Response of the Marine Microalga Dunaliella tertiolecta to Nutrient Concentration and Salinity Variations in Batch Cultures

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Sumary

The marine microalga Dunaliella tertiolecta is known for its ability to tolerate a wide range of salt concentrations. Cultures were grown under 56 different nutrient concentration-salinity conditions. Optimal growth conditions were between 25 and 35 ‰ salinity and with nutrient concentrations between 8 and 32 times higher than the standard concentrations, resulting in maximum cellular densities between 8.41×10^6 and 16.74×10^6 cells/ml. Growth is more affected by nutrient concentration than by salinity. No growth was obtained with the lowest salinities tested (0 and 5 ‰) at any of the nutrient concentrations used. Variations in salinity and in nutrient concentration had a greater effect on the final biomass than on the velocity of growth. Chlorophyll-a/ml was affected by salinity and nutrient concentrations and maximum values were found with 30 ‰ salinity and nutrient concentrations between 8 and 64 mM of $NaNO_3$. Chlorophyll-a/cell reached maximum values between 2.02 and 3.51 pg/cell and is only significantly affected by the nutrient concentration. These maximum values were reached with low nutrient concentrations (1-2 mM of $NaNO_3$). Protein per ml of culture and protein per cell were closely related to salinity and nutrient concentrations. Maximum protein per ml occurred at 20-25 ‰ salinity and 64mM of $NaNO_3$, with values between 926 and 957 µml. Maximum protein/ cell concentrations were obtained also at 64 mM of $NaNO_3$ for all the salinities. The nitrate-protein transformation rate was related to nutrient concentration and was independent of salinity. Maximum rate was

100% at 20 ‰ salinity and 1 mM of $NaNO_3$. This rate decreased as nutrient concentrations increased.

Keywords

Chlorophyll a; Dunaliella; Microalga; Nutrients; Protein; Salinity

Introduction

Dunaliella is a genus of green algae, the cells of which are wall-less and respond to changes in the osmotic pressure of the outer medium behaving as a perfect osmometer (Ginzburg, 1981; Riisgard, 1979). The marine species Dunaliella tertiolecta Butcher is commercially useful. It may be possible to produce glycerin from *D. tertiolecta* cells (Evans et al., 1982; Frank and Weigmann, 1974). As food for organisms of the next trophic link, *D.* tertiolecta has been used, for instance, for feeding *Artemia,* rotifers (Mason, 1963), in the rearing of marine fish (Scot[and Baynes, 1979), in rearing of bibalve molluscs (Bayne, 1976; Walne, 1974), and recently it has been suggested as a new source of Single Cell Protein (SCP) (Fabregas and Herrero, 1985).

It has been pointed out that the chemical composition of a marine microalga can readily be manipulated by varying environmental factors such as the nutrient concentration (Goldman, 1976).

In the present work we analyzed the response of the m:orine microalga *D. tertiolecta* to 7 x 8 nutrient concentration-salinity conditions in bar::h cultures, maintaining constant pH and temperature, and light in saturation.

Materials and Methods

The marine microalga *Dunaliella* tertiolecta Butcher was obtained fromThe Culture Centre of Algae and Protozoa, Cambridge, England. The standard culture medium used was seawater filtered through a 0.45 µ Millipore filter, autoclaved at 120ºC for 20 minutes, and enriched with $NaNO_3$, 2 mM; NaH_2PO_4 100 µM; $ZnCl_2$, 1 µM; Na_2MoO_{a} 1 µM; $CoCl_3$, 0.1 µM; $CuSO_4$, 0.1 µM; ferric citrate, 20 µM; thiamine, 35 µg/l; biotin, 5 µg/l; B_{12}, 3 µg/l; EDTA, 26.4 µM; TRIS-HCI, 15mM; pH: 7.4..

The first nutrient concentration utilized in the tests was the one the composition of which was the half of that given above and which corresponds to $NaNO_3$ 1 mM. From this we followed a geometrical progression, using concertations corresponding to 2, 4,

8, 16, 32, and 64 mM of NaNO$_3$. Nutrient concentrations are expressed as NaNO$_3$ concentrations, but all the other nutrients were proportionally increased.

We used eight salinities: 35, 30, 25, 20, 15,10, 5, and 0‰. The salinity of the seawater (35 ‰) was reduced by the addition of appropriate volumes of fresh distilled water prior to preparation of the medium.

Cultures were carried out in Kimax screw-capped test tubes (15x2.5 cm) with 40 ml of medium. All cultures were maintained in a controlled environment incubator (New Brunswick) at 15 ± 1 °C and 3900 lux light from fluorescent lamps (Philip's TL DW /55). A 12: 12 light-dark regime was maintained in order to obtain synchronous cultures An inoculum of 2 x 10^6 logarithmic phase cells/ml was used.

Transmittance of the cultures was determined by using a Coleman II 6/20 spectrophotometer reading at 530 nm and values were expressed as (100-T). Cellular density was determined by counting culture aliquots in a Thoma chamber

The velocity of growth of the cultures, expressed as doublings/day was calculated from the following equation:

$$\text{doubling/day} = t_d^{-1} = \frac{\ln f(tn) - \ln f(ti)}{\ln 2(tn - ti)}$$

were f(t) is cellular density, ti and tn are the initial and final time of the logarithmic phase, both expressed in days, and td is the duplication time.

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

Protein was measured in the stationary phase by the dye-binding method (Bradford, 1976).

Stationary phases, corresponding to maximum biomass production, were compared by an overall multivariate one-way analysis of variance (ANOVA), and logarithmic phases, that indicate the velocity of growth of the cultures, were compared by a one-way analysis of covariance (ANCOVA).

A two-way analysis of variance was applied for comparing the effects of each variable, salinity and nutrient concentration, on growth, cellular density, chlorophyll-a, protein, and efficiency.
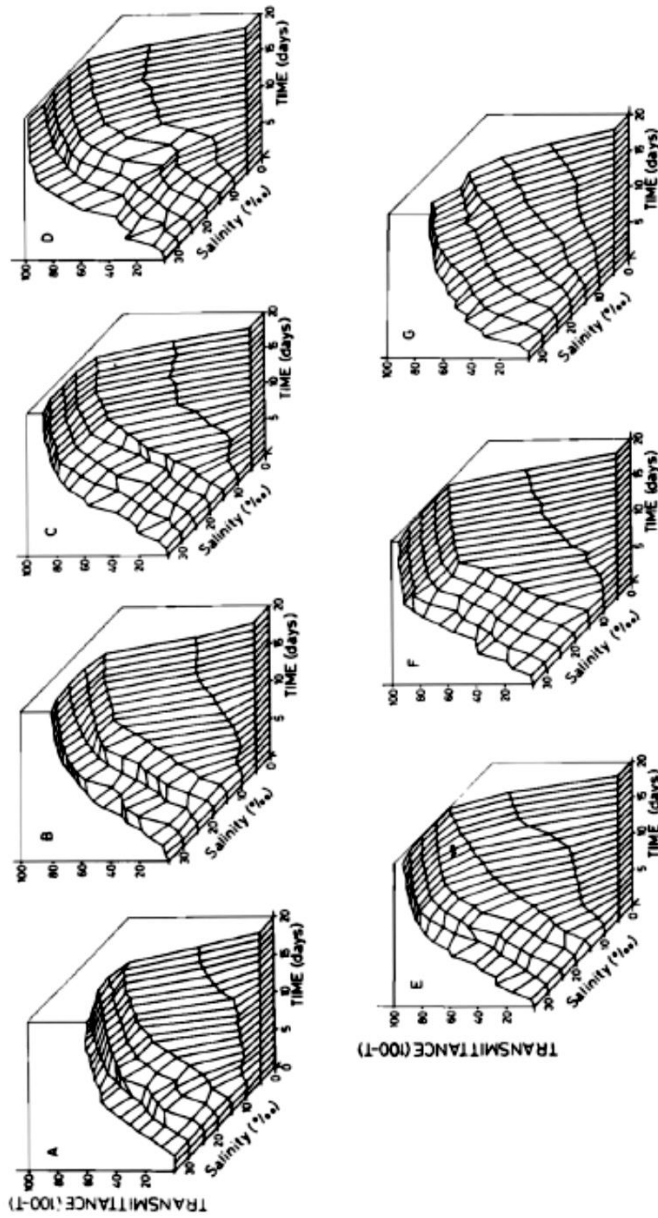

Fig. 1: Growth of *D. tertiolecta* at different salinities and nutrient concentrations, expressed as NaNO₃ concentrations. Transmittance at 530 nm is represented as (100-T) values. (A) Nutrient concentration 1 mM NaNO₃; (B) 2 mM; (C) 4 mM; (D) 8 mM; (E) 16 mM; (F) 32 mM; and (G) 64 mM.

Table 1: Statistical analysis of the growth curves of *D. tertiolecta* (represented as 100-T) at different salinities and nutrient concentrations. Values are expressed as mean ± standard deviation. Each value corresponding to a salinity and a nutrient concentration is compared by a one-way analysis of variance (ANOVA) with both the following salinity and nutrient concentration (p < 0.001).

Nutrient concentration*) (mM)	Salinities (‰)							
	2	5	10	15	20	25	30	35
1	–	–	32.00±2.83	<68.45± 4.95	=64.08± 7.64	=72.64± 2.62	=61.92±6.93	=57.73±3.55
			<	=		<	<	<
2	–	–	42.33±0.52	<79.31± 8.93	=79.27± 3.80	=81.00± 5.55	=86.64±3.53	>69.00±6.91
			=	=	<	=	=	<
4	–	–	45.27±2.41	<84.85± 9.81	=92.33± 3.04	=87.00±10.1	=93.27±4.88	=86.18±4.56
			<	=	=	=	=	<
8	–	–	59.55±3.45	<91.91± 6.39	=96.00± 2.71	=94.60± 4.17	=95.55±7.09	=95.64±3.67
			=	=	=	=	=	=
16	–	–	65.50±1.29	<97.27±10.8	=97.64±11.5	=98.85± 7.81	=98.58±6.92	=96.27±4.90
			>	=		=	=	=
32	–	–	52.67±4.18	<97.00± 1.84	=97.45± 1.75	=96.54± 7.59	=98.00±1.56	=96.27±3.04
			>	>	>	>	>	>
64	–	–	27.22±7.63	<52.86± 5.79	=62.00±10.4	=63.08± 7.68	=74.00±9.90	=71.27±3.74

*) Expressed as $NaNO_3$ concentration.

Results and Discussion

We plotted transmittance, expressed as (100-T), against time and against salinity for each nutrient concentration, obtaining three dimensional figures (Fig. 1). Statistical treatment of these figures is presented in Table 1.

We can establish the kinetics of cultures in the logarithmic and in the stationary phases from transmittance (100-T) measurements. In microalgal batch experiments, with both limited volume and supply of nutrients, a logistic curve is usually observed (Schantz and Zahler, 1981). In the stationary phases, transmittance measurements can be transformed into cellular densities, since it is generally accepted that there is a relation between optical density and cellular density (Fabregas et al., 1984 a, 1985 a; Lyon and Woo, 1980). This relation can be fitted to a power curve (Fabregas et al., 1984 a), but considering only the stationary phase a linear relation can be obtained (Fabregas et al., 1985 a).

In our experiments with D. tertiolecta, the relation between transmittance (100-T) and cellular density was salinity-dependent, and there was a linear relation for each salinity. It was observed that the correlation coefficients decreased when the salinity increased. For 15 -20 ‰ salinity, the relation between (100-T) measured at 530 nm in screw-capped test tubes (2.5 cm light run) and cellular density fitted to a linear equation y = 0.03x + 75.13, where y = transmittance expressed as (100-T) and x = cellular density, with a correlation coefficient of 0.95. This relation was only calculated for the stationary phase and for nutrient concentrations from 2 to 16 mM of $NaNO_3$. There was a strong relationship between salinity-nutrient concentration conditions and the final biomass production in the stationary phase (Figs. 1, 2).

Growth, expressed as 100-T, is affected by salinity (p<0.001) and nutrient concentration (p < 0.001). The effect of salinity is greater than the effect of nutrient concentration, but it must be taken into account that transmittance measurements are also affected by other variables such as changes in cellular volume associated with salinity and by the occurrence of exocellular products with absorption at 530 nm.

These facts can produce a bias and the effect of salinity is, thus, overestimated.
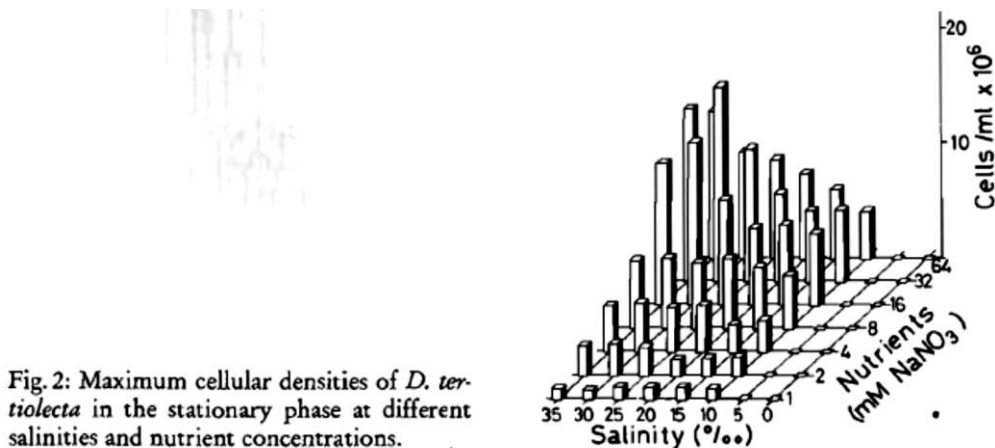

Fig. 2: Maximum cellular densities of *D. tertiolecta* in the stationary phase at different salinities and nutrient concentrations.

Marine unicellular algae are generally considered to be tolerant of and adaptable to a wide range of salinities (Fabregas et al., 1984a, 1985 a; McLachlan, 1961). D. tertiolecta is able to grow in salt media ranging from less than 0.5 M (approximately seawater) to saturated salt solutions (5M) (Ben-Amotz et al., 1982). However this marine micro alga did not grow at 0 an 5 %0 salinity for any of the nutrient concentrations used, although other unicellular marine microalgae, such as Tetraselmis suecica and Isochrysis galbana, were able to grow at this salinities (Fabregas et al., 1984 a, 1985 a).

Optimal growth conditions for obtaining maximum cellular density in the stationary phase were 25 - 35 ‰ salinity and 16-64 mM of $NaNO_3$, which corresponds to nutrient concentrations between 8 and 32 times higher than the standard concentrations (Fig. 2), with cellular densities between 8.41×10^6 and 16.74×10^6 cells/ml. An interaction can be observed between salinity and nutrient concentration, and, therefore, the increase in the cellular density with the salinity is greater at high nutrient concentrations than at low ones. The statistical analysis (two-way analysis of variance) shows that the cellular density is affected by nutrient concentration (p < 0.001) and by salinity (p < 0.005). The effect of the nutrient concentration on cellular density is greater than the effect of salinity.

Salinity and nutrient concentrations have little effect on the velocity of growth of D. tertiolecta in synchronous cultures in comparison with their effect on biomass production reached in the stationary phase. Maximum velocity of growth in the logarithmic phase was practically constant under all the conditions, with 0.45 doublings/ day, excepting for the lowest salinities (10-15 ‰) and highest nutrient concentrations (16- 64 mM of $NaNO_3$), in which this velocity was 0.28 doublings/ day.
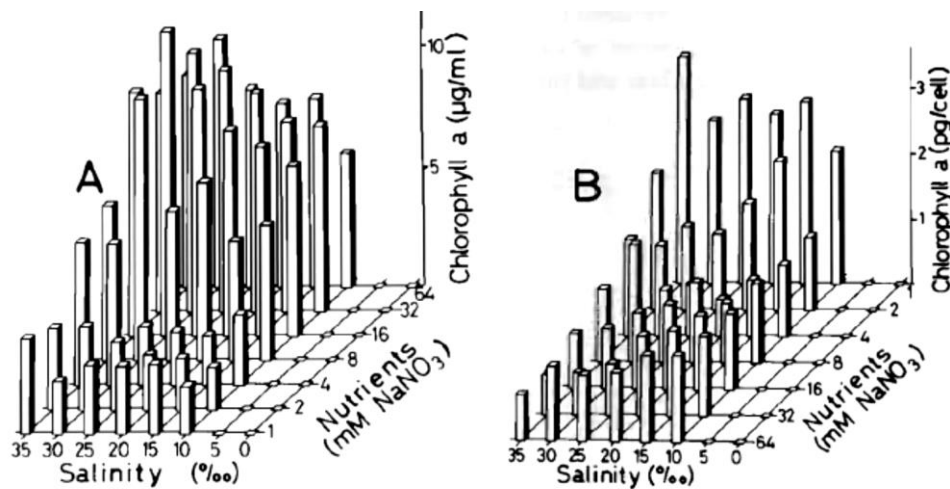

Fig. 3: Chlorophyll-a concentration in the stationary phase cultures of *D. tertiolecta* at different salinities and nutrient concentrations. (A) Chlorophyll-a per ml (μg/ml); (B) Chlorophyll-a per cell (pg/cell).
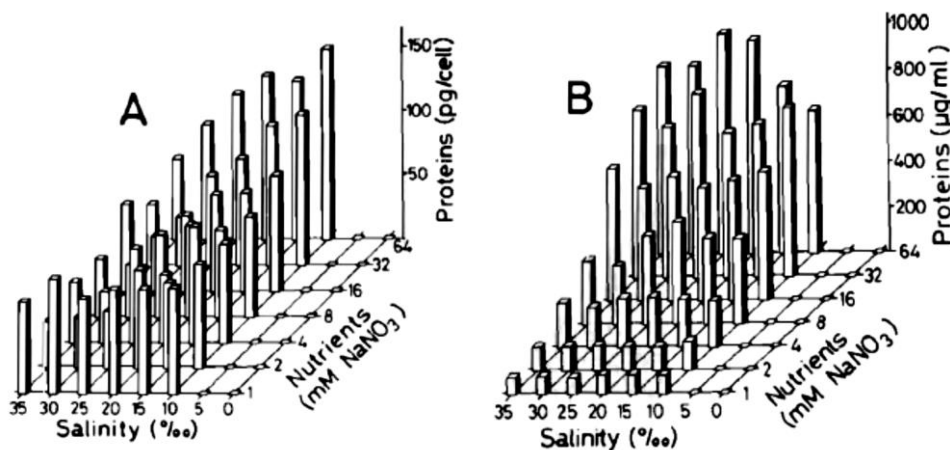

Fig. 4: Protein concentration in stationary phase cultures of *D. tertiolecta* at different salinities and nutrient concentrations. (A) Protein per ml (μg/ml); (B) Protein per cell (pg/cell).

Samples for chlorophyll-a and protein determination were always collected at the same time of the light period because protein concentration varies depending on the moment in the light period at which the sample is taken (Van Liere et al., 1979).

Maximum values of chlorophyll a ranged between 10.25 and 12.54 µg/ml at 30‰ salinity and 8-64mM of $NaNO_3$ (Fig. 3 A). Chlorophyll a/ml increased as salinity increased, up to 30 ‰. The nutrient concentration had a more marked effect on the

chlorophyll-a content per ml of culture than salinity, and the values obtained with nutrient concentrations higher than 8 mM are significantly greater than those obtained with concentrations less than this (Fig. 3 A).

Maximum concentrations of chlorophyll-a per cell were obtained at low (1-2 mM of $NaNO_3$) nutrient concentrations for all the salinities, with values between 2.02 and 3.51 pg/ cell (Fig. 3 B). Chlorophyll a/ ml concentration is affected by salinity and nutrient concentration ($p < 0.001$), the effect of the nutrient concentration being greater. However, chlorophyll-a/cell content is only affected by the nutrient concentration ($p<0.001$).

The nutrient concentrations affected the protein content of D. tertiolecta cultures (Fig. 4). When the nutrient concentration increased, the total protein content of the cultures and the protein per cell tended to increase. Salinity had less effect on the protein content than the nutrient concentration. Maximum protein content per ml were 926 and 957 µg/ml, found with 20-25 ‰ salinity and 64 mM of $NaNO_3$ (Fig. 4 A).

Maximum protein concentrations per cell were found at 64 mM of $NaNO_3$ for all salinity levels. Protein concentration/cell tended to diminish as the salinity increased, this fact being more marked at high than at low salinities (Fig. 4 B).

Protein content per ml of culture is affected by nutrient concentration ($p < 0.001$) and is independent of salinity.

Changes in the protein content are not necessarily related to cellular density in the culture because the biochemical composition of microalgal cells may change within more or less narrow limits depending on environmental action (Stross and Pemrick, 1974; Fabregas et a!., 1984 a, 1985 a).

D. tertiolecta showed considerable variability in its protein and chlorophyll-a content related to salinity and nutrient concentration. These data are in general agreement with those of other authors, indicating that the nutrient supply and the salinity influence the chlorophyll-a and the protein content of unialgal cultures (Myklestad, 1974; Fabregas et al., 1984a, 1985a, 1985 b).
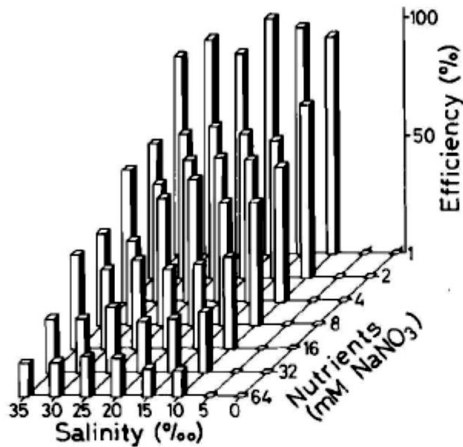
Fig. 5: Nitrate-N/protein-N transformation rate (efficiency) in stationary phase cultures of *D. tertiolecta* at different salinities and nutrient concentrations.
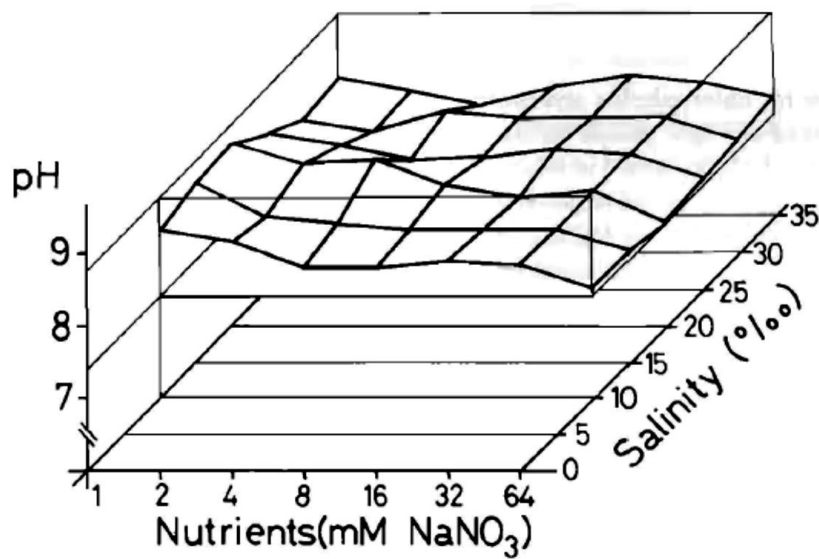

Fig. 6: pH values in stationary phase cultures of *D. tertiolecta* at different salinities and nutrient concentrations.

We established the efficiency of nitrate-N/protein-N transformation as the ratio between nitrogen added in nitrate form to the culture medium and the protein nitrogen produced per culture. Conversion was more efficient between 10-35 ‰ of salinity and between 1 and 8 mM of $NaNO_3$ (Fig. 5), with maximum values of 96 % and 100 % at 15 - 20 ‰ of salinity and 1 mM of $NaNO_3$. Maximum efficiencies were also found at 1 mM of $NaNO_3$ for the marine microalgae Tetraselmis suecica (Fabregas et al., 1984 a) and Isochrysis galbana (Fabregas et al., 1985 a) under the same culture conditions. This efficiency was nutrient concentration-dependent ($p < 0.001$) and salinity independent.

Culture conditions were aimed at obtaining maximum biomass production. In general, batch cultures are a helpful tool for establishing, in part or completely, the optimum parameters for a subsequent mass culture (Fabregas et al., 1984a, 1985a, 1985 b). When batch cultures are used, some of these parameters can become limiting. At high nutrient concentrations, the most important limiting factor is the carbon source, which is

not included in the culture medium, although in seawater there is an inorganic carbon concentration of about 2 mM (Burris, 1977). In batch cultures, without aeration, CO_2 is not supplied as carbon source. Carbon dioxide is closely related to the pH of the cultures and because CO_2 is not added pH can appear as a limiting factor. But in our growing system the culture medium was buffered with TRIS (Guillard and Ryther, 1962) which maintained the pH below 8.5 (Fig. 6).

Acknowledgements

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