Study on the Regulation of Cell Growth and the Synthesis of Alkenones by Temperature in Coccolithophorids

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Science

Joy M. SORROSA

Table of Contents

Abbreviations

ABA: abscisic acid

ACP: acetoacetyl-[acyl carrier protein]

CPC: coccolith-producing compartment

$C_{37:2}$: C_{37} methyl alkenones with 2 double bonds

$C_{37:3}$: C_{37} methyl alkenones with 3 double bonds

DCM: dichloromethane

DMS: dimethyl sulfide

DMSP: dimethylsulfoniopriopionate

DR: dilution rate

DRE: dehydration-responsive element

EE: $C_{36:2}$ ethyl alkenoate

EE/K: $C_{36:2}$ ethyl alkenoate/total alkenones

EE/K_{37}: $C_{36:2}$ ethyl alkenoate/total C_{37} alkenones

EK: ethyl ketone

ESM: Erd-Schreiber's medium

FAS: fatty acid synthase

GC: gas chromatography

HEBP: (1-hydroxyethylidene) biphosphonic acid

K: total alkenones

KAS: *β*-ketoacyl-ACP synthase

K_{37}: C_{37} alkenones

K_{38}: C_{38} ethyl and methyl alkenones

MA: Marine Art

ME: $C_{36:2}$ methyl alkenoate

MeOH: methanol

MK: methyl ketone

OD: optical density

PCV: packed cell volume

PUFA: polyunsaturated fatty acid

$\mathbf{U^{K'}_{37}}$: unsaturation index of alkenones

$\mathbf{U^{K'}_{38EK}}$: unsaturation index of C_{38} ethyl ketone

$\mathbf{U^{K'}_{38MK}}$: unsaturation index of C_{38} methyl ketone

SST: sea surface temperature

Abstract

Marine unicellular calcifying algae, coccolithophorids, strongly interact with the global climate and carbon cycle through their photosynthetic and calcite formation activities. They are exceptional in their ability to produce large blooms in the ocean and consequently contributed greatly to calcite deposition in the sediments during the past geologic era. As their distribution has been known to be common to high latitude in the ocean, response to low temperature may be unique in these organisms for supporting their growth and distribution. This thesis intended to elucidate the morphological and physiological responses of the two species of coccolith-producing haptophytes to temperature.

In Chapter 1, *Emiliania huxleyi* (Lohmann) Hay & Mohler (strain EH2) (Coccolithophorales, Prymnesiophyceae), a ubiquitous species and the closely related *Gephyrocapsa oceanica* Kamptner (strain GO1) were grown at various temperatures. *E. huxleyi* could grow at a wider temperature range (10-25ºC) while *G. oceanica* was limited at warmer temperature (20-25ºC). Those characteristics may explain the extensive distribution of *E. huxleyi* especially in the high latitude and *G. oceanica* distributes mostly in middle and low latitudes of the ocean. However, both strains failed to survive at 30ºC. At low temperature the enlargement of cells and chloroplasts and the stimulation of coccolith production were observed while cell growth was suppressed. These results clearly suggest the correlation between cell volume and calcification activity in coccolithophorids.

In Chapter 2, the coccolith-producing haptophytes, *E. huxleyi* and *G. oceanica* are known to be the chief source of alkenones found in both water column and sediment all over the world's oceans excepting the Arctic Ocean. Alkenones are long-chain ketones (C_{37} to C_{39}) that contain a carbonyl group and di-, tri- and tetra-*trans*-type-unsaturations. The number of double bonds in alkenones varies depending on the growth temperature at the time they are

synthesized in the cells. Most of the alkenones are degraded during their deposition into the oceanic sediments, yet some parts of the compounds tend to be preserved with maintaining the degree of unsaturation. Therefore, the molecules are widely used as a paleothermometer for the determination of the paleo sea surface temperature (SST) in paleoceanography. $U^{K'}_{37}$ is used as an index for the degree of unsaturation of C_{37} alkenones and calculated as: $U^{K'}_{37} = [C_{37:2}] / ([C_{37:2}] + [C_{37:3}])$, where $[C_{37:x}]$ denotes the concentration of alkenones consisting of 37 carbon atoms with x number of double bonds. In this study, *E. huxleyi* and *G. oceanica* were grown in batch and continuous cultures to assess factors affecting the production and the unsaturation index of alkenones. Continuous cultures are carried out where cell density is maintained constant by automated dilution of culture with fresh medium such that growth and physiological status of the cells are supposed to be maintained constant in comparison with batch cultures where growth status changes through the proceeding of growth phases.

The alkenone production by *E. huxleyi* was greatly stimulated at 10ºC where cell growth was greatly suppressed, resulting from the huge increase of $C_{37:3}$ alkenones in both batch and continuous cultures. When ^{14}C-bicarbonate was added as a substrate in *E. huxleyi*, ^{14}C-incorporation was especially higher into alkenones but slightly into other lipid fractions at 10ºC than at 20ºC, suggesting that the synthesis of alkenones is greatly enhanced at low temperature. C_{37} alkenones comprised about 50-60% of the total alkenones, the rest are C_{38} methyl and ethyl ketones and C_{39} ethyl ketones (C_{38MK}, C_{38EK} and C_{39EK}, respectively). The $U^{K'}_{37}$ and the ratio between $C_{36:2}$-ethyl alkenoate (EE) and total alkenones (K) (EE/K) changed during growing phase and remained nearly constant during the stationary phase at all temperatures tested in batch cultures, suggesting that those parameters were affected by both temperature and growth status of cells. Data in continuous culture where cell density was maintained constant showed

that the alkenones with 2 double bonds increased at high temperature while the alkenones with 3 double bonds decreased and the reverse change was clearly observed when temperature was decreased. $U^{K'}_{37}$ changed without any lag when temperature changed and needed approximately 2-6 days to attain the respective levels depending on the difference of temperature given. The final values of $U^{K'}_{37}$, $U^{K'}_{38EK}$ and $U^{K'}_{38MK}$ that were calculated using analogous equations of $U^{K'}_{37}$, respectively, obtained at stationary stage were similar between batch and continuous cultures at each temperature and the values increased with increasing temperature. The results strengthened that temperature is the major factor that influences the production and unsaturation of alkenones. In addition, the production of alkenes (including C_{29}, C_{31}, C_{33}) which comprised only about 4% of the total lipids was also greatly stimulated at 10ºC. Previous studies suggested that this lipid compound can also be used as indicator of paleo-SST but it is not commonly used because of the minimal amount produced in the cell. The present data show that data of alkenes are useful to confirm the results on C_{37}-alkenone studies in SST-estimation.

In Chapter 3, the effect of cerulenin on the synthesis of alkenones in *E. huxleyi* cells was determined. Cerulenin is a specific *β*-ketoacyl-ACP synthase (KAS)-inhibiting antibiotic isolated from *Cephalosphorium caerulens*, used to inhibit fatty acid synthesis by blocking chain elongation of fatty acid. In *E. huxleyi*, cerulenin greatly inhibited the biosynthesis of alkenones. The result suggests that alkenone producing steps may be at the downstream of fatty acid synthesis and/or the elongation reactions of alkenones may be catalyzed by KAS or KAS-like enzyme. On the other hand, desaturation reaction of alkenones may be greatly different from that of fatty acid since *cis/trans*-configuration of the unsaturation bond is different in between fatty acids and alkenones.

This study demonstrates that the coccolithophorid *E. huxleyi* increases the production and unsaturation of alkenones by stimulating their chain elongation and unsaturation reactions when the cells are stressed by low temperature. At the same time, the enlargement of cell volume and the calcification are also enhanced while cell growth is greatly suppressed. These changes are reversibly regulated at high temperature. The relationship among these phenomena is not clearly elucidated yet, but the results suggest that these phenomena may be closely associated under regulation by temperature-sensitive signal transduction system.

General Introduction

Temperature is an extremely important variable in the physical environment of living organism and its effect has been studied in a large number of different biological systems. Many living organisms including most marine life are susceptible to temperature stress and their survival and development generally limited. On the other hand, extreme increase in temperature may also have an adverse effect on marine habitats like the phenomenon of coral reef bleaching that has been found to be correlated with elevated sea surface temperature (Gates et al., 1992).

Photosynthetic organisms respond and adapt to temperature stress through various biochemical and physiological processes, thereby acquiring stress tolerance. Many genes are expressed by responding to cold stress at the transcriptional level and the gene products function in stress and its tolerance responses. It was found that several sets of *cis*- and *trans*-acting factors known to be involved in stress-responsive transcription are controlled by abscisic acid (ABA) in higher plants. An example of a *cis*-acting element named DRE (dehydration-responsive element) plays an important role in both dehydration- and low-temperature-induced gene expressions in *Arabidopsis* (Qiang et al., 1998; Shinozaki et al., 2003). A histidine kinase, Hik33, was also found to sense decrease in temperature and to regulate the expression of certain cold-inducible genes in the cyanobacterium *Synechosystis* sp. PCC6803 (Suzuki et al., 2001). The many stress-inducible genes include those encoding signaling molecules like enzymes involved in the metabolism of phospholipids.

Temperature also affects the integrity of the cell structure and control the direction of special metabolic processes. The effects of temperature on physiology and metabolism to marine organisms also influence their geographic distribution. Therefore, the enhancement of our knowledge on the effect of temperature to physiological and morphological properties such as growth, cell size and other

metabolic activities specific to certain groups of organisms is always interesting and gainful. Such knowledge would lead to a better understanding about the biological responses of the living organisms to environmental changes and how their processes are regulated by temperature in the ocean.

Coccolithophorids are the best known members of the Prymnesiophyceae (Haptophyta) which are strongly interactive with the global environment. The Prymnesiophyceae are primarily marine organisms that make up a major part of the marine nanoplankton. Members of this group are primarily unicellular and are photosynthetic. They are often important sources of food for aquatic organisms. Most Prymnesiophyte cells are covered with scales and in many cases the scales are calcified. The fossil record of the prymnesiophyceae is known from the Carboniferous, approximately 300 million years ago (Lee, 1999). Recently, they gained much attention as important players in global climate change and carbon cycle through their activities of fixing CO_2 by photosynthesis and intracellular calcification. Their activities and interactions with the physical environment are believed to be important example for studying a marine ecosystem (Winter et al., 1994; Harris, 1996). The cells of coccolithophorids are covered by coccoliths with complex embellishment either with one kind of calcified plate or two. Investigations by Young and Ziveri (1999) have shown that the mineral component of coccolith is calcium carbonate ($CaCO_3$) in the form of calcite. Experimental evidences suggest that calcite formation requires the uptake of HCO_3^- together with Ca^{2+} into the coccolith vesicle with the extrusion of H^+ (Nimer and Merrett, 1993; Sekino and Shiraiwa, 1995; Shiraiwa, 2003). The calcification reaction proceeds as shown: $2HCO_3^- + Ca^{2+} \fallingdotseq CaCO_3 + CO_2 + H_2O$. Carbon dioxide is released during calcification, and this may make coccolithophorids more competitive by reducing the cost of energy for transporting CO_2 from outside the cells and to stimulate CO_2 fixation by ribulose-

1,5-biphosphate carboxylase/oxygenase and consequently enhancing the photosynthetic carbon fixation (Sikes et al., 1980; Nimer and Merrett, 1993; Anning et al., 1996; Lee, 1999). When coccolithophorids died, their coccoliths greatly contributed to the calcite deposited underneath the ocean and even provided the major component of Mesozoic (Jurassic and Cretaceous) and Tertiary chalks and marks (Balch and Kilpatrik, 1995; Lee, 1999).

The biogeochemical influence of coccolithophorids is possibly most pronounced during the occurrence of blooms which give a milky appearance to the surface water. The high production and increased concentration of detached coccoliths during blooms increase the light reflectance, so that they are easily detected from space. This phenomenon also influences the albedo of the seawater, as such it reduces the temperature (Westbroek et al., 1993; Brown and Yoder, 1994). Coccolithophorids also play a significant role in the global environment by releasing dimethylsulfoniopropionate (DMSP), an osmoregulant of coccolithophorid cells that is immediately transformed to dimethyl sulfide (DMS) in the seawater. DMS and its oxidized products are recognized to function as cloud-condensation nuclei (Eppley et al., 1969) where their density influences droplet size, cloud albedo, and consequently climate (Charlson et al., 1987).

Coccolithophorids *Emiliania huxleyi* and *Gephyrocapsa oceanica* are the recognized predominant source of the long-chain methyl and ethyl alkenones (C_{37}-C_{39} ketones) recorded from the marine sediment (Volkman et al., 1980; Marlowe et al., 1984; Marlowe et al., 1990). These compounds contain a carbonyl group and di-, tri- and tetra-unsaturations with a rare *trans* double configuration. The numbers of double bonds of alkenones are known to vary depending on the growth temperature at the time when they are synthesized in the cells. About 90% of alkenones are degraded during transport to the sediment but the degree of unsaturation during the time when they are synthesized at the sea surface are

not affected by diagenetic process. Hence, the unsaturation index of alkenones ($U^{K'}_{37}$) has been widely employed in the late Quarternary period as biomarkers in the estimation of the paleo sea surface temperature (SST) (e.g. Prahl et al., 1989; Brassell, 1993; Müller, et al., 1998). $U^{K'}_{37}$ was calculated by dividing the concentration of alkenones containing 37 carbons with 2 double bonds by the total concentration of C_{37} alkenones containing 2 and 3 double bonds (Brassell et al., 1986; Prahl et al., 1988).

The worldwide distribution of *E. huxleyi* and *G. oceanica* (Okada and Honjo, 1973; Brand, 1982; Roth, 1994) and their impact to climate have attracted many researchers doing studies on these species in various fields (*e.g.* Nimer and Merrett, 1993; Westbroek et al., 1993; Sekino et al., 1996; Danbara and Shiraiwa, 1999; Shiraiwa et al., 2004; Obata et al., 2004). The morphological and physiological properties of these species had remained an interesting field to explore in order to thoroughly understand their remarkable ecological success. In this study a detailed examination on the effect of temperature to growth, cell size, and calcification was conducted. Such data will be deemed essential to understanding how growth status and mass production of coccolithphorids are regulated by temperature in the ocean.

The production and unsaturation of alkenones are also greatly affected by temperature. Alkenone compounds are important tools in the reconstruction of paleotemperature. *E. huxleyi* and *G. oceanica* are two of the rare species that are capable of producing alkenones. Several studies showed that the calibration curve between the unsaturation index of alkenones and growth temperature slightly vary (Prahl et al., 1989; Sawada et al., 1996), suggesting that other factors may also influence the $U^{K'}_{37}$ value. Hence, in Chapter 2 of this thesis, I tried to assess factors that may possibly influence the production and unsaturation of alkenones. It was intended also to show how the production and

the unsaturation of alkenone molecules are regulated during growth in batch and continuous cultures. Besides, alkenone study with a chemostat culture has rarely been performed, therefore the data obtained here should provide new insight and will verify the data obtained from the batch cultures.

Studies that would likely elucidate the metabolic pathway for the synthesis and the mechanism for changing the degree of unsaturation in alkenone molecules are needed. This knowledge will also lead to the understanding of the physiological function of alkenones in the coccolithophorid cells. Due to non-availability of such data, the effect of cerulenin, an antibiotic inhibitor of elongating enzymes in fatty acids (Funabashi et al., 1989; Morisaki et al., 1993) on the synthesis of alkenones was determined by ^{14}C-labeling experiment. In Chapter 3 of this thesis, I aimed to unlock the understanding of the alkenone biosynthesis that would enable also the organic geochemists to make use of the alkenones records from the sediment as paleoceanographic tool with great accuracy.

Considering the importance of *E. huxleyi* and *G. oceanica* in the modern ocean (Holligan et al., 1983; Winter et al., 1994), this study on the regulatory effect of temperature on the morphology, physiology and the synthesis of alkenones by these two coccolithophorids is deemed essential.

Chapter 1

Effect of temperature to growth, cell size and intracellular calcification of coccolithophorids, *Emiliania huxleyi* and *Gephyrocapsa oceanica*

Introduction

There is a growing awareness of the role of marine microorganisms in biogeochemical processes. Recently, coccolithophorids have gained much attention as important players in global climate change and carbon cycle because of the production of huge amounts of bloom in the ocean. Their activities are believed to be essential in studying a marine ecosystem especially at high latitude where water temperature is relatively low (Harris, 1996; Winter et al., 1994). Coccolithophorids are unique since they are capable of fixing carbon by both photosynthesis for organic matter production and calcification for $CaCO_3$ crystal (coccolith) formation. The coccolith production had contributed greatly on the calcite deposited in the sediments during geological era. These algae are known as organisms that produced huge amount of limestone especially at Cretaceous era (Balch and Kilpatrik, 1995). In addition, coccolith production is known to be influenced by light, salinity and availability of nutrients (Linschooten et al., 1991; Green et al., 1998; Shiraiwa et al., 2003).

Coccolithophorid bloom-forming species are limited to a few, suggesting that species specific physiological characteristics are important to such limitation. And as mentioned in the general introduction, temperature may also be an essential factor that regulates the physiological characteristics of these species. However, there is no data yet on morphological and physiological changes in properties that are affected by temperature in bloom-forming coccolithophorids. Therefore, the present study aimed to enhance our knowledge on the effect of temperature to physiological and morphological properties such as growth, cell size, and calcification that is an important character of coccolithophorids because it greatly influence the global biogeochemical cycle by culturing two Gephyrocapsean coccolithophorids, *E. huxleyi* and *G. oceanica*. Such knowledge

is essential to understanding how the growth activity of the cells and mass production of $CaCO_3$ crystals as coccoliths by coccolithophorids are regulated by temperature in the ocean.

Materials and Methods

Algae and culture maintenance

Stock cultures of the coccolith-producing haptophytes, *Emiliania huxleyi* (Lohmann) Hay & Mohler (strain EH2) (Coccolithophorales, Prymnesiophyceae), *Gephyrocapsa oceanica* Kamptner (strain GO1) isolated from Great Barrier Reef, SW Pacific, Australia and Mutsu Bay, NW Pacific, Japan, respectively, during a cruise of Sohgen-maru of the Marine Biotechnology Institute, Japan in 1990. Strains are maintained as stock cultures in natural seawater with Erd-Schreiber's Medium (ESM) as described by Sekino and Shiraiwa (1994). Temperature of stock cultures was maintained at 20 ± 0.3°C in an incubator (Advantec IS-2300, Tokyo, Japan) equipped with a temperature controller. Light was provided by 20-Wwhite fluorescent tubes (Toshiba Co. Ltd., Tokyo, Japan) with 16-h light/8-h dark regime. The light intensity determined by a photon sensor (IKS-25, Koito Manufacturing Co. Ltd., Tokyo, Japan) was 30 μmole $m^{-2}s^{-1}$.

Growth parameters and cell size determination

For experimental culture, cells were transferred to a 500-ml flat oblong glass vessel (inner height, 26 cm; inner width, 10.5 cm; inner thickness, 2.8 cm, Fujimoto Rika Co. Ltd., Tokyo, Japan) containing 400 ml of artificial seawater, Marine Art SF-1 (Senju Pharmaceutical Co., Osaka, Japan) enriched with modified ESM in which soil extract was replaced with 10 nmol l^{-1} sodium selenite (MA-ESM) (Danbara and Shiraiwa, 1999). At the initiation of experimental culture, the concentration of cells was approximately 0.05 of the optical density at 750 nm (OD_{750}). OD_{750} was determined by a UV-VIS recording

spectrophotometer (UV-2200, Shimadzu, Kyoto, Japan). The experimental cultures were maintained at a temperature range of 10-30ºC (fluctuation of temperature at any temperature was ± 0.1ºC) in a thermostated water bath where the temperature was continuously monitored by a thermometer attached to a digital temperature recorder. Cultures were under continuous illumination by white fluorescent tubes at an intensity of 35-40 μmol m^{-2} s^{-1} and bubbled with air at 260 ml min^{-1}.

At intervals, aliquots of the cultures were harvested and several variables were measured. The pH, OD_{750} and cell number were determined by a pH meter (HM-30S, TOA Electronics, Ltd., Tokyo, Japan), a UV-VIS spectrophotometer and microscopic counting with an MC-31 video monitor (Scalar, Tokyo, Japan). Size of a cell including or excluding coccoliths and the average value of thickness of coccosphere were determined by micrometer scales of a hemocytometer under the microscope and from photographs. The thickness of the coccosphere was used as a parameter for estimating the extent of calcification. Microscopic observations of cell size, coccolith formation and cell viability were also performed under Nomarsky and fluorescence microscope (BX50, OLYMPUS, Tokyo, Japan). At the same time micrographs were taken using a digital camera (Fujix HC-2000 3CCD, Fujifilm Co. Ltd., Tokyo, Japan). Growth constant (k_g day^{-1}) was calculated from growth rate at the logarithmic phase on the graph with semi-logarithmic plots according to Yamamoto et al. (2000).

In another experiment, the effect of temperature changed to growth, cell size and calcification was also determined in cultures first grown at 20ºC and then divided and transferred to 15 and 25ºC, respectively. Experimental conditions like medium, culture vessels and light were the same in the previous experiments as well as the parameters measured.

Results

Best growth of *E. huxleyi* was observed at 25ºC with a growth constant (k_g day^{-1}) of 0.26 and 0.36 for changes in turbidity (OD_{750}) and cell number, respectively (Fig. 1A). However, growth at 25°C was critical and a little shift in temperature may change the growth rate. *E. huxleyi* still grew with a high rate at 15ºC and to some extent even at 10ºC. The optimum growth for *G. oceanica* was also observed at 25ºC with a growth constant (k_g day^{-1}) of 0.25 and 0.19 for changes in OD_{750} and cell number, respectively, but failed to grow below 15ºC (Fig. 1B). Both species were not able to survive at 30ºC. These profiles of growth activity were very similar to those of the activity of photosynthetic O_2 evolution in *E. huxleyi* determined during an hour-order reaction (unpublished data by Sekino and Shiraiwa). When growth temperature was decreased, the symplast and coccosphere sizes increased in both *E. huxeyi* and *G. oceanica* (Figs. 1C-D).

The degree of calcification as calculated by the ratio of OD_{750}/cell number (10^9 ml^{-1}) was greatly stimulated at 10ºC in *E. huxleyi* and almost similar at 20 and 25ºC in both species (Fig. 2).

E. huxleyi cells transferred from 20 to 15ºC continue to grow while *G. oceanica* cells gradually died. On the other hand, *E. huxleyi* transferred at 25ºC continue to grow, and then the cell number started to decline, whereas, *G. oceanica* exhibited a faster growth (Fig. 3). The extent of the increase in symplast/whole cell in diameter were 20/23% and 32/36% in *E. huxleyi* and *G. oceanica*, respectively, when temperature was changed from 25 to 15ºC. Data also showed that *G. oceanica* has 17-27% larger cell size and thicker coccospheres than *E. huxleyi* (Figs. 1 and 4). The change in cell size was reversible when temperature was changed from high to low and vice versa (Fig. 4). The cells

became larger with thicker coccospheres at low temperature and smaller with thinner coccosphere at high temperature in both species.

These physiological data on changes in cell size and coccolith production in *E. huxleyi* were morphologically confirmed by microscopic observations under fluorescent and polarization microscopes (Fig. 5). The increase in size of chloroplast emitting red fluorescence was obvious under fluorescent microscope when *E. huxleyi* was grown at 10 and 15ºC. The same changes were clearly observed in *G. oceanica*, even when algal growth was greatly suppressed at 15ºC (data not shown). Coccolith production was not obvious at 10ºC as compared to higher growth temperatures since cells were still actively dividing.

Discussion

E. huxleyi is known as a ubiquitous species that distributes globally in the ocean and can produce huge blooms, especially at high latitude (Winter et al., 1994; Fritz and Balch, 1996). Maximum growth rates of *E. huxleyi* cultures were obtained in the range of 10-26ºC, depending on the difference in clones that were isolated from different habitats and have different amounts of chlorophyll content in relation to cell biomass (summarized by Paasche, 2002). Saitoh et al. (2001) and Shin et al. (2002) reported that the temperature during blooms in the Bering Sea in 2000 was above 10ºC and the bloom gradually disappeared when the temperature decreased to 7-9ºC. The wide biogeographic distribution and the occurrence of blooms in *E. huxleyi* can be attributed to its ability to grow in such a wide temperature range, especially at low temperature of 10 to 15ºC as shown in Fig. 1. Distribution of *G. oceanica*, however, has been reported to be limited to tropical and subtropical waters (Roth, 1994; Winter et al., 1994), and this should be due to its preference to warm conditions.

Cell size and the thickness of coccospheres in both *E. huxleyi* and *G. oceanica* were increased by decreasing growth temperature and the phenomena were accompanied with the suppression of the increase in cell number, namely, the suppression of cell division (Figs. 1, 5). The average diameter of *E. huxleyi* and *G. oceanica* at 25ºC was 22% and 24% smaller than that at 10 and 15ºC, respectively. Shiraiwa et al. (2003) and Shiraiwa (2003) reported that in cultures where nutrient (NO_3^- and PO_4^{3-}) was deficient, cell division was suppressed which resulted in a larger cell volume and enhancement of intracellular calcification. Furthermore, Shiraiwa et al. (2003) showed that the addition of hydroxyethylidene-bisphosphonic acid (HEBP), an inhibitor of $CaCO_3$ crystallization, stimulated increase in cell number during growth and actively

dividing cells in control samples were smaller in size with poor calcification. These results further reinforced the suggestion that the enlargement of cell volume is closely related to calcification.

The enhancement of coccolith production at low temperature may be one of key reasons why coccolithophorid bloom has been mostly observed by a satellite in the ocean at high latitude since coccoliths enhance the reflectance of light. The inverse relationship between growth rate and calcification shown in this study may suggest that a time lag separates the phase for increasing cell number of coccolithophorids and the phase for coccolith production during coccolithophorid bloom.

Anning et al. (1996) showed that the establishment of the molecular machinery for calcification is an energy-requiring process and that the production of coccoliths utilizes energy for the active transport of Ca^{2+} and bicarbonate. Karwath et al. (2000) showed that at low temperature (16ºC), about 75% of the dinophycean *Thoracospaera heimii* cells were well calcified, which can be attributed to low division rates where the organism is living. These results support the suggestion that calcification is enhanced when cell division was suppressed at low temperature.

Comprehensive knowledge on the response of the coccolithphorids to varying temperatures is essential, considering that *E. huxleyi* and *G. oceanica* are major primary producers that chiefly facilitate the conversion of inorganic carbon dissolved in the ocean to water-insoluble crystals, $CaCO_3$ (Steinmetz, 1994; Winter et al., 1994). The high production of coccoliths by coccolithophorids at low temperature may also increase the probability of survival because the coccosphere, as suggested by Paasche (2002), might be important to maintaining a suitable microenvironment around the cell surface as well as modifying the light environment. The presence of coccolith on the cell surface might be

advantageous because of ecological reasons rather than physiological ones, since artificially produced protoplasts (naked cells) of *E. huxleyi* showed the same growth curve as that of calcified cells in the laboratory culture (Sekino et al., 1996). However, the coccolith production reaction itself might have important functions to protecting cells from photo-damage by consuming excess energy at low temperatures under strong light when photosynthesis is oversaturated because of the suppression of energy-consuming processes (Nimer and Merrett, 1993).

Further studies are necessary to elucidate the role of coccoliths in coccolithophorids. Holligan (1992) stated that reduction in oceanic alkalinity was associated with increased calcification under conditions of higher global mean temperature. This will tend to cause a net shift of CO_2 from the oceans to the atmosphere and more coccolith production would mean greater light scattering which has implications in terms for the efficiency of surface layer heating. From this point of view, change in cell size and coccosphere thickness may also affect light scattering and may indicate the past climatic conditions under which where huge amounts of $CaCO_3$ were deposited as oceanic sediments.

Chapter 2

Production and unsaturation index of alkenones in response to temperature during batch and continuous cultures of the coccolithophorids, *Emiliania huxleyi* and *Gephyrocapsa oceanica*

Introduction

Alkenones are organic compounds of long-chain unsaturated methyl and ethyl ketones that were discovered 20 years ago (Boon et al., 1978; de Leeuw et al., 1980; Volkman et al., 1980). This compound contains a carbonyl group and di-, tri- and tetra-unsaturations with a rare *trans* configuration of double bonds in a C_{37} to C_{39} compound (Fig. 6). They have been found throughout the world's oceans, excepting the Arctic Ocean in both water column and sediment. Lately, alkenones are identified to be a major component of living cells of some specific haptophytes like *E. huxleyi* and *G. oceanica*, thus they are the acknowledged predominant source of alkenones (Volkman et al., 1980; Marlowe et al., 1984; Brassell et al., 1986). More than 90% of the produced alkenones in the open ocean are degraded during transport to the sediment surface, but they tend to retain the degree of unsaturation fixed at the time of synthesis at the sea surface. Hence, the unsaturation index of alkenones became an important tool in paleoceanography (Brassell et al., 1986; Prahl and Wakeham, 1987; Prahl et al., 1989).

Alkenone paleothermometry has been widely employed in the assessment of sea surface temperature in the late Quarternary since it was proposed in the mid-1980s (e.g., Brassell, 1993; Müller et al., 1998). $U^{K'}_{37}$ was used as an index for the degree of unsaturation of C_{37} alkenones, and the value was calculated by the equation, $U^{K'}_{37}$ = [37:2] / ([37:2] + [37:3]), where [37:x] denotes the concentration of alkenones consisting of 37 carbon atoms with x number of double bonds (Brassell et al., 1986; Prahl et al., 1988). The production of alkenones and the number of double bonds varied depending on the growth temperature. A linear $U^{K'}_{37}$-temperature relationship has been established in batch culture experiments with *E. huxleyi* (Prahl and Wakeham, 1987; Prahl et al., 1988).

However, the calibration lines presented in those studies differed from those in subsequent studies by Volkman et al. (1995), Sawada et al. (1996), and Conte et al. (1998). This fact suggests the existence of some unknown factors affecting $U^{K'}_{37}$ apart from the temperature. Previous studies suggested several factors that may possibly influence the content and composition of alkenones in the algal cells such as genetic make-up, changes in the physiological status, nutrient deficiency and salinity (Conte et al., 1995; Conte et al., 1998; Epstein et al., 1998; Popp et al., 1998; Yamamoto et al., 2000; Goñi et al., 2001).

$C_{36:2}$ ethyl (EE) and methyl (ME) alkenoates are intrinsic constituents of the long-chain alkenones biosynthesized by coccolithophorid cells (Prahl et al., 1988). But it is only the EE which is chromatographically well-resolved, unlike the ME which is impossible to quantify with certainty because it appears only as a small peak at the base of $C_{37:2}$ (Fig. 9). Therefore, the ratio between $C_{36:2}$ ethyl alkenoate against total alkenones (EE/K) and total C_{37} alkenones (EE/K_{37}) were calculated to quantify how the relative proportion of alkenoates to alkenones varies in the cells as a function of growth temperature.

The metabolic pathway for the synthesis and the mechanism for changing the degree of unsaturation in alkenone molecules by temperature effect are not clear yet. Considering its importance in the reconstruction of paleotemperature, there is a need to establish thorough knowledge about various factors that may possibly influence the value of $U^{K'}_{37}$ and the mechanism for production and degradation of alkenones. Most importantly, this study tried to elucidate the effect of temperature to the synthesis and unsaturation reaction of alkenones. In addition, the present study is intended to show how the production of alkenone molecules and $U^{K'}_{37}$ are regulated during growth in batch and continuous cultures of *E. huxleyi* and *G. oceanica*. Physiological condition of cells in the batch culture changed with growth period due to nutrient depletion. But such

phenomenon can be prevented in the continuous culture because of incessant dilution of fresh medium to the culture, thus maintaining the physiological condition of the cells constant. Data obtained from the continuous cultures will verify the data from the batch cultures. Besides, alkenone study with a chemostat culture has rarely been performed, therefore, the data obtained here should provide new insight. Results from this study will be important for studying the physiological functions of alkenones in Gephyrocapsean coccolithophorids.

Materials and Methods

Algae and culture maintenance

Algal materials used in this study were *E. huxleyi* and *G. oceanica*. Both species had been maintained in a stock culture as described in Chapter 1. The strains were the same as those used by Sawada et al. (1996).

For pre-experimental cultures, cells harvested from the stock culture at the late logarithmic phase were inoculated into a 500-ml Sakaguchi flask containing 300 ml of MA-ESM in which the soil extract was replaced with 10 nM selenite were used (Danbara and Shiraiwa, 1999). The cultures were maintained at various temperatures (10°C, 15°C, 20°C and 25°C) under continuous illumination at an intensity of 35 μmol m^{-2} s^{-1} and gently hand-shaken once a day to re-suspend the sedimented cells. After 8 days, the cells were transferred to experimental Sakaguchi flasks.

Various parameters like the cell number, the OD_{750} and the pH were measured at intervals as described in Chapter 1. The packed cell volume (PCV) was calculated from a calibration curve of OD_{750} versus PCV determined in a previous experiment (Yamamoto et al., 2000). The cells were harvested by filtration through a Whatman GF/F glass fiber filter precombusted at 400°C for 3 h, and stored frozen at –20°C until being used for extraction and analysis.

A continuous culture of the same strains was carried out at various temperatures in a photobioreactor (Able, Tokyo, Japan). Cells were grown in a pre-culture at 20°C with air bubbling at a flow rate of 260 ml min^{-1} under continuous illumination of 30 μmol m^{-2} s^{-1} in a 500 ml oblong glass vessel, then inoculated into the bioreactor and allowed to grow at the desired cell density. Constant cell density was maintained in the culture by automated dilution with

the same fresh medium as that used in the batch culture. The dilution rate was automatically controlled according to the algal growth rate by changing the amount of fresh medium input through a peristaltic pump connected to a turbidometer. The reaction vessel was a Pyrex glass cylinder of 150 mm i.d. x 300 mm height with a sufficient volume for 4 l of an medium, this being surrounded by twelve 10-W white fluorescent lamps and equipped with a pH stat system, a thermometer, and a turbidometer. The light intensity determined at the center of the vessel was 70 μmol m^{-2} s^{-1}. pH of 8.2 was automatically maintained by adding 0.1 N HCl or 0.1 N NaOH. The desired constant temperature was maintained by using a heater or by controlling the flow of cold water, as necessary. The culture was agitated at 50 rpm by a magnetic stirrer, bubbled with air at a flow rate of 0.4 l min^{-1} under continuous illumination and continuously monitored and controlled by a computer, all data being recorded. At appropriate intervals, 20 ml and 150 ml aliquots of the cell suspension were harvested from the culture to determine the cell number under a microscope, the optical density and for the analysis of alkenones, respectively.

Analytical procedures

Extraction of lipids

Lipids were extracted by four, 5-min rounds of sonication with 6 ml each of dichloromethane-methanol (6:4) and concentrated by rotary evaporation until the volume of solvent is reduced to less than 0.5 ml. Samples were passed through a short bed of Na_2SO_4 (precombusted at 400°C for 3 h) with glass wool to remove the water by using a Pasteur pipet where a 4 ml vial was set underneath. The inside of the flask was washed with dichloromethane/methanol (DCM/MeOH)

(6:4) and transferred to the column several times. Solvent from the vials was dried by nitrogen gas but not for long then, added with a tiny amount of *n*-hexane and stored in the refrigerator for separation into lipid fractions.

Column chromatography

An aliquot of the lipid extract was separated into three fractions (F1: alkenes; F2: alkenones and alkenoates; F3: polar lipids) by column chromatography using an emulsion of silica gel (SiO_2 with 5% distilled water, 5.5 mm i.d. x 45 mm long). The column was washed first with 3 ml of *n*-hexane. Then the F1 labeled vial was set underneath added with 30 µl of 0.1 g/l *n*-$C_{24}D_{50}$ as an internal standard. The extract was transferred into the column by a long Pasteur pipet and the vial containing the extract was washed with 3 ml of hexane:toluene (3:1). Afterwhich, the vial beneath the column was changed with F2 added with 50 µl of 0.1 g/l *n*-$C_{36}H_{74}$ (internal standard) and washed the sample vial by 4 ml of toluene. Then the vial was replaced with F3 and eluted with 3 ml toluene:methanol (3:1). F1 and F2 samples were dried under nitrogen gas stream then added with 50 µl and a certain amount of *n*-hexane calculated based on PCV, respectively. About 50 µl of samples was transferred into the GC vials for analysis.

Gas chromatography

The conditions used for the GC analysis of the alkenones were the same as those described by Yamamoto et al. (2000), as follows: Gas chromatography was conducted using a Hewlett Packard 5890 series II gas chromatograph with on-column injection and electron pressure control systems and a flame ionization

detector (FID). Samples were dissolved in hexane. Helium was used as a carrier gas with a flow rate of 30 cm/s. The column used was a Chrompack CP-Sil 5CB (length 60 m: i.d., 0.25 mm; thickness, 0.25 μm). For the analysis of alkenone samples, the oven temperature was programmed from 70 to 310°C at 20°C/min and then held at 310°C for 40 minutes. While for the alkene samples, the oven temperature was programmed from 70 to 130°C at 20°C/min, from 130 to 310°C at 4°C/min and then held at 310°C for more than 20 minutes.

Results

Algal growth in batch cultures

E. huxleyi and *G. oceanica* showed optimum growth at 20°C and 25°C, respectively, in both batch and continuous cultures (Figs. 7, 11 & 13). *E. huxleyi* could grow even at 25°C and 10°C while the growth of *G. oceanica* was limited to warmer temperatures (20°C and 25°C). The growth rate in *G. oceanica* was much lower than that in *E. huxleyi* cells and failed to grow at temperatures 15°C and below.

Changes in alkenones and alkenoates contents in batch cultures

The alkenone contents (pg cell^{-1}) in cells grown at 15°C, 20°C and 25°C changed during growing phase and attained at almost similar level at the stationary phase during batch culture (Figs. 8A-B). On the other hand, at 10°C that is a critical temperature for growth of *E. huxleyi*, alkenones were rapidly synthesized for about 6 days and continued further with a slow rate. This conspicuous increase of alkenone content at 10°C is the consequence of the apparent increased production of $C_{37:3}$ alkenones even with a decrease in $C_{37:2}$ alkenones (Figs. 8C-E). The ratio of content of $C_{37:3}$ to $C_{37:2}$ alkenones showed clear temperature-dependence. The alkenone contents per cell between the two species at 20°C and 25°C were almost similar. The extraordinary increase in the calculated alkenone content after the stationary growth phase was due to remarkable decrease in cell number resulting from cell death. Changes in the composition of alkenones and alkenoates can clearly been observed in gas chromatogram profiles in cells grown at 10°C and 25°C (Fig. 9).

Changes in $U^{K'}_{37}$ and EE/K ratio in batch cultures

The $U^{K'}_{37}$ (unsaturation index) value and the EE/K ($C_{36:2}$ ethyl alkenoate/total alkenones) ratio varied during the first 4-6 days and then reached a steady level that is specific to the growth temperature in batch cultures (Fig. 10). The average values of $U^{K'}_{37}$ at the steady level were 0.07, 0.23, 0.49 and 0.97 at 10°C, 15°C, 20°C and 25°C, respectively, in *E. huxleyi*, and 0.80 and 0.96 at 20°C and 25°C, respectively, in *G. oceanica*. Ratios for EE/K in both species at the steady level increased with decreasing temperatures (Figs. 10C-D).

Growth, changes in alkenone contents and $U^{K'}_{37}$ in continuous cultures

To make clear of the data obtained from batch cultures, the continuous culture system was applied and introduced to the present alkenone study. The cell density in the continuous culture of *E. huxleyi* remained nearly constant by automated dilution of fresh medium such that the growth conditions and physiological status of the cells were supposed to be maintained constant during culture after switching from different temperatures namely: from 20 to 25°C, 25 to 15°C, 15 to 20°C and 20 to 10°C (Figs. 11A-D). The variations in the counting of cell number are shown by error bars where the standard error ranges from 0.22 to 0.35. The calculated values in cell number and turbidity were also plotted by introducing the dilution rates. The dilution rates (DR) were calculated first at a given time by this equation: *DR* (ml/min) = actual fresh medium injected (ml)/ (time in h x 60 min/h). It was considered that a portion of the fresh medium injected was also included in the outflow of algal culture, hence, another equation was introduced to determine the calculated fresh medium (ml) added as follows: Calculated fresh medium added (ml) = volume of culture x (1-[1-DR/volume of

culture] ^ time). Then to ascertain the calculated cell number and optical density, the following equations were used:

Old medium remained$_t$ (ml) = ([old medium remained$_{tp}$ – calculated fresh medium added$_t$] x old medium remained$_{tp}$) / volume of culture

Calculated cell number or optical density = (measured cell number$_t$ or OD_{750t} x volume culture) / old medium remained$t$

where: t is the given time (h); tp is the given previous time (h); and volume of culture is 4000 ml

The values calculated exhibit the putative changes in growth parameters if the cultures were not diluted with fresh medium. Growth constant (Kg day^{-1}) at all temperatures tested in the continuous culture was very low. The highest Kg day^{-1} measured was 0.11 at culture transferred from 15 to 20ºC and the rest was much lower than it, especially when culture was transferred from 20 to 10ºC, the value was 0.01. The slow growth can be due to a high cell density already maintained at the start of the experiment. Temperature change likely upsets the physiological condition of the cells which probably also induced a low rate of growth. In addition, the sensitivity of the turbidometer that continuously monitors the optical density of the culture was slightly reduced by the disturbance of water bubbles and the coagulated cells that sometimes adhered to it. It resulted to overestimation of cell density and to increase in input of fresh medium and more cells were washed out causing a slight decrease in cell density as shown in Figs. 11A-D. Similar experiments have been performed in *G. oceanica* by changing the temperatures from 20 to 20°C, 20 to 25°C, 25 to 15°C

and 15 to 20°C (Figs. 13A-D). It was difficult to maintain a constant density of cells in the *G. oceanica* culture because of its sensitivity to temperature changes especially at low temperature. Cells at 15°C started to die and were not able to recover even when they were transferred back to higher temperature of 20°C.

Alkenone contents (pg cell^{-1}) in both *E. huxleyi* and *G. oceanica* cells changed in response to temperature change (Figs. 11 and 13). C_{37} alkenones (K_{37}) constituted 50-60% of the total alkenones (K) and the remainders were C_{38} and C_{39} alkenones (Table 1). The K_{37}/K ratio was maintained nearly constant during culture. Consequently, total alkenones change in accordance with the changes in C_{37} alkenones (Figs. 11E-H, 12A-D and 13E-H). The C_{37} alkenones was mainly composed of $C_{37:2}$ and $C_{37:3}$ alkenones in both species (Figs. 11I-L and 13I-L). The values calculated by assuming no dilution of fresh medium to the *E. huxleyi* cultures are also shown in total and C_{37} alkenones as well as $C_{37:2}$ and $C_{37:3}$ alkenones (Figs. 11E-L). The amount of $C_{37:3}$ alkenones was greatly reduced when temperature was increased (Figs. 11I and 12E). The results show that $C_{37:3}$ were degraded or metabolized when temperature was increased from 20 to 25°C and resulted to a slight decrease in the amounts of total and C_{37} alkenones in the culture.

Data in Fig. 11 were recalculated on a per cell basis including the measured and the expected $U^{K'}_{37}$ values (Fig. 12). When the temperature was increased from 20 to 25ºC and 15 to 20ºC, the total alkenone content decreased by resulting from large decrease in $C_{37:3}$ and slight increase in $C_{37:2}$ alkenones. On the other hand, $C_{37:3}$ alkenones largely increased while $C_{37:2}$ alkenones slightly decreased in response to the decrease in temperature from 25 to 15ºC and from 20 to 10ºC. As a result, $U^{K'}_{37}$ decreased and total C_{37} alkenone content gradually increased. As the temperature is increased, $C_{37:3}$ might be metabolized or biochemically modified into other compounds, since the degree of increase in $C_{37:2}$

was less than that of decrease in $C_{37:3}$. Even in *G. oceanica* cultures where difficulty in maintaining the constant cell density was encountered, yet alkenone contents (pg cell^{-1}) increased when growth temperatures decrease, and decreased when temperature was increased (Figs. 13E-L).

The $U^{K'}_{37}$ value began to change rapidly without any obvious lag and gradually attained a steady level that is specific at a given temperature after 2-6 days (Figs. 11I-L and 13M-P). Upon a sudden drop of temperature with a 10°C difference, such as from 25 to 15°C or from 20 to 10°C, the change in $U^{K'}_{37}$ value took about 4 days to reach the steady level.

The final values of $U^{K'}_{37}$, $U^{K'}_{38EK}$ and $U^{K'}_{38MK}$ that were calculated using analogous equations of $U^{K'}_{37}$, respectively, obtained at stationary phase were similar between batch and continuous cultures at each temperature (Fig. 14). The values increased with increasing temperature. The relationship between growth temperature and the values of $U^{K'}_{37}$ and EE/K_{37} were similar between batch and continuous cultures in this study (Fig. 15).

Changes in alkenes and fatty acids

Production of lipid fraction, alkenes (C_{29}, C_{31}, C_{33}) which constitute only about 4 % of the total lipids was also greatly stimulated at 10°C as compared to higher temperatures in *E. huxleyi* (Fig. 17). Changes in the amount of alkenes during culture at various temperatures (10-25°C) followed the same pattern as in alkenones.

Result of GC analysis showed that the bulk of the lipids at stationary level in *E. huxleyi* cells grown at 20°C is composed of alkenones (70%) followed by fatty acids (20%) and the rest is composed of alkenes and sterols. On the other hand, fatty acids comprised about 50% of the total lipids in *G. oceanica* cells while

alkenones make up about 30-40% and the remaining lipids are alkenes and sterols (Fig 18).

Discussion

Temperature dependence of growth

The wide temperature tolerance of *E. huxleyi* can explain its broad distribution (Roth, 1994; Winter et al., 1994) as compared to that of *G. oceanica*. Sawada et al. (1996) used the same strains in their alkenone study in 1994 where cell growth was observed in *G. oceanica* at 15°C, although the growth rate was very low as compared to *E. huxleyi*. The strains used in this study were isolated in 1990 and after that they have been maintained in the stock culture. Such change in optimum growth temperature was obvious in *G. oceanica* than in *E. huxleyi*. These results indicate that cultivation of coccolithophorid cells under constant stock culture conditions with a constant temperature may alter the physiological properties of the strain and limited its ability to acclimate to non-optimum conditions.

Synthesis of alkenones and alkenoates

In both coccolithophorid species, a continuous production of alkenones was observed throughout the culture period. The data further show that alkenones could be detected even in broken cells as indicated by the decline in the cell number (Figs. 7 and 8). This is thought to be due to that alkenones are not easily degraded in broken cells of *E. huxleyi*. The results agreed to the previous report by Rontani et al. (1997).

Figures 1C-D revealed that *G. oceanica* cells are bigger than the cells of *E. huxleyi*, however the alkenone content per cell between these two species at same temperature (20 and 25°C) are similar (Figs. 8A-B). This indicates that cell size

is not a major factor that will influence the quantity of alkenones synthesized within the cell (Conte et al., 1998).

Chapter 1 showed that intracellular calcification in *E. huxleyi* is greatly enhanced at 10°C in comparison with 20°C. As alkenones are known to locate mainly in the coccolith-producing compartment (Sawada and Shiraiwa, 2004), the stimulation of alkenone production at low temperature may closely be associated with that of the calcification physiologically. Alkenones may also be important for the survival of cells at low temperature critical for its growth.

Factors affecting $U^{K'}_{37}$ value and EE/K ratio

In batch culture, nutrient conditions start to change through logarithmic and linear growth phases and then to induce some physiological modifications of alkenones' status, such as the unsaturation degree of alkenones (Epstein et al., 1998; Yamamoto et al., 2000; Versteegh et al., 2001) and the EE/K ratio (Yamamoto et al., 2000). The final values of $U^{K'}_{37}$ and EE/K were obtained near the stationary growth phase in this study (Fig. 10). Therefore, the possibility of nutrient depletion cannot be completely excluded as a reason for the changes in the alkenone parameters, since no exact data on intracellular physiological and metabolic status during batch culture could be obtained. The increasing EE/K values with decrease in temperature showed a systematic decrease in the ethyl alkenoates relative to alkenone content of the cell over the temperature range studied.

The instant change in $U^{K'}_{37}$ value upon temperature change in the continuous cultures clearly showed that the requirement of day-order period for the complete change of alkenone unsaturation reaction is not the complex effect of changes in growth status and temperature (Figs.12I-L and 13M-P). Prahl et al.

(1988) showed that the $U^{K'}_{37}$ value progressively changed 1-4 days after a temperature change in the batch culture of *E. huxleyi*. The result obviously showed that temperature is the major factor that influenced the change in value of the unsaturation index of alkenones.

A comparison between the measured and the expected $U^{K'}_{37}$ was conducted to clearly understand how the unsaturation of alkenones responds to temperature changes (Figs. 12I-L). The expected $U^{K'}_{37}$ was calculated based on the equation by Prahl et al. (1988): $U^{K'}_{37}$ (expected) = $[U^{K'}_{37\ \text{old temp setting}}\, CD_0 + U^{K'}_{37\ \text{new temp setting}}\, (CD_t - CD_0)]/\, CD_t$, where $U^{K'}_{37\ \text{old temp setting}}$ and $U^{K'}_{37\ \text{new temp setting}}$ are the $U^{K'}_{37}$ at time zero and at the end of culture period (time when the value reached a steady level) in the new temperature setting, respectively, and CD_0 and CD_t are the cell densities at time zero and at any time, respectively. The cell densities used in the calculation of the $U^{K'}_{37}$ expected were the calculated values assuming no dilution of fresh medium to the culture. Results show that the measured and the expected $U^{K'}_{37}$ values did not fit. The differences between the results from this study and that of Prahl et al. (1988) could be due to low growth rate in the *E. huxleyi* culture in this study as compared to the latter. Low growth rate implies low number of new cells produced in the new temperature setting, thus the ratio is widely dominated by the old cells containing the alkenones which are typical of the old temperature setting. But, it could also be assumed that the degradation of $C_{37:3}$ are stimulated than the rate of its synthesis when the temperature was increased (from 20 to 25ºC and from 15 to 20ºC). Thus, the measured values were much higher than the expected one. In contrast, when the temperature decreased (from 25 to 15ºC and from 20 to 10ºC), the expected values were much higher than the measured one (Fig. 12). This suggests that the change in $U^{K'}_{37}$ is not just the result of simple admixture of old and new cells producing different alkenones typical to their respective temperature settings. It

can be speculated that the previously produced alkenones prior to temperature change undergo some metabolic changes like unsaturation reaction or degradation at low and high temperature, respectively. Data further implies that the production and unsaturation of $C_{37:2}$ to $C_{37:3}$ were greatly stimulated at low temperature.

The relationships between the growth temperatures and the values of $U^{K'}_{37}$, $U^{K'}_{38ET}$, and $U^{K'}_{38ME}$, and EE/K_{37} are shown in Figs. 14 and 15. These different values were calculated in order to characterize how the overall composition of the long-chain, unsaturated lipid mixture varies as a function of growth temperature (Sawada et al., 1996). The response of $U^{K'}_{37}$, $U^{K'}_{38EK}$, and $U^{K'}_{38MK}$ to temperature in the present study was similar between the batch and continuous cultures, strengthening the suggestion that temperature is the major factor that influences the production and unsaturation of the alkenones. Such a relationship is in contrast with that found by Popp et al. (1998), who reported a marked difference in $U^{K'}_{37}$ between a chemostat and a batch culture of *E. huxleyi* grown at the same temperature. However, at the very beginning of their experiments, the media compositions between the chemostat and batch cultures were different. In addition, they also employed different light conditions between the two experimental set-ups. The examination performed in the chemostat cultures used a nitrate-limited medium under continuous illumination. On the other hand, it was implied that cells in the batch cultures were grown in a nutrient-replete medium on a 14:10 light:dark cycle. Thus, differences in those values particularly the $U^{K'}_{37}$ may be the result of differences in nutrient and light conditions used in the two studies, since these factors were previously suggested by Versteegh et al. (2001) and Epstein et al. (2001) to influence the unsaturation ratio of alkenones. In addition, Liu and Lin (2001) reported that changes in media composition and light intensity changed lipid class and fatty acid compositions. This evidence

further reinforced the suggestion as to the reason for the differences between this study and that of Popp et al. (1998).

Very similar change in unsaturation degree was observed in $C_{37:2}$ and $C_{37:3}$, $C_{38:2MK}$ and $C_{38:3MK}$, $C_{38:2EK}$ and $C_{38:3EK}$, and $C_{39:2EK}$ and $C_{39:3EK}$ alkenones when temperature was changed variously (Table 1). The data clearly show inverse relationship between the contents of alkenones with 2 and 3 double bonds and the temperature tested in this study (e.g. Figs. 8, 12-13, Table 1).

Results of the present study particularly from the batch cultures were compared to the data of Sawada et al. (1996) considering that both studies used the same strains (*E. huxleyi* strain EH2 and *G. oceanica* strain GO1). Those strains had been maintained for approximately 3 and 11 years under constant stock culture conditions until use by Sawada et al. (1996) and in this study, respectively. In *E. huxleyi*, the variations of $U^{K'}_{37}$ and EE/K_{37} among experiments were smaller than the analytical error between 15 and 25°C but the difference was significant at 10°C (Figs. 15A and C). A significant difference especially at low temperature was observed in *G. oceanica* (Figs. 15B and D). Culture conditions between this study and that by Sawada et al. (1996) were similar, the only difference was the age of the strain (i.e., time after isolation). When isolated strains have been maintained under constant conditions for a long time, some physiological changes in cells might develop, leading to changes in the response of cells to environmental conditions. According to Fig. 15, *G. oceanica* strain GO1 may be more sensitive than *E. huxleyi* to such modification by exposure to the constant environment and to alteration of physiological response to temperature change.

Scheme for the regulation of alkenone synthesis by temperature

Based on the presented data, there are two possible ways by which temperature-dependent ratio of di- and tri-alkenones are given in the cell. The first one is that after temperature change the newly produced cells synthesized either with more di- or tri-unsaturated alkenones depending upon the temperature setting such as, from low to high or from high to low, respectively. This speculation is in agreement with the suggestion of Prahl et al. (1988) that the newly produced cells already fully acclimated to the new temperature synthesize different new alkenones resulting to a new $U^{K'}_{37}$ value. The second one is that tri-unsaturated alkenones might be synthesized from di-unsaturated one by unsaturation reaction as described in discussion on the difference between $U^{K'}_{37}$ measured and expected in Figs. 12I-L. In membrane lipids, compound with 3 double bonds such as 18:3 is generally known to be synthesized from 18:2 by a desaturation reaction and to change membrane fluidity in higher plants and algae (Somerville et al., 2000; Sato et al., 2003). The several desaturases and the genes encoding them had already been found and their involvement in the unsaturation reaction is well known (Murata et al., 1992). In non-coccolith-producing haptophyte alga *Isochrysis galbana*, Qi et al. (2002) reported that Δ^8 desaturation pathway is involved in the major route for polyunsaturated fatty acid (PUFA) synthesis. From these evidences we may expect that a coccolith-producing haptophyte alga *E. huxleyi* may also involve such desaturation pathway, since *I. galbana* and *E. huxleyi* are 2 of the rare species synthesizing alkenones.

If analogy of the desaturation mechanism of membrane lipids and the PUFA synthesis is applied to the speculation of alkenone biosynthesis, 3 double bond alkenones such as $C_{37:3}$ can be speculated to be synthesized from 2 double bond ones such as $C_{37:2}$. In this case, the decrease in $U^{K'}_{37}$ when temperature was decreased might be due to the stimulation of the synthesis and desaturation of

$C_{37:2}$ but the suppression of the degradation of $C_{37:3}$. On the other hand, at high temperature the desaturation reaction is suppressed but the degradation of $C_{37:3}$ alkenones is stimulated (Fig. 16). According to Table 1, all alkenone components exhibit similar change in the compositions of C_{37} alkenones. This model in Fig. 16 may also be applied to other alkenone compounds.

Synthesis of alkenes and fatty acids

Although alkenes and alkenones in *E. huxleyi* are structurally and biochemically related, the biological functions and the modes of synthesis of alkenes are still unknown and also deserve further attention. Alkenes represent only about 3-4% of the total lipids, yet the synthesis of this compound was also greatly increased at 10°C similar to alkenones. These compounds are also potential tool as biomarkers for paleoceanography and might be used to confirm results in alkenone studies (Volkman et al., 1980; Grossi et al., 2000). Furthermore, data showed that the percentage of alkenones and fatty acids changed differently within the cell which implies that these compounds may play the same physiological function in the cell and that their metabolic pathways are closely related. Further studies are needed to analyze the modes of synthesis, the degradation, and the physiological roles of alkenones in order to elucidate the importance of this compound in the coccolithophorid cells.

Chapter 3

Inhibitory effect of cerulenin on the synthesis of alkenones in *Emiliania huxleyi* at low temperature

Introduction

Emiliania huxleyi became dominant in coccolith assemblages around 70,000 years ago and accounts for 20-50 % of the total coccolithophore community in the oceans (McIntyre and Bé, 1967). It is commonest in temperate waters from where most of the blooms were recorded that could have persisted for 3-6 weeks. This species gave characteristic sedimentary records through its coccolith production and the suite of organic biomarkers, the long chain-alkenones (*n* C_{37}-C_{39}) and alkenoates (Holligan et al., 1983; Marlowe et al., 1984; Westbroek et al., 1993; Winter et al., 1994; Harris, 1996; Bjima et al., 2001; Paasche, 2002). In *E. huxleyi*, lipids constitute for 50% or more of the organic cell carbon as compared to other marine phytoplanktons (Fernandez et al., 1994). In addition, a high proportion of the lipids are composed of the polyunsaturated fatty acids (Pond & Harris, 1996) and alkenones (Yamamoto et al., 2000). Alkenones with di-, tri- and tetra-unsaturated methyl and ethyl ketones are important biological markers in the oceanic sediments since the unsaturation degree of this compound is not affected by diagenetic process (Volkman et al., 1980; Prahl et al., 1988; Marlowe et al., 1990). Recent studies suggested several possible physiological functions of alkenones like it may play a role in regulating membrane fluidity (Prahl et al., 1988; Brassell, 1993). Alkenones may serve as storage lipids (Epstein et al., 2001; Prahl et al., 2003) and as one of the important players in the photosynthetic pathway (Versteegh et al., 2001). However, unless the metabolic pathway of alkenones will be elucidated, its function should remain unclear.

In Chapter 2, it was described that the production and unsaturation reaction of alkenones were stimulated at low temperature. But previous study suggested no unsaturation reaction in alkenones and that 2 or 3 double bond alkenones are synthesized independently by the cell (Prahl et al., 1988).

Therefore, there is a need to check the reliability of the proposed scheme for the regulation of the synthesis of alkenones. In addition, according to data in Chapter 2 of this thesis, the amount of fatty acids in the cells of *E. huxleyi* and *G. oceanica* was inversely correlated with alkenones. With that, it was presupposed that the metabolic pathways of alkenones and fatty acids are closely similar and/or related. The ratio of saturated to unsaturated fatty acids in phospholipids of *E. coli* is influenced by growth temperature, and is, in part, controlled by enzyme activities of the fatty acid synthase (Marr and Ingraham, 1962; Cronan, 1975). This control has been attributed to a temperature dependence of *β*-ketoacyl-ACP synthase II in the last elongation reaction of the unsaturated pathway of fatty acid (Garwin et al., 1980). In the studies on fatty acid synthesis, cerulenin, an antibiotic produced by *Cephalosphorium caerulens*, is usually used as a tool. Cerulenin specifically inhibit the fatty acid synthase (FAS) to prevent the further elongation of fatty acid chain (Wakil and Stoop, 1983; Funabashi et al., 1989; Morisaki et al., 1993). Hence, in this study I also used cerulenin to determine its inhibitory effect on the synthesis of alkenones by ^{14}C-labeling experiment. Results will contribute to the enlightenment of the biosynthesis of alkenones that would later lead to the possible identification of the enzymes involving in the synthesis. In addition, results will also provide answer to the question as to the relationship on the metabolic pathways between fatty acid and alkenones.

Materials and methods

^{14}C-labeling experiment

Emiliania huxleyi (Lohmann) Hay & Mohler (strain EH2) was used as the algal material. Cells from the stock cultures were inoculated into the 500-ml Erlynmeyer flasks containing 300 ml of MA-ESM. Then the experimental cultures were grown in an incubator (Advantec IS-2300, Tokyo, Japan) equipped with a temperature controller at 20 or 10°C (fluctuation of temperature was ± 0.1ºC) for 4 days under continuous illumination of 30 μmol m^{-2} s^{-1} irradiance. After 3 days, each culture was divided into two and added with 14.6 μM $NaH^{14}CO_3$ (74 MBq/ml, 24.5 MBq/mg, Amersham, Biosciences) for the initiation of ^{14}C-labeling experiment. One of the flasks was added with cerulenin (final concentration, 20 μM) to test its effect on the growth of the cells and the synthesis of alkenones by the amount of ^{14}C-uptake.

At appropriate intervals, an aliquot of cells were harvested from the experimental cultures for the determination of cell number, OD_{750} and ^{14}C-uptake by cells. For the determination of ^{14}C-uptake by the cells, 400 μl of culture was harvested and transferred into the Ultrafree-MC filter unit (Millipore, Billerica, MA) (pore size: 0.65 μm; 8mm diameter,). The cells were filtered by centrifugation, then washed with 400 μl of MA-ESM twice at 5000 x*g* for 5 min at room temperature. The filter on which cells were trapped was cut off from the insert and transferred into a scintillation vial containing 1 ml of a scintillation cocktail, Scintisol EX-H (Dojindo, Kumamoto, Japan), and 80 μl distilled water. Radioactivity was determined by a liquid scintillation counter (LS-6500TA, Beckmann, USA).

In another experiment, cells were first grown at 20ºC for 4 days, then

divided and added with 14.6 μM $NaH^{14}CO_3$ for the initiation of ^{14}C-labeling experiment. The other half was added with 20 μM cerulenin and then transferred to 10ºC. This was done to determine the combined effects of temperature change and cerulenin to the synthesis of alkenones. Moreover, the effect of the different concentration of cerulenin on the growth and synthesis of alkenones was also tested.

Analysis of ^{14}C-uptake by alkenones and other lipid fractions

At the end of culture period, samples were filtered by precombusted (400°C for 3 h) Whatman GF/F filter and stored in frozen at –20°C for further extraction and analysis. Methods used in extraction and separation of various lipid fractions were the same as described in Chapter 2. After elution by different solvents for extraction of the lipid fractions, all samples were added with 100 μl *n*-hexane and the 40 μl aliquot was transferred to a scintillation vial containing 1 ml of a scintillation cocktail, Scintisol EX-H (Dojindo, Kumamoto, Japan). Radioactivity was determined by a liquid scintillation counter.

Preparation of cerulenin solution

Cerulenin (2,3-epoxy-4-oxo7,10-dodecadienamide, Sigma, St. Louis, MO) isolated from *Cephalosphorium caerulens*, was dissolved in 96% acetone and stored in frozen at -20°C in a vial.

Results

The effect of various concentrations (20, 30 and 50 μM) of cerulenin was tested on maintaining cell viability and lipids biosynthesis at 20ºC (Fig. 19). Cerulenin inhibited the biosynthesis of alkenes and alkenones completely at 20 μM. However, the biosynthesis of polar lipids was not so much affected at 20 μM and required 50 μM for strong inhibition. According to these results, 20 μM was used for further experiments. *E. huxleyi* grew well at 20ºC with a growth rate (μ) of 0.18 day^{-1} as compared to a growth rate of 0.10 day^{-1} at 10ºC (Fig. 20). The growth was obviously inhibited by the addition of 20 μM cerulenin. The initial rate of ^{14}C-incoporation into cells was as about twice as higher at 20ºC than 10ºC, but almost similar, irrespective of the temperature tested. The ^{14}C-incoporation for 47 h was reduced by the addition of 20 μM cerulenin to 40 and 70 % of control at 10ºC and 20ºC, respectively. Percent of ^{14}C-incorporation into total lipids as determined by radioactivity was 32% and 21% of the total ^{14}C-uptake at 10ºC and 20ºC, respectively (Fig. 20). In total lipids, there were alkenones (61 % and 18 %), polar lipids (35 % and 82 %) and alkenes (4 % and 0.3 %) in control and cerulenin-treated cells, respectively, at 10ºC. Similarly at 20ºC, alkenones there were (51 % and 24 %), polar lipids (44 % and 76 %) and alkenes (4 % and 0.3 %) in control and cerulenin-treated cells, respectively. The decline in the amount of alkenones due to the addition of cerulenin was compensated by the increase of polar lipids at both temperatures. Cerulenin significantly inhibited 82 % and 63 % of the synthesis of alkenones at 10ºC and 20ºC, respectively, but the inhibition was relatively small in alkenes and polar lipids (Fig. 21).

The effect of cerulenin (20 μM) on alkenone biosynthesis was also determined in another experimental set up where the *E. huxleyi* culture was first incubated at 20ºC for 72 h in pre-culture, then transferred to 10ºC. $NaH^{14}CO_3$

was added as carbon source at the time when temperature was decreased. Growth in terms of change in cell number was suppressed 8-31% during experiment in the presence of cerulenin (Fig. 23). The suppression of ^{14}C-incorporation into cells was about 40% and almost same as that into total lipids. In lipids, the synthesis of alkenones was suppressed 69-87 % by cerulenin, but that of polar lipids did not (16-28%). Therefore, the inhibitory effect of cerulenin in this experiment was due to the inhibition of alkenone biosynthesis and cerulenin-sensitive enzymes are involved in alkenone biosynthesis pathway.

Discussion

The synthesis of alkenones was significantly increased at 10°C as compared to other lipid fractions such as polar lipids and alkenes (Figs. 21, Chapter 2). Intracellular calcification was also accelerated at 10ºC (Chapter 1). Additionally, Sawada and Shiraiwa (2004) reported that alkenones are predominantly located in the coccolith-producing compartment (CPC) of the cell. These data may indicate that the synthesis of alkenones is associated with coccolith production and that both processes are necessary for coccolithophorid cells to survive at low temperature. As the distribution of alkenones is strongly limited to certain species of haptophyte algae such as *E. huxleyi, G. oceanica, I. galbana* and *Chrysotila lamellosa*, relationship between coccolith production and alkenones is still unclear. Generally, lower growth temperatures are known to lead to an increase in alkenone unsaturation (Volkman et al., 1980; Prahl et al., 1998; Conte et al., 1998). The same stimulation of unsaturation by low temperature was also common to fatty acids (Hardwood and Russell, 1984). Fatty acids grow by consecutive elongation of acyl chain with C_2 units derived from malonyl-CoA. The elongation of fatty acid is facilitated by three different enzymes namely: the acetoacetyl-[acyl carrier protein] (ACP), the *β*-ketoacyl-ACP synthase I (KAS I) and the *β*-ketoacyl-ACP synthase II (KAS II) which are differentially sensitive to cerulenin (Vance et al., 1972; Shimakata and Stumpf, 1982; MacKintosh et al., 1989). The use of cerulenin irreversibly inhibits FAS of both multifunctional and unassociated enzymes, thus preventing the further elongation of fatty acid (Wakil and Stoop, 1983; Jaworski et al., 1989; MacKintosh et al., 1989). Evidence of the greater inhibitory effect of cerulenin on alkenone synthesis at 10ºC than at 20ºC suggests that the enzymes for the synthesis of alkenones were also susceptible to cerulenin (Figs. 21 and 23). It

further implies that the metabolic pathway of alkenones may be closely associated to and/or connected to the downstream of the fatty acids synthesis. The slightly lower suppression by cerulenin on alkenone synthesis in *E. huxleyi* cells grown first at 20ºC then transferred to 10ºC as compared to cells continuously grown at 10ºC supports the suggestion in Chapter 2, that at low temperature the alkenones synthesis is greatly enhanced through the production of new alkenones as well as the unsaturation reaction of double bonds to three double bonds alkenones.

The lesser suppression by cerulenin on polar lipids than on alkenones (Figs. 21 and 22) might be due to the fact that polar lipids are composed of not only fatty acids but also sterols, chlorophylls and other membrane lipids which are synthesized on a different pathway. As such, even if the elongation of fatty acid was inhibited by cerulenin as reported in previous studies (Funabashi et al., 1989; Siggaard-Andersen et al., 1991; Child and Shoolingin-Jordan, 1998), the percent inhibition should be much lower than that of the alkenones.

The elucidation of metabolic pathway for alkenone biosynthesis is very important to increase reliability of the alkenone paleothermometer by determining unknown factors affecting the calibration curve of the unsaturation index versus temperature. Further, it is very important to make clear how such long-chain ketones were synthesized in limited haptophyte species and how such metabolism has been acquired by the organisms evolutionally. Genetic analysis of cerulenin-binding protein and unsaturation enzymes on alkenone biosynthesis will give important information to those questions.

General Discussion

The widespread distribution of *E. huxleyi* in the global ocean can be explained by its ability to grow and survive at a wide temperature range (10-25°C). Low temperature reduced growth rate of the coccolithophorid cells but enhanced the enlargement of cell size and the coccolith production (Chapter 1). The result supports the suggestion by Shiraiwa (2003) that the enlargement of cell volume is closely related to calcification. The differing relationship between the growth rate and the calcification may suggest that the phase for increasing cell number of coccolithophorids and for the calcite formation may be separated with a time lag during blooms. Calcification is stimulated when the slowly growing cells can spare the energy required for the transport of Ca^{2+} and bicarbonate (Anning et al., 1996). These results suggest that calcification is enhanced when cell division was suppressed at low temperature.

Survival of coccolithophorids at low temperature may be enhanced by the synthesis of more coccoliths because it creates a suitable microenvironment that can modify the light environment around the cell surface (Paasche, 2002). Coccoliths can protect the cells from photo-damages by utilizing surplus energy at low temperature where photosynthesis is oversaturated due to suppression of energy consuming growth processes. Calcification may also have an ecological impact since coccoliths enhance the light scattering (Holligan et al., 1983) and may have an implication on the efficiency of surface layer heating. The increased coccolith production at low temperature may elucidate the past climatic condition where huge depositions of $CaCO_3$ as oceanic sediments were observed.

Alkenones produced by *E. huxleyi* and *G. oceanica* are chemically stable compounds because they could still be detected in cells that started to be broken due to cell death (Rontani et al., 1997). The similar alkenone content between *E. huxleyi* and *G. oceanica* cells whose diameters without coccosphere are arround 4 and 6 μm, respectively, at same temperatures, implies that cells size is not a

major factor that determines the amount of alkenones synthesized by the cell (Conte et al., 1998). The bulk of alkenones is composed of C_{37} (50-60 %) and the rest was represented by C_{38} and C_{39} alkenones. Alkenone production was significantly stimulated at 10°C which was a consequence of increased production of $C_{37:3}$ alkenones. It can be speculated that alkenones may be essential for the continued existence of the cells at low temperature which is critical for their growth. In Chapter 1, I showed that calcification is enhanced at low temperature (10ºC), and as alkenones are chiefly localized in the coccolith-producing compartment (Sawada and Shiraiwa 2004), this may imply that the physiological function(s) of alkenones is closely associated with calcification.

The unsaturation index of alkenones ($U^{K'}_{37}$) decreased in parallel with the decrease of temperature but the ratio of ethyl alkenoate ($C_{36:2}$) against total alkenones (K) increased with decreasing temperature. This result showed the systematic decrease in alkenoates relative to alkenone content in the cell. $U^{K'}_{37}$ instantly changed in accordance with the change in temperature without any lag period in the continuous culture. $U^{K'}_{37}$, $U^{K'}_{38EK}$ and $U^{K'}_{38MK}$ were similar between batch and continuous cultures, suggesting that temperature is the key factor that induced the unsaturation reaction to progress and determines the production of alkenones in the cells, and the factors that change during a batch culture are not so effective for $U^{K'}_{37}$.

Since the expected shift in $U^{K'}_{37}$ values as calculated by the equation of Prahl et al. (1988) did not fit with the measured values in the continuous culture of this study, the unsaturation reaction of alkenones is suggested to be stimulated at low temperature. Lower growth temperatures generally lead to an increase in fatty acid unsaturation in plants, algae and fungi (Hardwood and Russell, 1984). Fatty acids in membrane lipids such as 18:3 are known to be synthesized from 18:2 by a desaturation reaction to regulate the fluidity of the

membrane (Somerville et al., 2000; Sato et al., 2003). In addition, Δ^8 desaturation pathway was reported in *Isochrysis galbana*, a non-coccolith-forming haptophyte and one of the rare species that synthesize alkenones to be involved in the PUFA synthesis (Qi et al., 2002). If all these evidences will be applied to the mechanism by which alkenones are synthesized in the cells, it also can be speculated that alkenones with 3 double bonds are synthesized by the unsaturation of alkenones with 2 double bonds. So, the decrease of $U^{K'}_{37}$ at low temperature is the result of stimulation of synthesis and desaturation of $C_{37:2}$. While the increase of $U^{K'}_{37}$ at high temperature is the result of enhanced degradation of $C_{37:3}$ but not by the suppression of the unsaturation reaction of $C_{37:2}$ to $C_{37:3}$ alkenones.

The synthesis of alkenes was also greatly stimulated at 10ºC which only confirmed their close structure and biochemical relations to alkenones. Although it was reported that alkenes are potential as biomarkers in paleoceanography (Grossi et al., 2000), the minimal amount synthesized by the cell limited its use. Nevertheless, result in this study suggests that alkenes are useful to confirm the results on C_{37}-alkenone studies in paleo-SST estimation.

The proposed scheme on the increased synthesis and unsaturation reaction of alkenones at low temperature was further studied by using cerulenin, an antibiotic commonly used to inhibit the condensing enzymes (acetoacetyl-[acyl carrier protein] (ACP), the *β*-ketoacyl-ACP synthase I (KAS I) and the *β*-ketoacyl –ACP synthase II (KAS II)) for fatty acid elongation (Vance et al., 1972; Shimakata and Stumpf, 1982; MacKintosh et al., 1989). The addition of 20 μM cerulenin inhibited the growth and the synthesis of alkenones. The results suggest that physiological status and metabolic processes of the cells were inhibited by cerulenin. The ^{14}C-incoporation of cells at 10 and 20°C were similar in control which implies that certain amount of carbons incorporated into cells at

10ºC were not utilized for growth but maybe used for the synthesis of other compounds like alkenones of which the production was greatly enhanced at this temperature. The synthesis of alkenones was greatly suppressed as compared to alkenes and polar lipids at 10°C. This implies that the biosynthesis of alkenones may be catalyzed by cerulenin-sensetive enzymes. In addition, the alkenone producing steps may be at the downstream of fatty acid synthesis and/or the elongation reaction of alkenones may be catalyzed by KAS or KAS-like enzymes.

Further studies are recommended on the effect of cerulenin to alkenone synthesis and identification of the enzymes involving in this process. Results from such studies might elucidate the physiological role of alkenones in the cell. Furthermore, data obtained in this research may also confirm the dependability and accuracy of alkenones as paleoceanographic tool in the determination of paleo sea surface temperature.

In summary, this study reveals that low temperature stress enhanced the enlargement of coccolithophorid *E. huxleyi* cells and stimulate the coccolith production but greatly suppressed the cell growth. These changes are reversible at high temperature. Data also implies that enlargement of cell volume is directly correlated with increased calcification. Simultaneously, an increased production and unsaturation of alkenones were stimulated at low temperature but the degradation of $C_{37:3}$ alkenones was enhanced at high temperature. Although the relationship among these phenomena is not yet clear, the results suggest that they may be closely associated with regulation by temperature-sensitive signal transduction system. It can further be speculated that increased coccolith and alkenone production at low temperature may be essential for the survival of this coccolithophorids at low temperature critical for its growth.

References

Anning, T., Nimer, N., Merrett, J., and Brownlee, C. (1996). Costs and benefits of calcification in coccolithophorids. *J Mar Syst* 9: 45-56.

Balch, W.M., and Kilpatrick, K. (1995). Calcification rates in the equatorial Pacific along 140°W. *Deep-Sea Res* 43: 971-993.

Bjima, J., Altabet, M., Conte, M., Kinkel, H., Versteegh, G..J.M., Volkman, J.K., Wakeham, S.G., and Weaver, P.P. (2001). Primary signal: ecological and environmental factors – report from Working Group 2. *Geochem Gephys Geosys* 2: 2000GC000051.

Blackwelder, P.L. (1984). Coccolithophore temporal and regional population dynamics and calcification in warm core rings and NW Atlantic Water. *Eos* 65, 912.

Boon, J.J., van der Meer, F.W., Schuyl, P.J., de Leeuw, J.W., and Schenck, P.A. (1978). Organic geochemical analyses of core samples from site 362 Walvis Ridge, DSDP Leg 40. *Initial Report of the Deep Sea Drilling Project* 40: 627-637.

Brand, L.E. (1982). Genetic and spatial patterns of genetic differentiation in the reproduction rates of the marine coccolithophores *Emiliania huxleyi* and *Gephyrocapsa oceanica*. *Limnol Oceanogr* 27: 236-245.

Brassell, S.C. (1993). Applications of biomarkers for delineating marine paleoclimatic fluctuations during the Pleistocene. In: Engel, M.H., and Macko, S.A. (eds.) Organic Geochemistry, Principles and Applications.

Plenum Press, New York, pp. 699-738.

Brassell, S.C., Eglinton, G., Marlowe, I.T., Plaufmann, U., and Sarnthein, M. (1986). Molecular stratigraphy: a new tool for climatic assessment. *Nature* 320: 129-133.

Brown, C.W., and Yoder, J.A. (1994). Coccolithophorid blooms in the global ocean. *J Geophy Res* 99: 7467-7482.

Charlston, R.J., Lovelock, J.E., Andreae, M.O., and Warren, S.G. (1987). Oceanic phytoplankton, atmospheric sulfur, cloud albedo and climate. *Nature* 326: 655-661.

Child, C.J., and Shoolingin-Jordan, P.M. (1998). Inactivation of the polyketide synthase, 6-methylsalicylic acid synthase, by the specific modification of Cys-204 of the *β*-ketoacyl synthase by the fungal mycotoxin cerulenin. *Biochem J* 330: 933-937.

Conte, M.H., Thompson, A., Eglinton, G., and Green, J.C. (1995). Lipid biomarker diversity in the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae) and the related species *Gephyrocapsa oceanica*. *J Phycol* 31: 272-282.

Conte, M.H., Thompson, A., Lesley, D., and Harris, R.P. (1998). Genetic and physiological influences on the alkenone/alkeoate versus growth temperatures relationship in *Emiliania huxleyi* and *Gephyrocapsa oceanica*. *Geochim Cosmochim Acta* 62: 51-68.

Cronan, J.E. Jr. (1975). Thermal regulation of the membrane lipid composition of *Escherichia coli*. Evidence for the direct control of fatty acid synthesis. *J Biol Chem* 250: 7074-7077.

Danbara, A., and Shiraiwa, Y. (1999). The requirement of selenium for the growth of marine coccolithophorids, *Emiliania huxleyi*, *Gephyrocapsa oceanica* and *Helladosphaera* sp. (Prymnesiophyceae). *Plant Cell Physiol* 40: 762-766.

Eppley, R.W., Rogers, J.N., and MacCarthy, J.J. (1969). Half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. *Limnol Oceanogr* 14: 912-920.

Epstein, B.L., Hondt, S.D'., and Hargraves, P.E. (2001). The possible metabolic role of C_{37} alkenones in *Emiliania huxleyi*. *Org Geochem* 32: 867-875.

Epstein, B.L., Hondt, S.D'., Quinn, J.G. and Zhang, J. (1998). An effect of dissolved nutrient concentrations on alkenone-based temperature estimates. *Paleoceanogr* 13: 122-126.

Fernandez, E., Balch, W.M., Marañon, E., and Holligan, P.M. (1994). High rates of lipid biosynthesis in cultured, mesocosm and coastal populations of the coccolithophore *Emiliania huxleyi*. *Mar Ecol Prog Series* 114: 13-22.

Fritz, J.J., and Balch, W.M. (1996). A light limited continuous culture study of *Emiliania huxleyi*: determination of coccolith detachment and its relevance to cell sinking. *J Expt Mar Biol Ecol* 207: 127-147.

Funabashi, H., Kawaguchi, A., Tomoda, H., Omura, S., Okuda, S., and Iwasaki, S. (1989). Binding site of cerulenin in fatty acid synthetase. *J Biochem* 105: 751-755.

Garwin, J.L., Klages, A.L., and Cronan, Jr. J.E. (1980). Structural, enzymatic, and genetic studies of *β*-ketoacyl-acyl carrier protein synthase I and II of *Escherichia coli*. *J Biol Chem* 255: 11949-11956.

Gates, R.D., Baghdasarian, G., and Muscatine, L. (1992). Temperature stress causes host cell detachment in symbiotic cnidarians: implication for coral bleaching. *Biol Bull* 182: 324-332.

Goñi, M.A., Hartz, D.M., Thunell, R.C., and Tappa, E. (2001). Oceanographic considerations for the application of the alkenone-based paleotemperature $U^{K'}_{37}$ index in the Gulf of California. *Geochim Cosmochim Acta* 65: 545-557.

Green, J.C., Heimdal, B.R., Paasche, E., and Moate, R. (1998). Changes in calcification and the dimensions of coccoliths of *Emiliania huxleyi* (Haptophyta) grown at reduced salinities. *Phycologia* 37:121-131.

Grossi, V., Raphel, D., Aubert, C., and Rontani, J.-F. (2000). The effect of growth temperature on the long-chain alkenes composition in the marine coccolithophorid *Emiliania huxleyi*. *Phytochemistry* 54; 393-399.

Harris, R.P. (1996). Coccolithophorid dynamics: The European *Emiliania huxleyi* programme, EHUX. *J Mar Syst* 9: 1-11.

Harwood, J.L., and Russell N.J. (1984). Distribution of lipids. In: Allen, G., and Unwin (eds.) Lipids in Plants and Microbes. University College of Wales, Cardiff, p. 63.

Holligan, P.M. (1992). Do marine phytoplankton influence global climate? In: Falkowski P.G., and Woodhead, A.D. (eds.) Primary Productivity and Biogeochemical Cycles in The Sea. Plenum Press, New York, pp. 487-501.

Holligan, P.M., Viollier, M., Harbour, D.S., Camus, P., and Champagne-Philippe, M. (1983). Satellite and ship studies of coccolithophore production along a continental shelf edge. *Nature* 304: 339-342.

Jaworski, J.G., Clough, R.C., and Barnum, S.R. (1989). A cerulenin insensitive short chain 3-ketoacyl-acyl carrier protein synthase in *Spinacia oleracia* leaves. *Plant Physiol* 90: 41-44.

Karwath, B., Janofske, D., Tietjen, F., and Willems, H. (2000). Temperature effects on growth and cell size in the marine calcareaous dinoflagellate *Thoracospaera heimii*. *Mar Micropal* 39: 43-51.

Lee, R.E. (1999). Phycology. Cambridge University Press, Cambridge, U.K., pp. 558-565.

de Leeuw, J.W., van der Meer, F.W., Rijpstra, W.I.C., and Schenck, P.A. (1980). On the occurrence and structural identification of long chain unsaturated ketones and hydrocarbons in sediments. In: Douglas, A.G., and Maxwell, J.R. (eds.) Advances in Organic Geochemistry 1979. Pergamon Press, Oxford, pp.

211-217.

Linschooten, C., van Bleijswijk, J.D.L., van Emburg, P., de Vrind, J.P.M., Kempers, E.S., Westbroek, P., and de Vrind-de Jong, E.W. (1991). Role of the light-dark cycle and medium composition on the production of coccoliths by *Emiliania huxleyi* (Haptophyceae). *J Phycol* 2: 82-86.

Liu, C.-P., and Lin, L.-P. (2001). Ultrastructural study and lipid formation of *Isochrysis* sp. CCMP 1324. *Bot Bul Acad Sin* 42: 207-214.

MacKintosh, R.W., Hardie, D.G., and Slabas, A.R. (1989). A new assay procedure to study the induction of *β*-ketoacyl-ACP synthase I and II, and the complete purification of *β*-ketoacyl-ACP synthase I from developing seeds of oilseed rape (*Brassica napus*). *Biochim Biophys Acta* 1002: 114-124.

Marlowe, I.T., Brassell, S.C., Eglinton, G., and Green, J.C. (1984). Long chain unsaturated ketones and esters in living algae and marine sediments. *Org Geochem* 6: 135-141.

Marlowe, I.T., Brassell, S.C., Eglinton, G., and Green, J.C. (1990). Long-chain alkenones and alkyl alkenoates and the fossil coccolith record of marine sediments. *Chem Geol* 88: 349-375.

Marr, A.G., and Ingraham, J.L. (1962). Effect of temperature on the composition of fatty acids in *Escherichia coli*. *J Bacteriol* 84: 1260-1267.

McIntyre, A.J., and Bé, A. (1967). Modern coccolithophoridae in the Atlantic

Ocean. I. Placoliths and Crytoliths. *Deep-Sea Res* 14: 561-597.

Morisaki, N., Funabashi, H., Shimazawa, R., Furukawa, J., Kawaguchi, A., Okuda, S., and Iwasaki, S. (1993). Effect of side-chain structure on inhibition of yeast fatty acid synthase by cerulenin analogues. *Eur J Biochem* 211: 111-115.

Murata, N., Wada, H., and Gombos, Z. (1992). Modes of fatty acid desaturation in cyanobacteria. *Plant Cell Physiol* 33: 933-941.

Müller, P.J., Kirst, G., Ruhland, G., von Storch, I., and Rosell-Melé, A. (1998). Calibration of the alkenone paleo-temperature index $U^{K'}_{37}$ based on core-tops from eastern South Atlantic and the global ocean (60°N-60°S). *Geochim Cosmochim Acta* 62: 1722-1757.

Nimer, N.A., and Merrett, M.J. (1993). Calcification rate in *Emiliania huxleyi* Lohmann in response to light, nitrate and availability of inorganic carbon. *New Phytol* 123: 673-677.

Obata, T., Araie, H., and Shiraiwa, Y. (2004). Bioconcentration mechanism of selenium by a coccolithophorid, *Emiliania huxleyi*. *Plant Cell Physiol* 45: 1434-1441.

Okada, H., and Honjo, S. (1973). The distribution of oceanic coccolithophorids in the Pacific. *Deep Sea Res* 26, 355-374.

Okada, H., and Honjo, S. (1979). Distribution of coccolithophores in marginal

seas along the western Pacific Ocean and in the Red Sea. *Mar Biol* 31: 271-285.

Paasche, E. (2002). A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. *Phycologia* 40: 503-529.

Pond, D.W., and Harris, R.P. (1996). The lipid composition of the coccolithophores *Emiliania huxleyi* and its possible ecophysiological significance. *J Mar Biol Assoc UK*, 76: 579-594.

Popp, B.N., Kenig, F., Wakeham, S.G., Laws, E.A., and Bidigare, R.R. (1998). Does growth rate affect ketone unsaturation and intracellular carbon isotopic variability in *Emiliania huxleyi*? *Paleoceanogr* 13: 35-41.

Prahl, F.G., de Lange, G.J., Lyle, M., and Sparrow, M.A. (1989). Post-depositional stability of long-chain alkenones under contrasting redox conditions. *Nature* 341: 434-437.

Prahl, F.G., Laurel, A., Muehlhausen, A., and Zanhle, D.L. (1988). Further evaluation of long-chain alkenones as indicators of paleoceanographic conditions. *Geochim Cosmochim Acta* 52: 2303-2310.

Prahl, F.G., and Wakeham, S.G. (1987). Calibration of unsaturation patterns in long-chain ketone compositions for paleotemperature assessment. *Nature* 330: 367-369.

Prahl, F.G., Wolfe, G.V., and Sparrow, M.A. (2003). Physiological impacts on alkenone paleothermometry. *Paleoceanography* 18: 1025-1029.

Qi, B., Beaudoin, F., Fraser, T., Stobart, A.K., Napier, J.A., and Lazarus, C.M. (2002). Identification of a cDNA encoding a novel C18-Δ^9 polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isochrysis galbana*. *FEBS Lett* 510: 159-165.

Qiang, L., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP / AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* 10: 1391-1406.

Rontani, J.F., Cuny, P., Grossi, V., and Beker, B. (1997). Stability of long-chain alkenones in senescing cells of *Emiliania huxleyi*:effect of photochemical and aerobic microbial degradation on the alkenone unsaturation ratio ($U^{K`}_{37}$). *Org Geochem* 26: 503-509.

Roth, P. (1994). Distribution of coccoliths in oceanic sediments. In: Winter A., and Siesser, W. G. (eds.) Coccolithophores. University Press Cambridge, Cambridge, pp. 199-218.

Sato, N., Tsuzuki, M., and Kawaguchi, A. (2003). Glycerolipid synthesis in *Chlorella kessleri* 11h I. Existence of a eukaryotic pathway. *Biochim Biophys Acta* 1633: 27-34.

Saitoh, S., Shiga, N., Mizobata, K., Miyamura, T., Imai, K., and Toratani, M. (2001). The observation of coccolithophore bloom in the southeastern Bering Sea, in Summer 2000. The Spring Meeting of the Oceanographic Society of Japan (Abstract), p. 230.

Sawada, K., Handa, N., Shiraiwa, Y., Danbara, A., and Montani, S. (1996). Long-chain alkenones and alkyl alkenoates in the coastal and pelagic sediments of the northwest North Pacific, with special reference to the reconstruction of *Emiliania huxleyi* and *Gephyrocapsa oceanica* ratios. *Org Geochem* 24: 751-764.

Sawada, K., and Shiraiwa, Y. (2004). Alkenone and alkenoic acid compositions of the membrane fractions of *Emiliania huxleyi*. *Phytochemistry* 65: 1299-1307.

Sekino, K., and Shiraiwa, Y. (1994). Accumulation and utilization of dissolved inorganic carbon by a marine unicellular coccolithophorids, *Emiliania huxleyi*. *Plant Cell Physiol* 35: 353-361.

Sekino, K., and Shiraiwa, Y. (1995). CO_2 release from the culture of calcifying and noncalcifying marine unicellular algae. *J Mar Biotech* 3: 101-104.

Sekino, K., Kobayashi, H., and Shiraiwa, Y. (1996). Role of coccoliths in the utilization of inorganic carbon by a marine unicellular coccolithophorid, *Emiliania huxleyi*: a survey using intact cells and protoplasts. *Plant Cell Physiol* 37: 123-127.

Shimakata, T., and Stumpf, P.K. (1982). Isolation and function of spinach leaf β-

ketoacyl-[acyl-carrier-protein] synthases. *Proc Natl Acad Sci USA* 79: 5808-5812.

Shin, K.H., Tanaka, N., Harada, N., and Marty, J.C. (2002). Production and turnover rates of C_{37} akenones in the eastern Bering Sea: implication for the mechanism of a long duration of *Emiliania huxleyi* bloom. *Prog Oceanogr* 55: 113-129.

Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. *Plant Biol* 6: 410-417.

Shiraiwa, Y. (2003). Physiological regulation of carbon fixation in the photosynthesis and calcification of coccolithophorids. *Comp Biochem and Physiol Part B* 136: 775-783.

Shiraiwa, Y., Hatano-Sugimoto, Y., and Satoh, M, (2003). Regulation of intracellular calcification and algal growth by nutrient supply in coccolithophorids. In: Kobayashi I., and Ozawa, H. (eds.) Biomineralization (BIOM 2001): Formation; Diversity, Evolution and Application. Tokai University Press, Kanagawa, Tokyo, pp. 241-246.

Siggaard-Andersen, M., Sakari, K., and Wettstein-Knowles, P. von. (1991). Primary structure of a cerulenin-binding *β*-ketoacyl-[acyl carier protein] synthase from barley chloroplasts. *Proc Natl Acad Sci USA* 88: 4114-4118.

Sikes, C.S., Roer, R.D., and Wilbur, K.M. (1980). Photosynthesis and coccolith

formation. Inorganic carbon sources and net inorganic reaction of deposition. *Limnol Oceanogr* 25: 248-261.

Somerville, C., Browse, J., Jaworski, J.G., and Ohlrogge, J.B. (2000). Lipids. In: Buchanan, B.B, Gruissem, W., and Jones, R.L. (eds.) Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, Maryland, pp.456-527.

Steinmetz, J.C. (1994). Sedimentation of coccolithophores. In: Winter, A., and Siesser, W.G. (eds.) Coccolithophores. University Press Cambridge, Cambridge, pp. 179-197.

Suzuki, I., Kanesaki, Y., Mikami, K., Kanehisa, M., and Murata, N. (2001). Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. *Mol Microbiol* 40: 235-244.

Vance, D., Goldberg, I., Mitsuhashi, O., Block, K., Omura, S., and Nomura, S. (1972). Inhibition of fatty acid synthetases by the antibiotic cerulenin. *Biochem Biophys Res Commun* 48: 649-656.

Versteegh, G.J.M., Riegman, R., de Leeuw, J.W., and Jansen, J.H.F. (2001). $U^{K'}_{37}$ values for *Isochrysis galbana* as a function of culture temperature, light intensity and nutrient concentrations. *Org Geochem* 32: 785-794.

Volkman, J.K., Barrett, S.M., Blackburn, S.I., and Sikes, E.L. (1995). Alkenones in *Gephyrocapsa oceanica*: Implications for studies of paleoclimate. *Geochim Cosmochim Acta* 59: 513-520.

Volkman, J.K., Eglinton, G., Corner, E.D.S., and Forsberg, T.E.V. (1980). Long-chain alkenes and alkenones in the marine coccolithophorid *Emiliania huxleyi*. *Phytochemistry* 19: 2619-2622.

Volkman, J.K., Eglinton, G.., Corner, E.D.S., and Sargent, J.R. (1980). Novel unsaturated straight-chain C_{37}-C_{39} methyl and ethyl ketones in marine sediments and a coccolithophore *Emiliania huxleyi*. In: Douglas, A.G., and Maxwell, J.R. (eds.) Advances in Organic Geochemistry 1979. Pergamom Press, Oxford, pp. 219-227.

Wakil, S.J., and Stoop, J.K. (1983). The enzymes. In: Boyer, P.D. (ed.) Academic press, New York, pp. 3-61.

Westbroek, P., Brown, C.W., van Bleijswijk, J., Brummer, G.J., Conte, M.H., Egge, J., Fernandez, E., Jordan, R., Knappertsbusch, M., Stefels, J., Veldhuis, M., van der Wal, P., and Young, J. (1993). A model system approach to biological climate forcing: the example of *Emiliania huxleyi*. *Global Planet Change* 8: 27-46.

Winter, A., Jordan, R.W., and Roth, P.H. (1994). Biogeography of living coccolithophores in ocean waters. In: Winter, A., and Siesser, W.G. (eds.) Coccolithophores. University Press Cambridge, Cambridge, pp. 161-177.

Yamamoto, M., Shiraiwa, Y., and Inouye, I. (2000). Physiological responses of lipids in *Emiliania huxleyi* and *Gephyrocapsa oceanica* (Haptophyceae) to growth status and their implications for alkenone paleothermometry. *Org Geochem* 31: 799-811.

Young, J.R., and Ziveri, P. (1999). Calculation of coccolith volume and its use in calibration of carbonate flux estimates. *Deep Sea Res* II 47: 1679-1700.

Table and Figures

Table 1. Changes in the amounts (pg cell^{-1}) of various alkenone compounds and their percent of total alkenones after the transfer of cells to different temperatures in a continuous culture of *Emiliania huxleyi*. Cells were pre-grown for 8 days at 20°C, then transferred to 25°C, 15°C, 20°C and 10°C in this sequence, as shown in the table.

Temperature change	Time (h)	Amount of alkenones (pg cell^{-1}) and its percent of total alkenones (%)															
		$C_{37:2}$		$C_{37:3}$		$C_{38:2}$MK		$C_{38:3}$MK		$C_{38:2}$EK		$C_{38:3}$EK		$C_{39:2}$EK		$C_{39:3}$EK	
20 to 25ºC	0	0.135	(34)	0.133	(33)	0.011	(3)	0.015	(4)	0.061	(16)	0.029	(17)	0.005	(1)	0.002	(1)
	16	0.192	(45)	0.061	(14)	0.019	(4)	0.010	(2)	0.116	(27)	0.020	(5)	0.008	(2)	0.001)	(1)
	113	0.154	(54)	0.014	(5)	0.016	(4)	0.003	(1)	0.091	(33)	0.004	(2)	0.003	(1)	nd	
	135	0.174	(55)	0.011	(3)	0.018	(6)	0.002	(1)	0.105	(32)	0.003	(1)	0.006	(2)	nd	
25 to 15ºC	0	0.174	(55)	0.011	(3)	0.018	(6)	0.002	(1)	0.105	(32)	0.003	(1)	0.006	(2)	nd	
	20	0.379	(41)	0.146	(16)	0.052	(6)	0.025	(3)	0.244	(26)	0.048	(5)	0.024	(2)	0.005	(1)
	39	0.185	(30)	0.171	(27)	0.027	(4)	0.030	(5)	0.133	(21)	0.069	(10)	0.014	(2)	0.007	(1)
	88	0.083	(20)	0.139	(34)	0.014	(3)	0.026	(6)	0.070	(17)	0.070	(17)	0.008	(2)	0.007	(1)
	113	0.064	(16)	0.151	(38)	0.011	(3)	0.028	(6)	0.057	(14)	0.080	(20)	0.007	(1)	0.008	(2)
15 to 20ºC	0	0.064	(16)	0.151	(38)	0.011	(3)	0.028	(6)	0.057	(14)	0.080	(20)	0.007	(1)	0.008	(2)
	40	0.080	(26)	0.107	(35)	0.007	(2)	0.013	(4)	0.058	(19)	0.039	(12)	0.003	(1)	0.002	(1)
	64	0.083	(27)	0.102	(33)	0.008	(3)	0.013	(4)	0.062	(20)	0.036	(11)	0.004	(1)	0.002	(1)
	166	0.082	(27)	0.092	(31)	0.010	(3)	0.015	(5)	0.061	(21)	0.029	(10)	0.005	(2)	0.002	(1)
20 to 10ºC	0	0.082	(27)	0.092	(31)	0.010	(3)	0.015	(5)	0.061	(21)	0.029	(10)	0.005	(2)	0.002	(1)
	9	0.087	(28)	0.095	(31)	0.007	(2)	0.011	(4)	0.067	(22)	0.034	(11)	0.005	(1)	0.003	(1)
	24	0.084	(21)	0.145	(36)	0.008	(2)	0.019	(5)	0.065	(16)	0.076	(17)	0.004	(1)	0.005	(2)
	72	0.058	(10)	0.245	(44)	0.007	(1)	0.037	(7)	0.060	(11)	0.140	(24)	0.004	(1)	0.012	(2)

nd : not detected by GC

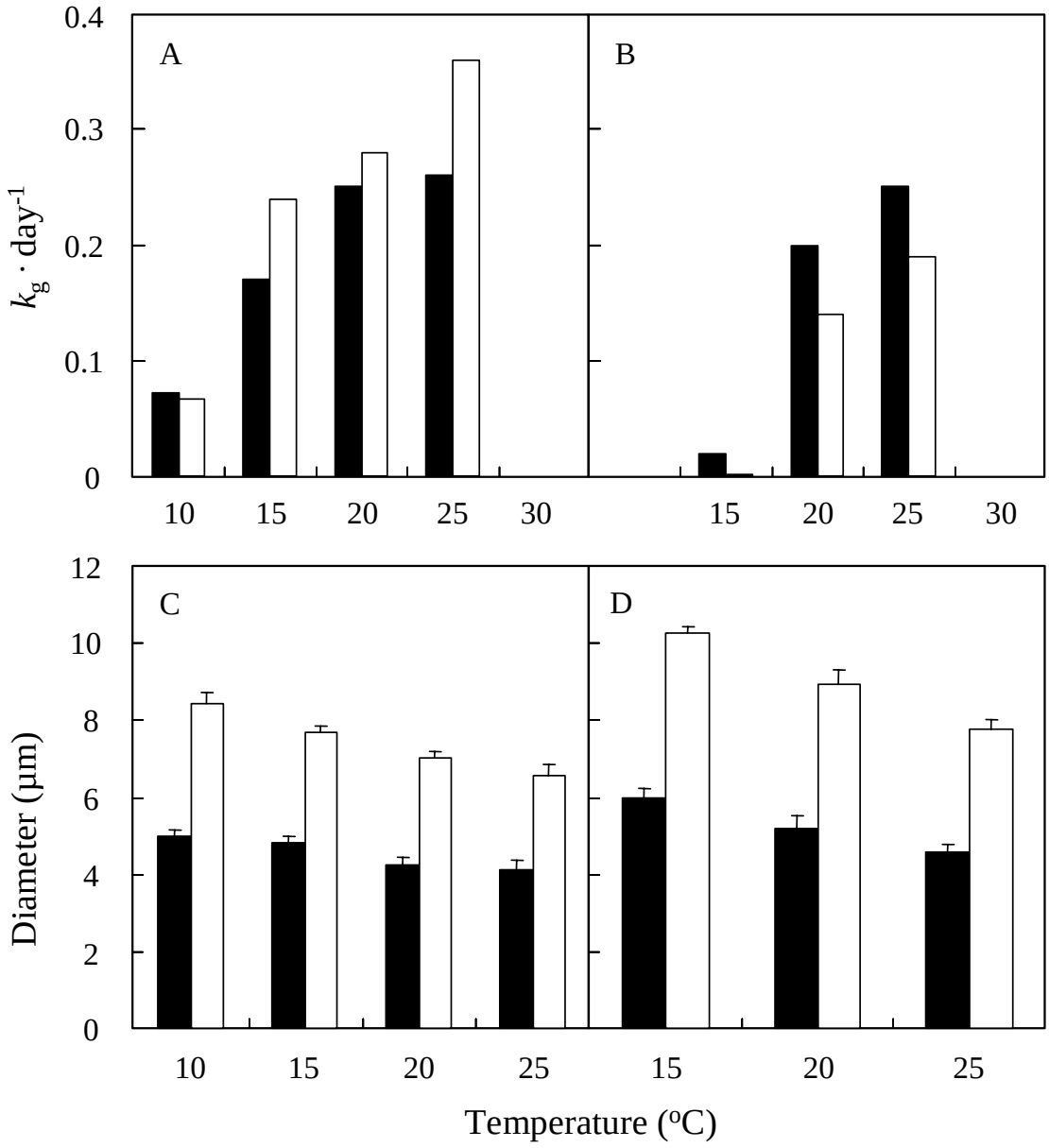


Figure 1. Temperature dependence of the growth constant (kg day^{-1}) (A) in *Emiliania huxleyi* strain EH2 and *Gephyrocapsa oceanica* strain GO1 (B), calculated from changes in OD_{750} (■), and cell number (□). Diameters of symplast (■) and whole cell with coccosphere (□) of *E. huxleyi* (C) and *G. oceanica* (D). Growth periods varied depending on conditions from 1 to 6 days in both species. The values presented for diameter were the average of at least 30 cells. Error bars are shown in C and D.

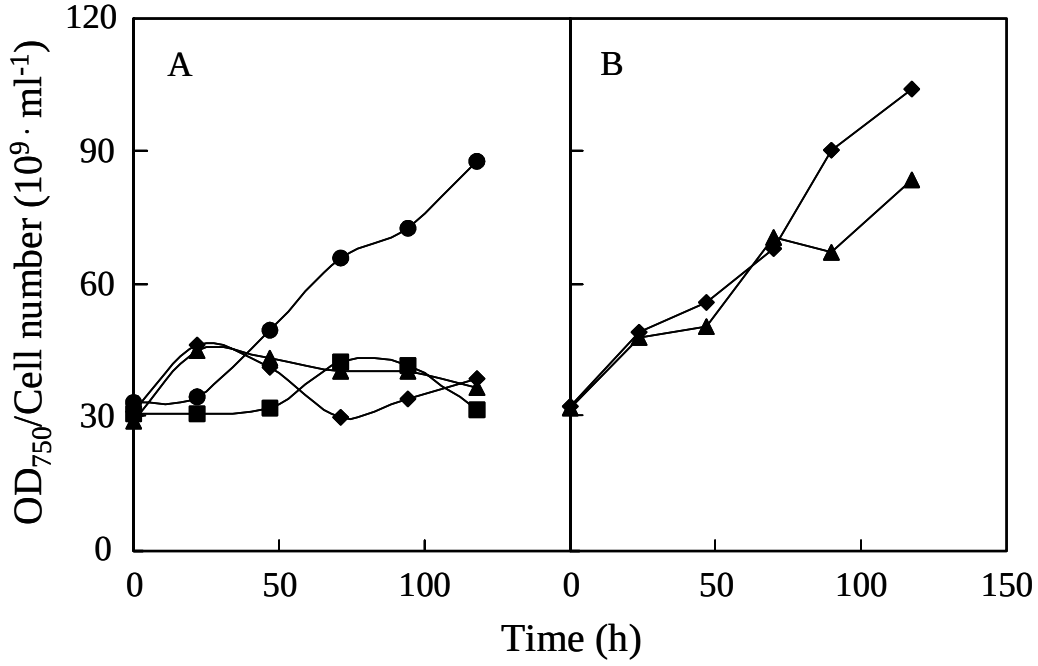


Figure 2. Temperature dependence of optical density (OD_{750}) per cell number of *Emiliania huxleyi* (A) and *Gephyrocapsa oceanica* (B) grown at various temperatures of 10ºC (●); 15 ºC (■); 20 ºC (▲) and 25 ºC (◆). *G. oceanica* failed to grow at 15 ºC and below.

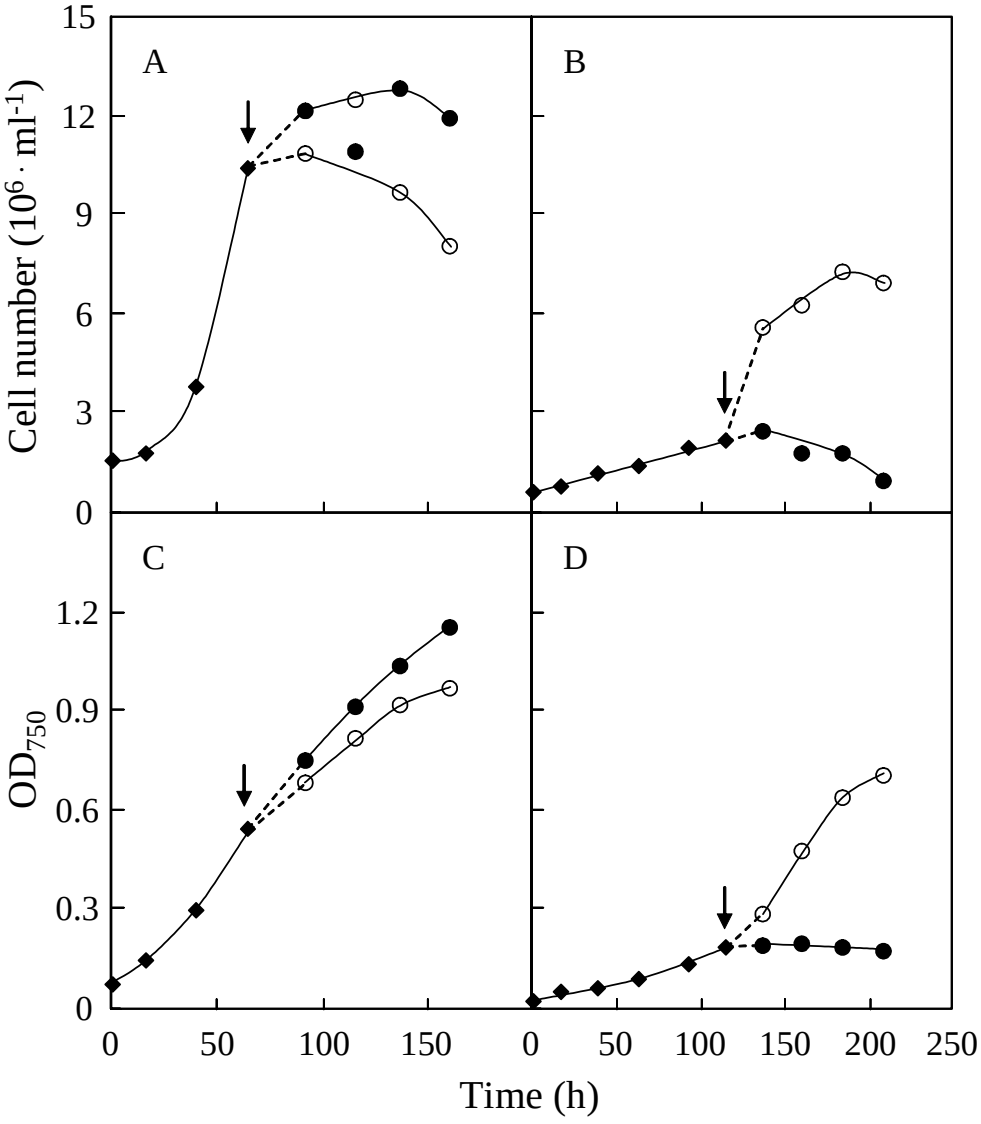


Figure 3. Effect of temperature change on growth as a function of cell number (A, B) and optical density (OD_{750}) (C, D) in *Emiliania huxleyi* (A, C) and *Gephyrocapsa oceanica* (B, D). Arrows indicate the time when the culture at 20ºC (◆) was divided into two and transferred to 15ºC (●) and 25ºC (○), respectively.

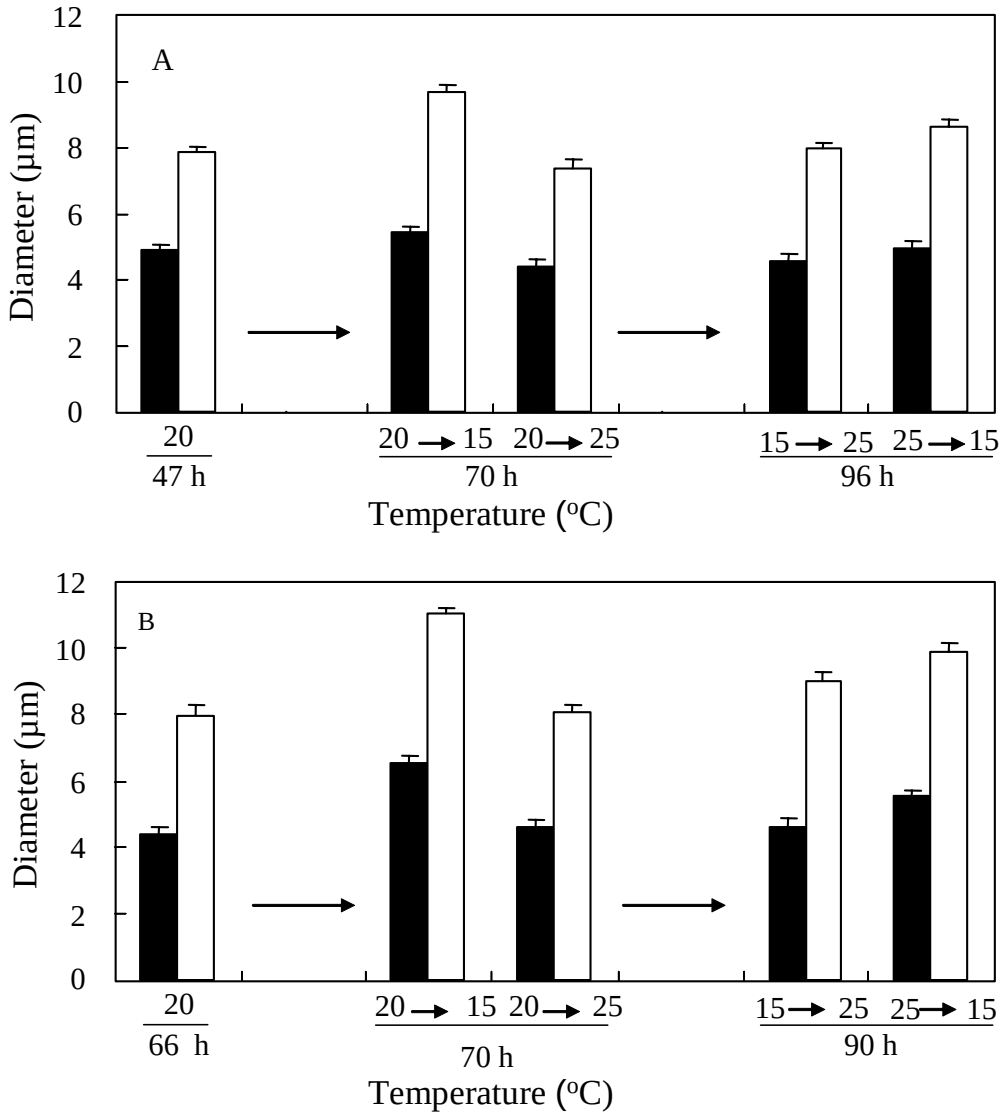


Figure 4. Changes in diameter of symplasts and whole cells with coccosphere of *Emiliania huxleyi* (A) and *Gephyrocapsa oceanica* (B) in response to temperature changes. Cultures of both strains were grown first at 20°C for 47 h (A) or 66 h (B) and then transferred to 15 or 25°C. Cultures were maintained at such temperatures for 70 h, and then temperature was changed to 25 or 15°C, respectively, and maintained for 96 h (A) or 90 h (B). Times indicated were period after transfer to new temperature settings. The values presented were the average of at least 30 cells. For symbols: ■, symplast; □, whole cell. Error bars are shown.

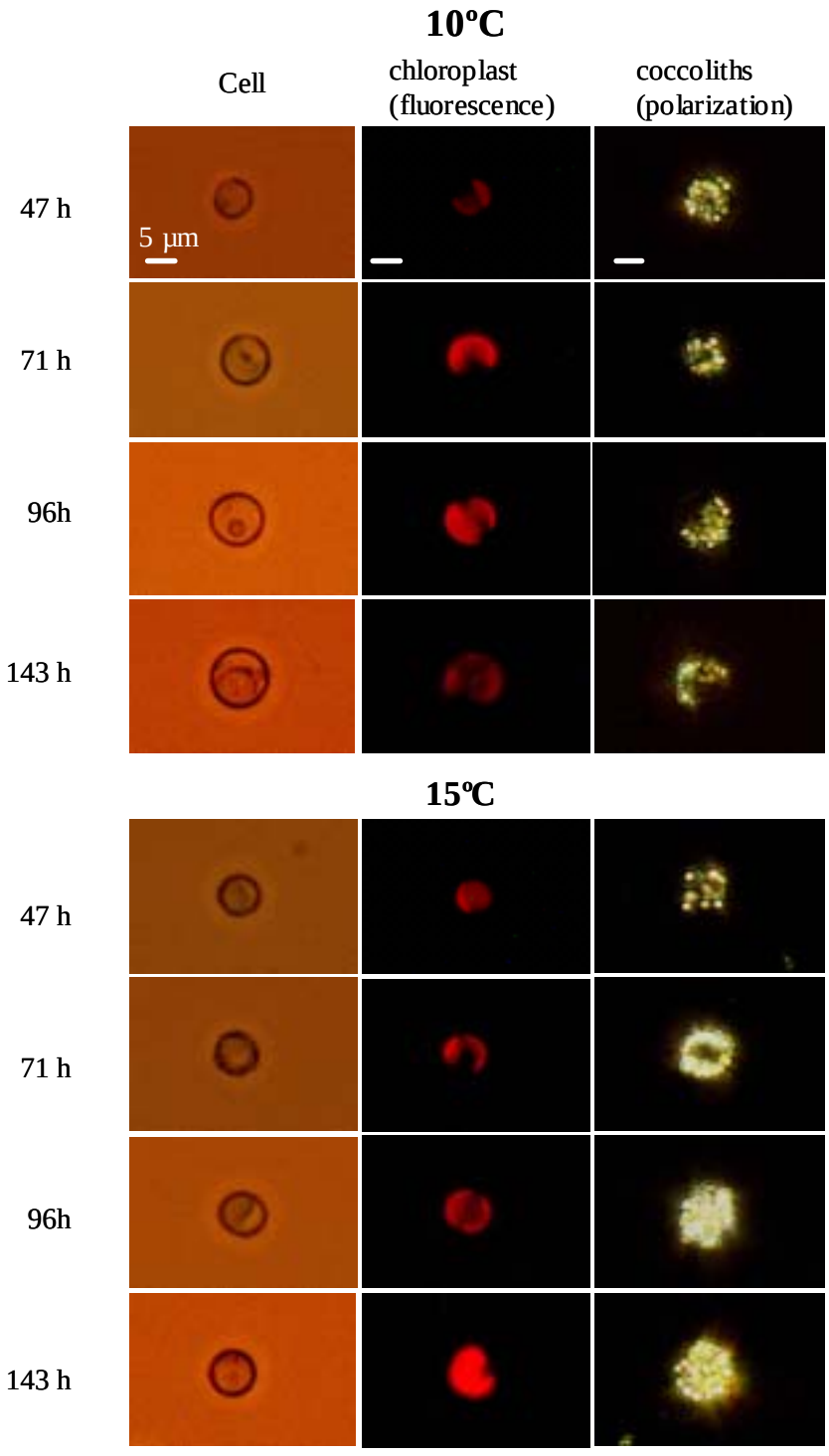


Figure 5A. Microscopic observations of *Emiliania huxleyi* grown at 10 and 15°C. At least 30 cells were observed and one typical cell was shown. Bar = 5 µm.

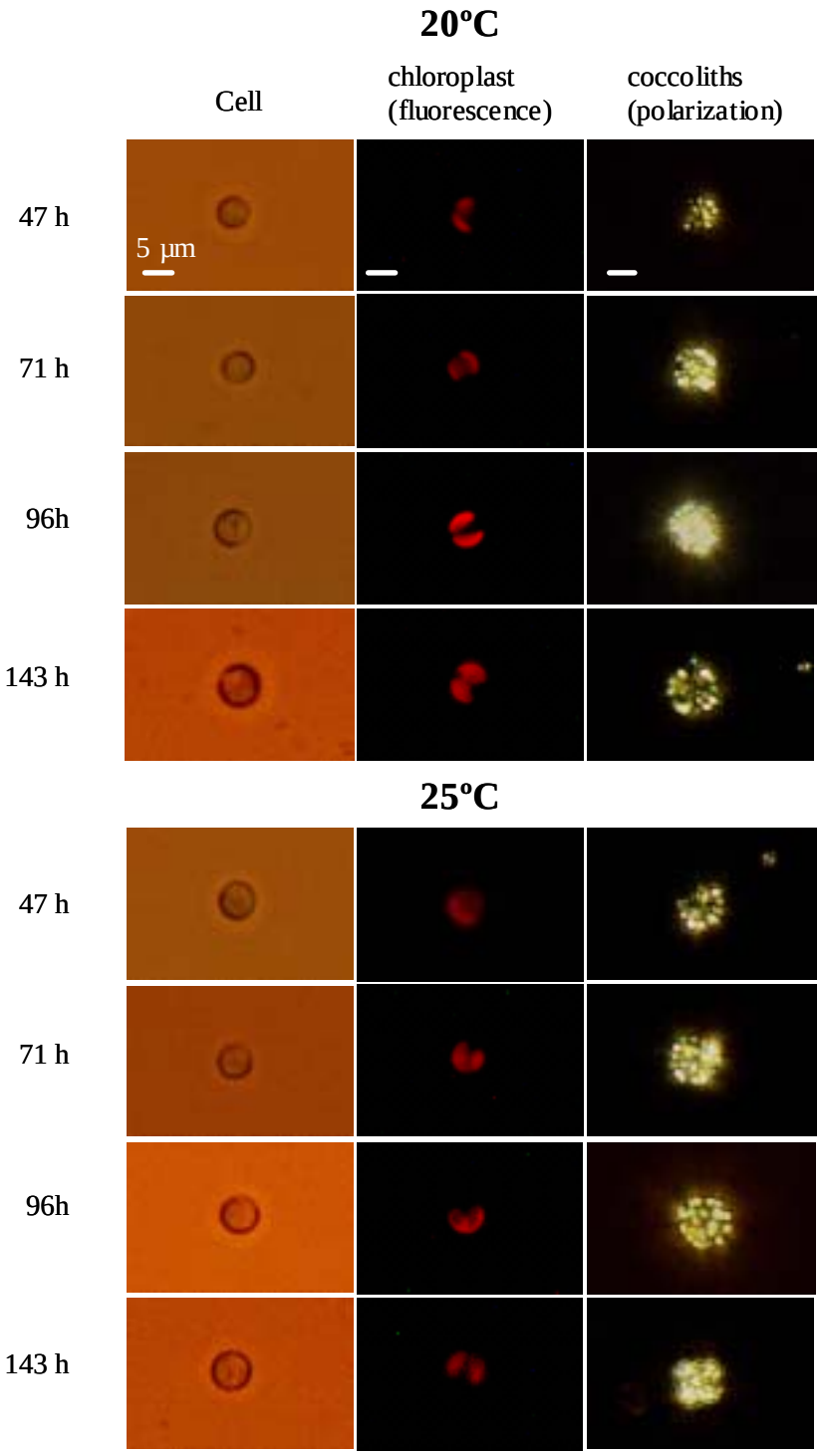
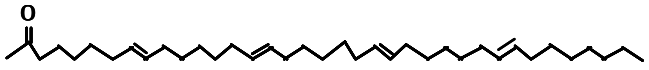


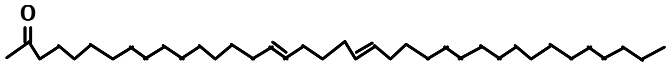
Figure 5B. Microscopic observations of *Emiliania huxleyi* grown at 20 and 25°C. At least 30 cells were observed and one typical cell was shown. Bar = 5 µm.

$C_{37:2}$ MK heptatriaconta- 15E,22E-dien-2-one

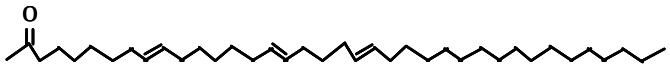
$C_{37:3}$ MK heptatriaconta- 8E,15E,22E-trien-2-one



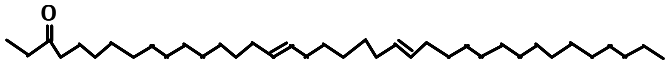
$C_{37:4}$ MK heptatriaconta- 8E,15E,22E,29E-tetraen-2-one



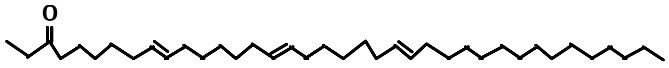
$C_{38:2}$ MK octatriaconta- 16E,23E-dien-2-one



$C_{38:3}$ MK octatriaconta- 9E,16E,23E-trien-2-one



$C_{38:2}$ EK octatriaconta- 16E,23E-dien-3-one



$C_{38:3}$ EK octatriaconta- 9E,16E,23E-trien-3-one

$C_{39:2}$ EK nonatriaconta- 17E,24E-dien-3-one

$C_{39:3}$ EK nonatriaconta- 10E,17E,24E-trien-3-one

Figure 6. Structures of the different long-chain alkenone compounds (C_{37}-C_{39}).

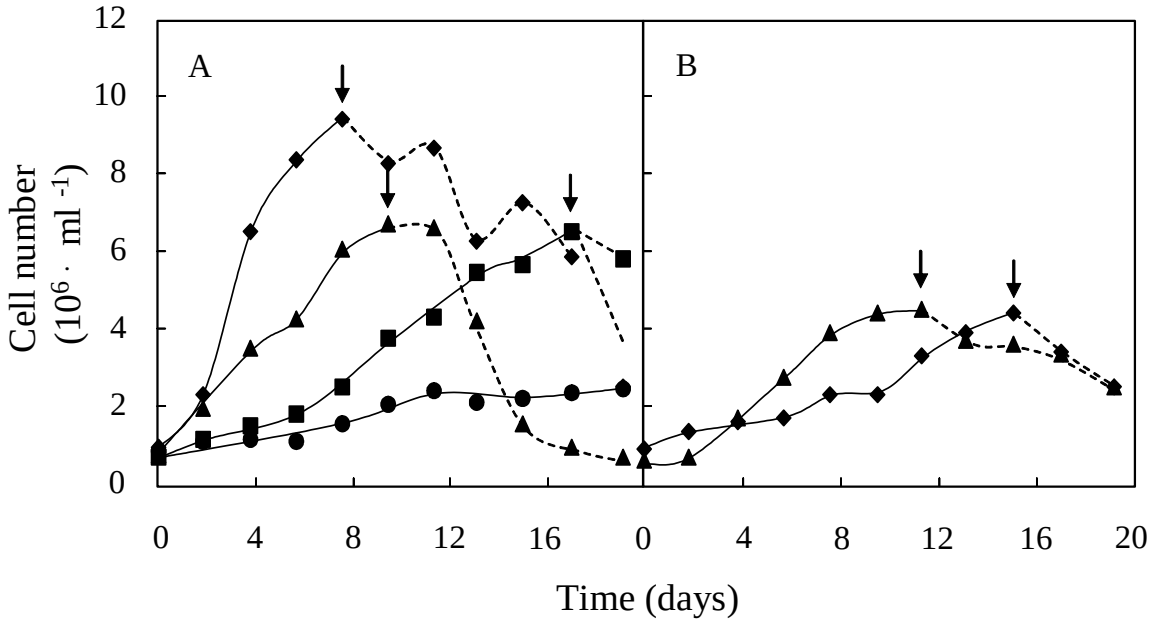


Figure 7. Changes in cell number of *Emiliania huxleyi* (A) and *Gephyrocapsa oceanica* (B) grown at various temperatures including 10°C (●), 15°C (■), 20°C (◆) and 25°C (▲). Arrows indicate the time at which the growth reached stationary phase. Broken lines indicate the values after the time when cell death commenced.

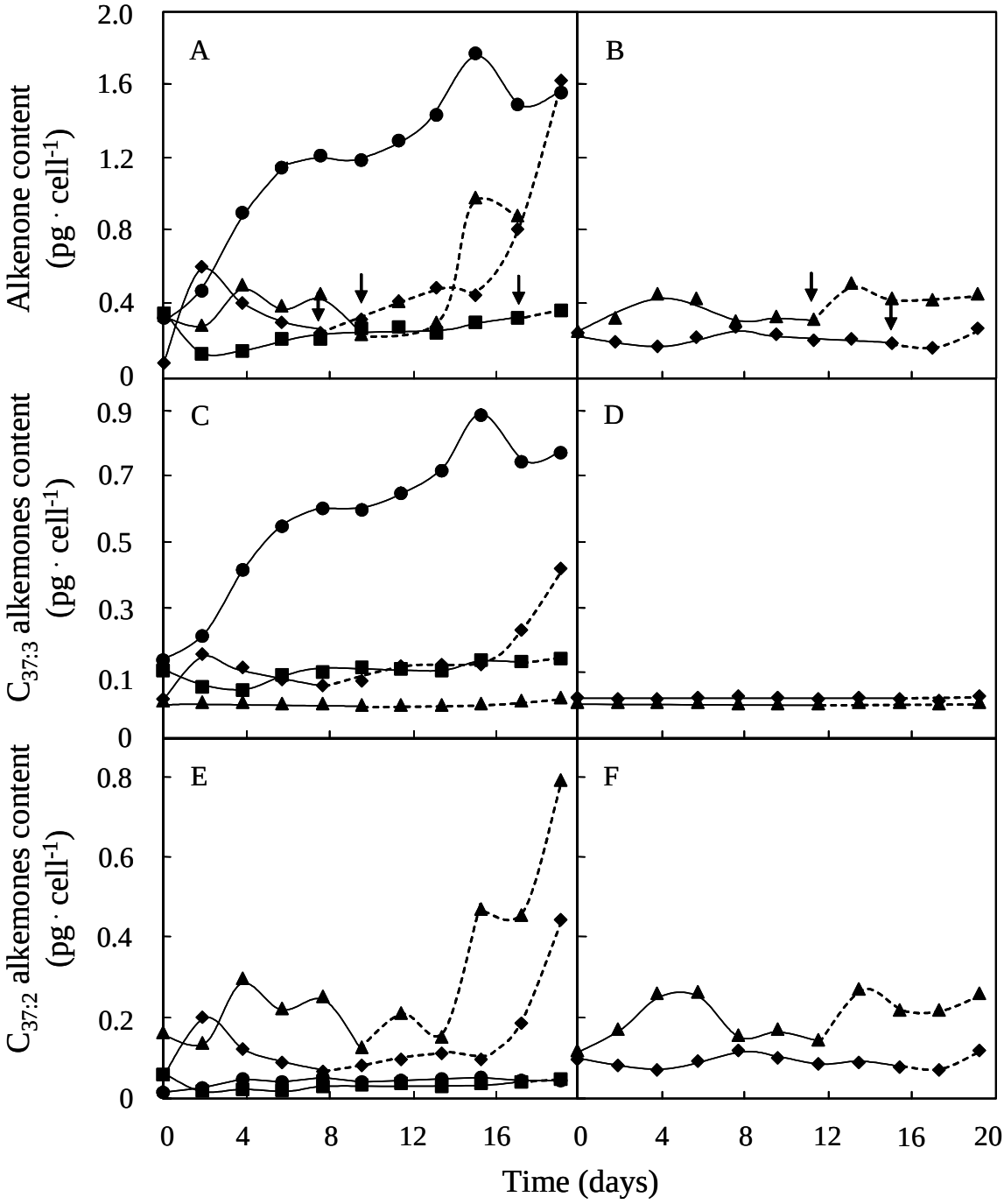


Figure 8. Changes in total alkenone content (A, B), $C_{37:3}$ alkenone content (C, D) and $C_{37:2}$ alkenone content (E, F) of *Emiliania huxleyi* (A, C, E) and *Gephyrocapsa oceanica* (B, D, F) grown at 10°C (●), 15°C (■), 20°C (◆) and 25°C (▲). For arrows and broken lines, see Fig. 7.

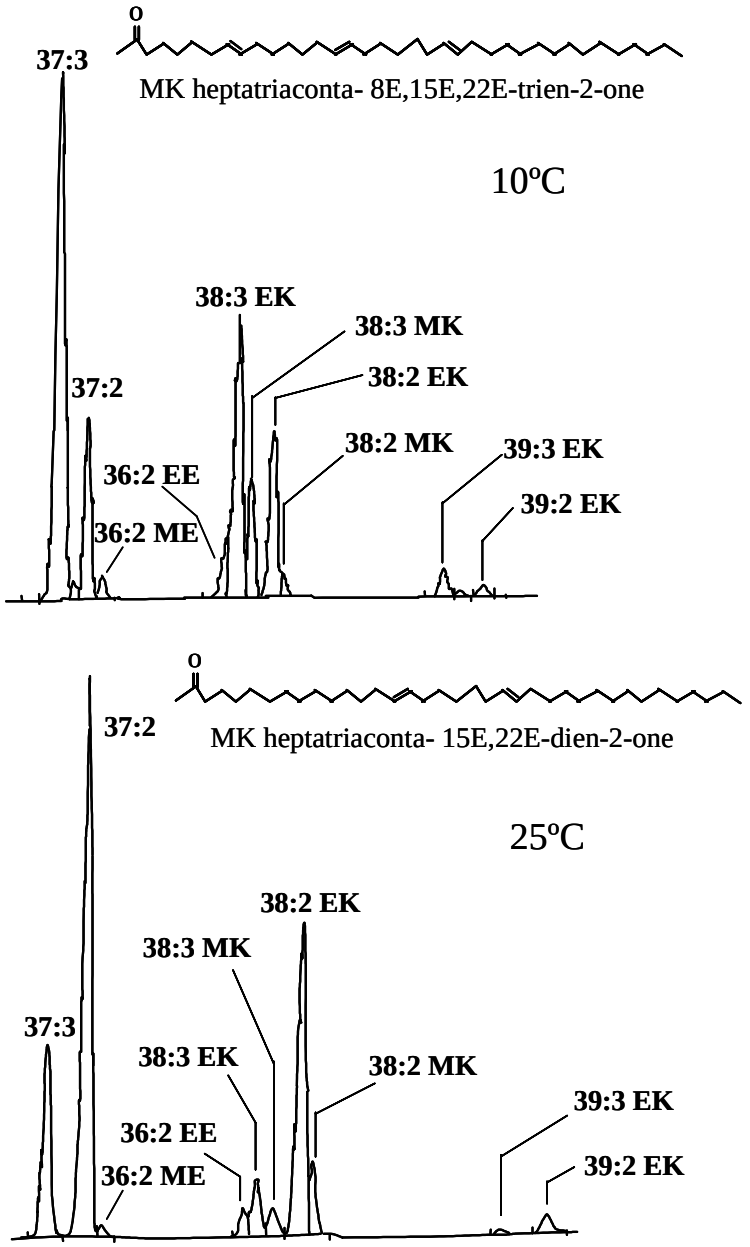


Figure 9. Gas chromatograms of long-chain alkenones and alkenoates in *Emiliania huxleyi* grown at 10°C and 25°C. EK, ethyl ketone; MK, methyl ketone, EE, ethyl alkenoate; ME, methyl alkenoate with C*x:y,* where *x* is carbon number and *y* is the number of double bonds.

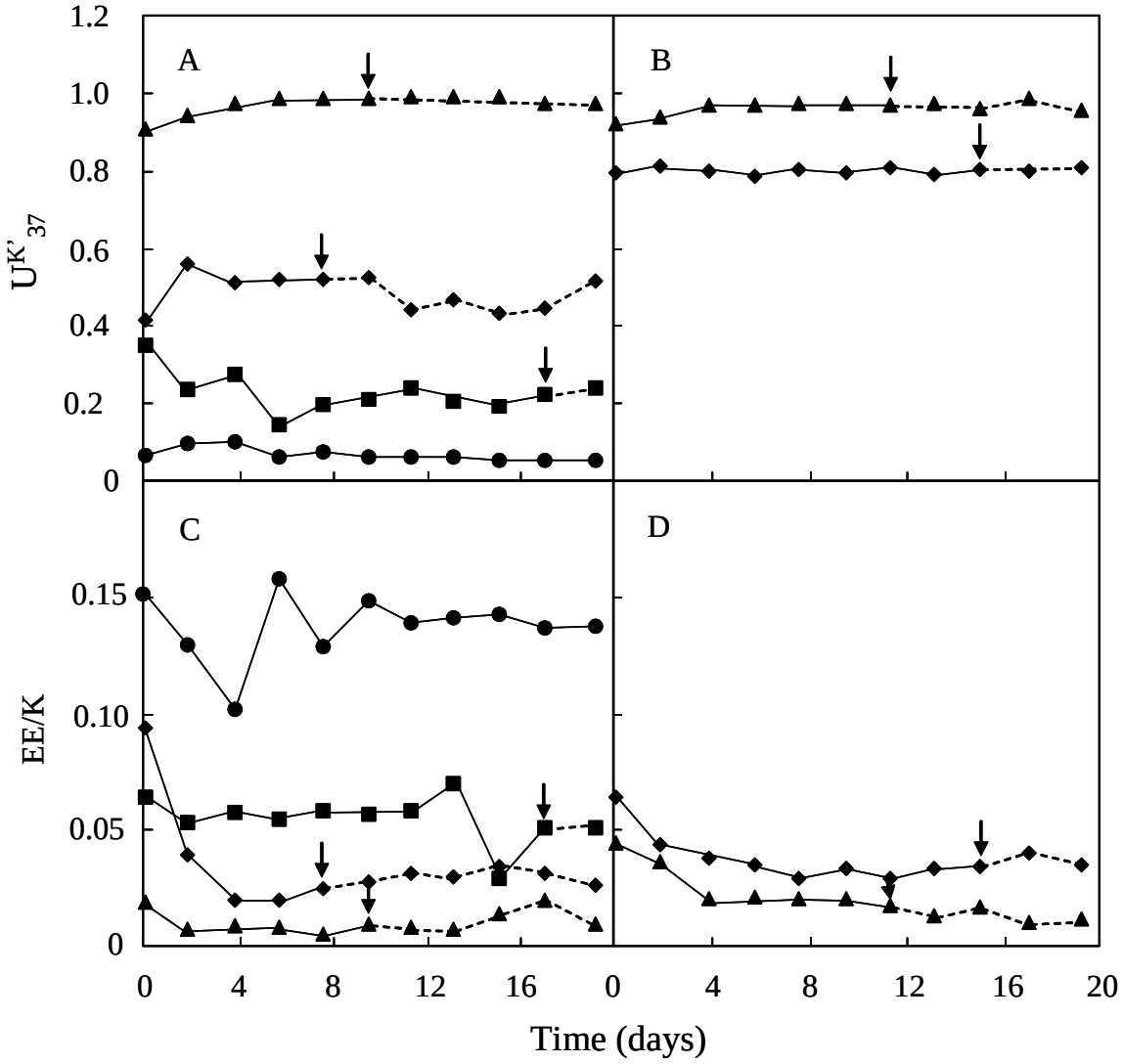


Figure 10. Changes in U^{K}_{37} (A, B) and EE/K (C, D) of *Emiliania huxleyi* (A, C) and *Gephyrocapsa oceanica* (B, D) grown at 10°C (●), 15°C (■), 20°C (◆) and 25°C (▲). For arrows and broken lines, see Fig. 7.

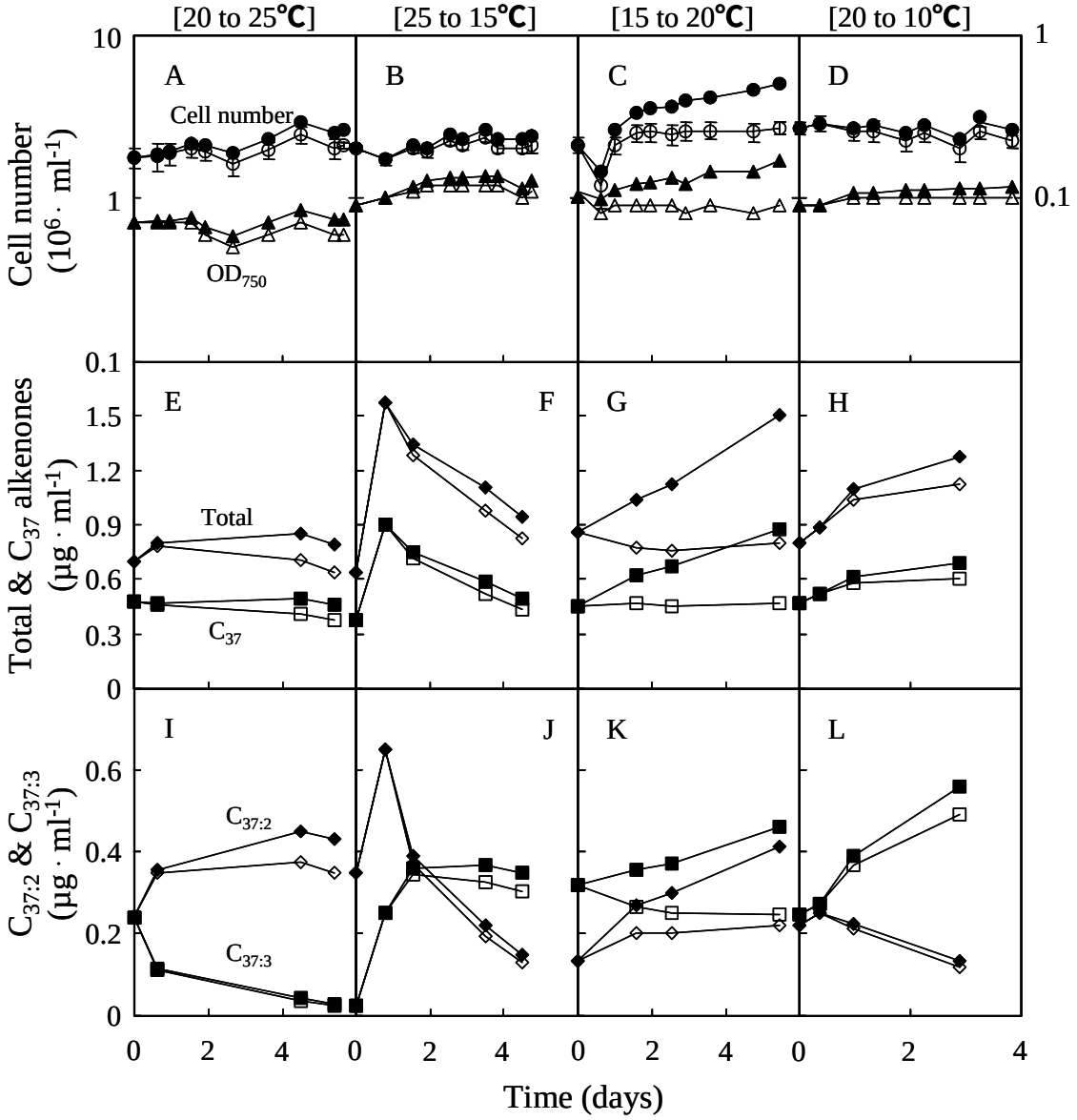


Figure 11. Changes in cell number (circles) and turbidity (triangles) (A-D); the amounts (µg ml^{-1}) of total alkenones (diamonds) and total C_{37} alkenones (squares) (E-H); the amounts (µg ml^{-1}) of $C_{37:2}$ (diamonds) and $C_{37:3}$ (squares) alkenones (I-L) after the transfer of cells to different temperatures in a continuous culture of *Emiliania huxleyi*. Cells were pre-grown for 8 days at 20°C, then transferred to 25°C (A, E, and I), from 25 to 15°C (B, F and J), from 15 to 20°C (C, G and K) and from 20 to 10°C (D, H and L) in this sequence. Open symbols, measured values; closed symbols, calculated values assuming no dilution.

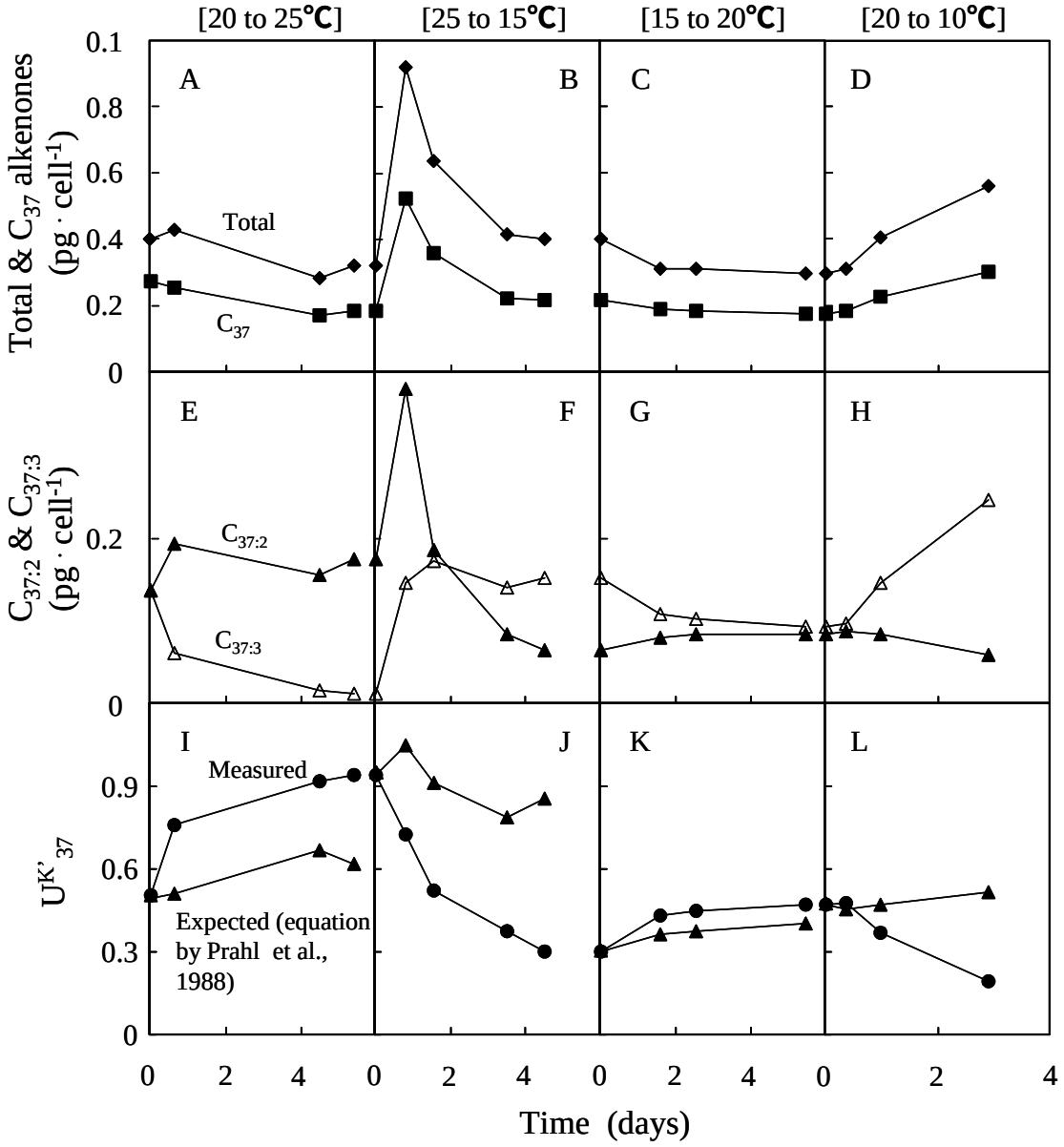


Figure 12. Changes in the amounts (pg cell^{-1}) of total alkenones (◆) and total C_{37} alkenones (■) (A-D); the amounts (pg cell^{-1}) of $C_{37:2}$ (▲) and $C_{37:3}$ (△) alkenones (E-H); the unsaturation index: ●, measured; ▲, expected based on the calculated values of cell number assuming no dilution (I-L) after the transfer of cells to different temperatures in a continuous culture of *Emiliania huxleyi*.

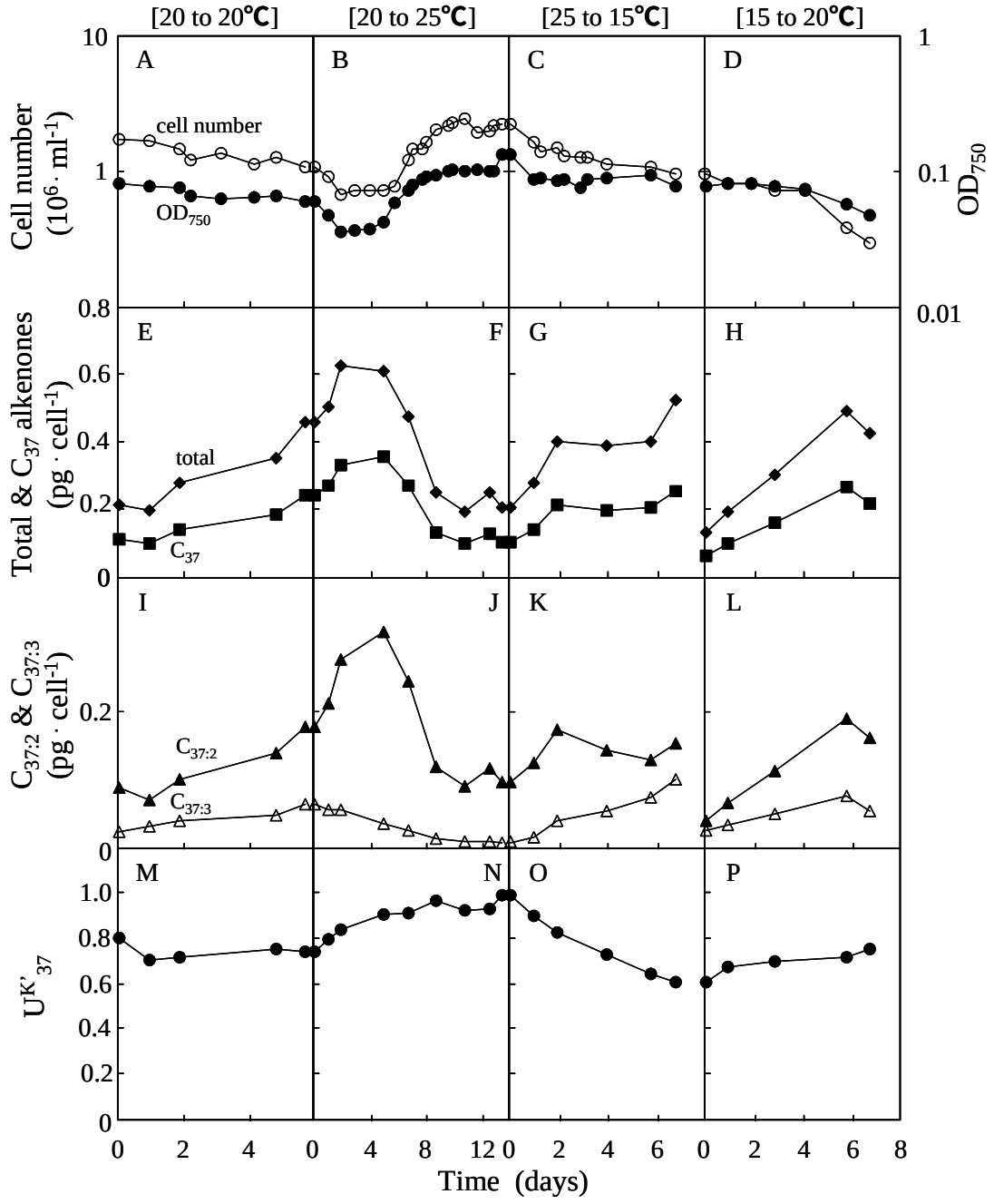


Figure 13. Changes in cell number (●) and turbidity (○) (A-D); the amounts (pg cell^{-1}) of total alkenones (□) and total C_{37} alkenones (■) (E-H); the amounts (pg cell^{-1}) of $C_{37:2}$ (▲) and $C_{37:3}$ (△) alkenones (I-L); the unsaturation index (●) (M-P) after transfer of cells to different temperatures as indicated in the graph in a continuous culture of *Gephyrocapsa oceanica*.

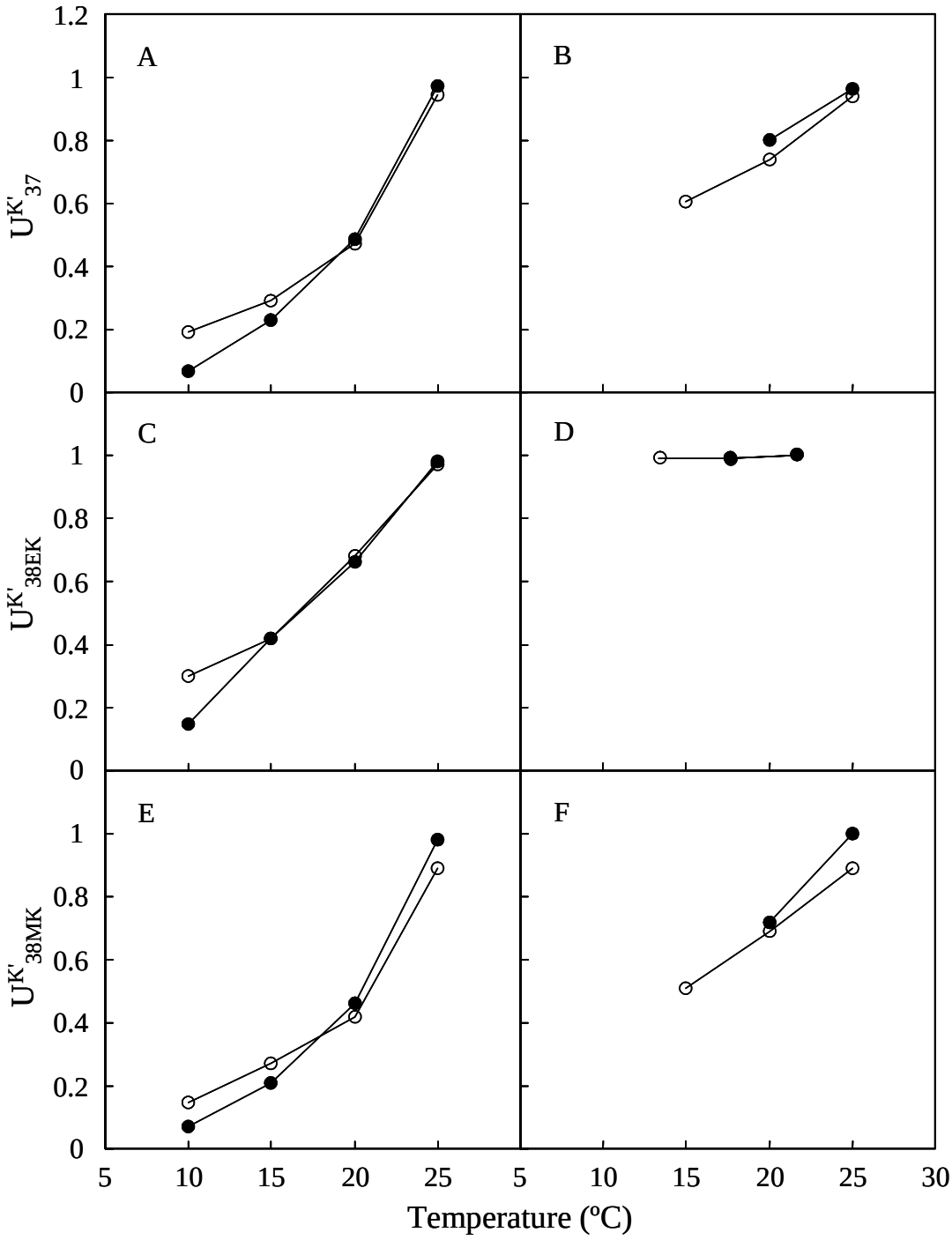


Figure 14. Relationship between $U^{K'}_{37}$ (A, B), $U^{K'}_{38EK}$ (C, D) and $U^{K'}_{38MK}$ (E, F) against growth temperature in *Emiliania huxleyi* (A, C, E) and *Gephyrocapsa oceanica* (B, D, F). For symbols: ●, data from batch cultures; ○, data from continuous cultures.

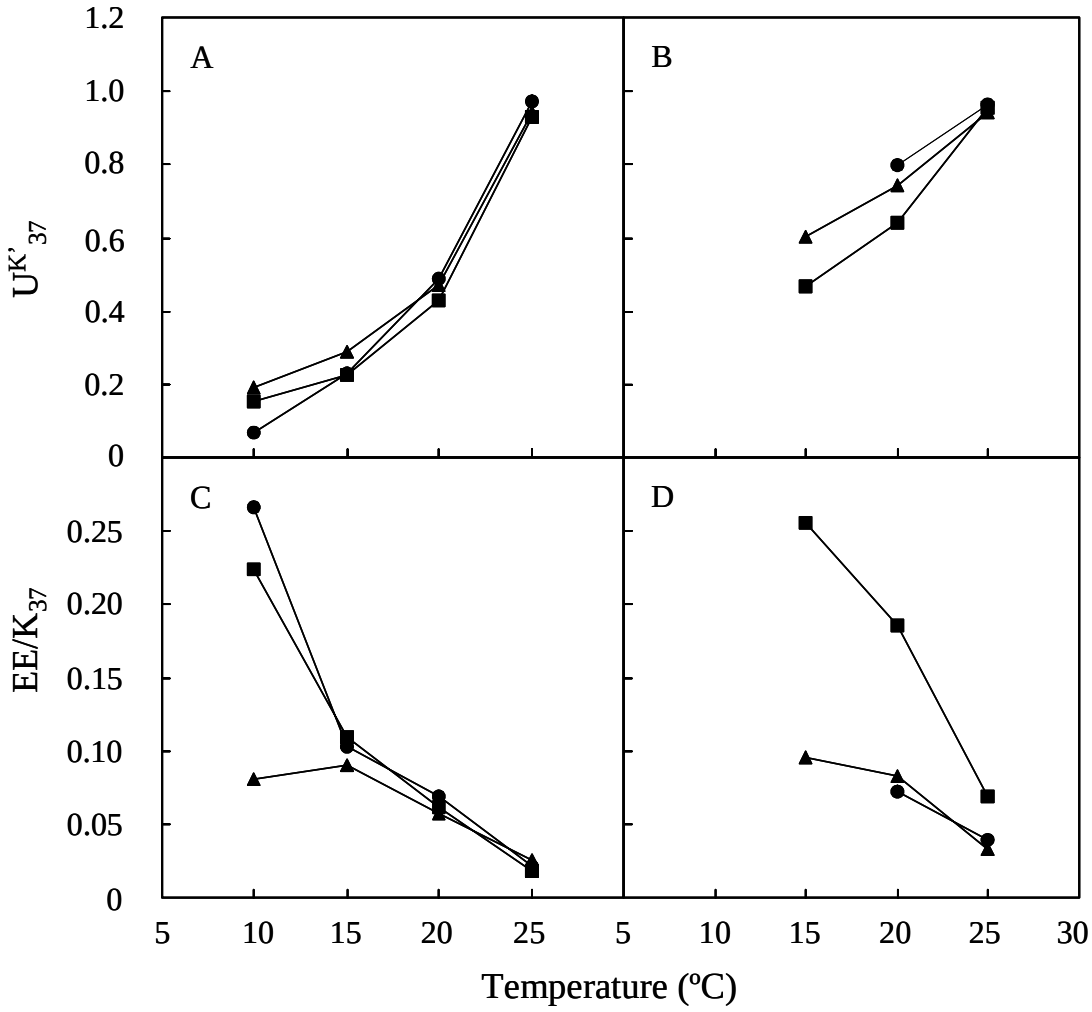


Figure 15. Relationship between $U^{K'}_{37}$ (A, B) and EE/K_{37} (C, D) of *Emiliania huxleyi* (A, C) and *Gephyrocapsa oceanica* (B, D) versus growth temperature. For symbols: ●, batch cultures in this study; ▲, continuous cultures in this study and ■, batch culture by Sawada et al. (1996) who used the same strains in their alkenone study.

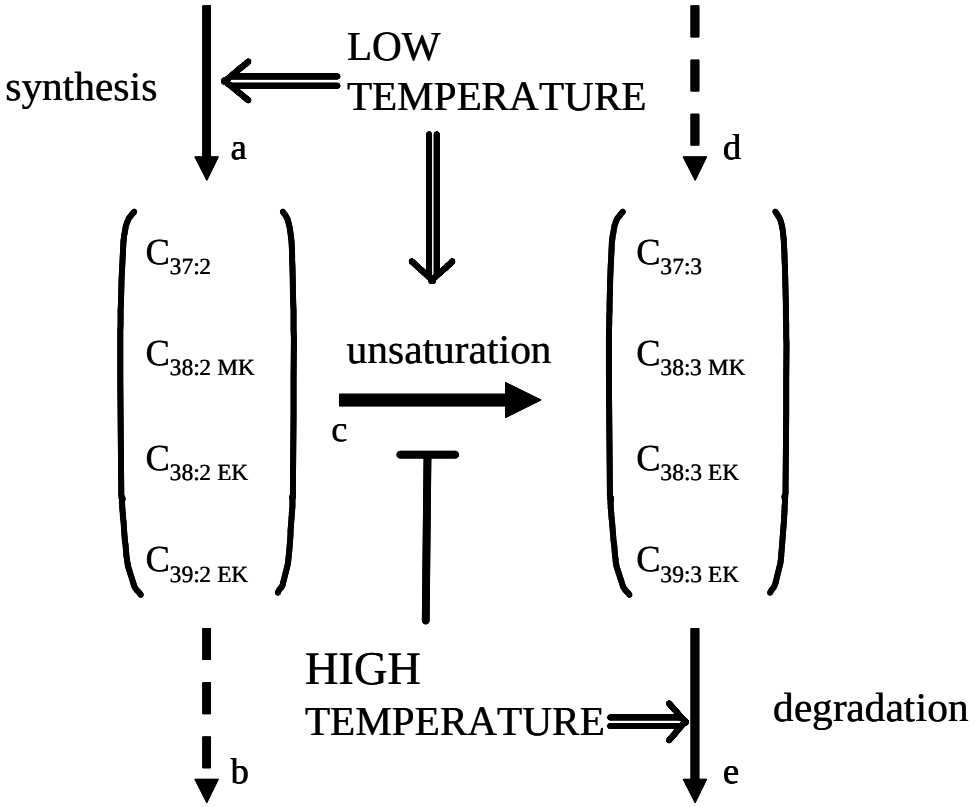


Figure 16. Scheme for the regulation of the synthesis, degradation and unsaturation of alkenones in *Emiliania huxleyi* by low temperature. Arrows a~e, major (solid arrows) and minor (dotted arrows) metabolic direction; ⇓, stimulation; ⊤, suppression.

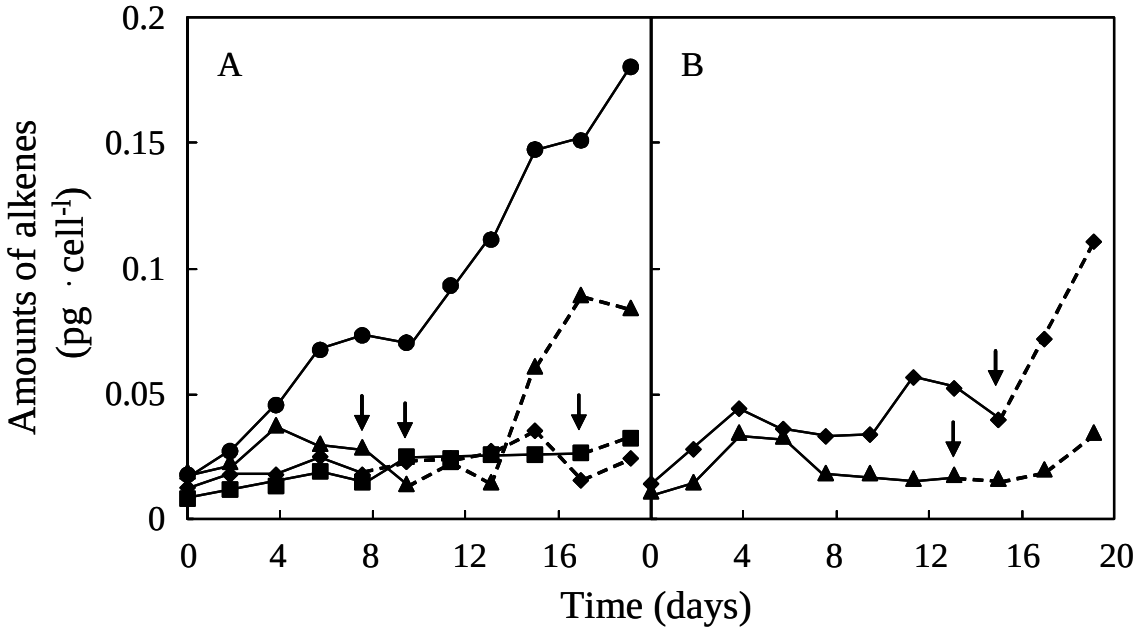


Figure 17. Changes in the alkene content (pg cell^{-1}) in *Emiliania huxleyi* (A) and *Gephyrocapsa oceanica* (B) grown at various temperatures including 10°C (●), 15°C (■), 20°C (◆) and 25°C (▲). For arrows and broken lines, see Fig. 7.

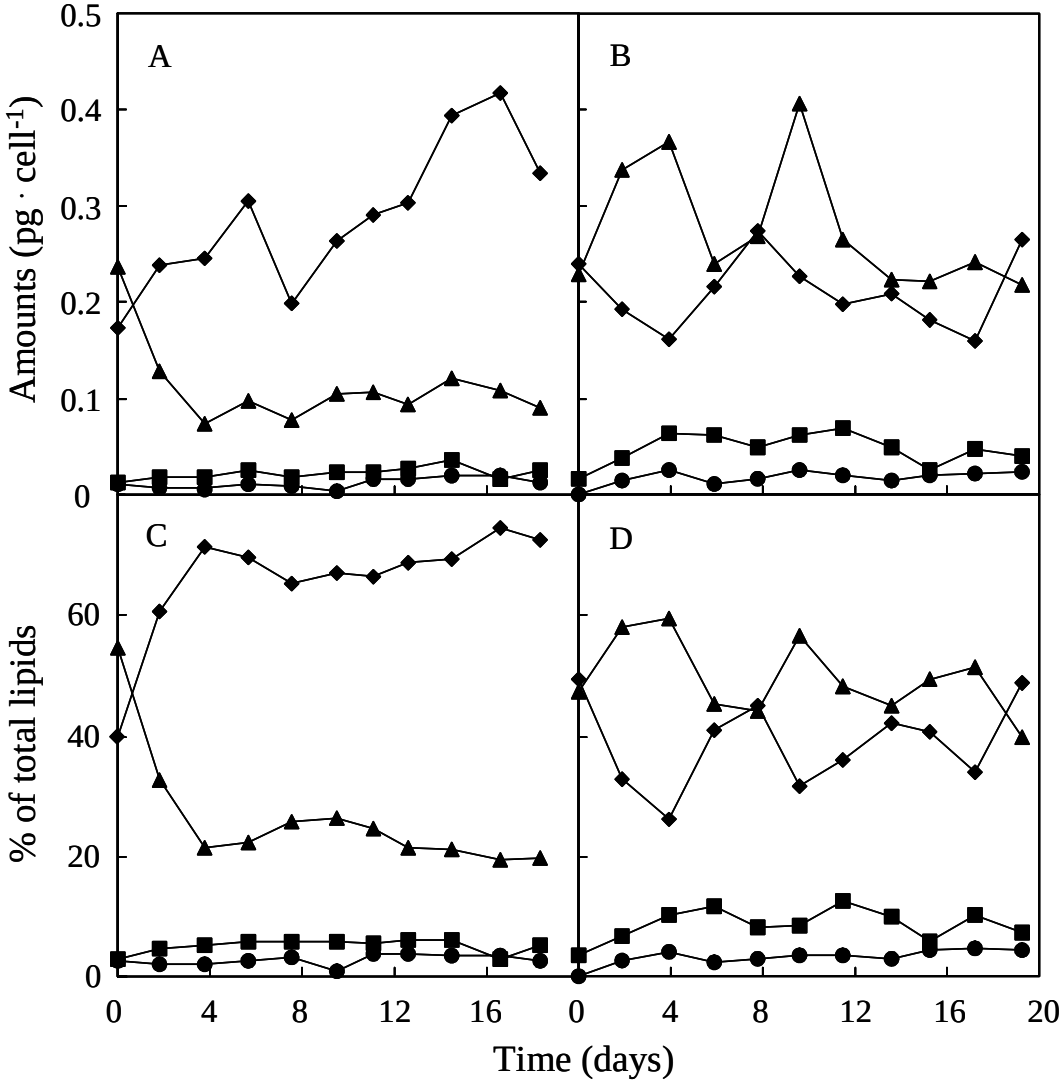


Figure 18. Changes in the amounts (pg cell^{-1}) of various lipid compounds (A, B) and their percent to total lipids (C, D) in *Emiliania huxleyi* (A, C) and *Gephyrocapsa oceanica* (B, D) grown at 20°C. For symbols: ◆, alkenones; ▲, fatty acids; ■, alkenes and ●, sterols (cholesterols and brassicasterols).

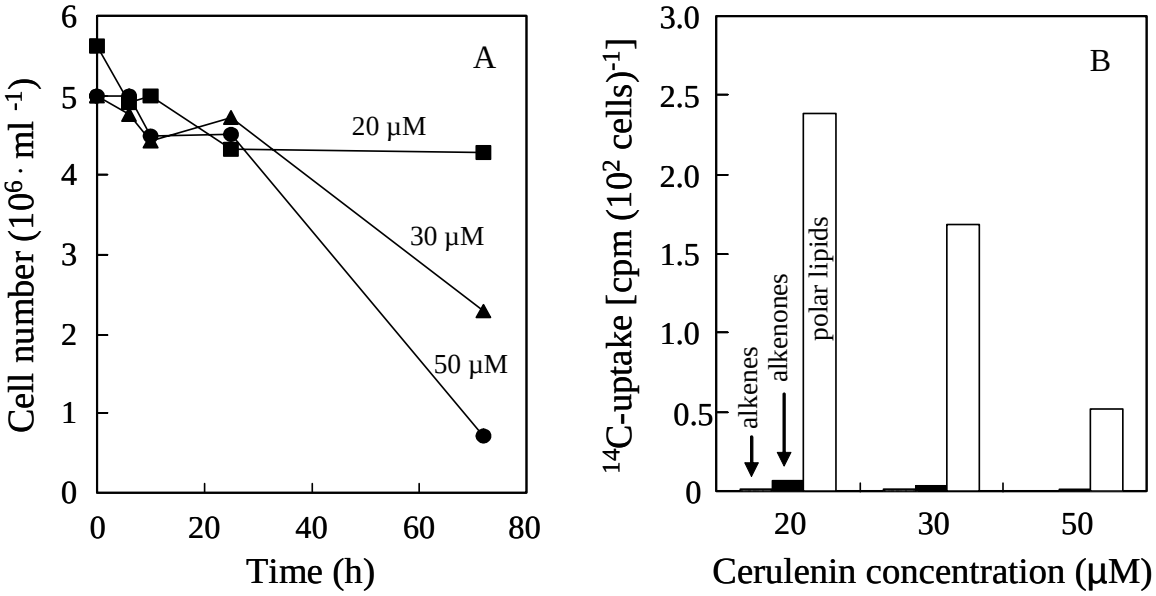


Figure 19. Effect of various concentration of cerulenin on the growth as measured by cell number (A) and the synthesis of various lipid fractions (B) in *E. huxleyi* cells grown first at 20°C, then transferred to 10°C in a ^{14}C-labeling experiment. In Fig. 19B, analyzed samples were harvested at 67 h. For symbols: ■, 20 µM; ▲, 30 µM; ●, 50 µM; ■, alkenones; □, polar lipids and ▨, alkenes.

.

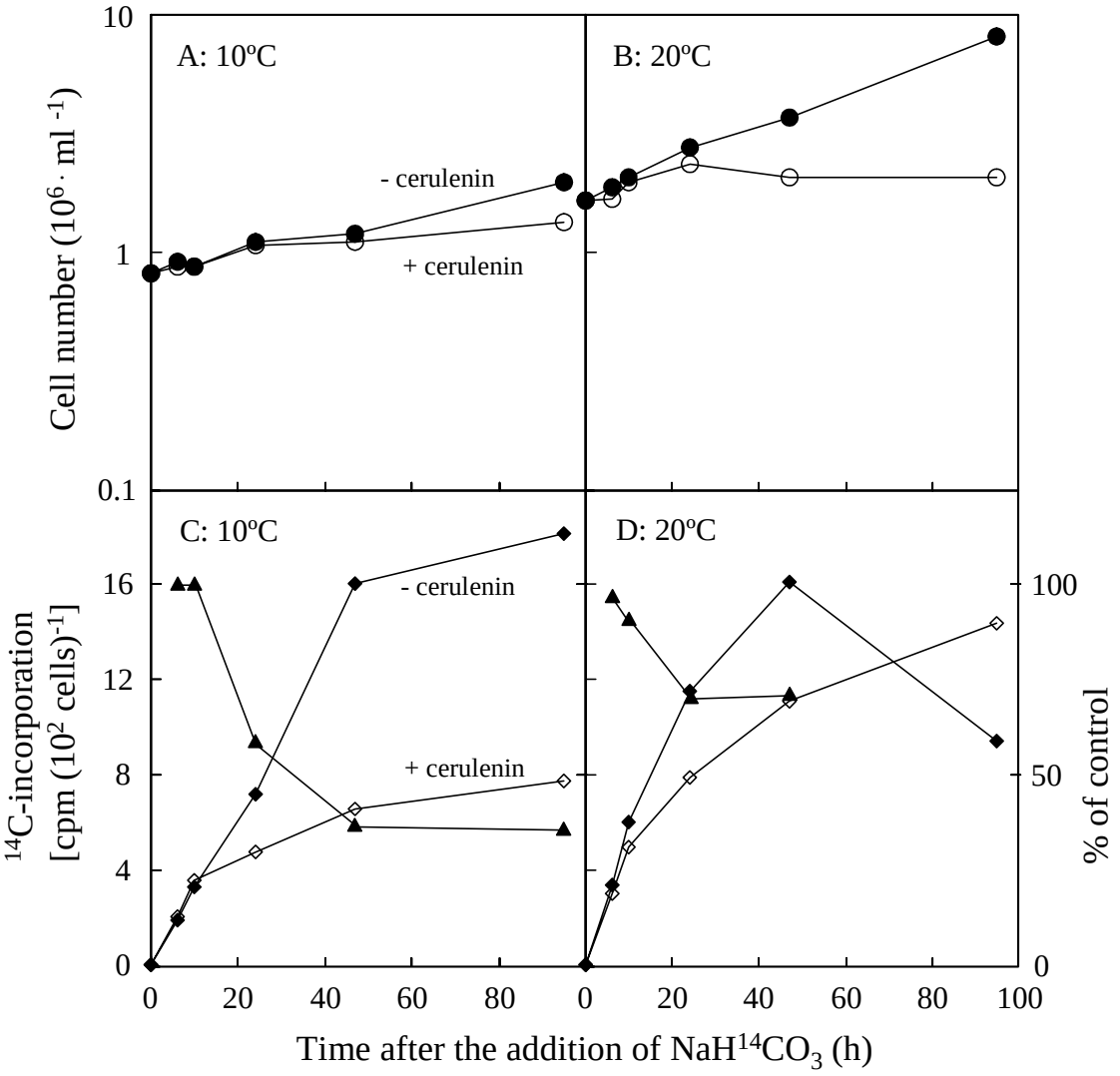


Figure 20. Effect of cerulenin on growth (A, B) and ^{14}C-incorporation (C, D) into cells of *E. huxleyi* cultures at 10°C (A, C) and 20°C (B, D). Algal cells were pre-incubated at respective temperature for 72 hr and then incubated for 95 h for the ^{14}C-labeling experiment with $NaH^{14}CO_3$. For symbols: ● and ◆,control; ○ and ◇; with 20 μM cerulenin; ▲, % of control.

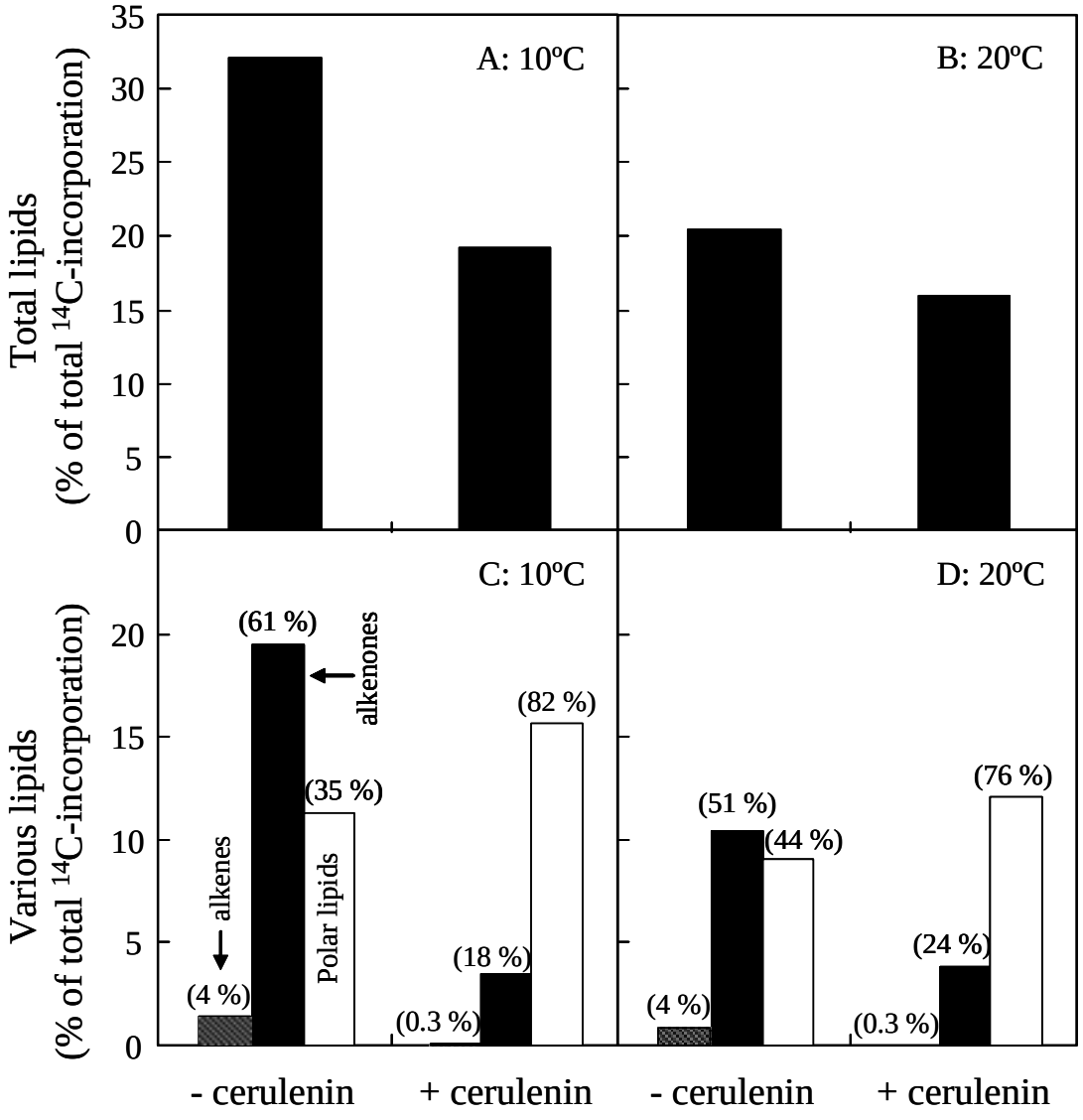


Figure 21. Effect of cerulenin on the ^{14}C-incorporation into total lipids (A, B) and various lipids (C, D) on the basis of percent of total ^{14}C-uptake by *E. huxleyi* cells in cultures at 10°C (A, C) and 20°C (B, D) for 47 h. Culture condition is same in Fig. 19. For symbols: ▧, alkenes; ■, alkenones and □, polar lipids. Numbers in parenthesis indicate percent of total lipids.

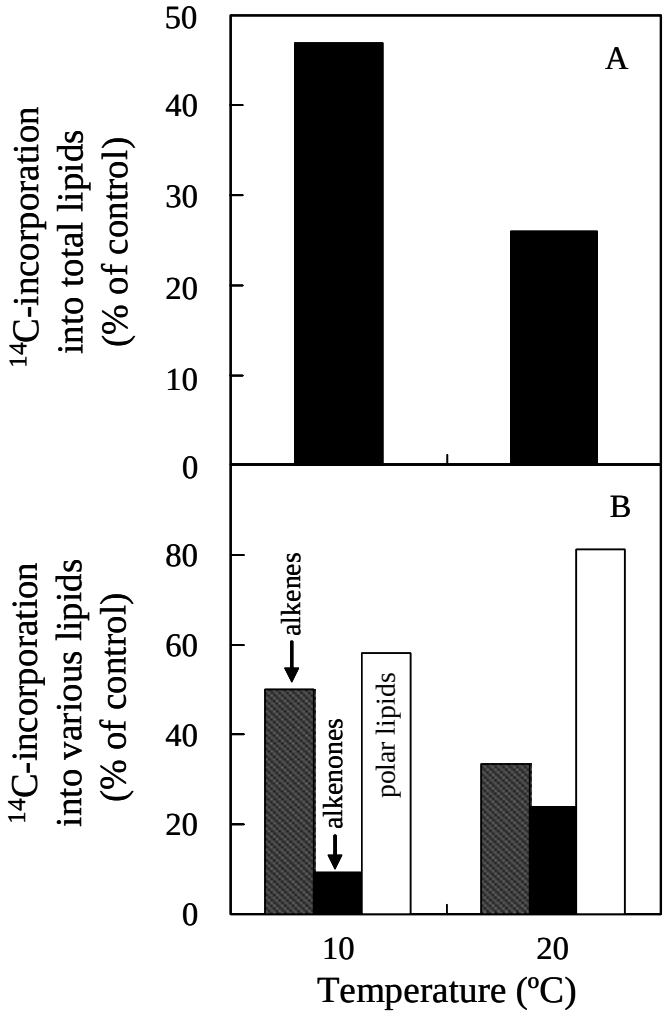


Figure 22. Percent inhibition by cerulenin of ^{14}C-incorporation in total lipids (A) and ^{14}C-incorporation into various lipids (B) in *E. huxleyi* at 10 and 20ºC. Samples are same as shown in Fig. 21. For symbols: ▧, alkenes; ■, alkenones and □, polar lipids.

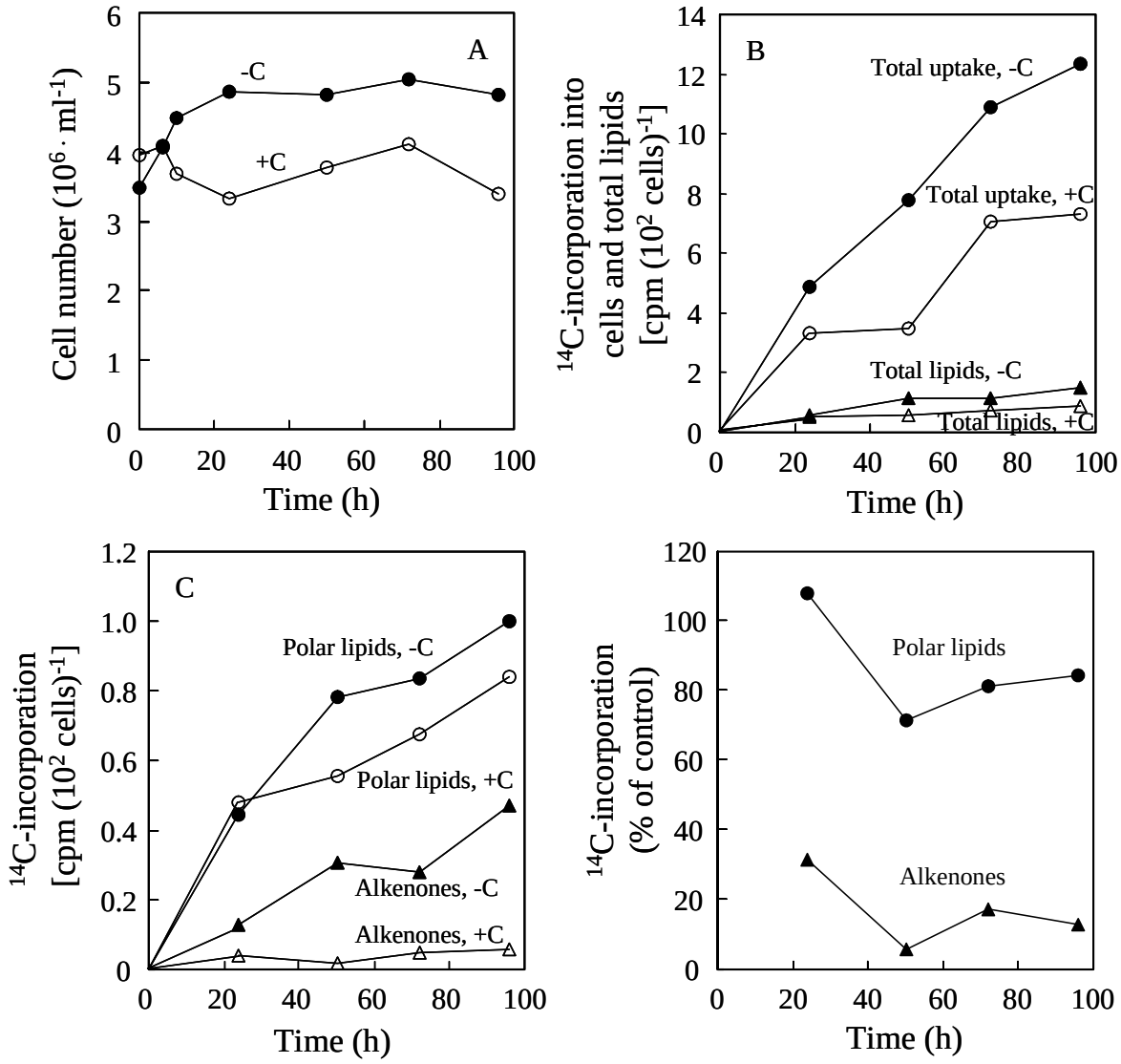


Figure 23. Effect of cerulenin on time courses of change in cell number (A), total ^{14}C-uptake (circles) and ^{14}C-uptake into total lipids (triangles) (B), ^{14}C-incorporation into polar lipids (circles) and alkenones (triangles) (C) and percent of control in ^{14}C-incorporation into polar lipids (circles) and the extent of inhibition expressed as alkenones (triangles) (D) when *E. huxleyi* cells were grown first at 20°C in a pre-culture and then transferred to 10°C during ^{14}C-labeling experiments. Closed symbols, control (-C); open symbols, with 20 μM cerulenin (+C).

Acknowledgements

The author would like to extend her deepest gratitude to Professor Yoshihiro Shiraiwa, Ph.D., her academic adviser, for his valuable guidance and support during her studies and the conduct of this research at the University of Tsukuba.

She is also indebted to Dr. Masanobu Yamamoto of the University of Hokkaido for teaching her the methods of alkenone analysis and for letting her analyzed her samples in his laboratory and for his helpful discussion. She is also thankful to Dr. Takanori Higashihara for being an instrument in introducing her to Dr. Shiraiwa and for always giving her advices and encouragements.

She would like also to thank Dr. Isao Inouye, Dr. Hiroshi Kamada and Dr. Takeo Hama of the University of Tsukuba for taking their time in reading this thesis and for their valuable comments and suggestions.

The continued support of the Davao Oriental State College of Science and Technology through its incumbent President, Dr. Jonathan A. Bayogan, by allowing the author to be on a study-leave is greatly appreciated. Indebtedness and gratitude are extended to the Japanese government through the Ministry of Education, Culture, Sports, Science and Technology for the scholarship granted to her.

Profound appreciation is extended to Yoko Kanke, her Japanese tutor for helping her especially when she had her entrance examination to the graduate program. She is also thankful to Dr. Koji Iwamoto for always being ready in extending his helping hands during her laboratory works and for his comments and suggestions for the improvement of this manuscript; Toshihiro Obata for his kind assistance every time she needed his help; the present and past members of the Plant Physiology and Metabolism Laboratory (Shiraiwa laboratory) who in

one way or another provided friendship, helped and clever suggestions for the improvement of this study.

A heartfelt gratitude is extended to her friends, Buena and Fe, for their generosity that enabled and encouraged her to finish her PhD.

The author deeply extends her gratitude to her family and friends for they are her prayer warriors, source of encouragements and inspiration.

And above all, thanksgiving and praise are given to the Almighty GOD. His sustaining grace and mercy enabled the author to accomplish this very difficult task and so she is offering this work to Him.