

## **Optimal Growth Conditions for *Isochrysis galbana***

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### *ABSTRACT*

*Environmental and nutritional growth conditions of the unicellular microalga Isochrysis galbana were studied under laboratory conditions. The information obtained was used for cultivating the alga in outdoor miniponds. Outdoor cultures stayed monoalgal and free of predators as long as the temperature did not fall below 19°C and the rate of dilution did not exceed 40% of the culture's volume. Isochrysis galbana grown in outdoor cultures provided lipid concentrations of 24-28% of ash free dry matter.*

*Key words: Isochrysis galbana, optimal growth conditions, outdoor cultivation, lipid accumulation.*

### INTRODUCTION

*Isochrysis galbana* is a free living marine unicellular phytoflagellate of the order *Chrysoomonadales*. Like other members of this order it is rich in polyunsaturated fatty acids<sup>1</sup> which are of nutritional value for marine fish larvae<sup>2</sup> and juvenile stages of molluscs.<sup>3</sup> Limited experience of growing this microalga for feeding oyster larvae proved very successful.<sup>4,5</sup>

The purpose of the work described here was to define optimal growth conditions for outdoor culture. The effects of various environmental and nutritional parameters on the growth rate of the alga and its resistance to predators were studied.

## MATERIALS AND METHODS

*Isochrysis galbana* (originally from the Hawaii Institute of Marine Biology) was kindly provided by Dr Hillel Gordin (Limnological Oceanographic Research Laboratory, Eilat) and used throughout the study. Axenic cultures were used throughout all laboratory studies, and monoalgal cultures in field studies.

### *Growth conditions*

The following artificial sea water medium (ASW) was used (in g litre<sup>-1</sup>): NaCl 32.0; MgCl<sub>2</sub> 7H<sub>2</sub>O 6.7; MgSO<sub>4</sub>6H<sub>2</sub>O 5.5; CaCl<sub>2</sub> 1.1; KNO<sub>3</sub> 1.0; KH<sub>2</sub>PO<sub>4</sub> 0.07; NaHCO<sub>3</sub> 0.04; trace elements and vitamins (in µg litre<sup>-1</sup>): ZnCl<sub>2</sub> 4; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>4H<sub>2</sub>O 36.8; CuCl<sub>2</sub>2H<sub>2</sub>O 3.4; MnCl<sub>2</sub>4H<sub>2</sub>O 40; H<sub>3</sub>BO<sub>3</sub> 60; FeCl<sub>3</sub>6H<sub>2</sub>O 240; EDTA (di-Na salt) 100; thiamine HCl 400; Biotin 50; and B<sub>12</sub> 10. Unless otherwise stated, all growth experiments were performed in batch cultures at 27°C, with constant illumination of 150 µE m<sup>-2</sup> s<sup>-1</sup> at the surface of the flasks, provided by fluorescent lamps (cool white) on a rotary shaker (180 rev min<sup>-1</sup>), 100 ml in 250 ml Erlenmeyer flasks. Larger volumes of algae were grown in 10 litre flasks sparged with CO<sub>2</sub> (1%)-enriched air maintaining a pH of 7.0–7.5 under continuous light of 200 µE m<sup>-2</sup> s<sup>-1</sup> at the surface of the flasks. Effect of temperature on growth was studied in an aluminium temperature block maintaining a temperature gradient between 13–42°C with 1.5°C increments between adjacent test tubes containing the algal cultures. Light was provided by fluorescent lamps (cool white) placed under the block, providing light intensity of 11 µE m<sup>-2</sup> s<sup>-1</sup>, at the bottom surface of the tubes.

### *Outdoor growth experiments*

Miniponds (1 m<sup>2</sup>, 150 litres) were used for outdoor studies of algal growth kinetics using the same growth medium. The cultures were stirred by a paddle wheel so as to maintain a homogeneous suspension. The pH was maintained at 7.2–7.9 by sparging pure CO<sub>2</sub> gas through the culture.

### *Analytical procedures*

Algal growth was followed by measuring optical density at 540 nm, cell counts in a haemocytometer, chlorophyll *a* concentration<sup>6</sup> and AFDM (ash free dry matter). Lipids were extracted using a modified Bligh-Dyer procedure:<sup>7</sup> freeze dried samples were extracted with 3 volumes of chloroform:methanol:water (10:5:4 v/v) solution at room temperature and centrifuged. Water and chloroform were added to the combined

supernatants so as to form a chloroform:methanol:water mixture of 10:10:9. After extraction the chloroform phase was separated and evaporated to dryness under  $N_2$ . Protein was determined according to Lowry *et al.*<sup>8</sup>

### Quantitative determination of algal contaminants

*Isochrysis*, as do other chrysophytes, contains fucoxanthin as a major xanthophyll. Acidification of a fucoxanthin extract followed by heating, causes isomerization of the 5,6-epoxide to the 5,8-epoxide which has a strong absorbance in the red, far beyond 700 nm.<sup>9</sup> The absorption spectrum of a methanolic extract of *Isochrysis galbana* pigments before and after acidification and heating is shown in Fig. 1. Acidification and heating produces a shift of the Soret band and a shoulder at 700–750 nm, which makes the methanolic extract appear blue. In addition, a shift in chlorophyll *a* absorption peak occurs from 670 to 660 nm. Similar treatments of methanolic extracts of possible contaminants such as *Monolanthus* sp., *Chlorella* sp., *Scenedesmus obliquus*, and *Chroococcus* sp. did not produce this effect. This phenomenon enabled us to use a simple 'purity index' for estimating contaminants' concentrations in outdoor ponds, based on the differences in xanthophyll composition among algae. For pure cultures of *Isochrysis galbana*

$$\frac{\text{mg protein}}{A_{710} \times 100} = 1.4 \pm 0.3$$

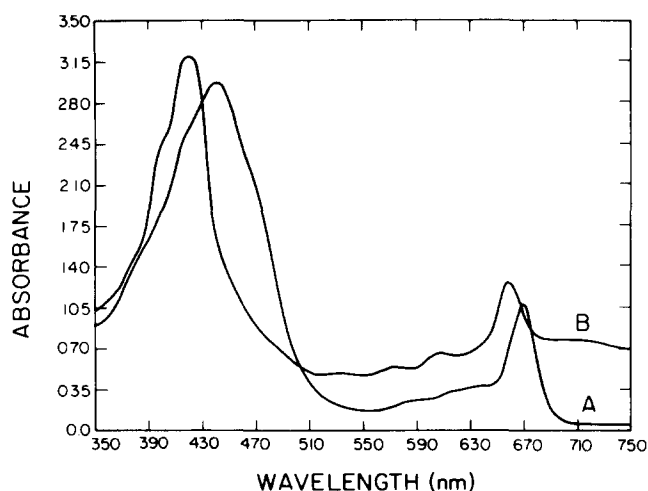


Fig. 1. Absorption spectrum of a methanolic extract from *Isochrysis galbana*. (A) control, (B) after acidification and heating.

and therefore

$$\frac{x - 1.4}{1.4} = \% \text{ contamination of culture,}$$

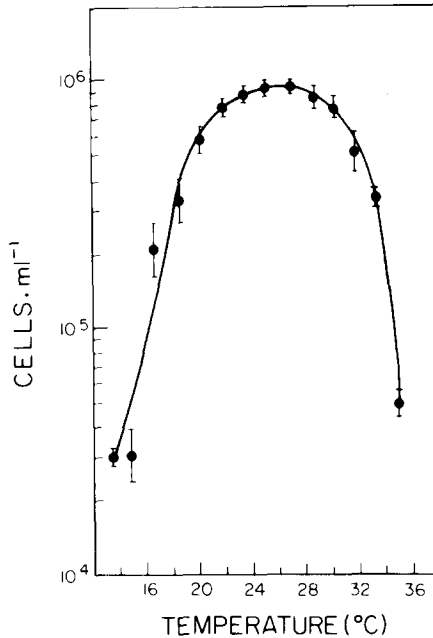
where  $x$  is the absorbance at 710 nm of the treated methanolic extract of an outdoor culture. Obviously, this method cannot be applied when the contaminants, such as diatoms, contain fucoxanthin.

## RESULTS AND DISCUSSION

### Laboratory studies

#### Temperature effects

The optimal temperature for achieving highest algal yield was 27°C (Fig. 2), while temperatures higher than 32°C or lower than 19°C reduced algal yield markedly. Effect of temperature on growth rate was deter-



**Fig. 2.** Effect of temperature on yield of *Isochrysis galbana*. Tubes containing 10 ml of medium were inoculated with algae ( $5 \times 10^4$  cells ml<sup>-1</sup>) and placed in temperature gradient block. Cells were counted after allowing growth for 10 days.

mined for several selected temperatures (Fig. 3), and the temperature for maximal growth rate was also 27°C. However, it should be noted that the low light intensity available for the alga under the experimental conditions present in the temperature block accounts for the slow growth rates measured (38 h doubling time at 27°C).

#### *Effect of pH*

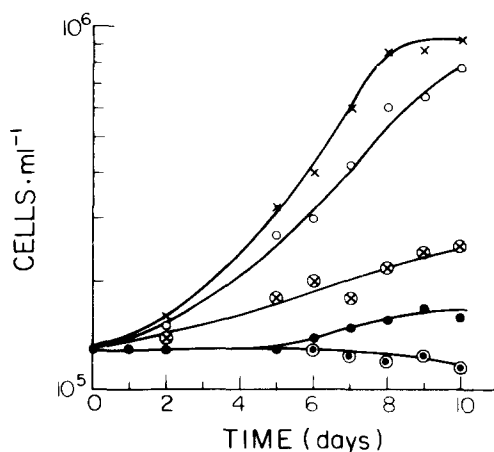
Within the range of 5.0–9.0 the pH in itself had no significant effect on yield and growth rate of the alga, provided that there was sufficient concentration of  $\text{Fe}^{3+}$  available in the growth medium (Fig. 4). There was a marked pH effect on algal yield whenever supply of  $\text{Fe}^{3+}$  was limited, so that no growth occurred at  $\geq$  pH 9.1. This effect of pH is enhanced by the presence of carbonates in the medium (Fig. 5).

#### *Effect of light*

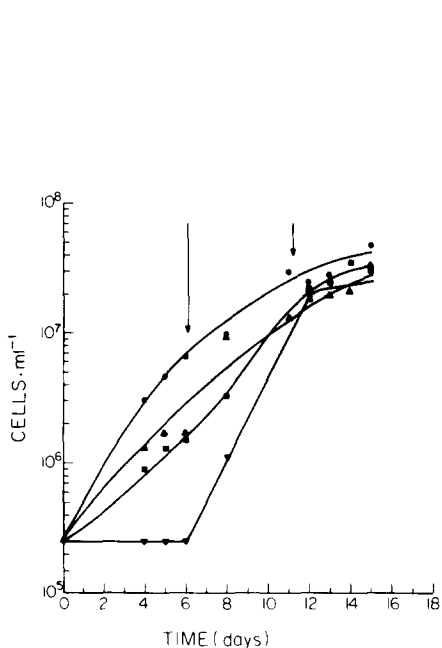
The effects of increasing light intensity from 50 to 150  $\mu\text{E m}^{-2} \text{s}^{-1}$  are presented in Table 1. Growth rate was doubled by increasing light intensity  $\times 3$ .

#### *Effect of salinity*

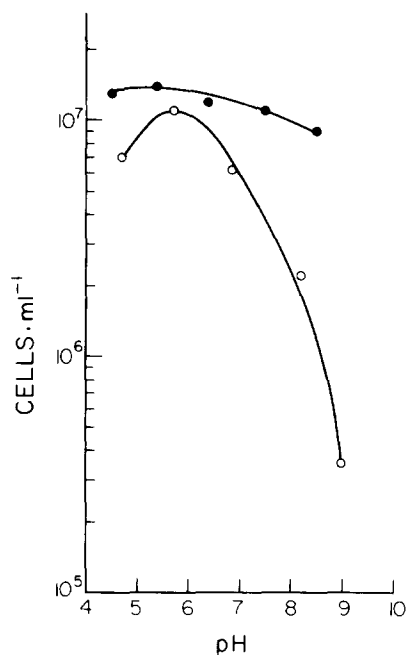
*Isochrysis galbana* exhibited resistance to a wide range of NaCl concentrations. Only a slight reduction in growth rates was observed upon dilut-



**Fig. 3.** Effect of temperature on growth rate of *Isochrysis galbana*. Experimental conditions as described in Fig. 2, except that cell density was monitored daily.  $\odot$ , 15°C and 35°C;  $\bullet$ , 17°C;  $\circ$ , 22°C;  $\times$ , 27°C;  $\otimes$ , 33°C.



**Fig. 4.** Effect of pH on growth of *Isochrysis galbana*. The pH of the growth medium was monitored and adjusted daily. At the times indicated by arrows  $1.2 \text{ mg litre}^{-1}$  of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was added to the medium. ●, pH 5.7; ▲, pH 6.8; ■, pH 8.2; ▼, pH 9.1.

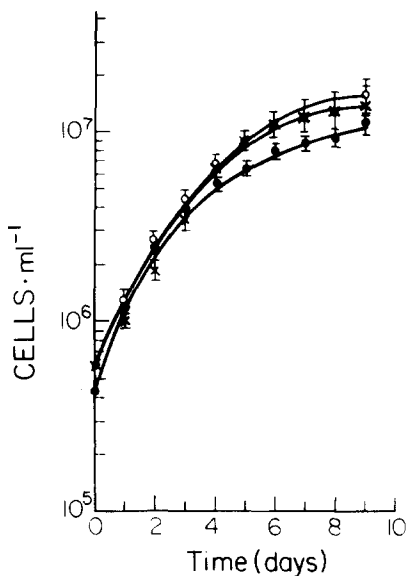


**Fig. 5.** Effect of pH and addition of carbonates on algal yield in  $\text{Fe}^{3+}$  limited culture. See Materials and Methods for experimental conditions. The pH was monitored and adjusted daily. Yield is presented after 10 days of growth. (●), without  $\text{NaHCO}_3$ ; (○), with  $42 \text{ mg litre}^{-1}$   $\text{NaHCO}_3$ , in the growth medium.

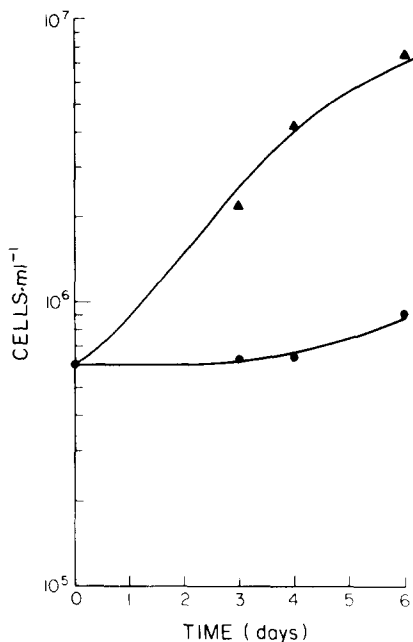
**TABLE 1**  
Effect of Light Intensity on Growth<sup>a</sup> of *Isochrysis galbana*

Light intensity ( $\mu\text{E m}^{-2} \text{ s}^{-1}$ )	Time (days)			
	0	4	5	7
50	$3.0 \times 10^5$	$2.0 \times 10^6$	$3.7 \times 10^6$	$6.1 \times 10^6$
150	$3.0 \times 10^5$	$3.0 \times 10^6$	$5.0 \times 10^6$	$1.2 \times 10^7$

<sup>a</sup>Growth is presented as cells  $\text{ml}^{-1}$ . For growth conditions see Materials and Methods.



**Fig. 6.** Effect of changing NaCl concentration in growth medium on growth rate of *Isochrysis galbana*. See Materials and Methods for growth conditions. ASW medium contained 5 (●), 30 (○) and 60 (×) g litre<sup>-1</sup> NaCl.

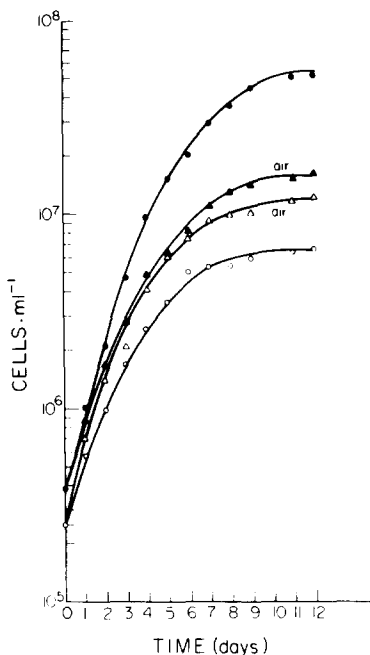


**Fig. 7.** Effect of agitation on growth rate. Cultures were grown stationary, ●; or on rotary shaker ▲, 150 rev min<sup>-1</sup> at identical light regime (see Methods).

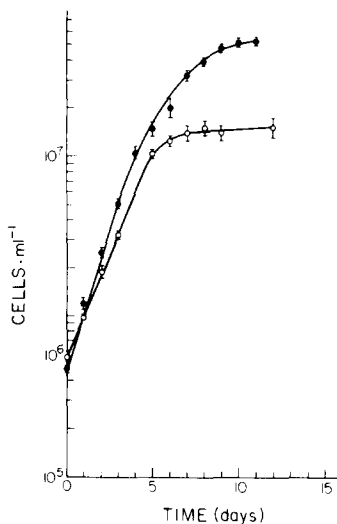
ing the growth medium to 15% sea water, while increasing salinity to twice that of sea water had no effect (Fig. 6).

#### *Effect of aeration*

Agitation of the cultures had a strong effect both on growth rate and on algal yield, as seen in Fig. 7. Sparging of cultures with CO<sub>2</sub> (1%) enriched air was even more effective in increasing growth rate and yield, as long as the culture medium was supplemented with excess Fe<sup>3+</sup> (Fig. 8), otherwise, Fe<sup>3+</sup> starvation developed rapidly. Apparently excess CO<sub>2</sub> causes precipitation of Fe<sup>3+</sup> as insoluble carbonates. Addition of saturating concentrations of Fe<sup>3+</sup> chelates restores high growth rates. This phenomenon might also explain the decrease growth rates at high pHs when high NaHCO<sub>3</sub> concentrations are present in the growth medium, shown in Fig. 5.



**Fig. 8.** Effects of aeration with and without  $\text{CO}_2$  and  $\text{Fe}^{3+}$  on growth rate of *Isochrysis galbana*. Algae were grown in 10 litre flasks with (closed symbols) and without (open symbols)  $\text{Fe}^{3+}$  supplement. Cultures were sparged with air ( $\Delta$ ,  $\blacktriangle$ ) or with air with 1%  $\text{CO}_2$  ( $\bullet$ ,  $\circ$ ).



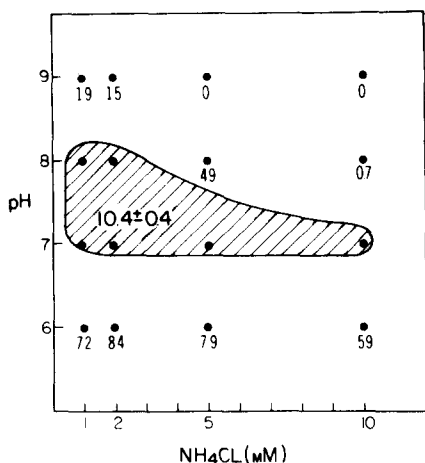
**Fig. 9.** The effect of initial concentrations of  $\text{KNO}_3$  on growth rate and yield of *Isochrysis galbana*. Cultures were grown in 10 litre flasks on improved ASW medium ( $\times 5$  [ $\text{Fe}^{3+}$ ]) and were sparged with air enriched with 1%  $\text{CO}_2$ .  $\bullet$ , 1000  $\text{mg litre}^{-1}$ ;  $\circ$ , 200  $\text{mg litre}^{-1}$   $\text{KNO}_3$ .

### *Nitrate vs ammonia as nitrogen source*

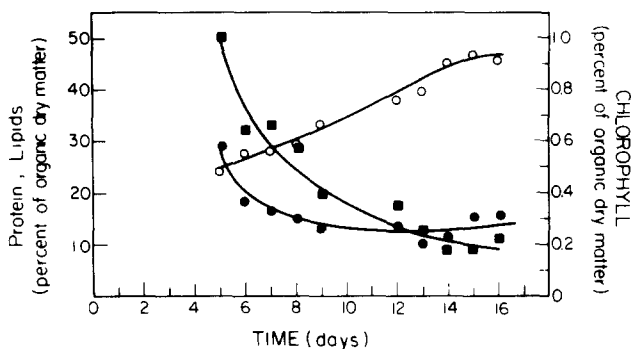
The effects of increasing initial concentrations of  $\text{KNO}_3$  in the growth medium on growth rate and yield of *Isochrysis galbana* are presented in Fig. 9. Initial growth rates were only slightly affected by the difference in  $\text{NO}_3^-$  concentrations, but total yield at the end of logarithmic phase of growth shows differences of about an order of magnitude.  $\text{NH}_3$  can replace  $\text{NO}_3^-$  as nitrogen source, but special attention must be given to the relation between pH and concentration of  $\text{NH}_3$  supplied, as the combination of high pH and high  $\text{NH}_3$  concentration was found to be toxic to *Isochrysis galbana* (Fig. 10), as already described in previously reported cases.<sup>10</sup> Growth rates on  $\text{NH}_3$  were slightly lower than the corresponding figures for  $\text{NO}_3^-$ .

### *Effects of addition of microelements on growth*

Of all microelements tested ( $\text{Mn}^{2+}$ ;  $\text{Zn}^{2+}$ ;  $\text{Mo}^{6+}$ ;  $\text{Cu}^{2+}$ ;  $\text{B}^{3+}$  and  $\text{Fe}^{3+}$ ) by increasing their concentration up to 5 times their concentration in the



**Fig. 10.** Growth of *Isochrysis galbana* at various combinations of pH and  $\text{NH}_4\text{Cl}$  concentrations. Algae were grown in Ehrlenmeyer flasks as described in Material and Methods. The numbers represent concentration of cells  $\text{ml}^{-1} \times 10^6$  at the 10th day of growth. Shaded area indicates optimal growth conditions with respect to pH and  $\text{NH}_3$  concentrations.



**Fig. 11.** Effect of nitrogen starvation on lipid, protein and chlorophyll *a* content. Cultures were grown in 10 litre flasks on ASW medium containing  $200 \text{ mg litre}^{-1} \text{ KNO}_3$ . Growth ceased at day 5 because of N depletion. O, lipids; ●, proteins; ■, chlorophyll *a*.

original growth medium, the algae responded only to the addition of  $\text{Fe}^{3+}$  (see above). All the rest were ineffective in enhancing growth rate.

#### *Effects of nitrogen starvation*

According to Fogg,<sup>11</sup> nitrogen starvation affects not only algal yields, but also shifts anabolic pathways from protein synthesis to carbohydrate or lipid accumulation. When *Isochrysis galbana* was subjected to conditions of nitrogen starvation, concentration of lipids increased from 24% to 47% of total AFDM. This increase in lipid concentration was coupled to a decrease in both chlorophyll *a* and total protein content. Carbohydrate concentration did not change significantly (Fig. 11).

## Outdoor culture of *Isochrysis galbana*

### *Growth conditions*

Outdoor growth experiments in miniponds were started by inoculating them with 10% of their volume by indoor cultures. Outdoor light intensities reached  $2500 \mu\text{E m}^{-2} \text{s}^{-1}$  at noon throughout the summer. To protect the low-light-adapted cultures the ponds were covered by screens cutting 50% of the light intensity on the surface of the ponds. The screens were gradually removed as the culture density increased. After initial adaptation new ponds could be started without using any protective means against photo-oxidative damage. The ponds were operated as semicontinuous reactors: when cultures reached the stationary phase of growth they were diluted by replacing up to 40% of the pond's volume with fresh medium. Doubling time of *Isochrysis galbana* in outdoor cultures was 6 days, and maximal cell density was  $5 \times 10^7$  cells  $\text{ml}^{-1}$ , which was the highest density obtained under any experimental conditions we tried, suggesting that light was the limiting factor preventing higher growth rates and higher population densities in the laboratory.

Cultures were maintained essentially monoalgal for over one month, providing yields of  $11\text{--}12 \text{ g m}^{-2} \text{ day}^{-1}$  as determined spectrophotometrically (see Methods), and by microscopic observation.

### *Effect of nitrogen starvation on outdoor cultures*

Algal lipid content in outdoor cultures was 22–25% of AFDM, similar to what was found for cultures grown indoors. However, nitrogen depletion had only a marginal effect on lipid accumulation by outdoor cultures when maintained in nitrogen-free medium.

After a period of about 2 weeks without the addition of  $\text{NO}_3^-$  nitrogen was exhausted from the medium, causing cessation of growth and reduction in chlorophyll *a* content, but no significant increase in lipid content. Lipid content of outdoor cultures, after 30 days of nitrogen starvation was 27–28% of AFDM. The discrepancy between indoor and outdoor cultures in respect to nitrogen starvation might possibly be a result of conditions suitable for the development of a nitrogen fixing microbial population.

### *Predators*

Outdoor cultures of *Isochrysis galbana* were found to be sensitive to various predators. Among these, ciliates such as *Paramecium* sp., *Euplotes* sp., as well as various rotifers were identified. Infections developed primarily whenever growth conditions deteriorated; in

particular high light intensity shocks or prolonged low night temperatures ( $< 18\text{--}19^{\circ}\text{C}$ ) affected the cultures adversely. It should be pointed out here that Thomas *et al.*<sup>12</sup> were also careful to maintain cultures of *Isochrysis* at  $> 22^{\circ}\text{C}$ .

## CONCLUSION

The series of experiments described here were aimed at defining optimal conditions for outdoor mass culture of *Isochrysis galbana*. The organism was found sensitive to  $\text{Fe}^{3+}$  starvation and temperatures  $\leq 19^{\circ}\text{C}$ . Low light adapted cultures were sensitive to photodynamic damage. Therefore, if growth conditions are properly designed and these precautions are taken into consideration, the alga can be grown in outdoor ponds at good yields with high lipid concentrations (24–28% of AFDM) without any particular difficulty.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Wood, B. J. B. (1974). Fatty acids and saponifiable lipids. In: *Algal physiology and biochemistry*, W. D. P. Stewart (ed.), Blackwell Scientific Publications, pp. 236–65.
2. Scott, A. P. & Middleton, C. (1979). Unicellular algae as a food for turbot (*Scophthalmus maximus* L.) larvae — the importance of dietary long chain polyunsaturated fatty acids. *Aquaculture*, **18**, 227–40.
3. Rhodes, E. W. & Landers, W.C. (1973). Growth of oyster larva *Grassorted virginica* of various sizes in different concentrations of the Chrysophyte *Isochrysis galbana*. *Proc. Natn. Shellfish Assoc.*, **63**, 53–9.
4. Walne, P. R. (1966). Experiments in the large-scale culture of the larvae of *Ostrea edulis* L. *Fish Invest. London Ser. 2*, **25** (4) 53pp.
5. Walne, P. R. & Helm, M.M. (1974). The routine culture of the Pacific oyster (*Crassostrea gigas*) at Lonwy during 1973. *Shellfish Inf. Leaflet MAFF Fish Lab. Burnham No. 32*, 10pp.
6. Parson, T. R. & Strickland, J. D. H. (1963). Discussion of spectrophotometric determination of marine plant pigments, with revised equation for ascertaining chlorophylls and carotenoids. *J. Mar. Res.*, **21**, 155–63.

7. Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**, 911–17.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R.J. (1951). Protein measurements with folin–phenol reagent. *J. Biol. Chem.*, **193**, 265–75.
9. Goodwin, T. W. (1976). Distribution of carotenoids. In: *Chemistry and biochemistry of plant pigments*, Vol. 1, T. W. Goodwin (ed.), Academic Press, London, New York, San Francisco.
10. Abeliovich, A. & Azov, Y. (1976). Toxicity of ammonium to algae in sewage oxidation ponds. *Appl. Envir. Microbiol.*, **31**, 801–6.
11. Fogg, G. E. (1959). Nitrogen nutrition and metabolic patterns in algae. *Symposia. Soc. Exptl. Biol.*, **13**, 106–25.
12. Thomas, W. H., Seibert, D. L. R., Alden, M., Neori, A. & Eldridge, P. (1984). Yields, photosynthetic efficiencies and proximate composition of dense marine microalgal cultures. III. *Isochrysis* sp. and *Monollantus salina* experiments and comparative conclusions. *Biomass*, **5**, 299–316.