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**Article**

# Change of the unsaturation degree of alkenone and alkenoate during acclimation to salinity change in *Emiliania huxleyi* and *Gephyrocapsa oceanica* with reference to palaeosalinity indicator.

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**Abstract**

Laboratory cultured strains of *Emiliania huxleyi* EH2 (identical to NIES-837) and *Gephyrocapsa oceanica* GO1 (NIES-838) were grown at 20ºC under various salinity conditions ranging from 15‰ to 34‰, and were analyzed for long-chain ($C_{37}$ - $C_{39}$) alkenones and ($C_{37}$ - $C_{38}$) alkyl alkenoates. In both *E. huxleyi* EH2 and *G. oceanica* GO1, there were no tetra-unsaturated ($C_{37:4}$) alkenones, which were frequently identified at low salinity waters in literatures. The alkenone unsaturation index ($U^{k'}_{37}$) in *E. huxleyi* clearly changed under lower salinities such as 27‰ and 32‰, while the values in *G. oceanica* were almost constant in various salinities. From these results, the $U^{k'}_{37}$ variations affected by salinity should be paid attention in paleotemperature reconstruction from natural samples that *E. huxleyi* predominates, especially in lower saline environments. Interestingly, our culture experiments also showed that the alkenone chain-length ratio ($K_{37}/K_{38}$) for *E. huxleyi* and *G. oceanica* increased with decreasing salinities, although the ranges of these variations were small. These results suggest that the $K_{37}/K_{38}$ values were affected by the cellular and physiological factors in a single haptophyte cell, although this value varies mainly depending on the changes of haptophyte species and/or strains in natural environments. Hence, we suggest that the $K_{37}/K_{38}$ ratio can be more reliable as a paleosalinity indicator.

## 1. Introduction

Long-chain ($C_{37}$ - $C_{39}$) alkenones, which are derived from Haptophycean algae such as Gephyrocapsaceae and Isocrysidaceae, have been well used as a proxy for paleotemperatures of sea surface water, as reviewed by Brassell (1993, and references therein). Recently, it was reported that the relative abundance of tetra-unsaturated alkenones (*e.g.* $C_{37:4}$ alkenone) increased with the decrease in salinity, and therefore, the ratio of tetra-unsaturation and the distributions of alkenones could be potentially used as a proxy for paleosalinity of ambient waters (Rosell-Melé, 1998; Schulz et al., 2000; Harada et al., 2003). Furthermore, the relative ratio of the $C_{37}$ and $C_{38}$ tetra-unsaturated alkenones were remarkably higher in the alkenones identified in lacustrine environments (Thiel et al., 1997; Zink et al., 2001; Chu et al., 2005). In addition, it was demonstrated that the alkenone chain-length ratios ($C_{37}$ / $C_{38}$) increased with decreasing salinity in marginal sea such as Baltic Sea (Schulz et al., 2000) and lacustrine sediments (Chu et al., 2005, and references therein). The facts indicate that

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there is potential in the abundances of tetra-unsaturated alkenones and the alkenone chain-length ratios for estimating the paleosalinity of lake water. We have been investigated the variations of the unsaturation ratios of the alkenones for a culture strain of Haptophycean alga *Isochrysis galbana*, which is a source organism for alkenones in coastal and shallow-marine environments, against the changes of salinity to clarify the relationships between alkenone unsaturation ratio and salinity (Sawada et al., 2009). Nevertheless, the tetra-unsaturated alkenones could not be detected in both low and high salinity conditions of this algal strain.

In this study, we analyzed the unsaturation ratios and distributions of alkenones and alkenoates from a culture *Emiliania huxleyi* (Lohman) Hay and Mother and *Gephyrocapsa oceanica* Kampther, which are typical alkenone producers in marine environment, and examined a potential of those parameters for paleosalinity indicator.

## 2. Materials and methods

### 2.1. Cultures

*Emiliania huxleyi* strain EH2 (identical to NIES-837, The Culture Collection of the national Institute of Environmental Sciences (NIES), Japan) and *Gephyrocapsa oceanica* strain GO1 (identical to NIES-838) was obtained from the Great Barrier Reef and Mutsu Bay in northern Japan (41ºN, 141'E), respectively, during the November 1990 cruise of Sogen-maru of the Marine Biotechnology Institute, Japan (Sekino and Shiraiwa, 1994; Sawada et al., 1996; Iwamoto and Shiraiwa, 2003). The strain names of EH2 and GO1 for *E. huxleyi* and *G. oceanica* are referred from Sawada et al. (1996). In the NIES Culture Collection, both species have been maintained in the IMK medium (Epply et al., 1967). Both species were cultured separately in batch culture systems at 21ºC and 34‰ under the cool white fluorescent light and continuous bubbling of sterilized air. The strain of *E. huxleyi* was transferred into 500-ml Mericron flasks (Iwaki Co. Ltd., Tokyo, Japan) containing 300 ml of artificial seawater Marine Art SF-1 (MA; produced by Tomita Pharmaceutical Co. Ltd., Naruto, Tokushima, Japan and purchased from formerly Senju

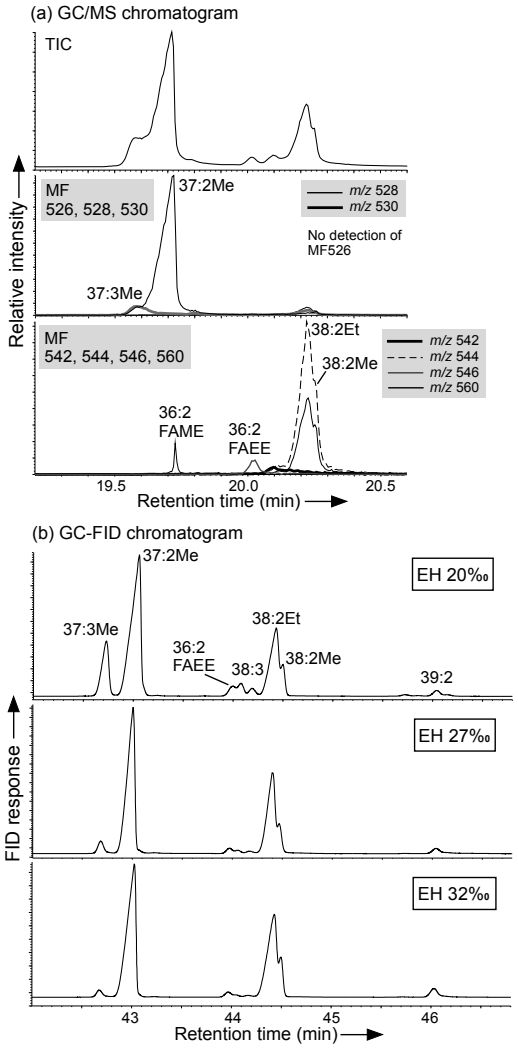


**Fig. 1.** (a) Total ion chromatogram (TIC) and mass fragmentgrams (MFs) of a fraction 1&2 from a culture sample of *E. huxleyi* EH2 grown at 32‰ of salinity, and (b) partial gas chromatograms of the fractions 1&2 from *E. huxleyi* EH2 grown at 20‰, 27‰ and 32‰ of salinities.

Pharmaceutical Co. Ltd. and lately Osaka Yakken Co. Ltd.,Osaka, Japan) enriched with modified ESM in which soil extract was replaced by 10 nM sodium selenite (Danbara and Shiraiwa, 1999) at 20ºC under 15‰, 20‰, 27‰, and 32‰ of salinity, but this strain could not grow under 15‰. *G. oceanica* was transferred into 500-ml Mericron flasks containing the same medium as used for *E. huxleyi* at 20ºC under 15‰, 20‰, 25‰,

and 34‰ of salinity. These were harvested at 2th (48 hrs), 4th (96 hrs) and 8th (192 hrs) day. Each medium with different salinity was made by dilution of natural seawater. The concentration of micronutrient enrichments was maintained constant in all cultures without dilution. At intervals, cells (20-50 ml cell suspension each) were harvested and then the optical density at 750 nm ($OD_{750}$; Danbara and Shiraiwa, 1999) and the cell number were determined using the UV-VIS spectrophotometer (Shimadzu, UV mini-1240) and under the microscope, respectively.

### 2.2. Lipid extraction and analytical procedures

Extraction and separation of lipids were performed according to Sawada et al. (1996) and Sawada and Shiraiwa (2004). After extraction, the lipids were separated through a silica gel column, and then fractions 1&2 (hexane and hexane / ethyl acetate (9/1, v/v)) were analyzed by gas chromatography with a flame ion detector (GC-FID) and gas chromatography - mass spectrometer (GC-MS); GC with a Hewlett Packard 6890 attached to a capillary GC column (50m x 0.32 mm i.d. CPSil5CB, Chrompack), GC-MS with a Hewlett Packard 6890 attached to a capillary GC column (30m x 0.25 mm i.d. DB-5HT, J&W Scientific) directly coupled to a Agilent MSD quadrupole mass spectrometer (electron voltage, 70 eV; emission current, 350 μA; mass range, m/z 50-650 in 1.3 s). The alkenones were detected in fractions 1&2 (Fig. 1). The GC temperature was programmed as follows: 60ºC for 5 min, 60-120ºC at 4ºC/min, 260-320ºC at 3ºC/min and 320ºC for 25 min.

Alkenone unsaturation ratio ($U^{k'}_{37}$) was calculated by the equation of Prahl et al. (1988) as follows, $U^{k'}_{37}$ = [37:2] / ([37:2] + [37:3]). In addition, alkenone chain-length ratio (the ratio of total $C_{37}$ alkenones to total $C_{38}$ alkenones; $K_{37}/K_{38}$) and the ratio of alkyl alkenoates to alkenones (the ratio of fatty acid ethyl esters (FAEE) to total $C_{37}$ alkenones; $EE/K_{37}$) were calculated by the following equations,

$K_{37}/K_{38}$ = ([37:2Me] + [37:3Me]) / ([38:2Et] + [38:2 Me] + [38:3Et] + [38:3Me]) and $EE/K_{37}$ = [36:2FAEE] / ([37:2Me] + [37:3Me], respectively (Prahl et al. 1988).

## 3. Results and Discussion

### 3.1. Cell growth, alkenone abundances, and intercellular concentrations of alkenones

The growth curves of *E. huxleyi* EH2 and *G. oceanica* GO1 by cell number and $OD_{750}$ are shown in Fig. 2 and Fig. 3, respectively. *E. huxleyi* grew exponentially during 48 - 192 hours (2-8 days) under 27‰ and 32‰, and grew linearly under 20‰. *G. oceanica* also grew exponentially during 48 - 192 hours under 20‰, 25‰ and 34‰, but hardly grew under 15‰. It was found that *G. oceanica* showed a better growth under 25‰ than 34‰ (salinity of seawater in open ocean). *G. oceanica* has been known to predominate exclusively in coastal, inland and marginal sea areas of the monsoonal regions, the Pacific and the Indian Ocean (Houghton and Guptha, 1991), but seldom in oligotrophic regions of the open ocean. Thus, our result is reasonable for the property of growth against salinity condition for *G. oceanica*.

The changes of alkenone abundances, which calculated by using the sum of total $C_{37}$ - $C_{39}$ alkenones, were concordant with those of cell number and $OD_{750}$ in *E. huxleyi* and *G. oceanica* (Figs. 2 and 3). However, the alkenone abundances in *G. oceanica* under 34‰ were much higher than those under 25‰ (Fig. 3), which disagreed with the results from cell number and $OD_{750}$. The intercellular concentrations of alkenones in *E. huxleyi* were nearly constant in all salinity conditions, while those in *G. oceanica* were higher in 15‰ and 34‰ (2.21 - 5.37 pg/cell; Table 1). Recently, it was demonstrated that the alkenone is thought to function as storage lipid within cell (Sawada and Shiraiwa, 2004; Eltgroth et al., 2005), so that the high concentrations of alkenones in 15‰ possibly show the physiological response associated with the storage of compounds in very low salinity for *G. oceanica*.

### 3.2. Tetra-unsaturated alkenones

As shown in Fig. 1a, there is no peak in the mass fragmentogram of m/z 526.5, which is a molecular ion of $C_{37}$ tetra-unsaturated ($C_{37:4}$) alkenone, in all samples of *E. huxleyi* EH2 and *G. oceanica* GO1. This result

clearly indicates that these two strains do not produce $C_{37:4}$ alkenone, and it is the same as results that in *I. galbana* (strain UTEX LB 2307) as reported by Sawada et al. (2009). Previous studies (Conte et al., 1995; Rosell-Melé, 1998) reported that the abundance of $C_{37:4}$ alkenone was remarkably high in *E. huxleyi* strains collected from coastal and neritic environments, especially at high latitude. In contrast to the previous study, our experiments show a case that no $C_{37:4}$ alkenone is produced by *E. huxleyi* and *G. oceanica*, which can live in coastal waters, even in cells that had been grown under low salinity conditions. From these results, it is unlikely that one haptophyte species are favorably synthesized the tetra-unsaturated alkenones under low salinity condition. Furthermore, we suggested that the tetra-unsaturated alkenones were abundantly appeared in coastal and lacustrine environments, resulting from the occurrence of the peculiar species that can synthesized such tetra-unsaturated compounds rather than physiological response in single haptophyte cell against the environmental change as low salinity condition.

### 3.3. Relationship between salinity and alkenone unsaturation index ($U^{k'}_{37}$)

The $U^{k'}_{37}$ values in *E. huxleyi* EH2 were found to increase rapidly from 0.38 to 0.96 at 20ºC under 27‰ and 32‰, and from 0.38 to 0.78 at 20ºC under 20‰ (Table 1 and Fig. 2). The results indicate that the $U^{k'}_{37}$ values in *E. huxleyi* cells grown at low salinity vary as a result of the acclimation of cells to respective salinity conditions, or changes in the alkenone synthesis due to physiological stress against low salinity even under sufficiently active growth of cells. The $U^{k'}_{37}$ value of the cell samples harvested in 192 hours at 20‰ was lower than those at 27‰ and 32‰, probably due to no acclimation of the cell to low salinity as 20‰, because the growth of *E. huxleyi* was suppressed at 20‰ (Fig. 2). Such effect on cell growth may be an important

**Table 1.** Alkenone abundance (K37-39; μg/ml), intercellular concentration of alkenone (K37-39/Cell; pg/cell), alkenone unsaturation index ($U^{k'}_{37}$), alkenone chain-length ratios ($K_{37}/K_{38}$), and alkenoates / alkenone ratio ($EE/K_{37}$) in *E. huxleyi* EH2 and *G. oceanica* GO1 grown at several salinity conditions.

| Samples | Salinity (‰) | Time (hours) | K37-39 (μg/ml) | K37-39/Cell (pg/cell) | $U^{k'}_{37}$ | $K_{37}/K_{38}$ | $EE/K_{37}$ |
|---|---|---|---|---|---|---|---|
| EH-0 | 34 | 0 | <0.01 | - | 0.38 | 1.36 | 0.472 |
| EH20-4 | 20 | 96 | 0.25 | 0.92 | 0.72 | 2.15 | 0.036 |
| EH20-8 | 20 | 192 | 2.12 | 0.58 | 0.78 | 2.04 | 0.038 |
| EH27-4 | 27 | 96 | 1.45 | 0.72 | 0.92 | 1.92 | 0.032 |
| EH27-8 | 27 | 192 | 8.73 | 0.86 | 0.94 | 1.52 | 0.027 |
| EH32-2 | 32 | 48 | 1.03 | 0.71 | 0.77 | 1.98 | 0.037 |
| EH32-4 | 32 | 96 | 3.23 | 0.79 | 0.94 | 1.60 | 0.025 |
| EH32-8 | 32 | 192 | 12.15 | 1.37 | 0.96 | 1.26 | 0.028 |
| GO-0 | 34 | 0 | <0.01 | 0.53 | 0.69 | 0.87 | 0.189 |
| GO15-4 | 15 | 96 | 0.01 | 5.37 | 0.62 | 2.13 | 0.124 |
| GO15-8 | 15 | 192 | 0.06 | 3.39 | 0.64 | 1.98 | 0.166 |
| GO20-4 | 20 | 96 | 0.01 | 1.40 | 0.61 | 2.35 | 0.135 |
| GO20-8 | 20 | 192 | 0.08 | 0.91 | 0.69 | 1.76 | 0.153 |
| GO25-4 | 25 | 96 | 0.04 | 1.90 | 0.63 | 2.16 | 0.139 |
| GO25-8 | 25 | 192 | 0.12 | 0.41 | 0.70 | 1.42 | 0.281 |
| GO34-2 | 34 | 48 | 0.02 | 5.03 | 0.71 | 1.89 | 0.119 |
| GO34-4 | 34 | 96 | 0.08 | 3.85 | 0.73 | 1.95 | 0.118 |
| GO34-8 | 34 | 192 | 0.20 | 2.12 | 0.75 | 1.49 | 0.140 |

factor for changing $U^{k'}_{37}$. Moreover, from this insight, we should consider and pay attention to such variations of the $U^{k'}_{37}$ values obtained from *E. huxleyi*-dominant waters of which salinity was low in paleotemperature reconstruction.

On the other hand, the $U^{k'}_{37}$ values in *G. oceanica* GO1 did not significantly change at 20ºC under 20‰, 25‰, and 34‰, although these values decreased in 96 hours at low salinity conditions such as 20‰ and 25‰ (Table 1 and Fig. 3). From these results, it is presumed that this *G. oceanica* strain was sufficiently acclimated to salinities of 20‰ and 25‰, and no physiological stress against such conditions, so that the $U^{k'}_{37}$ values were not affected by salinity. However, the $U^{k'}_{37}$ values of this strain decreased from 0.69 to 0.62 at 21ºC under 15‰ (Fig. 3). This thing might occurr as a result of no acclimation of cells to low salinity of 15‰ due to less growth of cells (Fig. 3). Hence, it is concluded that the $U^{k'}_{37}$ in *G. oceanica* do not or less vary depending on salinity.

### 3.4. Alkenone chain-length ratio ($K_{37}/K_{38}$) and alkenoate / alkenone ratio (EE/$K_{37}$)

We examined the potential of alkenone chain-length ratio ($K_{37}/K_{38}$) as a proxy for paleosalinity, which has been suggested by Schulz et al. (2000). In this study, the $K_{37}/K_{38}$ ratio of cells harvesting in 192 hours increased from 1.26 to 2.04 for *E. huxleyi* and from 1.49 to 1.98 for *G. oceanica* with decreasing salinities (Table 1). However, these variations cannot explain the variations of $K_{37}/K_{38}$ ($\Sigma C_{37}/\Sigma C_{38}$) ratios, which were widely ranging from <1 to 7 as reported by Shultz et al. (2000) and Chu et al. (2005). Shultz et al. (2000) reported that varied depending on salinities of surface waters in the Baltic Sea, and decreased exponentially below 9‰ and were constant from 9 to 30‰. They inferred that such differences might be attributed to the variations of species and/or strains of alkenone producers. However, our experiments showed that the $K_{37}/K_{38}$ ratios slightly varied with changing salinities in a single strain. These

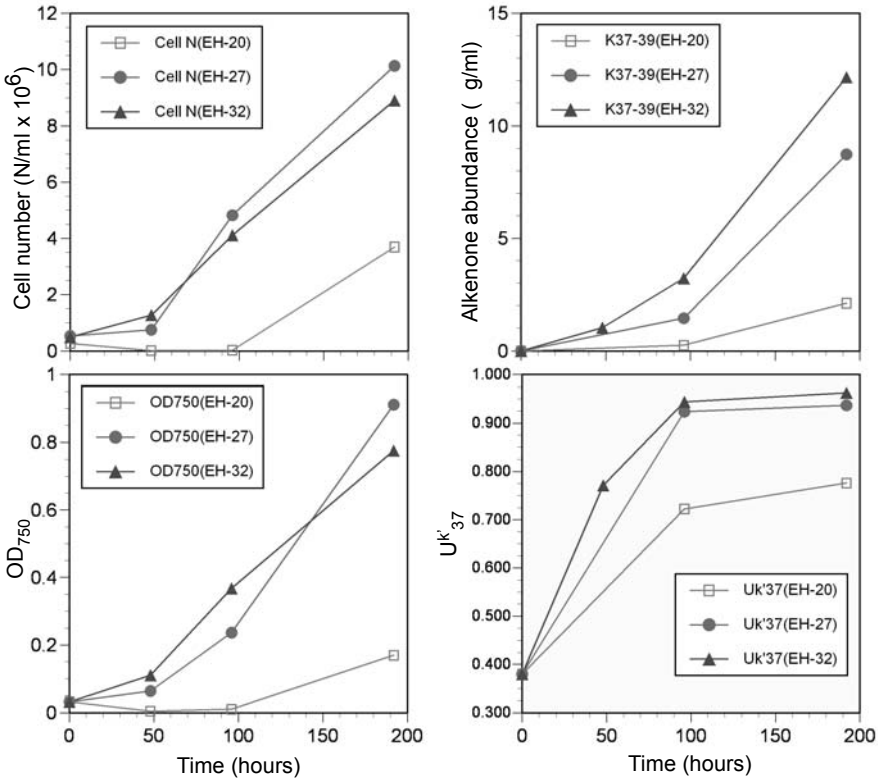


**Fig. 2.** Changes in the cell numbers (N/mlx$10^6$), $OD_{750}$, alkenone abundances (μg/ml), and $U^{k'}_{37}$ in *E. huxleyi* EH2 grown at various salinity conditions (20‰, 27‰, and 32‰).

results suggest that these variations are mainly attributed to the differences of haptophyte species and/or strains, but also might be slightly controlled by cellular and physiological factors in a single haptophyte strain.

In addition, the ratios of ethyl alkenoates to $C_{37}$ alkenones ($EE/K_{37}$) are also examined as a salinity proxy. However, these values were nearly constant in both *E. huxleyi* and *G. oceanica* at various salinities, and were not correlated with the salinities (Table 1). Sawada et al. (1996) suggested that $EE/K_{37}$ ratio vary dependence on taxonomic variation such as *E. huxleyi* and *G. oceanica*. However, Conte et al. (1998) demonstrated that the variability of alkenone / alkenoate ratio reflected differences in genetic makeup and physiological status of alkenone and alkenoate producers. The crucial factor(s) for variability of alkenoate / alkenone ratio is still unknown. In this study, it can be concluded that the $EE/K_{37}$ ratio is not affected by salinity in *E. huxleyi* and *G. oceanica*.

## 4. Conclusions

In this study, the tetra-unsaturated alkenones could not be identified in the strains of *E.huxleyi* EH2 and *G. oceanica* GO1 grown at both low and high salinity conditions. This result implied that tetra-unsaturated alkenones, which were abundantly appeared in coastal and lacustrine environments, might occur as a result from the production by the peculiar species and/or strains that can synthesized such alkenones rather than physiological response against the environmental change as low salinity. On the other hand, we could observe that the alkenone chain-length ratio ($K_{37}/K_{38}$) for strains of *E. huxleyi* and *G. oceanica* increased with decreasing salinities, although the ranges of these variations were small. These results suggest that the $K_{37}/K_{38}$ values were affected by the cellular and physiological factors in single haptophyte cell, although this value varies mainly depending on the changes of haptophyte

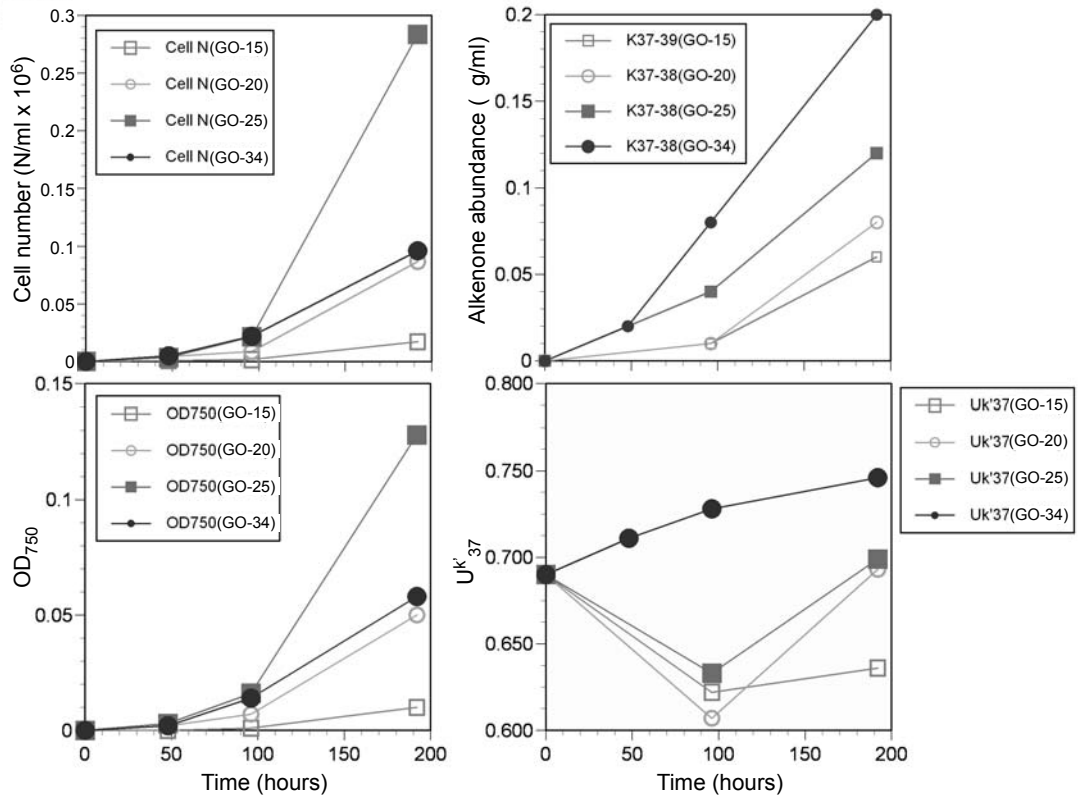


**Fig. 3.** Changes in the cell numbers (N/mlx$10^6$), $OD_{750}$, alkenone abundances (μg/ml), and $U^{k'}_{37}$ in *G. oceanica* GO1 grown at various salinity conditions (15‰, 20‰, 25‰, and 34‰).

species and/or strains. Hence, we suggest that $K_{37}/K_{38}$ ratio can be used as more reliable paleosalinity proxy, although further examination is necessary. In addition, we found that the alkenone unsaturation index ($U^{k'}_{37}$) in *E. huxleyi* EH2 varied under low salinity conditions. From this insight, we should pay attention to such variations of the $U^{k'}_{37}$ values obtained from *E. huxleyi*-dominant waters of which salinity was low in paleotemperature reconstruction.

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