

Biomass production and biochemical variability of the marine microalga *Dunaliella tertiolecta* (Butcher) with high nutrient concentrations

Fabregas, J., Herrero, C., Abalde, J., Liaño, R., Cabezas, B.

Departamento de Microbiología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

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ABSTRACT

Mass cultures of *Dunaliella tertiolecta* were carried out in 10-l flasks with four nutrient concentrations in order to obtain a maximum biomass production and to find out its biochemical variability. Using these cultural conditions equations were calculated by a multiple non-linear least squares regression of order four, enabling predictions to be made of growth kinetics and chemical composition.

Maximum cellular densities between 12.45×10^6 and 14.23×10^6 cells/ml were obtained with 4, 8 and 16 mM of NaNO_3 . Growth velocity ranged between 0.50 and 0.61 doublings/day.

Protein content reached maximum values in the stationary phase of 442 $\mu\text{g/ml}$ and 31 pg/cell at 16 mM of NaNO_3 . In the logarithmic phase protein concentration per cell was not related to nutrient concentration. Maximum efficiencies of transformation from nitrate to protein were 100%, obtained at 2 and 4 mM of NaNO_3 .

Chlorophyll a/cell reached values between 1.03 and 1.95 pg/cell in the stationary phase. There was no relationship between nutrient concentration and chlorophyll a/cell in the logarithmic phase, the values for the different nutrient concentrations being very similar.

The maximum value of carbohydrates, 185 µg/ml, was obtained with 4 mM of NaNO₃. Carbohydrates/cell reached the maximum values of 8.94 and 10.05 pg/cell with 8 and 16 mM of NaNO₃, respectively, in the stationary phase. RNA/cell ranged from 4.28 to 5.40 pg/cell in the logarithmic phase and from 4.72 to 5.80 pg/cell in the stationary. The level of DNA/cell was constant in all the nutrient concentrations tested and in both growth phases, and ranged from 0.05 to 0.12 pg/cell.

Great variability in the chemical composition of *D. tertiolecta* has been shown. Growth in mass cultures is closely coupled to changes in nutrient concentration, and variations occur in protein, chlorophyll *a*, carbohydrates and RNA content, showing differences of 197%, 255%, 142% and 150%, respectively. This biochemical variability must have a marked effect on the value of this microalga as source of single cell protein, chemicals or as feed in mariculture.

INTRODUCTION

Dunaliella tertiolecta (Butcher) is a biflagellate unicellular green alga with a cell structure typical of the members of the order Volvocales (class Chlorophyceae) (Hoshaw and Maluf, 1981), though lacking a cell wall (Oliveira et al., 1980). It is normally found in marine habitats, where it can survive in a wide range of salinities (Ben-Amotz and Avron, 1973; Ben-Amotz et al., 1982; Gilmour et al., 1985).

The cultivation of microalgae is a part of the technology of growing marine molluscs, crustaceans and fish. Much attention has been given to algae of the genus *Dunaliella*. One of the merits of these algae is the absence of a thick cellulose membrane (Oliveira et al., 1980). Besides its use in aquaculture, *D. tertiolecta* is useful in the production of chemicals, e.g. glycerol, β-carotene and high-protein material (Ben-Amotz and Avron, 1973, 1978, 1980; Gibbs and Duffus, 1976; Ben-Amotz et al., 1982), as a source of single-cell protein (SCP) (Fabregas and Herrero, 1985) and as a source of minerals in fish diets (Fabregas and Herrero, 1986). Mass culture of the organism and improved knowledge of its composition, growth and chemical variability lead to its better use and allow high algal yields to be obtained.

We report here the response of a mass culture of *Dunaliella tertiolecta* to a series of nutrient concentrations. Data are presented on its chemical composition under the different conditions and during the different growth phases. The work was done in order to establish the conditions required for maximum production, to predict the response of the organism and to estimate its biochemical variability, since this variability can affect its nutritive and commercial value when this species is used as feed in mariculture, as SCP or in the production of chemicals.

MATERIALS AND METHODS

The marine microalga *Dunaliella tertiolecta* was obtained from The Culture Centre for Algae and Protozoa, Cambridge, England. It was cultured in seawater which had been filtered through a 0.45 µm Millipore filter, autoclaved at 120°C for 60 min, and enriched with NaNO₃, 2 mM; NaH₂PO₄, 100 µM; ZnCl₂, 1 µM; MnCl₂, 1 µM; Na₂MoO₄, 1 µM; CoCl₃, 0.1 µM; CuSO₄, 0.1 µM; ferric citrate, 20 µM; thiamine, 35 µg/l; biotin, 5 µg/l; B₁₂, 3 µg/l; EDTA, 26.4 µM; Tris-HCl, 5 mM. Salinity of the seawater was 35‰ and the initial pH of the cultures was 7.6.

The nutrient solution with the composition given above was the first to be used. From this we followed a geometrical progression, using concentrations corresponding to 4, 8 and 16 mM of NaNO₃. Nutrient concentrations are expressed as NaNO₃ concentrations, but all the other nutrients were increased proportionally.

Culture conditions were established in order to obtain a maximum biomass production and to ensure that light, pH and carbon source did not become limiting, even with high cellular densities.

Cultures were contained in 10-1 flasks with 9 l of culture medium. All cultures were maintained in a controlled environment incubator at 15 ± 1°C and illuminated with 11 fluorescent lamps (Osram daylight L55/10), five of which were placed under the flasks and six alongside them. A light:dark regime of 12 h :12 h was maintained in order to obtain synchronous cultures. An inoculum of 6 X 10⁵ logarithmic phase cells/ml was used. Cultures had air continuously bubbled through them at a rate of 15 l/min

Cellular density was determined by counting culture aliquots in a Thoma chamber.

Chlorophylls were extracted in acetone-methanol 2:1 at 4°C for 48 h. The extracts were filtered through a Fluoropore Millipore filter (Fabregas et al., 1984b), and the absorbance of the pigment extract at specific wavelengths was recorded. The concentration of chlorophyll *a* was determined by the formula of Parsons and Strickland (1965).

Protein and carbohydrates were measured in the crude extract obtained after collecting the microalgal cells by centrifugation, resuspending them in distilled water and breaking them in an ultrasonic disintegrator. After sonication the extracts were centrifuged again, the pellets were discarded and protein and carbohydrates were measured in the supernatants. Protein was measured by the dye-binding method (Bradford, 1976) and carbohydrates by the phenol-sulfuric acid method (Kochert, 1978a).

Nucleic acids were extracted with perchloric acid, and ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined as described by Kochert (1978b).

Stationary phases were compared by an overall multivariate one-way analysis of variance (ANOVA) and logarithmic phases were compared by a oneway analysis of covariance (ANCOVA).

A multiple non-linear least squares regression of order 4 was applied to all the curves. The resultant equations were as follows:

$$f(t) = a + bt + ct^2 + dt^3 + et^4$$

where $f(t)$ is cellular density or proteins/ml or carbohydrates/ml or chlorophyll α /ml, t is time in days, and a, b, c, d and e are the coefficients of the equation.

From the growth equation we calculated doublings/day:

$$\text{dbls/day} = t_d^{-1} = \frac{\ln f(t_n) - \ln f(t_i)}{\ln 2 (t_n - t_i)}$$

where t_i and t_n are the initial and final time of the logarithmic phase, both expressed in days, and t_d is the duplication time.

RESULTS AND DISCUSSION

The uptake of NaNO_3 during photosynthesis generates alkalinity (Goldman et al., 1972; Brewer and Goldman, 1976). In our growing system, at low CO_2 concentrations (0.03% V/V in the air), an air flow of 15 l/min maintained a transference of CO_2 to the culture medium that kept the pH within the optimum range for *D. tertiolecta* (Spectorova et al., 1982). In addition, these culture conditions ensure that the carbon source did not become limiting. Grown at low CO_2 concentrations (0.03% V/V in the air) green and bluegreen algae are much more efficient in utilizing inorganic carbon from the medium as compared with cells grown at high CO_2 concentrations (5% V/V in the air) (Zenvirth and Kaplan, 1981).

TABLE 1

Statistical analysis (ANOVA) of the growth curves of *D. tertiolecta* at different nutrient concentrations in mass culture. Each value corresponding to a nutrient concentration is compared only with the succeeding concentration.

	Growth phase	P	Nutrient concentration ^a						
			2 mM	4 mM	8 mM	16 mM			
Cell/ml·10 ⁶	Stationary	0.001	10.14 ± 0.84	<	12.45 ± 0.30	=	14.12 ± 1.20	=	14.23 ± 0.58
Days interval ($t_n - t_i$)			5-2		6-2		7-2		13-3
Doublings/day	Logarithmic	0.001	0.61	=	0.61	>	0.50	=	0.50
Protein ($\mu\text{g/ml}$)	Stationary	0.001	173.4 ± 7.77	<	342.6 ± 9.89	<	400.0 ± 6.75	<	442.0 ± 18.71
Chlorophyll α ($\mu\text{g/ml}$)	Stationary	0.001	10.49 ± 0.60	<	23.57 ± 1.90	=	26.67 ± 1.75	=	25.20 ± 0.84
Carbohydrates ($\mu\text{g/ml}$)	Stationary	0.001	153.71 ± 8.96	<	185.00 ± 8.52	>	130.80 ± 3.03	=	136.75 ± 4.86
Efficiency (%)	Stationary	0.001	104		100		59		32

^aExpressed as NaNO_3 concentration.

The growth kinetics model (cells/ml) of the mass culture of *D. tertiolecta* was similar to one of mass culture of *T. suecica* (Fabregas et al., 1985b). We plotted cellular density, protein ($\mu\text{g/ml}$), chlorophyll *a* ($\mu\text{g/ml}$) and carbohydrates ($\mu\text{g/ml}$) against time for each nutrient concentration, obtaining three-dimensional figures (Figs. 1, 2, 3, 4). Statistical treatment of these figures is presented in Table 1. From the equations calculated, we can establish the growth kinetics for each culture and predict the growth of the microalgal population. The values of *a*, *b*, *e*, *d* and *e* are presented in Table 3. From this equation we calculated doublings/day or the inverse of the duplication time (t^{-1}_d). The initial time (t_i) and final time (t_f) of the logarithmic phase were established for each nutrient concentration (Table 1).

After a lag phase of 2-3 days, the cells entered into exponential growth, which lasted 4, 5 and 6 days for the cultures with 2, 4 and 8 mM of NaNO_3 , respectively, whereas it lasted 10 days for the culture with 16 mM of NaNO_3 . Optimal nutrient concentrations to produce a maximum cellular density were 4, 8 and 16 mM of NaNO_3 , which gave 12.45×10^6 , 14.12×10^6 and 14.23×10^6 cells/ml, respectively (Fig. 1). Statistically, there are no significant differences among these three cellular densities ($P < 0.001$), but they are all significantly greater than 10.14×10^6 cells/ml which was obtained with a nutrient concentration of 2 mM of NaNO_3 . A nutrient concentration of 2 mM of NaNO_3 is commonly used (McLachlan, 1964).

The maximum growth velocity of *D. tertiolecta* cultures in the logarithmic phase was between 0.50 and 0.61 doublings/day (Table 1). These results are similar to those found for *T. suecica* in batch and in mass cultures (Fabregas et al., 1984a, 1985b). At the end of the culture period (16 days) the protein content had reached maximum values of 442 $\mu\text{g/ml}$ and 31.06 $\mu\text{g/cell}$ (Tables 1 and 2) with a nutrient concentration of 4 mM of NaNO_3 . The protein content of the cultures increased with the nutrient concentration to 255%, this increase being greater at low than at high nutrient concentrations (Table 1). These differences in the protein content were more acute than those found in *Dunaliella salina* grown in different culture media, nitrate or glutamine, with a 12% higher protein content (Kosmakova and Prozumenshahicova, 1983). During the logarithmic phase, protein was not regulated by the nutrient concentration, and protein/cell was independent of the nutrient concentration (Table 2), the differences appearing when the cultures entered into the stationary phase. Great differences in the protein content such as those shown here must be taken into account when *D. tertiolecta* is going to be used as SCP or in aquaculture, since these variations can seriously affect, for instance, the success of a culture of mollusc larvae.

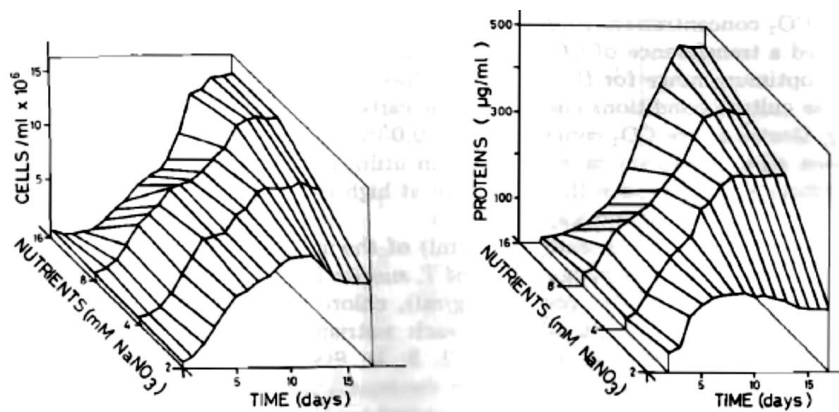


Fig. 1. Growth of *D. tertiolecta* at different nutrient concentrations, expressed as NaNO_3 concentrations.

Fig. 2. Protein concentration in mass cultures of *D. tertiolecta* at different nutrient concentrations.

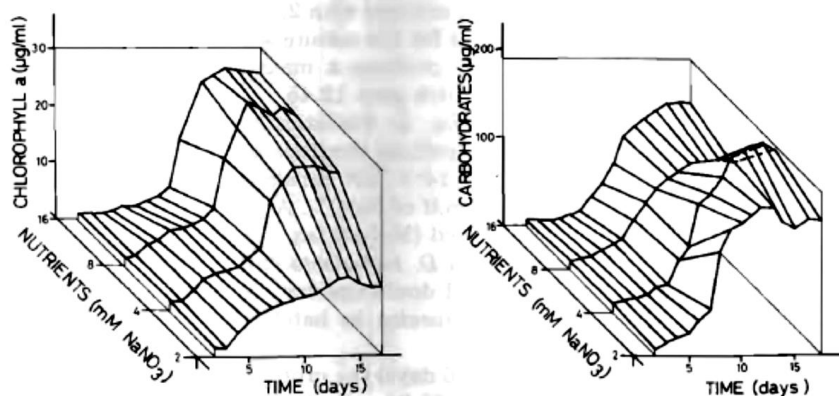


Fig. 3. Chlorophyll *a* concentration in mass cultures of *D. tertiolecta* at different nutrient concentrations.

Fig. 4. Carbohydrates concentration in mass cultures of *D. tertiolecta* at different nutrient concentrations.

The protein/carbohydrate ratio ranged between 1.26 and 2.94 in the stationary phase and between 3.11 and 3.91 in the logarithmic phase (Table 2). These ratios were similar to those found for other microalgae (Parsons et al., 1961; Myklestad, 1974; Hitchcock, 1980; Fabregas et al., 1985b).

Maximum efficiencies of nitrogen transformation of 104% and 100% were reached with 2 and 4 mM of NaNO_3 , respectively. The efficiency of nitrogen transformation decreased as the nutrient concentration increased. We established this efficiency as the ratio between the nitrogen added in the form of nitrate to the culture medium and the protein nitrogen produced per culture. Values greater than 100% can be due to residual nitrogen, present in the seawater used in the preparation of the culture medium, and to the possible increase in the availability of such nitrogen after the seawater has been autoclaved. An efficiency of 100% means that all the inorganic nitrogen in the culture medium has been absorbed by the cells. Among the three nutrient concentrations which produced the maximum biomass, 4, 8 and 16 mM of NaNO_3 , cultures with 4 mM had the greatest efficiency. These data indicate that the cheapest production is obtained with a nutrient

concentration of 4 mM of NaNO₃ (Fig. 5). With higher nutrient concentrations, the inorganic nitrogen is not entirely absorbed by the cells. This increases the costs without producing better yields.

Protein/chlorophyll *a* ratios ranged between 14 and 18 in the stationary phase and between 20 and 25 in the logarithmic phase. These ratios are similar to those obtained for *T. suecica* under the same conditions (Fabregas et al., 1985b).

The protein/RNA ratio was practically constant during the logarithmic phase, but increased with the nutrient concentration in the stationary phase.

Chlorophyll *a*/ml and chlorophyll *a*/cell were constant in the stationary phase for 4, 8 and 16 mM of NaNO₃, with values between 23.57 and 26.67 µg/ml and between 1.80 and 1.95 pg/cell. These values were significantly greater than those obtained with 2 mM of NaNO₃ (10.49 µg/ml and 1.03 pg/cell). In the logarithmic phase, chlorophyll *a*/cell was constant for all the nutrient concentrations (Tables 1, 2).

TABLE 2

Statistical analysis of the cellular content of *D. tertiolecta* grown at different nutrient concentrations in mass culture

	Growth phase	P	Nutrient concentration ^a						
			2 mM	4 mM	8 mM	16 mM			
Protein/cell (pg)	Stationary ^b	0.001	18.39 ± 1.16	<	27.69 ± 0.35	=	27.24 ± 1.18	<	31.06 ± 0.20
	Logarithmic ^c	0.001	21.46 ± 3.68	=	16.76 ± 3.85	=	18.79 ± 2.62	=	23.22 ± 3.54
Chlorophyll <i>a</i> /cell (pg)	Stationary	0.001	1.03 ± 0.05	<	1.95 ± 0.20	=	1.84 ± 0.11	=	1.80 ± 0.08
	Logarithmic	0.001	0.81 ± 0.12	=	0.86 ± 0.06	=	0.79 ± 0.09	=	0.93 ± 0.12
Carbohydrates/cell (pg)	Stationary	0.001	14.58 ± 0.55	=	14.85 ± 0.60	>	8.94 ± 0.57	=	10.05 ± 0.51
	Logarithmic	0.001	4.36 ± 1.22	=	4.43 ± 1.33	=	4.14 ± 0.98	<	8.68 ± 1.71
RNA/cell (pg)	Stationary	0.001	5.41 ± 0.28	=	5.80 ± 0.36	>	4.72 ± 0.38	=	5.33 ± 0.28
	Logarithmic	0.001	4.28 ± 0.20	=	5.00 ± 0.43	=	4.88 ± 0.39	=	5.40 ± 0.60
DNA/cell (pg)	Stationary	0.001	0.09 ± 0.06	=	0.08 ± 0.03	=	0.06 ± 0.03	=	0.09 ± 0.04
	Logarithmic	0.001	0.12 ± 0.08	=	0.06 ± 0.04	=	0.05 ± 0.03	=	0.11 ± 0.04
Protein/carbohydrates	Stationary		1.26		1.86		2.84		2.94
	Logarithmic		3.91		3.78		3.95		3.11
Protein/chlorophyll <i>a</i>	Stationary		18		14		15		17
	Logarithmic		22		20		24		25
Protein/RNA	Stationary		3.79		4.74		5.4		5.70
	Logarithmic		4.89		3.62		4.01		4.40
DNA/RNA	Stationary		0.016		0.014		0.013		0.017
	Logarithmic		0.027		0.012		0.010		0.020

^a Expressed as NaNO₃ concentration.

^b One-way analysis of variance (ANOVA).

^c One-way analysis of covariance (ANCOVA).

Maximum concentration of carbohydrates per ml in the stationary phase occurred at 4 mM of NaNO₃, with 185 µg/ml. Maximum carbohydrates/cell was obtained at low nutrient concentrations (2, 4 mM NaNO₃) in the stationary phase, whereas in the logarithmic phase it was obtained at high nutrient concentrations (8 mM NaNO₃) (Table 2). Carbohydrates/cell values were significantly lower in the logarithmic than in the stationary phase for all the nutrient concentrations. This suggests an adaptive mechanism that increased the level of storage products when the cultures aged, since carbohydrates occur mainly as storage products of cellular metabolism (Parsons and Takahashi, 1973).

RNA/cell was fairly constant in the logarithmic phase and ranged between 4.28 and 5.40 pg/cell. There was no obvious relationship between RNA and doublings/day, since different values of doublings/day presented similar values of RNA/cell (Tables 1 and 2). Therefore, we cannot relate the growth velocity of the cultures to the RNA/cell in the present conditions. In the stationary phase, RNA/cell values were also constant for all the nutrient concentrations, except for 8 mM of NaNO₃ which presented a lower value of RNA/cell. In this phase RNA/cell was not related to biomass production (cells/ml) either. However, in other systems the RNA has been said to serve as a measure of active biomass (Koliander et al., 1984).

TABLE 3

Values of the coefficients *a*, *b*, *c*, *d* and *e* of the equation $f(t) = a + bt + ct^2 + dt^3 + et^4$ calculated by a multiple non-linear least squares regression. (A) Cells/ml; (B) protein/ml; (C) chlorophyll *a*/ml; (D) carbohydrates/ml

(A)	Nutrient concentration			
	2 mM	4 mM	8 mM	16 mM
<i>a</i>	-128.306	-323.416	-73.8943	-788.547
<i>b</i>	+214.451	+278.507	+141.852	+489.341
<i>c</i>	-16.8643	-26.7765	-2.75735	-97.7316
<i>d</i>	+1.19915	+1.79461	—	+8.77726
<i>e</i>	-0.051591	-0.051213	—	-0.251289
SD	47.87	36.96	59.76	56.73

(B)	Nutrient concentration			
	2 mM	4 mM	8 mM	16 mM
<i>a</i>	-106.072	-43.3823	-164.746	-159.026
<i>b</i>	+91.4303	+28.3401	+127.592	+112.167
<i>c</i>	-10.7671	+0.22957	-23.0127	-25.0849
<i>d</i>	+0.572262	+0.135181	+2.07627	+2.41398
<i>e</i>	-0.012804	-0.010268	-0.062395	-0.069858
SD	12.95	8.28	9.64	12.25

(C)	Nutrient concentration			
	2 mM	4 mM	8 mM	16 mM
<i>a</i>	-9.74096	-6.10446	-9.34951	-14.8007
<i>b</i>	+7.19043	+5.54849	+7.90608	+10.8427
<i>c</i>	-1.27881	-1.22515	-1.78647	-2.55406
<i>d</i>	+0.112626	+0.127428	+0.175774	+0.240257
<i>e</i>	-0.003549	-0.004039	-0.005353	-0.007035
SD	0.90	1.78	2.02	1.48

(D)	Nutrient concentration			
	2 mM	4 mM	8 mM	16 mM
<i>a</i>	+143.413	+3.44385	+53.7379	+95.7067
<i>b</i>	-102.292	+8.40258	-31.8145	-40.1831
<i>c</i>	+23.1285	-3.32007	+6.72394	+4.89271
<i>d</i>	-1.68179	+0.56089	-0.386708	-0.071439
<i>e</i>	+0.039806	-0.021046	+0.006847	-0.004133
SD	12.58	6.56	4.90	6.97

The concentration of DNA/cell was constant in all the nutrient concentrations tested and in both growth phases; DNA/cell was, therefore, not related to doublings/day. These data are in

general accordance with those of other authors who have found that the rate of DNA synthesis is independent of division rate (Martin and Gonzalez, 1978).

The DNA content of *D. tertiolecta* cells ranged from 0.05 to 0.12 pg/cell. These values are lower than those presented by *T. suecica* (Fabregas et al., 1985b) and similar to those in other smaller marine microalgae such as *Monochrysis lutherii* and *Naucula pelliculosa* which contain approximately 0.1 pg DNA per cell (Holm-Hansen, 1969).

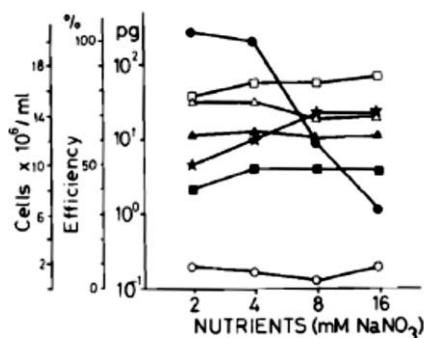


Fig. 5. Cellular density and chemical composition of *D. tertiolecta* in the stationary phase, at different nutrient concentrations. (★) cells/ml; (□) protein/cell; (△) carbohydrates/cell; (■) chlorophyll *a*/cell; (▲) RNA/cell; (○) DNA/cell; (●) efficiency (nitrate-N/protein-N transformation rate).

A correlation has been shown between the rates of RNA, DNA and protein synthesis, and the rate of cell growth (Leick, 1968; Pritchard et al., 1969; Nierlich, 1978). But such a correlation was not observed here for the DNA/RNA ratio and this is due to the great standard deviation in the DNA/ cell measurements. Perhaps the method we used for estimating DNA in this marine microalga was not the most suitable, and more accurate measurements might be obtained by using another method.

Great variability in the chemical composition of *D. tertiolecta* grown in mass culture has been shown to result from changes in the nutrient concentration. Such variations occur in protein, chlorophyll *a*, carbohydrates and RNA content, showing differences of 197%, 255%, 142% and 150%, respectively, in the stationary phase. It has already been reported elsewhere that variations in the chemical composition of microalgae are linked to changes in nutrient concentration (Myklestad and Haug, 1972; Goldman, 1976; Fabregas et al., 1985b), to salinity and nutrient concentration (Fabregas et al., 1984a, 1985a) and to growth rate (Goldman et al., 1979; Rhee, 1980), and also that the calorific value of the cells varies with changes in specific growth rate (Scott, 1980). *D. tertiolecta* is at present widely used in aquaculture, in the production of chemicals or as single cell protein (SCP). It must be taken into account that the nutritional value of the algal food is of more importance for the growth of herbivores, as pointed out by Parsons et al. (1961), than the species themselves. Most of the literature dealing with the food value of different algae for shellfish has been limited to comparisons of species, with virtually no consideration given to the nutritional state and corresponding chemical composition of the algae themselves (Ryther and Goldman, 1975). By altering the chemical composition of a commercially important microalgal

species such as *D. tertiolecta* in mass culture it may be possible not only to obtain high algal yields, but also to transfer this food efficiently to higher components of the food chain so as to maximize the conversion of dissolved nutrients into biomass of commercially important marine food crops.

The chemical variability shown by *D. tertiolecta* in relation to the nutrient concentration must have a marked effect on the use of this microalga as source of single-cell protein, chemicals or as feed in mariculture.

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