Cah. Biol. Mar. (1994), **35** : 213-223
Roscoff

Variations in the biochemical profile of the marine microalga *Dunaliella tertiolecta* (Butcher) cultured with different nutrient concentrations and urea as a nitrogen source

C. Herrero*, J. Abalde, A. Cid, P. Fidalgo and J. Fabregas**
Laboratorio de Microbiología, Facultad de Ciencias - Universidad de La Coruña -
Campus de la Zapateira s/n - E-15071 La Coruña - Spain
**Laboratorio de Microbiología - Facultad de Farmacia
Universidad de Santiago - E-15706 Santiago de Compostela - Spain

Abstract : Variations in the biochemical profile of the marine microalga *Dunaliella tertiolecta* (Butcher) cultured with different nutrient concentrations and urea as a nitrogen source.
Cultures of the marine microalga *Dunaliella tertiolecta* were grown in a urea-based medium, using four nutrient concentrations following a geometrical progression of order 2, expressed as x1, x2, x4 and x8, in order to obtain a maximum biomass production and to find out its biochemical variability. The nutrient concentration had little effect on the growth rate in the logarithmic phase, with values between 0.40 and 0.44 doublings.day^{-1}. Maximum cellular densities of 7.53×10^6 and 7.96×10^6 cells.ml^{-1} were obtained in x2 and x4 cultures (corresponding to 2 and 4 mM of urea). Differences in biochemical composition were found in function of the nutrient concentration. Protein content reached maximum values in the stationary phase in x4 and x8 cultures, with 29.56 and 29.28 pg.cell^{-1}. The maximum value of carbohydrates was obtained in xl cultures, with 14.38 pg.cell^{-1}. Maximum lipids per cell were obtained in the stationary phase in cultures with the higher nutrient concentration asssayed (x8), with 23.12 pg.cell^{-1}. Cultures at the lower nutrient concentration assayed (xl) showed a nitrogen deficiency, with cells with low protein and high carbohydrate content at the stationary phase.

Résumé : Variations du profil biochimique de la microalgue marine *Dunaliella tertiolecta* (Butcher) cultivée avec différentes concentrations de nourriture et dans un milieu d'urée.
Afin d'obtenir les plus grandes quantités de biomasse et de trouver la variabilité biochimique de la microalgue marine *D. tertiolecta*, nous avons effectué des cultures dans un milieu d'urée, sous quatre concentrations différentes de nourriture, suivant une progression géométrique d'ordre (x1, x2, x4, x8). La concentration en nutriment azoté n'a guère d'effet sur le taux de croissance dans la phase logarithmique (0,40 à 0,44 division.jour^{-1}). Les densités cellulaires maximales (7,53 x 10^6 et 7,96 x 10^6 cell.ml^{-1}) ont été obtenues dans les cultures x2 et x4, correspondant respectivement à 2 et 4 mM d'urée. Des différences au niveau de la composition biochimique ont été observées en fonction de la concentration en nourriture. Les contenus en protéines les plus élevés ont été observés dans les cultures x4 et x8, soit 29,56 et 29,28 pg. cell^{-1}. La valeur maxima en carbohydrates a été obtenue dans les cultures x1 (14,38 pg.cell^{-1}). Les contenus en lipides par cellule les plus élevés ont été observés dans les cultures ayant les plus grandes concentrations de nourriture (x8 : 23,12 pg.cell^{-1}). Les cultures effectuées à la plus faible concentration en nutriment ont conduit à la production de cellules aux contenus peu élevés en protéines mais élevés en carbohydrates, liés au manque d'azote.

INTRODUCTION

The genus *Dunaliella* of the order Volvocales includes a variety of species of unicellular green algae. The chief morphological characteristic of *Dunaliella*, in contrast to other members of the chlorophyta, is the lack of a rigid polysaccharide wall. Instead, the cell is a natu-

* Author for correspondence

ral protoplast, enclosed by only a glycocalix-type envelope (Oliveira *et al.*, 1980 ; Klut *et al.*, 1983). *Dunaliella* demonstrates a remarkable degree of environmental adaptation to salt (Ben-Amotz & Avron, 1978 ; Fabregas *et al.*, 1986b ; Ginzburg, 1987 ; Borowitzka & Borowitzka, 1988) and is widely distributed in natural habitats. The absence of a rigid polysaccharide cell wall in *Dunaliella* permits a rapid adjustment of the intracellular osmotic pressure by fluxes of water through the cytoplasmic membrane. As food for organisms of the next trophic link, *D. tertiolecta* has been used for instance for feeding *Artemia*, and rotifers, or in the rearing of marine fishes and in the rearing of bivalve molluscs (De Pauw & Persoone, 1988). Besides its use in aquaculture systems, *D. tertiolecta* can be also used as raw material for Single Cell Protein (SCP) (Fabregas & Herrero, 1985), as supply of minerals in fish diets (Fabregas & Herrero, 1986) or as potential source of vitamins (Fabregas & Herrero, 1990 ; Abalde *et al.*, 1991).

The biochemical content of microalgae can vary with changes in the environmental conditions. Variations in the nutrient concentration of the culture medium can cause changes in the biochemical content of marine microalgae (Fabregas *et al.*, 1986a ; Richmond, 1986). *D. tertiolecta* showed significant differences in carbohydrate and protein composition in different media (Wikfors *et al.*, 1984) and growth of this marine microalga in mass culture was coupled to changes in nutrient concentration, occurring variations in chlorophyll *a*, protein, carbohydrates and RNA content (Fabregas *et al.*, 1986a).

After carbon, nitrogen is quantitatively the most important element contributing to the dry matter of algal cells. Although the most generally used nitrogen source in microalgal cultures is nitrate, urea appears as a good potential nitrogen source, since it is usually hydrolyzed and its nitrogen is directly incorporated into microalgal cells. Besides the metabolic advantage in using urea as nitrogen source, this compound is easily available in great amounts and at low cost.

In the present work we analyze the growth and biochemical composition in the different growth phases of the marine microalga *Dunaliella tertiolecta* cultured with different nutrient concentrations and urea as the sole nitrogen source, maintaining constant temperature, salinity, carbon source, light and the pH within the optimum range for this microalga.

MATERIALS AND METHODS

The marine microalga *Dunaliella tertiolecta* was obtained from The Culture Centre of Algae and Protozoa, Cambridge, England. It was cultured in seawater filtered through a 0.45 µm Millipore filter, autoclaved at 120 °C for 60 minutes and enriched with urea ($(NH_2)CO$] 1 mM, $NaH_2PO_4.2H_2O$ 0.2 mM, ferric citrate 40.0 µM, $ZnCl_2$ 2.0 µM, $MnCl_2.4H_2O$ 2.0 µM, $Na_2MoO_4.5H_2O$ 2.0 µM, $CoCl_3$ 0.2 µM, $CuSO_4.5H_2O$ 0.2 µM, thiamine 70.0 $\mu g.l^{-1}$, biotine 10.0 $\mu g.l^{-1}$, vitamine B_{12} 6.0 $\mu g.l^{-1}$, EDTA 92.8 µM, TRIS-HCl (pH 7.4) 1.0 mM.

We used four initial nutrient concentrations, following a geometrical progression, corresponding to x1, x2, x4 and x8 of that given above.

Cultures were carried out in 6 l flasks with 5.5 l of culture medium. All cultures were maintained in a controlled environmental incubator at 18±1 °C, 35 ‰ salinity and 110 $\mu E.m^{-2}s^{-1}$ light from fluorescent lamps (Osram daylight L55/10). A 12:12 light-dark cycle was maintained. Cultures were continuously bubbled with 0.22 µm-filtered air at a rate of 15 $l.min^{-1}$. An inoculum of $75x10^4$ logarithmic phase $cells.ml^{-1}$ was used. Cultures were axenic ; axenic conditions were tested daily.

Cellular density was determined by counting culture aliquots in a Thoma chamber. Growth rate was calculated as $doublings.day^{-1}$ as follows :

$$dbls\ day^{-1} = \frac{\ln N(n) - \ln N(i)}{Ln2\ (tn-ti)}$$

where ti and tn are the initial and final time of the logarithmic phase, both expressed in days, and N(i) and N(n) are the initial and final cellular density, respectively.

Microalgal growth is characterized by a sigmoid or logistic function. The form of the logistic growth function was (Schanz & Zahler, 1981) :

$$y(t) = K/[1 + B^{-zt}]$$

where y(t) represents the cellular density at time t and K is the ultimate limiting value ("carrying capacity"). B is a biologically unimportant constant, and its value was calculated by the following equation : $(K-y_0)/y_0$. The parameter z is related with growth rate. All the parameters of the equation were fitted by non-linear regression using Marquardt's algorithm.

Samples for biochemical determinations were taken in the logarithmic (at the 5th, 6th and 7th days of starting the exponential growth) and in the stationary phases (the last five days of culture). Samples were always collected at the same time of the light period because biochemical composition of microalgal cells can vary depending on the moment in the light period at which the sample is taken (Ganf *et al.*, 1986). Chlorophylls were determined spectrophotometrically 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 desintegrator. 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 as described by Kochert (1978a). Lipids were measured by the charring method of Marsh and Weinstein (1966), after extraction with methanol : chloroform : water. Ribonucleic acid (RNA) was extracted with perchloric acid and determined as described by Kochert (1978b).

Data were statistically compared by an overall multivariate one-way analysis of variance (ANOVA) and by a one-way analysis of covariance (ANCOVA).

RESULTS AND DISCUSSION

Urea is the cheapest N source among those generally used, so the ability of growing on urea is a desirable microalgal feature for mass culture. Most of the culture media for marine microalgae use nitrate as N source and many studies have been reported about the effect of these media on growth and biochemical composition of microalgae. However, the use of urea as N source has been less studied. Previously, the ability of *D. tertiolecta* for growing on urea, with a better growth than in nitrate and nitrite (Fabregas *et al.*, 1989) has been shown in batch cultures. After these results, we try to optimize the mass production of *D. tertiolecta* in an urea-based medium in order to obtain a maximum biomass production and to find out its biochemical variability.

Cellular density was plotted against time and against nutrient concentrations. The growth curves and their mathematical functions are shown in Fig. 1.

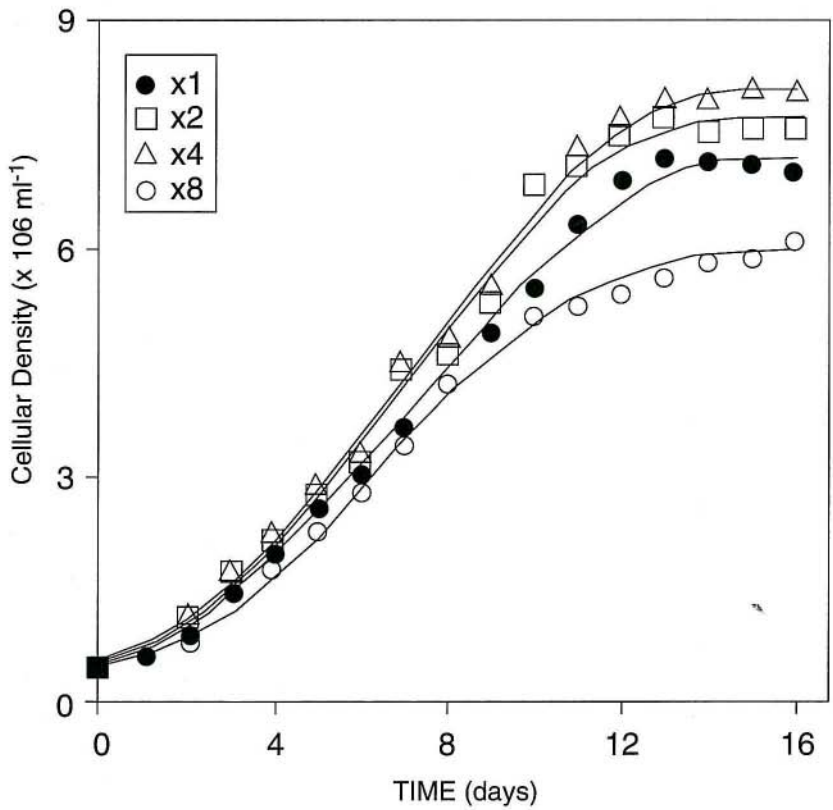

Fig. 1 : Growth of *D. tertiolecta* cultures with different concentrations of nutrients in a urea-based medium. The logistic equations are :

x1 : $Nt = 7.481/1 + 14.61^{-0.393t}$ r = 0.986
x2 : $Nt = 8.023/1 + 15.05^{-0.403t}$ r = 0.997
x3 : $Nt = 8.327/1 + 16.56^{-0.409t}$ r = 0.997
x4 : $Nt = 6.152/1 + 12.22^{-0.406t}$ r = 0.992

Cellular densities yielded in the stationary phase were affected by the nutrient concentration (Table I). Higher values were reached in cultures with nutrient concentrations of x2 and x4, with 7.53×10^6 and 7.96×10^6 cells.ml^{-1}, respectively, without significant differences between them ($P<0.01$). Significant lower cellular densities were obtained in cultures with x1 and x8 concentrations. A similar pattern occurred with the carrying capacity (K) obtained from the logistic function (Table I).

TABLE I

Maximum growth rates, maximum cell densities in the stationary phases and carrying capacities obtained in *D. tertiolecta* cultures with different concentrations of nutrients in a urea-based medium.

	x1		x2		x4		x8
Doublings day	0.42		0.43		0.44		0.40
Cells($x10^6$ ml^{-1})	7.09 ± 0.14	<	7.53 ± 0.23	=	7.96 ± 0.11	>	5.87 ± 0.45
Carrying capacity (K)	7.48		8.02		8.32		6.15

Maximum growth rates of *D. tertiolecta* cultures in the logarithmic phase were between 0.42 and 0.44 doublings.day^{-1} for cultures with x1, x2 and x4, decreasing to 0.40 doublings. day^{-1} at x8 concentration (Table I). Therefore, the nutrient concentration used in the culture media had little effect on the growth rate of *D. tertiolecta* cultures in the logarithmic phase. In a same way, growth rates of *I. galbana* were only slightly affected by the increase in initial concentrations of nitrate in the growth medium (Kaplan *et al.*, 1986a).

Cellular densities increased in cultures with the nutrient concentration up to x4, but decreased at the higher concentration assayed. This decrease suggests a variation in the cellular volume, cellular composition or the toxicity of the N source, since cultures of this species with such high nitrate concentrations and all the nutrients at the same concentrations than those used here did not show any toxic effect (Fabregas *et al.*, 1986a). Toxic effects of urea at high concentrations are not known. In fact, urea concentrations as high as 37.5 and 75 mM have been used in *Chlorella* cultures without any toxic effects (Mak & Trevan, 1989). Variations in the cellular volume were observed, and cells grown in x8 cultures appeared clearly higher than those grown in lower nutrient concentrations. Their composition was also different, as will be seen below.

Differences in the biochemical composition of *D. tertiolecta* were found in function of the nutrient concentration used, with a urea-based medium, although these changes were not necessarily related to cellular density in the culture.

Cellular contents of chlorophyll a, protein, carbohydrates, lipids and RNA in cells growing exponentially and in the stationary phase are represented in Fig. 2A and 2B, respectively, and yields per volume of culture in the stationary phase are shown in Table II.

The main cellular constituent in all cultures and in both growth phases was protein (Fig. 2). Comparing the cellular content in protein in both growth phases for each culture, cell protein concentration was significantly higher in the logarithmic than in the stationary

TABLE II

Ratios between different cellular constituents at logarithmic and stationary phases of *D. tertiolecta* cultures, with different concentrations of nutrients in a urea-based medium.

	Nutrients concentration							
	x1		x2		x4		x8	
Ratios	log.	stat.	log.	stat.	log.	stat.	log.	stat
Protein/Lipids	2.06	1.24	1.84	1.29	1.93	1.51	1.44	1.27
Protein/Carboh.	3.51	1.43	3.14	2.54	3.13	3.09	3.08	2.75
Prot./Lip+Carb.	1.30	0.66	1.16	0.86	1.19	1.01	0.98	0.87
Lipids/Carboh.	1.70	1.15	1.71	1.96	1.62	2.04	2.13	2.17

phase for nutrient concentrations of xl, x2 and x4 cultures, whereas in x8 cultures the cellular protein content was higher in the stationary than in the logarithmic phase. With respect to the effect of nutrient concentration on the protein content per cell, different patterns occurred in logarithmic and stationary phases (Fig. 2). Cell protein concentration tended to decrease in the logarithmic phase when the nutrient concentration increased, with values between 39 pg.cell^{-1} in x1 cultures and 34 pg.cell^{-1} in x4 cultures, and decreased significantly to 25.52 ± 1.32 pg.cell^{-1} in x8 cultures. Conversely, in the stationary phase cell protein concentration significantly increased in cultures with the nutrient concentration up to x4, remaining constant in x8 cultures ($20.51 \pm 1.46 < 25.95 \pm 1.56 < 29.56 \pm 1.17 = 29.28 \pm 1.19$ pg.cell^{-1} in x1, x2, x4 and x8 cultures, respectively).

Cell carbohydrate concentration in the logarithmic phases did not show significant differences among the different cultures, with values about 10 pg.cell^{-1} (Fig. 2A). In the stationary phase, the cellular content of carbohydrates was significantly higher in x1 cultures (14.38 ± 0.63 pg.cell^{-1}) than in the remaining ones, and these values were more similar than those obtained in the logarithmic phase. Therefore, the cellular content of carbohydrate in both growth phases for each culture was found significantly higher in the stationary than in the logarithmic phase for x1 cultures, but differences were not significant for the remaining nutrient concentrations.

Lipids were the second constituents in both growth phases and in all the conditions assayed. Lipid concentrations per cell were very similar for the different nutrient concentrations at the logarithmic phase, with values between 17.47 ± 1.35 and 19.16 ± 1.82 pg.cell^{-1} (Fig. 2). In the stationary phase cell lipid values tended to increase with the nutrient concentration, differences being significant between xl and x2 cultures, and x4 and x8. Maximum cellular content in lipids at the stationary phase were obtained in x8 cultures, 23.12 ± 1.71 pg.cell^{-1}. Cellular lipid content was significantly higher in the stationary phase than in the logarithmic one for x8 cultures.

Cellular contents in chlorophyll a were very similar for the different nutrient concentrations at the logarithmic phase,with values between 3.86 ± 0.15 and 4.87 ± 0.47 pg.cell^{-1}. At

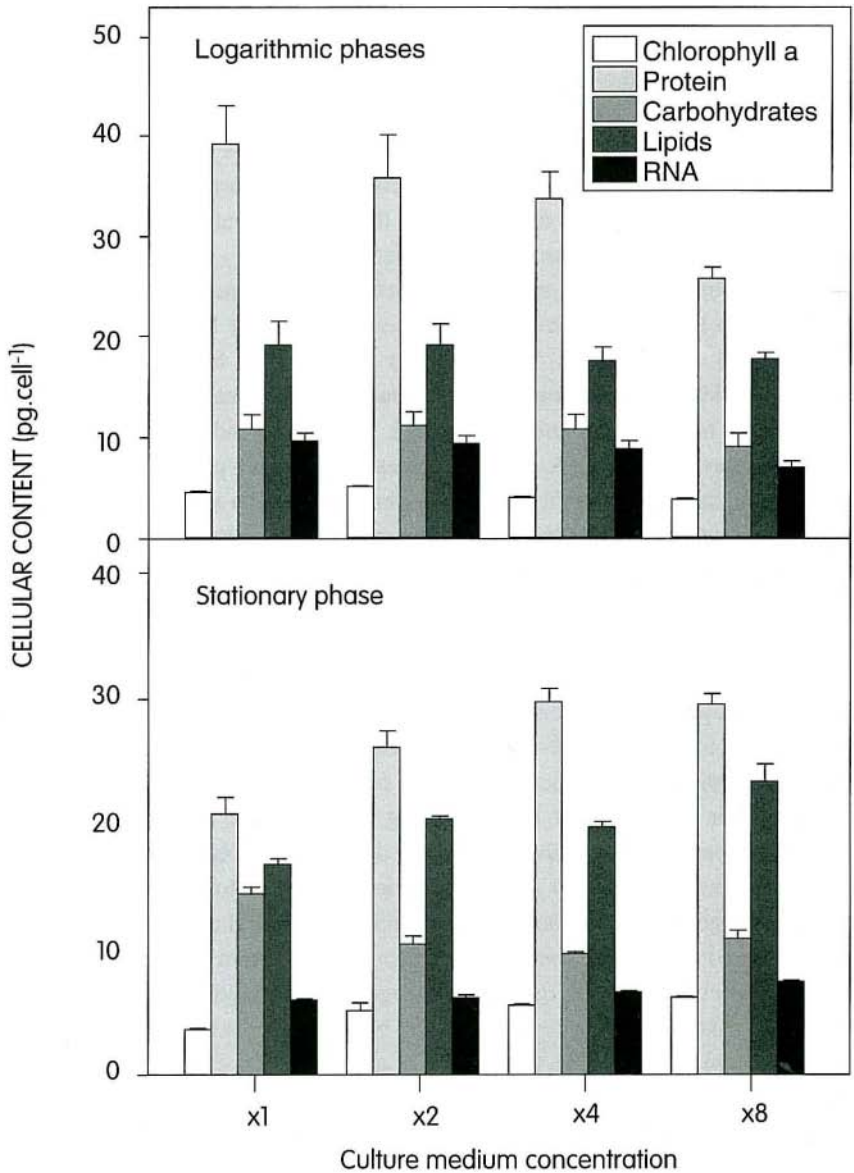

Fig. 2 : Mean values and variations of cellular contents (expressed as pg.cell^{-1}) of *D. tertiolecta* cells grown with different concentrations of nutrients in a urea-based medium, during the logarithmic (A) and stationary (B) phases of growth.

the stationary phase, chlorophyll a/cell ratio tended to increase with the nutrient concentration and significant differences occurred between x1 and x2 cultures, and x4 and x8

(Fig. 2). Chlorophyll a content per cell was higher at the stationary than at the logarithmic phase for all the concentrations assayed, except for x1.

There were not great differences in the cellular RNA content due to the nutrient concentration in both growth phases, although a slight tendency to increase with the nutrient concentration was observed in the stationary phase, whereas RNA per cell tended to decrease in the logarithmic phase, but significant differences only occurred in x8 cultures in both phases (Fig. 2). For each nutrient concentration, the cellular content of RNA was significantly higher in the logarithmic than in the stationary phase.

In non-aerated batch cultures of *D. tertiolecta* with different nitrogen sources and concentrations, two kinds of cultures were defined : cultures at low N concentration and cultures at high N concentration, for N values less than or higher than 2 mM $N.l^{-1}$ (Fabregas *et al.*, 1989). In the present cultures a clear difference can be noted in the biochemical composition between the cultures at xl (2 mM $N.l^{-1}$) and the remaining cultures, mainly in the stationary phase. A strong decrease in cellular protein and an important increase in carbohydrate in the stationary phase were observed in x1 cultures ; a decrease in chlorophyll a content is also shown at the stationary phase in these cultures (Fig. 2). When an algal culture is deprived of an essential nutrient, cell division promptly ceases and the fraction of carbon allocated into storage rnacromolecules can be greatly increased at the expense of protein synthesis, that decreased due to the low availability of N (Myklestad, 1988, Livne & Sukenik 1992). Under N limiting conditions, cellular protein content decreased and carbohydrates and/or lipids and fatty acids increased (Utting, 1985, Sukenik & Livne 1991). Microalgal cells with low protein and high carbohydrate contents have been often associated with nitrogen deficiency both in culture (Wikfors 1986, Myklestad 1988), and in nature under conditions of blooms (Haug *et al.*, 1973). Under nitrogen deficient conditions the content of photosynthetic pigments decreases (Kaplan *et al.*, 1986b).

The remaining cultures (x2, x4 and x8) did not present a similar biochemical pattern since nitrogen was not a limiting factor. The cellular biochemical composition in the stationary phase in x2 and x4 cultures was similar except for the protein content, that was significantly higher in x4 cultures due to the higher nitrogen concentration. In x8 cultures, cellular density was lower but cells were higher with the higher contents in protein, lipids, chlorophyll a and RNA ; in these cultures the content of all the cellular components was higher in the stationary than in the logarithmic phase.

Ratios between different components were also calculated (Table II). Some of these ratios have been proposed as indicators of physiological state of microalgal populations. Protein/lipids ratio (P/L) ranged between 1.44 and 2.06 in the logarithmic phase and between 1.24 and 1.51 in the stationary phase. In the stationary phase, the P/L ratio increased with the nutrient concentration up to x4, decreasing at x8. This ratio was higher in the logarithmic than in the stationary phase for all the nutrient concentrations used. The protein/carbohydrates (P/C) ratio varied between 3.08 and 3.51 in the logarithmic phase and between 1.43 and 3.09 in the stationary phase, being higher in the logarithmic than in the stationary for all the cultures. The P/C ratio was higher than the P/L ratio in all the cultures and in

both growth phases. The ratio between protein and storage products (carbohydrates + lipids) was between 0.98 and 1.30 in the logarithmic phase, decreasing in the stationary phases, with values between 0.66 and 1.01. Ratios between lipids and carbohydrates (L/C) were very similar in the logarithmic phase (1.71-1.62), except for the x8 cultures (2.13). In the stationary phase this relationship increased with the nutrient concentration, between 1.15 and 2.17. Regarding the growth phases, this ratio increased in the stationary phase for all cultures except for the x1 culture.

The nitrogen deficiency in the x1 cultures was confirmed by the ratios between different components (Table II) : protein/carbohydrates falls from 3.51 to 1.43, protein/lipids + carbohydrates from 1.30 to 0.66, and lipids/carbohydrates from 1.70 to 1.15. The protein/carbohydrate ratio has been defined as a convenient parameter for characterizing the physiological state of a microalgal cell and the nutrient deficiency because it responds to the availability of the most commonly limiting nutrients, N and P, decreasing under 2 when exhausted ; in deficient cells there is a fall in the protein/carbohydrate ratio primarily because of a build-up of storage products (Ganf *et al.*, 1986). This ratio is independent of light conditions, since this ratio can be used as an indicator of both the kind and severity of the nutrient limitation occurring (Wynne & Rhee, 1986). All these facts indicate a N limitation of this culture in the stationary phase.

The lipids/carbohydrates ratio had shown a relationship more linear with the N concentration than P/C and P/L ratios for *D. tertiolecta* cultures with nitrate, nitrite and urea (Fabregas *et al.*, 1989), acting as a suitable indicator of the microalgal physiological state. In the present results, there was also a correlation between this ratio and the nutrient concentration in the stationary phase (Table II). However, this ratio will increase or decrease as a function of the storage products of each species, and for the same species as a function of the age of the culture. In addition to this, this ratio can vary for the same species when growth is limited by different environmental factors.

In reference to the yields of different components in the stationary phase (Table III), when the nutrient concentration increased the total protein content of the cultures significantly increased up to x4 cultures, whereas a statistically significant decrease occurred at x8 cultures. Maximum values of protein per liter of culture were obtained in x4 cultures, with 235.40 $mg.l^{-1}$. Maximum values of carbohydrates per liter of culture in the stationary phase occurred in x1 cultures, with 102.16 ± 6.02 $mg.l^{-1}$. Carbohydrate concentration decreased significantly at higher nutrient concentrations. There was a strong increase in the lipid l^{-1} concentration at the stationary phase for nutrient concentrations between x1 and x2, decreasing with the highest concentration assayed. Maximum values of chlorophyll $a.1^{-1}$ were obtained in x2 and x4 cultures, with 39.54 ± 4.60 $mg.l^{-1}$ and 42.54 ± 2.40 $mg.1^{-1}$, respectively. RNA content per liter of culture reached maximum values in x4 cultures, with 52.11 ± 0.74 $mg.l^{-1}$. Overall, cultures with a x4 nutrient concentration showed better yields of all constituents except carbohydrates. However, these differences were not significant with x2 cultures, except for protein and RNA ; but even in these cases differences were lower than 20 %, indicating that the improvements in the yields between x2 and x4 cultures were not balanced with the increase in the nutrient concentration (100 %).

Urea is one of the lowest cost nitrogen source for microalgal culture, making it an ideal growing medium for mass microalgal cultivation. Urea is generally hydrolysed by urease or UALase (urea amydolyase) (Bekheit & Syrett, 1977) rendering two moles of ammonium and one of CO_2. Therefore, urea is a source of both carbon and nitrogen. Carbon is often a limiting nutrient in mass cultivation of microalgae and aeration with CO_2 enriched air is needed. The use of urea in the culture medium can avoid, at least in part, this aeration.

The possible and desired development of aquaculture will require the availability of low-cost algal nutrients and the knowledge of concentrations which lead to the lowest nutrient losses. But, besides their use in aquaculture, microalgae have other potential biotechnological uses and immobilized microalgae have been successfully used in different processes (Trevan & Mak, 1988 ; Bedell & Darnall, 1990). A problem found with many immobilized cells is their peripheral distribution in gel beads, suggesting diffusional limitations within the matrix. In the case of photosynthetic algae, CO_2 limitations appear to be particularly important. Immobilized cells of *Chlorella emersonii* were provided with urea as a dual source of N and C and co-immobilized with the enzyme urease to increase the urea hydrolysis. This co-immobilization increased biomass retention in media of high urea concentrations (Mak & Trevan, 1989).

Urea appears as a potential nutrient of high quality and low cost for mass cultivation of microalgae.

ACKNOWLEDGEMENTS

This work was supported by a grant of CICYT (Ref. n° AGF92-0736)

REFERENCES

ABALDE, J., J. FABREGAS & C. HERRERO, 1991. B-carotene, vitamin C and vitamin E content of the marine microalga *Dunaliella tertiolecta* cultured with different nitrogen sources. *Biores. Technol.* 38 : 121-125.

BEDDELL, G.W. & D.W. DARNALL, 1990. Immobilization of nonviable, biosorbent, algal biomass for the recovery of metal ions. In : B. Volesk y (eds), Biosorption of Heavy Metals. CRC Press, Boca Raton 313-326.

BEKHEIT, I.A. & P.J. Syrett, 1977. Urea degrading enzymes in algae. *Br. Phycol. J.* 12 : 137-143.

BEN-AMOTZ, A. & M. AVRON, 1978. On the mechanism of osmoregulation in *Dunaliella*. In : S.R. Kaplan & M. Ginzburg (eds), Energetics & Structure of Halophilic Microorganisms. Elsevier/North Holland Biomedical Press, Amsterdam : 529-541.

BOROWITZKA, M.A. & L.J. BOROWITZKA, 1988. *Dunaliella*. In : M.A. Borowitzka & L.J. Borowitzka (Editors), Microalgal Biotechnology. Cambridge University Press, New York : 27-58.

BRADFORD, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein dye-binding. *Anal. Biochem.* 72 : 248-254.

DE PAUW, N. & G. PERSOONE, 1988. Microalgae for aquaculture. In : M.A. Borowitzka & L.J. Borowitzka (Editors), Microalgal Biotechnology. Cambridge University Press, New York : 197-221.

FABREGAS, J., J. ABALDE, B. CABEZAS & C. HERRERO, 1989. Changes in protein, carbohydrates and gross energy in the marine microalga *Dunaliella tertiolecta* (Butcher) by nitrogen concentrations as nitrate, nitrite and urea. *Aquacultural Eng.* 8 : 223-229.

FABREGAS, J. & C. HERRERO, 1985. Marine microalgae as a potential source of single cell protein (SCP). *Appl. Microbiol. Biotechnol.* 23 : 110-113.

FABREGAS, J. & C. HERRERO, 1986. Marine microalgae as a potential source of minerals in fish diets. *Aquaculture* 51 : 237-243.

FABREGAS, J. & C. HERRERO, 1990. The potential use of marine microalgae as source of vitamins in nutrition. *J. Ind. Microbiol.* 5 : 259-264.

FABREGAS, J., C. HERRERO, J. ABALDE, R. LIAÑO & B. CABEZAS, 1986a. Biomass production and biochemical variability of the marine microalga *Dunaliella tertiolecta* (Butcher) with high nutrient concentrations. *Aquaculture* 53 : 187-199.

FABREGAS, J., C. HERRERO, B. CABEZAS, R. LIAÑO & J. ABALDE, 1986b. Response of the marine microalga *Dunaliella tertiolecta* to nutrient concentration and salinity variations in batch cultures. *J. Plant Physiol.* 125 : 475-484.

GANF, G.G., S.J.L. STONE & R.L. OLIVER, 1986. Use of protein to carbohydrate ratios to analyse for nutrient deficiency in phytoplankton. *Aust. J. Mar. Freshw. Res.* 37 : 183-197.

GINZBURG, M. 1987. *Dunaliella* : a green alga adapted to salt. *Adv. Bot. Res.* 14 : 93-183.

HAUG, A., S. MYKLESTAD & E. SAKSHAUG, 1973. Studies on the phytoplankton ecology of the Trondheimsfjord. I. The chemical composition of phytoplankton populations. *J. Exp. Mar. Biol. Ecol.* 11 : 15-26.

KAPLAN, D., Z. COHEN & A. ABELIOVITCH, 1986a. Optimal growth conditions for *Isochrysis galbana. Biomass* 9 : 37-48.

KAPLAN, D., A.E. RICHMOND, Z. Dubinsky & S. AARONSON, 1986b. Algal nutrition. In : A. Richmond (eds), Handbook of Microalgal Mass Culture. CRC Press, Boca Raton : 147-197.

KLUT, M.E., T. BISALPUTRA, & N.J. ANTIA, 1983. Agglutination of the chlorophyceaen flagellate *Dunaliella tertiolecta* by treatment with lectins or divalent cations at alkaline pH. *J. Phycol.* 19 : 112-115

KOCHERT, G., 1978a. Carbohydrate determination by the phenol-sulfuric acid method. In : J.A. Hellebust & J. S. Craigie (eds), Handbook of Phycological Methods. Physiological and Biochemical Methods. Cambridge University Press, London : 95-97.

KOCHERT, G., 1978b. Quantitation of the macromolecular components of microalgae. In : J.A. Hellebust & J. S. Craigie (eds), Handbook of Phycological Methods. Physiological and Biochemical Methods. Cambridge University Press, London : 189-195.

LIVNE, A. & A. SUKENIK, 1992. Lipid synthesis and abundance of acetyl CoA carboxylase in *Isochrysis galbana* (Prymnesiophyceae) following nitrogen starvation. *Plant Cell Physiol.* 33 : 1 175-1 181.

MAK, A.L. & M.D. TREVAN, 1989. Urea as a nitrogen source for immobilized *Chlorella*. In : Biochemistry of Algae and Cyanobacteria. *Proc. Phytochem. Soc. Eur* . 28 : 362-363.

MARSH, J.B. & D.B. WEINSTEIN, 1966. Simple charring method for determination of lipids. *J. Lipid Res.* 7 : 574-576.

MYKLESTAD, S., 1988. Production, chemical structure, metabolism, and biological function of the (1->3)-linked, ß-D-glucans in diatoms. *Biol. Oceanogr.* 6 : 313-326.

OLIVEIRA, L., T. BISALPUTRA & N.J. ANTIA, 1980. Ultraestructural observation of the surface coat of *Dunaliella tertiolecta* from staining with cationic dyes and enzyme treatments. *New Phytol.* 85 : 385-265.

PARSONS, T.R. & J.D.H. STRICKLAND, 1965. Particulate organic matter. III. I Pigment analysis. III. II. Determination of phytoplankton pigments. *J. Fish. Res. Bd. Can.* 18 : 117-127.

RICHMOND, A. 1986. Cell response to environmental factors. In : A. Richmond (ed), Handbook of Microalgal Mass Culture. CRC Press, Boca Raton : 69-99.

SCHANZ, F. & U. ZAHLER, 1981. Prediction of algal growth in batch cultures. Schweiz. *Z. Hydrol.* 43 : 103-113.

SUKENIK, A. & A. LIVNE, 1991. Variations in lipid and fatty acid content in relation to acetyl CoA carboxylase in the marine prymnesiophyte *Isochrysis galbana. Plant Cell Physiol.* 32 : 371-378.

TREVAN, M.D. & A.L. MAK, 1988. Immobilized algae and their potential for use as biocatalyst. *Trends Biotech.* 6 (3) : 68-73

UTTING, S.D. 1985. Influence of nitrogen availability on the biochemical composition of three unicellular marine algae of commercial importance. *Aquaculture Eng.* 4 : 175-190.

WYNNE, D. & G.Y. RHEE, 1986. Effects of light intensity and quality on relative N and P requeriment (the optimun N:P ratio) of marine planktonic algae. *J. Plank. Res.* 8 : 91-103.

WIKFORS, G.H., J.W.Jr. TWAROG & R. UKELES, 1984. Influence of chemical composition of algal food sources on growth of juvenile oysters, *Crassostrea virginica. Biol. Bull* 167 : 251-263.