広海域分布型ラン藻ピコプランクトン *Synechococcus* spp.の生理学的特性 －光および栄養塩環境に対する特性－

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Physiological characteristics of the picophytoplankton of cyanopytes *Synechococcus* spp., common phytoplankton in warm waters of open ocean, in relation to the light and nutrient environments in their natural habitat

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List of Abbreviations

α : affinity index

γ : rate of effective fraction of carotenoid absorption which drives PS I

λ_1 : monochromatic light which is mainly absorbed by PS I

λ_2 : monochromatic light which is mainly absorbed by PS II

A : area of one uptake site

APC : allophycocyanin

BGL : blue-green light model

Chl *a* : chlorophyll *a*

Chl a_I : number of Chl *a* molecules included in PS I complex

Chl a_{II} : number of Chl *a* molecules included in PS II complex

Chl a_T : total number of Chl *a* molecules

Chl *b* : chlorophyll *b*

d : day

DCMU : 3-(3',4'-dichlorophenyl)-1,1-dimethylurea

F(X) : fractional absorption of a given pigment component, X

h : handling time per ion

hr : hour

K_s : half saturation constant

M : mol per litter

min : minute

n : number of ion uptake sites per cell

PBP : phycobiliprotein

PBS : phycobilisome

PC : phycocyanin

PCB : phycocyanobilin

PE : phycoerythrin

PEB : phycoerythrobilin

P*i* : inorganic orthophosphate

PS I : photosystem I

PS II : photosystem II

PUB : phycourobilin

RC I : reaction center I

RC II : reaction center II

r : relative ratio of PS I and PS II

s : second

S : concentration of nutrient

SOW : Standard Ocean Water

v : mass transfer efficiency

V : rate of nutrient uptake

V_{max} : maximum velocity (of phosphate uptake)

WL : white light model

1 General introduction

Phytoplankton are major photosynthetic organisms in marine ecosystems, except in close proximity to land where benthic macrophytes and seagrasses inhabit. Total area of ocean covers about 70% of the surface of the earth. The vastness of ocean includes different types of water masses and diverse species of phytoplankton. There exist various temporal and spatial environmental gradients and variations in the ocean, such as light (ca. 200 W/m^2 to infinitively small in photosynthetically active radiation between 350 and 750 nm in wavelengths), nutrients (a few to 10^{-3} micromoles per liter in phosphate) and temperature (36 to -2 °C), whereas the range of habitat of each species of phytoplankton is limited to narrower scale than that. Phytoplankton include various phyla of species which have different evolutional background (Taylor 1980). Some groups of phytoplankton tend to appear in specific geographical areas or seasonal successional stages, and the others showed ubiquitous distribution (Guillard and Kilham 1977; Kilham and Kilham 1980; Parsons et al. 1984). Such characteristic distribution of phytoplankton species could partly be due to possible differences in the characteristics of each species for the surrounding environment and habitat segregation of different types of species. Consequently, the present study is focussing on the evaluation of some essential characteristics of phytoplankton to determine their possible distribution in the ocean environment. Marine cyanophytes, *Synechococcus* spp., have been chosen for the group to study

because they have been known to distribute in a large area of ocean.

The oceanic forms of cyanophytes were believed to be confined to filamentous *Trichodesmium* and *Richelia intracellularis* in cells of diatoms such as *Rhizosolenia* although some descriptions of possible presence of chroococcoid forms in marine waters had been reported since the beginning of this century (cf. Johnson and Sieburth 1979), whereas various species of terrestrial and freshwater forms were known (Fogg 1973, 1982). Thus, the apparent paucity of cyanophytes in the ocean has been puzzling (Fogg 1982; Glover 1985). This was because floristic studies were restricted to rather large size fractions of the phytoplankton community due to the limited resolution of ordinary microscopes (ca. > 5 μm) (Takahashi and Hori 1984). As a result, minute phytoplankton tended to be overlooked. Following the quantitative observations with fluorescent microscopy (Waterbury et al. 1979), the microscopic observations revealed the existence of chroococcoid cyanophyte in oceanic waters (Johnson and Sieburth 1979; Takahashi and Hori 1984; Joint and Pipe 1984). These findings led to further accumulation of many evidences which indicate the ubiquitous existence of minute forms including eukaryotic algae in the world ocean (Joint 1986; Stockner 1988). These minute forms have been named ultraphytoplankton (< 5-10 μm) (Sverdrup et al. 1942; Jorgensen 1966; Throndsen 1976), (< 5 μm) (Shapiro and Guillard 1986) or (< 3 μm) (Takahashi and Bienfang 1983; Bienfang and Takahashi 1983), or picophytoplankton (< 2 μm) (Sieburth et al. 1978). The analysis of size structure of the

phytoplankton biomass indicated that this small size class shared substantial portion (Li et al. 1983; Takahashi and Bienfang 1983; Takahashi and Hori 1984; Takahashi et al. 1985b; Glover et al. 1985, 1986; Furuya 1990) and chroococcoid cyanophytes often became its major constituent (Takahashi and Bienfang 1983; Takahashi and Hori 1984; Joint and Pipe 1984; Murphy and Haugen 1985; Takahashi et al. 1985b; Tsuji et al. 1986; Tsuji and Ohki 1989; Furuya 1990). According to the cellular morphology and fluorescence property of intact cells, the genus name *Synechococcus* has been assigned to these organisms.

The first isolated strain of marine *Synechococcus* collected by Guillard was a red-colored clone Syn-48 (cf. Waterbury et al. 1979). There are, at present, over forty clones listed in the Woods Hole Culture Collection (Waterbury et al. 1986). Isolated strains were also obtained by others (Perkins et al. 1981; Takahashi et al. 1985b; Ohki et al. 1987). Those culture strains are small coccoid to rod-shaped cells, 0.6-0.8 x 0.6-1.6 μm in size with peripherally located thylakoids, and reproduce by binary transversal fission in a single plain, which are characteristics of the genus *Synechococcus* (Rippka et al. 1979). All strains thus far tested have elevated growth requirements for Na^+, Cl^-, Mg^{2+} and Ca^{2+}, are obligate phototrophs, and utilize nitrate and ammonia as the sole nitrogen source (Waterbury et al. 1986). Examination of nitrogenase synthesis showed that the possibility of nitrogen fixation was unlikely (Waterbury et al. 1986). All strains isolated from oceanic water are commonly reddish-orange to purplish-red due to the predominant phycobili-

protein of phycoerythrin (PE, hereafter) (Kursar et al. 1981; Ong et al. 1984; Alberte et al. 1984; Takahashi et al. 1985b; Waterbury et al. 1986; Ohki et al. 1987). These strains have been grouped into marine *Synechococcus*-Cluster A (Waterbury and Rippka 1989).

In spite of those common features, clonal differences were presented in the followings; mobility (Waterbury et al. 1985), possession of spinae (Perkins et al. 1981), urea utilization as a sole nitrogen source (Waterbury et al. 1986), different growth responses to nitrogenous nutrients (Glibert et al. 1986), and iron requirement (Rueter and Unsworth 1991). Several variations of PE's which are different in chromophore composition have also been found in different strains (Kursar et al. 1981; Ong et al. 1984; Alberte et al. 1984). Substantial genetic diversity between and even within such phenotypically defined groups was shown by the analysis of restriction fragment length polymorphism in four loci; psbA (coding D1 peptide in reaction center II), cpe (coding peptides of phycoerythrin), rRNA (coding ribosomal RNA) and rbcL (coding large subunit of ribulose 1,5-bisphosphate carboxylase) (Douglas and Carr 1988; Wood and Townsend 1990).

Heterogeneous serogroups of marine *Synechococcus* which could be identified with cell surface antigenic properties were discriminated in natural communities (Campbell et al. 1983; Glover et al. 1986; Campbell and Carpenter 1987; Campbell and Iturriaga 1988; Olson et al. 1988). It has been proposed that such widely divergent lineage of marine *Synechococcus* may expand the adaptive capacity of *Synechococcus* as a whole group against various envi-

ronmental conditions (Campbell et al. 1983; Wood and Townsend 1990).

Recently, it has been reported that *Synechococcus* spp. distribute from surface to the bottom of euphotic zone in the Kuroshio area and the neighboring North Central Pacific Gyre (Takahashi and Hori 1984; Tsuji et al. 1986; Tsuji and Ohki 1989; Furuya 1990). Since the presence of *Synechococcus* throughout the water column have also been observed in coastal water off Japan (Takahashi et al. 1985b; Tsuji et al. 1986; Tsuji and Ohki 1989), the distribution of *Synechococcus* extends horizontally from coastal to oceanic water, and vertically from the surface to the bottom of euphotic zone. Such wide distribution of *Synechococcus* was also reported elsewhere (Waterbury et al. 1979; Johnson and Sieburth 1979; Murphy and Haugen 1985; Glover et al. 1985, 1986; Joint 1986; Tsuji and Ohki 1989). Since there exist substantial environmental gradients in the ocean as mentioned previously, these results rise a question how *Synechococcus* can establish such a wide distribution in the world ocean environment. In this study, *Synechococcus* was evaluated their physiological characteristics in conjunction with the actual environmental conditions of the ocean.

For the growth of phytoplankton, some special environmental conditions are required; most important factors are light for energy source, nutrients for materials of cellular components and temperature which determines physical conditions for various metabolic reactions. In this study, light and nutrients were particularly concerned. A large differences in light quality and

quantity are observed vertically in the water column due to the selective absorption and scattering of radiant energy by different components in water, as well as noticeable geographic differences (Jerlov 1976; Kishino et al. 1986). If the compensation depth, that is the base of euphotic zone, is approximated with 1% of the surface irradiance (Parsons et al. 1984), the depth of euphotic zone corresponds to 70 to 100 m in the Kuroshio area (cf. Kishino et al. 1986). The light energy will be sufficient for photosynthesis at the surface whereas light energy strictly limits photosynthesis near the bottom of euphotic zone (Iturriaga and Mitchell 1986; Kana and Glibert 1987). Then, some special tactics of utilization of light will be necessary to carry out the growth near the bottom of euphotic zone.

On the other hand, there exist a large variations of concentration of nutrients both in horizontally and vertically in the ocean. The nutrient availability in the sea is largely related to hydrographic variability of the water column (Parsons and Harrison 1983). In coastal and upwelling regions, there are frequent vertical upward transports of deeper waters which contain relatively high concentrations of nutrient into the euphotic zone. Then, the primary production in these regions is high (> 200 $gC/m^2/yr$). In subtropical and temperate (in summer) ocean such as the Kuroshio area, on the contrary, the euphotic zone is occupied by the surface mixed layer into which upward diffusion of nutrients across the seasonal thermocline is restricted by thermal stratification (Walsh 1976). The ambient nutrient concentrations are, therefore, extremely low and hence the primary

production is low (< 50 $gC/m^2/yr$). The autotrophs inhabiting in the surface mixed layer depend mostly on the nutrients which recycle within the mixed layer and yet whose concentrations are extremely low for prolonged periods (Eppley and Peterson 1979; Harrison 1980; Parsons et al. 1984; Takahashi et al. 1985a; Fujita 1985; Kanda et al. 1988; Furuya 1990). Then, some special utilization capability of nutrients will be essential in order to absorb nutrients at low concentrations but having possible rapid flux to maintain the active growth in the oceanic water in the Kuroshio area.

It was, thus, highly required to examine the utilization characteristics of light at low energy and confined energy distribution to extend the distribution to deeper depths in the ocean, and that of nutrients at low concentrations to extend the distribution to open ocean particularly in/near the surface for *Synechococcus*. Since the natural samples include a large variety of species and the biomass of organisms is small, larboratory cultures were used for the present study. Consequently, several culture strains of *Synechococcus* were isolated from ocean water samples and were cultured under simulated light and nutrient conditions as the oceanic environment in the laboratory. With using the laboratory cultures, a large quantity of algal sample grown under simulated ocean environments can be obtained for various kinds of analyses and rate measurements. This is almost impractical by using field samples.

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2 Photosynthetic characteristics of the picophytoplankton *Synechococcus* spp. with special reference to light environments near the bottom of euphotic zone in open ocean

2.1 Introduction

It has been reported that *Synechococcus* spp. distribute and often become dominant throughout the euphotic zone in oligotrophic open ocean such as the Kuroshio area (Takahashi and Hori 1984; Tsuji et al. 1986; Tsuji and Ohki 1989; Furuya 1990). Since light energy attenuates exponentially in water column, the extended distribution of *Synechococcus* toward the base of euphotic zone implicates that they have to overcome a great decrease in light energy from surface to the base of euphotic zone. Since photosynthesis is strictly limited by light near the base of euphotic zone (see Chap. 1), the light-harvesting is of great importance in maintaining active autotrophic growth there. Spectral distribution of the light energy is also changed with attenuation and is restricted to wavelengths in the blue-green region at the lower part of euphotic zone (ca. > 40 m) in open ocean (Jerlov 1976; Kishino et al. 1986). Such chromatic change of light may affect the efficiency of photosynthesis because light absorption of photosynthetic pigments is not consistent throughout the photosynthetically active radiation (PAR) but changes with wavelengths. Furthermore, a great diversity of spectral property of photosynthetic pigments and their relative composition have been recognized widely among different phytoplankton species (e.g. Glazer 1983).

The pigment system of *Synechococcus* consists of phycobiliproteins (PBP) such as phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC)) in addition to carotenoids and chlorophyll *a* (Chl *a*). A large fraction of Chl *a* belongs to photosystem I (PS I) and a small fraction of Chl *a* belongs to photosystem II (PS II) (Mimuro and Fujita 1977; Fujita and Murakami 1987). Both fractions are integrated in thylakoid membranes. Phycobiliproteins form a supra-molecular structure which is attached to the surface of thylakoids and is called phycobilisome (PBS) (Gantt 1975). The PBS transfers light energy principally to PS II with some fraction of energy transferred to PS I via spillover processes, whereas the most of light energy absorbed by Chl *a* is transferred to PS I (Wang et al. 1977; Ley and Butler 1977; Butler 1978). Carotenoids have two major functional roles, light-harvesting and protection from harmful effects of high irradiance (Jeffrey 1980; Larkum and Barrett 1983). Carotenoids locate in thylakoid membranes and cytoplasmic membranes (Omata and Murata 1983). The light energy absorbed by PS I drives reaction center I (RC I) to reduce $NADP^+$ whereas that absorbed by PS II drives reaction center II (RCII) to reduce plastoquinone, which then supplies electrons to RC I. Water is oxidized into H^+ and O_2 in RC II to donate electrons. As a consequence of series of these reactions, $NADPH_2$ and ATP are produced. The quantum yield of photosynthesis is maximized when the equal amount of absorbed light energy is distributed to the two photosystems.

Pigment composition determined with a microscopic spectrofluorometer indicates that *Synechococcus* contained PE exclusively

as a main pigment among phycobiliproteins for light-harvesting for photosynthesis (Tsuji et al. 1986; Tsuji and Ohki 1989). PE specifically absorbs light in the green wavelength region, although the predominant light in the deeper depths of oligotrophic open ocean is blue-green as mentioned above. Then, *Synechococcus* appears not to be able to efficiently absorb the light energy there. Further, the blue-green light may facilitate the absorption by Chl *a* and may cause a preferential excitation of PS I. It results in the decrease in quantum yield of photosynthesis as has been known as "red drop" phenomenon (Emerson and Rabinowitch 1959), and the light environment at the lower part of euphotic zone seems not to be favorable to these organisms. Therefore, it seems that the extended distribution of *Synechococcus* toward the base of euphotic zone appears to be rather curious.

To know the photosynthesis of *Synechococcus* particularly under such a light environment as at the lower part of euphotic zone in the open ocean blue water, pigmentation and nature of photosynthesis in the isolated strains of *Synechococcus* spp. were examined, using the light which was simulated to the spectral property at low intensities.

2.2 Material and methods

2.2.1 Isolation and culture of picophytoplankton strains

A subsurface water sample was obtained during a research cruise of R/V *Tansei-maru* (KT 87-18) in the Kuroshio area, north east of Torishima Island (30° 49' North, 140° 59' East) on Novem-

ber 21, 1987. The water sample was collected by a Niskin bottle from 70 m depth and transferred into plastic bottles which were previously cleaned by concentrated HCl and distilled water. All processes of water-sampling were made aseptically as far as possible. In the laboratory, the water was enriched with nutrients of the *f* medium of Guillard and Ryther (1962) at 1/50 strength. Ten milliliters of enriched seawater was placed in sterilized test tubes and incubated at 25 °C under a weak natural light (max. 2 W/m^2). The colony initially formed in each subculture was isolated and transferred to a fresh enriched seawater successively at very low cell concentrations. Some of strains were further isolated from a single colony developed on 0.8 % agar plate. Cells isolated were maintained in the *f* medium at 1/7 strength.

Two *Synechococcus* strains NIBB1059 and NIBB1071, isolated from surface waters of the Gulf Stream and of the Kuroshio area, respectively, by Dr. K. Ohki of the National Institute for Basic Biology, were also used. The strains grown in an artificial seawater medium of the ASP_7 medium (Pintner and Provasoli 1958) were used in the present experiments because the experiments were performed before establishment of culture systems using the modified 'aquil' medium as described in Chap. 3. Cells were grown in an oblong flat bottle (light path was 25 or 55 mm) at 23 °C in the ASP_7 medium with slight modifications; nitrate, phosphate and vitamin B_{12} were increased 10 times of the original recipe, respectively. Both air and illumination were supplied continuously. Light source was prepared as described later

(Chap. 2.2.5). Light intensity was measured with a 2π pyranosensor (Crump, No. 580). Cells were grown at least for the times of 3 generations during the exponential growth phase under given conditions, and final cell density was kept below 1.3×10^8 cells/ml (absorbance of 0.1 at 750 nm at 1 cm of light path).

Freshwater cyanophyte strain of *Synechocystis* ATCC27170 was also used and grown in the MDM medium (Watanabe 1960) at 25 °C under green light (480-580 nm, obtained by a green plastic filter, Mitsubishi-Rayon, Sinkolite A, No. 363) (2.0 W/m²), which induces synthesis of PE in this strain (cf. Ohki et al. 1987).

2.2.2. Measurements of absorption spectra of intact cells

Cells at the late exponential growth phase were suspended in fresh medium and the light absorption spectra were determined by a Hitachi 340 spectrophotometer in a split beam mode. All measurements were done at room temperature (ca. 25 °C).

2.2.3 Determinations of the reaction centers I and II, and phycobiliproteins

Abundance of RC II was determined for intact cells from a flash yield of O_2 evolution as described previously (Kawamura et al. 1979; Fujita et al. 1985) with a slight modification. As the background illumination, far red light with wavelengths longer than 680 nm was given to the sample at 10 W/M².

Abundance of RC I and phycobiliproteins was determined spectrophotometrically. Cells were suspended in 100 mM phosphate buffer(pH 6.2) and disrupted using a French pressure cell (1,500

Kg/cm^2). After a brief sonication (5 s treatment with 10 s interval, 2 cycles), unbroken cells were removed by low speed centrifugation (1,600 x *g*, 15 min). Clear supernatants were obtained after ultracentrifugation (180,000 x *g*, 90 min) and membranes were also obtained as precipitates. The membranes were further washed three times with 50 mM Tricine-NaOH buffer (pH 7.5). The content of RC I was determined from P700 levels as described by Fujita and Murakami (1987). The abundance of P700 was measured from the absorption difference at 700 nm between ascorbate-reduced and ferricyanide-oxidized samples in a two-wavelength mode (reference wavelength, 730 nm) by a Hitachi 557 spectrophotometer. Calculation of P700 concentration was made by the absorption coefficient of Hiyama and Ke (1972).

Concentrations of PE, PC and APC were estimated by the method described by Ohki et al. (1987). APC content in strains NIBB1059 and NIBB1071, and PC content in the strain NIBB1059 were determined from the absorption spectra of clear supernatant using the absorption coefficients reported by Hattori and Fujita (1959).

PE in *Synechococcus* NIBB1059 and NIBB1071 is unstable after extraction and easily unfolded even at low temperatures. Levels of PE in these strains were determined from PEB and PUB, chromophores of PE. PEB, PCB and PUB contents were determined from the absorption spectra in 8 M urea (pH 3.0) using the absorption coefficients reported by Glazer and Hixson (1975) for PEB and PCB, and that reported by Klotz and Glazer (1985) for PUB. PE content in NIBB1059 was calculated under the assumption that each

PE monomer contains 6 bilins (Glazer and Hixson 1975). PE and PC contents in the strain NIBB1071 were calculated under the assumption that chromophore compositions of PE and PC are both the same as those found by Ong and Glazer (1987) in the marine *Synechococcus* strain WH8103.

Chl *a* contents were determined spectrophotometrically with 80% acetone extracts for membrane samples and 100% methanol extracts for intact cells using the absorption coefficient of Mackinney (1941). Absorption spectra were determined by a Hitachi 340 spectrophotometer.

2.2.4 Measurements of photosynthetic activity

Photosynthetic O_2 evolution was measured using a Clark-type O_2 electrode (YSI, 4004) at 23 °C. A cross-illumination system was set in order to illuminate the sample simultaneously with two types of light. In addition to the light model described below (Chap. 2.2.5), chromatic light was prepared with an interference filter (Nihon-Shinku-Kogaku, S-type, 550 nm or 560 nm) and a sharp cut-off filter (Toshiba, R-69). Monochromatic lights passed through a monochrometer (Ritu-Oyo-Kogaku, MC20-L) were also used for measuring the quantum yield at defined wavelengths. The light energy was determined with a radiometer (YSI, Model 65A). The spectral distribution of light energy was determined with a spectro-radiometer (Optical Science, Model OSMO-504C). The light energy at each wavelength was converted into photon flux density using the obtained spectrum. Absorption of cell suspension under each experimental light condition was determined

with a thin layer of cell suspension placed on an opal glass with respective experimental lights. Absorbed quanta were estimated from the photon flux density and the absorption by cells.

2.2.5 Construction of light models

Model of blue-green light in the deep euphotic zone (BGL) was constructed to simulate the spectral distribution of light energy at the bottom of euphotic zone in open ocean. Two types of simulation were made. One was prepared for light source of experimental cultures. The light source for experimental cultures was required to illuminate a large area of the target homogeneously due to the large surface size of vessels used for the culture. This requirement can be satisfied by using the light source which gives a scattered emission of large area. Reflector-lamps of flat-type have often been used for this purpose. However, it was found not to be adequate to the light source for the present blue-green light because of its low color-temperature. Fluorescent lamp is another type of light source which fits for the requirement. After examination of emission spectra of various fluorescent lamps, a combination of blue fluorescent tube (Toshiba, FL20B) and blue-green colored film (Nakagawa-Chemical Inc., Cutting-Sheet, 431C) was found to produce a light similar to that penetrating to the lower part of euphotic zone in the sea (Fig. 1a). However, there occur two strong line emissions (Hg lines) in this light model, and the line in green region is absorbed effectively by PE, main light-harvesting antenna of *Synechococcus* (cf. Fig. 3). Further, the

Fig. 1 Spectral energy distribution of the model light simulated the light quality at the bottom of euphotic zone in open ocean (BGL) and that of white light (WL). a: light source for cultures; b: light source for photosynthesis measurements. Dashed lines indicate spectral distribution of the light at the lower part of euphotic zone (66 m) in the Kuroshio area (data were replotted from Kishino et al. 1986).

luminous flux of fluorescence tube is weak as the light source for photosynthesis experiments. Thus, this light model was considered not to be suitable to the study of photosynthesis determination. In order to measure the photosynthesis under various intensities of simulated light, another combination of a set of Corning-filters (4-72 and two pieces of 4-96) and Xenon lamp light was developed (Fig. 1b). White light (WL) was also obtained with tungusten lamp light (Toshiba, Chuko-ranpu) passed through a 5 mm thick water filter containing 2% $CuSO_4$ (Figs. 1a, 1b).

2.2.6 Determinations of fractional light absorption by each pigment

Total light absorption of intact cells was divided into fractional absorptions of PBS, and Chl *a* and carotenoids. The intact PBS fraction was isolated according to Gantt et al. (1979) and Ohki and Fujita (1987). One to 2 g (wet weight) cells were suspended in 7 ml of 9 M phosphate buffer (pH 6.8) and broken with a French pressure cell at 700 kg/cm^2. Triton X-100 was added to the broken cells to a final concentration of 1%, and the mixture was incubated for 20 min with gentle stirring. Crude PBS was obtained from the supernatant after centrifugation for 30 min at 25,000 x *g*. The crude sample was layered on a buffered step-wise-gradient with sucrose concentrations, 2.0, 1.0, 0.5 and 0.25 M. After centrifugation for 3 hours in an angle head rotor at 136,000 x *g*, PBS was obtained from the interface between 1.0 and 2.0 M sucrose layers. All procedures were conducted at room

temperature.

Membrane fraction was isolated from intact cells by the following method. Cells suspended in 50 mM Tricine buffer (pH 7.5) containing 10 mM NaCl, 2 mM $MgCl_2$, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride were disrupted using a French pressure cell at 1,500 kg/cm^2. After removal of unbroken cells, membranes were obtained as the precipitates of ultracentrifugation (180,000 x *g*, 90 min). All treatments were conducted under ice-water temperature. Light absorption spectra of PBS and membranes were measured by a Hitachi 340 spectrophotometer with an opal glass method. The spectrum of total cell absorption was then reconstructed by adding absorption spectra of PBS and membranes. The difference between the spectrum of original intact cell and reconstructed one was 5 and 6 % for the strains NIBB1059 and NIBB1071, respectively.

Chl *a* was purified with the thin layer chromatography using light-petroleum containing 1% n-propanol, after extraction from the isolated membrane fraction with 100% ethanol at -20 °C. Original spectrum of Chl *a* was determined with purified Chl *a* suspended in 0.2% Triton X-100. To simulate the spectrum of Chl *a* in the thylakoid membranes, original spectrum of Chl *a* was corrected to adjust the γ and the α peaks of Chl *a* to the corresponding peaks of the isolated membranes, respectively (from 436 to 440 nm, and from 668 to 680 nm). Each peak position was determined from second derivative of respective spectrum (Savitzky and Golay 1964). Spectrum of carotenoids was determined by subtraction of corrected spectrum of Chl *a* from that of

membrane fraction which contains the equal amount of Chl *a*.

Relative absorption of each fraction under a given light regime was obtained by multiplying % light absorption of each pigment fraction and the photon flux density at an interval of 1 nm.

2.3 Results

2.3.1 Light absorption characteristics of picophytoplankton isolated from subsurface and surface waters

Trials to isolate *Synechococcus* strains from natural habitat were made for a water sample collected from the subsurface layer in the Kuroshio area, and 12 picophytoplankton strains were obtained from a water sample enriched with nutrients at low concentrations.

Nine isolates were pink-colored algae, which are unicellular and 1-2 μm in their cell diameter. They showed characteristics that (1)cell division occurs at one plane, (2)cells do not form sheath, and (3)predominant content of PE in their light-harvesting system for photosynthesis as described below. The above characteristics strongly indicate that these strains are cyanophyte of *Synechococcus* (Rippka et al. 1979; Waterbury et al. 1986; Alberte et al. 1984). Absorption spectra in three different strains were compared with other *Synechococcus* isolated from surface water (Figs. 2a, 2b, 2c, 2d, 2e). A common feature among those strains is the prominent peak at around 546-569 nm, indicating that they all contain PE. With or without having an addi-

Fig. 2 Absorption spectra of PE-containing cyanophytes.

a: *Synechococcus* Z-12; b: *Synechococcus* WES; c: *Synechococcus* Z-11; d: *Synechococcus* NIBB1059; e: *Synechococcus* NIBB1071; f: *Syenchocystis* ATCC27170. Strains a, b, c were isolated from subsurface of the Kuroshio area and were grown in the *f* medium at 1/7 nutritional strength at ca. 25 °C under natural light at max. 2.0 W/m². Strains d and e were isolated by Dr. K. Ohki from surface of the Kuroshio and the Gulf stream, respectively and were grown in the ASP_7 medium at 23 °C under BGL at 2.0 W/m². Strain f was grown in the MDM medium at 25 °C under green light at 2.0 W/m². Dashed line: same as Fig. 1.

tional peak at 500 nm in the spectra, *Synechococcus* can be classified into two types; those which have obvious peak at 500 nm such as a, b, c and e in Fig. 2, and that which has no such obvious peak as d in Fig. 2. The extinction of the peak at 500 nm relative to that at around 546 nm is further somewhat variable among the strains examined. It suggests that they have different pigment proportions in their chromophore composition, that is the proportion of PEB to PUB, and/or different in their intramolecular environments at the sites for chromophores.

The two strains, NIBB1059 and NIBB1071 were chosen as representatives of the two types of *Synechococcus* with and without the additional peak at 500 nm and were further investigated the characterisrtics of the spectral property of PBS, the light-harvesting pigment complex containing PBP. As expected from the spectral property of the intact cells, the spectral property of their isolated PBS were also significantly different (Fig. 3). The maximum absorption peak occurred at 568 and 546 nm for the strains NIBB1059 and NIBB1071, respectively. A weak bump at 500 nm was observed only in the strain NIBB1071. When the extracted phycobiliproteins were denatured in acidic urea, a clear peak was observed at 500 nm in the strain NIBB1071, whereas no such peak was found in the strain NIBB1059 (Fig. 4). This indicates that PE of the strain NIBB1071 contained PUB as an additional chromophore besides PEB, whereas the strain NIBB1059 contained only PEB. The content of PUB relative to that of PEB was 0.1 in the strain NIBB1071.

Fig. 2f also shows the absorption spectrum of intact cells

Fig. 3 Absorption spectra of phycobilisomes (PBS) isolated from *Synechococcus* spp. Samples were suspended in 0.9 M potassium phosphate buffer (pH 6.8). a: strain NIBB1059; b: strain NIBB1071. Experimental details, see the text.

Fig. 4 Absorption spectra of PBS of *Synechococcus* spp. denatured with 8 M urea (pH 3.0). a: strain NIBB1059; b: strain NIBB1071. Experimental details, see the text.

of *Synechocystis* ATCC27170. Absorption peaks in green wavelength region located at 569 and 622 nm, which corresponded to the absorption of PE and PC, respectively. The absorption-extinction of PE relative to that of Chl *a* (α band, at 683 nm) was far less than those in the marine *Synechococcus* strains: the extinction of PE relative to that of Chl *a* in *Synechococcus* was about 2 and about two times as high as that of *Synechocystis* ATCC27170 (Fig. 2). Abundance of PE in *Synechococcus* must be much greater than that of *Synechocystis* ATCC27170. Since these high values were observed in all *Synechococcus* isolated from the surface and subsurface water, the high content of PE seems to be a common feature of the marine minute *Synechococcus*.

There were three other strains besides *Synechococcus* which were green-colored coccoid species. Their sizes varied from 2 to 4 um in length. A high absorption peak at 476 nm was observed together with a peak at 654 nm as shown the most pronounced case in Fig. 5. It indicates that they contained Chl *b* and were not cyanophyte. Since a cup-shaped chloroplast was observed with fluorescent microscopy, they were likely to be eukaryotic algae. The low ratio of Chl *a/b* such as 0.3 was measured for this strain. It indicates that this strain contains high amount of Chl *b*. High absorption at 476 and 654 nm is common feature in the three strains though the other 2 strains contain less amount of Chl *b* when compared with the former strain. In addition to the characteristics mentioned above, one of the latter 2 strains is motile and is likely to be *Micromonas* sp.

Fig. 5 Absorption spectra of intact cells of eukaryotic picophytoplankton having the Chl *a* and *b* pigment system. It was isolated from the subsurface water in the Kuroshio area and was grown in the *f* medium at 1/7 nutritional strength at ca. 25 °C under natural light at max. 2.0 W/m². The arrow indicates the absorption peak due to Chl *b*.

It is apparent that the γ peak of Chl *b* locates in the blue-green wavelength region in which the maximum energy distribution occurs in the light regime at the depth of euphotic zone in the Kuroshio area (Kishino et al. 1986), whereas the main absorption peak of PE in both strains does not. The blue-green wavelength region is located between the absorption maxima of PE and Chl *a* in *Synechococcus* (cf. Fig. 2).

2.3.2 Growth of *Synechococcus* NIBB1059 and NIBB1071 under low intensities of BGL

The strains NIBB1059 and NIBB1071 were cultured under low light intensities of BGL in order to assess their growth capabilities under light energy of such spectral distributions. These light intensities correspond to less than 3% of sunny noon light intensities at the sea surface, and are as low as those at the base of euphotic zone. Growth constants were calculated from the cell-increase during the exponential growth phase and were plotted against the light intensity (Fig. 6). Growth rate of both strains was strictly limited by light at these intensities, although these light conditions were enough to support their autotrophic growth. The growth constant and the slope of the strain NIBB1071 were both slightly higher than those of the strain NIBB1059. This suggests that PUB of the strain NIBB1071 might act as a more effective chromophore to capture such a blue-green light. High growth constants at lower light intensities were noticed in the both strains, which could be partly due to

Fig. 6 Growth constants of *Synechococcus* NIBB1059 (a) and NIBB1071 (b) under various light intensities of BGL. Experimental details, see the text.

possible efficient enhancement of growth at such low light intensities. However, there could occur a rapid decrease in the growth constant at further low light intensities.

2.3.3 Effects of light regime on the composition of photosystems and phycobiliproteins in *Synechococcus* spp.

Pigment composition could change in stoichiometry among the components involved in electron transport in the photosystem, photosystem I and II complexes and their light-harvesting antenna including cytochrome b_6/f complex, when cells of cyanophytes grow under a light regime which causes imbalance of energy distribution between the two photosystems; regulational change of the composition of the two photosystems can compensate the imbalance of light harvesting capability (Fujita et al. 1985, 1987b, 1988; Fujita and Murakami 1987; Murakami and Fujita 1988). If BGL causes imbalance of energy distribution between the two photosystems, composition of the two photosystems in cells grown under BGL would be different from that under WL having a broad energy spectral distribution in photosynthesis-effective wavelengths and being distributed to both photosystems (cf. Fig. 1). To evaluate this possibility, pigment composition of cells grown under either BGL or WL were compared. Since pigment composition is affected by the intensity (Kawamura et al. 1979) as well as by the light-quality due to changes in photosystem stoichiometry (Fujita and Murakami 1987), growth irradiance of both BGL and WL was set at the intensity giving equal growth constants under both light conditions (0.3 day^{-1}). Examinations of the pigment composition

Table 1 Stoichiometric composition of RC I, RC II and PBP in cells grown under different light conditions.

Strain	Growth conditions (W/m²)	Chl a abundance per RC I (mols/mol)	Chl a abundance per RC II (mols/mol)	RC I abundance relative to PS II (mols/mol)	Abundance relative to PS II (monomer mols/mol) PE	PC	APC
Synechococcus NIBB1059	BGL (2.4)	125	349	2.8	75	16	3
	WL (2.3)	122	358	2.9	77	16	4
Synechococcus NIBB1071	BGL (2.0)	125	502	4.0	69	37	6
	WL (2.3)	124	515	4.2	71	22	6

were made for the cells which grew at least for 3 generation times under respective conditions and fully adapted to the light regime.

Table 1 shows relative compositions of RC I and RC II, as the representative of respective PS I and PS II, and of PBP in cells grown under BGL and WL. Values of RC I and RC II are expressed as relative contents based on the number of Chl *a* molecules, and those of PBP are expressed as those based on the number of RC II molecules, which are the direct acceptor of excitation energy captured by PBS. Stoichiometry between the two photosystems was not affected by the growth light regime. Abundance of RC I relative to that of RC II under BGL and WL conditions were 3 and 4 in the strains NIBB1059 and NIBB1071, respectively. Constant stoichiometry was also observed in the stoichiometric relationship between PBP and RC II. Difference in PC abundance in the strain NIBB1071 appears to be significant. Since the abundance of PC was calculated based on that of PCB which is as low as one-tenth of PEB, observed variation seems to be a result of errors in measurements for such a low level of PCB. Molar ratio of APC to PS II obtained for the strain NIBB1059 are smaller than those reported by Ohki et al. (1987), which suggests that a slight underestimation occurred in the present case. However, APC level in the strain NIBB1071 is the same as that previously determined. Since APC forms the core of PBS, these results indicate that the number of PBS relative to RC II and the size of PBS were not affected by the light regime for cell growth. In conclusion, it has been obvious that any regula-

tory changes in pigment composition is not caused by BGL.

Table 1 also indicates extremely high contents of PE in PBS in both strains. The ratios of PE to APC, which is contained in PBS at constant amount, were 12 to 13 for the strains NIBB1059 and NIBB1071, respectively, when APC is assumed to be contained at 6 mols per mol PS II in both strains. These values are 4 to 5 times greater than those in freshwater strains of cyanophytes (cf. Ohki et al. 1987; Ikeya et al. 1991).

2.3.4 Photosynthetic oxygen-evolution under a light regime simulated to that at the deep euphotic zone

To know the photosynthesis of *Synechococcus* under the light regime at the lower part of euphotic zone, rates of photosynthetic oxygen-evolution was firstly measured. Light-photosynthesis curve was determined for the cells grown under BGL using BGL and WL as the actinic light. Photosynthetic rates per incident quantum of BGL and WL were compared for the initial slopes of light-photosynthesis curves, and were expressed as relative values (Table 2). Results indicate that photosynthetic rate was somewhat lower under BGL than under WL in both strains. The values were as high as 67% and 74% for the strains NIBB1059 and NIBB1071, respectively. The latter showed a slightly higher rate than that of the former.

Since photosynthetic O_2 evolution is an overall reaction driven by co-operative actions of PS I and PS II, the overall efficiency is affected by light energy distribution between the two photosystems, and is the highest under balanced energy dis-

Table 2 Relative rates of oxygen evolution per incident quantum under different light conditions.

Strain	Light regime for growth[a]	Rate[b] of O_2 evolution under BGL
Synechococcus NIBB1059	BGL (2.4)	67
Synechococcus NIBB1071	BGL (2.0)	74

[a] Figures in parentheses are intensities of respective lights (W/m^2).

[b] Values are relative to those under WL (%).

tribution (Wang and Myers 1976). To know the photosynthetic efficiency for the absorbed energy under BGL and WL, photosynthetic rate per absorbed quantum was calculated from the above data. If the light energy absorbed by the pigment system is evenly distributed between the two photosystems under BGL, the efficiency under BGL should be as high as that under WL. However, when data were expressed as the efficiency, the value under BGL was low such as 40% of that under WL in both strains (Table 3). Low efficiency under BGL suggests a possibility that the energy supply to PS II is limited far more than supply to PS I under BGL, and that there is some imbalance between the actions of PS I and PS II.

2.3.5 Excitation-energy distribution between PS I and PS II under BGL

Imbalance between actions of the two photosystems may be caused by a biased distribution of excitation-energy between the two photosystems, with excessive excitation of PS I. To examine the excitation-energy distribution between PS I and PS II under BGL, the Emerson's enhancement effect was evaluated. The quantum yield of monochromatic light which is absorbed by either Chl *a* of long wavelength form (λ_1, > 690 nm) or PBS (λ_2, 550 nm) was measured with and without background illumination. The former exclusively excites PS I, and the latter excites mainly PS II. The reason why the Emerson's enhancement is adopted to determine the nature of BGL is the following. If BGL excites preferentially PS I, addition of BGL does not enhance photosynthetic quantum

Table 3 Photosynthetic efficiencies under different light conditions.

Strain	Light regime for growth[a]	Photosynthetic efficiency[b] under BGL
Synechococcus NIBB1059	BGL (2.4)	39
Synechococcus NIBB1071	BGL (2.0)	40

[a] Figures in parentheses are intensities of respective lights (W/m^2).

[b] Values are relative to those under WL (%).

yield of λ_1 light because the excitation of PS II is limited. However, the addition of BGL must enhance the yield of λ_2 light in this case. In the reverse case, BGL must enhance the yield of λ_1 light. Occurrence of enhancement of either yield of λ_1 or λ_2 light can determine the nature of BGL in the sense of the energy distribution to the two photosystems.

Table 4 shows results obtained for the two strains of *Synechococcus*, which have been grown under BGL. When λ_1 light was illuminated as background light, it enhanced the quantum yield of λ_2 light by about 1.5 times for both strains of NIBB1059 and NIBB1071. When λ_2 was illuminated as background light, it enhanced the quantum yield of λ_1 light more than 10 times for both strains. This indicates that a typical "red drop" occurs in both strains (Emerson and Ravinowitch 1959). If BGL excite PS I more than PS II, addition of BGL should cause the enhancement of the yield of λ_2 light upto 1.5 times. However, when BGL was illuminated as background light, the enhancement was far smaller in the strain NIBB1071. In the strain NIBB1059, a significant enhancement was observed. Results indicate that excitation-energy distribution was biased to PS I in some extent under BGL in this strain.

In conclusion, the decline of photosynthetic quantum yield under BGL in the strain NIBB1059 is at least partly due to the imbalance of excitation-energy distribution. However, in the case of the strain NIBB1071, such an imbalance is insignificant.

2.3.6 Fractional absorption of pigment components

Table 4 Enhancement effects of BGL, λ_1 (> 690 nm) and λ_2 (560 nm) background lights on oxygen evolution of under λ_1 and λ_2 lights.

Strain	Probe light	Enhancement of photosynthesis under		
		BGL light	λ_1 light	λ_2 light
Synechococcus NIBB1059[a]	λ_1 light	1.02	-	> 10
	λ_2 light	1.32	1.62	-
Synechococcus NIBB1071[b]	λ_1 light	1.43	-	> 10
	λ_2 light	1.09	1.54	-

[a] Cells were grown under BGL of 2.4 W/m².

[b] Cells were grown under BGL of 2.0 W/m².

As an approach of another aspect to examine the distribution of absorbed light-energy under BGL, contribution of respective pigments to the total light-absorption was determined for *Synechococcus* strains grown under BGL. The *in vivo* absorption spectra of intact cells were resolved into spectral components of three pigment groups, PBS, Chl *a* and carotenoids. The fractional absorption spectra of these pigment groups were shown in Fig 7. Light-absorption by three types of pigments was associated in the blue-green region. It is notable that a significant fraction of BGL was absorbed by PBS although the highest absorption of PBS located in the green region. Light energy of BGL absorbed by PBS was as high as 44% in the strain NIBB1071, and 36% of total absorption was made by PBS even in the strain NIBB1059, in which PE contains only PEB but not PUB having the absorption maximum at shorter wavelengths (Table 5). On the contrary, absorption of Chl *a* was small in BGL wavelength region. The fractional absorption of Chl *a* was a half and one-third of that of PBS in the strains NIBB1059 and NIBB1071, respectively. Location of the absorption band of carotenoids coincided well with the energy distribution of BGL. Nearly 50% of BGL was absorbed by carotenoids in both strains. Results indicate that a significant fraction of light energy is absorbed by PBS, and that the fractional absorption of Chl *a* is not so great as to cuause preferential excitation of PS I in both strains as far as concerned with absorption by PBS and Chl *a*.

Since the energy absorbed by carotenoids are not necessarily utilized by photosynthesis (Jeffrey 1980; Larkum and Barrett

Fig. 7 Spectral fractional absorption by PBS, Chl *a* and Carotenoids. a: *Synechococcus* NIBB1059; b: *Synechococcus* NIBB1071. Dotted lines indicate spectral energy distribution of BGL used for determination of photosynthesis.

Table 5 Relative absorption of each pigment component in *Synechococcus* spp.[a] under BGL and WL.

Strain	Light regime	Fraction (%) absorbed by PBS	Chl *a*	Carotenoids
Synechococcus	BGL	36.2	16.3	47.5
NIBB1059	WL	61.8	24.4	13.7
Synechococcus	BGL	43.6	14.6	41.7
NIBB1071	WL	58.8	26.1	15.0

[a] Cells have been grown under BGL of 2.4 and 2.0 W/m² for the strains NIBB1059 and NIBB1071, respectively.

1983), it is essential to determine how much energy absorbed by carotenoids is utilized by photosynthesis. As one of ways to evaluate this, the quantum yield of oxygen evolution was measured at two wavelengths. One was made for the light at 480 nm where light is absorbed mostly by carotenoids and PE, and the others at 560 and 545 nm in the strains NIBB1059 and NIBB1071, respectively, where there is the absorption maximum of PE and therefore most of light energy is absorbed by PE. Table 6 shows fractional absorption of each pigment component at the two wavelengths and relative quantum yield for oxygen evolution at respective wavelengths. Fractional absorption of carotenoids was about 1% at the wavelength for the absorption maximum of PE. However, it was as high as 68 and 56% at 480 nm in the strains NIBB1059 and NIBB1071, respectively. On the contrary, the quantum yield at 480 nm decreased to 35% and 41% of that at the wavelength for absorption maximum of PE in the strains NIBB1059 and NIBB1071, respectively. A correlation between the decline of quantum yield and the increase of fractional absorption of carotenoids indicates that most of energy absorbed by carotenoids is not utilized by the photosynthetic reaction measured as O_2 evolution, i.e., the reaction driven by PS II and PS I.

Fractional absorption of carotenoids under BGL was as high as 3.5 and 2.8 times of that under WL in the strains NIBB1059 and NIBB1071, respectively (Table 5). If the energy absorbed by carotenoids was ineffective, the energy available for photosynthesis under BGL should be decreased to 61% and 69% of that under WL in the strains NIBB1059 and NIBB1071, respectively. Although

Table 6 Relative absorption by each pigment component and quantum yield of photosynthesis in *Synechococcus* spp.[a] at wavelength of 480, and 560 or 545 nm.

Strain	Wavelength of light (nm)	Fraction (%) absorbed by			Quantum yield[b]
		PBS	Chl *a*	Carotenoids	
Synechococcus NIBB1059	480	28.0	3.7	68.3	35
	560	96.6	2.4	1.1	100
Synechococcus NIBB1071	480	41.1	3.0	55.9	41
	545	96.2	2.5	1.2	100

[a] Cells have been grown under BGL at 2.4 and 2.0 W/m^2 for the strain NIBB1059 and NIBB1071, respectively.

[b] Values are relative to those at 560 or 545 nm.

these figures do not completely explain the low value of quantum yield of BGL, the main cause of the low quantum yield of BGL for the two strains of *Synechococcus* must be the dissipation of carotenoid-absorbed energy not coupling with O_2 evolving photosynthetic reaction. The reason for that the quantum yield is lower than that predicted may be due to 1)most of carotenoid-absorbed energy is utilized by the reaction driven by PS I, 2)errors in estimation of quantum yields or fractional absorption of pigments, 3)loss in the energy transfer from PE to APC, and 4)loss in the energy transfer from PBS to antenna Chl *a*. However, a rather high value (0.90 to 0.95) was actually reported for the efficiency of energy transfer from PBS to Chl *a* (Ley and Butler 1976), and from PE to APC (Grabowski and Gantt 1978), and thus, possibilities 3) and 4) are feasible. Since fractional absorption of Chl *a* and carotenoids were determined by the absorption spectra of Chl *a* in aqueous phase with first-order approximation, some error may occur in the estimation of fractional absorption of Chl *a* and carotenoids. Feasibility of the possibility 1) will be discussed later (Chap. 2.4).

A further estimation of the energy distribution under BGL was made by calculation of absorption fractions by antenna pigments acting for each photosystem under the assumption that the sizes of PS I Chl *a* (Chl a_I) and PS II Chl *a* (Chl a_{II}) are 120 and 60, respectively (Fujita and Murakami 1987). Since molar ratios between the two photosystems are determinable as shown in Table 1, total number of Chl *a* (Chl a_T) can be resolved into Chl a_I and Chl a_{II} as follows;

$$\text{Chl } a_{I} = \frac{120 \times r}{120 \times r + 60} \times \text{Chl } a_{T}$$

$$\text{Chl } a_{II} = \frac{60}{120 \times r + 60} \times \text{Chl } a_{T}$$

where r is the relative ratio of PS I to PS II. Since the relationship between carotenoids and the two photosystems has not been clear yet, the energy absorbed by carotenoids is not considered in this calculation. Since almost all energy absorbed by PBS is primarily transferred to PS II (Wang et al. 1977; Ley and Butler 1977; Butler 1978), the energy absorbed by PS I and that by PS II can be calculated as follows;

$$F(\text{PS I}) = F(\text{Chl } a_{I})$$

$$F(\text{PS II}) = F(\text{Chl } a_{II}) + F(\text{PBS})$$

in which F(X) is a fractional absorption of a given pigment component, X. Respective absorption fractions by each pigment for the two photosystems under BGL and WL can be calculated using these equations (Table 7). Results indicate that the absorption of BGL by the pigments of PS II is greater than that by PS I pigments in both strains of NIBB1059 and NIBB1071. However, the energy distribution was found to be somewhat biased to PS I under BGL in the strain NIBB1059 in the determination using the Emerson's enhancement effect (cf. Table 4). Difference may come

Table 7 Relative light absorption of BGL and WL by the pigments of each photosystem in *Synechococcus* spp.[a)]

Strain	Light regime	Fraction (%) absorbed by PS I (Chl a_I)	PS II (PBS + Chl a_{II})	PS II/PS I
Synechococcus NIBB1059	BGL	14.0	38.5	2.7
	WL	20.9	65.3	3.1
Synechococcus NIBB1071	BGL	13.0	45.3	3.5
	WL	23.2	61.7	2.7

[a)] Cells have been grown under BGL at 2.4 and 2.0 W/m² for the strain NIBB1059 and NIBB1071, respectively.

from exclusion of carotenoid-absorption in the present calculation. Or, numbers of Chl *a* in PS I and PS II in the two *Synechococcus* strains may be different from those in *Synechococcus* PCC6714 used for the calculation. If the former is the case, at least a half of the light-energy absorbed by carotenoids may be transferred to PS I in the strain NIBB1059. Similar contribution of carotenoid-absorption may also occur in the strain NIBB1071. However, even when such a contribution occurs, absorption of BGL by PS II pigments is still greater than that by PS I pigments consistently with the results of Emerson's enhancement. Results for the strain NIBB1071 indicate that BGL causes an excitation biased to PS II. However, the energy absorbed by PS II can be transferred to PS I up to 59% of the total even when RC II is active in photoreaction, that is in an open state, (Ley and Butler 1980). In the strain NIBB1071, the imbalance of absorbed energy is probably compensated by the spillover from PS II to PS I.

At least as for the energy absorbed by respective pigment components, there are no significant difference between BGL and WL in the relative ratio of the energy absorbed by the pigments for the two photosystems, but BGL and WL are different in the fractional absorption of carotenoids. This suggests that the primary cause of the decline of quantum yield under BGL is due to the absorption by carotenoids inactive at least in O_2-evolving photosynthetic reaction in both *Synechococcus* strains. As noted above, at least a half of the energy absorbed by carotenoids is possibly transferred to PS I. As far as we determine the photo-

synthetic quantum yield using O_2 evolution, we cannot evaluate such a contribution of carotenoid-absorption.

2.4 Discussion

The analysis of the pigment system of *Synechococcus* have shown that *Synechococcus* can maintain photosynthesis so as to support active autotrophic growth under the blue-green light and it will support the extended distribution of *Synechococcus* toward the base of euphotic zone. As indicated by absorption spectra of intact cells, a high content of PE was observed in all the strains collected from surface and subsurface waters (cf. Fig. 2). Thus, a large size of light-harvesting pigment system is not special characteristics to the strains living at the subsurface depth but this is probably a common characteristics of marine minute *Synechococcus* (Kishino et al. 1986; Takahashi et al. 1989). These implicate a high competence of *Synechococcus* to utilize light in green region, which is the light at the lower part of euphotic zone in coastal water. In fact, high efficiency of photosynthetic rate under green light has been reported for PE-containing *Synechococcus* by Glover et al. (1986). On the contrary, the blue-green light, the light at the lower part of euphotic zone in oligotrophic open ocean, appears not to be efficiently utilized by photosynthesis in *Synechococcus* spp. because of biased energy-distribution at shorter wavelengths from the range of PE-absorption. However, calculated energy distribution between the two photosystems under BGL indicates that a

large amount of energy can be absorbed by PBS and transferred to PS II. Even when the PE contains only PEB, a high content of PE in PBS enables *Synechococcus* to absorb BGL. The fraction of light absorption of PBS under BGL was as high as 36 and 44% of the total light absorption for the strains of NIBB1059 and NIBB1071, respectively (Table 5). In the case of a freshwater strain, such as *Synechocystis* ATCC27170, which contains less amounts of PE, the value should be much smaller. In the case of *Anacystis nidulans* which contains only PC and APC, for example, the energy distribution was biased to PS I at wavelengths shorter than 530 nm (Jones and Myers 1964). Since efficient energy-transfer from PE to APC occurs in *P. cruentum* (Grabowski and Gantt 1978), such a great amount of PE of *Synechococcus* provides a marked increase in absorption cross-section of PBS even in BGL region. These results indicate that a high content of PE, common characteristics of pigment composition in *Synechococcus*, expands a range of wavelengths of light absorbed by PBS toward blue-green region.

Determinations of the energy distribution by the Emerson's enhancement effect have indicated that the energy distribution under BGL is balanced in the strain NIBB1071 which contains PUB together with PEB in PE. In the strain NIBB1059, however, results indicate that BGL excites PS I more than PS II to some extent. As described above, the discrepancy between the results obtained from the two types of experiments suggests that some of carotenoid absorption is effective in driving PS I, but not PS II. If it is the case, the light-absorption for PS I in Table 7

must be corrected by adding such an effective fraction of carotenoid absorption. The corrected value must be greater than the absorption for PS II in the strain NIBB1059, but smaller than that in the strain NIBB1071. If the effective fraction relative to the total absorption by carotenoids is the same in the two strains, we can roughly estimate the size of the effective fraction using the data presented in Tables 5 and 7. In the strain NIBB1059, BGL could excite PS I greater than PS II, and so,

$$F(PBS) + F(Chl\ a_{II}) < F(Chl\ a_{I}) + \gamma \times F(Carotenoid),$$

where γ is the rate of effective fraction of carotenoid absorption which drives PS I. This derives $\gamma > 0.52$. Similarly, we can obtain the relationship, $\gamma < 0.77$ in the case of the strain NIBB1071. As noted above, enhancement effect of BGL in the strain of NIBB1059 indicate that BGL is not a typical λ_1 light. Thus, F(PS II) is not far less than F(PS I), indicating that γ is not very much larger than 0.52 but is a little greater than this value. On the other hand, the enhancement of quantum yield of λ_1 light by BGL is much greater in the strain NIBB1071, which indicates that γ is far smaller than 0.77. These features suggest that γ takes a value in a range from 0.52 to 0.77 but it is close to 0.52.

Energy absorbed by β-carotene is known to be efficient for photosynthesis (Goedheer 1969) and both PS I and PS II complexes contain β-carotene. Then, some fraction of light energy absorbed by carotenoids may be transferred to PS II. Since the content of

β-carotene in RC II protein is usually about 2 times greater than that in RC I protein (Siefermann-Harms 1985), the content of β-carotene in RC II was estimated to be one-third and one-fifth of total β-carotene in the strains of NIBB1059 and NIBB1071, in which the molar ratio of RC I to RC II is 3 and 4, respectively (Table 1). Assuming that the content of β-carotene is 20% according to Kana et al. (1988), 2-3% of total energy absorbed by carotenoids will be available to PS II under BGL. This indicates that this smaller fraction causes a marked drop in the quantum yield as far as determined by O_2 evolution.

Guillard et al. (1985) examined the composition of carotenoids in marine *Synechococcus* strains. Though clonal variations have been observed in some extent, common feature is that zeaxanthin is dominant species (50 to 80% of total carotenoids). Kana et al. (1988) investigated the effects of light intensity on carotenoid composition for a strain of *Synechococcus*. They have been reported that the major species is always zeaxanthin (> 75%) and that most of carotenoids did not act as photosynthetic light harvesting pigments at least for the O_2-evolving reaction. Energy ransfer from β-carotene to PS I with nearly 100% efficiency was observed for a cyanophyte strain whereas no indication was observed for energy transfer from carotenoids to PS II (Goedheer 1969). As explained above, however, more than 50% of carotenoid-absorption is probably utilized by the reaction driven by PS I. This high value cannot be explained by the absorption by β-carotene in PS I. Some other carotenoids may act as the light-harvesting antenna for PS I. If zeaxanthin is such a species, the

active species is present in thylakoid membranes in a molecular state which allows the energy transfer to PS I. If the energy transfer is as high as that among other antenna pigments, the amount of such a species is around 50% of total zeaxanthin. This interpretation about antenna role of carotenoids, however, is needed to be confirmed by a more direct analysis.

If our argument is correct, the energy absorbed by carotenoids is probably utilized partly to the reaction driven by PS I, which cannot be detected by O_2-evolution such as the cyclic electron transport by PS I and converts the light energy to ATP. Concentrations of major nutrients such as NO_3^-, PO_4^{3-} are not sufficiently high near the bottom of euphotic zone even though they are far higher than at the surface, so that nutrient uptake needs to depend on an uptake system which allows to concentrate nutrients within cells. Since such a system depends on ATP supply from photosynthesis, cyclic photophosphorylation depending on carotenoid-absorption may be a great support in this aspect.

When *Synechococcus* was grown under BGL and WL, no significant difference was observed in the pigment composition. The stoichiometric balance between the two photosystems and between PBS and PS II did not change greatly (Table 1). It has been known that the ratio of PS I to PS II abundance is markedly reduced and so the ratio of PE to Chl *a* becomes greater when *Synechococcus* NIBB1059 is grown under red light which is absorbed preferentially by Chl *a* and excites preferentially PS I (Fujita et al. 1987a). The present results on stoichiometry of the two photosystems under BGL or WL indicate that BGL is not a typical λ_1

light in the case of the strain NIBB1059. This is consistent with the conclusion derived from analysis of photosynthetic efficiency under BGL.

PE level is variable depending on the light intensity of the growth environments; the lower level at the stronger light intensities. Variation reaching 20 times have been reported for the marine *Synechococcus* strain (Kana and Glibert 1987). The decrease in β-carotene up to 5 times has also been recognized with the decrease in PE level. Thus, such variations can adjust the light-harvesting in response to the light intensity in a wide range. Smaller antenna size will lower the light absorption under the strong light energy near the surface of water column. On the other hand, a very high content of PE in PBS at low light energy enables the light-harvesting at such short wavelengths. Furthermore there is a possibility that more than a half of energy absorbed by carotenoids drives PS I. These characteristics indicate that more than 60-70% of total absorbed quanta is converted to biological energy und utilized for growth of *Synechococcus* under BGL. These evidences suggests that these characteristics enable PE-containing *Synechococcus* to extend their distribution down to the bottom of euphotic zone in the Kuroshio area as observed by Tsuji et al. (1986) and Tsuji and Ohki (1989).

The absorption spectra of these strains showed an additional peak at 500 nm as seen in the strain NIBB1071. The relative extinction at 500 nm to that at 546 nm is variable among the strains (Fig. 1), which suggests that proportion of PUB to PEB is

different in different strains. Unusually high content of PUB in PE is also reported for a strain isolated from warm waters of Atlantic Ocean (Ong et al. 1984). PUB absorption at shorter wavelengths than PEB must give further advantage to the photosynthesis under blue-green light at the bottom of euphotic zone. The value of fractional absorption of PBS under BGL was a little higher in the strain NIBB1071 than that in the strain NIBB1059 (Table 5). It indicate that inclusion of PUB is effective in collecting blue-green light. The results of the photosynthetic rate and the growth constant obtained for these two strains further suggest an effectiveness of PUB (Table 2, Fig. 6). These results suggest us that there may be some possible differences in the utilization of light near the bottom of euphotic zone, and may be some possible differences in the vertical distributions between strains. Evaluation for those posibilities is left behind for further work.

A characteristic pigmentation was also found in green-colored picophytoplankton which were isolated from subsurface in the Kuroshio area in this study. As shown in Fig. 2, they had a large amount of Chl *b*. Since Chl *b* is the chromophore of light-harvesting chlorophyll-protein complex (Glazer 1983), such a large abundance of Chl *b* indicates that these strains can form light-harvesting chlorophyll protein complex in much larger sizes than that in the Chl *a*-*b* system of the ordinary green algae, or that the light-harvesting chlorophyll proteins in these strains contain Chl *b* in much larger amounts than in the other green algae.

Interestingly, common characteristics of extremely large size of pigment system can be found in the organisms of the two different phyla in the subsurface water. Ability to form a very large size of light-harvesting system is probably one of adaptation for low light environment such as the subsurface depths in the ocean.

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3 Culture of the picophytoplankton *Synechococcus* spp. in artificial seawater medium and characterization of isolated strains

3.1 Introduction

Picophytoplankton of cyanophytes *Synechococcus* spp. have been known to distribute widely from eutrophic coastal water to oligotrophic open ocean water in the sea. It is very much interesting how the *Synechococcus* make their wide distribution under such a great change in the essential nutrient(s) availability. A particular attention was given to extremely low nutrient concentrations, such as nanomoles per liter in inorganic nitrogen and phosphorus compounds as observed in the oligotrophic open surface water, although turnover of those nutrients occurs at significant rates due to a rapid remineralization through biological pathways including microbial loop activity (see Chap. 1). Analysis of the nutrient utilization of *Synechococcus* inhabiting in such low nutrient concentrations is particularly important for understanding why they can inhabit commonly in oligotrophic ocean and often became dominant in the phytoplankton community. Although the understanding of characteristics of functional activities occurring in natural environment is final objective, there are difficulties to perform experimental analyses using natural population at least in the following points: 1)extraction of the information on a particular species is not easily made from field data because the natural phytoplankton community consists of various species; 2)it is difficult to set reproducible experimental

conditions by using natural samples due to a large variation of environmental conditions; 3)evaluation of experimental results using natural samples is often difficult because of uncertainty of physiological history of the organism in question; and 4)experimental analyses are rather confined because the population density is usually lower than that required for most of analyses. Consequently, it is essential to isolate such target organisms and examine their biological characteristics in the laboratory.

To achieve this task, it is required to establish a culture system of the strain(s) of the target organisms in the laboratory using a defined medium, especially for understanding nutritional characteristics of the phytoplankton. However, most of phytoplankton species isolated from the sea in the past, especially from the open ocean, have not been cultured sufficiently in defined media but in the media based on natural seawater enriched with various macronutrients, minor metals and vitamins (Provasoli 1968; Guillard and Ryther 1962; Keller et al. 1987). For the purpose of study of nutrient requirements mentioned above, such types of culture media with natural seawater are not adequate because the composition of nutrients in the seawater is not easily defined as a basal medium for nutritional experiments. Further variations in nutritional composition of seawater occur depending on area for collection of the water. Precipitation by autoclaving is an another difficulty for the preparation of artificial seawater medium (Jones 1967; Harrison et al. 1980). Thus, the development of chemically defined artificial seawater

medium is a primary requirement for the study of physiological nature and mechanism of nutrient utilization of oceanic species.

Chemically defined medium is based on artificial seawater which has the chemical composition of natural seawater. Morel et al. (1979) have developed the 'aquil' medium based on the chemical composition of the Standard Ocean Water (SOW). The 'aquil' medium was chosen as a possible candidate for the development of defined medium for the phytoplankton *Synechococcus* spp. The buffering system of HCO_3^---CO_3^{2-} was used for the pH-buffer of the medium as similar to the natural seawater, because artificial buffer system such as Tris-HCl has been known to be toxic for several open ocean species (Ohki et al. 1987). Two cautions have been specially paid for preparation of the medium. The first is protection from precipitate formation during autoclaving. Since occurrence of precipitation depends on pH in the medium, the effect of change in pH of the medium during autoclaving was examined. Concentrations of minor metals such as iron were also examined to find out the level not to cause precipitation but to allow cell growth upto the cell density required by the experiments. The second caution is protection of the medium from heavy metal contaminants comming from chemical reagents and glass wares, because oceanic phytoplankton species have been reported to be very sensitive to heavy metals (Carpenter and Lively 1980; Rueter et al. 1979; Ohki et al. 1986).

In this study, development of culture for marine *Synechococcus* isolated from the Kuroshio area was tried using the modified 'aquil' medium. Characteristics of growth of *Synechococcus*

in such medium were also investigated.

3.2 Material and methods

3.2.1 Preparation of culture medium for oceanic picophytoplankton

Table 1 shows composition of the modified 'aquil' medium. The recipe of the Standard Ocean Water (SOW) (cf. Morel et al. 1979) was adopted to the basal composition of artificial seawater. Composition of nutrients added to SOW was modified from the 'aquil' medium (Morel et al. 1979) and was basically equivalent to that of the *f* medium (Guillard and Ryther 1962) at 1/5 nutritional strength with slight modifications. Concentrations of Fe-EDTA and manganese were reduced to one-fifth and a half of the original composition, respectively, and molybdenum and vitamin B_{12} concentrations were increased up to ten times. Silicate was not added.

Precipitation during autoclaving (120 °C, 20min) was eliminated either by autoclaving the medium at lowered pH or by separating divalent cations from other salts as reported by Harrison et al. (1980). In the former case, HCl was added to the medium containing all the chemicals immediately before autoclaving. Adequate amount of HCl for this purpose was determined as described later. In the latter case, Solutions I and II were prepared as described in Table 1. HCl was added to Solution I before autoclaving to adjust pH, and both solutions were autoclaved separately. After cooling, the two solutions were mixed

Table 1 Composition of modified 'aquil' medium[a)].

Chemicals	Concentration (M)	Chemicals	Concentration (M)
Solution I			
$NaCl$	4.20×10^{-1}	Fe-EDTA	9.50×10^{-7}
$NaSO_4$	2.88×10^{-2}	$MnCl_2$	1.82×10^{-7}
NaF	7.14×10^{-5}	$CuSO_4$	1.57×10^{-8}
H_3BO_3	4.85×10^{-4}	$ZnSO_4$	3.06×10^{-8}
KCl	9.39×10^{-3}	$CoCl_2$	1.68×10^{-8}
KBr	8.40×10^{-4}	$NaMoO_4$	1.04×10^{-7}
$NaHCO_3$	2.38×10^{-3}	Vitamin B_{12}	1.48×10^{-9}
$NaNO_3$	3.53×10^{-4}	Biotin	8.19×10^{-10}
NaH_2PO_4	variable	Thiamin HCl	1.19×10^{-7}
Solution II			
$CaCl_2$	1.05×10^{-2}	$MgCl_2$	5.46×10^{-2}
$SrCl_2$	6.38×10^{-5}		

[a)] Medium pH was adjusted to 8.0.

aseptically. In both cases, stable pH was obtained after 1 to 2 days (see Chap. 3.3.1).

All glasswares were washed carefully with alkaline detergent and were soaked in 1N HCl over night to remove toxic heavy metals. Then, they were rinsed thoroughly with distilled water. As the phosphate source, analytical grade reagent (Merk, Darmstadt) was used in order to reduce probable contamination by heavy metals supplied from the reagents as suggested by Ohki et al. (1986). Nitrate and phosphate solutions were further passed through a Chelex-100 column to minimize metal contaminations.

3.2.2 Culture of oceanic picophytoplankton in the artificial seawater medium

All strains isolated from the Kuroshio area (see Chap. 2) were firstly grown in the enriched seawater of the *f* medium at 1/5 nutritional strength. Cells were then inoculated into 10 ml of the artificial seawater medium (the modified 'aquil' medium) in culture test tubes and were grown at 25 °C under continuous white fluorescent light (1.4 W/m^2). Three other oceanic strains of *Synechococcus* NIBB1059, NIBB1070 and NIBB1071 isolated by Dr. K. Ohki from the surface waters of the Gulf Stream and the Kuroshio area were also grown in the modified 'aquil' medium. Growth of algal cells was followed by turbidometric measurements, and the growth constants and the maximal population densities were determined.

3.2.3 Measurements of turbidity of intact cells in test tube cultures

For determination of cell density in test tube cultures, a special attachment was devised and set to a Hitachi 100-50 spectrophotometer: a transparent waterbath, in which the culture tube was embedded, was placed in the cell compartment of the spectrophotometer. The test tube (inside diameter, 18 mm) was placed at a fixed position in the light path. Since light reflection at the boundary between glass and water is smaller than that between glass and air, it can reduce light scattering at a curved surface of the test tube. Apparent absorption of cell suspension at 750 nm was determined by subtracting blank value measured before inoculation for each tube from the obtained value.

3.3 Results

3.3.1 Conditions for sterilization of culture medium

Since divalent cations are used to form insoluble precipitates with CO_3^{2-} and PO_4^{3-} in alkaline pH conditions, and since the present medium contains divalent Mg^{2+} and Ca^{2+} cations at high concentrations, it is necessary to keep the medium pH in acidic but moderate range during autoclaving and also to adjust to a given pH value after autoclaving. When the mixture of Solutions I and II which had been adjusted to pH 8.0 was autoclaved, the medium pH increased during autoclaving and decreased slowly thereafter (Fig. 1). Changes in pH were greater in the medium of the smaller volume. It took a rather long time period

Fig. 1 pH changes in culture medium after autoclaving. The modified 'aquil' medium, which had been adjusted to pH 8.0, was autoclaved (120 °C, 20 min) in 500 ml flask with a cotton plug. Volumes of medium contained in each flask were 100, 200, 300 and 400 ml as described in the figure, respectively.

Table 2 Effects of the addition of different amounts of 1N HCl on the pH in the artificial seawater[a] after autoclaving.

Volume (μl)	pH	
	-Si	+Si
0	8.26	8.31
20	8.19	8.21
40	8.14	8.21
60	8.03	8.05
80	7.98	7.93

[a]Various amounts of 1N HCl were added to 50ml of the modified 'aquil' medium, pH of which was adjusted to 8.0 before autoclaving. -Si: silicate free; +Si: $NaSiO_3$ was added at 2.11 x 10^{-5} M. Media were autoclaved (120 °C, 20 min) in 100 ml screw capped flasks. pH value in each medium was determined at 72 hrs after autoclaving.

usually from 48 to 72 hrs to reach a stable pH value after autoclaving.

Decrease in pH after autoclaving never reached to the initial value before the autoclaving. In addition, slight precipitation was often observed. Table 2 shows the effect of 1 N HCl addition on the pH of autoclaved medium containing Solutions I and II. When 60 µl of 1N HCl was added to the 50 ml medium, the medium pH became at 6.1 before autoclaving and was changed to 8.0 after autoclaving. Effects of the flask size and the medium volume on the pH values were also tested (Figs. 2, 3). Smaller ratios of medium volume to flask size tended to give higher pH values immediately after autoclaving, and slow pH changes occurred thereafter in order to compensate a large shift caused by the autoclaving until reaching a stable pH around 8.0. Type of plugs also affected the pH changes. Screw cap caused greater pH changes than the cotton plug. In the former case, pH return after autoclaving became small and did not reach to 8.0 within 72 hrs (Fig. 3d, 3e). Fig. 4 shows replots of the results shown in Fig. 2 in relation to the ratios of the surface areas to the volumes of media in flasks. A clear relationship between the ratios of surface areas to volumes and the pH values at 6 hrs after autoclaving was observed. Higher ratios of the surface area to the volume resulted in higher pH values.

Autoclaving with the addition of HCl can protect from making precipitation in most cases. However, a slight precipitation occasionally occurred. When the Solution II, which contains divalent cations, was separated from the Solution I during auto-

Fig. 2 Changes in pH in the culture medium in flask plugged with cotton after autoclaving. Before autoclaving, 1.2 ml of 1 N HCl was added into 1 liter of medium. Media with various volumes were placed in various sizes of flask plugged with cotton, and were autoclaved (120 °C, 20 min). a: 100 ml flask; b: 300 ml flask; c: 500 ml flask; d: 1000 ml flask. Medium volumes (ml) are indicated by numerals in the figure.

Fig. 3 Changes in pH in the culture medium in flask plugged with screw cap after autoclaving. Before autoclaving, 1.2 ml of 1 N HCl was added into 1 liter of medium. Media with various volumes were placed in various sizes of flask plugged with screw cap, and were autoclaved (120 °C, 20 min). a: 20 ml test tube; b: 100 ml flask; c: 300 ml flask; d: 500 ml flask; e: 1000 ml flask. Medium volumes (ml) are indicated by numerals in the figure.

Fig. 4 Relationships between surface-volume ratio of the medium and pH value at 6 hrs after autoclaving. Data in Fig. 3 were applied.

claving and was mixed after cooling, no precipitates were found. Therefore, the latter method is more reliable. Since the pH changes occurred similarly to the case for the former method during autoclaving, HCl was added to Solution I in order to adjust final pH to 8.0 after autoclaving.

3.3.2 *Synechococcus* growth in the modified 'aquil' medium

Using the modified 'aquil' medium, stability and reproducibility of the growth of *Synechococcus* strains were examined. Tests were made for 9 strains isolated in the present study and 3 strains isolated by Dr. K. Ohki. Each strain growing in the *f* medium was inoculated in the modified 'aquil' medium. Among 12 strains tested, 7 strains grew in the modified 'aquil' medium as well as in the *f* medium.

Four strains showed positive growth in some extent in the early period but followed by no successive growth thereafter. One strain did not show any positive growth in the modified 'aquil' medium.

Nutritional availability of the modified 'aquil' medium was further tested using phosphate as a limiting nutrient component for the three strains which showed active growth in the modified 'aquil' medium and *Synechococcus* NIBB1071. Growth constants at the exponential growth phase were not much affected by the diffrences in initial phosphate concentrations within the range from 1.8 to 29.2 μM in the three strains except for the strain Z-11 which showed about 50 % reduction in the growth constant at the lowest phosphate concentration (Fig. 5).

Fig. 5 Growth constants as a function of initial phosphate concentrations in the modified 'aquil' medium at 25 °C under continuous white fluorescent light of 1.4 W/m². Initial phosphate concentrations were adjusted at 1.8, 3.7, 7.3, 14.6 and 29.2 μM, respectively. All phosphate solutions except for b and f were treated with Chelex-100 before the experiment. a and b: strain T1; c: T11; d: Z-11; e and f: NIBB1071. In b and f, phosphate solution without Chelex-100 treatment. Closed and open circles indicate mean values of duplicate samples and single sample, respectively.

Fig. 6 Maximum cell density at the stationary growth phase as a function of initial phosphate concentrations in the medium. Data were obtained by the same experiments as those in Fig. 5. Maximum cell densities were determined from each growth curve and were expressed as light absorption unit. The absorption of 0.10 corresponded to 7.9×10^7 cells/ml in the case of *Synechococcus* NIBB1071. a and b: strain T1; c: T11; d: Z-11; e and f: NIBB1071. Symbols indicated in the figure were the same as those in Fig. 5.

The maximum cell density at the stationary growth phase was affected by the initial phosphate concentration, and the level was different depending upon different strains (Fig. 6). The strains NIBB1071 and Z-11 showed almost linear increase with increasing the initial phosphate concentrations within the tested range, which indicates that there occurred no nutrient limitation other than phosphate within the tested phosphate concentrations. Results further indicate that the nutrient composition of the modified 'aquil' medium is sufficient for the growth of these two strains. Two other strains, T1 and T11, showed a saturation of increase in the cell density within the tested phosphate concentrations, and even decrease in the cell density occurred at the highest phosphate concentration.

Same kinds of experiments but using phosphate stock solution without the Chelex-100 treatment were performed with the strains of NIBB1071 and T1 (Figs. 5b, 5f, 6b, 6f). Growth constants and maximum cell densities in both strains were not significantly reduced by phosphate without the Chelex-100 treatment for these two strains.

3.4 Discussion

As mentioned earlier, it is essential to establish a defined culture medium based on artificial seawater for the nutritional study of oceanic phytoplankton. Artificial seawater is a key for such a defined medium, and the recipie of SOW (Morel et al. 1979), whose chemical composition is similar to that of natural seawater, has been recommended for open sea phytoplankton by Ohki

et al. (1986). The 'aquil' medium developed by Morel et al. (1979) was then adopted to the present experiments after slight modifications. Generally, sterilization of the medium causes difficult problems for the preparation of artificial seawater medium. Although autoclaving is convenient and common to sterilize culture medium, control of pH is difficult, and precipitation of inorganic ingredients often occur during autoclaving especially in alkaline medium such as artificial seawater (Jones 1967; Harrison et al. 1980). The modified 'aquil' medium does not contain inorganic phosphate at such a concentration as high as for pH buffering. It also does not contain buffer reagents such as Tris-HCl but contains a large amount of HCO_3^-. Thus, pH of the medium is determined by the HCO_3^--CO_3^{2-} system in the modified 'aquil' medium. The change in pH induced by autoclaving is probably caused by a leakage of CO_2 from the medium. A larger boundary area between liquid phase and gaseous phase per unit volume may enhance the leakage of CO_2 from the medium supersaturated with CO_2 under high temperature, which could result in the observed large pH changes (Fig. 4). These results suggest that the medium must be autoclaved with the minimum surface area. Since cotton plug forms a stronger resistance for exchange of gaseous materials between inside and outside of flask during autoclaving, a large amount of HCO_3^- can remain in the medium in the cotton-pluged flasks, and the pH change is smaller than that with screw cap. An addition of HCl before autoclaving has two functions. The first is compensation of pH increase after autoclaving. The second is protection from precipitation which occurs

in alkaline pH. Separated autoclaving of the solution containing divalent cations from others is further effective to avoid precipitation.

Among *Synechococcus* strains examined, the strains NIBB1071 and Z-11 were found to grow stably and reproducibly in the modified 'aquil' medium. The evidence that the maximum cell density at the stationary growth phase increased proportionally with the increase of initial phosphate concentrations indicates that other nutrients do not limit their growth at least within the cell population attained. Thus, the modified 'aquil' medium can be used as a complete medium at least for the two *Synechococcus* strains, NIBB1071 and Z-11.

Some isolated strains were unable to cultured in the modified 'aquil' medium. There are at least two possible reasons as following to explain this failure. The one is possible contaminations of heavy metal(s) in the reagents used. As Ohki et al. (1986) reported, some of phytoplankton in open ocean such as *Trichodesmium* is extremely susceptive to heavy metals(s). It is possible that the sensitivity for metals is different in different strains. Since the treatment with the Chelex-100 for phosphate had no significant effect on the growth of the strains NIBB1071 and T1, it is probable that the phosphate reagent used in this experiments does not contain toxic metals so much. The other possible reason is the requirement of some essential growth factor(s) such as vitamins. The strains showing only a limited growth or no growth may require nutrient(s) which is contained at very limited amounts or is not contained in the medium. If the

latter case occurred, use of defined medium can open a way to analyze nutrient-requirements of phytoplankton. Although the question as to which possibility is the case is still remained, results obtained from the present study imply that strains of *Synechococcus* examined are heterogenous in nutritional characteristics.

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4 Characteristics of phosphate-uptake in the picophytoplankton *Synechococcus* spp. in relation to oligotrophic environments in open ocean

4.1 Introduction

Nutrient availability in euphotic zone for phytoplankton growth is different in different areas of ocean due to physically dynamic nature of ocean. More than a half of the ocean area is covered with barren region. Since ambient concentrations of nutrients are kept extremely low and the primary production in such region is far lower than those in coastal and upwelling regions (cf. Chap. 1), it is called oligotrophic region. It has been assumed that growth of phytoplankton inhabiting in such a region is limited by nutrients (Eppley et al. 1973; Perry 1972). However, there has been controversy as to such an assumption (Goldman et al. 1979; Eppley 1980), since rather rapid growth of phytoplankton has been reported in the oligotrophic ocean (Goldman et al. 1979; Bienfang and Takahashi 1983; Li et al. 1983; Douglas 1984; Laws et al. 1984; Iturriaga and Mitchell 1986) and since the physiological state of natural populations of phytoplankton did not always indicate nutrient deficiency in such region (Kanda et al. 1988).

Distribution of *Synechococcus* spp. is extended from eutrophic to oligotrophic regions and the organisms often become dominant biomass species within the phytoplankton community (Takahashi and Hori 1984; Tsuji et al. 1986; Tsuji and Ohki 1989; Furuya 1990). There have been several papers reporting active

reproduction (more than 1 division per day) of *Synechococcus* in oligotrophic region (Campbell and Carpenter 1986a, b; Iturriaga and Mitchel 1986; Landry et al. 1984; Hagström et al. 1988; Iturriaga and Marra 1988; Kudoh et al. 1990). These results imply that *Synechococcus* spp. may have some special ability for efficient utilization of nutrients at such low concentrations. However, the kinetic analyses of nutrient utilization of such organisms have not been examined well (Takahashi and Bienfang 1983).

The rate of nutrient uptake (V) can be expressed with a hyperbolic function of the concentration of nutrient (S);

$$V = V_{max} \times \frac{S}{K_m + S}$$

where V_{max} is the maximum velocity, and K_m is the half saturation constant with the value of S when $V = V_{max}/2$ (Caperon 1967; Dugdale 1967). Successful description of nutrient uptake with the Michaelis-Menten kinetics has been confirmed by many researchers (cf. Raymont 1980; Healey 1982). Dugdale (1967) stated a competitive ability to utilize nutrients by means of kinetic parameters; a lower value of K_m can compensate for a lower V_{max} up to a certain nutrient concentration. Since then, studies on kinetics of nutrient uptake have shown variability in the ability to use nutrients at low concentrations among different species. Natural communities in oligotrophic region show lower K_m for ammonia and nitrate uptake than that of eutrophic communities

(MacIsaac and Dugdale 1969, 1972; Eppley et al. 1973). Species or clones isolated from oceanic region tend to show lower K_m for ammonia and nitrate uptake than that of neritic species or clones of the same species (Eppley and Thomas 1969; Eppley et al. 1969, 1973; Carpenter and Guillard 1971; Koike et al. 1973). Based on these results, it has been recognized that the value of K_m is specific to respective uptake systems and that oceanic species inheritantly have mechanisms to utilize nutrients at low concentrations.

In a freshwater strain of *Synechococcus* (*Anacystis nidulans*), NO_3^- uptake system (K_m < 10 μM), which can concentrate NO_3^- within cells, has been found to play an important role in nitrate assimilation; elevation of intracellular NO_3^- concentration enables the nitrate reductase of a high half saturation constant (0.7 mM) to reduce NO_3^- (Flores 1983). There have been also several reports suggesting an enhancement of the activity for uptake of nitrate (Zevenboom and Mur 1979), phosphate (Healey and Hendzel 1975; Grillo and Gibson 1979; Falkner et al. 1984), sulfate (Jeanjean and Broda 1977; Green and Grossman 1988) and bicarbonate (Omata and Ogawa 1986) upon substrate depression for freshwater cyanophytes. These evidences rise questions whether marine strains of *Synechococcus* have such a capability to concentrate external phosphate within cells, and how they respond to changes in nutrient concentrations from eutrophic to oligotrophic environments, and whether the characteristics of enhanced uptake activity is common in other cyanophytes.

In this study, analysis was made to characterize the uptake

system of marine *Synechococcus* for inorganic orthophosphate (P*i*), which is a major form of phosphate taken up by cyanophyte (cf. Healey 1982). Experiments were performed in well-defined chemical conditions using a chemically defined medium, the modified 'aquil' medium. A freshwater cyanophyte, *Synechococcus* PCC7942 (*A. nidulans* R2), was also used as a control organism.

4.2 Material and methods

4.2.1 Organisms and culture conditions

The strain of *Synechococcus* NIBB1071 was grown in the modified "aquil" medium (see Chap. 3.2.1), at 25 °C under continuous white fluorescent light, in which this organism can grow stably and reproducibly as described in Chapter 3.

In the experiments for determination of growth constant, cells at 8 x 10^6 cells/ml were incubated in the medium containing various phosphate concentrations (7.2, 3.7, 1.9 and 1.0 μM) under fluorescent light of 2.8 W/m² (measured with 2π sensor). Cultures were successively diluted with an equal volume of fresh medium at every 24 hrs in order to dilute the cell density down to a half and to maintain a given phosphate concentration in the culture. After establishment of steady state (at least for the times of 3 generations), the growth constant was determined from cell increase during the incubation.

For preparation of cells acclimated to high concentration of phosphate, cells at 2.4 x 10^7 cells/ml were incubated in the medium containing phosphate at 14 μM under illumination of 3.5 W/m² (measured with 2π sensor), which brings about the cell

growth at 1 division per day. Cultures were successively diluted with an equal volume of fresh medium containing phosphate of 14 μM at every 24 hrs. The phosphate concentration in this culture was maintained at more than 10 μM even at the end of incubation. After incubation at least for 3 days, cells were used for experiments. For preparation of cells acclimated to low phosphate concentration, cells at 6 x 10^6 cells/ml were incubated in the same way as that for cells acclimated to high phosphate concentration but the dilution medium contained phosphate of 1 μM. Cells were used after incubation for 5 days.

Cells of both types were also prepared with a continuous culture system. The growth chamber (500 ml) had a water jacket, in which water thermostated at 25 °C was circulated, and was continuously illuminated at the intensity of 7.7 W/m^2 (measured with 4π sensor). These conditions brought about the growth at 1 division per day. Cells of 6.3 x 10^7 cells/ml were maintained with a continuous supply of medium. For cells acclimated to high phosphate, the medium containing 15 μM phosphate was supplied at a dilution rate of 1.0 d^{-1}, under which 10 μM phosphate was found to be present in the medium in growth chamber. For cells acclimated to low phosphate, the medium containing 5 μM phosphate was supplied at a dilution rate of 0.5 d^{-1} in order to maintain a low phosphate condition.

The strain of *Synechococcus* PCC7942 was grown in a defined inorganic medium, MDM medium (Watanabe 1960), buffered with 2.38 mM $NaHCO_3$ (pH 8.0). Other culture conditions were the same as those for *Synechococcus* NIBB1071 (25 °C, continuous illumination

at 3.5W/m^2).

Cell density was determined by counting with a hematocytometer or by turbidometry.

4.2.2 Measurments of the activity for phosphate-uptake

Cells were collected by a low speed centrifugation and were resuspended in the assay medium which contained NaCl at 479 mM, KCl at 9.39 mM, $MgCl_2$ at 54.6 mM, $CaCl_2$ at 10.5 mM and $NaHCO_3$ at 2.38 mM (pH 8.0) for *Synechococcus* NIBB1071, and NaCl at 1.71 mM, KCl at 13.3 mM, $MgCl_2$ at 1.01 mM, $CaCl_2$ at 0.68 mM and $NaHCO_3$ at 2.38 mM (pH 8.0) for *Synechococcus* PCC7942, respectively. The cell density was adjusted to 6 x 10^6 cells/ml. Incubation for phosphate uptake was made at 25 °C under illumination of 3.5 W/m^2. In the standard assay, phosphate of 2 μM was added to the cell suspension. When necessary, phosphate concentration was changed as described in the text. Two ml of suspension was withdrawn at time intervals after phosphate addition and was centrifugated for 3 min at 8,500 *g*. Supernatants were stored at -80 °C until determination of phosphate concentration.

Phosphate concentration was determined by the method originally described by Murphy and Riley (1962) (cf. Strickland and Parsons 1972). Phosphate in the samples was detected by colorimetry with one-tenth volume of the mixed reagent, which contains $(NH_4)_6Mo_7O_{24}$ at 4.86 mM, H_2SO_4 at 1.4 M, ascorbic acid at 61.4 mM and $K(SbO)C_4H_4O_6$ at 0.407 mM. The mixed reagent was prepared immediately before each measurement. Absorption at 885 nm was measured at 10 min after the addition of mixed reagent to the sam-

ples. The detection-limit of phosphate was ca. 0.03 μM.

For determination of activity for phosphate uptake, rate constants were calculated from decrease in phosphate concentration in the medium during a given time period under assumption that phosphate decrease follows the first order kinetics.

In the determination of kinetic parameters in cells acclimated to low phosphate concentration, the initial phase of phosphate uptake was determined from phosphate decrease in the 10 min incubation with cell suspensions at 6×10^6 cells/ml. In the case of cells acclimated to high phosphate concentration, the cell density increased to 6×10^7 cells/ml. In the latter case, an abrupt decrease occurred in the first 2.5 min. In such cases, the second phase of phosphate decline was used for the analysis.

4.3 Results

4.3.1 Growth rate under various phosphate concentrations

To examine growth of *Synechococcus* NIBB1071 under low phosphate concentrations, cells were grown in the medium which contained phosphate at various concentrations. Results indicated that growth was not reduced significantly even at low phosphate concentrations (Fig. 1). Growth constant was 0.85 division per day at phosphate concentrations from 6.2 to 1 μM. Growth constant decreased slightly at 0.26 μM, the lowest concentration of phosphate, under which phosphate was not detected at the end of incubation.

Cellular phosphate content (cell quota), which was calculated from decrease in phosphate in the culture medium during the

Fig. 1 Growth constants of *Synechococcus* NIBB1071 as a function of external phosphate concentrations. Cells were grown at 25 °C under continuous white fluorescent light of 2.8 W/m², and cultures were successively diluted with fresh medium containing respective phosphate concentrations once every 24 hrs. Growth constants were calculated after establishment of steady state and were plotted against phosphate concentrations. Since phosphate concentration was changed during each incubation, average concentrations were adopted.

incubation, was 1.0×10^{-16} mol P*i*/cell at all phosphate concentrations.

4.3.2 Effects of phosphate concentrations in culture medium on phosphate-uptake activity

Growth experiments described above revealed that cell growth is maintained at high level for long time period (> 3 generations) even at low phosphate concentrations. Thus, the cell growth observed seems not to be supported by the surplus pool of phosphate within the cells, but by the phosphate in the medium. These features indicate that *Synechococcus* NIBB1071 has an inherent high activity of phosphate uptake or a regulatory mechanism to enhance phosphate uptake under conditions of low phosphate concentrations.

To examine the effects of phosphate concentrations on the activity of phosphate uptake, cells grown in a high phosphate medium (10 μM) were transferred to the medium containing a low phosphate concentration. Cells at 8×10^6 cells/ml were incubated with successive dilutions by equal volume of fresh medium containing 1 μM phosphate once every 24 hrs. Fig. 2 shows changes in the phosphate concentration in the culture medium and growth constants during incubation. Phosphate concentration decreased to 0.64 μM at the end of the first dilution treatment and was less than detectable level at the end of the second dilution treatment. Since the culture medium was diluted with an equal volume of fresh medium containing 1 μM phosphate, the estimation of phosphate concentration of the culture medium after

Fig.2 Phosphate-limited growth of *Synechococcus* NIBB1071 after shift of phosphate concentration from 10 μM to 1.7 μM. Cells were grown at 25 °C under continuous white fluorescent light of 3.5 W/m², and culture medium was successively diluted with fresh medium containing 1 μM phosphate once every 24 hrs. a: external phosphate concentration; b: growth constant.

the dilution can be made by the value before the dilution as indicated by horizontal bars in Fig. 2a. The phosphate concentration just after the first dilution was 0.75 μM, which was close to the expected value of 0.82 μM. However, the concentration just after the second dilution was less than a half of the expected value. The difference between the observed and the expected values became further greater at the third dilution. Thereafter, no increase in phosphate concentrations was observed upon dilution with the fresh medium. In spite of decrease in phosphate level in the medium, growth constant was maintained at a high level during the experimental period. Since cells growing at a rate of 1 division per day were diluted two-fold once a day, the cell density remained at a constant level during the incubation. These results suggest that the activity for phosphate uptake per cell increases after the second dilution treatment and that phosphate in the added fresh medium is consumed so rapidly as the phosphate level in the samples becomes lower than that of the expected value.

Fig. 3 shows time courses of phosphate uptake by cells before and after shift of phosphate concentration to a low level. The phosphate uptake in cells after shift-down culture was far faster than that in cells grown under 10 μM phosphate. These results clearly indicate that the activity for phosphate uptake is enhanced by the shift-down of phosphate concentration in the culture medium. Time courses of phosphate uptake were found to be approximated to a single exponential function. The rate constant was 5.6×10^{-8} and 1.1×10^{-9} ml/cell/hr for the former

Fig. 3 Time courses of inorganic phosphate uptake by *Synechococcus* NIBB1071 grown under high and low phosphate conditions. Two μM phosphate was added to cells grown under high and low phosphate medium. Cell density was 7 x 10^6 cells/ml in both cases. The time cource of phosphate uptake was found to be approximated to single exponential function. The rate constant was 1.0 x 10^{-9} and 5.6 x 10^{-8} ml/cell/hr for the former (open circle) and the latter cells (closed circle), respectively.

and the latter cells, respectively.

To characterize properties of phosphate uptake in cells grown under a high and a low phosphate media, kinetic parameters of phosphate uptake were determined. Fig. 4 shows Lineweaver-Burk plots of phospate uptake by cells of the two types determined from the initial rate of phosphate uptake. In both cases, plots can be approximated to the Michaelis-Menten kinetics. However, the kinetic parameters were dramatically changed by the phosphate concentrations in the medium (Table 1). The V_{max} of cells grown in a low phosphate medium was 65-fold higher than that of cells grown in a high phosphate medium, whereas K_m of the former cells was one-fourth of that of the latter.

The energy dependency of phosphate uptake system in cells of high activity was also examined. The rate constant of phosphate uptake was measured with and without occurrence of photosynthesis (Table 2). When measurements were made in the dark, the rate of phosphate uptake decreased to about a half of the value obtained in the light. Inhibition of photosynthesis by 10^{-4} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) also resulted in a reduction of phosphate uptake to almost the same extent as in the dark. Results indicate that phosphate uptake depends on photosynthesis, although the coupling seemed not to be strict. The phosphate uptake probably requires energy supply from photosynthesis. Such a supply may be replaced by respiration in the dark but in a limited range.

Fig. 4 Lineweaver-Burk plots for the inorganic phosphate uptake of *Synechococcus* NIBB1071 under various phosphate concentrations. a: cells grown in phosphate-replete chemostat (dilution rate = 1.0 d^{-1}, 10 μM phosphate was detected in growth chamber); b: cells grown in phosphate-limited chemostat (dilution rate = 0.5 d^{-1}). Note that the scale of ordinate is different by two-orders between a and b. The rate constant of phosphate uptake was 9.5 x 10^{-10} and 2.9 x 10^{-8} ml/cell/hr for the former and the latter cells, respectively.

Table 1 Kinetic parameters of inorganic phosphate uptake in *Synechococcus* NIBB1071 grown under a high and a low phosphate media.[a]

Cell type	K_m[b]	V_{max}[c]
Grown under		
high phosphate[d]	0.47	0.99
low phosphate[e]	0.11	64

[a] Data were taken from Fig. 4.

[b] μM phosphate.

[c] 10^{-17} mol P*i*/cell/hr.

[d] cells grown in phosphate-replete chemostat (dilution rate = 1.0 d^{-1}. 10 μM phosphate was detected in growth chamber.).

[e] cells grown in phosphate-limited chemostat (dilution rate = 0.5 d^{-1}. No phosphate was detected in growth chamber.).

Table 2 Effect of light on inorganic phosphate-uptake in cells grown under low phosphate conditions[a].

Conditions	% [b]	(S.D.)
Light (3.5 W/m^2)	100	(18.4)
Light (3.5 W/m^2) + DCMU (10^{-4} M)	40.9	(2.1)
Dark	51.7	(4.6)

[a] Cells were grown in phosphate-limited chemostat (dilution rate = 0.5 d^{-1}). Rate constant of cells under light conditions was 4.0 x 10^{-8} ml/cell/hr.

[b] Each value was averaged for three sets of data.

4.3.3 Time course of activation of phosphate-uptake

To clarify the relationship between the external phosphate concentration and the activation of phosphate uptake, time course of the increase in the activity for phosphate uptake with decrease in the phosphate in the medium was followed. Cells grown in the medium containing 10 μM phosphate were transferred to the medium containing either 1 μM or 0.5 μM phosphate, and these cultures were successively diluted with an equal volume of fresh medium containing 1 μM phosphate once every 24 hrs. When cells were transferred to 0.5 μM phosphate medium, phosphate was again not detectable after incubation for 24 hrs and, at the same time, the activation of phosphate uptake occurred (Fig. 5b). When cells were transferred to 1 μM phosphate medium, however, 0.2 μM phosphate remained in the culture medium after incubation for 24 hrs, and the activity of phosphate uptake remained at low level. In this case, the activation of phosphate uptake occurred after 2 days, when the phosphate concentration decreased to less than 0.2 μM (Fig. 5a). These results suggest that the activation is induced by a certain low level of external phosphate concentration (around 0.2 μM). The activation occurred rapidly, and the activity increased to the maximum level within a generation time, though the value obtained in these experiments (2.8×10^{-8} ml/cell/hr) was somewhat less than those obtained in the previous experiments (cf. Fig. 3). Marked difference between the expected (indicated as horizontal bars in Fig. 5a, 5b) and the measured phosphate concentration was observed when the activation of phosphate uptake occurred. It is now clear that the

Fig. 5 Effect of external phosphate concentration on the inorganic phosphate uptake of *Synechococcus* NIBB1071. Cells grown under 10 μM were transferred to the medium containing either 1 μM (a, c) or 0.5 μM (b, d). Cells were grown at 25 °C under continuous white fluorescent light of 3.5 W/m², and culture medium was successively diluted with fresh medium containing 1 μM phosphate once every 24 hrs. a: inorganic phosphate uptake activity (closed circle) and external phosphate concentration (open circle) obtained from transferring to 1 μM phosphate; b: obtained from transferring to 0.5 μM phosphate; c: cell density corrected for respective dilutions in a; d: cell density in b.

difference is a result of a rapid phosphate uptake by the activated system as assumed above. In both cultures, cells increased exponentially throughout the incubations (Fig. 5c, 5d), which indicated that activated phosphate uptake maintains cell growth at high level even under very low phosphate levels.

4.3.4 Activation of phosphate-uptake in other cyanophyte species

As described above, it was suggested that the enhancement of phosphate-uptake supports phosphate utilization of *Synechococcus* under the conditions from high to low phosphate concentration. For the further characterization of the phosphate uptake system of *Synechococcus* which enables their efficient phosphate-utilization in a wide range, it is highly required to clarify the molecular system of phosphate-uptake. To perform such analyses, application of biochemical methodology must be examined, such as isolation of memebrane, formation of mutant and genetic engineering techniques. However, such problems have not yet been solved for this orgamism, such that the prefect culture system for the marine *Synechococcus* has not been completed and the growth on a agar plate, which is needed for the preparation of mutant, is particularly difficult.

Recently, several protein components involved in the nutrient uptake were identified for nitrate (Omata et al. 1989) and sulfate (Green and Grossman 1988) in a freshwater strain of *Synechococcus* PCC7942 (*A. nidulans* R2). For this strain, the method of isolation of membrane have been eatablished (Omata and Murata 1983). Furthermore, this strain is transformable and the

techniques of genetic engineering have been applied to this strain (Porter 1988; Houmard and Tandeau de Marsac 1988). The strain PCC7942 is also an unicellular coccoid cyanophyte and is included in same generic group of *Synechococcus*. Then, *Synechococcus* PCC7942 may serve as a model system to analyze the uptake system of marine *Synechococcus*. To confirm whether the enhancement of phosphate uptake also occur in the strain PCC7942, similar experiments were made for *Synechococcus* PCC7942.

Cells grown under 11.4 μM phosphate were transferred into the modified MDM medium containing 0.5 μM phosphate (see Chap. 4.2.1). The cell density was adjusted to 3.1×10^6 cells/ml (approximately a half of that of *Synechococcus* NIBB1071) at the beginning of incubation, since before the transfer (0 hr), the rate constant of phosphate uptake of *Synechococcus* PCC7942 was 1.9×10^{-9} ml/cell/hr, which is about twice higher than that of *Synechococcus* NIBB1071 (1.0×10^{-9} ml/cell/hr). After the transfer, cells continued to grow at a rate of 1 division per day with successive dilutions with equal volume of fresh medium containing 1 μM phosphate once every 24hrs (Fig. 6b).

Fig. 6a shows changes in phosphate concentration and the activity for phosphate uptake and lillustrates that the enhancement of phosphate uptake occurs also in *Synechococcus* PCC7942. After 1 day, phosphate concentration decreased to nearly zero, and the activity for phosphate uptake started to increase. The rate constant at this time (ca. 1.1×10^{-8} ml/cell/hr) was about a half of that of *Synechococcus* NIBB1071 (Fig. 5b vs 6a). However, the increase in activity for phosphate uptake further

Fig. 6 Effect of external phosphate concentrations on the inorganic phosphate uptake of *Synechococcus* PCC7942. Cells grown under 11.4 μM were transferred to the medium containing 0.5 μM phosphate. Cells were grown at 25 °C under continuous white fluorescent light of 3.5 W/m², and the culture medium was successively diluted with fresh medium containing 1 μM phosphate once every 24 hrs. a: rate constant of phosphate uptake (closed and open circles indicate rate constants of the initial and the second phases, respectively) and external phosphate concentrations (triangles); b: cell density corrected for respective dilutions.

continued and reached a maximum after 4 days. The time course of phosphate uptake was apparently altered from monophasic to biphasic. Thus, the rate constants were calculated from the first (0-15 min) and the second phases (30-120 min), respectively. Both rate constants increased, but the extent of increase was different. Fig. 7 shows time courses of phosphate uptake by cells before and after (6th day) the shift-down of phosphate. After the enhancement of phosphate-uptake, the maximum rate constant of the first phase (8.4×10^{-8} ml/cell/hr) incerased 44 times which was as high as that of *Synechococcus* NIBB1071, whereas the magnitude of increase in the second phase (3.7×10^{-8} ml/cell/hr) was 19 and one-third of that of latter cells (Fig. 3 vs 6a). The difference between the measured and expected phosphate concentrations after dilution increased with increase in the activity for phosphate uptake, but it was not so great as that observed in *Synechococcus* NIBB1071. It is probably due to lower cell density in this case.

4.4 Discussion

The present study has revealed that the enhanced uptake of phosphate occurs in a marine strain of *Synechococcus* upon decrease in the external phosphate concentration. With this capability, *Synechococcus* can grow under a wide range of nutrient conditions covering both eutrophic and oligotrophic waters. Following evidences indicate that the activity of phosphate uptake is regulated by some biological mechanism but not by

Fig. 7 Time courses of inorganic phosphate uptake by *Synechococcus* PCC7942 grown under a high and a low phosphate conditions. Two μM phosphate was added to cells grown under a high and a low phosphate media. Cell density was 7×10^6 cells/ml in both cases. The time cource of phosphate uptake by the cells grown under a high phosphate medium was found to be approximated to single exponential function. The rate constant was 1.9×10^{-9} ml/cell/hr for the former cells (open circles). For the latter cells (closed circles), the phosphate uptake was approximated biphasic exponential function. The rate constant was 8.4×10^{-8} and 3.7×10^{-8} ml/cell/hr for the intitial (0-15 min) and the second phase (30-120 min), respectively.

changes in rate of physical diffusion induced by changes in the gradient of phosphate concentration between outside and inside of the cell: 1)the enhanced activity of phosphate uptake is energy-dependent process, 2)kinetic characteristics of phosphate uptake changed markedly before and after the activation, 3)the magnitude of activation is so high that it cannot be explained by a simple physical diffusion depending on the gradient between the intra- and the extracellular concentration, and 4)the enhancement of phosphate uptake seems to occur rapidly (within a generation time) upon decrease in phosphate concentration below 0.2 μM (Figs. 5a, 5b). Preliminary experiments for the determination of changes in phosphate uptake upon shift-down to phosphate-free conditions have suggested that the enhancement occurs depending on energy-supply.

Enhancement of phosphate uptake in *Synechococcus* NIBB1071 is accompanied with marked changes in both V_{max} and K_m. Since the affinity index ($\alpha = V_{max}/K_m$) is the initial slope of rectangular hyperbola of functional relationship between the substrate concentration and the uptake rate, α correlates the relative uptake rate under limiting substrate concentration (Button 1978; Healey 1980). The value of α increased nearly 300 times by simultaneous changes in V_{max} and K_m, which indicates that the activity of phosphate uptake is increased especially in the lower range of phosphate concentrations. This type of enhancement of phosphate uptake may fit for the requirement for active growth under very low phosphate concentrations.

The K_m of phosphate uptake determined for *Synechococcus*

NIBB1071 was 0.1 μM. The value is markedly lower than that reported for the marine cyanophyte *Trichodesmium thiebautii* (9.0 μM) (McCarthy and Carpenter 1979) and is comparable to that of high affinity system of *Escherichia coli* (Yagil 1987). These evidences suggest that the uptake system of a marine strain of cyanophyte can utilize phosphate as effectively as bacterial system.

Aksnes and Egge (1991) have proposed a hypothetical model of nutrient uptake process in phytoplankton. According to them, the kinetic parameters can be expressed:

$$V_{max} = n \times h^{-1}$$

$$K_m = (A \times v \times h)^{-1}$$

where n = number of ion uptake sites per cell; h = handling time per ion (s), during which the uptake of another ion is prevented; A = area of one uptake site (m^2); v = mass transfer efficiency (m/s), which is velocity between ions and uptake sites. The combined term of A x v means the affinity of uptake site (Aksnes and Egge 1991). Changes in h mean the changes in the activity of uptake site or the changes in the mechanism of transport of the ion across the membrane (Raven 1980; Aksnes and Egge 1991). Since the increase in V_{max} indicates the increase in n and/or the decrease in h, and since the decrease in K_m indicates the increase in A x v and/or the increase in h, the observed changes in kinetic parameters in *Synechococcus* NIBB1071 imply the following

possibilities: 1)The changes occur in the activity of uptake site, h, and/or 2)The increase occurs in the number of uptake site, n, and/or 3)The induction of different type (of reaction time, h and/or affinity, A x v) of transport system occurs. The enhancement of phosphate uptake activity in cells grown in low phosphate medium cannot be explained only by the possibility 1). The increase in the number of uptake site and in the affinity of uptake site must occur. If it is correct, a new transport system is inducibly developed under low phosphate concentrations.

Similar magnitude of enhancement of phosphate uptake was observed in both *Synechococcus* NIBB1071 and *Synechococcus* PCC7942. It suggests that the enhancement of phosphate uptake is common characteristics in the generic group of *Synechococcus*. The increase in V_{max} of phosphate uptake was also reported for another freshwater strains of *Synechococcus* (*A. nidulans*) (Grillo and Gibson 1979; Falkner et al. 1984). However, the value of K_m did not change by phosphate-limitation, and they have interpreted that differential expression of a single system is involved.

Occurrence of the cases 2) and 3) have been postulated for sulfate uptake in *Synechococcus* PCC7942 by the evidences that synthesis of new polypeptides is specifically induced by sulfate deprivation (Green and Grossman 1988). They have assumed that the induced transport system is not a type of higher affinity to sulfate, since K_m did not change before and after sulfate-deficiency whereas V_{max} increased. Thus, the pattern is different from that in the present case for phosphate uptake. As noted above, marked changes in K_m in the present case cannot be ex-

plained by a simple increase in the number of uptake site (the case 2). Pattern of regulation may be different between phosphate and sulfate uptakes. Further examination is necessary to solve the questions whether the uptake activity is regulated by differential expression of a single uptake system or by development of a new high affinity uptake system, and whether the mechanism is common among cyanophytes and among substrates.

The response to shift-down of external phosphate concentration of *Synechococcus* PCC7942 was rather slower than that of *Synechococcus* NIBB1071 (Fig. 5b vs 6a). This is probably attributed to the difference in internal phosphate pool, such as polyphosphates. Polyphosphates are usually formed under phosphate-replete conditions in cyanophyte (Fogg et al. 1973; Grillo and Gibson 1979) whereas little phosphate was found in polyphosphate fractions in marine *Synechococcus* (Cuhel and Waterbury 1984). A difference between the two strains was also observed in the time course of phosphate uptake in the cells of high activity cell. Biphasic phosphate uptake in *Synechococcus* PCC7942 may be caused by a limitation of intracellular phosphate-assimilation. Further investigations are required to examine such hypotheses.

In this study, it was found that an enhancement of phosphate uptake activity can allow *Synechococcus* NIBB1071 to grow continuously at a relatively high rate such as 1 division per day even after shift-down to low level of phosphate. A quantitative estimation was further made to evaluate the growth rate under natural environments in oligotrophic ocean where the phosphate is kept at low concentrations. Assuming that cellular phosphate content is

1.0×10^{-16} mol P*i*/cell as determined above, the growth rate of either type of cells under a given phosphate concentration can be estimated using kinetic parameters described in Table 1. The phosphate concentration which gives the growth rate of 1 division per day is as low as 7.2×10^{-4} μM in cells grown under low phosphate medium, whereas 0.34 μM in cells grown under high phosphate medium under the assumptions of no leakage of phosphate from the cells and of no changes occurring in the intracellular phosphate content. Results from the present study showed that cellular phosphate content was maintained at a constant level under the phosphate concentrations, at least, higher than 0.26 μM. This tentative estimation also suggests that the enhancement of phosphate uptake may occur below 0.34 μM, at which the rate of phosphate uptake cannot support cell growth. Since the activation occurs when the phosphate concentration becomes less than 0.2 μM (Fig. 5a, 5b), the above estimation is consistent with the observed change. If saturating concentration of phosphate (> 1 μM) is present, it takes only 30 minutes for uptake of phosphate of the amount equivalent to that contained in the cell (Fig. 3). Since the enhanced uptake rate seems to continue longer time than the time length required for uptake of the amount equivalent to cellular phosphate content (Fig. 3), *Synechococcus* NIBB1071 may transiently accumulate a surplus phosphate within the cell. Such an increased nutrient uptake capacity may be advantageous for nutrient accumulation if the cells with high activity meet minute patches with elevated nutrient level in natural habitats (McCarthy and Goldman 1979). Results obtained from the present

study suggest that the enhancement of phosphate uptake could permit the active growth of *Synechococcus* under nanomoles in phosphate in oligotrophic ocean such as Kuroshio area where regenerated phosphate is supplied at a sufficiently rapid rate.

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5 General discussion: characteristics of *Synechococcus* in its autotrophic growth and distribution in natural marine environment

Distribution of a given species of organisms such as phytoplankton in a natural habitat is determined by a series of complicated processes; individual growth, mortality, predation, virulence and physical transportation of cells. Among them, the establishment of growth capability is at least prerequisite for the existence and proliferation. Physiological attributes determine the existence of organisms to a certain range of physical and chemical environmental conditions. Resource availability in a given environment may be possible essential selective factor for the species distribution, because the distribution of resources such as light and nutrients is largely disproportioned: light energy rapidly decreases in waters with the increase in depth, and nutrient availability is low in the surface mixed layer in thermally stratified waters as compared with coastal and upwelling regions (see Chap. 1). The least requirement for *Synechococcus* to distribute vertically from the surface to the bottom of euphotic zone and horizontally from the coastal to the oceanic waters is to overcome the low availability of light and nutrients. The present study was conducted to clarify such physiological characteristics, which permit a wide distribution of *Synechococcus* using isolated strains of *Synechococcus* under simulated marine environment.

Since the maximum absorption of PE locates around 546-569 nm, the marine *Synechococcus* apparently favors green light, which

is the dominant radiant energy penetrating into the base of euphotic zone in coastal waters, but is longer in wavelengths than blue-green light near the bottom of euphotic zone in open ocean such as the Kuroshio area. Thus, it was expected that there occurs preferential excitation of PS I, to which Chl *a* delivers most of energy, and hence quantum efficiency of photosynthesis may decrease because of poor absorption of light energy by PE under the blue-green low radiant environment near the bottom of euphotic zone in open ocean. However, isolated strains of *Synechococcus* from both surface and subsurface waters commonly had a high content of PE; the extinction of PE relative to of Chl *a* in *Synechococcus* was about 2 times as high as that in ordinary freshwater cyanophyte. Based on the fractional absorption of photosynthetic pigments under the simulated blue-green light, BGL, it was found that PBS of *Synechococcus* can absorb 36-44% of total absorbed quanta of BGL because of the high content of PE and presence of PUB in PE. Such a substantial absorption of light-energy by PBS enables *Synechococcus* to maintain a rather high photosynthetic rate even under BGL. Further, comparison of the Emerson's enhancement effect of BGL and the absorption fraction of PS II pigments, PBS and Chl *a* in PS II complex, under BGL has indicated that at least a half of the light-energy absorbed by carotenoids, which is more than 25% of total absorbed quanta, is utilized for PS I excitation. These features indicate that more than 60-70% of total absorbed quanta is converted to biological energy and utilized for growth of *Synechococcus* under BGL. It is then concluded that such photosynthetic characteristics can

extend the range for an active growth of *Synechococcus* toward the base of euphotic zone even in open ocean as well as in coastal region as far as other environmental conditions permit the formation of such a large PBS.

Beneath the secondary (or seasonal) thermocline which often locates near the base of euphotic zone in open ocean, water temperature sharply declines, whereas the concentration of nutrients increases in a reverse way. Fig. 1 shows the effects of temperature on the absorption spectra of *Synechococcus* NIBB1071 grown under BGL at 2 W/m^2. When temperature decreased from 23 to 11 °C, the absorption of PE relative to that of Chl *a* markedly decreased with a significant increase in the absorption at shorter wavelength region where the absorption band of carotenoids is located, when compared with that of 23 °C. However, at 19 °C, which was commonly observed at the base of euphotic zone in the Kuroshio area during the summer stratification (Takahashi et al. 1985; Fujita 1985; Furuya 1990), only a little change was observed in the relative extinction of PE with a slight increase in carotenoids absorption. Growth constants at different temperatures indicate that the decrease due to temperature differences from 27 to 19 °C was not very significant, and a nearly linear decrease with decreasing temperature was observed below 19 °C (Fig. 2). These results further support the idea that *Synechococcus* spp. will maintain active growth down to the base of euphotic zone beneath the thermocline in warm open ocean such as the Kuroshio area (Takahashi and Hori 1984; Takahashi et al. 1989; Furuya 1990).

Fig. 1 Effects of temperature on the absorption spectra of *Synechococcus* NIBB1071. Cells were grown at least for 3 generations in the ASP_7 medium under BGL at 2.0 W/m^2 at 11, 15, 19 and 23 °C, as stated in figure.

Fig. 2 Growth constant of *Synechococcus* NIBB1071 as a function of temperature. Cells were grown at 7, 11, 15, 19, 23, 27, 31 and 35 °C. Otherwise, same as Fig. 1.

One of the isolated strains of *Synechococcus*, NIBB1071, was found to efficiently utilize ambient phosphate from high to low phosphate concentrations. This capability could be essential for the cells to carry out their growth from eutrophic coastal waters to phosphate-depleted surface mixed layer of open ocean. Efficient phosphate uptake system of *Synechococcus* at low concentrations was found to be activated upon the decrease in external phosphate concentration. Phosphate uptake was enhanced nearly 300 times at low phosphate concentrations by development of the rapid uptake system. An estimation of actual phosphate uptake in the activated cells have clearly suggested that *Synechococcus* can accumulate enough phosphate in order to maintain their active growth in oligotrophic open ocean where phosphate is continuously but at low concentrations supplied by the rapid regeneration process in the water (Eppley and Peterson 1979; Harrison 1980; Parsons et al. 1984). Since several other essential nutrients including nitrogenous compounds are also low in concentration in the surface mixed layer of thermally stratified open ocean, possible rapid uptake mechanisms for those nutrients are also required for further evaluation. Since the area of oligotrophic open ocean occupies about 40% of the total area of ocean (Koblentz-Mishke et al. 1970), the rapid uptake capability of low nutrient concentration observed in the present study will substantially contribute for the wide areal geographic distribution of *Synechococcus*. It may also support the formation of large light harvesting pigment complex, PBS, which facilitates efficient light utilization near the base of euphotic zone.

As described above, it has been clarified that the wide distribution of *Synechococcus* particularly for low green and blue-green radiant environment and for high and low nutrient environment is both supported by their unique physiological characteristics on the utilization of light and nutrient. Maintenance of photosynthetic activity even near the bottom of euphotic zone from coastal to oceanic water is realized by the common characteristics of pigmentation in *Synechococcus*, extremely high content of PE. Since the enhancement of phosphate uptake induced by low concentration of ambient phosphate was found not only in the marine *Synechococcus* but also in the freshwater strain *Synechococcus* PCC7942, such an ability seems to be common in the generic group of *Synechococcus*. Thus, it is plausible that the extended distribition of the genus *Synechococcus* toward the bottom of euphotic zone from coastal to open ocean waters is attained not by the occurrence of populations of different species with different characteristics in light and nutrient utilization, but by a population of *Synechococcus*. Occurrence of these two advantageous characteristics in one strain, NIBB1071, supports this idea. However, the culture of *Synechococcus* in the modified 'aquil' medium is successful only a part of isolated strains at present time, since some difference exists in nutritional requirement among the *Synechococcus* strains. It is also plausible that these occur genetically different strains in *Synechococcus* in the aspect of nutritional requirement and that among them, some strains show obligate-oligotrophic nutrient requirement. Question remains as to whether the ability to

expand the distribution toward oligotrophic environments is common in *Synechococcus* or limited to some members of *Synechococcus*.

Among the *Synechococcus* strains used in the present study, we found a clear diversity of phenotype in the chromophore composition of PE, main light-harvesting pigment, and in nutrient requirement. As described in Chap. 2.3.1, strains can be classified into two groups, the one containing PE with chromophores of PEB and PUB, and the other, PE with only PEB. Further, the proportion of PEB to PUB is also somewhat variable among the former strains. These characteristics remain unaltered in respective strains in the successive cultures as far as has been made. As described in Chap. 3.3.2, some of strains cannot grow in the modified 'aquil' medium, suggesting that the strains require some other nutrient(s) which is not contained in this defined medium. Further, the strains growing in the modified 'aquil' medium showed different requirement of ambient phosphate, as represented by the strains, NIBB1071 and Z-11. These characteristics also remain unaltered as far as examined. Such variations suggest that the marine *Synechococcus* strains, which show common phenotypes of cell morphology and pigmentation, a very high content of PE in PBS, do not necessary belong to a single species. Plural species may occur in the marine *Synechococcus*. Taxonomical diversity is another important aspect for understanding the community of *Synechococcus* in the ocean. The possible different activities between strains and the potential role of the diversity of *Synechococcus* in the oceanic environment should

be further investigated. To perform such investigation, it is essential to obtain representatives having respective adaptive forms. However, the present culture technique seems to be selective to a certain extent. A further elaboration for the isolation of such representatives and the scrutiny of culture system are required.

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