA. Seawater was collected
from the Gulf of Mexico (28°16'N, 91°59'W; in September 2006 and 28°10'N, 91°36'W; in May 2008) and from the South Atlantic Bight (31°13'N, 79°35'W; in September 2014) by directly pumping surface (1 m) water into carboys (10 liters and 20 liters). The seawater used for medium preparation was filtered through the 0.7µm pore size Whatman GF/F (WHA1825047 ALDRICH, Whatman glass microfiber filters, Grade GF/F circles, 47 mm, 100/pk) glass microfiber filters (precombusted at 450 °C for 4 h) and then sterilized by autoclaving. Medium f/2 was used as maintenance and basal experimental medium for *Thalassiosira pseudonana*. Medium f/2 contains nutrients, trace metal and vitamin for phytoplankton growth. The concentrations of nutrients, trace metal solution and vitamin solution and the preparation of f/2 medium was conducted according to the protocols recommended by the NCMA (Guillard and Ryther, 1962; Guillard, 1975). The seawater is stored in carboys until culturing experiments.

2.2 EXPERIMENTAL SETUP

In order to test the two hypotheses of this research, two series of experiments were designed.

The first series of experiment was the batch culture of *Thalassiosira pseudonana*. It was designed to determine the heterogeneity in molecular stable carbon isotopic compositions of fatty acids bound in different cellular structures. *Thalassiosira pseudonana* was batch cultured (in duplicate) in ~250 mL f/2 medium in two 500 mL conical flasks at a constant temperature of 17°C under a light:dark (12 h:12 h) regime with a constant light intensity (105±5 µmol photons m⁻² s⁻¹). Illumination was provided by cool-white fluorescent lamps. The cultures was hand shaken once daily and cell
densities in each culture was counted using Z2 particle count and size analyzer (Beckman Coulter, Brea, CA, USA) every day. Algal cells were collected at exponential growth stage (day 5) and stationary stage (day 18) respectively.

The second series of this study was designed to examine the influence of growth rates of phytoplankton cells at the exponential growth stage on the molecular isotopic compositions of phytoplankton-produced fatty acids. Continuous culture was used in this series of experiment to make sure that the growth chemical environment is at chemostat. A chemostat is a bioreactor to which fresh medium is continuously added, while culture liquid is continuously removed to keep the culture volume constant (Novick et al., 1950). By controlling the rate with which medium is added to the bioreactor, the growth rate of microorganisms can be controlled. In steady state, growth occurs at a constant rate and all culture parameters remain constant (nutrient and product concentrations, pH, cell density, etc.). Microorganisms grown in chemostats naturally strive to steady state: if a low amount of cells are present in the bioreactor, the cells can grow at growth rates higher than the dilution rate, as growth is not limited by the addition of the limiting nutrient. However, if the cell concentration becomes too high, the amount of cells that are removed from the reactor cannot be replenished by growth, as the addition of the limiting nutrient is insufficient. This results in a steady state. At steady state, the specific growth rate of the microorganism is equal to the dilution rate. And the dilution rate is defined as the rate of flow of medium over the volume of culture in the bioreactor:

\[
\text{Growth rate} = \text{Dilution rate} = \frac{\text{Medium flow rate}}{\text{Culture volume}}
\]

The design of continuous culture flasks is shown in Fig. 1. Two Pyrex 2 L (R1, R2 and R3)/4L (R4) conical reaction flasks served as continuous culturing chambers. The
both flasks have an S shape tube extended from the bottom of the flask to the 1 L (R1, R2 and R3)/2L (R4) scale height, which enabled surplus medium with algal cells flowing out from the chambers. Air can come into the flask through the 0.2 µm pore size syringe filter (Thermo Scientific Nalgene Syringe Filters – 25 mm Diameter; Acrylic housing; SFCA; 0.2 µm pore; Sterile; 50/CS 190-2520). *Thalassiosira pseudonana* cells were batch cultured in the two continuous culturing chambers for 5 days first. On day 5, the stirring plates were open and cultures were stirred with Teflon coated magnetic stirring bars and the fresh medium was introduced into the growth chamber through autoclaved 0.89 mm silicone tubing (Microbore Tubing 0.89 mm Silicone for Carter Pump) at different controlled rates by Carter Pump (Thermo Scientific FH100M Multichannel Peristaltic Pumps; Flow capacity: 0.007 to 126 mL/min). The velocity of medium introduced into the flask was controlled by the Carter pump. Continuous culture was conducted four times in duplicate with four different sets of medium dilution rates, which were the same to the growth rates of *Thalassiosira pseudonana* cells in the continuous culture chambers. The actual dilution rates are shown in Table.1. Sampling of the cells in growth chamber was done until four doubling times have elapsed at each dilution rate, by which time cell counts have stabilized (Laws et al., 2011).

2.3 SAMPLING AND CELL COUNTING

Culture growth was monitored daily using the measurements of cell concentrations. During both the batch culture and continuous culture processes, cell density of each growth chamber was counted by Z2 particle count and size analyzer (Beckman Coulter, Brea, CA, USA) every day by taking 1 mL culture sample from the
chamber.

Transfer and sampling of cultures were carried out in a clean hood, which was pre-irradiated with a UV lamp for 10 min. Before sampling, the flasks were gently shaken by hand for a while to make the cell culture uniform. Subsamples (30 or 60 mL, depending on cell densities) were collected at certain time points (for the batch culture, the middle point of exponential growth stage and stationary stage; for the continuous culture, when the growth chamber is at steady state) by filtering the cultures through 1.5 µm pore size glass microfiber filters (Whatman 934-AH) and then stored at −40 °C for future lipid extraction and analysis.

2.4 EXTRACTION, SEPARATION AND ANALYSIS OF LIPIDS

Lipid extraction, separation, and derivatization were done following the procedure described in previous studies (Sun et al., 1998; Sun and Wakeham, 1999). Lipids in experimental samples was extracted with 3 × 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 ~75 mL 5% NaCl solution. The volume of total lipid extract (TLE) was reduced to nearly dry by rotary evaporation. Then 3×1.33 mL hexane was used to wash and transfer TLE into clean vials. The total volume of TLE is 4 mL.

1 mL TLE was directly saponified at 92 °C with 6 mL 0.5 M KOH in MeOH:H₂O (95:5, v:v) for 2 h. After cooling and the addition of 1 mL 5% NaCl solution, the neutral lipids, including sterols, phytol and fatty alcohols, was first extracted from the basic solution (pH > 13) with hexane. Since the focus of this research is fatty acids, the neutral
Lipids extractions were stored in 4 °C refrigerator and not analyzed. Subsequently, the solution was acidified (pH < 2) by adding 36 drops of HCl and the fatty acids was extracted with $3 \times \sim 6 \text{ mL}$ hexane. The fatty acids was methylated with 5% BF$_3$-MeOH at 92 °C for 2 h to form fatty acid methyl esters (FAMEs).

1 mL TLE was passed through Si-gel column (6 mm i.d. and 14 cm in length) to separate membrane and intracellular fatty acids. Si-gel (Whatman Science Company, 63-200 mesh) was pre-cleaned by solvent extraction and high temperature combustion (at 450°C) and deactivated by 5% water. This 1 mL TLE was separated into six fractions by eluting with the following solvents: (1) 10 ml hexane; (2) 10 ml 10% ethyl acetate+90% hexane; (3) 10 ml 20% ethyl acetate+80% hexane; (4) 10 ml 50% ethyl acetate+50% hexane; (5) 10 ml ethyl acetate; (6) 10 ml methanol. Fractions (2) and (3) are for intracellular lipids and fraction (6) is for membrane lipids respectively. We confirmed the separation of two standards as representatives of intracellular and membrane components (tripalmitin eluting 70% in fraction 2 and 30% in fraction 3 and L-α-phosphatidylcholine-β-oleoyl-γ-myristoyl~100% in fraction 6). Six fractions were further saponified with KOH/MeOH to separate neutral and acidic lipids. Neutral lipids were extracted from the basic solution and fatty acids were extracted after addition of HCl (pH < 2). Fatty acids in the extracts were methylated with BF$_3$-methanol to form FAMEs.

The other 2 mL of TLE was sealed in 4 mL clean vials and stored in 4 °C refrigerator.

Concentrations of fatty acids were quantified by capillary gas chromatography using a Hewlett-Packard 6890 GC with an on-column injector and a flame ionization detector. Compound separation was achieved by a 30 m × 0.25 mm i.d. column coated
with 5%-diphenyl-95%-dimethylsiloxane copolymer (HP-5, Hewlett-Packard). An internal standard (nonadecanoic acid methyl ester) was added to the samples immediately prior to GC analysis to aid in quantification. The operation temperature program was: 50-170ºC at 20ºC/min, followed by 170-310ºC at 4ºC/min and held at 310ºC for 5 min. Internal standard nonadecanoic acid methyl ester were added to each corresponding sample before the GC analysis to aid in quantification.

Selected samples were analyzed by gas chromatography-mass spectrometry (GC-MS) to identify fatty acid structures. GC-MS analysis was performed on an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C mass selective detector (GC-MS). A DB5-MS capillary column (30 m x 0.25 mm I.D. and film thickness of 0.25 mm) was used with helium as the carrier gas at a constant flow rate of 1.3 mL min⁻¹. The injector and MS source temperatures were maintained at 280 ºC and 230 ºC, respectively. The column temperature program consisted of injection at 65 ºC and hold for 2 min, temperature increase of 6 ºC min⁻¹ to 300 ºC, followed by an isothermal hold at 300 ºC for 15 min. The MS was operated in the electron impact mode with an ionization energy of 70 eV. The scan range was set from 50 to 650 Da. The samples were analyzed in the splitless mode.

2.5 ISOTOPIC ANALYSIS OF FATTY ACID COMPOUNDS

Compound-specific stable carbon isotopic analyses was carried out using Thermo Scientific GC (Trace 1310)-Isolink (GC Isolink II)-Interface (ConFlo IV)-IRMS (Mat 253), which is available at the University of Georgia’s Center for Applied Isotope Studies. Specifically, the δ¹³C values of lipid compounds were determined using a GC-C
(combustion of separated compounds into CO₂)-IRMS. The carbon isotopic values were calculated based on working standards (UHP CO₂ for δ¹³C), calibrated to international reference materials PDB for δ¹³C, and corrected for addition of extra carbon atoms during their derivatizations. In addition, internal standards, of well-defined isotopic abundance, were used to confirm accuracy during GC-IRMS analyses. Analytical precisions in δ¹³C, including GC preparation, on-line combustion, and IRMS analyses, are respectively in the ranges of 0.5‰ (for δ¹³C). The carbon isotopic values were also corrected using mass balance calculation for the influences of Si-gel blank.
CHAPTER 3
RESULTS

3.1 VARIATIONS OF CELL DENSITY DURING BATCH CULTURE

The batch culture started at an initial cell density of $\sim 10^3$ cells mL$^{-1}$ and reached a maximum density of $\sim 2.4 \times 10^6$ cells mL$^{-1}$ at around day 8. From day 0 to day 8, the culture was at exponential stage, with a cell division rate of $\sim 0.97$ times d$^{-1}$. After day 8, the cell density remained a nearly constant level with a slight fluctuation around the maximum density, which indicates that the cells are at stationary growth stage (Fig. 2). Algal cell samples of exponential stage were collected at day 5 while algal cell samples of stationary stage were collected at day 18.

3.2 VARIATIONS OF CELL DENSITY DURING CONTINUOUS CULTURES

Four continuous cultures were conducted in f/2 medium with four different sets of flow rates (also called the dilution rate).

For continuous culture 1 (R1), the f/2 medium flow rate was controlled at $\sim 0.69$ mL min$^{-1}$, which means the division rate of cells is $\sim 1$ divisions d$^{-1}$. R1 started at an initial cell density of $\sim 10^3$ cells mL$^{-1}$ and reached the cell density of $\sim 5 \times 10^5$ cells mL$^{-1}$ at day 5. The f/2 medium was introduced into culturing chambers at day 5. Sampling was done at day 13, 8 days after the introduction of f/2 medium and over four doubling time at the f/2 medium flow rate of $\sim 1$ divisions d$^{-1}$ ($4 \times 1/1=4$ days) to make sure that the cell
density, cellular structures, cellular fatty acids and related isotopic compositions have stabilized in this environment. The cell density was \( \sim 7 \times 10^5 \) cells mL\(^{-1} \) at the sampling day.

For continuous culture 2 (R2), the f/2 medium flow rate was controlled at \( \sim 0.56 \) mL min\(^{-1} \), which means the division rate of cells is \( \sim 0.84 \) divisions d\(^{-1} \). R2 started at an initial cell density of \( \sim 10^3 \) cells mL\(^{-1} \) and reached the cell density of \( \sim 5 \times 10^5 \) cells mL\(^{-1} \) at day 5. The f/2 medium was introduced into culturing chambers at day 5. Sampling was done at day 16, 11 days after the introduction of f/2 medium and over four doubling time at the f/2 medium flow rate of \( \sim 0.85 \) divisions d\(^{-1} \) (\( 4 \times 1/0.85 = 4.71 \) days) to make sure that the cell density, cellular structures, cellular fatty acids and related isotopic compositions have stabilized in this environment. The cell density was \( \sim 8 \times 10^5 \) cells mL\(^{-1} \) at the sampling day.

For continuous culture 3 (R3), the f/2 medium flow rate was controlled at \( \sim 0.42 \) mL min\(^{-1} \), which means the division rate of cells is \( \sim 0.62 \) divisions d\(^{-1} \). R3 started at an initial cell density of \( \sim 3 \times 10^3 \) cells mL\(^{-1} \) and reached the cell density of \( \sim 5 \times 10^5 \) cells mL\(^{-1} \) at day 7. The f/2 medium was introduced into culturing chambers at day 7. Sampling was done at day 20, 13 days after the introduction of f/2 medium and over four doubling time at the f/2 medium flow rate of \( \sim 0.62 \) divisions d\(^{-1} \) (\( 4 \times 1/0.62 = 6.45 \) days) to make sure that the cell density, cellular structures, cellular fatty acids and related isotopic compositions have stabilized in this environment. The cell density was \( \sim 5 \times 10^5 \) cells mL\(^{-1} \) at the sampling day.

For continuous culture 4 (R4), the f/2 medium flow rate was controlled at \( \sim 0.28 \) mL min\(^{-1} \), which means the division rate of cells is \( \sim 0.17 \) divisions d\(^{-1} \). R4 started at an
initial cell density of ~3×10³ cells mL⁻¹ and reached the cell density of ~5×10⁶ cells mL⁻¹ at day 5. The f/2 medium was introduced into culturing chambers at day 5. Sampling was done at day 30, 25 days after the introduction of f/2 medium and over four doubling time at the f/2 medium flow rate of ~0.17 divisions d⁻¹ (4×1/0.17=23.5 days) to make sure that the cell density, cellular structures, cellular fatty acids and related isotopic compositions have stabilized in this environment. The cell density was ~3.5×10⁵ cells mL⁻¹ at the sampling day.

The growth curves of four continuous cultures are shown in Fig. 3.

3.3 VARIATIONS OF ALGAL FATTY ACID CONCENTRATIONS AND COMPOSITIONS IN BATCH CULTURE

In the total fatty acids extraction of day 5 and day 18 from batch culture of *Thalassiosira pseudonana*, 8 fatty acids were identified (Fig. 4). These fatty acids were: saturated fatty acids 14:0, 16:0 and 18:0, monounsaturated fatty acid 16:1, and polyunsaturated fatty acids 16:4, 16:3/2, 18:4, 18:2/1 and 20:5/4. This is consistent with the research done by Volkman et al. (1989). Total fatty acid content of cells in day 18 (4.04±0.06 pg cell⁻¹) was ~8 times higher than that in day 5 (0.56±0.04 pg cell⁻¹). Fig. 5 shows that on day 5, the dominant fatty acids were saturated fatty acids, such as 14:0 (5.6%), 16:0 (37.7%) and 18:0 (49.4%). There was only a little monounsaturated fatty acid 16:1(7.3%) in day 5. By contrast, on day 18, the concentrations of unsaturated fatty acids increased a lot. Unsaturated fatty acids 16:4/3 (13.9%), 16:1 (25.9%), 18:4 (2.8%), 18:2/1 (10.5%) and 20:5/4 (11.4%) were more abundant than saturated fatty acids 14:0.
(11%), 16:0 (18.3%) and 18:0 (6.1%). Table 2 shows the proportions of unsaturated fatty acids to saturated fatty acids on day 5 and day 18 of batch culture.

3.4 VARIATIONS OF ALGAL FATTY ACID COMPOSITIONS IN CONTINUOUS CULTURES

In continuous cultures, the 8 fatty acids identified at batch culture were still treated as targeted fatty acids. From R1 to R4, the concentrations of total fatty acids changed from 1.5 pg cell\(^{-1}\) (R1) to 0.7 pg cell\(^{-1}\) (R2) to 3.8 pg cell\(^{-1}\) (R3) to 6.3 pg cell\(^{-1}\) (R4). The general pattern of change was increase in total fatty acids concentrations with the decrease in growth rate (Fig. 6).

For R1, 14:0, 16:1, 16:0, 18:2/1 and 18:0 existed in the total fatty acids extraction with the dominant fatty acids being 16:0 (27.7%) and 18:2/1 (27.8%). For R2, fatty acids 14:0, 16:1, 16:0, 18:4, 18:2/1, 18:0 and 20:5/4 were detected in total fatty acids extraction, with 16:0 (29.1%) and 18:0 (26.3%) being the most abundant fatty acids. For R3, all the 8 targeted fatty acids were detected, and the dominant fatty acids were 16:0 (25.9%), 18:2/1 (23.3%) and 18:0 (15.8%). R4 was the culturing with the slowest cell division rate. All the 8 fatty acids existed in this culture and the most abundant fatty acids were 16:1 (28.0%), 16:0 (18.9%) (Fig. 7).

Based on the relative percentages of unsaturated and saturated fatty acids in the cells, the proportions of unsaturated to saturated fatty acids were calculated. The proportions of unsaturated to saturated fatty acids changed from 0.69 (R1) to 0.48 (R2) to 0.96 (R3) to 1.74 (R4). With the decrease of growth rate, the proportion of unsaturated
fatty acids to saturated fatty acids generally increased, with R2 being a little bit abnormal (Table. 3).

3.5 UNSATURATED FATTY ACIDS ASSOCIATED WITH MEMBRANE AND INTRACELLULAR COMPONENT IN BATCH CULTURE

The membrane and intracellular fatty acids in *Thalassiosira pseudonana* cells were separated by silica gel column chromatography. Owing to the fact that the silica gel blanks also contained the detected fatty acids 14:0, 16:0, 18:2/1 and 18:0, focus was placed on the unsaturated fatty acids 16:4/3, 16:1, 18:4 and 20:5/4 for silica gel separated fatty acid compositions and corresponding δ¹³C values.

On day 5, there was only a little monounsaturated 16:1 (0.033 pg cell⁻¹) existed in intracellular fatty acids. 16:1 also existed in membrane fatty acids, but the concentration was even lower (0.029 pg cell⁻¹). The proportion of membrane unsaturated fatty acids to intracellular unsaturated fatty acids was 0.88. On day 18, the relative percentages and concentrations of unsaturated fatty acids changed noticeably in both membrane fatty acids and intracellular fatty acids. 16:4/3, 16:1, 18:4 and 20:5/4 were detected in samples from day 18. The concentrations in both membrane and intracellular fatty acids increased. For membrane fatty acids, 16:4/3 had a concentration of 0.114 pg cell⁻¹, 16:1 was 0.132 pg cell⁻¹, 18:4 was 0.008 pg cell⁻¹ and 20:5/4 was 0.072 pg cell⁻¹. For intracellular fatty acids, 16:4/3 was 0.249 pg cell⁻¹, 16:1 was 0.688 pg cell⁻¹, 18:4 was 0.108 pg cell⁻¹ and 20:5/4 was 0.210 pg cell⁻¹. The membrane unsaturated fatty acids accounted for 24.4% of the total unsaturated fatty acids while the intracellular unsaturated fatty acids accounted for 75.6% of the total unsaturated fatty acids. The fatty acid ratio of membrane to
intracellular components was 0.32 on day 18. On day 18, 16:1 was the most abundant unsaturated fatty acid in both membrane unsaturated fatty acids (19.3%) and intracellular unsaturated fatty acid (32.5%) (Fig. 8).

3.6 UNSATURATED FATTY ACIDS ASSOCIATED WITH MEMBRANE AND INTRACELLULAR COMPONENT IN CONTINUOUS CULTURES

For continuous cultures, the membrane and intracellular fatty acids in *Thalassiosira pseudonana* cells were also separated by silica gel column chromatography.

For R1, only 16:1 was detectable by gas chromatography with a concentration of 0.127 pg cell\(^{-1}\) in storage and 0.062 pg cell\(^{-1}\) in membrane. The ratio of membrane unsaturated fatty acids to unsaturated intracellular fatty acids is 0.49.

For R2, 16:1, 18:4 and 20:5/4 were detected and quantified by gas chromatography. 16:1 had a concentration of 0.03 pg cell\(^{-1}\) in membrane and 0.091 pg cell\(^{-1}\) in intracellular storage. 18:4 had a concentration of 0.004 pg cell\(^{-1}\) in membrane while 0.009 pg cell\(^{-1}\) in intracellular storage. 20:5/4 had a concentration of 0.005 pg cell\(^{-1}\) in membrane and 0.021 pg cell\(^{-1}\) in intracellular storage. The membrane unsaturated fatty acids compromised 24.4% of the total unsaturated fatty acids and the intracellular unsaturated fatty acids composed 75.6% of the total unsaturated fatty acids. The ratio of unsaturated fatty acids to unsaturated intracellular fatty acids was 0.32.

For R3, 16:4/3, 16:1, 18:4 and 20:5/4 were all identified and quantified. 16:4/3 had a concentration of 0.031 pg cell\(^{-1}\) in membrane and 0.091 pg cell\(^{-1}\) in intracellular storage. 16:1 had a concentration of 0.130 pg cell\(^{-1}\) in membrane and 0.234 pg cell\(^{-1}\) in
intracellular storage. 18:4 had a concentration of 0.032 pg cell\(^{-1}\) in membrane and 0.043 pg cell\(^{-1}\) in intracellular storage. 20:5/4 had a concentration of 0.057 pg cell\(^{-1}\) in membrane and 0.161 pg cell\(^{-1}\) in intracellular storage. The membrane unsaturated fatty acids consisted 32.1% of the total unsaturated fatty acids while the intracellular unsaturated fatty acids composed 67.9% of the total unsaturated fatty acids. The ratio of unsaturated membrane fatty acids to unsaturated intracellular components was 0.47.

For R4, all the four target unsaturated fatty acids were identified and quantified. 16:4/3 had a concentration of 0.327 pg cell\(^{-1}\) in membrane and 0.381 pg cell\(^{-1}\) in intracellular storage. 16:1 had a concentration of 0.384 pg cell\(^{-1}\) in membrane and 1.4 pg cell\(^{-1}\) in intracellular storage. 18:4 had a concentration of 0.071 pg cell\(^{-1}\) in membrane and 0.08 pg cell\(^{-1}\) in intracellular storage. 20:5/4 had a concentration of 0.249 pg cell\(^{-1}\) in membrane and 0.266 pg cell\(^{-1}\) in intracellular storage. The membrane unsaturated fatty acids consisted 32.6% of the total unsaturated fatty acids while the intracellular unsaturated fatty acids composed 67.4% of the total unsaturated fatty acids. The ratio of unsaturated membrane fatty acids to unsaturated intracellular components was 0.48.

Overall, from R1 to R4, with the decrease in growth rate, the concentrations of unsaturated fatty acids increased in both membrane and intracellular structures (Fig. 9). The proportions of membrane to intracellular most unsaturated fatty acids (16:4/3, 18:4, 20:5/4) became bigger with the decrease of growth rate. But for 16:1, it seemed to have some fluctuations. The ratio decreased first, then increased, and then decreased again. The ratio of total membrane to total intracellular unsaturated acids did not change a lot though over the change in growth rate (Table. 4).
3.7 VARIATIONS OF UNSATURATED FATTY ACID δ\textsuperscript{13}C VALUES IN BATCH CULTURE

Owing to the fact that the concentrations of unsaturated fatty acids 16:4/3, 18:4 and 20:5/4 were not detectable by gas chromatography on day 5 samples, the compound-specific stable carbon isotopic values (δ\textsuperscript{13}C) were not available for these fatty acids on day 5. For the total fatty acids extraction on day 5 and day 18, 16:1 was the only comparable unsaturated fatty acid. 16:1 from day 5 total fatty acids extraction had a δ\textsuperscript{13}C of -22.8 ‰ compared to -20.8 ‰ on day 18. There was an enrichment of 2 ‰ from exponential stage to stationary stage (Fig. 10).

For silica get separated samples on day 18, δ\textsuperscript{13}C values were measured for membrane unsaturated fatty acids and intracellular unsaturated fatty acids respectively. 16:4/3 had δ\textsuperscript{13}C of -20.1‰ on membrane and -19.1‰ on intracellular storage. 16:1 had δ\textsuperscript{13}C of -21.3‰ on membrane and -20.7‰ on intracellular storage. 18:4 had δ\textsuperscript{13}C of -22.2‰ on membrane and -20.1‰ on intracellular storage and 20:5/4 had δ\textsuperscript{13}C of -21.0‰ on membrane and -19.4‰ on intracellular storage. The result shows that membrane unsaturated fatty acids had more depleted δ\textsuperscript{13}C values than intracellular unsaturated fatty acids, and the difference is around 1-2‰ (Fig. 11).

3.8 VARIATIONS OF UNSATURATED FATTY ACID δ\textsuperscript{13}C VALUES IN CONTINUOUS CULTURES

The δ\textsuperscript{13}C values of unsaturated fatty acids 16:4/3, 16:1, 18:4 and 20:5/4 of total fatty acids extraction samples are shown in Fig. 12. There were some variations in the δ\textsuperscript{13}C values with the decrease of growth rate. For example, for 16:1, from R1 to R4, the
\( \delta^{13}C \) values remained pretty constant from 1 to 2, and then increased \(~3.5\%\) from R3 to R4.

The \( \delta^{13}C \) values of membrane and intracellular unsaturated fatty acids 16:4/3, 16:1, 18:4 and 20:5/4 are shown in Fig. 13. It is obvious from the figure that \( \delta^{13}C \) values of storage associated unsaturated fatty acids were higher than membrane associated unsaturated fatty acids, which again proved the conclusion that intracellular fatty acids are enriched in \( \delta^{13}C \) values than membrane fatty acids. Besides, Fig. 13 also shows that unsaturated fatty acids from R4 had most enriched \( \delta^{13}C \) values compared with unsaturated fatty acids from other continuous cultures. However, the \( \delta^{13}C \) values of unsaturated fatty acids of R1, R2 and R3 did not show a very obvious changing pattern.
4.1 INFLUENCES OF CELLULAR STRUCTURES ON MOLECULAR STABLE CARBON ISOTOPIC COMPOSITION OF FATTY ACIDS

It has been documented that marine microalgae produce both polar and nonpolar fatty acids in different structures over different growth stages. The proportions of fatty acids bound in different cellular structures are dependent on algal cell physiological states and growth phases (Sukenik and Carmeli, 1990; Bell and Pond, 1996; Brown et al., 1996; Mansour et al., 2003). For cells at exponential stage, most marine algae species produce phospholipids for membrane use (Volkman et al., 1989; Sukenik and Carmeli, 1990; Brown et al., 1996; Mansour et al., 2003); while for cells at stationary stage, most marine algae species generally produce triglycerides for intracellular storage use (Sukenik and Carmeli, 1990; Mansour et al., 2003). It was also recently studied by Lv et al. (2010) using two common marine algae (Isochrysis sp. and Gymnodinium sp.) that most fatty acids (84-100%) were used for membrane structures in exponential growth stage. While in stationary stage, the proportions of intracellular storage fatty acids increased significantly to 33-59%. The result of our research drew similar conclusion with the previous studies, that Thalassiosira pseidonana mainly produced phospholipids for membrane use during exponential stage while mainly produced triglycerides for intracellular storage use during stationary stage.
Our results showed that fatty acids were produced in different amount and for different cellular structure uses through exponential stage to stationary stage (Fig. 4 and Fig. 5). The total fatty acid concentrations on day 18 was ~8 timers higher than that in day 5 with more storage fatty acids generated. Fatty acid compositions also differed with growth stages. On day 5, most fatty acids were produced as saturated fatty acids (83.4%) and were used for membrane structures. On day 18, the percentage of unsaturated fatty acids increased (from 16.6% to 61.9%) and more intracellular storage fatty acids were produced. On day 5, saturated fatty acids 14:0, 16:0, and 18:0 dominated with only a little 16:1. But on day 18, unsaturated fatty acids 16:4/3, 16:1, 18:4, 18:2/1 and 20:5/4 (61.9%) were more abundant than saturated fatty acids (38.1%).

Our research also showed that the isotopic composition of phytoplankton fatty acids varied between different growth phases (Fig. 10). For most fatty acids (14:0, 16:1, 16:0 and 18:2/1), the δ¹³C values were 1-2 ‰ higher (enriched) on day 18 than those on day 5. The only exception was 18:0, the δ¹³C value on day 18 was 1‰ lower (depleted) than that on day 5. However, since pCO₂ was not monitored during the culturing processes, it is also possible that pCO₂ influenced the stable carbon isotopic compositions of fatty acids.

For isotopic compositions related with unsaturated fatty acids bound in membrane and intracellular structures, silica gel separation was done to parallel samples taken from day 18, and membrane and intracellular unsaturated fatty acids δ¹³C values were measured separately. For all measured unsaturated fatty acids (16:4/3, 16:1, 18:4 and 20:5/4), the δ¹³C values for intracellular storage use were 1-2 ‰ higher (enriched) than those for membrane use.
Hayes (1993, 2001) pointed out that different precursors and synthesis pathways are two major causes for heterogeneous isotopic compositions of organic compounds. Wong et al. (1985) indicated that the major constituents of the triglycerides are various kinds of fatty acids that are polyunsaturated and derived through aerobic desaturation and chain elongation from the ‘precursor’ fatty acids palmitic (16:0) and oleic (18:1x9) acids. According to Hu et al. (2008), in algae, the de novo synthesis of fatty acids occurs primarily in the chloroplast. Overall, the pathway produces a 16- or 18-carbon fatty acid or both. These are then used as the precursors for the synthesis of chloroplast and other cellular membranes. According to these studies, the precursors for membrane fatty acids and intracellular storage fatty acids have some differences. For membrane phospholipids, the primary two precursors are fatty acids 16:0 and 18:1x9. While for intracellular triglycerides, the precursors have a wider range (16-carbon or 18-carbon fatty acids). For the formation pathways, the pathways of generating membrane phospholipids and intracellular triglycerides are also different. The pathway of synthesizing membrane phospholipids is aerobic desaturation and chain elongation. The generation of intracellular triglycerides is called de novo synthesis of fatty acids. Therefore, the difference of $\delta^{13}C$ values in membrane associated unsaturated fatty acids and intracellular storage associated unsaturated fatty acids might be caused by the difference in precursors or synthesis pathways in our study.

Based on above discussion, we can speculate that: (1) marine phytoplankton synthesized fatty acids for different cellular structure uses over different growth stages; (2) total fatty acid content of cells at stationary stage is much higher than that in exponential stage, with more storage fatty acids generated; (3) fatty acid composition differs with
growth stages. In exponential stage, the dominant fatty acids are saturated fatty acids while in stationary stage, the unsaturated fatty acids are more abundant; (4) $\delta^{13}$C values of unsaturated fatty acids in intracellular storage are enriched than those in membrane structures.

4.2 INFLUENCES OF CELL GROWTH RATE ON MOLECULAR STABLE CARBON ISOTOPIC COMPOSITION OF FATTY ACIDS

Four continuous cultures were performed at four sets of growth rates in the research. From R1 to R4, the growth rate decreased. Our results showed that fatty acids were produced differently in concentration and composition through the four cultures. From R1 to R4, the general pattern of the change of concentrations for both saturated fatty acids and unsaturated fatty acids was increase. For example, saturated fatty acid 16:0 had a concentration of 0.41 pg cell$^{-1}$ in R1, 0.21 pg cell$^{-1}$ in R2, 0.99 pg cell$^{-1}$ in R3 and 1.19 pg cell$^{-1}$ in R4. And unsaturated fatty acid 16:1 had a concentration of 0.19 pg cell$^{-1}$ in R1, 0.11 pg cell$^{-1}$ in R2, 0.38 pg cell$^{-1}$ in R3 and 1.77 pg cell$^{-1}$ in R4. The concentrations of fatty acids increased with the decreased growth rates with an exception of R1 to R2, where a slight decrease was shown in the experiment than increase. With the decrease of growth rate, the proportion of unsaturated fatty acids to saturated fatty acids increased in general, from 0.69 (R1) to 0.48 (R2) to 0.96 (R3) to 1.74 (R4). The only exception was R1 to R2 again, where a slight drop happened instead of increase.

For the carbon isotopic compositions of fatty acids in four continuous cultures, the most enriched $\delta^{13}$C values were in R4 for most of the fatty acids with an exception of 18:2/1 and 20:5/4, in which the most enriched fatty acids were in R3. For silica gel
separated unsaturated fatty acids, the changes of $\delta^{13}$C values from R1 to R3 were not obvious. However, from R3 to R4, there were noticeable positive shifts for all the four detected unsaturated fatty acids in both membrane structure and intracellular storage structure. Besides, it was evident that the $\delta^{13}$C values of intracellular storage associated unsaturated fatty acids were higher (2-6 ‰) than membrane associated unsaturated fatty acids, which is consistent with our result in the batch culture.

Previous researches (Popp et al., 1989; Rau et al., 1989; Freeman and Hayes, 1992) have implied that the availability of aqueous CO$_2$ plays an important role in determining the stable carbon isotopic compositions of marine algae. Besides, Laws et al. (1995) found that cell growth rate together with aqueous CO$_2$ concentration determine the stable carbon isotopic compositions of phytoplankton. Laws et al. (1995) conducted a series of experiment with marine diatom *Phaeodactylum tricornutum*, both carbon isotopic composition ($\delta^{13}$C values), growth rates and aqueous CO$_2$ were measured over the growth of cells. And they concluded that a linear relationship was found between the biological fractionation associated with carbon fixation and $\mu$/[CO$_2$]$_aq$ (the division of cell growth rate over aqueous CO$_2$ concentration). In our experiment, the result shows that the isotopic compositions of algal fatty acids had differences among different growth rates, however, the differences were not that clear and obvious. The difference between R1 and R4, the fastest growth rate and the slowest growth rate was clear, for all the detected unsaturated fatty acids, and for all the forms of them (membrane, intracellular and total fatty acids extraction), the $\delta^{13}$C values were enriched in R4 than R1. However, there were some fluctuations from R1 to R3. And the fluctuations might be caused by the change in aqueous CO$_2$ concentrations mentioned by the above researches, since CO$_2$
control was not performed in this research. Also, the sampling timing may need to be
extended for future studies. With longer continuous culture time, the structural, chemical
and isotopic compositions of algal cells can be more stable.

Based on above discussion, we can speculate that: (1) fatty acid content of cells in
lower growth rates are generally higher than that in higher growth rates; (2) fatty acid
compositions differ with growth rates. When the growth rate is lower, there are generally
more unsaturated fatty acids produced; (3) $\delta^{13}C$ values of unsaturated fatty acids in
intracellular storage are enriched than those in membrane structures. (4) $\delta^{13}C$ values of
unsaturated fatty acids from lower growth rates are generally enriched than those from
higher growth rates; (5) the isotopic compositions of fatty acids is not only determined by
growth rate, other factors such as aqueous CO$_2$ concentrations may also influence the
$\delta^{13}C$ values.

4.3 IMPLICATIONS

In the introductory part we mentioned that in spite of the wide applications of
biomarkers and associated molecular isotopic compositions, the fundamental assumption
of the applications 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). However, in order to use isotopic signals of biomarkers
to study carbon cycling, ecology and paleontography, it is actually very necessary to
understand how and when the biomarkers with specific isotopic signals are generated by
phytoplanktons in euphotic zone and how the carbon cycling modifies the signals from
euphotic zone to sediments. Our research indicates that it is essential to figure out when (in what growth stage) and what (fatty acids from what cellular structure, membrane or intracellular storage) are transported from euphotic zone to sediments from the phytoplankton cells in order to use the isotopic signals of lipid biomarkers for ecological or paleoceanographic studies.

The actual environment for marine phytoplankton is changing constantly, so no laboratory culture method can mimic the exactly conditions in nature. However, the basic understanding and studying of factors such as irradiance, temperature, growth rate affecting the algal physiology and composition in a steady state is necessary (Laws et al., 2001). According to Conte et al. (1998), the average physiological state of algae in the open ocean is analogous to the late stationary phase of batch culture; Prahl et al. (2003) pointed out that the growth of algae in nutrient sufficient environments resembles the pattern in continuous culture. Also, the exponential stage of phytoplankton is normally corresponding to the bloom in the ocean, when the nutrients are abundant and the concentration of cell is relatively low; while the stationary stage of phytoplankton can be seen as the post-bloom time in the ocean, where the concentrations of algal cells are high and nutrients are becoming limited (Benthien et al., 2007).

From the results of our research, it is clear that cellular structures and cell growth rates both have an influence on the stable carbon isotopic compositions of marine phytoplankton. Consequently, the heterogeneity of δ^{13}C values of cellular lipids can be caused by either of them. And this may influence the applications of fatty acid biomarker. For example, the stable carbon isotopic compositions of fatty acids are often used to study the sources of organic matters for estuarine samples. Thornton et al. (1998) used
the stable carbon isotopic composition to study the sources of organic matter in estuarine system in Tay Estuarine, Scotland, and for all the samples along the estuarine system, the biggest difference in stable carbon isotopic signals is 3‰. And they calculated the terrestrial fraction and marine fraction of estuarine sediment samples using this difference. But this calculation can be problematic if the difference of stable carbon isotopic composition is caused by the different growth stages of marine phytoplankton (1-2‰ difference according to this study). A similar problem can also influence the application of using fatty acid biomarker to study food web. Kharlamenko et al. (2001) studied the food sources of invertebrates by stable carbon isotopic analysis. And their study was based on the fact that the seagrasses and other primary producers usually differ in the isotopic composition of organic carbon. However, whether the difference is big enough to distinguish the food sources is doubtable. Zooplanktons may graze and digest lipids and other organic matters from different cellular structures of plankton, and this can also cause difference in stable carbon isotopic signals.

In summary, more and more evidence is showing that there are diversified changes in isotopic signals owing to biogeochemical cycling processes in the ocean. Our findings indicate that the internal factors such as cellular structures and cell growth rate of phytoplankton can have influence on the stable carbon isotopic compositions of cellular lipid compounds. This is a possible explanation for the heterogeneity of carbon isotopic changes in the ocean. However, other factors such as aqueous CO₂ concentration, irradiance and temperature may also influence the heterogeneity together with the factors we studied in this research. More studies are needed to further clarify the influences of
these factors separately and collaboratively on the heterogeneity of stable carbon isotopic compositions.
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<table>
<thead>
<tr>
<th>Continuous culture number</th>
<th>Growth rate/Dilution rate of algae (times/day)</th>
<th>f/2 Medium flow rate (mL/min)</th>
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Table. 1. Actual 4 sets of f/2 medium flow rates and their corresponding *Thalassiosira pseudonana* growth rates for continuous culture 1 to 4.
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<th>Batch culture</th>
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</tr>
<tr>
<td>Day 18</td>
<td>38.1%</td>
<td>61.9%</td>
<td>1.62</td>
</tr>
</tbody>
</table>

Table 2. The percentages of saturated fatty acids, unsaturated fatty acids and the proportions of unsaturated fatty acids to saturated fatty acids in total fatty acids extractions of batch culture day 5 and day 18
<table>
<thead>
<tr>
<th>Continuous culture number</th>
<th>Percentage of saturated fatty acids</th>
<th>Percentage of unsaturated fatty acids</th>
<th>Proportion of unsaturated fatty acids to saturated fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>59.3%</td>
<td>40.7%</td>
<td>0.69</td>
</tr>
<tr>
<td>R2</td>
<td>67.5%</td>
<td>32.5%</td>
<td>0.48</td>
</tr>
<tr>
<td>R3</td>
<td>51.1%</td>
<td>48.9%</td>
<td>0.96</td>
</tr>
<tr>
<td>R4</td>
<td>36.5%</td>
<td>63.5%</td>
<td>1.74</td>
</tr>
</tbody>
</table>

Table 3. The percentages of saturated fatty acids, unsaturated fatty acids and the proportions of unsaturated fatty acids to saturated fatty acids in total fatty acids extractions of continuous cultures 1 to 4
<table>
<thead>
<tr>
<th>Continuous culture rate</th>
<th>Fatty acid compounds</th>
<th>16:4/3</th>
<th>16:1</th>
<th>18:4</th>
<th>20:5/4</th>
<th>total unsaturated membrane to unsaturated intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>NA</td>
<td>0.49</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.49</td>
</tr>
<tr>
<td>R2</td>
<td>NA</td>
<td>0.33</td>
<td>0.44</td>
<td>0.24</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>0.34</td>
<td>0.56</td>
<td>0.74</td>
<td>0.35</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>R4</td>
<td>0.86</td>
<td>0.27</td>
<td>0.89</td>
<td>0.94</td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>

Table. 4. The proportions of membrane to intracellular unsaturated fatty acids of continuous cultures 1 to 4
Fig. 1. Design of continuous culture chamber

light : dark = 12h : 12h
Fig. 2. Batch culture growth curve of *Thalassiosira pseudonana*
Fig. 3. Continuous culture growth curves of *Thalassiosira pseudonana*. 
Fig. 4. Fatty acid compounds concentrations on day 5 and day 18 of batch culture
Fig. 5. Fatty acid compounds compositions on day 5 and day 18 of batch culture
Fig. 6. The comparison among the concentrations of fatty acid compounds in four continuous cultures
Fig. 7. The comparison among the compositions of fatty acid compounds in four continuous cultures
Fig. 8. Comparison of silica gel separated unsaturated fatty acid compounds concentrations bound in intracellular structures and membrane structures on batch culture day 5 and day 18
Fig. 9. Comparison of silica gel separated unsaturated fatty acids concentrations bound in intracellular structures and membrane structures from continuous culture 1 to 4.
Fig. 10. $\delta^{13}$C values of total fatty acids extraction on batch culture day 5 and day 18
Fig. 11. $\delta^{13}$C values and concentrations of silica gel separated unsaturated fatty acids 16:4/3, 16:1, 18:4 and 20:5/4 bound in intracellular structures and membrane structures.
Fig. 12. $\delta^{13}$C values of total fatty acids extraction from continuous cultures 1 to 4
Fig. 13. Variations of $\delta^{13}C$ values of silica gel separated unsaturated fatty acids 16:4/3, 16:1, 18:4 and 20:5/4 bound in intracellular and membrane structures of continuous cultures 1 to 4