Variations in Intracellular Phosphorus and Growth of the Halotolerant Alga *Dunaliella parva* as Influenced by Environmental Conditions

Takahiro SUZUKI[a], Masahiro KUROZUME[b], Yoshiyuki NAKAMURA[b], Francesco POMATI[c], and Brett A. NEILAN[c]

Abstract

The effects of various environmental and culture conditions on the cellular content of phosphorus and growth rate were studied in a halotolerant green alga *Dunaliella parva*, growing in laboratory cultures. The factors considered included the cultivation temperature, light intensity, CO_2 concentration in the flushing gas, medium components (P, NaCl, Ca^{2+}, and N/P atomic ratio), P concentration in the preculture, age of culture, and a light/dark cycle. The intracellular compartments of P-containing molecules, inorganic phosphate, ATP, phospholipids, and polyphosphate, were followed by *in vivo* ^{31}P-nuclear magnetic resonance (NMR) spectroscopy. On the basis of the experimental data, the relative significance of the experimental factors was analyzed by constructing second-order polynomial models which describe the system of the experimental factors and the responses of the algal cells. The models obtained showed that for the cellular P content, the most significant factor was N/P atomic ratio, with P concentration in the preculture, NaCl, CO_2 concentration, and the interaction between temperature and light close next in this order. Just two factors, P and Ca^{2+} concentrations in the medium, were statistically significant for the growth rate at 30℃ under the experimental domain studied. In addition, other two factors, age of culture and the light/dark cycle, were found to have affected considerably algal P content. The *in vivo* ^{31}P-NMR studies showed that the composition of the intracellular phosphate compounds was significantly affected by age of culture. It was found that the length of the chain of the polyphosphates was decreasing with increasing cell age from the observed data indicating the increase in terminal phosphate group and free inorganic phosphate levels.

Key words: *Dunaliella*, Inorganic phosphate, Micro-algae, Photosynthesis, Polyphosphates, ^{31}P-nuclear magnetic resonance (NMR)

[a] Natural Science Laboratory, Toyo University, 11-10, Oka 2, Asaka-shi, Saitama 351-8510, JAPAN
[b] Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan
[c] School of Microbiology and Immunology, The University of New South Wales, Sydney 2052, N. S. W., Australia

1. Introduction

Occurrence of algal blooms and scums, especially if dominated by toxic species, are one of the principal concerns about eutrophication of waters (Ferguson et al., 1996). Phosphorus (P), together with nitrogen, is generally accepted as being the critical nutrient that contributes to the eutrophication of lakes and oceans (Austin, 1988; Kmet and Straskraba, 1989). The significance of P to biological systems is illustrated by its presence in phospholipids and nucleic acids and the turnover time is often very short (Holm-Hansen, 1970). In algal cells, inorganic phosphate acts both as a substrate and as a controlling factor in photosynthesis-related metabolism (Mimura, 1995). Besides, it is well known that phytoplankton are able to store excess P (more than metabolic need) in the form of condensed phosphates, inorganic polyphosphates (Sakshaug et al., 1983), like other microorganisms. Such luxury consumption probably plays a major role in the P cycles in most aquatic ecosystems.

The cycling of P in natural waters is also dependent on the phytoplankton population which in turn is strongly influenced by the growth or climate conditions, in particular temperature and sunlight. Therefore, there is a need of the data of the effects of various environmental factors on algal P content and growth to control the algal populations.

Members of the marine green alga *Dunaliella* (division Chlorophycophyta, order Volvocales, family Polyblepharidaceae) are unicellular, oval or lobed organisms which lack a rigid cell wall. They have the capacity to adapt to a wide range of salt concentrations (0.1−5.5 M NaCl), adjusting to the extracellular osmotic pressure by accumulating glycerol as an osmolyte and compatible solute (Avron, 1986). Besides changes other intracellular factors such as inorganic phosphate, ATP (Belmans & van Laere, 1987), and pH (Goyal et al., 1987) have been reported as possible effectors. Following a hyperosmotic shock, a very rapid increase in the endogenous inorganic phosphate level was observed (Gimmler & Möller, 1981). In general, algal intracellular phosphate levels vary widely, depending on whether the algae are growing under phosphorus-rich or phosphorus-limited environment.

In a previous study (Suzuki et al., 1997) exploring the relative contributions of some environmental factors to the P content in *Dunaliella parva*, laboratory cultures under controlled conditions were performed. The results revealed that the P concentration in the preculture medium was the most significant factor under lower P concentrations in the medium. The effects of light intensity, temperature and NaCl concentration in the medium on the algal P content were not so significant in the system.

The purpose of this study was to obtain more quantitative information on algal P content of *D. parva* during different growth phases. The effects of Ca^{2+} concentration of the medium, CO_2 concentration in the flushing gas, a light-dark cycle, and the algal growth phases on algal P content have been examined in addition to the previous factors explored. Besides, the cultivation allowed us to analyze the time courses of changes in amounts of intracellular phosphate compounds by the application of *in vivo* ^{31}P-NMR studies. ^{31}P-NMR spectroscopy has been used as a useful tool to examine metabolism in algae (Bock et al., 1996; Kuchitsu et al., 1989; Meneses et al., 1993; Mitsumori & Ito, 1984; Ratcliffe, 1994; Vogel, 1988). There have been several reports of the kinetics of the phosphorus and carbon metabolites in the *Dunaliella* species using ^{31}P-NMR (Oren-Shamir, M. et al, 1988; Bental, M. et al., 1988). However, unfortunately, little is known about the effect of culture age on the distribution of the intracellular phosphate compounds in *Dunaliella*.

2. Materials and Methods

2.1. Organisms

The marine microalga *Dunaliella parva* Lerche (IAM C-527) was obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan.

2.2. Experimental system

The alga was grown autotrophically in 0.1 dm^3 cotton-stoppered oblong flat flasks specially manufactured for algal photo-autotrophic growth. Details of the experimental apparatus have been given in the previous studies (Suzuki et al., 1995; Suzuki et al., 1997). The basal mineral medium used was a modified Erd-Schreiber medium (Suzuki et al., 1995) and unless otherwise described, this medium was used in all experiments. The medium contained (per 1 dm^3): NaCl 40.0 g; $MgCl_2 \cdot 6H_2O$ 8.34 g; $MgSO_4 \cdot 7H_2O$ 11.4 g; $CaCl_2 \cdot 2H_2O$ 0.41 g; $NaNO_3$ 0.50 g; KCl 4.17 g; H_3BO_3 0.02 g; K_2HPO_4 0.03 g; $Na_2HPO_4 \cdot 12H_2O$ 0.01 g; $Na_2SiO_3 \cdot 9H_2O$ 0.1 g; NaBr 0.07 g; $FeCl_3 \cdot 6H_2O$ 2 mg; Na_2EDTA 0.05 g; $MnCl_2 \cdot 4H_2O$ 2 mg; $ZnSO_4 \cdot 7H_2O$ 0.2 mg; $(NH_4)_6Mo_7O_{24}$ 0.2 mg; $CuSO_4$ 0.02 mg; $Co(NO_3)_2 \cdot H_2O$ 0.5 mg.

The experiments investigating the effect of P concentration in the medium on the P content of algal biomass were performed by inoculating aliquots of cells that were adapted to four levels of P concentrations in preculture media for over 30 days: reduced phosphate media containing 0.05 and 0.10 mM of P, the standard basal medium containing 0.2 mM of P, and a phosphate-enriched medium containing 0.5 mM of P. Culture pH was adjusted to 7.6.

2.3. Measurement of total P content

The microalga was harvested by centrifugation and washed with water. The slurries obtained were dehydrated in an oven at 80℃ for 2-4 h. Total P in the de-

hydrated alga (0.5−3.0 mg) was analyzed according to the assay method (Bartlett, 1959). All the measurements were carried out in triplicate and the mean values were employed.

2.4. ^{31}P-NMR measurements

^{31}P-NMR spectra were measured on a JEOL JNM-La-500 spectrometer (Tokyo, Japan) operating at 202.35 MHz in the pulsed Fourier transformation mode at 6 ℃. Our preliminary study revealed that the amount of phosphate compounds were very sensitive to temperatures and changeable at around room temperature.

Samples were analyzed with proton broad-band decoupling to eliminate ^{1}H-^{31}P NMR multiplets. Each spectral resonance correspond to a single phosphorus functional group under these conditions Chemical-shift data are reported relative to the standard of 85% inorganic orthophosphoric acid. For the primary internal chemical-shift and concentration standard, tris(2-methoxyphenyl)phosphine($C_{21}H_{21}O_3P$) was employed.

2.5. Preparation of cells for ^{31}P-NMR measurements

Cells were harvested by centrifugation (1,500×*g*, 5 min) after the growth for five days. Then the cells were washed three times by fresh culture medium without K_2HPO_4. Just before the NMR measurements, the cells were suspended in 1 ml of 10% D_2O in an NMR sample tube (diameter, 10 mm) and hold in ethanol at −30℃.

3. Results and Discussion

3.1. Effect of culture age

In the previous study (Suzuki et al., 1997), the algal cells in a late-logarithmic growth phase were harvested and the P contents were measured. Inorganic phosphate in the culture consumed in the photosynthetic reaction is regenerated during starch formation within the chloroplasts (Furbank & Taylor, 1995). Therefore, accumulation of P may be fluctuated in response to age of culture. It was reported that solute concentration (glycerol, Na^+, K^+, Mg^{2+}, Cl^-, and phosphate in the cells) in two strains of *Dunaliella* including *D. parva* vary over a wide range and age of culture was one of the significant factors affecting the solute component (Ginzburg & Ginzburg, 1985).

In order to follow the fluctuations in the cellular content of P, it is necessary to make determinations of P content with time. The results (Fig. 1) confirmed that the age of the algal culture is a key factor affecting the cellular P content. At 168 h cultivation, the P content reached a highest value of 0.77% and then decreased to a value of 0.3% at the end of the experiment at 240 h. It was verified that the harvested cells in the previous study was just around at the cultivation time for the highest P content. For this reason, the subsequent determination of P were

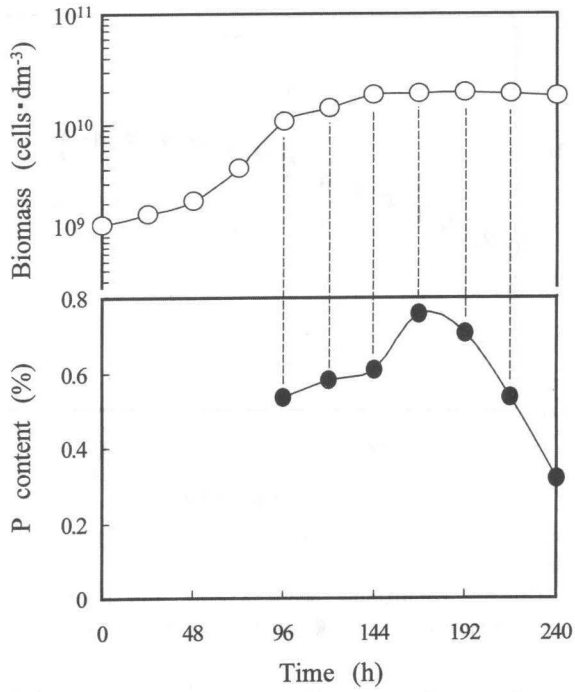

Fig. 1 Variation of intracellular P content (on dry weight basis) of *Dunaliella parva* in relation to the growth curve. Culture conditions: 30℃, light intensity 2 klux (continuous illumination with white fluorescent lamps) and flushing with air.

carried out using the cells in 168 h of culture.

3.2. Algal P content in a light/dark cycle incubation

The effect of an alternating light/dark cycle incubation on the growth and cellular P content was investigated with compared to those under conditions of continuous illumination (Fig. 2). The light-dark cycle is defined as 12 hours light and 12 hours dark. The growth rate under the light-dark cycle conditions fell to just 50% of that under conditions of continuous illumination since there was no increase in biomass in the dark period. The resulting P content of the cells grown in the light/dark incubation was significantly decreased in comparison with that of the cells grown in continuous illumination culture.

From the observation, the P content appears dependent of the growth rate. The effect of growth rate on the cellular P/C ratio has been observed by a laboratory study. In the both case of N-limited and P-limited growth of *Dunaliella tertiolecta*, the cellular P/C ratios increased markedly with increasing growth rates (Goldman et al., 1979). In addition, the behavior of *D. parva* in an alternating light/dark

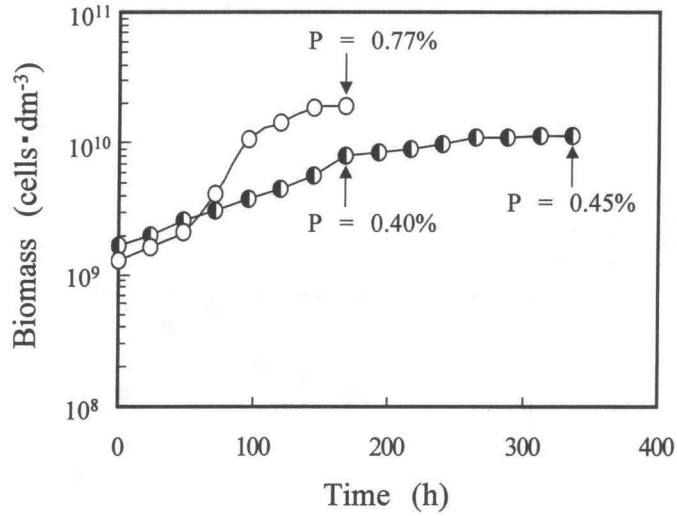

Fig. 2 Comparison of growth curves and P contents of *Dunaliella parva* under a continuous light and an alternating light/dark cycle conditions. Culture conditions: 30℃, 2 klux and flushing with air. Symbols: ○ Continuous light; ◐ 12-h light/dark cycle.

cycle incubation may have some resemblance to that of activated sludge polyphosphate accumulating microorganisms with the phosphorus release in anaerobic phase and uptake in aerobic phase (Wang et al., 1998).

3.3. Effect of P concentrations in preculture and culture media

The effect of P concentration in the preculture medium (P_{pre}) on the P content in the biomass (P_{bio}) was evaluated under two-levels, 0.05 mM and 0.20 mM of P_{pre} in the previous study (Suzuki et al., 1997). The experimental domain was extended to include four-levels, 0.05, 0.1, 0.2 and 0.5 mM of P_{pre}, in this study, and the effects of P_{pre} on P_{bio} and growth were tested with varying P concentrations in the culture (P_{cul}) at 30℃ , 2 klux and flushing with plain air (Fig. 3). The significant changes in P_{bio} due to preculture treatments were verified in lower P_{cul} (<0.5 mM). The algal cells grown in the P-deficient media (including 0.05 mM and 0.1 mM) were characterized by their lower P content when they were cultured with medium containing lower than 0.5 mM of P. The broken lines in this figure show the values of bio-concentration factor (BCF) which is defined as the ratio of P_{bio} to initial P_{cul}. The BCF value attained a maxium value of around 3×10^3 under the experimental conditions tested.

Figure 4 represents the specific growth rates (μ) as a function of P_{cul} for three different levels of P_{pre}: 0.1, 0.2, and 0.5 mM. At around 0.2 mM of P_{cul}, the μ values were maximum and increases P_{cul} resulted in significant inhibition of the algal

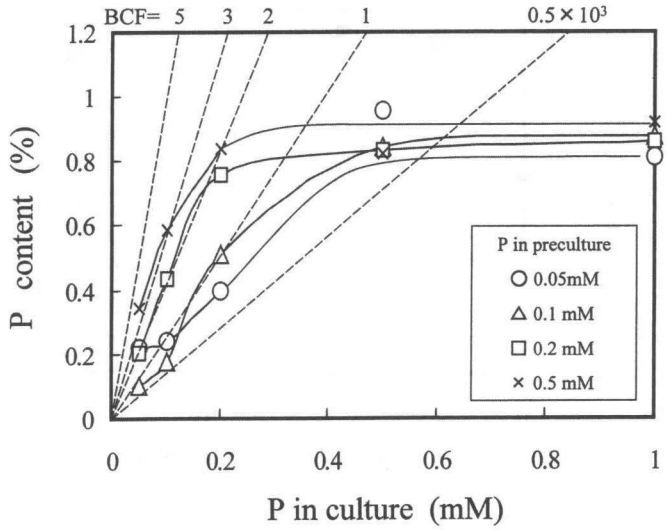

Fig. 3 Effects of P concentrations in both preculture and culture media on the intracellular P content in *Dunaliella parva*. Culture conditions: 30℃, 2 klux and flushing with air.

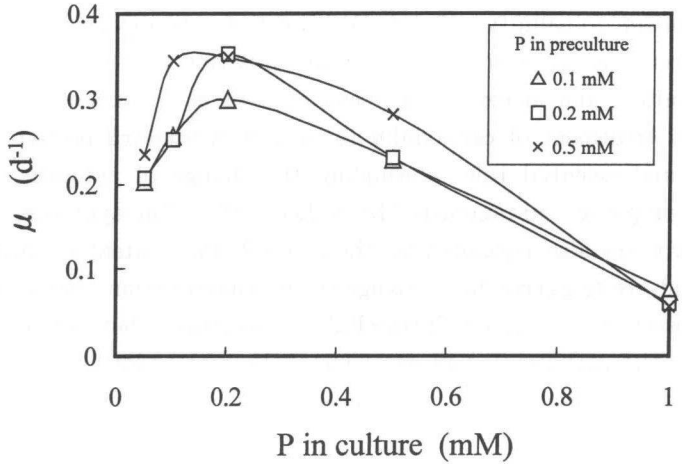

Fig. 4 Effects of P concentrations in both preculture and culture media on the growth of *Dunaliella parva*. Culture conditions: 30 ℃, 2 klux and flushing with air.

growth over the P range 0.2–1.0 mM. There were relatively small differences between the μ values at three levels of P_{pre}.

3.4. Effect of Ca^{2+} concentration

Ca^{2+} ion is an important factor in the stress response of the halotolerant green

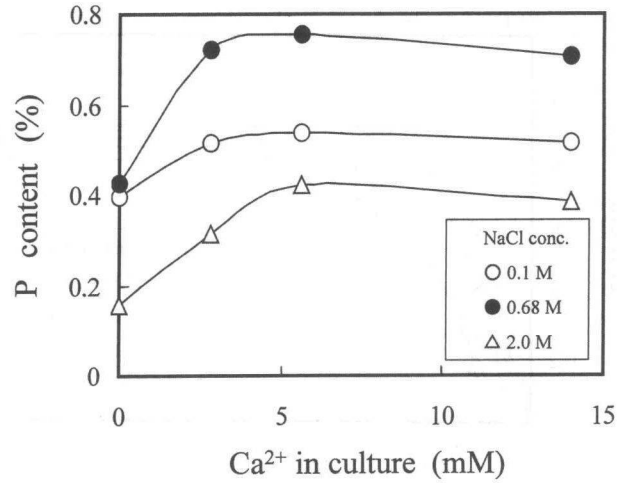

Fig. 5 Effect of Ca^{2+} concentration on intracellular P content of *Dunaliella parva* in relation to NaCl concentration in the culture. Culture conditions: 30℃, 2 klux and flushing with air.

alga of *Dunaliella* to salinity. *Dunaliella* adjusts to the external salinity by accumulating glycerol as an osmolyte. The osmoregulation is affected by extracellular Ca^{2+} concentration, and increase of glycerol content as an osmoticum is inhibited about 50% by treatment of calmodulin (a calcium modulated protein which plays a significant and essential role in coupling the change of cytosolic Ca^{2+} level to physiological responses) antagonists (Ko & Lee, 1995). The synthesis and degradation of glycerol are also regulated by the intracellular content of inorganic polyphosphate, and are triggered by a change in its concentration (Bental et al., 1990). The above observations suggest intracellular associations between Ca^{2+} and polyphosphates in the pathway for glycerol metabolism. In addition, Ca^{2+} has a positive role in the photosynthetic oxygen evolution and dark oxygen uptake in *Dunaliella* cells (Issa, 1996).

The effect of Ca^{2+} concentration on algal P content and growth was tested under three different levels of NaCl concentration at the same time (Fig. 5). The results demonstrate that the concentration of Ca^{2+} markedly affect the P content in the Ca^{2+} concentration range of less than 5 mM. In contrast, the specific growth rate observed is shown as a function of Ca^{2+} concentration in the culture (Fig. 6). At a lower Ca^{2+} concentration (2.8 mM) the specific growth rate was relatively higher. The variations of the specific growth rate seemed largely unaffected by NaCl concentration with compared to the result in Fig. 5.

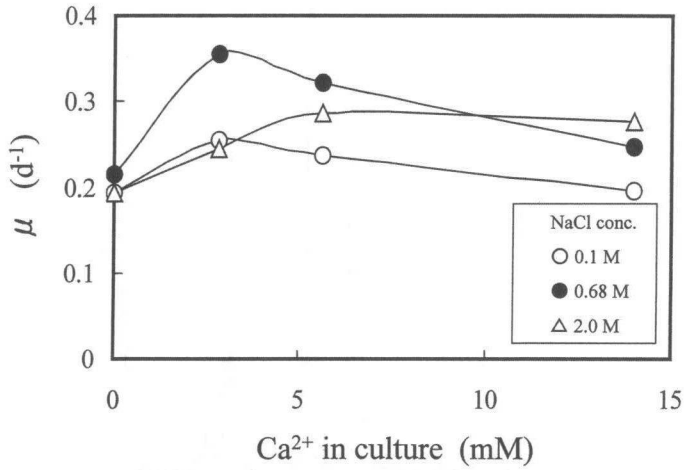

Fig. 6 Effect of Ca^{2+} concentration on the specific growth rate of *Dunaliella parva* in relation to NaCl concentration in the culture. Culture conditions: 30℃, 2 klux and flushing with air.

3.5. ^{31}P-NMR spectra of intact cells

^{31}P-NMR enables us to follow changes in intracellular ATP (adenosine triphosphate), inorganic phosphate (P_i), sugar phosphates and polyphosphates. The amount of phosphate compounds was estimated by comparing the signal intensity with the signal of external standard, and expressed in % relative to that in the control cells.

Figure 7 is a ^{31}P-NMR spectra of cells cultured at 30℃, light intensity 2 klux and flushing with air for 168 h. Under the culture conditions, the following prominent resonances appear in the ^{31}P-NMR spectrum: (a) phospholipids (2.8 ppm); (b) extracellular inorganic phosphate, PO_4 (1.19 ppm); (c) intracellular inorganic phosphate, PO_4 (0.59 ppm); (d) two terminal phosphate groups of polyphosphate chains, $\underline{P}$-$(P)_n$-$\underline{P}$, and γ-ATP (−6.31 ppm); (e) α-ATP (−10.0 ppm); (f) the middle phosphate group of tripolyphosphate, $P\underline{P}P$ (−18.39 ppm); (g) β-ATP (−20.13 ppm); (h) the core phosphate residues of polyphosphate chains, P-$(\underline{P})_n$-P (−22.0 ppm); and (i) tris(2-methoxyphenyl)phosphine (−35.17 ppm). The peak assignments were consulted to the work by Bental et al. (1988) on intracellular phosphorus and carbon metabolites in the algal cells of *Dunaliella salina*.

To observe the changes in the above phosphate compartments at different culture age, measurements were carried out for the samples harvested at 120 h, 240 h, and 312 h, respectively, in addition to the data (Fig. 7) at 168 h. The time dependent change of the spectra is shown in Fig. 8. The difference of the magnitude of the

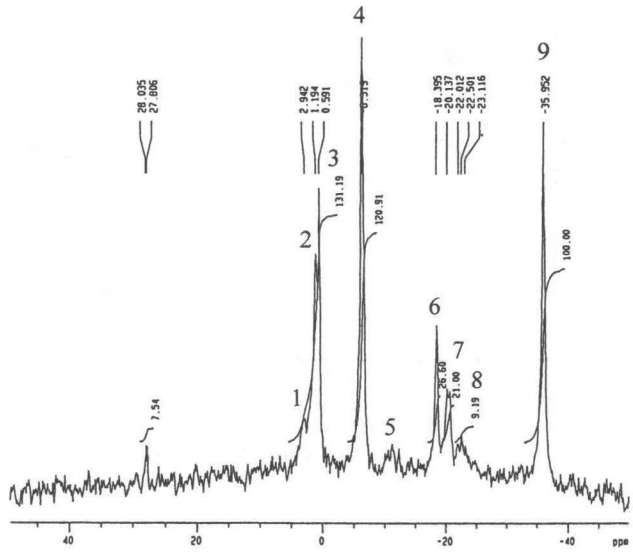

Fig. 7 202.35 MHz ^{31}P–NMR spectra of intact autotrophically grown *Dunaliella parva* cells at 30℃, light intensity 2 klux and flushing with air for 168 h. Spectral assignments are 1, phospholipids; 2, extracellular inorganic phosphate (PO_4); 3, intracellular inorganic phosphate (PO_4); 4, two terminal phosphate groups of polyphosphate chains (P–(P)$_n$–P) and γ-ATP; 5, α-ATP; 6, the middle phosphate of tripolyphosphate (PPP); 7, β-ATP; 8, the core phosphate residues of polyphosphate chains (P–(P)$_n$–P); 9, tris(2-methoxyphenyl) phosphine. External inorganic phosphates were removed by washing cells with phosphate free medium three times.

signals of external tris(2-methoxyphenyl)phosphine at −35.17 ppm means the difference of the concentrations of the biomass measured. The intracellular distribution of phosphorus compounds are summarized in Table 1. In 120 h cells (at late logarithmic growth phase), the most dominant form is P–(P)$_n$–P. Subsequently, the amount of the core phosphate residues decreased and terminal phosphate group levels in polyphosphate chains P–(P)$_n$–P or PO_4 increased, suggesting the breakdown of long-chain polyphosphate molecules into low-molecular shorter chains and free inorganic phosphate, during the stationary growth phase after 168 h of cultivation.

3.6. Statistical evaluation

All of the experimental conditions tested and the two responses, total P content in biomass and specific growth rates, which have been discussed above are listed (Table 2; nos. 18–50), together with those obtained in the previous study (nos. 1–17).

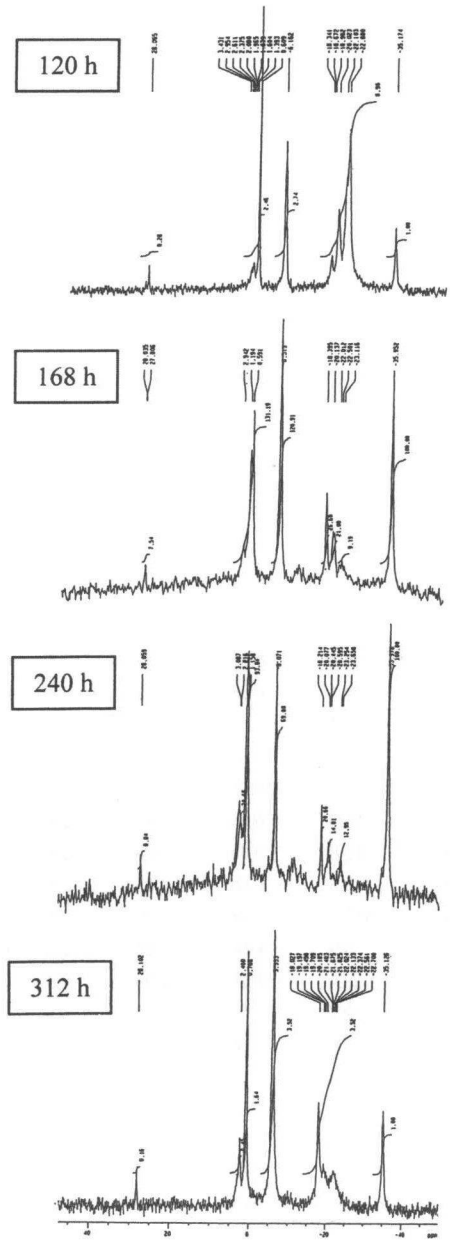

Fig. 8 Comparison of ^{31}P–NMR spectra of autotrophically grown *Dunaliella parva* cells at different culture time. Experimental conditions as described for Figure 1. The resulting growth curve was shown in Figure 2.

Table 1 Percentage of P-containing components in *Dunaliella parva* cells at different culture time, on dry weight basis.

Component	Culture time			
	120 h	168 h	240 h	312 h
phospholipids (%)	3.8	6.8	10.4	4.9
PO_4 (%)	13.5	32.0	34.0	18.0
P-$(P)_n$-P and γ-ATP (%)	19.4	35.7	25.4	38.6
α-ATP (%)	0	5.6	12.6	0
PPP (%)	5.1	7.8	7.5	17.0
β-ATP (%)	14.5	6.2	5.4	8.7
P-$(P)_n$-P (%)	43.7	5.9	4.7	12.8

Table 2 Summary of experimental conditions and responses

Run no.	Experimental conditions								Responses	
	Temp. (℃)	Light (klux)	P conc. (mM)	N/P ratio (atom/atom)	NaCl (mM)	P in preculture (mM)	Ca^{2+} (mM)	CO_2 (%)	P cont. (%)	Specific growth rate (d^{-1})
1.	35	7	0.20	29.4	0.68	0.05	2.8	0.03	0.35	–
2.	35	2	0.20	29.4	0.68	0.05	2.8	0.03	0.59	–
3.	25	7	0.20	29.4	0.68	0.05	2.8	0.03	0.52	–
4.	25	2	0.20	29.4	0.68	0.05	2.8	0.03	0.60	–
5.	30	4	0.20	29.4	0.68	0.05	2.8	0.03	0.58	–
6.	30	1	0.20	29.4	0.68	0.05	2.8	0.03	0.40	0.356
7.	30	2	0.05	117.6	0.68	0.05	2.8	0.03	0.22	0.260
8.	30	2	0.10	58.8	0.68	0.05	2.8	0.03	0.24	0.412
9.	30	2	0.50	11.8	0.68	0.05	2.8	0.03	0.96	0.325
10.	30	2	1.00	5.9	0.68	0.05	2.8	0.03	0.81	0.200
11.	30	2	0.05	117.6	0.68	0.20	2.8	0.03	0.89	–
12.	30	2	1.00	5.9	0.68	0.20	2.8	0.03	1.14	–
13.	30	2	0.20	29.4	0.10	0.05	2.8	0.03	0.79	0.187
14.	30	2	0.20	29.4	0.30	0.05	2.8	0.03	0.47	0.242
15.	30	2	0.20	29.4	0.68	0.05	2.8	0.03	0.40	0.350
16.	30	2	0.20	29.4	2.00	0.05	2.8	0.03	0.48	0.232
17.	30	4	0.05	117.6	0.68	0.05	2.8	0.03	0.24	–
18.	30	2	0.05	117.6	0.68	0.10	2.8	0.03	0.10	0.203
19.	30	2	0.10	58.8	0.68	0.10	2.8	0.03	0.18	0.256
20.	30	2	0.20	29.4	0.68	0.10	2.8	0.03	0.51	0.300
21.	30	2	0.50	11.8	0.68	0.10	2.8	0.03	0.84	0.228
22.	30	2	1.00	5.9	0.68	0.10	2.8	0.03	0.87	0.074
23.	30	2	0.05	117.6	0.68	0.20	2.8	0.03	0.20	0.207
24.	30	2	0.10	58.8	0.68	0.20	2.8	0.03	0.44	0.252
25.	30	2	0.20	29.4	0.68	0.20	2.8	0.03	0.76	0.353
26.	30	2	0.50	11.8	0.68	0.20	2.8	0.03	0.83	0.231

Table 2 Continued

Run no.	Experimental conditions								Responses	
	Temp. (℃)	Light (klux)	P conc. (mM)	N/P ratio (atom/atom)	NaCl (mM)	P in preculture (mM)	Ca^{2+} (mM)	CO_2 (%)	P cont. (%)	Specific growth rate (d^{-1})
27.	30	2	1.00	5.9	0.68	0.20	2.8	0.03	0.86	0.061
28.	30	2	0.05	117.6	0.68	0.50	2.8	0.03	0.34	0.235
29.	30	2	0.10	58.8	0.68	0.50	2.8	0.03	0.59	0.344
30.	30	2	0.20	29.4	0.68	0.50	2.8	0.03	0.84	0.349
31.	30	2	0.50	11.8	0.68	0.50	2.8	0.03	0.82	0.282
32.	30	2	1.00	5.9	0.68	0.50	2.8	0.03	0.92	0.058
33.	30	10	0.20	29.4	0.68	0.20	2.8	0.03	0.49	0.283
34.	30	2	0.20	29.4	0.68	0.20	2.8	1.00	0.76	0.326
35.	30	2	0.20	29.4	0.68	0.20	2.8	8.00	0.82	0.374
36.	30	2	0.20	29.4	0.68	0.20	2.8	15.00	0.80	0.314
37.	30	10	0.20	29.4	0.68	0.20	2.8	6.00	0.56	0.336
38.	30	2	0.20	29.4	0.10	0.20	0.0	0.03	0.40	0.194
39.	30	2	0.20	29.4	0.10	0.20	2.8	0.03	0.52	0.254
40.	30	2	0.20	29.4	0.10	0.20	5.6	0.03	0.54	0.237
41.	30	2	0.20	29.4	0.10	0.20	14.0	0.03	0.52	0.197
42.	30	2	0.20	29.4	0.68	0.20	0.0	0.03	0.43	0.216
43.	30	2	0.20	29.4	0.68	0.20	2.8	0.03	0.72	0.355
44.	30	2	0.20	29.4	0.68	0.20	5.6	0.03	0.76	0.322
45.	30	2	0.20	29.4	0.68	0.20	14.0	0.03	0.71	0.248
46.	30	2	0.20	29.4	2.00	0.20	0.0	0.03	0.16	0.195
47.	30	2	0.20	29.4	2.00	0.20	2.8	0.03	0.32	0.246
48.	30	2	0.20	29.4	2.00	0.20	5.6	0.03	0.42	0.286
49.	30	2	0.20	29.4	2.00	0.20	14.0	0.03	0.38	0.277
50.	30	2	0.20	29.4	2.00	0.20	2.8	0.03	0.79	0.347

It is assumed that the observed responses can be expressed by using second-order polynomials:

$$Y_i = a_0 + \sum_{i=1}^{8} a_i x_i + \sum_{i=1}^{7} \sum_{j=2}^{8} a_{ij} x_i x_j + \sum_{i=1}^{7} a_{ii} x_i^2 \qquad (1)$$

where Y_i are the dependent variable (i.e. either P content in biomass or specific growth rates), x_i the normalized experimental factors; x_1=temperature, x_2=light intensity, x_3=P concentration in the medium, x_4=N/P atomic ratio in the medium, x_5=NaCl concentration in the medium, x_6=P concentration in the preculture medium, x_7=Ca^{2+} concentration, and x_8=CO_2 concentration in the flushing gas, and a_0……a_{ii} regression coefficients calculated from the experimental data. The normalized, dimensionless variables x_i were obtained based on the following formula where the minimum value of a factor (u_i(min)) is set to zero and the values of the factors are divided by the range of the factor:

$$x_i = \{u_i - u_i(\min)\} / \{u_i(\max) - u_i(\min)\} \quad (2)$$

The following statistically significant model with the correlation coefficient of 0.897 and the root mean squares error of 0.10 could be obtained for P content in biomass (Y_1):

$$Y_1 = -1.687\, x_4 + 0.188\, x_6 + 0.237\, x_8 + 1.01\, x_4^2 - 0.208\, x_5^2 - 0.160\, x_1 x_2 + 0.89 \quad (3)$$

Five data points (nos. 9, 11, 38, 46, and 50) were removed in the regression as outliers or exceptions, although the reason is not known. The Eq. (3) contains only 6 terms (others were insignificant from statistical test) and the resulting relative significance of the terms was in the following order from F test: $x_4 > x_4^2 > x_6 > x_5^2 > x_8 > x_1 x_2$.

For specific growth rates (Y_2) at 30℃, the following equation was obtained for 35 data points (seven data points, nos. 8, 10, 13, 16, 38, 42, and 46 were left out as outliers) after neglecting the statistically insignificant terms:

$$Y_2 = 0.387\, x_3 - 0.078\, x_7 - 0.595\, x_3^2 + 0.28 \quad (4)$$

The equation gave the correlation coefficient of 0.847 and the root mean squares error of 0.04. The resulting relative significance of the terms was in the following order from F test: $x_3^2 > x_3 > x_7$. The non-linear dependence of Y_2 on x_3 and the negative coefficient for x_7 could be expected from the results of Figs. 4 and 6, respectively.

4. Conclusions

To determine the effects of some environmental or culture conditions in various combinations on both the cellular P content and growth rate of *D. parva*, laboratory cultures under controlled conditions were performed. The temperature, light intensity, CO_2 concentration in the flushing gas, some medium components (P, NaCl, Ca^{2+}, and N/P atomic ratio), P concentration in the preculture, age of culture, and a light dark cycle were chosen as the significant factors for the cellular P content and growth rate.

The P content of the algal cells varied, depending on experimental conditions as well as upon physiological state, between 0.1 and 1.2% on dry weight basis. The cells at the late-logarithmic growth phase accumulated the highest amount of P. The most abundant form of the intracellular P at the age is the core phosphate residues of long polyphosphate chains from ^{31}P-NMR data. The stored long-chain polyphosphate was found to be hydrolyzed into either shorter chains or free inorganic phosphate in the algal cells in older stationary cultures. The cells cultured

in a 12-h light/dark cycle had a markedly reduced P content in comparison with a continuously illuminated control.

Non-linear mathematical models were developed to examine the relative effects of the factors on the cellular P content and the specific growth rate, respectively. Within the range of the factors tested, all factors, except P and Ca^{2+} concentrations in the medium exhibited significant effects on the P content. On the other hand, just two factors, P and Ca^{2+} concentrations in the medium, were statistically significant for the growth rate at 30℃.

Acknowledgements

This work was supported by a Grant-in Aid for Scientific Research (Project No. 09650841) from the Ministry of Education, Science, Sports and Culture of Japan.

References

Austin, B., 1988. Marine Micobiology, Cambridge University Press, Cambridge, Chap. 5, pp. 99-102.

Avron, M., 1986. The osmotic components of halotolerant alga. *Trends Biochem. Sci.* 11, 5-6.

Bartlett, G. R., 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234, 466-468.

Belmans, C., van Laere, A., 1987. Glycerol cycle enzymes and intermediates during adaptation of *Dunaliella tertiolecta* cells to hyperosmotic stress. *Plant Cell Environ.* 10, 185-190.

Bental, M., Oren-Shamir, M., Avron, M., Degani, H., 1988. ^{31}P and ^{13}C-NMR studies of the phosphorus and carbon methabolites in the halotolerant alga, *Dunaliella salina*. *Plant Physiol.* 87, 320-324.

Bental, M., Pick, U., Avron, M., Degani, H., 1990. The role of intracellular orthophosphate in triggering osmoregulation in the alga *Dunaliella salina*. *Eur. J. Biochem.* 188, 117-122.

Bock, C., Jacob, A., Kirst, G. O., Leibfritz, D., Nayer, A., 1996. Methabolic changes of the Antarctic alga *Prasiola crispa* subjected to water stress investigated by *in vivo* ^{31}P NMR. *J. Exp. Botany* 47, 241-249.

Ferguson, A. J. D., Pearson, M. J., Reynolds, C. S., 1996. Eutrophication of Natural Waters and Toxic Algal Booms. In: R. E. Hester and R. M. Harrison (eds), Agricultural Chemicals and the Environment, The Royal Society of Chemistry, Cambridge, 1996, pp. 27-41.

Furbank, R. T., Taylor, W. C., 1995. Regulation of photosynthesis in C_3 and C_4 plants: a molecular approach. *Plant Cell* 7, 797-807.

Gimmler, H., Möller, E.-M., 1981. Salinity-dependent regulation of starch and glycerol metabolism in *Dunaliella parva*. *Plant Cell Environ*. 4, 367-375.

Ginzburg, M., Ginzburg, B. Z., 1985. Influence of age of culture and light intensity on solute concentrations in two *Dunaliella* strains. *J. Exp. Bot*. 36, 701-712.

Goldman, J. C., McCarthy, J. J., Peavey, D. G., 1979. Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature* 279, 210-215.

Goyal, A., Brown, A. D., Gimmler, H., 1987. Regulation of salt-induced starch degradation

in *Dunaliella tertiolecta*. *J. Plant Physiol.*, 127, 77–96.
Holm-Hansen, O., 1970. ATP levels in algal cells as influenced by environmental conditions. *Plant Cell Physiol*. 11, 689–700.
Issa, A. A., 1996. The role of calcium in the stress response of the halotolerant green alga *Dunaliella bardawil* Ben-Amotz et Avron. *Phyton* (Horn, Austria) 36, 295–302.
Kmet, T., Straskraba, M., 1989. Global behavior of a generalized aquatic ecosystem model. *Ecological Modelling* 45, 95–110.
Ko, J. H., Lee, S. H., 1995. Role of calcium in the osmoregulation under salt stress in *Dunaliella salina*. *J. Plant Biol.* 38, 243–250.
Kuchitsu, K., Katsuhara, M., Miyachi, S., 1989. Rapid cytoplasmic alkalization and dynamics of intracellular compartmentation of inorganic phosphate during adaptation against salt stress in a halotolerant unicellular green alga Dunaliella tertiolecta: ^{31}P-nuclear magnetic resonance study. *Plant Cell Physiol.* 30, 407–414.
Meneses, P., Navarro, J. N., Glonek, T., 1993. Algal phospholiphids by ^{31}P NMR: comparing isopropanol pretreatment with simple chloroform/methanol extraction. *Int. J. Biochem.*, 25, 903–910.
Mimura, T., 1995. Homeostasis and transport of inorganic phosphate in plants. *Plant Cell Physiol*. 36, 1–7.
Mitsumori, F., Ito, O., 1984. Phosphorus-31 nuclear magnetic resonance studies of photosynthesizing Chlorella. *FEBS Letters*, 174, 248–252.
Oren-Shamir, Avron, M., Degani, H., 1988. *In vivo* NMR studies of the alga *Dunaliella salina* embedded in beads. *FEBS Letters*, 233, 124–128.
Ratcliffe, R. G., 1994. *In vivo* NMR studies of higher plants and algae. *Advances in Botanical Research* 20, 44–123.
Sakshaug, E., Andersen, K., Myklestad, S., Olsen, Y., 1983. Nutrient status of phytoplankton communities in Norwegian waters (marine, brackish, and fresh) as revealed by their chemical composition. *J. Plankton Res*. 5, 175–196.
Suzuki, T., Matsuo, T., Ohtaguchi, K., Koide, K., 1995. Gas-sparged bioreactors for CO_2 fixation by *Dunaliella tertiolecta*. *J. Chem. Tech. Biotechnol.* 62, 351–358.
Suzuki, T., Imai, M., Ishida, M., 1997. Influence of environmental factors on phosphorous content in the marine microalga *Dunaliella*. *Toxicol. Environ. Chem.* 64, 173–181.
Wang, B., Li, J., Wang, L., Nie, M., Li, J., 1998. Mechanism of phosphorus removal by SBR submerged biofilm system. *Wat. Res.* 32, 2633–2638.