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Xanthophyll-cycle of ice algae on the sea ice bottom in Saroma Ko lagoon, Hokkaido, Japan

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Abstract: Using the ice algal community prevailing on the sea ice bottom in Saroma Ko lagoon, Hokkaido, Japan, the response of a photosynthetic system to exposure to light was investigated, focusing on xanthophyll-cycle features, diel changes of the pool size of xanthophyll-cycle pigments and the effective quantum yield of PS II in early February 1998. By pigment analysis, β-carotene, chlorophylls *a* and *c,* diadinoxanthin, diatoxanthin and fucoxanthin were detected as major pigments, which suggests that diatoms dominated as ice algae during this study. When such ice algae were exposed to irradiance nearly 4 times higher than the daily maximum level at the ice bottom, the interconversion between diadinoxanthin and diatoxanthin continued for *ca.* 20 min immediately after the onset of irradiation in spite of the sub-zero Celsius ambient temperature. Although the pool size of this xanthophyll-cycle (relative amount of diadinoxanthin plus diatoxanthin per chlorophyll *a*) was not so large compared to that of mesophilic diatoms, it showed a circadian change increasing during the daytime and decreasing at night. This change correlated well with the effective quantum yield of PS II. These results suggest that ice algae at the sea ice bottom possess a relatively effective xanthophyll-cycle to regulate light energy usage. However, the xanthophyll-cycle in ice algae may be poor compared to that of algae living in intermediate irradiance, which can be interpreted from the point of view of bioenergetic aspects of shade adapted ice algae.

key words: diel change, ice algae, light acclimation, light stress, xanthophyll-cycle

Introduction

Ice algae, which spread and bloom at the sea ice bottom, are one of the most important primary producers of sea ice ecosystems (reviewed in Horner, 1985; Legendre *et al.*, 1992). They have excellent ability to develop massive bloom even under a rather weak light environment such as the sea ice bottom. Hence, many studies concerning their shade-adaptation ability have been reported based on *in situ* observations and

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Abbreviations: Chl, Chlorophyll; HPLC, high performance liquid chromatography; DD, diadinoxanthin; DT, diatoxanthin; DTT, dithiothreitol; F, the measured fluorescence yield at any given time; F_m, maximal fluorescence yield of dark-adapted sample; F_m', maximal fluorescence yield reached in a pulse of saturation light with an illuminated sample; NPQ, coefficient of non-photochemical quenching determined by $[F_m–F_m']/F_m'$; Y, effective quantum yield of PS II ($[F_m'–F]/F_m' = \Delta F/F_m'$); PAR, photosynthetically active radiation; PS II, photosystem II

analytical and experimental demonstrations since the 1960's. Such work includes research on the photosynthetic response to light (Bunt, 1964a, b; Palmisano *et al.*, 1985; Smith *et al.*, 1994; Suzuki *et al.*, 1997), the light absorption efficiency (Barlow *et al.*, 1988; Cota and Horne, 1989), the analysis of pigment composition (Kashino *et al.*, 1998; Ikeya *et al.*, 2000), and the photosynthetic performance (Cota, 1985; Smith *et al.,* 1988; Gleiz and Kirst, 1991) using natural ice algae as well as isolated cultures.

The sea ice bottom as a habitat for ice algae is a generally shaded environment due to high light reflection and attenuation by snow and sea ice (SooHoo *et al.*, 1987; Kishino, 1993). Yet, the light intensity that penetrates to the sea ice bottom should fluctuate with diel changes of sun angle, weather conditions (sunshine, cloud and snow), especially in sea ice at lower latitude since clear day/night changes occur even during winter (nearly 12L/12D cycle with 0 to 35 μmol $m^{-2}s^{-1}$ of photon flux; see Kudoh, 1993; Kudoh *et al.*, 1997). Here are some questions: "How do ice algae detect such environmental light fluctuations? Do ice algae respond to the change of irradiance by adjusting their photosystems?" According to recent physiological studies on photosynthesis, which have investigated the photo-damages to and the light protection of photosystems, photosynthetic organisms have several strategies to regulate the flow of light energy into their photosystems so as to protect them from unrecoverable damage, which is easily induced under low temperature (reviewed by Aro *et al.*, 1993; Sonoike, 1998; Ort, 2001). Although ice algae efficiently perform enough photosynthesis to support their growth under low temperature (–1 to 5°C), fluctuation of irradiance might be a problem if ice algae possess shade-adapted photosystems, and the light intensity fluctuates in excess of their capacity.

The xanthophyll-cycle, especially the diadinoxanthin-cycle (DD-cycle), is one of the most studied protection mechanisms in phytoplankton ecophysiology (Sakshaug *et al.*, 1987; Demers *et al.*, 1991; Olaizola *et al.*, 1994; Lohr and Wilhelm, 1999), which dissipates excess light energy as heat. For psychrophilic algae in surface water of polar seas, the xanthophyll-cycle is very important during the spring-summer season because of two conflicting environmental conditions: surrounding low temperature and high availability of light. However, in spite of this potential importance, there are few papers that report the function of the xanthophyll-cycle observed in psychrophilic algae at the polar sea surface (Olaizola *et al.*, 1992; Kashino *et al.*, 2002).

This is the first report that analyzes the characteristics of the xanthophyll-cycle in natural ice algae (its activity, response rate and diel variability of pool size) in the southernmost sea ice ecosystem in the northern hemisphere, Saroma Ko lagoon, Hokkaido, Japan.

Materials and methods

Ice algal sampling for illumination experiments

Three ice algae samples were collected from the bottom of sea ice at *ca.* 1 km offshore from the Saroma Research Center for Aquaculture, which locates at eastern shore of Saroma Ko lagoon, from 4 through 6 February 1998 (Fig. 1). During this sampling period, the weather was calm and fine and a maximum PAR intensity of *ca.* 1000 μmol $m^{-2}s^{-1}$ was detected on the ice at around noon. An ice auger (inside

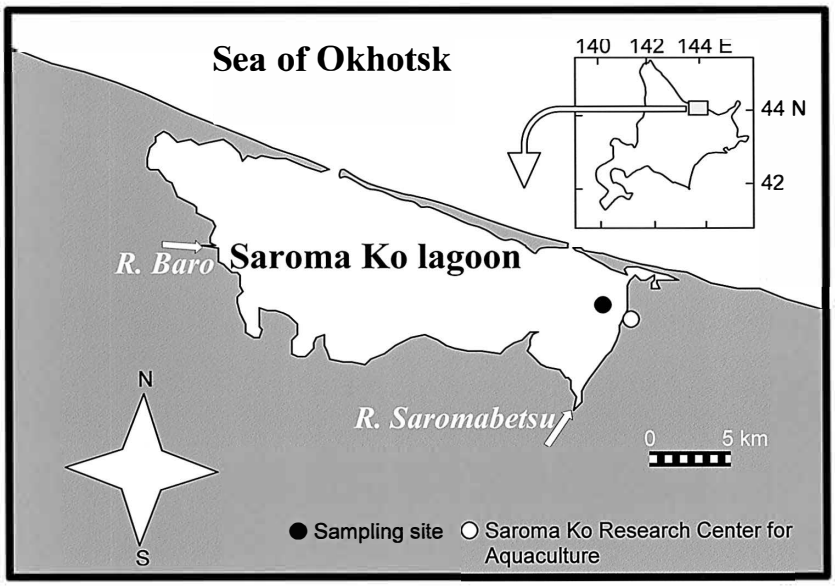

Fig. 1. Location of sampling site.

diameter, *ca.* 75 mm) and an ice saw were used to cut the sea ice whose thickness was *ca.* 40 cm with 5–8 cm snow cover. The collected bottom part of the sea ice showed a clearly brown color. The colored part of 3–5 cm thickness was immediately sliced horizontally using an ice saw. The sliced sea ice was kept in filtered seawater (GF/F, Whatman) at near-freezing temperature in a thermos box during transportation to the Center laboratory, which took less than 30 min. During these procedures, the sample was kept in the dark to avoid exposure to any direct sunlight.

The collected ice samples were crushed and allowed to melt for one hour [less for the fluorescence quenching experiments, see below] in filtered seawater under dark condition. The sample was then stirred gently, and sieved through a *ca.* 0.5 mm nylon mesh to remove remained ice pieces. The resulting filtrate (ice algal suspension) was used in the following experiments. Temperature in the suspension was occasionally checked using a thermometer; it never increased above zero during these procedures.

Illumination experiment

The ice algal suspension was transferred in conical beakers that were kept below 0℃ in an ice bath. The ice algal samples were exposed to irradiance at 0 (dark), 50 and 100 μmol $m^{-2}s^{-1}$ provided by a halogen lamp (Trad HL-500, Sankyo) with gentle stirring. Light intensities selected for this experiment were intended to simulate roughly the daily maximum level obtained in the upper sea ice (50 μmol $m^{-2}s^{-1}$) and its two-fold magnitude of the maximum (100 μmol $m^{-2}s^{-1}$, *e.g.* Kudoh *et al.*, 1997). After illumination for various periods (0–>120 min), a part of them was divided (10 m*l*) and filtrated onto a glass fiber filter (GF/F, Whatman) after addition of 300 μM dithiothreitol (DTT) (final concentration) so as to stop xanthophyll-cycle activity

(Olaizola *et al.*, 1994; Kashino and Kudoh, 2003). Then, they were immediately frozen by liquid nitrogen and stored at –80℃ until pigment analysis. Those illumination experiments were continued for >2 hrs to check the kinetics of changes in ice algal xanthophyll pigments.

Fluorescence quenching analysis

Sea ice bottom samples collected at 0920, 1105, 1340, 1600 on 5 February and 0955 on 6 February (local time and date) were immediately transported to the laboratory under cool and dark condition within 30 min as described above. The sea ice bottom sample was crushed, mixed with filtered seawater (at *ca.* 0℃), and stirred gently for 30 s. Remaining ice pieces were then removed by sieving with a plastic mesh basket. An aliquot of ice algal suspension was immediately collected onto a glass fiber filter for pigment analysis after addition of 300 μM DTT, and another aliquot was used for fluorescence quenching experiments.

A pulse-amplitude-modulated chlorophyll fluorometer (Teaching PAM, Walz) was used for determination of the Chl fluorescence quenching properties in ice algae (Schreiber *et al.*, 1997; Kashino *et al.*, 2002). The temperature of the equipment was kept at *ca.* 0℃ by cooling the equipment with ice and snow during the measurement. Ice algal suspension was concentrated (about 100 mg-Chl *a*/L) by a portable centrifuge just before the measurements. A drop of the concentrated ice algal suspension was set on a special suspension cuvette (TEACH-SC, Walz) and allowed to remain further in the dark for a few min.

After the determination of F_0 under a low-intensity modulated measuring light beam (3 μs with 32 Hz delivered from a light-emitting diode of 660 nm emission peak) that was weak enough to not induce any significant variable fluorescence, a high-intensity saturating light pulse of 3500 μmol $m^{-2}s^{-1}$ with a duration of 0.5–1.0 s was applied to the sample in order to close all reaction centers for the determination of F_m. Then, an actinic light of 165 μmol $m^{-2}s^{-1}$ was turned on and the fluorescence signal was recorded for 5 min, during which, a train of single saturation pulses of the same intensity and duration as the former was supplied at intervals of 20 s. The parameters of non-photochemical quenching (NPQ) and quantum yield of PS II were determined using the following equations with averaged data from the last 1 min when the signal was rather stable,

$$NPQ = (F_m - F_m') / Fm'$$
$$\text{PS II yield} = (F_m' - F) / F_m' = \Delta F / F_m'$$

where F_m is maximum fluorescence yield after dark adaptation, F_m' is the maximum fluorescence yield by saturation pulse under actinic illumination, and F is the fluorescence yield under illumination.

Pigment analysis

Algal pigments were analyzed according to Kashino *et al.* (1998) with slight modifications. Ice algae filtrated onto the glass fiber filters were extracted by sonicating algal cells for one min (Bransonic 2200, Branson) in 90% acetone followed by incubation overnight at –20℃ in the dark.

After further sonication for one min and succeeding centrifugation, the extracts were then subjected to reverse-phase HPLC analysis with solvents; 20 mM of ammonium acetate/80% methanol, and 30% ethylacetate/70% methanol gradient protocol (Kashino *et al.*, 1998). Absorption patterns of pigments, such as Chlorophyll (Chl) *a* and *c,* β-carotene, diadinoxanthin, diatoxanthin and fucoxanthin, were detected by a photodiode array detector (SPD-M10AV, Shimadzu), and the amounts of these pigments were determined using external standards, which were purchased from the Water Quality Institute, Denmark.

Results

Pigment composition of ice algae

Table 1 summarizes the relative amount of pigments against Chl *a* in the ice algal samples, as determined by HPLC analysis. The pigment composition of ice algae, which was measured after dark incubation for >1.5 hrs under sub-zero temperature, showed similar values among the three samples. The HPLC analysis clearly detected β-carotene, Chl *a,* Chl *c,* diadinoxanthin, diatoxanthin and fucoxanthin. Other pigments that are frequently detected in seawater samples, such as Chl *b,* lutein, alloxanthin or peridinin, were negligible in these ice algal samples. This suggests that chromophytes such as diatoms dominated in the ice algal community and composed the main biomass of the community during the season of the present study.

Response of DD-cycle pigments (diadinoxanthin and diatoxanthin) against irradiance

Remarkable changes of DD-cycle pigments occurred immediately after the illumination was started, whereas almost no significant changes were observed for 2 hrs in the samples that were not illuminated (Figs. 2a, b, c). Dark incubated ice algae (>1.5 hrs) contained 0.035 and 0.002 mol/mol-Chl *a* of diadinoxanthin and diatoxanthin, respectively. The relative amounts of these pigments were kept constant for >2 hrs (Fig. 2a). On the other hand, a rapid increase of diatoxanthin and concomitant decrease of diadinoxanthin were recognized in the illuminated samples (Figs. 2b, c). These rapid changes were observed during the period of 10–20 min immediately after

Table 1. Pigment composition (mol/mol-Chl *a*) of ice algae collected from the sea ice bottom (0–5 cm) in Saroma Ko lagoon. Samples were collected on 4 (1000 LT), 5 (0920 LT) and 6 (0955 LT) February 1998, and stayed under dark condition for >1.5 hr before pigment fixation.

Pigments	Relative content (SD) (mol/mol-Chl *a*)
β-carotene	0.017 (0.005)
Chl *c*	0.211 (0.034)
Diadinoxanthin	0.035 (0.006)
Diatoxanthin	0.002 (<0.001)
Fucoxanthin	0.481 (0.031)

SD, standard deviation ($n=3$)
LT, local time

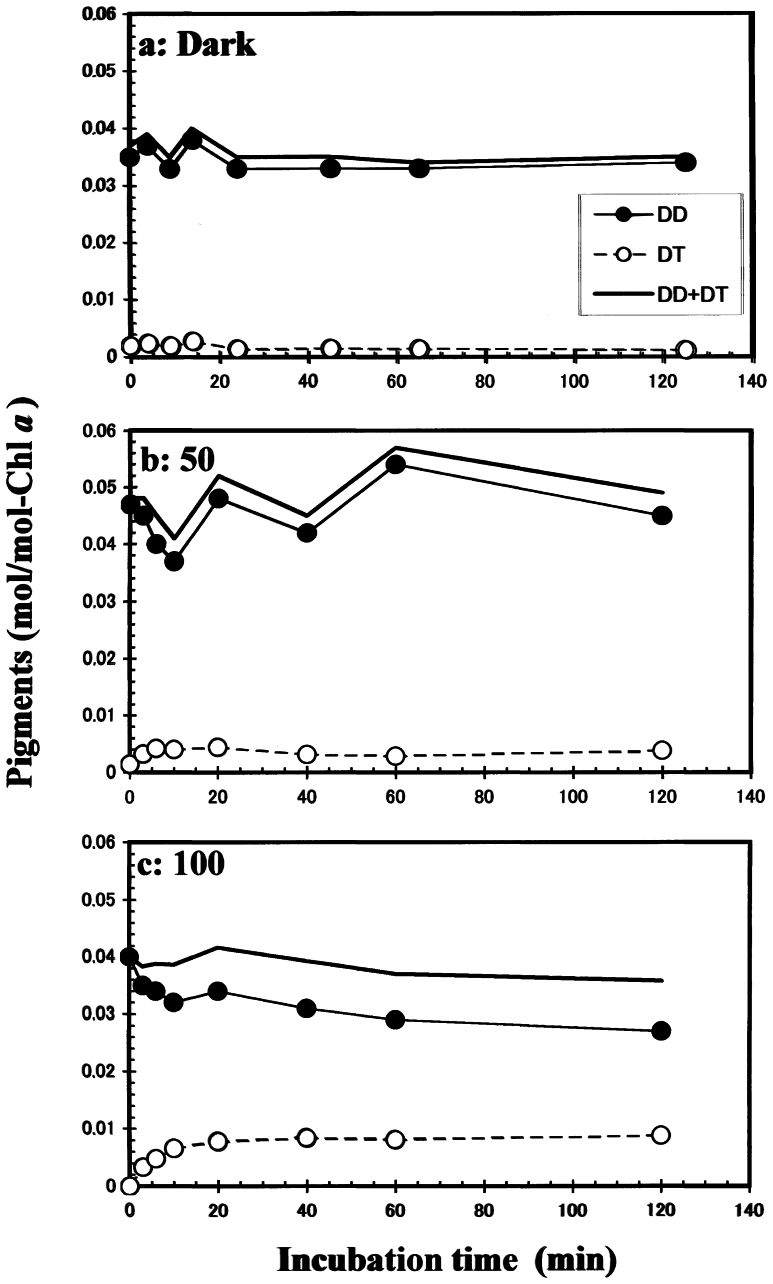

Fig. 2. Changes of DD-cycle pigments occurred immediately after the onset of illumination. Ice algal suspensions, which were kept below 0°C in an ice bath, were exposed to (a) 0 (dark), (b) 50 and (c) 100 μmol $m^{-2}s^{-1}$ of irradiance with gentle stirring. Solid circles: diadinoxanthin (DD), open circles: diatoxanthin (DT), bold line: diadinoxanthin plus diatoxanthin (DD+DT). Each experiment was carried out separately using the sample collected on 5 (0920 LT), 6 (0955 LT) and 4 (1000 LT) February 1998, respectively.

turning the light on regardless of the light intensities used in the present experiments (50 and 100 μmol $m^{-2}s^{-1}$). After this period, the relative amounts of both pigments were kept fairly constant. In the ice algae, which were illuminated at the irradiance of 50 μmol $m^{-2}s^{-1}$, the relative amount of diatoxanthin increased to around twice the initial level. It increased to >4 times the initial level in ice algae which experienced 100 μmol $m^{-2}s^{-1}$ irradiance.

Diel change of DD-cycle pigments and fluorescence quenching

The pool size of the ice algal DD-cycle (diadinoxanthin plus diatoxanthin, thick line in Fig. 3a) showed a clear diel variation. This analysis was closely correlated to the sample for the following fluorescence quenching analysis. Because the procedure to

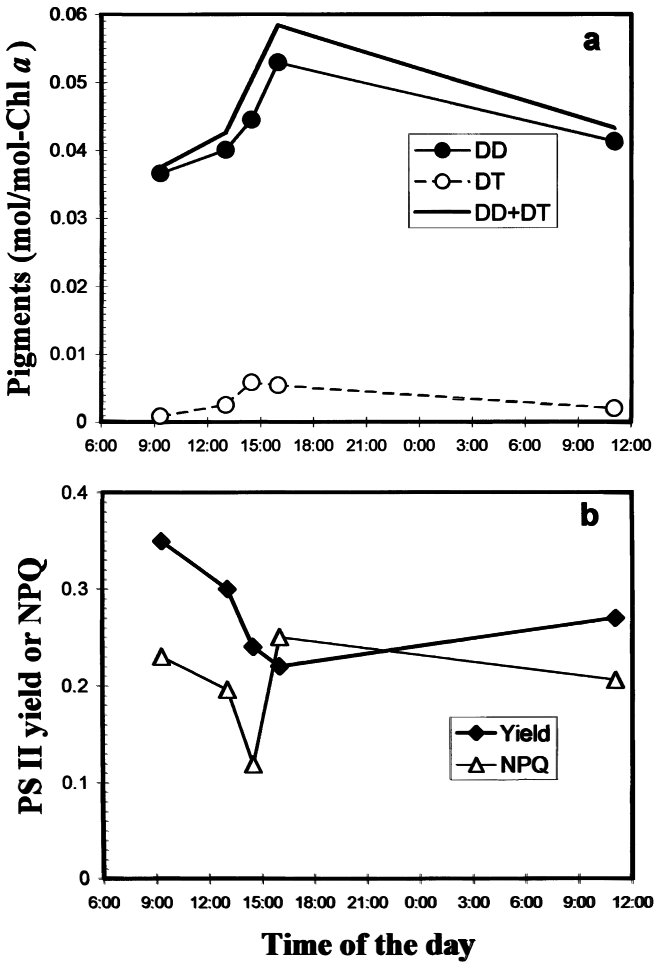

Fig. 3. Diel change of DD-cycle pigments (a), and Diel changes of the effective quantum yield and the NPQ (b). Sea ice bottom samples were collected at 0920, 1105, 1340, 1600 on 5 February and 0955 on 6 February (local time and date).

extract ice algae from the sea ice and the following filtration were performed as quickly as possible, it is reasonably considered that the detected amount of these pigments reflects the real *in situ* amounts. The pool size gradually increased during daytime and then reached a maximum of 0.058 mol/mol-Chl *a* at sunset (*ca.* 1630 LT). After that, the pool size seemed to decrease gradually during the night, and reached 0.043 mol/mol-Chl *a* by the next noon. Maximum amounts of both diadinoxanthin and diatoxanthin were 0.053 and 0.006 mol/mol-Chl *a,* respectively; the former value was obtained at 1600 whereas the latter value was observed *ca.* 2 hours before the former one.

The effective quantum yield of PS II, which was measured under 165 μmol $m^{-2}s^{-1}$ of actinic illumination by a PAM fluorometer, showed clear reverse kinetics of the diel change in the pool size of DD-cycle pigments (Fig. 3b); the PS II yield gradually decreased during daytime, reached a minimum at around the sunset, and seemed to recover during the night. The parameter of NPQ, however, showed rather complex fluctuation at the value of around 0.2 in this measurement.

Discussion

DD-cycle pigments protect the photosystems against excess irradiance by dissipating excess light energy as heat through the de-epoxidized pigment, diatoxanthin, which is inter-converted from epoxidized xanthophyll, diadinoxanthin (Olaizola and Yamamoto, 1994; Arsalane *et al.*, 1994). It is an efficient quenching mechanism, which does not affect the light harvesting efficiency (Schubert *et al.*, 1994) and lessens the cost of synthesizing other caroteniods (Brunet *et al.*, 1993). The averaged relative amount of the present ice algal DD-cycle pigments (pool size) was around 0.04 mol/mol-Chl *a,* which showed a clear diel change of 20% of the total amount (Table 1 and Fig. 3a). Several papers which measured DD-cycle pigments of marine mesophilic diatoms have reported that the pool size was 5–10 times larger than the value obtained in this work; *e.g.*, 0.1–0.7 mol/mol-Chl *a* (Demers *et al.*, 1991; Olaizola *et al.*, 1994; Kashino and Kudoh, 2003) when they were cultivated under somewhat higher irradiance (50–200 μmol $m^{-2}s^{-1}$). Compared with these data, the ice algae in the present study, which were living under dim light condition at the sea ice bottom, contained fewer amounts of DD-cycle pigments. Ikeya *et al.* (2000) have clearly demonstrated that, in psychrophilic diatoms, the total amount of diadinoxanthin and diatoxanthin was decreased below 0.05 mol/mol-Chl *a* when they were grown under extremely dim light (5 μmol $m^{-2}s^{-1}$), while it gradually increased to 0.1 mol/mol-Chl *a* along the increment of growth irradiance up to 42 μmol $m^{-2}s^{-1}$. A similar trend in low content of xanthophyll pigments under lowering light irradiance was also reported by Moisan *et al.* (1998) and Moisan and Mitchell (1999) using an isolated polar alga, *Phaeocystis antarctica.* The low content of these xanthophyll pigments may indicate that the ice algae collected here had not experienced high irradiance recently enough to exhibit high activity of the DD-cycle to protect their photosystems under their natural light condition at the sea ice bottom in Saroma Ko lagoon. Kudoh *et al.* (1997) reported that the PAR intensity at the sea ice bottom in the lagoon was less than 10 μmol $m^{-2}s^{-1}$, even at noon under similar ice thickness and snow coverage as the present study, that is, present ice algal sample seemed to experience as such dim light.

In spite of the low content of DD-cycle pigments, rapid interconversion from diadinoxanthin to diatoxanthin was clearly detected when the dark-adapted ice algae were exposed to irradiances at 50 and 100 μmol $m^{-2}s^{-1}$ (Figs. 2b, c). Under these conditions, diadinoxanthin in the dark-adapted ice algae was immediately de-epoxidized into diatoxanthin within 10–20 min, a rate comparable to those of some mesophilic diatoms which have been reported (Olaizola *et al.*, 1994; Lohr and Wilhelm, 1999; Kashino and Kudoh, 2003). This de-epoxidation activity has a close correlation with the *in vivo* fluorescence quenching, *i.e.*, the thermal dissipation of absorbed light energy (Sakshaug *et al.*, 1987; Demers *et al.*, 1991; Olaizola and Yamamoto, 1994; Olaizola *et al.*, 1994). Taking this close correlation into account, the ice algae in the present study also possess the ability to dissipate excess light energy with rapid function of the DD-cycle. An example of the rapid interconversion under low temperature was also reported by Moisan *et al.* (1998); the results including the present one strongly suggest that the DD-cycle can be effective in quenching the excess energy even under low temperature condition such as sea ice habitat.

The interconversion rate itself was comparable to that found in some mesophilic diatoms. However, the steady-state ratio of DT/DD during light exposure after 20 min was not so large in the present ice algal samples (Figs. 2a, b, c). The maximum value of the DT/DD ratio, 0.3, was obtained in the cells exposed to 100 μmol $m^{-2}s^{-1}$ irradiance for 2 hrs. The maximum ratio of DT/DD reported by several researchers using cultivated diatoms was sometimes much larger: >0.5 (Olaizola *et al.*, 1994; Fujiki and Taguchi, 2001; Kashino and Kudoh, 2003). Considering the somewhat lower pool size in conjunction with the lower maximum ratio of DT/DD shown in the present study, it seems that this ice algal community could dissipate only a limited level of excess light energy in a short time, and therefore, the energy dissipation system is not efficient compared to the previously reported cultivated mesophilic diatoms.

In the present study, we intended to evaluate (1) the kinetics of individual DD-cycle pigments upon the onset of irradiance as well as (2) the diel changes of individual DD-cycle pigments in natural ice algae. To assess the *in vivo* amount of such pigments individually, the interconversion of those should be considered. Because the rate of epoxidation from DT to DD is 5–10 times slower than that de-epoxidation (Olaizola *et al.*, 1994; Lohr and Wilhelm, 1999; Kashino and Kudoh, 2003), we only applied DTT as an inhibitor of the rapid de-epoxidase activity, in addition to quick filtration and freezing handling. The concentration of DTT (300 μM, in final) in the present study was selected to stop the de-epoxidation activity completely according to the experimental result of Kashino and Kudoh (2003). On the assessment of the former purpose (1), the de-epoxidase activity was expected to be blocked immediately by adding DTT after a certain period of exposure to light and the much slower epoxidase activity in the dark could also be hampered by quick filtration and freezing. Therefore, the contents of the pigments in the illumination experiment were correctly estimated. But, in evaluating the latter feature (2), it took several ten of minutes for the collection of samples and the following processing before freezing although the sample was kept in the dark during these procedures. This somewhat long process might affect the precise evaluation of the relative amount of diadinoxanthin and diatoxanthin in Fig. 3a; natural ice algae might have more diatoxanthin because diatoxanthin might be converted to

diadinoxanthin during the handling process before pigment fixation in the dark. This is one of the limitations of our analytical research on ice algae, which inhabit sea ice; *i.e.* we have to release algal cells from sea ice crystals for further analyses and this will take some time.

In spite of such technical limitation in handling the natural ice algae, the observed diel changes in the DD-cycle pool size (total amount of DD and DT) seem to reflect nearly the true amount of natural ice algae, since our procedure from sampling to pigment fixation was completed at most within one hour while *de novo* synthesis of the xanthophylls takes much longer (order of hours to days, Olaizola *et al.*, 1994; Kashino and Kudoh, 2003). Moisan *et al.* (1998) reported that the *de novo* synthesis of the xanthophylls of a polar prymnesiophycean alga, *Phaeocystis antarctica,* did not occur within an hour of the irradiance shift, but only after prolonged light irradiance shifts. Quick handling (within an hour) may make it possible to estimate the pool size of natural samples, but estimation of ratios such as DT/DD or DT/(DD+DT) as an index of DD-cycle activity could not be correctly estimated because of rapid interconversion between diadinoxanthin and diatoxanthin, yet the changes of the ratio showed clear diel changes with a peak at around noon (calculated data are not shown).

As suggested by Fujiki and Taguchi (2001), the increase of relative amount of the xanthophylls (pool size) can diminish the light energy coming into photosystems by competing with other light harvesting pigments. The diel changes of the pool size in the present study, therefore, may suggest that the ice algae regulate light energy flux into their photosystems by means of the rapid dissipation activity of DD-cycle pigments upon onset of higher irradiance, as well as filtering by those pigments. As a result, the effective yield of PS II, which was evaluated by a PAM fluorometer in this study, showed a clear reverse correlation with the DD-cycle pool size (Figs. 3a, b). The rather complex diel change of the NPQ may indicate that not only the activity of excess light dissipation into heat through DD-cycle interconversion, but also light quenching due to increased xanthophylls, reduced the energy flux into their photosystems (Fujiki and Taguchi, 2001) without inducing changes in the NPQ value; however, further confirmation is required.

The protection mechanisms from excess irradiance are, no doubt, important for the maintenance of photosystems of microalgae even in cold environments (Moisan *et al.*, 1998; Kashino *et al.*, 2002). Present results that have clearly shown the occurrence of rapid DD-cycle activity and diel changes of the pool size may suggest that the ice algae have the ability to regulate the light flux through the DD-cycle; however with the evidences of low content of the pool size and less DT/DD ratio in the present ice algae, the algae that have not experienced high irradiance very recently do not possess enough protection system(s) against excess light energy as do the ones reported in some mesophilic diatoms. This can be explained in terms of bioenergetic economy, the efficient capture of light energy and the low cost of maintenance of the DD-cycle.

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