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Biochemical composition and growth of the marine microalga Dunaliella tertiolecta (Butcher) with different ammonium nitrogen concentrations as chloride, sulphate, nitrate and carbonate.

Jaime Fábregas, Julio Abalde and Concepción Herrero

Laboratorio de Microbiología, Facultad de Farmacia, Universidad de Santiago, Spain

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Abstract

Cultures of the marine microalga Dunaliella tertiolecta were grown in ammonium chloride, sulphate, nitrate and carbonate at concentrations ranging from 0.25 to 16 mg.atom N/l. Cells were harvested in the stationary phase and cell density and biochemical composition determined. Biomass production at the end of the stationary phase, expressed as cell density, was affected by the concentration of ammonium-N in the medium but not by the ammonium compound used. Optimal growth conditions for obtaining maximum cell density, between 1.86×10^6 and 2.81×10^6 cells/ml, were 2, 4 and 8 mg.atom N/l. The compound and concentration of ammonium-N had little effect on the growth velocity of D. tertiolecta cultures in the logarithmic phase, with values of 0.35±0.06 doublings/day under all the conditions assayed. The ammonium compound and the concentration of nitrogen affected the concentration of different cellular constituents such as protein, carbohydrate, lipid and chlorophyll a, although these changes were not necessarily related to cell density in the culture. Protein, the most affected fraction, tended to increase with an increase in the

nitrogen concentration for all the ammonium compounds used. Maximum protein/ml was obtained with ammonium carbonate at all the nitrogen concentrations used. Maximum protein/cell occurred at the higher nitrogen concentrations (16 and 32 mg/atom N/l) for all the ammonium compounds. Considering the optimum growth interval (2-8 mg.atom N/l), maximum protein/cell concentrations were also obtained in the cultures with ammonium carbonate. Carbohydrate and lipid concentrations varied less than protein concentration. Maximum values of carbohydrate/ml were also found in the ammonium carbonate cultures. Maximum lipid/cell concentrations occurred at the lowest nitrogen concentrations, in contrast to protein values. As a percentage of the total organic matter, protein increased and lipid decreased with the nitrogen concentration, whereas carbohydrate remained constant. Consequently, lipid seemed to be the storage product in this marine microalga. Gross energy values in the different cultures were a function of nitrogen concentration, maximum differences occurring in the ammonium carbonate cultures. The biochemicalvariability of this microalga must have a marked effect on its value as a source of single-cell protein, as 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), 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, 1983; Fabregas et al., 1986c). This marine microalga is used in the production of chemicals such as glycerol and β-carotene (Ben-Amotz and Avron, 1983; Borowitzka, 1986), as a source of single-cell protein (Fabregas and Herrero, 1985), as a source of minerals in fish diets (Fabregas and Herrero, 1986), in aquaculture for feeding *Artemia* and rotifers (Mason, 1963), in the rearing of marine fishes (Scott and Baynes, 1979) and in the rearing of bivalve molluscs (Wikfors et al., 1984).

The manner in which microalgae are cultivated varies widely, depending not only on the specific organism, but also on the intended use of the culture. Variations in nutrient availability can produce changes in the microalgal biochemistry (Parsons and Takahashi, 1973; Kaplan et al., 1986). For example, nitrogen deficiency in the culture medium affects the chemical composition of microalgae (Myklestad and Haug, 1972; Utting, 1985). Large variations in protein, chlorophyll and RNA contents have been shown in cultures of different marine microalgae at high nutrient concentrations

(Fabregas et al., 1985, 1986a, b). After carbon, nitrogen is quantitatively the most important element contributing to the dry matter of algal cells.

A variety of nitrogen compounds, both inorganic and organic, can serve as sole nitrogen sources for the growth of various microalgae. The ability to use nitrate (NO^-_3), nitrite (NO^-_2) or ammonium (NH^+_4) appears to be general among microalgae (Kaplan et al., 1986), although ammonium is usually the nitrogen source preferred. The ammonium ion can be supplied to the cultures in different compounds which can themselves affect the development of a microalgal culture, by affecting the pH or by being, themselves, sources of elements other than nitrogen. Chloride and sulphate are the ammonium compounds most generally used, but ammonium nitrate can supply two forms of nitrogen, and the carbonate supplies a carbon source besides the nitrogen.

In the present work we analyzed the growth, the biochemical composition and the gross energy potentially available from the marine microalga *Dunaliella tertiolecta* cultured with different sources and concentrations of ammonium-N, and adjusting the phosphorus concentrations in order to maintain a constant N /P ratio.

MATERIALS AND METHODS

The marine microalga *Dunaliella tertiolecta* was obtained from The Culture Centre of Algae and Protozoa, Cambridge, England. *D. tertiolecta* was cultured, with different sources and concentrations of ammonium-N, in seawater filtered through a 0.45 *µm* Millipore filter, autoclaved at 120ºC for 60 min and enriched with $ZnCl_2$, 1 µM; $MnCl_2$, 1 µM; Na_2MoO_4, 1 µM; $CoCl_3$, 0.1 *µ*M; $CuSO_4$, 0.1 *µM;* ferric citrate, 20 µM; thiamine, 35 *µ*g/l; biotin, 5 *µ*g/l; B_{12}, 3 *µ*g/l; EDTA, 26.4 mM; Tris-HCl, 15 mM; pH 7.6. The ammonium-N sources were ammonium chloride (NH_4Cl), ammonium sulphate [$(NH_4)_2SO_4$], ammonium nitrate (NH_4NO_3) and ammonium carbonate [$(NH_4)_2CO_3$]. The phosphorus concentrations, as NaH2P04, were adjusted in order to maintain a constant N /P ratio.

Seven concentrations of each ammonium -N compound were used: 0.25, 0.50, 1, 2, 4, 8 and 16 mg.atom N/I and, respectively, phosphorus concentrations of 12.5, 25, 50, 100, 200, 400 and 800 *µ*g.atom P /1. The 7 X 4 resulting media were expressed as a function of the ammonium-N concentration but phosphorus concentration was proportionally changed.

Cultures were grown in Kimax screw-capped test tubes (15 X 2.5 cm) with 40 ml of medium. All cultures were maintained in a controlled-environment incubator at 18± 1°C, 35‰ salinity, pH between 7 and 8 and 3900 lux light from fluorescent lamps (Osram daylight L55/10). A 12 h : 12 h light-dark cycle was maintained. An inoculum of 5 X 10^4 logarithmic phase cells/ml was used. All the experiments were carried out in duplicate. Cultures were unialgal.

Transmittance of the cultures was determined at 530 nm with a Colemann II 6/20 spectrophotometer and values were expressed as (100-T). Cell density was determined by counting culture aliquots in a Thoma chamber. Growth velocity was calculated as doublings/day (dbls/day):

$$\text{dbls/day} = \frac{\ln N_n - \ln N_i}{\ln 2\ (t_n - t_i)}$$

where t_i and t_n were the initial and final time of the logarithmic phase, in days, and N_i and N_m were the initial and final cell densities, respectively.

Samples for biochemical determinations were always collected in the stationary phase 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 aI., 1986).

Chlorophylls were determined spectrophotometrically by the formula of Parsons and Strickland (1965). Protein was measured by the dye-binding method (Bradford, 1976) and carbohydrate by the phenol-sulphuric acid method as described by Kochert (1978a). Lipid was measured by the charring method of Marsh and Weinstein (1966). Ribonucleic acid (RNA) was extracted with perchloric acid and determined as described by Kochert (1978b). The biochemical composition has been expressed as a percentage of the total organic content in some comparisons. Total organic content was equivalent to the sum (in pg/cell) of the protein, carbohydrate and lipid recovered in the chemical analysis (Utting, 1985).

Gross energy of the microalgal cells under the different conditions was calculated in the stationary phase using the formula of the National Research Council (1977):

GE (kcal/kg) =5.72 (% protein) + 9.50 (% lipid) +4.03 (% carbohydrate)

Stationary phases, corresponding to maximum biomass production, were compared by an overall multivariate one-way analysis of variance (ANOVA).

RESULTS

Transmittance, expressed as (100-T), was plotted against time and against nitrogen concentration for each source of nitrogen tested (Fig. 1). Statistical treatment of these figures is presented in Table I.

The marine microalga *D. tertiolecta* grew with each ammonium compound tested and in a wide range of nitrogen concentrations. Growth, expressed as 100-T, tended to increase with nitrogen concentration up to 1 mg.atom N/l for each ammonium compound used; at higher ammonium concentrations there were no significant differences in growth due to nitrogen concentrations, except for the cultures with ammonium carbonate, in which there was a significant *(P* < 0.01) decrease in growth at higher concentrations (8,16,32 mg.atom N/l*)* (Fig. 1, Table 1).

TABLE 1

Statistical analysis of the growth curves of *D. tertiolecta* (represented as 100−T) with different sources and concentrations of ammonium nitrogen. Values are expressed as mean ±standard deviation. Each value corresponding to a nitrogen concentration is compared by a one-way analysis of variance (ANOVA) with the following nitrogen concentration

Ammonium-N (mg.atom N/l)	Ammonium source			
	Chloride	Sulphate	Nitrate	Carbonate
0.25	23.42 ± 2.08	25.13 ± 2.37	58.50 ± 1.86	21.04 ± 2.16
	<	<	=	<
0.50	57.33 ± 2.96	60.50 ± 3.08	59.42 ± 3.64	50.63 ± 2.81
	<	<	<	<
1	68.17 ± 4.95	70.33 ± 4.88	71.50 ± 4.25	69.50 ± 4.04
	=	=	=	=
2	72.17 ± 5.31	74.67 ± 4.67	76.58 ± 4.08	75.00 ± 4.52
	=	=	=	=
4	77.92 ± 4.53	78.33 ± 4.30	78.08 ± 3.31	77.75 ± 5.08
	=	=	=	>
8	74.17 ± 4.61	75.08 ± 4.54	74.92 ± 3.04	69.92 ± 3.09
	=	=	=	>
16	69.25 ± 4.55	67.83 ± 5.16	71.22 ± 4.49	55.67 ± 4.76
	=	=	=	>
32	68.75 ± 5.95	64.42 ± 5.30	69.50 ± 3.19	34.00 ± 4.59

Considering the effect ofthe different ammonium compounds at similar nitrogen concentrations on the growth of *D. tertiolecta,* differences appeared only at extreme nitrogen concentrations. There were no significant *(P<0.01)* differences at concentrations between 1 and 8 mg.atom N/l At 0.25 mg.atom *N/l* growth was significantly higher in ammonium nitrate cultures, whereas at 0.5, 16 and 32 mg.atom *Nil* growth was significantly lower in ammonium carbonate cultures.

Optimal growth conditions for obtaining maximum cell density in the stationary phase were 2, 4, 8 mg.atom N/l (Fig. 2), with cell densities between 1.86 X 10^6 and 2.81 X 10^6 cells/ml. Cell densities obtained with the different ammonium compounds for the

same nitrogen concentration were very similar, as had also occurred with the transmittance.

The compound and concentration of ammonium had no effect on the growth velocity of *D. tertiolecta.* Maximum growth velocity in the logarithmic phase was 0.35 ± 0.06 doublings/day under all conditions, with no correlations between growth and ammonium compound or nitrogen concentration.

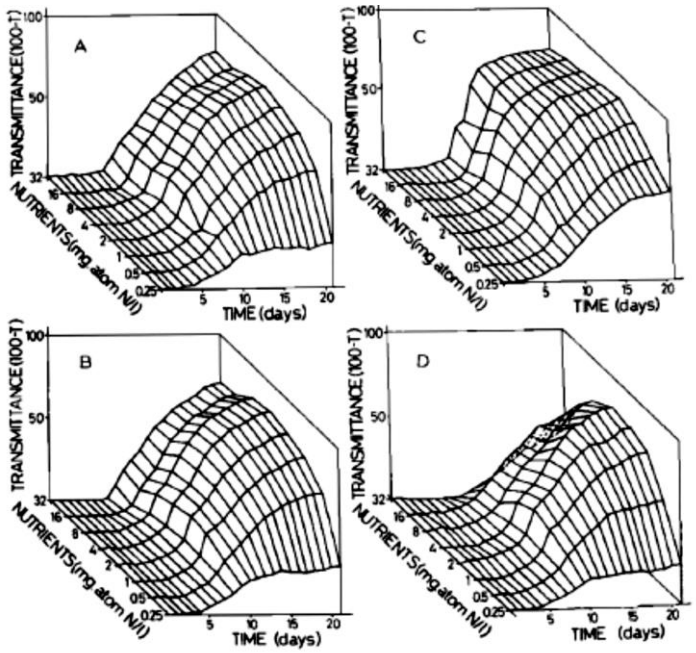

Fig. 1. Growth of *D. tertiolecta* with different sources and concentrations of ammonium nitrogen. Transmittance at 530 nm is expressed as (100 – T) values. (A) Cultures with ammonium chloride; (B) ammonium sulphate; (C) ammonium nitrate; and (D) ammonium carbonate.

The source and concentration of ammonium-N affected the protein content of D. tertiolecta cultures (Fig. 3) . When the ammonium-N concentration increased, the total protein content of the cultures tended to increase, except in cultures with chloride and carbonate in which nitrogen concentration higher than 4 mg.atom N/l reversed the process (Fig. 3a). Maximum values of protein per ml of culture were 86.77 µg/ml in the cultures with ammonium chloride at 4 mg.atom N/l, 85.03 *µ*g/ml in ammonium sulphate at 32 mg.atom N/l, 94.03 *µ*g/ml in ammonium nitrate at 32 mg.atom N/l and 107.41 *µ*g/ml in ammonium carbonate at 4 mg.atom N/l Comparing the effect of the different ammonium compounds for the same nitrogen concentration, the highest values of protein/ml occurred in the ammonium carbonate cultures, except for the extreme nitrogen concentrations (0.25 and 32 mg.atom N/l).

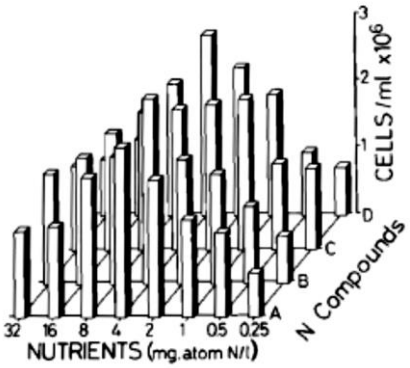

Fig. 2. Maximum cellular density of *D. tertiolecta* in the stationary phase in cultures with different sources and concentrations of ammonium nitrogen. (A) Chloride; (B) sulphate; (C) nitrate; and (D) carbonate.

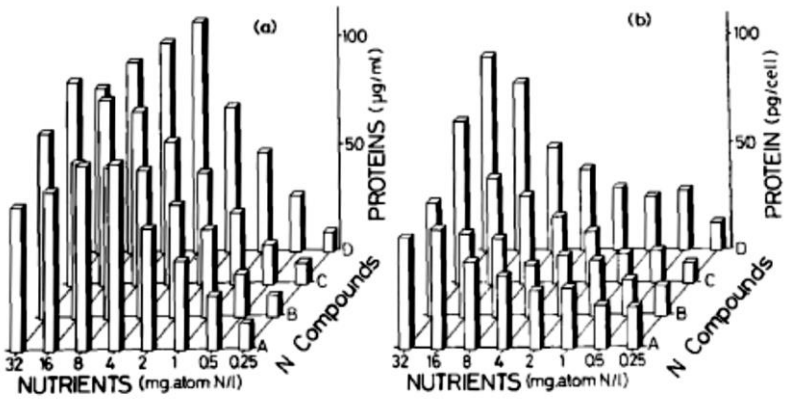

Fig. 3. Protein concentration in the stationary phase of *D. tertiolecta* cultures with different sources and concentrations of ammonium nitrogen. (a) Protein per ml (µg/ml); (b) protein per cell (pg/cell). (A) Chloride; (B) sulphate; (C) nitrate; and (D) carbonate.

Protein/cell increased as nitrogen concentration increased, with all the ammonium-N sources (Fig. 3b). Maximum protein/cell values occurred at the higher ammonium-N concentrations (16 and 32 mg.atom N/l) for all the ammonium sources, with values of 52.16, 75.22,90.78 and 55.27 pg/cell in the cultures with ammonium sulphate, nitrate, carbonate and chloride, respectively. Protein/cell concentrations were very similar at nitrogen concentrations up to 4 mg.atom N/l, but at higher N concentrations protein/cell values were higher in ammonium nitrate and carbonate than in ammonium chloride or sulphate.

The compound and concentration of ammonium-N affected the carbohydrate content per cell and per ml of culture of *D. tertiolecta* (Fig. 4), although differences were lower than those with protein. When the ammonium-N concentration increased, the total carbohydrate in the cultures increased proportionally to nitrogen concentrations up to 2-4 mg.atom N/l, for the four ammonium compounds, but higher nutrient concentrations

reversed the process. Maximum values of carbohydrate/ml were 36.99 μg/ml in ammonium chloride cultures, 41.89 μg/ml in ammonium sulphate cultures, 40.49 μg/ml in ammonium nitrate cultures and 42.40 μg/ml in ammonium carbonate cultures (Fig. 4a). Carbohydrate/cell ratio varied with the nitrogen concentration in the same way as carbohydrate/ml, with maximum values at nitrogen concentrations higher than 4 mg.atom N/l. Maximum carbohydrate / cell values were 23.78 pg/cell, 18.52 pg/cell, 23.23 pg/cell and 17.17 pg/cell, in ammonium chloride, sulphate, nitrate and carbonate, respectively.

The carbohydrate fraction was less affected by the ammonium compound than was the proteinaceous fraction. There were few differences in the carbohydrate contents among the different ammonium compounds for a specific nitrogen concentration.

Maximum values of lipid concentration per ml of culture oc curred between 1 and 8 mg.atom N /1 (Fig. 5). These maximum values were 48.26 μg/ml in ammonium chloride cultures at 4 mg.atom N/l, 47.14 μg/ml in ammonium sulphate cultures at 8 mg.atom N/l, 38.58 μg/ml in ammonium nitrate cultures at 2 mg.atom N/l and 46.45 μg/ml in ammonium carbonate cultures at 2 mg.atom N/l. Maximum lipid/cell concentrations occurred at the lowest nitrogen concentrations (0.25-0.5 mg.atom N/l), and were 25.65 pg/cell, 26.86 pg/cell, 21.03 pg/cell and 29.18 pg/cell, respectively. In comparison with protein and carbohydrate, lipid values both per cell and per ml showed little variation.

Chlorophyll *a* content per ml increased with the nitrogen concentration to 2-4 mg.atom N/l, but decreased at higher nitrogen values (Fig. 6). Maximum values of chlorophyll a/ml were 8.86 μg/ml in ammonium chloride cultures at 4 mg.atom N/l, 9.98 μg/ml in ammonium sulphate cultures at 4 mg.atom N/l, 8.58 μg/ml in ammonium nitrate cultures at 2 mg.atom N/l and 10.41 μg/ml in ammonium carbonate cultures at 4 mg.atom N/l. Maximum concentrations of chlorophyll *a* per cell were found between 0.5 and 2 mg.atom N/l, with values of 5.41 pg/cell in ammonium chloride cultures, 5.17 pg/cell in ammonium sulphate cultures, 4.44 pg/cell in ammonium nitrate cultures and 4.54 pg/cell in ammonium carbonate cultures. Chlorophyll *a* content per cell tended to diminish at higher ammonium-N concentrations.

If we consider the percentages of the different cellular compounds in relation to the total organic matter (protein + lipid + carbohydrate), in all the ammonium compounds the percentage of protein increased with the nitrogen concentration, whereas the percentage of lipid decreased and the percentage of carbohydrate increased up to 2-4 mg.atom N/l and tended to diminish at higher ammonium -N concentrations (Table 2).

Maximum percentages of protein were found in ammonium carbonate cultures for all nitro gen concentrations.

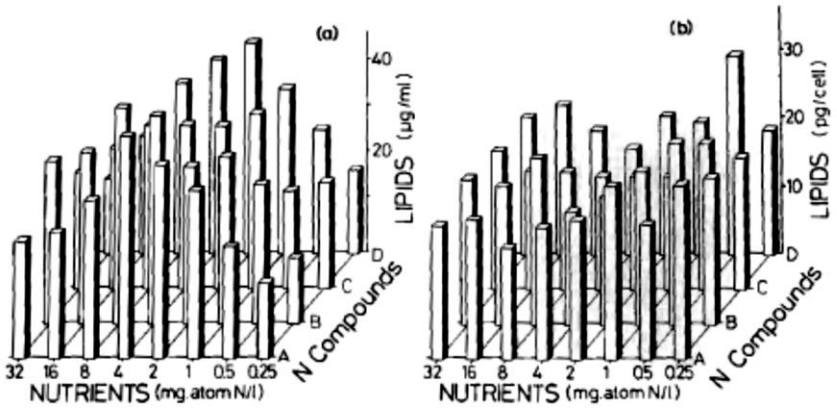

Fig. 5. Lipid concentration in the stationary phase of *D. tertiolecta* cultures with different sources and concentrations of ammonium nitrogen. (a) Lipids per ml (µg/ml); (b) lipids per cell (pg/cell). (A) Chloride; (B) sulphate; (C) nitrate; and (D) carbonate.

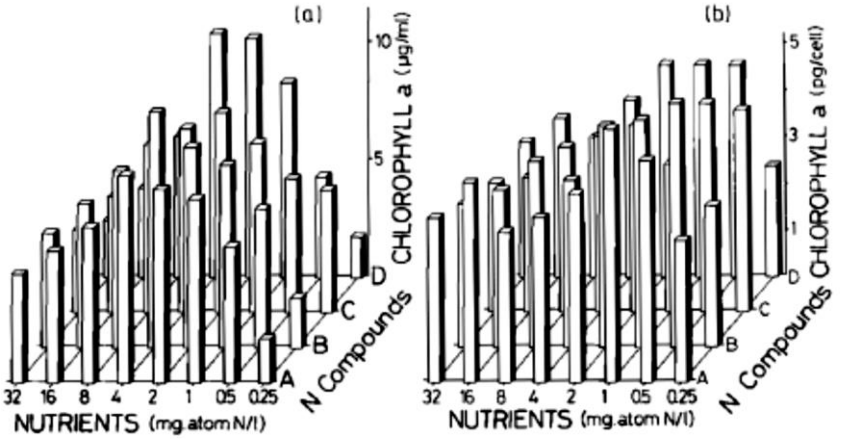

Fig. 6. Chlorophyll *a* concentration in the stationary phase of *D. tertiolecta* with different sources and concentrations of ammonium nitrogen. (a) Chlorophyll *a* per ml (µg/ml); (b) chlorophyll *a* per cell (pg/cell). (A) Chloride; (B) sulphate; (C) nitrate; and (D) carbonate.

TABLE 2

Protein (P), lipid (L) and carbohydrate (C) content, as a percentage of the total organic matter, of *D. tertiolecta* cultured with different sources and concentrations of ammonium nitrogen

Ammonium-N (mg.atom N/l)	Ammonium source											
	Chloride			Sulphate			Nitrate			Carbonate		
	P	L	C	P	L	C	P	L	C	P	L	C
0.25	19.9	60.1	19.9	34.6	49.5	15.8	17.5	64.2	18.1	36.9	51.6	11.3
0.50	41.5	39.7	18.7	39.2	46.1	23.4	35.4	42.6	21.8	43.4	44.6	11.9
1	41.4	36.1	22.3	36.4	35.5	28.0	33.0	37.8	29.1	44.3	34.5	21.1
2	42.7	32.1	25.1	44.8	29.4	25.7	40.6	27.5	31.8	44.9	31.0	23.9
4	50.4	28.0	21.5	43.7	29.3	26.8	49.5	26.3	24.0	55.9	21.9	22.0
8	57.7	23.0	19.2	45.8	31.0	23.1	57.0	24.0	18.8	58.8	22.7	18.4
16	55.2	20.9	23.7	52.3	27.7	19.8	58.4	20.0	20.7	66.0	19.0	14.9
32	57.5	22.0	20.3	60.3	24.8	14.7	63.3	17.0	19.5	72.5	16.1	11.3

Energy measurements, based on the protein, carbohydrate and lipid content of the microalga, were calculated per litre of culture. Energy increased proportionally to the nitrogen concentration up to 4-8 mg.atom N/l (Table 3). Gross energy decreased at higher nitrogen concentrations in ammonium chloride and carbonate cultures, but was maintained in sulphate and nitrate cultures. Values were between 25.78 and 105.87 cal/l in ammonium chloride cultures, between 21.96 and 99.69 cal/l in ammonium sulphate cultures, between 32.28 and 93.04 cal/l in ammonium nitrate cultures and between 19.78 and 118.61 cal/l in ammonium carbonate cultures.

TABLE 3

Gross energy (cal/l) in cultures of *D. tertiolecta* with different sources and concentrations of ammonium nitrogen

Ammonium-N (mg.atom N/l)	Ammonium source			
	Chloride	Sulphate	Nitrate	Carbonate
0.25	25.78	21.96	32.28	19.78
0.50	42.27	46.92	41.35	44.76
1	67.56	67.75	54.32	70.97
2	86.24	74.69	79.75	97.05
4	105.82	99.69	84.03	118.61
8	92.93	99.16	93.04	104.10
16	81.28	87.73	90.83	82.71
32	72.00	90.39	89.58	64.51

DISCUSSION

Variations in the composition of the culture medium can cause changes in the biochemical content of marine microalgae, especially in the lipids, proteins and carbohydrates (Myklestad and Haug, 1972; Parsons and Takahashi, 1973; Shifrin and Chisholm, 1981; Fabregas et al., 1985, 1986a, b).

Among the different components of the culture medium, the source and concentration of nitrogen can provoke important changes in the growth and biochemical composition of microalgal species (Kaplan et aI., 1986). Protein content of microalgae depends on the nitrogen source used in the culture medium (Venkataraman and Nigan, 1979; Boussiba and Richmond, 1980). In the same way, the most important effect on the lipid fraction is due to nitrogen in the culture medium (Shifrin and Chisholm, 1980). Manipulation of the nitrogen concentration of the culture medium was found to be a simple technique to effect significant differences in the protein, carbohydrate and lipid content of three species of marine phytoplankton (Utting, 1985).

Studies with different nitrogen sources have been generally carried out at low nitrogen concentrations (0.25-1 mg.atom N/l), under conditions of limiting nitrogen. Nevertheless, there are some reports on microalgal cultures at non-limiting or high nitrogen concentrations (Abeliovich and Azov, 1976; Admiraal, 1977; Utting, 1985; Fabregas et aI., 1985, 1986a, b). Microalgal cultures at high nitrogen concentrations are usually made to obtain maximum production of total biomass or of a particular product. The nitrogen concentration generaHy used is 2 mg.atom N/l (McLachlan, 1964).

By altering the chemical composition of *Dunaliella tertiolecta,* 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 in aquaculture systems so as to maximize the conversion of dissolved nutrients into biomass in commercially important marine food crops. Taking into account the biochemical variability of marine microalgae, their nutritional state is as important for the growth of herbivores (Wikfors et al., 1984) as the species themselves, and this nutritional state is a function of the chemical composition.

In the present study an optimization of the culture of *D. tertiolecta* and the manipulation of its chemical composition improved the conversion of environmental nitrogen into microbial protein and resulted in a microalgal biomass with a specific chemical composition.

Changes in the sources and concentration of nitrogen were accompanied by variation in phosphorus concentration in order to maintain a constant N/P ratio, because the alteration of the N/P ratio can cause more drastic effects on a microalgal culture than the variation in nitrogen concentration (Wikfors, 1986; Wynne and Rhee, 1986).

All other culture parameters were kept constant, since variations in more than one parameter produce interrelationships in the microalgal response. For example, the

uptake of nitrate-N and ammonium-N is affected by salinity (Dohler and Biermann, 1985), so in our experiments salinity was kept constant at *35%0* which is optimal for *D. tertiolecta* (Fabregas et al., 1986c). Light was maintained at saturation level. Light can have secondary effects on primary production as an agent modifying nutrient requirements, in addition to its primary role in photosynthesis (Wynne and Rhee, 1986). In all cultures, media were buffered with TRIS and the pH of the cultures was maintained within the optimum range for this species (7-8) (Spectorova et al., 1982).

We can establish the kinetics of the cultures from transmittance measurements (100-T). These turbidity methods are the techniques most generally used to follow the growth of pure cultures of microalgae. In the stationary phase and under certain conditions, transmittance measurements can be transformed into cellular densities (Lyon and Woo, 1980; Fabregas et al., 1986c). However, these methods mainly give information about increase in biomass and not directly about the number of cells. In fact, in *D. tertiolecta* cultures with the different ammonium compounds, there was no direct relationship between cell density and optical density, although there was a certain parallelism (Figs. 1, 2) .

There was a significant increase in *D. tertiolecta* growth at nitrogen concentrations from 0.25 to 1 mg.atom N/l for all the ammonium compounds used (Fig. 1). Cultures of six different marine microalgae, including *D. tertiolecta,* showed an almost linear increase with increasing concentrations of ammonium sulphate from 0.05 to 0.2 mg.atom N/l (Hastings and Fujita, 1980). Similar linearity was observed for *D. tertiolecta* up to 1 mg.atom N/l under the present conditions. Growth increase was lower at nitrogen concentrations between 1 and 8 mg.atom N/l*,* and higher concentrations provoked a growth decrease with all the ammonium compounds.

Some microalgal species are sensitive to high ammonium concentrations and their growth may be inhibited by 1 mg.atom N/l of ammonium (Kaplan et al., 1986). Ammonium concentrations higher than 0.5 mg.atom N/l inhibited growth of 10 different species of benthic diatoms under certain conditions; this inhibition by ammonium was strongly enhanced by high irradiance and high pH (Admiraal, 1977). Special attention must be given to the relationship between pH and concentration of ammonium supplied, as the combination of high pH and high ammonium concentrations was found to be toxic for microalgae (Abeliovitch and Azov, 1976; Kaplan et al., 1986). In our cultures, light intensity was not high (Fabregas et al., 1986c) and pH was maintained below 8.0 in all the cultures. In the freshwater microalga *Scenedesmus obliquus,* growth was inhibited by ammonium concentrations at high pH values (pH = 9); growth

inhibition began at ammonium concentrations of 2-2.5 mg.atom N/l and no growth was observed at ammonium concentrations higher than 3 mg.atom N/l. However, if the cells were transferred to a medium with pH 7, inhibition was not observed even at ammonium concentrations higher than 3.0 mg.atom N/l. The upper pH limit for avoiding growth inhibition in ammonium cultures was 7.9 (Abeliovitch and Azov, 1976). Taking into account the effect of the pH on ammonium toxicity, inhibition was not observed in our growing systems because the pH did not surpass the upper safety limit.

On the other hand, ammonium uptake during photosynthesis generates a strong decrease in the pH (Kaplan et al., 1986) and if the pH decreased below 7, conditions for the growth of this marine microalga would be far from optimum (Spectorova et al., 1982). In our growing systems, the use of TRIS buffer at 15 mM kept the pH within the optimum range. TRIS buffer is commonly used in microalgal cultures both with nitrate and with ammonium. We used TRIS buffer with the four ammonium-N sources. This buffer has been used at 15 mM in cultures of the marine microalga *Phaeodactylum tricornutum* with ammonium (Syrett et al., 1986).

The biomass production at the end of the stationary phase, expressed as cell density (Fig. 2), was affected by the concentration of ammonium-N used but not by the compound utilized. In contrast, the compound and concentration of ammonium-N had little effect on the growth velocity of *D. tertiolecta* cultures in the logarithmic phase; growth velocity was practically constant under all the conditions assayed. Assays carried out with this and other microalgae showed that other variables, such as salinity, nutrient concentration, etc., one by one, have no effect on the growth velocity, but growth velocity is affected by the interaction ofthese variables (Terlizzi and Karlander, 1980).

The ammonium compound and the concentration of nitrogen can affect the concentration of certain cell constituents, such as protein, carbohydrate, lipid and chlorophyll *a.* These changes in the cell constituents of microalgae have been shown in different species, including *D. tertiolecta* (Myklestad, 1974; Fabregas et al., 1985, 1986a,b,c), although these changes are not necessarily related to cell density of the culture.

The fraction most affected by ammonium compound and concentration of nitrogen was the protein (Fig. 3). Protein concentration, both per mI and per cell, tended to increase with the increase in the nitrogen concentration for all the ammonium compounds used. These data are in general agreement with those of other authors who reported that protein content of microalgae depends on the nitrogen concentration used in the

culture medium (Venkataraman and Nigan, 1979; Boussiba and Richmond, 1980). Maximum protein/ml values were obtained with ammonium carbonate for all the nitrogen concentrations used. Maximum protein/cell values occurred at the higher nitro gen concentrations (16 and 32 mg.atom N/l) for all the ammonium compounds.

Considering the optimum growth interval (2-8 mg.atom N/l), maximum protein/cell concentrations were also obtained in the cultures with ammonium carbonate.

Carbohydrate and lipid concentrations showed less variation than protein concentration (Fig. 4, 5). Maximum values of carbohydrate/ml were also found in the ammonium carbonate cultures. It has been reported that the most important effect on the lipid fraction is due to nitrogen in the culture medium (Shifrin and Chisholm, 1980). In our study, maximum lipid/ cell values tended to occur at the lowest nitrogen concentration, when protein values were minimum, for each ammonium source (Fig. 5).

The determination of chlorophyll is one of the most rapid chemical methods for estimating the amount of living microalgae and, as a result, chlorophyll *a* can be used to measure growth. In our studies there were parallelisms between values for chlorophyll *a/ml* and growth (Figs. 1, 6). However, chlorophyll *a/*cell was practically constant above 1 mg.atom N/l for all the ammonium compounds used (Fig. 6b). Chlorophyll only accounts for a small part of the cytoplasm in microalgae and, therefore, changes in chlorophyll content are not necessarily indicative of changes in biomass or vice versa. Nitrogen, a component of the chlorophyll molecule, can stimulate the production of chlorophyll without affecting growth (White and Payne, 1977). In fact, there is an increase in microalgal chlorophyll when nutrients are increased (Shutter, 1979). However, one of the first symptoms of nitrogen deficiency is the decrease in chlorophyll content, and an increase in the chlorophyll fraction in response to a nutrient enrichment may prove the previous scarcity of such nutrient (White and Payne, 1977). In the cultures with the different ammonium compounds, the concentration of chlorophyll *a/*cell increased from the cultures at 0.25 mg.atom N /1 to those at 0.5 mg.atom N /1. This may indicate that nitrogen was limiting at 0.25 mg.atom N/l. The low protein and high lipid concentrations found at 0.25 mg.atom N/I are in agreement with this, because nitrogen starvation is coupled to lipid increase and protein and chlorophyll *a* decrease (Kaplan et al., 1986).

As a proportion of the total organic matter, the percentage of protein was the most variable (Table 2), increasing with the nitrogen concentrations for all the ammonium compounds. However, the percentage of carbohydrate was very similar with all the ammonium-N compounds and presented little variation with the nitrogen concentration.

The percentage of lipid presented considerable variations and, in contrast to protein, decreased with the nitrogen concentration for all the ammonium compounds. Lipids of marine microalgae can vary within a wide range (1-40% of dry matter) (Paoletti, 1976), showing maximum values at low nitrogen concentrations (Shifrin and Chisholm, 1980).

In our cultures of *D. tertiolecta,* the highest percentages of protein occurred at the higher nitrogen concentrations. In marine microalgal cultures, in any conditions where protein decreased, lipids and/or carbohydrates might be expected to increase, depending on the species of alga (Utting, 1985). In *D. tertiolecta,* lipids seem to be the storage products since they increased when protein decreased, whereas carbohydrates were more independent of the nutrient concentration and the protein content.

Gross energy values showed differences of 310, 353, 188 and 499% in ammonium chloride, sulphate, nitrate and carbonate, respectively, with the different nitrogen concentrations. As can be seen, minimum variations occurred in ammonium nitrate cultures and maximum in ammonium carbonate. Therefore, the calorific value of *D. tertiolecta* cultures was affected more by variations in ammonium carbonate than by variations in other nitrogen sources. It has been reported that *Tetraselmis* had 34% more energy available when grown in a high-nitrate medium compared to a nitrogen-deficient medium (Utting, 1985). Energy increased proportionally to the nitrogen concentration in all the cultures up to 4-8 mg.atom N/l, decreasing at higher nitrogen concentrations (Table 3).

Biochemical variability of the marine microalga *Dunaliella tertiolecta* in different sources and concentrations of nitrogen can change its nutritive value, with a potential effect on its value as single-cell protein (Fabregas and Herrero, 1985), as a source of chemicals (Ben-Amotz and Avron, 1983; Borowitzka, 1986) or as feed in mariculture (Scott and Baynes, 1979; Wikfors et al., 1984; Fabregas and Herrero, 1986). In mariculture it has already been proved that differences in growth media affect the gross chemical composition of algal food sources, which alone can account for differences in algal nutritional value to *Crassostrea virginica* (Wikfors et al., 1984).

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REFERENCES

Abeliovich, A. and Y. Azov. 1976. Toxicity of ammonia to algae in sewage oxidation ponds. *Applied and Environmental Microbiology* 31, no. 6: 801-806

Admiraal, W. 1977. Tolerance of estuarine benthic diatoms to high concentrations of ammonia, nitrite ion, nitrate ion and orthophosphate. *Marine Biology* 43, no. 4: 307-315.

Ben-Amotz, A. and M. Avron. 1983. Accumulation of metabolites by halotolerant algae and its industrial potential. *Annual Review of Microbiology* 37, : 95-119.

Borowitzka, M. A. 1986. Micro-algae as sources of fine chemicals. *Microbiological Sciences* 3, no. 12: 372-375.

Boussiba, S. and A. E. Richmond. 1980. C-phycocyanin as a storage protein in the blue-green alga spirulina platensis. *Archives of Microbiology* 125, no. 1-2: 143-147.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, no. 1-2: 248-254.

Döhler, G. and I. Biermann. 1985. Effect of salinity on 15N-ammonia and 15N-nitrate assimilation of bellerochea yucatanensis and thalassiosira rotula. *Biochemie Und Physiologie Der Pflanzen* 180, : 589-598.

Fabregas, J. and C. Herrero. 1986. Marine microalgae as a potential source of minerals in fish diets. *Aquaculture* 51, no. 3-4: 237-243.

———. 1985. Marine microalgae as a potential source of single cell protein (SCP). *Applied Microbiology and Biotechnology* 23, no. 2: 110-113.

Fabregas, J., C. Herrero, J. Abalde, R. Liaño, and B. Cabezas. 1986. Biomass production and biochemical variability of the marine microalga dunaliella tertiolecta (butcher) with high nutrient concentrations.*Aquaculture* 53, no. 3-4: 187-199.

Fabregas, J., C. Herrero, B. Cabezas, and J. Abalde. 1986. Biomass production and biochemical composition in mass cultures of the marine microalga isochrysis galbana parke at varying nutrient concentrations.*Aquaculture* 53, no. 2: 101-113.

———. 1985. Mass culture and biochemical variability of the marine microalga tetraselmis suecica kylin (butch) with high nutrient concentrations. *Aquaculture* 49, no. 3-4: 231-244.

Fabregas, J., C. Herrero, B. Cabezas, R. Liaño, and J. Abalde. 1986. Response of the marine microalga dunaliella tertiolecta to nutrient concentration and salinity variations in batch cultures. *Journal of Plant Physiology* 125, no. 5: 475-484.

Ganf, G. G., S. J. L. Stone, and R. L. Oliver. 1986. Use of protein to carbohydrate ratios to analyse for nutrient deficiency in phytoplankton. *Australian Journal of Marine & Freshwater Research* 37, no. 2: 183-197.

Kaplan, D., A. E. Richmond, Z. Dubinsky, and S. Aaronson. 1986. Algal nutrition. *Handbook of Microalgal Mass Culture*: 147-198.

Kochert, G. 1978. Carbohydrate determination by the phenol-sulfuric acid method. *Handbook of Phycological Methods.Physiological and Biochemical Methods*: 95-97.

———. 1978. Quantitation of the macromolecular components of microalgae. *Handbook of Phycological Methods.Physiological and Biochemical Methods*: 189-195.

Lyon, H. W. and C. S. Woo. 1980. Accelerated uptake response of the green alga, chlorella vulgaris, to high levels of phosphorus in mississippi river water. *Science of the Total Environment, the* 14, no. 3: 279-285.

Marsh, J. B. and D. B. Weinstein. 1966. Simple charring method for determination of lipids. *Journal of Lipid Research* 7, no. 4: 574-576.

Mason, D. T. 1963. The growth response of artemia salina (L.) to various feeding regimes. *Crustaceana* 5, no. 2: 138-150.

McLachlan, J. 1964. Some considerations of the growth of marine algae in artificial media. *Canadian Journal of Microbiology* 7, : 399-406.

Myklestad, S. 1974. Production of carbohydrates by marine planktonic diatoms. I. comparison of nine different species in culture. *Journal of Experimental Marine Biology and Ecology* 15, no. 3: 261-274.

Myklestad, S. and A. Haug. 1972. Production of carbohydrates by the marine diatom chaetoceros affinis var. willei (gran) hustedt. I. effect of the concentration of nutrients in the culture medium. *Journal of Experimental Marine Biology and Ecology* 9, no. 2: 125-136.

National Research Council. 1981. *Nutrient Requirements of Coldwater Fishes*.

Oliveira, L., T. Bisalputra, and N. J. Antia. 1980. Ultrastructural observation of the surface coat of dunaliella tertiolecta from staining with cationic dyes and enzyme treatments. *New Phytologist* 85, : 385-392.

Paoletti, C., B. Pushparaj, G. Florenzano, P. Capella, and G. Lercker. 1976. Unsaponifiable matter of green and blue-green algal lipids as a factor of biochemical differentiation of their biomasses: I. total unsaponifiable and hydrocarbon fraction. *Lipids* 11, no. 4: 258-265.

Parsons, T. R. and J. D. H. Strickland. 1965. Particulate organic matter. III.I. pigment analysis. III.I.I. determination of phytoplankton pigments. *J.Fish.Res.Board can.* 18, : 117-127.

Parsons, T. R., M. Takahashi, and B. Hargrave. 1984. *Biological Oceanographic Processes*.

Scott, A. and S. Baynes. 1979. The effect of unicellular algae on survival and growth of turbot larvae (scophthalmus maximus L.). *Finfish Nutrition and Fishfeed Technology* 1, : 423-433.

Shifrin, N. S. and S. W. Chisholm. 1981. Phytoplankton lipids: Interspecific differences and effects of nitrate, silicate and light-dark cycles. *J.Phycol.* 17, : 374-384.

———. 1980. Phytoplankton lipids: Environmental influences on production and possible commercial applications. *Algae Biomass*: 627-645.

Shuter, B. 1979. A model of physiological adaptation in unicellular algae. *Journal of Theoretical Biology* 78, no. 4: 519-552.

Spectorova, L. V., O. I. Goronkova, L. P. Nosova, and O. N. Albitskaya. 1982. High-density culture of marine microalgae - promising items for mariculture. I. mineral feeding regime and installations for culturing dunaliella tertiolecta butch. *Aquaculture* 26, no. 3-4: 289-302.

Syrett, P. J., K. J. Flynn, C. J. Molloy, G. K. Dixon, A. M. Peplinska, and R. C. Cresswell. 1986. Effects of nitrogen deprivation on rates of uptake of nitrogenous compounds by the marine diatom phaeodactylum tricornutum bohlin. *New Phytol.* 102, : 39-44.

Terlizzi, D., Jr. and E. Karlander. 1980. Growth of a coccoid nonoplankter (eustigmatophyceae) from the chesapeake bay as influenced by light temperature salinity and nitrogen source in factorial combination.*Journal of Phycology* 16, : 364-368.

Thomas, W. H., J. Hastings, and M. Fujita. 1980. Ammonium input to the sea via large sewage outfalls. part 2: Effects of ammonium on growth and photosynthesis of southern california phytoplankton cultures.*Marine Environmental Research* 3, no. 4: 291-296.

Utting, S. D. 1985. Influence of nitrogen availability on the biochemical composition of three unicellular marine algae of commercial importance. *Aquacultural Engineering* 4, no. 3: 175-190.

Venkataraman, L. V. and B. P. Nigam. 1979. Mass culturing of freshwater algae for utilization as protein source. *Phykos* 18, no. 1-2: 83-95.

White, E. and G. W. Payne. 1977. Chlorophyll production, in response to nutrient additions, by the algae in lake taupo water. *New Zealand Journal of Marine and Freshwater Research* 11, no. 3: 501-507.

Wikfors, G. H. 1986. Altering growth and gross chemical composition of two microalgal molluscan food species by varying nitrate and phosphate. *Aquaculture* 59, no. 1: 1-14.

Wikfors, G. H., J. W. Twarog, and R. Ukeles. 1984. Influence of chemical composition of algal food sources on growth of juvenile oysters, crassostrea virginica. *Biol.Bull.* 167, : 251-263.

Wynne, D. and G-Y Rhee. 1986. Effects of light intensity and quality on the relative N and P requirement (the optimum N:P ratio) of marine planktonic algae. *Journal of Plankton Research* 8, no. 1: 91-103.