

Aquaculture

Volume 49, Issue 3-4, 1 November 1985, Pages 231-244

## **Mass culture and biochemical variability of the marine microalga *Tetraselmis suecica* Kylin (Butch) with high nutrient concentrations**

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

Departamento de Microbiología, Facultades de Farmacia y Medicina, Universidad de Santiago de Compostela, Spain

Fabregas J, Herrero C, Cabezas B, Abalde J. Mass culture and biochemical variability of the marine microalga *Tetraselmis suecica* Kylin (Butch) with high nutrient concentrations. *Aquaculture* 1985;49(3-4):231-244.

ISSN: 00448486

DOI: 10.1016/0044-8486(85)90082-1

### **Abstract**

Mass cultures of *Tetraselmis suecica* were carried out with four nutrient concentrations, ranging from 2 to 16 mM of NaNO<sub>3</sub> and salinity 35‰. An air flow of 15 l/min maintained a CO<sub>2</sub> transference rate sufficient to keep the pH below 8.4. 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 of  $7.83 \times 10^6$  and  $7.15 \times 10^6$  cells/ml were obtained with 8 and 16 mM of NaNO<sub>3</sub>, respectively. Growth velocity ranged between 0.53 and 0.63 doublings (dbl)/day, although 0.98 dbl/day were reached with 16 mM of NaNO<sub>3</sub>. Volume increased with nutrient concentration from 252 to 905  $\mu\text{m}^3$ . Protein content reached maximum values of 306  $\mu\text{g/ml}$  or 59.8 pg/cell. In the logarithmic phase, protein was regulated by nutrient concentration and decreased according to this concentration. Maximum efficiency of transformation from nitrate to protein was 108%, obtained at 2 mM of NaNO<sub>3</sub>. Efficiency decreased, to 14%, when nutrient concentration increased. This fact indicates that the lowest cost of harvesting is obtained with a nutrient concentration of 2 mM NaNO<sub>3</sub>. Chlorophyll a cell reached values

between 3.1 and 3.8 pg/cell in the stationary phase. There was a relationship between nutrient concentration and chlorophyll  $\alpha$  cell in the logarithmic phase, with an increase from 2.15 pg/cell to 3.74 pg/cell. Changes in chlorophyll  $\alpha$  level are related to nitrogen depletion. Carbohydrate/cell was constant at values of 19.84-28.68 pg/cell in the logarithmic and stationary phases and was not related to nitrogen depletion. RNA/cell ranged from 4.17 to 5.48 pg/cell, except at 2 mM of NaNO<sub>3</sub> when it was 2.77 pg/cell, probably due to nitrogen depletion. The level of DNA/cell was constant in all the nutrient concentrations assayed and ranged from 0.1 to 1.09 pg/cell. Great variability in the chemical composition of *T. suecica* has been shown. Growth in mass cultures is closely coupled to changes in nutrient concentrations and variations occur in protein, chlorophyll  $\alpha$  and RNA content, showing differences of 215%, 190% and 203%, respectively, in the stationary phase. This biochemical variability, mainly in protein content, must have a marked effect on the nutritive value of this microalga as feed in mariculture.

## Introduction

Microorganisms are potentially useful in aquaculture, in the bioconversion of solar energy, in the production of chemicals and as food for human consumption (Kharatyan, 1978; Goldman, 1979). The marine micro alga *Tetraselmis suecica* Kylin (Butch) is at present widely used in aquaculture (Walne, 1974; Bayne, 1976; Laing and Utting, 1980). Knowing its ability to grow in a wide range of nutrient concentrations and salinity conditions, in batch cultures, we have previously established some of the parameters for mass production and this has enabled us to obtain maximum growth velocity and cellular density and we have also been made aware of its biochemical variability (Fabregas et al., 1984a). Our experience with laboratory mass cultures may, therefore, enable us to establish some of these parameters for outdoor mass cultures.

We report here the response of a mass culture of *Tetraselmis suecica* to a series of high nutrient concentrations. The work was done in order to establish the conditions for maximum production, to predict the response of the organism and to estimate its biochemical variability, since this variability can affect its nutritive value when this species is used as feed in mariculture.

## Materials and methods

*Tetraselmis suecica* Kylin (Butch), the marine microalga used here, was isolated from Ria de Arosa waters (NW of Spain). It was cultured in seawater which had been filtered through a

0.45  $\mu\text{M}$  Millipore filter, autoclaved at 120°C for 60 min and enriched with  $\text{NaNO}_3$ , 2 mM;  $\text{NaH}_2\text{PO}_4$ , 100  $\mu\text{M}$ ;  $\text{ZnCl}_2$ , 1  $\mu\text{M}$ ;  $\text{ZnCl}_2$ , 1  $\mu\text{M}$ ;  $\text{Na}_2\text{MoO}_4$ , 1  $\mu\text{M}$ ;  $\text{CoCl}_3$ , 0.1  $\mu\text{M}$ ;  $\text{CuSO}_4$ , 0.1  $\mu\text{M}$ ; ferric citrate, 20 mM; thiamine, 35  $\mu\text{g/l}$ ; biotin, 5  $\mu\text{g/l}$ ; B 12, 3 J.Lg/l; EDTA, 26.4  $\mu\text{M}$ ; 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, containing  $\text{NaNO}_3$  2 mM, was the first to be used. From this we followed a geometrical progression, using concentrations corresponding to 4, 8 and 16 mM of  $\text{NaNO}_3$ . Nutrient concentrations are expressed as  $\text{NaNO}_3$  concentrations, but all the other nutrients were proportionally increased.

Cultures were contained in 10-l flasks with 9 l of culture medium. All cultures were maintained in a controlled environment incubator at  $15 \pm 1^\circ\text{C}$  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  $50 \times 10^4$  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.

Cell volume was calculated by measuring the width and length of a significant number of cells under the microscope, and assuming the cell to be cylindrical.

Chlorophylls were extracted in acetone-methanol 2:1 at 4°C for 48 h. The extracts were filtered through a Fluoropore Millipore filter for clarification (Fabregas et al., 1984b), and the absorbance of the pigment extract at specific wavelengths was recorded. The concentration of chlorophyll  $\alpha$  was determined by the following formula (Parsons and Strickland, 1965): chlorophyll  $\alpha$  (mg/ l) =  $11.64 \times D_{663} - 2.16 \times D_{645} - 0.1 \times D_{630}$  ( $U/V$ ), where  $D_{630}$ ,  $D_{645}$  and  $D_{663}$  are the absorbances at 630, 645 and 663 nm respectively, read in a 1.0 cm cell;  $V$  is the sample volume, and  $U$  is the final acetone-methanol volume.

Protein and carbohydrates were measured in the crude extracts 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-sulphuric 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 one-way analysis of co variance (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  $\alpha$ /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  $t_j$  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 Prasinophyte *Tetraselmis suecica* is one of the microalgae being increasingly used in aquaculture. Mass culture of the organism and improved knowledge of its composition, growth and chemical variability will enable its better use in marine aquaculture.

TABLE 1

Statistical analysis of the growth curves of *T. suecica* 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 <sup>a</sup>			
			2 mM	4 mM	8 mM	16 mM
Cell/ml $\times 10^4$	Stationary <sup>b</sup>	0.01	476 $\pm$ 20	= 511 $\pm$ 41	< 783 $\pm$ 45	= 715 $\pm$ 34
Time interval, days ( $t_n - t_i$ )			6-3	6-2	5-3	5-2
Doublings/day	Logarithmic <sup>c</sup>	0.01	0.63	= 0.55	= 0.53	< 0.98
Protein ( $\mu\text{g/ml}$ )	Stationary	0.01	190 $\pm$ 23.6	< 306 $\pm$ 27	> 247 $\pm$ 24	> 196 $\pm$ 9.1
Chlorophyll $\alpha$ ( $\mu\text{g/ml}$ )	Stationary	0.01	9.9 $\pm$ 1.2	< 19.2 $\pm$ 2.8	= 21.9 $\pm$ 1.9	= 23 $\pm$ 1.9
Carbohydrates ( $\mu\text{g/ml}$ )	Stationary	0.01	130 $\pm$ 12	= 111.5 $\pm$ 3.11	< 193 $\pm$ 6.2	= 173 $\pm$ 8.2
Volume ( $\mu\text{m}^3$ )	Stationary	0.05	252 $\pm$ 59	< 753 $\pm$ 64	< 848 $\pm$ 51	< 905 $\pm$ 62
Efficiency	Stationary		108	87.5	27.5	14

<sup>a</sup> Expressed as  $\text{NaNO}_3$  concentration.

<sup>b</sup> One-way analysis of variance (ANOVA).

<sup>c</sup> One-way analysis of covariance (ANCOVA).

Cultural conditions were established in order to obtain a maximum biomass production and to ensure that light, pH and carbon source did not become limiting. Cultures were, therefore, illuminated with a sufficient number of fluorescent lamps (11) so that light did not become

limiting due to the high cellular densities obtained. It has been shown that there was little increase in the yields when illumination was provided by 6 to 8 lamps (Laing and Helm, 1981).

The growth of *T. suecica* generated a strong alkalinity in the cultures since the uptake of  $\text{NaNO}_3$  during photosynthesis generates alkalinity (Goldman et al., 1972; Brewer and Goldman, 1976). In our growing system, at low  $\text{CO}_2$  concentrations (0.03% v/v, in the air), an air flow of 15 l/min maintained a transference of  $\text{CO}_2$  to the culture medium that kept the pH below 8.4 (Fig. 1). In addition, these culture conditions ensured that the carbon source did not become limiting.

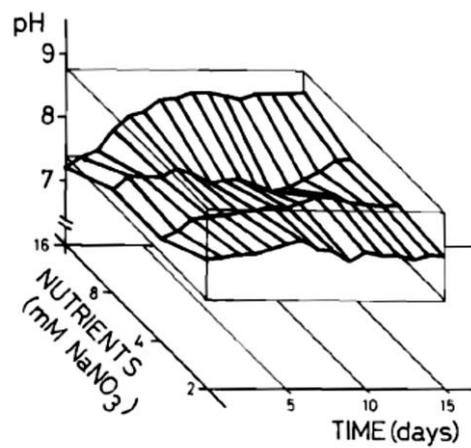


Fig. 1. pH values in mass cultures of *T. suecica* at different nutrient concentrations.

Carbon supply and sufficient light intensity are the two important factors for the production of mass cultures of microalgae with high cellular densities. The growth kinetics model (cells/ml) of the mass culture of *T. suecica* was similar to one of batch culture (Fabregas et al., 1984a). In algal batch experiments, with both limited volume and supply of nutrients, a logistic curve is usually observed (Schantz and Zahler, 1981).

We plotted cellular density, protein ( $\mu\text{g/ml}$ ), chlorophyll  $\alpha$  ( $\mu\text{g/ml}$ ) and carbohydrates ( $\mu\text{g/ml}$ ) against time for each nutrient concentration, obtaining three-dimensional figures (Figs. 2, 3, 4, 5). 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  $a$ ,  $b$ ,  $c$ ,  $d$  and  $e$  are presented in Table 3. From this equation we calculated doublings/day or the inverse of the duplication time ( $t_d^{-1}$ ). The initial time ( $t_i$ ) and final time ( $t_n$ ) of the logarithmic phase were established for each nutrient concentration (Table 1).

TABLE 2

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

	Growth phase	P	Nutrient concentration <sup>a</sup>			
			2 mM	4 mM	8 mM	16 mM
Protein/cell (pg)	Stationary <sup>b</sup>	0.01	41.77 ± 3.28	< 59.83 ± 7.88	> 32.50 ± 1.31	> 27.75 ± 1.89
	Logarithmic <sup>c</sup>	0.01	*52.20 ± 14.20 <sup>d</sup>	= 39.84 ± 11.65	= 31.33 ± 7.28 <sup>e</sup>	= 29.20 ± 1.92 <sup>e</sup>
Chlorophyll a/cell (pg)	Stationary	0.01	2.03 ± 0.32	< 3.80 ± 0.45	= 3.10 ± 0.32	= 3.19 ± 0.33
	Logarithmic	0.01	**2.15 ± 0.58 <sup>f</sup>	= 3.01 ± 0.40	= 3.20 ± 0.43 <sup>g</sup>	= 3.74 ± 0.56 <sup>g</sup>
Carbohydrates/cell (pg)	Stationary	0.01	26.43 ± 1.86	= 21.78 ± 3.36	= 27.82 ± 2.05	= 24.78 ± 2.13
	Logarithmic	0.01	27.98 ± 5.77	= 28.68 ± 5.40	= 24.70 ± 6.96	= 19.84 ± 3.80
RNA/cell (pg)	Stationary	0.01	2.77 ± 1.66	< 4.95 ± 0.91	= 5.48 ± 1.05	= 4.17 ± 0.25
	Logarithmic	0.01	6.98 ± 0.09	= 7.15 ± 0.22	> 5.99 ± 0.16	> 3.78 ± 1.19
DNA/cell (pg)	Stationary	0.01	0.13 ± 0.08	= 0.15 ± 0.09	= 0.10 ± 0.03	= 0.25 ± 0.18
	Logarithmic	0.01	1.09 ± 0.68	= 0.11 ± 0.02	= 0.12 ± 0.06	= 0.21 ± 0.25
Protein/carbohydrate	Stationary		1.58	2.74	1.20	1.12
	Logarithmic		1.87	1.39	1.27	1.47
Protein/chlorophyll a	Stationary		20.58	15.74	10.48	8.7
	Logarithmic		24.28	13.23	9.8	7.8
Protein/RNA	Stationary		15.08	12.08	5.93	6.65
	Logarithmic		7.48	5.57	5.23	7.72
DNA/RNA	Stationary		0.05	0.03	0.02	0.06
	Logarithmic		0.16	0.01	0.02	0.06

<sup>a</sup> Expressed as NaNO<sub>3</sub> concentration.<sup>b</sup> One-way analysis of variance (ANOVA).<sup>c</sup> One-way analysis of covariance (ANCOVA).

\*d &gt; e, P &lt; 0.01.

\*\*f &lt; g, P &lt; 0.01.

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				B	Nutrient concentration			
	2 mM	4 mM	8 mM	16 mM		2 mM	4 mM	8 mM	16 mM
a	+59.5875	+37.0820	+44.6376	+71.5005	a	+26.0839	+21.4348	+27.9490	+15.1608
b	-52.5082	-7.6925	-1.0054	-110.5700	b	-2.5189	-66.6777	-9.0007	-15.6242
c	+24.6674	+10.2548	+9.0626	+62.8148	c	+4.0207	+38.2386	+6.9192	+19.2188
d	-1.1259	-0.2567	+0.0461	-6.6173	d	+0.1342	-4.2563	-0.3717	-2.5659
e	-0.0402	-0.0223	-0.0294	+0.2103	e	-0.0347	+0.1398	-0.0003	+0.0970
SD	34.51	40.51	38.03	43.30	SD	14.33	23.39	17.03	11.60

C	Nutrient concentration				D	Nutrient concentration			
	2 mM	4 mM	8 mM	16 mM		2 mM	4 mM	8 mM	16 mM
a	+0.1775	+1.1252	+3.6189	+3.9864	a	+22.7203	+19.7111	+19.1450	+13.8951
b	+0.2025	+1.1285	+1.1793	-2.9632	b	-33.2628	-13.7921	-1.4554	-4.9147
c	+0.1059	-0.1335	-0.1304	+2.4333	c	+24.5309	+9.7264	+9.2451	+5.7753
d	+0.0235	+0.0543	+0.0351	-0.3131	d	-3.8042	-1.1238	-1.0240	-0.2170
e	-0.0027	-0.0033	-0.0018	+0.0117	e	+0.1818	+0.0377	+0.0306	-0.0143
SD	0.67	1.83	1.18	2.03	SD	8.08	7.71	14.92	15.72

The growth and biochemical composition of *T. suecica* were evaluated in the nutrient concentration range of 2, 4, 8 and 16 mM of NaNO<sub>3</sub>, and salinity of 35‰. These conditions had been demonstrated previously to be optimum for *T. suecica*, in batch cultures (Fabregas et al., 1984a).

After a lag phase of 1-2 days, the cells entered exponential growth, which lasted 2-6 days. Optimal nutrient concentrations to produce a maximum cellular density were 8 and 16 mM of NaNO<sub>3</sub>, which gave  $7.83 \times 10^6$  and  $7.15 \times 10^6$  cells/ml respectively (Fig. 2). Statistically, there are no significant differences between these two cellular densities ( $P < 0.01$ ). Cellular densities of  $4.76 \times 10^6$  and  $5.11 \times 10^6$  cells/ml were obtained with nutrient concentrations of 2 and 4 mM respectively. These densities are also statistically equal ( $P < 0.01$ ). A nutrient

concentration of 2 mM of  $\text{NaNO}_3$  is commonly used (McLachlan, 1964). In Table 3A the values of the growth-predicting parameters are presented.

The maximum growth velocity of *T. suecica* cultures in the logarithmic phase was between 0.53 and 0.63 doublings/day for 2 to 8 mM  $\text{NaNO}_3$ . These results are similar to those obtained in batch cultures (Fabregas et al., 1984a). In the culture with 16 mM of  $\text{NaNO}_3$  the maximum growth velocity reached 0.98 doublings/day (Table 1).

Cellular volume of *T. suecica* increased with nutrient concentration from 252.9 to 905.4  $\mu\text{m}^3$  with  $P < 0.05$ . The increase in the cellular volume appears not to have been produced by the increase in salinity of the culture medium, since maximum nutrient concentration (16 mM of  $\text{NaNO}_3$ ) produced an increase in the salinity only from 35‰ to 36.4‰. The variability is also light-independent, since the same cellular densities presented different cellular volumes. In other microalgae, such as *Dunaliella bioculata*, cell volume was also light-independent in a hypersaline medium (Grizeau et al., 1983). It may be that *T. suecica* has a volume-regulating mechanism in relation to the composition and concentration of the culture medium. In the present case, cellular volume was not affected by the carbon source, although the main effect of a limitation of inorganic carbon appears to be on cell size rather than on the chemical structure of the biomass (Goldman and Graham, 1981); but in our culture conditions carbon supply was always in excess and was never limiting.

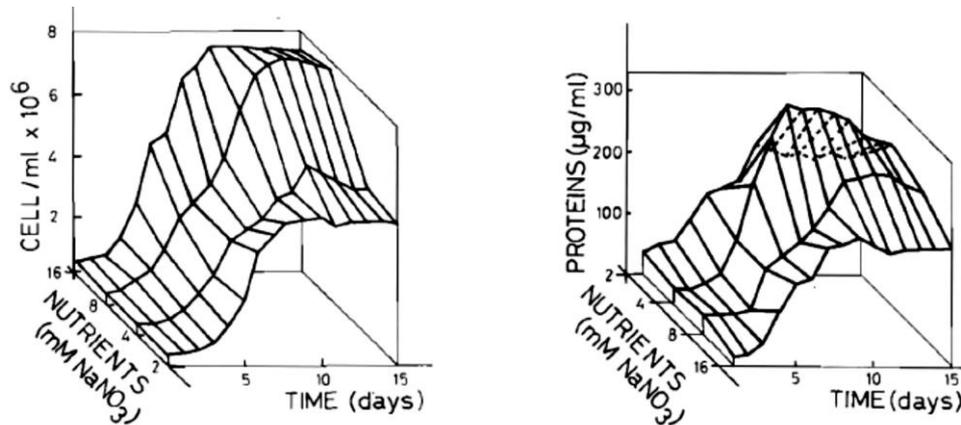


Fig. 2. Growth of *T. suecica* at different nutrient concentrations, expressed as  $\text{NaNO}_3$  concentrations.

Fig. 3. Protein concentration in mass cultures of *T. suecica* at different nutrient concentrations.

At the end of the culture period (15 days) the protein content had reached maximum values of 306  $\mu\text{g/ml}$  and 59.8  $\mu\text{g/cell}$  (Tables 1 and 2) with a nutrient concentration of 4 mM of  $\text{NaNO}_3$ , decreasing to 46% with higher concentrations. 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

Prozumenshchikova, 1983). During the logarithmic phase, protein was regulated by the nutrient concentration. Great differences in the protein/cell content such as those shown here can seriously affect the success of a culture of mollusc larvae.

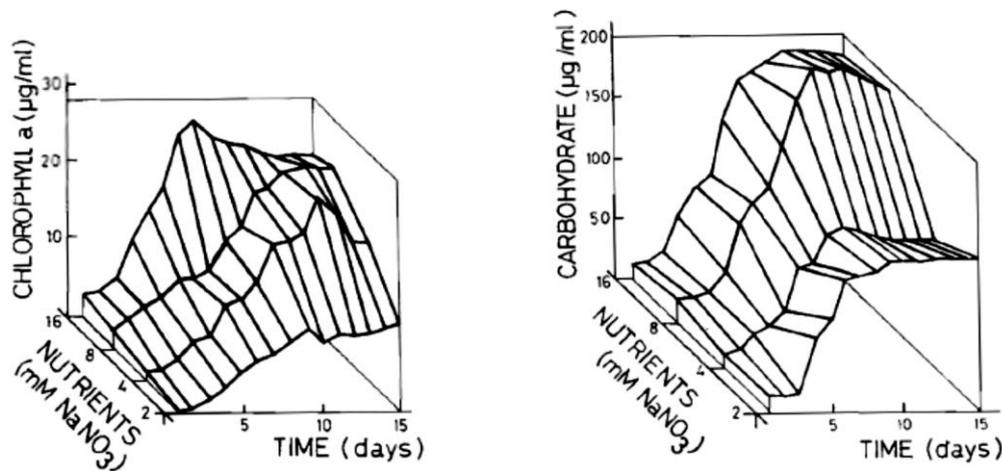


Fig. 4. Chlorophyll *a* concentration in mass cultures of *T. suecica* at different nutrient concentrations.

Fig. 5. Carbohydrates concentration in mass cultures of *T. suecica* at different nutrient concentrations.

The protein/carbohydrate ratio ranged between 1.12 and 2.74 in the stationary phase and between 1.39 and 1.87 in the logarithmic phase (Table 2). These ratios are similar to those found for other microalgae (between 0.23 and 2.0, Parsons et al., 1961), for *Skeletonema costatum* (from 0.5 to 2.0 over a diel cycle, Hitchcock, 1980) and for nine diatom species, where there was a decline from 2.0 to between 0.1 and 0.5 as nitrogen was depleted (Myklestad, 1974). These ratios are lower than the ratio of 1.58 for *T. suecica* obtained with a nutrient concentration of 2 mM of NaNO<sub>3</sub>. This represented an efficiency of 100% and, therefore, the nitrogen was depleted.

The efficiency of nitrogen transformation decreased as the nutrient concentration increased. We established this efficiency as the ratio between the nitrogen added in nitrate form to the culture medium and the protein nitrogen produced per culture. A maximum efficiency of 108% was reached with 2 mM of NaNO<sub>3</sub>. A value 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 sea water has been autoclaved. An efficiency of 100% means that all the inorganic nitrogen in the culture medium has been absorbed by the cells. Nutrient concentrations of 8 and 16 mM, which produced the maximum biomass, had the least efficiency at 27.5% and 14% respectively. These data indicate that the cheapest production is obtained with a nutrient concentration of 2 mM of NaNO<sub>3</sub> (Fig. 6).

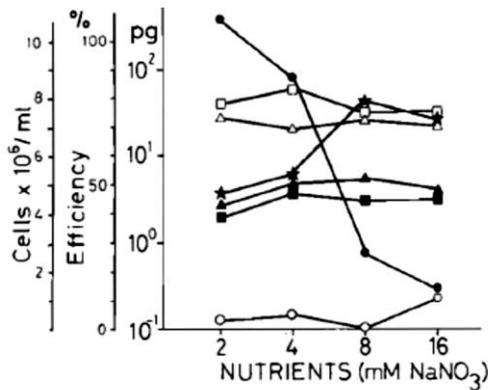


Fig. 6. Cellular density and chemical composition of *T. suecica* in the stationary phase, at different nutrient concentrations. (●) cells/ml; (□) protein/cell; (△) carbohydrates/cell; (■) chlorophyll *a*/cell; (▲) RNA/cell; (○) DNA/cell; (●) efficiency (nitrate-N/protein-N transformation rate).

Protein/chlorophyll *a* ratios ranged between 20.58 and 8.7 in the stationary phase and between 24.28 and 7.8 in the logarithmic phase. This ratio decreased similarly in both growth phases as the nutrient concentration increased.

In the logarithmic phase, protein/cell and chlorophyll *a*/cell appeared to be inversely related. The cellular concentrations of each compound were not related to nitrogen depletion in these cultural conditions.

The protein/RNA ratio was practically constant during the logarithmic phase and decreased at a concentration of 8 mM of NaNO<sub>3</sub> in the stationary phase.

Chlorophyll *a*/ml and chlorophyll *a*/cell were constant in the stationary phase for 4, 8 and 16 mM of NaNO<sub>3</sub>; chlorophyll *a*/cell reached values between 3.1 and 3.8 pg/cell. These values are significantly higher than the 2.03 pg/cell obtained with 2 mM of NaNO<sub>3</sub>. Changes in chlorophyll *a* levels were related to the nitrogen depletion which occurred in the stationary phase in the cultures with 2 mM of NaNO<sub>3</sub>. Large changes in chlorophyll levels have also been found under nitrate and ammonia limitations (Caperon and Meyer, 1972). This suggests an adaptive mechanism to increase the chlorophyll to a level of 3.1 and 3.8 pg/cell at the saturation level of nitrogen in the culture, whereas higher concentrations do not increase this value.

There was a relationship between nutrient concentration and chlorophyll *a*/cell in the logarithmic phase, with an increase from 2.15 pg/cell to 3.74 pg/cell. Similar results have been obtained (Falkowski and Stone, 1975) for fluctuations in chlorophyll *a* with nitrogen, suggesting an adaptive mechanism which involves increases in photosynthesis resulting from increased nitrogen-mediated chlorophyll *a* concentration. As the chlorophyll *a*/cell content in the stationary phase was constant, it is clear that there was no light-limitation due to a shading of the cells in the conditions of high cellular density.

Carbohydrate/cell was constant in both the logarithmic and the stationary phases and though it was not related to nitrogen depletion, others have related it to light and temperature (Hitchcock, 1980).

RNA concentrations ranged from 4.17 to 5.48 pg/cell with 4.8 and 16 mM of NaNO<sub>3</sub>, whereas the culture with 2 mM of NaNO<sub>3</sub> produced a significantly lower concentration (2.77 pg/cell). There could be a cellular mechanism which decreases the concentration of RNA/cell when nitrogen is depleted and which maintains the RNA/cell constant, and independent of the different nutrient concentrations, when nitrogen is at the saturation level in the stationary phase. In the logarithmic growth phase, RNA/cell values were similar in the 2 and 4 mM NaNO<sub>3</sub> nutrient concentrations with levels of 6.98-7.15 pg/cell, and these values were higher than those of the cultures with 8 and 16 mM of NaNO<sub>3</sub>. RNA/cell decreased as the nutrient concentration increased; it was directly related to protein and chlorophyll  $\alpha$  and inversely related to cellular density. However, there was no obvious relationship between RNA and doublings/day since the maximum doublings/day value of 0.98 corresponded to the minimum RNA/cell concentration. Therefore, we cannot relate the growth velocity of the culture to the RNA/cell concentration in the present conditions, although in other systems the RNA has been said to serve as a measure of active biomass (Koliander et al., 1984).

The concentration of DNA/cell was constant in all of the nutrient concentrations assayed 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 *T. suecica* cells ranged from 0.1 to 1.09 pg/cell. These values are similar to those in other, smaller, marine micro algae such as *Monochrysis lutheri* and *Navicula pelliculosa* which contain approximately 0.1 pg of DNA per cell (Holm-Hansen, 1969). There was no proportionality between DNA/cell and the increase in cellular volume during the stationary phase, under the cultural conditions described above.

A correlation has been shown between the rates of RNA, DNA and protein synthesis, and the rate of cell growth (Nierlich, 1978; Pritchard et al., 1969; Leick, 1968). 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 (Karl, 1981).

Great variability in the chemical composition of *T. suecica* has been shown to result from environmental conditions. Variations in the chemical composition of *T. suecica*, grown in mass culture, are closely related to changes in nutrient concentrations; such variations occur

in protein, chlorophyll a and RNA content, showing differences of 215%, 190% and 203%, respectively, in the stationary phase. It seems reasonable to suggest that variability in the protein content per cell is a most important factor in the use of *T. suecica* for animal feeding in aquaculture systems. Differences between 27.7 and 59.8 pg of protein/cell, in the stationary phase (Table 2), are equivalent to a 215% deviation in the protein content due solely to the nutrient concentration, and any system of mariculture where microalgae are used as feed would be severely affected by such variations. 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), to salinity and nutrient concentration (Fabregas et al., 1984a) 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).

## Acknowledgements

Thanks are due to Prof. Dr. B. Regueiro Varela for his interesting comments, to Prof. Dr. C. Ferreiros for excellent help in statistical analysis and to Prof. Dr. B. Regueiro Garcia for allowing us access to computational facilities.

This work was supported by a grant of Dirección General de Ordenación Pesquera, Madrid, Spain. Part of this work was supported by a grant to C.H. from Fundación Juan March, Madrid, Spain.

## References

1. Bayne BL. The biology of mussel larvae. *Marine Mussels: Their Ecology and Physiology* 1976;81-120.
2. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72(1-2):248-54.
3. Brewer PG, Goldman JC. Alkalinity changes generated by phytoplankton growth. *Limnol Oceanogr* 1976;21(1):108-17.
4. Caperon J, Meyer J. Nitrogen-limited growth of marine phytoplankton-I. changes in population characteristics with steady-state growth rate. *Deep-Sea Research and Oceanographic Abstracts* 1972;19(9):601-18.
5. Fabregas J, Abalde J, Herrero C, Cabezas B, Veiga M. Growth of the marine microalga *tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. *Aquaculture* 1984;42(3-4):207-15.

6. Fabregas J, Herrero C, Veiga M. Effect of oil and dispersant on growth and chlorophyll  $\alpha$  content of the marine microalga *tetraselmis suecica*. *Appl Environ Microbiol* 1984;47(2):445-7.
7. Falkowski PG, Stone DP. Nitrate uptake in marine phytoplankton: Energy sources and the interaction with carbon fixation. *Mar Biol* 1975;32(1):77-84.
8. Goldman JC. Outdoor algal mass cultures. I. applications. *Water Res* 1979;13(1):1-19.
9. Goldman JC, Graham SJ. Inorganic carbon limitation and chemical composition of two freshwater green microalgae. *Appl Environ Microbiol* 1981;41(1):60-70.
10. Goldman JC, McCarthy JJ, Peavey DG. Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature* 1979;279(5710):210-5.
11. Goldman JC, Porcella DB, Joe Middlebrooks E, Toerien DF. The effect of carbon on algal growth-its relationship to eutrophication. *Water Res* 1972;6(6):637-79.
12. Grizeau D, Jeanne N, Puiseux-Dao S. Effects of various factors and inhibitors on the regulation of cell volume in *dunaliella bioculata*. *Plant Science Letters* 1983;28:213-22.
13. Hitchcock GL. Diel variation in chlorophyll  $\alpha$ , carbohydrate and protein content of the marine diatom *skeletonema costatum*. *Mar Biol* 1980;57(4):271-8.
14. Holm-Hansen O. Algae: Amounts of DNA and organic carbon in single cells. *Science* 1969;163(3862):87-8.
15. Karl DM. Simultaneous rates of ribonucleic acid and deoxyribonucleic acid syntheses for estimating growth and cell division of aquatic microbial communities. *Appl Environ Microbiol* 1981;42(5):802-10.
16. Kharatyan SG. Microbes as food for humans. *Annu Rev Microbiol* 1978;32:301-27.
17. Kochert G. Carbohydrate determination by the phenol-sulfuric acid method. *Handbook of Phycological Methods. Physiological and Biochemical Methods* 1978:95-7.
18. Kochert G. Quantitation of the macromolecular components of microalgae. *Handbook of Phycological Methods. Physiological and Biochemical Methods* 1978:189-95.
19. Koliander B, Hampel W, Roehr M. Indirect estimation of biomass by rapid ribonucleic acid determination. *Appl Microbiol Biotechnol* 1984;19(4):272-6.

20. Kosmakova V, Prozumenshahicova L. Growth and biochemical composition of the algae *dunaliella salina* and *platymonas viridis* fed on organic and inorganic nitrogen. *Biol.Morya*. 1983;1:42-6.
21. Laing I, Helm MM. Factors affecting the semi-continuous production of *tetraselmis suecica* (kylin) butch. in 200-l vessels. *Aquaculture* 1981;22(C):137-48.
22. Laing I, Utting S. The influence of salinity on the production of two commercially important unicellular marine algae. *Aquaculture* 1980;21:79-86.
23. Leick V. Ratios between contents of DNA, RNA and protein in different micro-organisms as a function of maximal growth rate. *Nature* 1968;217(5134):1153-5.
24. Martin DF, Gonzalez MH. Effects of salinity on synthesis of DNA, acidic polysaccharide, and growth in the blue-green alga, *gomphosphaeria aponina*. *Water Res* 1978;12(11):951-5.
25. McLachlan J. Some considerations of the growth of marine algae in artificial media. *Can J Microbiol* 1964;10:769-82.
26. Myklestad S. Production of carbohydrates by marine planktonic diatoms. I. comparison of nine different species in culture. *J Exp Mar Biol Ecol* 1974;15(3):261-74.
27. Myklestad S, Haug A. Production of carbohydrates by the marine diatom *chaetoceros affinis* var. *willei* (gran) hustedt. I. effect of the concentration of nutrients in the culture medium. *J Exp Mar Biol Ecol* 1972;9(2):125-36.
28. Nierlich DP. Regulation of bacterial growth, RNA, and protein synthesis. *Annu Rev Microbiol* 1978;32:393-432.
29. Parsons TR, Strickland JDH. Particulate organic matter. III.I. pigment analysis. III.I.I. determination of phytoplankton pigments. *J.Fish.Res.Board can.* 1965;18:117-27.
30. Parsons T, Stephens K, Strickland J. On the chemical composition of eleven species of phytoplankton. *J.Fish.Res.Board can.* 1961;18:101-16.
31. Pritchard R, Barth P, Collins J. Control of DNA synthesis in bacteria. *Symp.Soc.Gen.Microbiol.* 1969;19:263-97.
32. Rhee GY. Continuous culture in phytoplankton ecology. *Advances in Aquatic Microbiology*;2:151-203.

33. Schanz F, Zahler U. Prediction of algal growth in batch cultures. Schweizerische Zeitschrift Für Hydrologie 1981;43(1):103-13.
34. Scott JM. Effect of growth rate of the food alga on the growth/ingestion efficiency of a marine herbivore. J Mar Biol Assoc U K 1980;60:681-702.
35. Walne PR. Culture of Bivalve Molluscs 1974.