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Biomass production and biochemical composition in mass cultures of the marine microalga *Isochrysis galbana* Parke at varying nutrient concentrations

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ABSTRACT

Mass cultures of Isochrysis galbana were carried out with four nutrient concentrations ranging from 2 to 16 mM of $NaNO_3$ and salinity 35‰. An air flow of 15 l/min maintained a CO_2 transference rate sufficient to keep the pH below 8.4. Using these 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 density of 65.5×10^6 cells/ml was obtained with 4 mM $NaNO_3$. Cellular volume was constant in the different nutrient concentrations. Protein content reached a maximum value of 374 µg/ml at 4 mM of $NaNO_3$, and this concentration also presented the maximum efficiency of transformation from nitrate to protein, i.e. 114%. As a result, lowest costs for harvesting are obtained at a nutrient concentration of 4 mM $NaNO_3$. Efficiencies decreased to 15% as nutrient concentration increased. Maximum values of chlorophyll a (21.9 µg/ml) and carbohydrates (213 µg/ml) were also obtained with 4 mM $NaNO_3$. In the logarithmic phase, the contents of protein, chlorophyll a, carbohydrates, RNA and DNA per cell were constant. Chlorophyll a reached values between 0.15 and 0.33 pg/cell in the stationary phase. Carbohydrate levels reached the maximum value of 3.16 pg/cell with 4 mM $NaNO_3$ in the

stationary phase. The levels of RNA/cell and DNA/cell were constant in all the nutrient concentrations tested and in both growth phases, and ranged from 1.15 to 1.71 pg/cell for RNA and from 0.006 to 0.014 pg/cell for DNA. Growth in mass cultures is closely coupled to changes in nutrient concentrations and variations occur in protein, chlorophyll a and carbohydrate contents, showing differences of 177%, 220% and 136%, 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 a feed in mariculture.

INTRODUCTION

The artificial cultivation of shellfish in hatcheries has provoked great interest in marine microalgal biomass production (Ukeles, 1980). Production of this biomass is considered the major bottleneck in nursery culturing of molluscs (Persoone and Claus, 1980; De Pauw et al., 1983). The marine micro alga *Isochrysis galbana* Parke (Haptophyceae) is a naked flagellate that provides excellent nutrition for larvae (Ukeles, 1980) and is at present widely used in aquaculture (Walne, 1974; Bayne, 1976; Laing and Utting, 1980). Using batch cultures of *I. galbana* we have previously established some of the conditions for mass production, and this has enabled us to obtain maximum growth velocity and cellular density and to assess its biochemical variability (Fabregas et al., 1985a). 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 *Isochrysis galbana* to a series of high nutrient concentrations, studied in order to establish the conditions for maximum production, to predict this response and to estimate biochemical variability, since this variability can affect the nutritive value when this alga is used as feed in mariculture.

MATERIALS AND METHODS

The marine microalga *Isochrysis galbana* was obtained from The Culture Centre for Algae and Protozoa, Cambridge, England. It was cultured in seawater filtered through a 0.45 µm filter, autoclaved at 120°C for 60 min and enriched with NaN03 , 2 *mM;* NaH_2PO_4 , 100 µ*M;* $ZnCl_2$, 1 µ*M;* $MnCl_2$, *1* µ*M;* Na_2MoO_4 , 1 µ*M;* $CoCl_3$, 0.1 µ*M;* $CuSO_4$, 0.1 µ*M;* ferric citrate, 20 µ*M;* thiamine, 35 µg/l; biotin, 5 µg/l; B12 , 3 µg/l; EDTA, 26.4 µ*M;* Tris-HC1, *5 mM.* Salinity of the seawater was 35%0 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 $NaNO_3$. Nutrient concentrations are expressed as $NaNO_3$ concentrations, but all the other nutrients were increased proportionally.

Cultures were carried out in 10-l 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 2 X 10^6 logarithmic phase cells/ml was used.

Cultures had air continuously bubbled through them at a rate of 15 l/min. Culture conditions were optimized in order to obtain a maximum biomass production and to ensure that pH and carbon source did not become limiting; in fact, these parameters can be considered among the most important for culturing marine microalgae. Cultures were illuminated with fluorescent lamps in order to maintain light in saturation even at high cell densities (Kain and Fogg, 1958; Laing and Helm, 1981). Temperature was maintained at the optimum value of 15 ± 1°C (Ukeles, 1961) and salinity at 35‰ (Fabregas et al., 1985a).

Cellular density was determined by counting culture aliquots in a Thoma chamber. Cell volume was calculated by measuring the diameter of a significant number of cells under the microscope, and assuming the cell to be spherical.

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 one-way 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 protein/ml or carbohydrates/ml or chlorophyll *a/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 *td* is the duplication time .

TABLE 1

Statistical analysis of the growth curves of *I. galbana* at different nutrient concentrations in mass culture. Each value corresponding to a nutrient concentration is compared only with the following concentration

			Nutrient concentration[a]											
	Growth phase	*P*	2 m*M*		4 m*M*			8 m*M*			16 m*M*			
Cell/ml · 10^6	Stationary[b]	0.001	53.8	± 3.4 <	65.5	± 3.9 >		47.7	± 5.9 >		29.3	± 3.5		
Days interval (t_n–t_i)			9—2		10—2			7—2			8—2			
dbls/day	Logarithmic[c]	0.001	0.54	=	0.53		<	0.70		=	0.69			
Protein (μg/ml)	Stationary	0.001	161	± 3.8 <	374	± 18.8 >		267	± 15.5 >		182	± 2.2		
Chlorophyll *a* (μg/ml)	Stationary	0.001	7.69	± 0.6 <	21.9	± 1.9 >		13.8	± 1.0 >		9.71	± 0.2		
Carbohydrates (μg/ml)	Stationary	0.001	121	± 20.9 <	213	± 7.9 >		112	± 14.6 >		70.3	± 9.5		
Volume (μm^3)	Stationary	0.001	71.7	± 6.7 =	80.0	± 10 =		75.2	± 4.5 =		74.0	± 6.9		
Efficiency	Stationary	0.001	109		114			41			15			

[a]Expressed as $NaNO_3$ concentration.
[b]One-way analysis of variance (ANOVA).
[c]One-way analysis of covariance (ANCOVA).

RESULTS AND DISCUSSION

The growth of *I. galbana* generated a strong alkalinity in the cultures, since the uptake of NaN03 during photosynthesis generates a strong base (Goldman et al., 1972; Brewer and Goldman, 1976). In our growing system an air flow of 15 l/min maintained a transfer of CO2 to the culture medium that kept the pH below the critical level of 8.5 (Fig. 1) above which growth in *I. galbana* is inhibited (Kain and Fogg, 1958). In addition, these culture conditions ensured that the carbon source did not become limited.

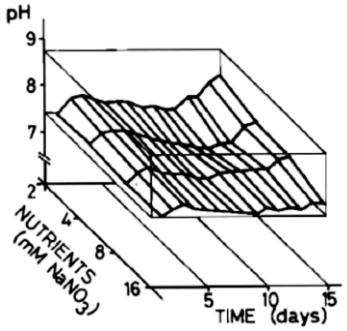

Fig. 1. pH values in mass cultures of *I. galbana* at different nutrient concentrations.

We plotted cellular density, protein (µg/ml), chlorophyll *a* (µg/ml) and carbohydrates (µg/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).

After a lag phase of 2 days, the cells entered into exponential growth, which lasted 5-8 days. The duration of the logarithmic phase is longer in *I. galbana* than in *Tetraselmis suecica,* under the same conditions (Fabregas et al., 1985b) and longer in mass cultures of *I. galbana* than in batch cultures of this microalga (Fabregas et al., 1985a). Optimal nutrient concentration to produce a maximum cellular density was 4 mM $NaNO_3$, which gave 65.5 X 10^6 cells/ml (Fig. 2). Statistically this value is significantly higher than those obtained with the remaining nutrient concentrations.

Above 4 mM $NaNO_3$, cellular density decreased proportionally to the increase in the nutrient concentrations. A nutrient concentration of 2 mM of NaN03 is commonly used (McLachlan, 1964). In Table 3A the growth predicting parameters are presented.

The maximum growth velocity of *I. galbana* cultures in the logarithmic phase was between 0.53 and 0.70 doublings/day (Table 1). Cellular volumen was not affected by nutrient concentrations, showing values between 71 and *80 µm^3.* The main influence of inorganic carbon limitation appears to be not on the chemical structure of the biomass, but rather on cell size (Goldman and Graham, 1981); but in our culture conditions carbon supply was always in excess, and probably for this reason cellular volume was not affected.

TABLE 2

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

	Growth phase	*P*	Nutrient concentration[a] 2 m*M*		4 m*M*		8 m*M*		16 m*M*
Protein/cell (pg)	Stationary[b]	0.001	3.19 ± 0.40	<	5.66 ± 0.31	=	5.65 ± 0.63	=	5.94 ± 0.12
	Logarithmic[c]	0.001	5.34 ± 1.33	=	5.63 ± 0.60	=	5.12 ± 0.89	=	5.65 ± 0.74
Chlorophyll *a*/cell (pg)	Stationary	0.001	0.15 ± 0.02	<	0.33 ± 0.02	=	0.28 ± 0.04	=	0.33 ± 0.03
	Logarithmic	0.001	0.21 ± 0.02	=	0.22 ± 0.04	=	0.21 ± 0.03	=	0.22 ± 0.02
Carbohydrates/cell (pg)	Stationary	0.001	2.31 ± 0.28	<	3.16 ± 0.31	>	2.36 ± 0.08	=	2.44 ± 0.09
	Logarithmic	0.001	2.29 ± 0.51	=	2.97 ± 0.68	=	2.53 ± 0.41	=	2.33 ± 0.72
RNA/cell (pg)	Stationary	0.001	1.15 ± 0.01	=	1.45 ± 0.21	=	1.44 ± 0.13	=	1.65 ± 0.11
	Logarithmic	0.001	1.71 ± 0.05	=	1.66 ± 0.16	=	1.69 ± 0.02	=	1.56 ± 0.15
DNA/cell (pg)	Stationary	0.001	0.006 ± 0.003	=	0.007 ± 0.002	=	0.011 ± 0.005	=	0.011 ± 0.006
	Logarithmic	0.001	0.011 ± 0.004	=	0.014 ± 0.010	=	0.012 ± 0.005	=	0.014 ± 0.005
Protein/carbohydrate ratio	Stationary		1.44		1.81		2.32		2.52
	Logarithmic		2.32		1.95		2.13		2.15
Protein/chlorophyll *a* ratio	Stationary		22.41		17.14		19.51		18.88
	Logarithmic		25.63		25.35		25.65		26.15
Protein/RNA	Stationary		2.88		3.97		3.83		3.66
	Logarithmic		3.76		3.52		3.27		3.37
DNA/RNA	Stationary		0.005		0.005		0.008		0.007
	Logarithmic		0.006		0.008		0.007		0.009

[a]Expressed as $NaNO_3$ concentration.
[b]One-way analysis of variance (ANOVA).
[c]One-way analysis of covariance (ANCOVA).

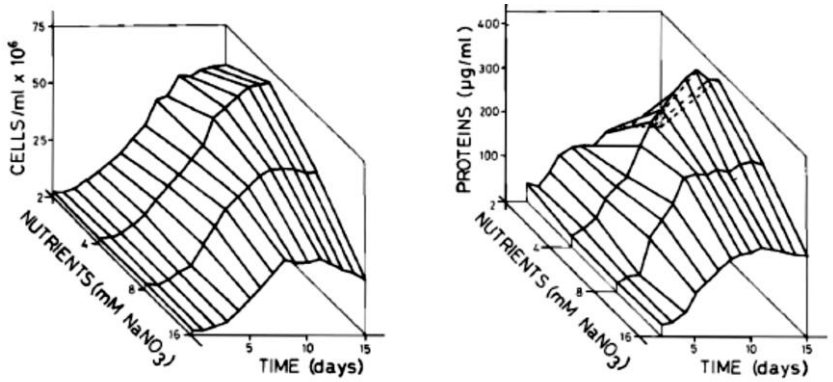

Fig. 2. Growth of *I. galbana* at different nutrient concentrations, expressed as $NaNO_3$ concentrations.

Fig. 3. Protein concentration in mass cultures of *I. galbana* at different nutrient concentrations.

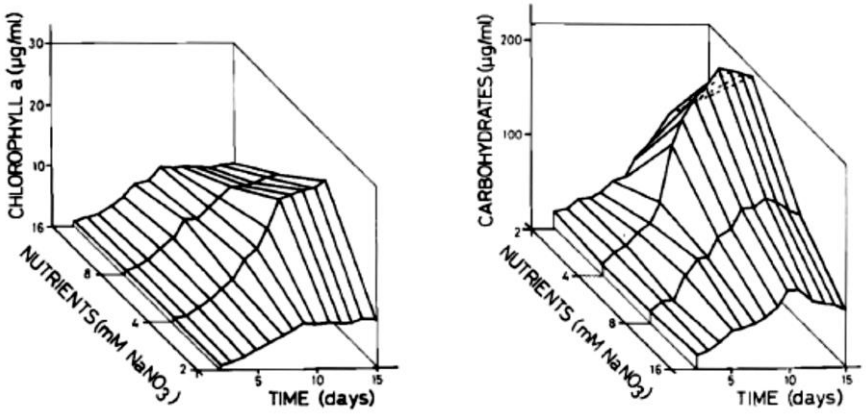

Fig. 4. Chlorophyll *a* concentration in mass cultures of *I. galbana* at different nutrient concentrations.

Fig. 5. Carbohydrates concentration in mass cultures of *I.galbana* at different nutrient concentrations.

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 m*M*	4 m*M*	8 m*M*	16 m*M*
a	+3.63978	-1.07212	+1.39695	+6.15540
b	-2.94001	+2.54075	-0.779589	-6.18315
c	+1.95087	+0.056046	+1.10544	+2.79683
d	-0.161486	+0.053939	-0.045946	-0.26720
e	+0.004142	-0.003219	-0.000819	+0.007605
SD	2.01	1.16	1.82	0.89

(B)	Nutrient concentration			
	2 m*M*	4 m*M*	8 m*M*	16 m*M*
a	+ 6.62832	+22.7627	+169.064	+163.785
b	+ 8.64136	- 7.69408	-147.808	-131.264
c	+ 3.18881	+ 4.27813	+ 44.8354	+ 38.536
d	- 0.35757	+ 0.12038	- 4.16847	- 3.6283
e	+ 0.010184	- 0.018087	+ 0.126139	+ 0.11144
SD	10.02	7.41	12.61	12.01

(C)	Nutrient concentration			
	2 m*M*	4 m*M*	8 m*M*	16 m*M*
a	-2.77662	-1.56142	+2.24279	+5.22698
b	+1.59549	+1.85934	-1.98077	-4.14145
c	-0.059682	-0.498404	+0.689668	+1.20017
d	—	+0.080985	-0.048491	-0.102662
e	—	-0.003263	+0.000981	+0.002835
SD	0.45	0.94	0.57	0.50

(D)	Nutrient concentration			
	2 m*M*	4 m*M*	8 m*M*	16 m*M*
a	- 2.01194	+62.5145	+ 3.40259	+ 16.0929
b	+17.0395	- 37.4424	+ 1.92128	- 2.95617
c	- 4.11159	+ 8.05818	+ 1.27003	+ 1.86863
d	+ 0.519333	- 0.250411	+ 0.02552	- 0.100268
e	- 0.018648	- 0.005742	- 0.006042	—
SD	5.56	10.29	4.53	4.38

At the end of the culture period the protein content had reached maximum values of 374.8 μg/ml and 5.66 pg/cell (Tables 1 and 2) with a nutrient concentration of 4 mM $NaNO^3$; the protein content was lower with higher nutrient concentrations. These differences in the protein content are more acute than those found in other micro algae such as *Dunaliella salina* grown in different culture media, nitrate or glutamine, with a 12% higher protein content (Kosmakova and Prozumenshahikova, 1983). In I. *galbana,* protein/cell concentration was similar for 4,8 and 16 mM of $NaNO_3$, and during the logarithmic phase, protein/cell was independent of the nutrient concentrations (Table 2).

The protein/carbohydrate ratio ranged between 1.44 and 2.52 in the stationary phase and between 1.95 and 2.32 in the logarithmic phase (Table 2). These ratios are similar to the range reported for *T. suecica (1.12-2.74,* Fabregas et al., 1985b), for other microalgae (between 0.23 and 2, Parsons et al., 1961), for *Skeletonema costatum* (from 0.5 to 2 over a diel cycle, Hitchcock, 1980) and in nine diatom species (declining from 2 to between 0.1 and 0.5 as nitrogen was depleted, Myklestad, 1974). These ratios are lower than the ratio of 1.44 obtained for *I. galbana* with 2 mM $NaNO_3$. This represented an efficiency of 100% and, therefore, nitrogen was depleted. This value obtained for 1. *galbana* is, however, very similar to that obtained for *T. suecica* (1.58) under the same conditions when nitrogen was depleted (Fabregas et al., 1985b).

Maximum efficiencies of nitrogen transformation of 109 and 114% were reached with 2 and 4 mM of $NaNO_3$, respectively. The efficiency decreased with higher nutrient concentrations. We established this efficiency as the ratio between the nitrogen added to the culture medium in the form of nitrate 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. Values slightly greater than 100% were also obtained in

mass cultures of *T. suecica* (Fabregas et al., 1985b). The nutrient concentration of 4 mM of $NaNO_3$, which produced the maximum biomass and the maximum protein concentration, also had the maximum efficiency. These data indicate that the cheapest production is obtained with a nutrient concentration of 4 mM $NaNO_3$ (Fig. 6).

Protein/chlorophyll *a* ratios ranged between 17.14 and 22.41 in the stationary phase and between 25.35 and 26.15 in the logarithmic phase. In both phases the values were very similar.

The protein/RNA ratio was practically constant during both growth phases, decreasing only at a concentration of 2 mM of $NaNO_3$ in the stationary phase.

Chlorophyll a/ml reached maximum values of 21.95 µg/ml in the stationary phase with 4 mM of $NaNO_3$. The chlorophyll *a* per culture increased with the nutrient concentration to 4 mM of $NaNO_3$; at higher concentrations the chlorophyll *a* decreased as the nutrient concentration increased (Table 1). Chlorophyll a/cell was constant in the logarithmic phase for all the nutrient concentrations and in the stationary phase for 4, 8 and 16 *mM* of $NaNO_3$. The chlorophyll a/cell content with 2 *mM* of NaN03 in the stationary phase was significantly lower than at higher nutrient concentrations.

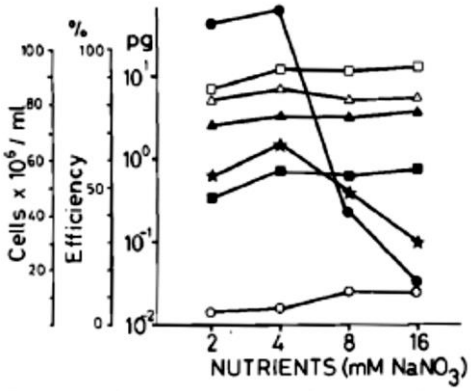

Fig. 6. Cellular density and chemical composition of *I. galbana* in the stationary phase, at different nutrient concentrations. (⋆) cells/ml; (□) protein/cell; (△) carbohydrates/cell; (-) chlorophyll *a*/cell; (▲) RNA/cell; (○) DNA/cell; (●) efficiency (nitrate-N/protein-N transformation rate).

Changes in chlorophyll *a* levels were related to nitrogen depletion which occurred in the stationary phase in the cultures with 2 *mM* of $NaNO_3$. 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 *a* from 0.15 pg/cell to a level of 0.33 pg/cell at the saturation level of nitrogen in the culture, whereas higher concentrations do not increase this value.

There was no relationship between nutrient concentration and chlorophyll a/cell in the logarithmic phase, since chlorophyll a/cell concentrations were constant with all the nutrient concentrations.

As the chlorophyll/cell content in the stationary phase was constant, there was no light-limitation due to shading of the cells at these high densities. Maximum carbohydrate concentration in the stationary phase occurred at 4 *mM* of $NaNO_3$ with 213 µg/ml and 3.16 pg/cell. This nutrient concentration also led to the maximum values of biomass (cells/ml), protein and chlorophyll *a.* In the logarithmic phase, carbohydrate content/cell was constant and was not related to nitrogen depletion, although it has been related to light and temperature (Hitchcock, 1980).

RNA/cell was almost constant in both growth phases, ranging between 1.15 and 1.65 pg/cell in the stationary phase and between 1.56 and 1.71 pg/cell in the logarithmic phase. There was no relationship between RNA and doublings/day, and different values of doublings/day presented the same RNA/cell values. Therefore, we cannot relate the growth velocity of the culture to RNA/cell in the present conditions. Similar results were obtained with *T. suecica* under the same conditions. However, in other systems RNA may serve as a measure of active biomass (Koliander et al., 1984).

DNA/cell was constant in all the nutrient concentrations assayed and in both growth phases; in the same way DNA/cell was 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).

DNA contents of I. *galbana* cells ranged from 0.006 to 0.014 pg/cell. These values are lower than those presented for *T. suecica* (Fabregas et al., 1985b) and than those for *Monochrysis lutherii* and *Navicula pelliculosa* that contain approximately 0.1 pg of DNA per cell (Holm-Hansen, 1969).

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.

Variability in the chemical composition of I. *galbana* has been shown to result from environmental factors. Variations in the chemical composition of I. *galbana* grown in mass culture are closely coupled to changes in nutrient concentration, and these variations occur in protein, chlorophyll *a* and carbohydrate contents, showing differences of 177%, 220% and 136%, respectively, in the stationary phase. The variability in the protein content per cell is

probably the most important for animal feeding in aquaculture systems. A difference of 3.19 and 5.66 pg of protein/cell, in the stationary phase (Table 2) is equivalent to 177% variation in the protein content due to nutrient concentrations, and any mariculture system using microalgae as feed can be affected by these variations. It has already been reported that variations in the chemical composition of microalgae are coupled to changes in nutrient concentration (Myklestad and Haug, 1972), salinity and nutrient concentration (Fabregas et aI., 1984a; 1985a, b) and growth rate (Goldman et aI., 1979; Rhee, 1980) and also that the calorific value of the cells varies with changes in specific growth rate (Scott, 1980).

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