STUDIES ON SALINITY TOLERANCE, GROWTH AND EXTRACELLULAR CARBOHYDRATE YIELD IN PRASINOCLADUS MARINUS (Cienk.) Waern.

Thesis presented by

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CONTENTS

	Page
Acknowledgements	i
SUMMARY	ii
Chapter 1. INTRODUCTION	1
Chapter 2. MATERIALS and METHODS	4
I. Materials	4
II. Culture methods	4
a - Culture media	4
b - Maintenance of cultures	9
c - Growth conditions	9
III. Technique for preparation of axenic culture	13
IV. Methods of analysis	16
a - Determination of dry weight	16
b - Wet packed cells volume determination	16
c - Colorimetric determination of total carbohydrate	16
d - Determination of extracellular carbohydrate	17
e - Calibration curve for carbohydrate	17
f - Extraction of pigments from cultures	19
g - Thin layer chromatography of pigments	19
h - Determination of chlorophyll *a* and total carotenoids	20
i - Determination of sugars	20
j - Paper chromatography of sugars	22
k - DNA determination	23
l - Determination of phosphate-phosporus in the medium	23
m - Determination of nitrate-nitrogen in the medium	24
Chapter 3. OBSERVATIONS and RESULTS	25
1. Morphology and mode of life	25
2. Estimation of growth	26
3. Effect of salinity variation on the amount of living/ non-living cell materials	26
4. Effect of various salinities on growth of *P. marinus*	28

Page

5. Effect of various salinities on extracellular liberation of carbohydrate 29

6. Extracellular carbohydrate release during the course of growth 33

7. Composition of the carbohydrate in *P. marinus* 38

8. Uptake of minerals (nitrate and phosphate) at various salinities 40

9. Pigments 42

Chapter 4. DISCUSSION 49

REFERENCES 53

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SUMMARY

Prasinocladus marinus (Cienk.) Waern. [Class Prasinophyceae] grows at high water mark on shores and in estuaries, regions of salinity fluctuation. The effects of salinity variation on the growth and some aspects of cell metabolism have been investigated. The alga was tolerant of both increased and reduced salinities but grew best in a salinity range close to that of normal sea water. The extracellular release of carbohydrate at both increased and reduced salinities was greater than that at normal salinity. The release was found to increase at the early and late stages of growth. The chloroplast pigments and the ratio of nitrogen to phosphorus uptake seem not to be affected by the salinity variation in the ranges in which considerable growth takes place.

Chapter 1

INTRODUCTION

Prasinocladus marinus grows as a non-motile pnase near high water mark on shores, in estuaries and in high-level rock pools (Fig. 1a). Quadriflagellate motile cells (Fig. 2a) are produced, which in their cell organization and possession of scale-bearing flagella, show all the characteristics of a member of the class Prasinophyceae (Christensen, 1962; Parke and Manton, 1965). It was originally described as Prasinocladus lubricus Kuckuck but is now regarded as P. marinus (Chihara, 1963; Parke and Dixon, 1964). The motile cells swim actively for a short while, then settle on suitable substrata by their flagellar poles and subsequently produce branched dendroid structures formed of empty thecae and terminating with living cells. Studies on the life history and various aspects of the biology of Prasinocladus have been made by Lambert (1930), Fritsch (1949), Proskauer (1950), Waern (1952) and Chihara (1963). A good deal of information about the fine structure has been given by Parke and Manton (1965).

It is known from field studies and in laboratory cultures that some proportion of the products of phytoplankton photosynthesis is liberated extracellularly. Extracellular products, which may be defined as soluble organic substances liberated from healthy, as distinct from injured or decomposing cells, are produced by algae in great variety and amount (Fogg, 1966). A substantial amount of work has been done on both the total amount liberated and the chemical nature of the products (Guillard and Wangersky, 1958; Hellebust, 1965; Marker, 1965; Huntsman, 1972; Myklestad, Haug and Larsen, 1972; Ignatiades, 1973; and Ignatiades and Fogg, 1973). Extracellular production was found to

vary from less than 0.5% up to 34% of the total photosynthetic products (Fogg, 1966). Various substances are produced extracellularly, viz. glycollic acid, carbohydrates, nitrogenous substances, vitamins, growth promoting substances and volatile compounds (Fogg, 1962 and 1966).

Carbohydrates appear to be a significant fraction of the extracellular products. A preliminary study made by Guillard and Wangersky (1958) on seven marine algae, showed that accumulation of carbohydrate in the medium increased at the end of the logarithmic phase of growth, and in most instances increased during the stationary phase with senescence of the cultures. Marker (1965) studied some factors affecting the liberation of extracellular carbohydrate in pure cultures of two marine Chrysophyceae, Isochrysis galbana and Prymnesium parvum. He found that increased liberation of carbohydrate occurred at reduced salinity and low light intensity. The rate of liberation was greatest in the early and late stages of growth. Marker favoured the assumption that the extracellular carbohydrates are passively released from dead or dying cells. Myklestad, Haug and Larsen (1972) studied the carbohydrate production by the marine diatom Chaetoceros affinis and concluded that the extracellular polysaccharide isolated from the culture medium is not produced by leakage from dead or dying cells, but is released from healthy cells of Chaetoceros affinis in the stationary phase of growth.

Because of the localization of Prasinocladus marinus at high water mark on rocky shores and in estuaries, it is expected to be affected by salinity fluctuation due to the evaporation of sea water, heavy rainfall and tidal movements. Salinity is considered an important ecological variable in the marine environment, particularly in inshore areas (Provasoli, 1958). The change in salinity, whether it is sudden or gradual, may interrupt directly or indirectly the metabolic activities

of the organism (Boney, 1966).

Little attention in the past appears to have been paid to the physiology of P. marinus. It was selected for the experimental study because of its ability to tolerate various salinity changes which are very common in its natural habitat, and because of the relatively short period of time required for its growth.

The proposed theme of study was planned to investigate the effect of salinity variation on the growth and some aspects of metabolic activity of Prasinocladus marinus. A series of experiments were carried out to investigate quantitatively the proportion of carbohydrate released in the extracellular form during the course of growth at normal salinity and with salinity variation. A preliminary investigation was made into the nature of the extracellular carbohydrate, and its relation to the intracellular fraction. Assessments were made of the pigment's composition and the effect of various salinities on the quantities of chlorophyll a and total carotenoids. The uptake of phosphate and nitrate from the medium at various salinities was also studied.

Chapter 2

MATERIALS and METHODS

I. Materials

A culture of Prasinocladus marinus (Cienk.) Waern. was obtained from the Culture Collection of Algae and Protozoa, Cambridge (Ref. No. 163/1) as a uni-algal culture. An axenic culture (successfully established by treatment with antibiotics as described later) was used as a test material for the various experiments.

II. Culture methods

a. Culture media.

i - Enriched sea water medium (Provasoli's sea water enrichment medium as quoted by West, 1966).

Stock solution

Na_2 glycerophosphate	500 mg	in 10 mls distilled water
$NaNo_3$	3.5 grams	in 10 mls distilled water
Vitamin B_{12}	100 µg	in 10 mls distilled water
Thiamine	5 mg	in 10 mls distilled water
Biotin	50 µg	in 10 mls distilled water
Tris buffer	5 grams	in 100 mls distilled water
$Fe(NH_4)_2(So_4)$. $6H_2O$	175.5 mg	in 250 mls distilled water
$[CH_2N\ (CH_2.COOH)\ .\ CH_2.COO\ Na]_2\ .\ 2H_2O$	165 mg	

Note. pH of Tris buffer adjusted to 7.6 - 8.0 with 1N NCl.

Trace metals solution

H_3Bo_3	285 mg
$FeCl_3 . 6H_2O$	12.25 mg
$MnSo_4 . 4H_2O$	41 mg
$ZnSo_4 . 7H_2O$	5.5 mg
$CoSo_4 . 7H_2O$	1.2 mg
Na_2 EDTA	250 mg
Distilled water	250 ml

The stock solution and the trace metal solutions were mixed, and the final volume was brought to 1250 ml with distilled water.

The final stock solution was sterilized by means of a bacteriological filter. 20 ml of the sterilized mixture was added aseptically to one litre of aged sea water (supplied by the Marine Biological Station, Millport) which was autoclaved at 15 pounds per square inch for 30 minutes. The salinity of the aged sea water was approximately $34^{o}/oo$. Culture media with salinities higher than that of the natural sea water were prepared by adding the salts NaCl, $MgSo_4.7H_2O$, KCl and $CaCl_2$ in concentrations proportional to the artificial sea water medium ASP_2.

ii - Artificial sea water medium ASP_2 (Provasoli, McLaughlin and Droop, 1957).

A full strength artificial sea water medium (salinity $35^{o}/oo$ was prepared as follows:-

1 - Major salt elements (variable)

NaCl	25.2 g
$MgSo_4 . 7H_2O$	7.0 g
KCl	0.84 g
$CaCl_2$	0.14 g
Distilled water	966 ml

2 - Constant components

$NaNo_3$	25 g/l	2 ml
$K_2H\ PO_4$	5 g/l	1 ml
$Na_2\ Sio_3 \cdot 9H_2O$	25 g/l	6 ml
TRIS	166.7 g/l	6 ml
B_{12}	3 mg/l	0.5 ml
Na_2 EDTA	15 g/l	2 ml
$FeCl_3$	0.8 g/l	1 ml
$ZnCl_2$	150 mg/l	1 ml
$MnCl_2$	1.2 g/l	1 ml
$CoCl_2$	3 mg/l	1 ml
$CuCl_2$	1.8 mg/l	0.5 ml
H_3Bo_3	3 g/l	2 ml
Vitamin mixture		10 ml
		34 ml / 1 litre medium

Vitamin mixture - prepared as follows:-

Thiamine HCl	50 mg
Nicotinic acid	10 mg
Ca pantothenate	10 mg
Biotin	1 mg
Inositol	500 mg
Folic acid	0.2 mg
Thymine	0.3 g
Distilled water	1000 ml

The constant components mixture was sterilized by passing through a bacteriological filter. The major salt elements were autoclaved at 15 pounds per square inch pressure for 30 minutes ($CaCl_2$ solution autoclaved separately). The three solutions were cooled and mixed aseptically.

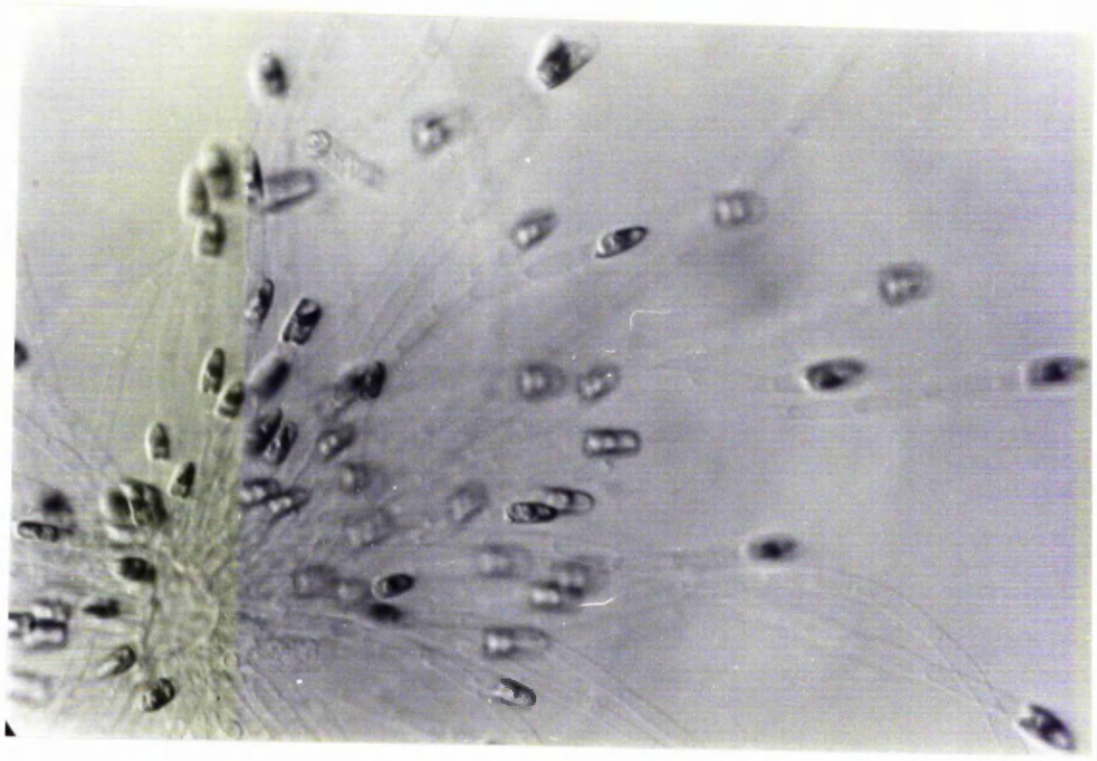
The pH of the major salts solution was adjusted in order to make the final pH of the medium 7.5 - 7.8

Figure 1

Non-motile cells of *Prasinocladus marinus*.

A - At normal salinity ($35^{o}/oo$)

B - At reduced salinity ($10^{o}/oo$)



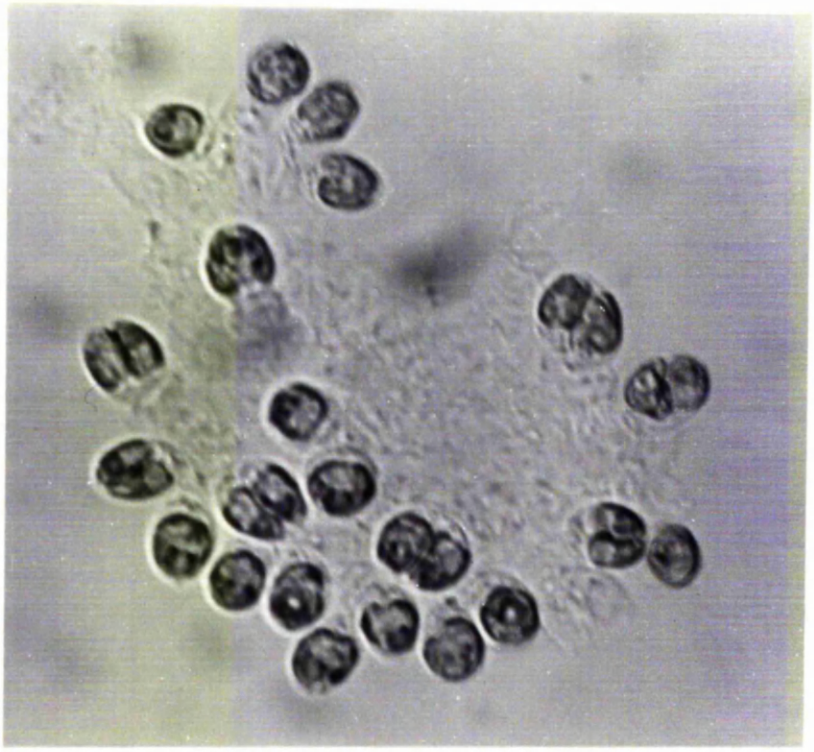
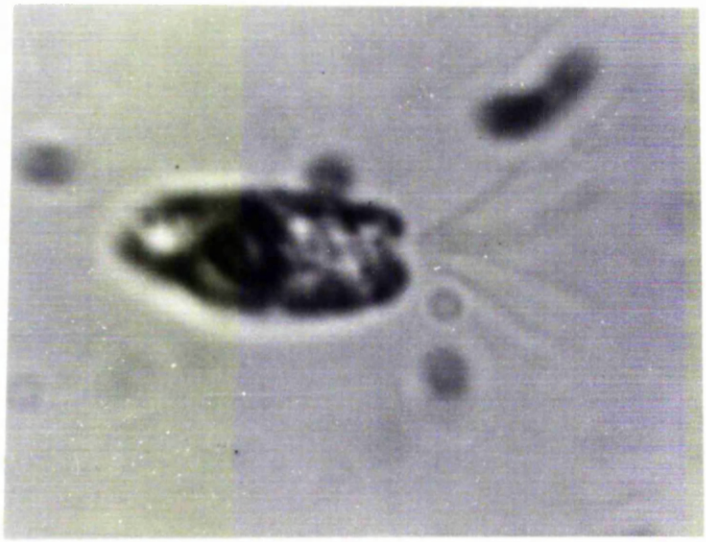


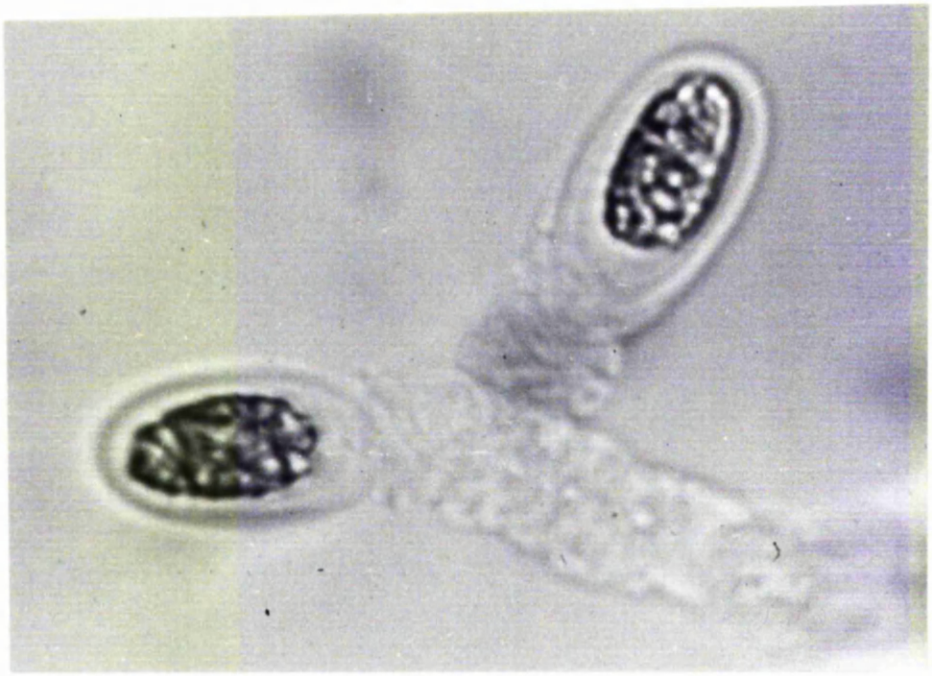
Figure 2

Cell of *Prasinocladus marinus*.

A - Motile cell

B - Cyst stage





b. Maintenance of cultures.

Stock cultures were maintained in the enriched sea water medium at a constant temperature of 15°C and an illumination of 2160 lux for 12 hours per day. Subcultures of bacteria-free Prasinocladus (see p. 13) were made routinely to fresh medium every month with regular tests for sterility, using the peptone sea water medium described by Spencer (1952):-

Bacto-peptone	5 grams
Ferric phosphate	0.1 gram
Aged sea water	750 ml
Distilled water	250 ml

c. Growth conditions.

The stock cultures and the cultures of some experiments which need a small volume of media and relatively large numbers of replicates were placed on glass shelves in the growth room. Illumination was provided by one 60 watt (5 feet long) Atlas "daylight" fluorescent tube beneath each shelf, which gave an illumination of 2160 lux at the flask level. The temperature in the growth room ranged between 15°C during the day-time and 14°C during the night.

More controlled conditions of temperature and aeration were achieved by the use of a culture tank which was specially designed and built for this purpose (Fig. 3). It consisted of a 4' x 1'6" x 1'6" glass tank filled with water. The temperature of the water was controlled at 15 $\pm$ 0.1°C by using two standard immersion heaters

placed on the bottom of the tank filled with water. An even temperature was maintained throughout the tank by continuous circulation of the water with a small water pump. The maximum variation of the water temperature was $\pm$ 0.1°C. Two 60 watt (5 feet length) Atlas "daylight" fluorescent tubes were fixed on each side of the culture tank to give an illumination of 2160 lux at the flask position. This was measured by immersing a culture flask without the neck in the water bath at the place where a culture flask would normally be suspended, and the illumination was measured inside the flask using a luxmeter. The culture flask was a 1 litre Pyrex Roux bottle with a central neck (Fig. 4), fitted with a standard multiple adapter, ADP, (Quickfit Cat. No. MA 1/3, size B 24/29). The central socket of the adapter carried an aeration tube, AT, (Quickfit Cat. No. MF 15/2, size B 19/26) reaching nearly to the bottom of the culture flask. The upper end of the aeration tube was connected with silicone rubber tubing which in turn was connected to the air supply pump (as described later). The off-set socket (OF) of the adapter was plugged with non-absorbent cotton-wool and served as an exit for the outgoing air. The cultures were aerated with air from a high-flow pump (Fig. 4); this was first passed through an aspirator filled with distilled water (oil trap), after that through a column of silica gel (moisture trap) and then through another aspirator filled with cotton-wool which was regularly autoclaved. The sterile air was then led into a manifold with a number of outlets which were connected to the individual culture flasks by silicone rubber tubing fitted with screw clamps to allow regulation of air in each culture flask. The air pressure was kept

Figure 3

Apparatus for controlled growth conditions of temperature, light and aeration.

(The two fluorescent tubes were removed from the front side for ease of photography.)

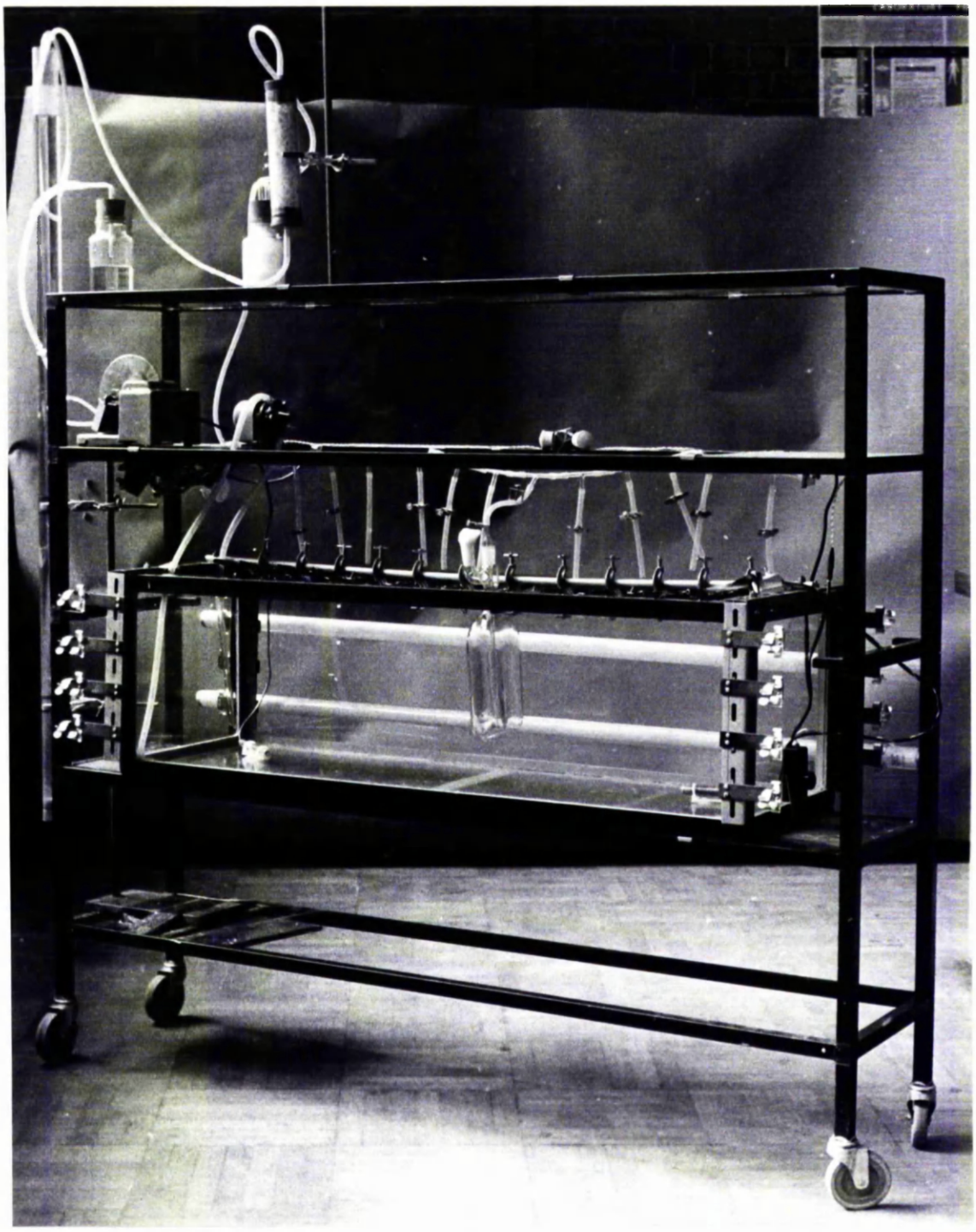
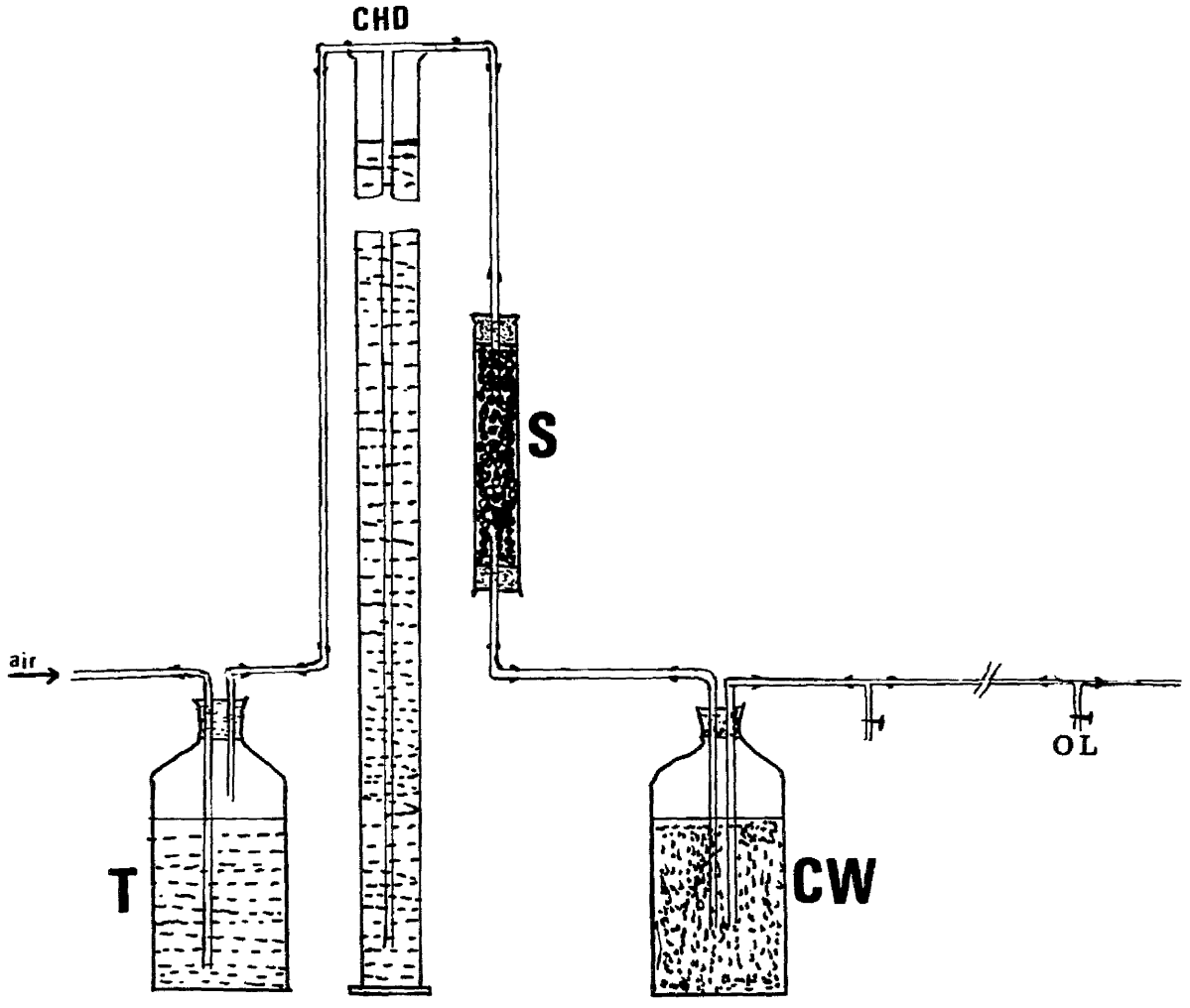
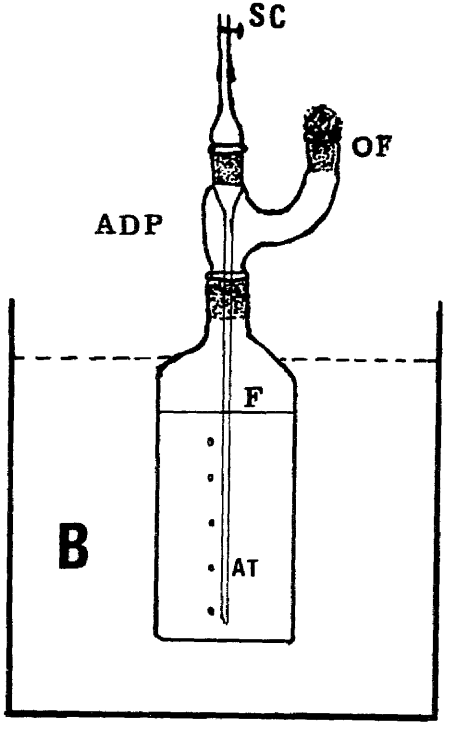


Figure 4

Diagram of the aeration system and the culture apparatus.

T = oil trap
CHD = constant head device
S = silica gel column
CW = aspirator with cotton-wool
OL = outlet for individual culture flask
B = constant temperature bath
SC = screw clips
OF = off-set socket
ADP = multiple adapter
F = culture flask
AT = aeration tube


CHD
S
air
T
CW
OL


SC
OF
ADP
F
B
AT

constant by allowing the excess to bubble off against a 150 cm column of water.

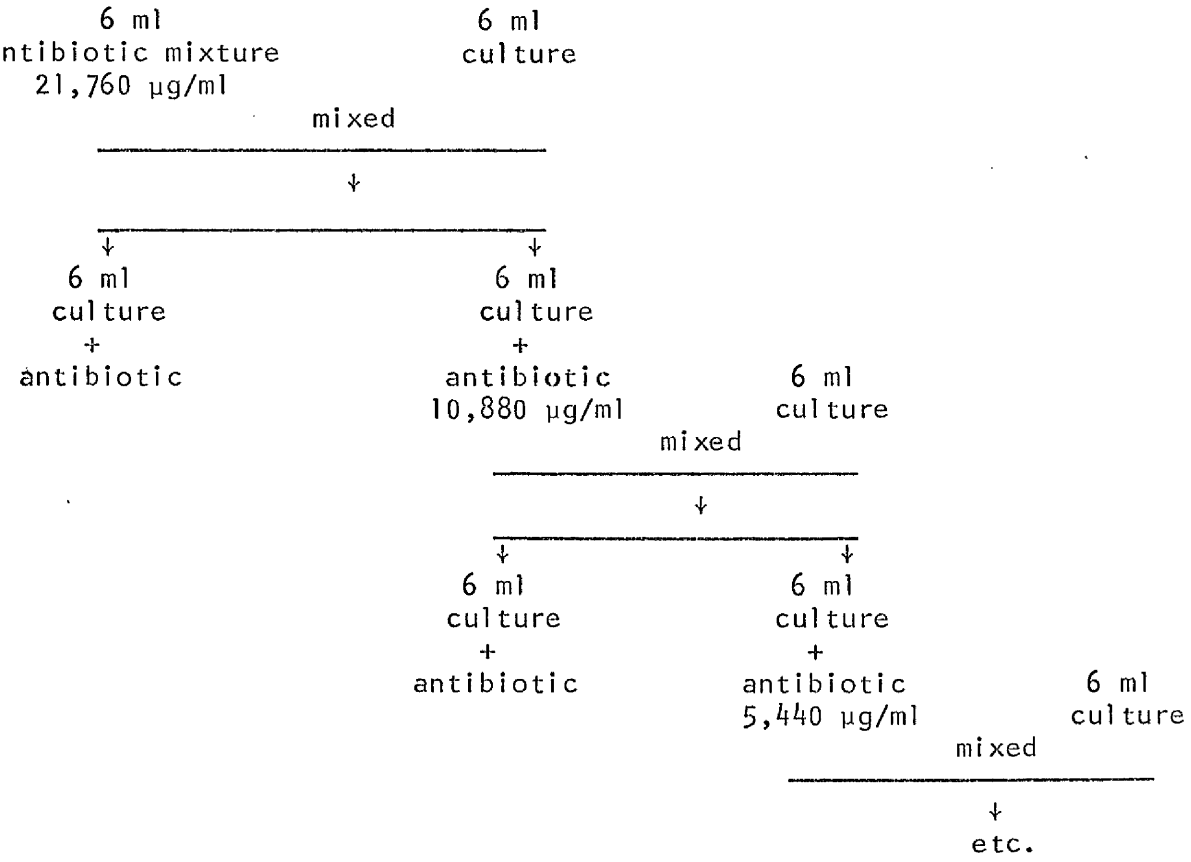
III. Technique for preparation of axenic cultures

P. marinus was obtained as a uni-algal culture but it was contaminated with mould and bacteria. To obtain a bacteria-free culture was the most crucial step in the present study. The standard plating technique used with the motile cells at the beginning was unsuccessful. The use of ultraviolet light was also investigated. Some of the motile cells were streaked on enriched sea water medium with 1.5% agar and exposed for varying periods at U.V. source emitting at 254 nm. Exposures of 5 and 10 minutes did not result in bacteria-free cultures. The longer exposure time was found to affect the algal cells.

Antibiotic methods were therefore necessarily applied. Droop's (1967) successful technique with a wide range of algae depends on exposure to high concentrations of antibiotics for a short time. He claimed that there was "seldom any difficulty in purifying the organisms; the hard part is continuing to grow them afterwards!". His mixture VI was applied to the culture of *P. marinus*. The result of 24 hours exposure was that both the bacteria and the algae were viable in all the antibiotic concentrations. Modifications were made by using a higher concentration of Chloramphenicol with the addition of neomycin, which is another broad spectrum antibiotic. Actedione was added to remove the mould contamination. The antibiotic mixture which gave a successful result was prepared as below:-

Benzyl penicillin So_4	16,000 µg
Streptomycin So_4	3,200 µg
Chloramphenicol	160 µg
Neomycin	800 µg
Actidione	1,600 µg
	21,760 µg per one ml

Six tubes of the uni-algal cultures numbered from 1 to 6 were prepared, each containing 6 ml of culture. A solution of antibiotic mixture was prepared and sterilized by filtration. 6 ml of this mixture was mixed with culture tube no. 1, after that half of it was mixed with tube no. 2 to dilute the antibiotic concentration to 1/4, and so on to get six different concentrations of the antibiotic mixture (Table 1, and as shown in the sequence below):-



After 24 hours incubation at light intensity of 2160 lux and temperature 15°C, sub-cultures were made into antibiotic-free media from each of the six tubes. Sterility tests were made on all the sub-cultures after three weeks using peptone sea water medium. Sterility tested cultures were incubated for at least two further weeks at 25°C to ensure confidence in any negative result obtained.

Table 1. Routine method of obtaining axenic cultures.

Tube number	1	2	3	4	5	6
Antibiotic conc. (µg/ml), 24 hours exposure	10,880	5,440	2,720	1,360	680	340
Viable bacteria in sub-cultures	-	-	-	+	+	+
Viable algae in sub-cultures	-	+	+	+	+	+

The results of the different treatments illustrated in Table 1 show that in tube no. 1 both the bacteria and the algae were killed. In tubes nos. 2 and 3 only the bacteria were killed. Other tubes, numbered 4, 5 and 6 were still contaminated with bacteria. No detectable changes occurred in the purified algal cells, and they grew well subsequently.

IV. Methods of analysis

a - Determination of dry weight.

Samples of cell suspension were taken in preweighed centrifuge tubes and were spun at 3000 g. The centrifugate, after a quick rinse with distilled water and recentrifugation at the same speed, was stored at 90°C for three or four days until it attained a constant weight. The tubes were reweighed and the differences in weight gave the assessment of the dry weight of the cells.

b - Wet packed cells volume determination.

Wintrobe (blood sedimentation) tubes were used for the estimation of wet packed volume (Dring, 1967). These are thick-walled tubes with a hole of about 2 mm. They are 11 cm long, and are graduated in mm. Since the total volume of the tubes was variable, they were calibrated before use by filling to the 100 mm mark with distilled water, and weighing. The reading had then to be corrected by a factor specific to each tube, to obtain the final volume. Samples of cell suspension were centrifuged and the centrifugate was transferred with a small volume of medium to a Wintrobe tube. Care was taken to ensure even packing and the absence of air-bubbles, and the tubes were then centrifuged at 1500 g for 5 minutes. The level of cells in the tubes was noted and the total volume was calculated.

c - Colorimetric determination of total carbohydrate.

Test samples were centrifuged at 3000 g. The centrifugate was mixed with 2ml of culture medium and homogenized by forcing the

mixture through a fine 1 ml sterile syringe needle. One ml of the homogenate was carefully layered on to 5 ml of ice cold anthrone reagent (Yemm & Willis, 1954), allowed to react for 5 minutes and then thoroughly mixed while still immersed in the ice water. The tubes were heated at 100°C in a constant level water bath for 10 minutes and then cooled in ice bath to stop the reaction. The optical density was measured using one cm fused silica cells against culture medium at 620 nm using a Unicam S.P.600 Spectrophotometer. Reagent blanks using normal culture medium were prepared in exactly the same way as described above.

Quantities of carbohydrate equivalent to the observed optical densities were evaluated by means of a calibration curve for glucose.

Anthrone reagent was prepared by dissolving 0.2 gm of anthrone (recrystallized from toluene) in 100 ml of diluted H_2So_4, made by adding 900 ml of concentrated acid to 100 ml of distilled water.

d - Determination of extracellular carbohydrate.

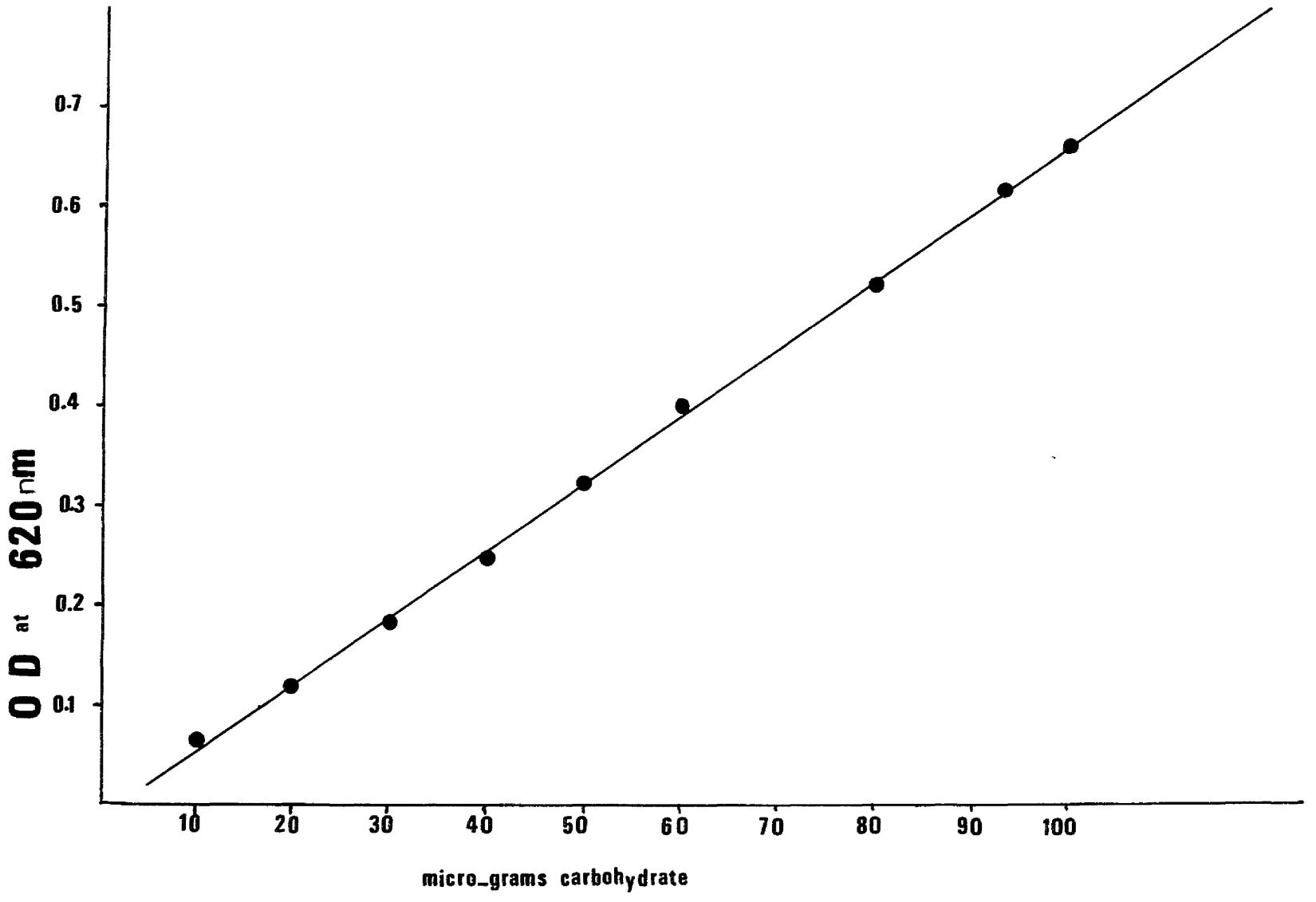
The cell suspension under test was centrifuged at 3000 g. The clear supernatant was filtered through a sintered glass crucible lined with Kieselguhr and passed through a 0.37 µ millipore filter. One ml replicates were tested for carbohydrate as described above.

e - Calibration curve for carbohydrate.

Using the same experimental procedure, optical densities of duplicate samples of known amounts of glucose (10, 20 ---- 100 µgs) were determined at 620 nm using an S.P.600 and plotted against quantities (Fig. 5).

Figure 5.

Calibration curve for anthrone reagent with standard glucose solutions.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
O D at 620nm
10
20
30
40
50
60
70
80
90
100
micro_grams carbohydrate

f - Extraction of pigments from cultures.

A sufficient quantity of cultures was centrifuged at 2000 g for 15 minutes. After decantation of the supernatant, the cells were extracted with 90% acetone in subdued light. The extract was centrifuged and the supernatant liquid was decanted. Extraction was repeated three times with 10 ml aliquots of 90% acetone. The four extracts were combined and partitioned with diethyl ether by the presence of 10% (w/v) NaCl solution. The ether layer containing the pigment was rewashed with 10 volumes of 10% (w/v) NaCl solution. The pigment extract was centrifuged at high speed to remove droplets of water and the solvent was evaporated under a stream of nitrogen.

g - Thin layer chromatography of pigments.

The dried pigments were dissolved in a minimal quantity of ether and loaded on to the thin layer plates coated with a 0.25 mm layer of silica gel G under a current of nitrogen. The development of the plates was carried out in darkness using a mixture consisting of 58 (v/v) petroleum ether (b.p. 60-80°C), 30% (v/v) redistilled ethyl acetate and 12% (v/v) diethylamine (Riley and Wilson, 1965). After the development was completed the plates were air dried and the Rf values of each band were determined.

The separated bands were eluted with a suitable solvent (viz. diethyl ether, hexane, petroleum ether, acetone), and redeveloped on thin layer plates using the same solvent system. The purified bands were re-eluted and characterized by their absorption spectra using a Unicam S.P.800. From the Rf information and the absorption maxima of the bands, the pigments composition was characterized and

identified by comparing their properties with the published authentic data.

h - Determination of chlorophyll a and total carotenoids.

Equal aliquots of wet packed volume of algal cells were extracted with 90% acetone. Each extract was subjected to ultra sonification for 30 minutes (Nelson, 1960) to rupture the cell wall. The sonificated samples were placed in a refrigerator in complete darkness overnight, and the extract was transferred to 1 cm fused silica cells to note the extinction at 665, 645, 750 and 480 nm. Concentrations of chlorophyll a and carotenoids were calculated according to the equations of Strickland and Parsons (1968).

$$\text{Chlorophyll } a = 11.6E_{665} - 1.31_{E645}$$

$$\text{Carotenoids} = 4.0E_{480}$$

The reading at 750 nm was always less than 0.012 and was subtracted from the other extinctions to correct for turbidity.

i - Determination of sugars.

1. Intracellular fraction.

The plant materials were separated from the culture medium by centrifugation and extracted by reflux overnight with 80% ethanol at 85°C. The extract was centrifuged at high speed and the supernatant (alcohol soluble fraction) was concentrated in vacuo at 35°C and partitioned between chloroform and water. The aqueous phase containing the sugars was hydrolysed in 3N sulphuric acid for 3 hours. The acid was neutralized with $BaCo_3$. Excess carbonate and sulphate was removed by millipore filtration.

The alcohol insoluble fraction was hydrolysed as above and the extract subjected to paper partition chromatography.

2. Extracellular fraction.

The culture medium, after the separation of the plant material, was passed through a 0.37 μ millipore filter to remove any fine particles of algae. The medium was reduced in volume to about 100 ml by evaporation at reduced pressure at 55°C since it was necessary to deal with a relatively large volume of liquid phase (2 litres) in order to detect the dissolved organic substances liberated into the surrounding medium. Difficulties were experienced in reducing the volume of this liquid phase in the presence of the massive inorganic salts, and in removing these salts which interfered with the chromatographic separation. Dialysis was found not to be satisfactory because after up to 50 hours dialysis against distilled water the medium was still considerably saline, and prolonged dialysis may have led to a loss of as much as 50% of the carbohydrate (Marker, 1965). The desalting with an ion retardation resin (AG 11A8) was tried and found inefficient.

Desalting by the use of cation and anion resin was found to be the most satisfactory method. The medium was passed through a column (50 cm long x 3.5 cm diameter) containing cation exchange resin (amberlite IR-120) in the H form, and then was passed through a column (50 cm x 3.5 cm) of anion exchange resin (amberlite IR-45) in the OH form. The desalted medium was reduced in volume at reduced pressure and hydrolized with 3N sulphuric acid for 3 hours. The hydrolysate was neutralized in $BaCo_3$, and the excess carbonate and sulphate

removed by millipore filtration. The volume of the extract was reduced in vacuo and subjected to paper chromatography.

j - Paper chromatography of sugars.

Descending paper chromatography was carried out in an air-tight glass tank (Panglas chromatank, Shandon) of a size suitable to take paper 18 x 22 inches. The tank was first saturated with the solvent vapours by covering the bottom with the solvent and replacing the lid. Sugar extracted from the plant material was spotted on Whatman No. 3MM paper and separated by downward displacement. Several solvent systems were tried, but two of them were selected which gave a good separation.

a) [v/v] n-butanol : ethanol : water (53 : 32 : 16).

b) [v/v] ethylmethyl ketone : acetic acid : water (saturated with boric acid) (9 : 1 : 1).

A marker of glucose was always used in parallel with the solution to be examined. After the development was completed the chromatogram was air dried, and the spots were stained and identified by means of Rg values.

$$Rg = \frac{\text{distance substance travels from the origin}}{\text{distance glucose travels from the origin}} \times 100$$

Staining reagent for paper chromatography

Periodic acid reagent was used as a staining reagent and prepared as follows:-

a - Periodic acid (H_5IO_6) 2.28 g in 100 ml distilled water .. 1 volume.

b - Benzidine 184 mg in 95 ml acetone with the addition of 4.4 ml distilled water and 0.6 ml acetic acid. .. .. .. 19 volumes

The dried chromatogram was dipped in reagent (a) and allowed to stand for 3-4 minutes, during which time any sugar present reduced the periodic acid. The damp paper was then dipped in reagent (b). After a few minutes a white spot appeared on a blue background.

k - DNA determination.

A known volume of packed cells was refluxed with 80% redistilled methanol to aid bleaching of the cells, then centrifuged at high speed, and the residue treated with 10% ice cold trichloroacetic acid for one hour at -15°C. The precipitate thus obtained was centrifuged at 5000 g and extracted with a solvent mixture consisting of ether : ethanol : chloroform (2 : 2 : 1) in order to remove fat and moisture. The precipitate was separated by centrifugation at 5000 g and the pellet was further dried *in vacuo*. The dried pellet was hydrolysed with 5 ml KOH (0.5N) for one hour at 37°C. The precipitate was separated by centrifugation at high speed at 0°C and a known volume of 10% perchloric acid was added, mixed, and the resulting slurry thus obtained was incubated at 90°C for 15 minutes. The clear supernatant, after centrifugation, was transferred to 1 cm fused silica cells and the optical density at 260 nm was noted using the Unicam S.P.800 Spectrophotometer.

l - Determination of phosphate-phosphorus in the medium.

The concentration of phosphate in the medium was measured using the technique described by Murphy and Riley (1962), 100 ml of the sample was measured with a graduated cylinder and poured into a

250 ml capacity polythene bottle. The sample was warmed to a temperature between 15 and 30°C, 10 ml of mixed reagent were added and mixed quickly. After 5 minutes the extinction of the solution was measured in 4 cm cells at 885 nm using the S.P.600. The extinction was corrected by subtracting the reagent blank. The phosphate concentration was measured in microgram-atoms of phosphate phosphorus per litre from the expression:-

μg - at p/litre = corrected extinction x F.

$$F = \frac{3.00}{E_s - E_b}$$

E_s = the mean extinction of four standards containing 3 μg - at p/l.

E_b = the mean extinction of two blanks.

m - Determination of nitrate-nitrogen in the medium.

The concentration of nitrate in the medium was measured using the method of Wood, Armstrong and Richards (1967), a method in which nitrate is quantitatively reduced to nitrite by passing the sample through a cadmium-copper column. The sample was then treated as a nitrite sample by the method of Bendscheider and Robinson (1952).

Chapter 3

OBSERVATIONS and RESULTS

1. Morphology and mode of life.

The newly settled motile cell is enclosed within a theca of unknown chemical composition (Boney, 1970), which is somewhat variable in size and shape, usually about 16-20 µm in length and 7-8 µm in diameter. The chloroplast is lobed or laciniate (Parke and Manton, 1965) in which a two-layered eye-spot clearly stands out. The pyrenoid is located near the centre or somewhat towards the posterior end of the chloroplast and surrounded with a cup-shaped starch shell. The nucleus is located just in front of the pyrenoid. Four flagella, which are about half the body length, arise from the bottom of a depression at the anterior end of the cell. The flagellar surface is covered with small plate-scales and hairs, which are considered to be one of the main characteristics of the class Prasinophyceae (Parke and Manton, 1965). The sedentary phase is initiated when the zooids settle down by their flagellar poles. They throw their flagella, and the protoplast secretes a new wall inside the previous one. The inner wall elongates and the previous wall is ruptured at the apex. The upward passage of the rejuvenated cell continues to a more or less considerable extent until its base is only enclosed in a collar-like strip of the previous wall. When this process of rejuvenation is repeated a number of times, a filament of empty walls (thecae) develops, terminating in a partly extruded cell. Branching is effected by longitudinal and oblique division of the terminal cell, and hence a branched dendroid colony may be

formed. There is no evidence of any sexual phase in the life of the organism.

2. Estimation of growth.

The irregularly branched dendroid nature of P. marinus, which consists of empty cell envelopes and the green cells, made the normal counting methods impossible. The growth in clumps prevents the use of optical density measurements. Measurement of the increase in chlorophyll content involves the quantitative extraction of the pigments by an organic solvent which is not easily accomplished. Also it does not give a measure of the overall production of organic matter by the organism. An alternative method for the estimation of the growth was developed which depends on the measurement of the compact volume of the algae, and which was sufficiently sensitive to give a direct measure of the overall increase of organic matter (both cells and empty thecae). In addition, measurements can be made in a short time interval.

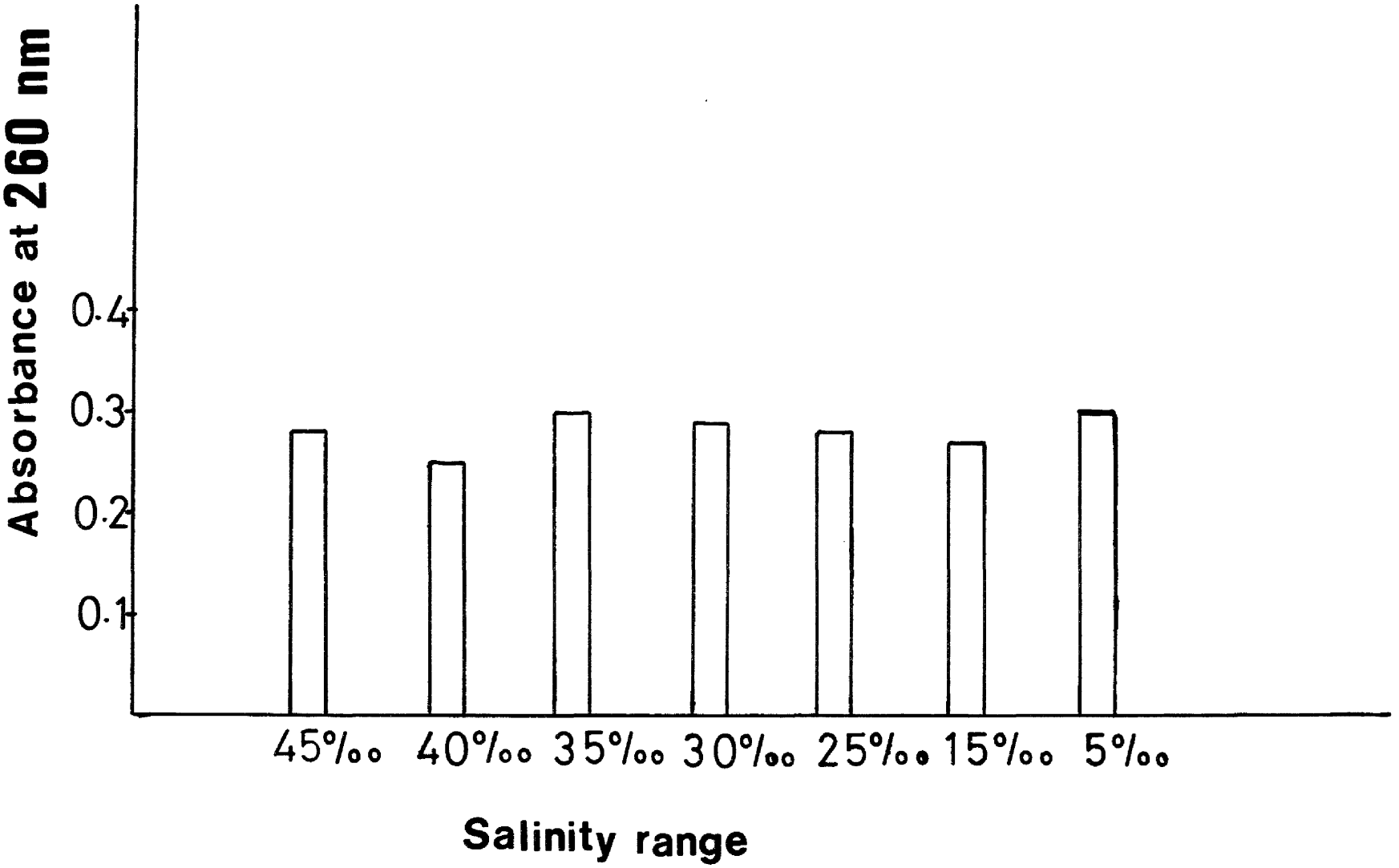
3. Effect of salinity variation on the amount of living/non-living cell materials.

The external morphology of P. marinus when grown at various salinities was not similar. In cultures of normal salinity the empty cell envelopes were usually in a filamentous form (Fig. 1A), while at reduced salinity, on the other hand, the empty thecae were mainly compact and irregular in their appearance (Fig. 1B). It was

Figure 6.

Quantities of DNA per 0.05 ml wet packed volume of *P. marinus* at various salinities.

Salinity range	Absorbance at 260 nm
45°/oo	0.28
40°/oo	0.26
35°/oo	0.295
30°/oo	0.29
25°/oo	0.28
15°/oo	0.27
5°/oo	0.295


Absorbance at 260 nm
0·4
0·3
0·2
0·1
45‰
40‰
35‰
30‰
25‰
15‰
5‰
Salinity range

therefore necessary to know the relative amount of living cell material per a known wet packed cell volume (and whether it is similar or not) for cultures growing at various salinities. The estimation was made on the basis of DNA content of the living cell materials in a known volume of cells. Ricketts (1966) stated that the DNA content per average cell is relatively constant in the same organism.

Equal aliquots (0.05 ml) of wet packed volume of P. marinus grown at salinities of 5°/oo, 15°/oo, 25°/oo, 30°/oo, 35°/oo, 40°/oo, and 45°/oo were extracted with 80% methanol and estimated quantitatively for the total DNA (see page 23). The results are illustrated in Fig. 6 and showed no significant differences in the amount of DNA from cultures growing at various salinities. This may lead to the conclusion that equal wet packed volumes taken from cultures at various salinities contain the same amount of cell materials, irrespective of the form taken by the thecae.

4. Effect of various salinities on growth of P. marinus.

A preliminary experiment was made to find the pattern of growth at various salinities. Inocula of 0.1 ml wet packed volume of P. marinus (taken from a culture growing in enriched sea water medium at 15°C and illumination of 2160 lux) were suspended in 500 ml of enriched sea water media of various salinities (10°/oo, 15°/oo, 20°/oo, 27.5°/oo, 35°/oo and 45°/oo). Two replicates of each salinity range were made. The culture flasks were 1 litre Roux flasks, and sterilized air was distributed through each culture.

The temperature was 15 $\pm$ 0.1°C and the illumination was 2160 lux for a period of 12 hours per day. After 10 days incubation the algal material was separated by centrifugation and the final crop determined by wet packed volume and dry weight.

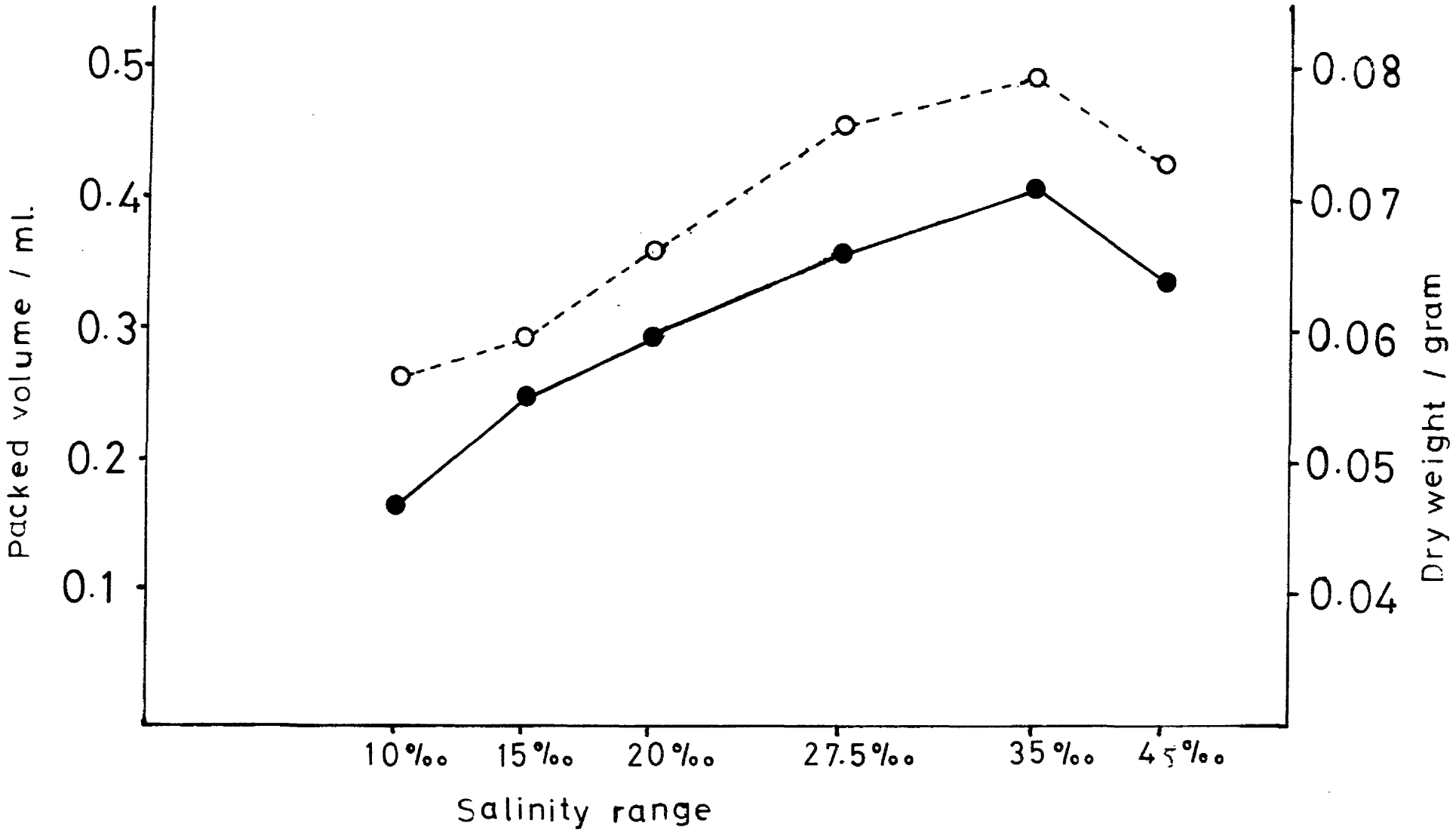
It can be seen from the results illustrated in Fig. 7 that a packed volume of 0.5 ml at normal salinity shows a five-fold increase from the original inoculum over the 10 days period. By contrast, growth in media of salinities $10^{o}/oo$, $15^{o}/oo$, $20^{o}/oo$, $27.5^{o}/oo$ and $45^{o}/oo$ was about 55%, 60%, 75%, 90% and 85% respectively of that in the normal salinity. However, the cells were green in colour and there were no significant differences in their appearance in all the salinities used.

5. Effect of various salinities on extra-cellular liberation of carbohydrate.

The object of this experiment was to find the total release of carbohydrate into the surrounding media during the growth at various salinities. The experiment was carried out by altering the concentration of the major salts (NaCl, $MgSo_4$, $7H_2O$, KCl and $CaCl_2$) proportionately in the artificial sea water medium ASP_2 (Provasoli et al., 1957). Various salinities were prepared ($5^{o}/oo$, $10^{o}/oo$, $15^{o}/oo$, $20^{o}/oo$, $25^{o}/oo$, $30^{o}/oo$, $35^{o}/oo$, $40^{o}/oo$ and $45^{o}/oo$) with pH adjusted between 7.5 - 7.8; other minerals and trace elements were kept constant in all the preparations. Cultures (100 ml each) were prepared by inoculation with equal amounts of P. marinus (in suspension) under aseptic conditions. After 3 weeks incubation at

Figure 7.

Effect of various salinities on growth of *P. marinus*. ——o——, dry weight as gm/500 ml cultures; ---o---, wet packed volume as mls. (Initial inoculum was 0.1 ml wet packed volume.)


0.5
0.4
0.3
0.2
0.1
Packed volume / ml.
0.08
0.07
0.06
0.05
0.04
Dry weight / gram
10‰
15‰
20‰
27.5‰
35‰
45‰
Salinity range

light intensity of 2160 lux and a temperature of 15°C (with gentle daily agitation), the cells were harvested by centrifugation and the supernatant filtered through a sintered glass crucible lined with Kieselguhr. The carbohydrate in both the medium (extracellular) and the cells (intracellular) was determined using the anthrone reagent method (Yemm and Willis, 1954), as described on page 17. Intra-cellular carbohydrate is here taken to mean all carbohydrate associated with the organism and not released in extracellular form.

The results are summarized in Table 2 and Fig. 8. They show that the higher release of carbohydrate to the medium occurred at both reduced (25^{o}/oo, 20^{o}/oo --- etc.) salinity and increased (45^{o}/oo) salinity levels.

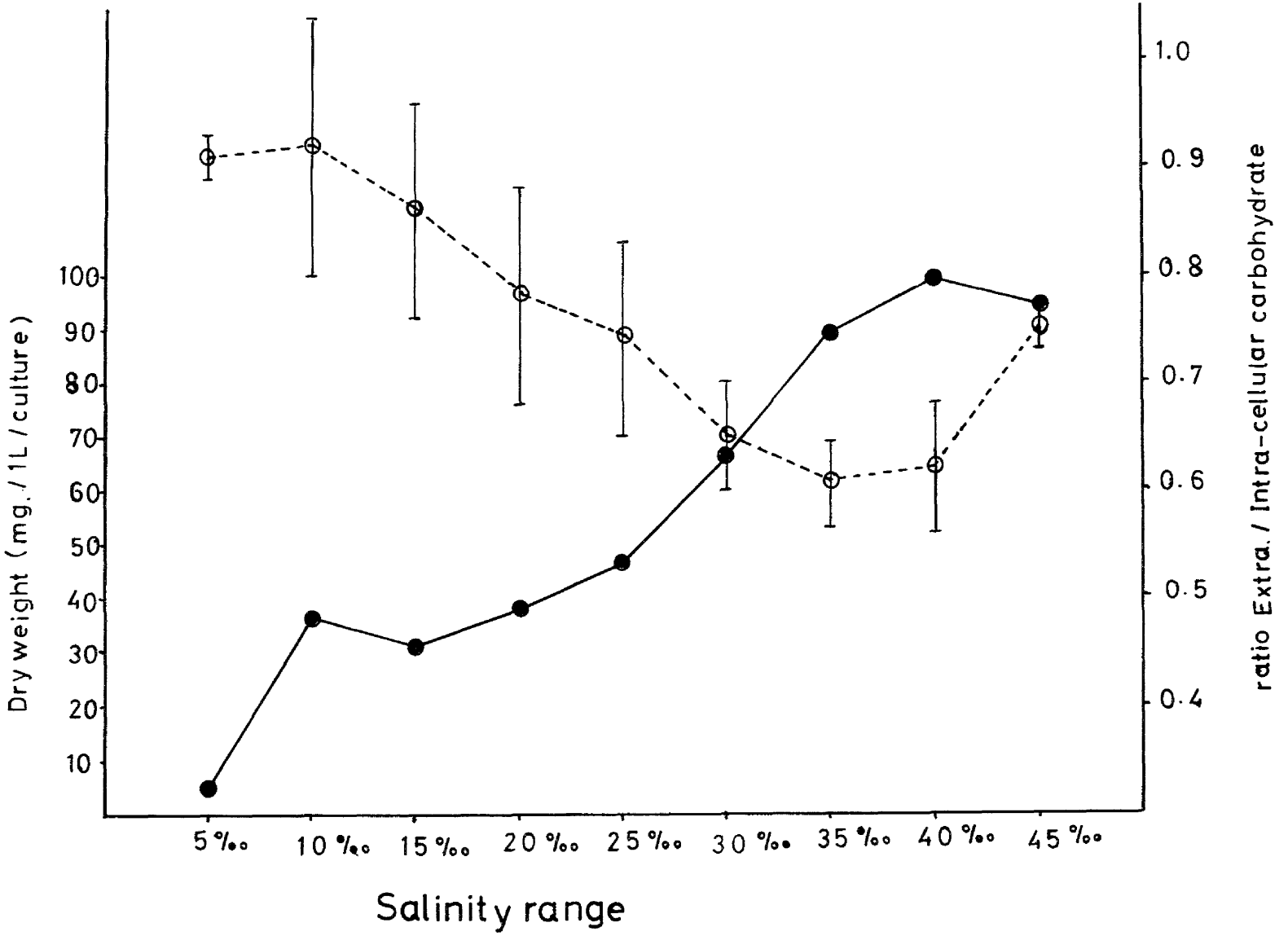
Table 2. Effect of various salinities on the carbohydrate release in P. marinus. Initial inoculum was 0.45 mg dry weight per 100 ml medium (4.5 mg/1L). Each reading was the mean of three replicates.

Salinity range °/oo	µg/ml extracellular carbohydrate	µg/ml culture intracellular carbohydrate	Ratio extra/intracellular carbohydrate	Dry weight mg/1L
.5	1.82	2.0	0.91	5
10	5.75	6.25	0.92	36.5
15	7.5	8.72	0.86	31.0
20	7.8	10.0	0.78	38.0
25	8.55	11.52	0.742	46.5
30	9.42	14.48	0.65	66.5
35	11.6	19.2	0.604	89.5
40	9.92	16.0	0.62	99.0
45	12.24	16.32	0.75	94.0

Figure 8.

Effect of various salinities on the carbohydrate release in P. marinus. ——o——, dry weight as mg/1 litre culture; ---o---, the ratio extracellular to intracellular carbohydrate.

 represents the standard deviation about the mean.


Dry weight (mg./1L/culture)
100
90
80
70
60
50
40
30
20
10
1.0
0.9
0.8
0.7
0.6
0.5
0.4
ratio Extra./Intra-cellular carbohydrate
5‰
10‰
15‰
20‰
25‰
30‰
35‰
40‰
45‰
Salinity range

6. Extracellular carbohydrate release during the course of growth.

The liberation of carbohydrate to the medium during the course of growth of P. marinus was investigated in this experiment. ASP_2 medium was prepared with salt concentrations of 15°/oo, 35°/oo and 45°/oo. Cultures (100 ml each) were inoculated with equal inocula of algae and incubated at 15°C with light intensity of 2160 lux. Over a period of 6 weeks, cultures were harvested and both the intracellular and extracellular carbohydrate quantity determined.

From the results in Table 3 and Figs. 9, 10 and 11), it can be seen that in the three salinities studied the extracellular liberation of carbohydrate was high at the early period of growth; later on, during the mid-growth phase, the release became less compared to the quantity of carbohydrate in the intracellular fractions, and increased again at the late stage of growth. The highest level of extracellular carbohydrate was found in the oldest cultures.

At any particular time there appeared to be greater extracellular production at reduced and increased salinities and this was marked at all times in growth.

Table 3 shows that the ratio Extra/Intracellular carbohydrate after 23 days incubation for the salinities studied was less than the ratio observed in the previous experiment after 3 weeks for the salinities 15°/oo, 35°/oo and 45°/oo, which is summarized in Table 2. This difference in the level of excretion may be mainly due to the difference in the population density and its effect on the excretion (Ignatiades & Fogg, 1973).

Table 3. A comparison of the levels of carbohydrate in P. marinus during the course of growth at various salinities.

Age days	Dry weight mg/L			Extracellular carbohydrate µg/ml			Intracellular carbohydrate µg/ml			Ratio Extra/ Intracellular carbohydrate		
	15°/oo	35°/oo	45°/oo	15°/oo	35°/oo	45°/oo	15°/oo	35°/oo	45°/oo	15°/oo	35°/oo	45°/oo
6	81.5	137	118	9.6	7.8	10.2	21.8	26.8	32	0.45	0.29	0.32
10	136	159.5	173	14	12.2	15.5	34.5	55.3	53.62	0.406	0.22	0.29
18	157	214.5	214.5	15.6	17.9	21.8	35.5	59.92	64.21	0.44	0.30	0.34
23	179	219	215	19.3	18.2	22.4	38.5	56.98	59.03	0.50	0.32	0.38
32	192	253	240	23.3	19.3	22.1	44	53.6 8	51.62	0.53	0.36	0.43
38	220	292	270	26.8	19.1	25.9	45.45	48.9	54.0	0.59	0.39	0.48
42	256	300	290.2	31.1	22.2	29.8	52.8	54.1	58.35	0.59	0.41	0.51

Figure 9.

Changes in the proportion of carbohydrate released in the extracellular form during the course of growth of *P. marinus* (at 35^{o}/oo). ——●——, dry weight measured as mg/1 litre culture; ---o---, the ratio of extra/intracellular carbohydrate.

Ϙ represents the standard deviation about the mean.

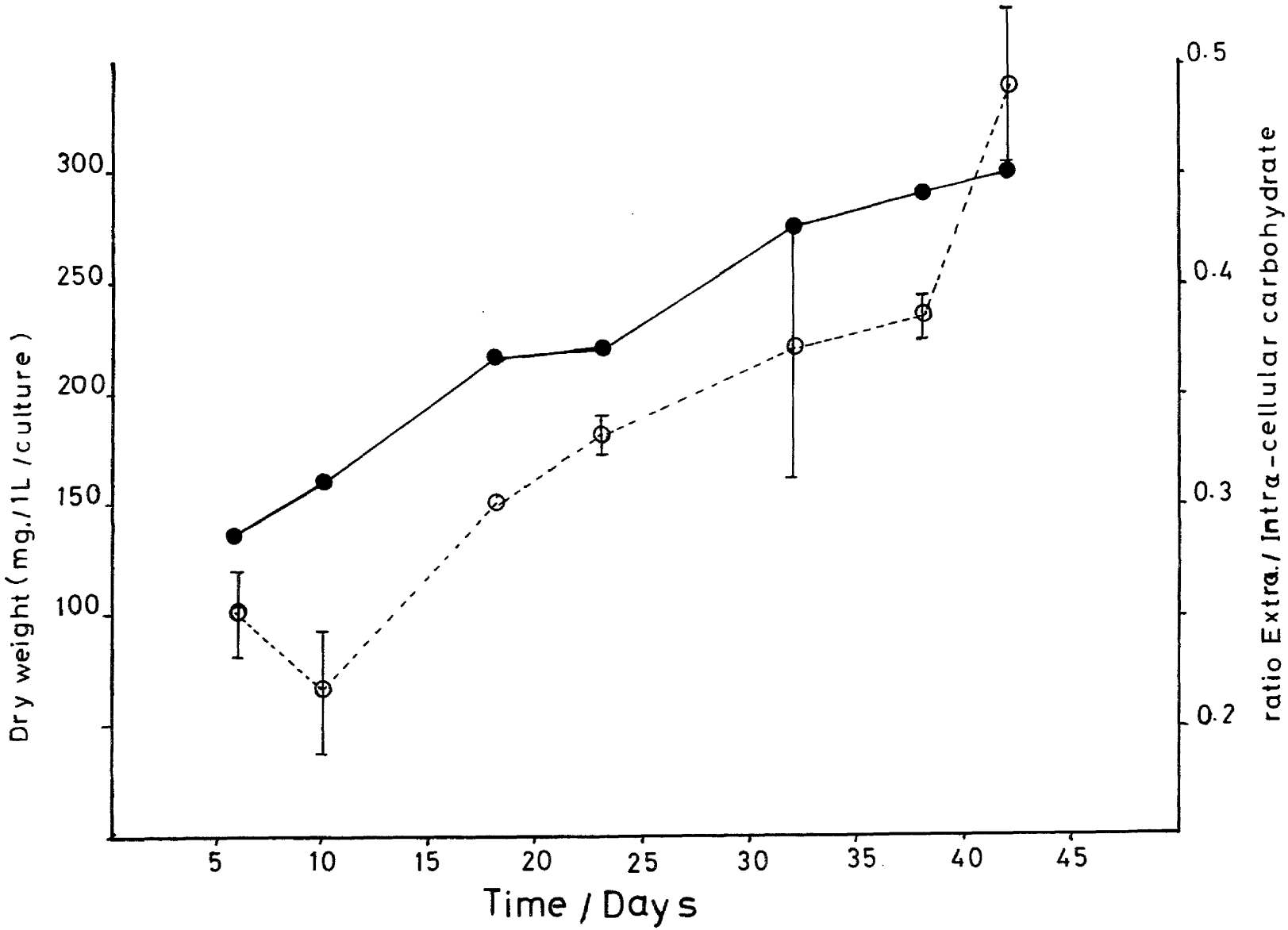

Dry weight (mg./1L /culture)
300
250
200
150
100
5
10
15
20
25
30
35
40
45
Time / Days
0.5
0.4
0.3
0.2
ratio Extra./ Intra-cellular carbohydrate

Figure 10.

Extracellular release of carbohydrate during the course of growth of P. marinus at reduced salinity (15°/oo). —⊙—, the dry weight measured as mg/1 litre culture; —o—, the ratio extra/intracellular carbohydrate. ⊥ represents the standard deviation about the mean.

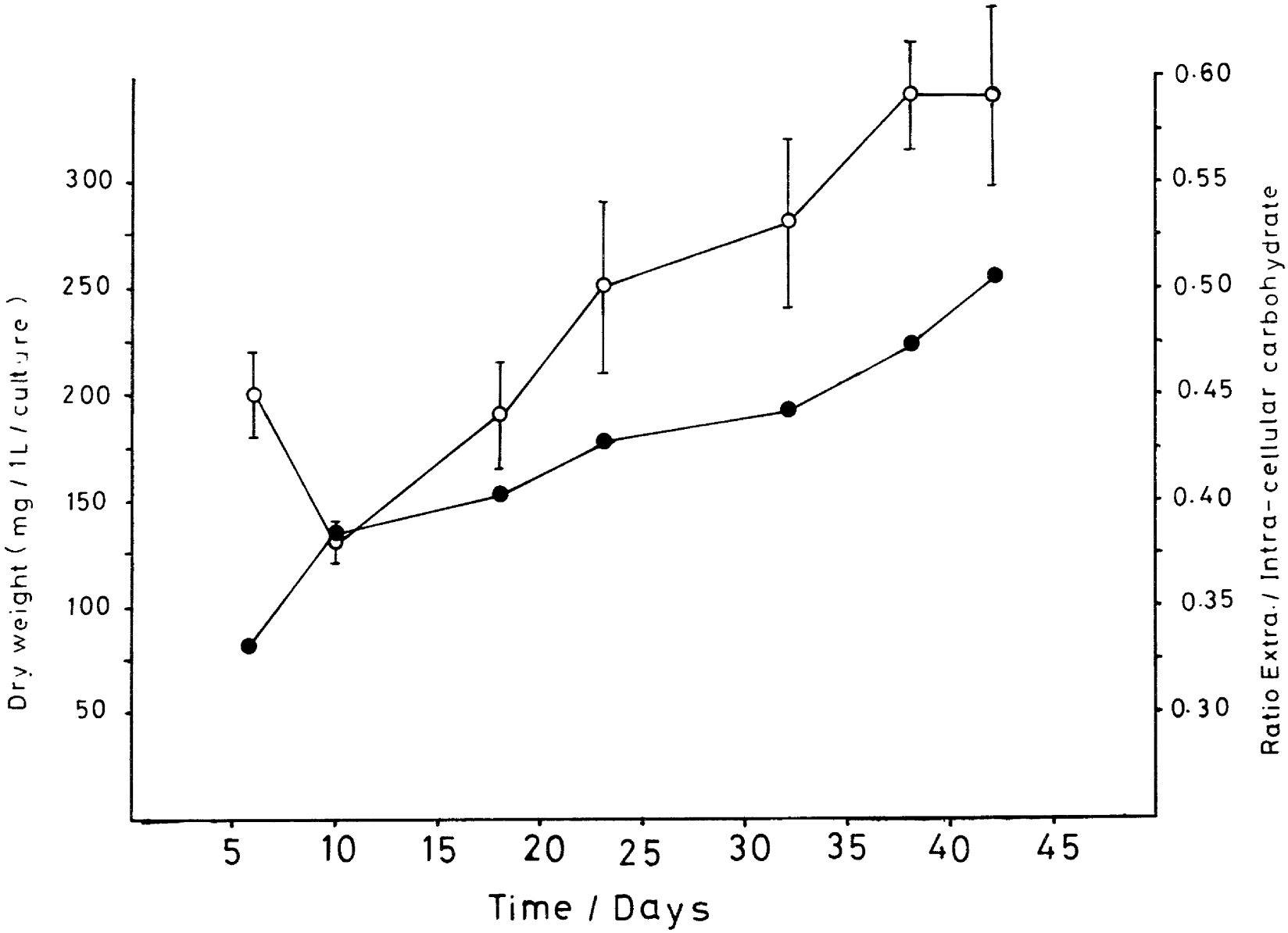
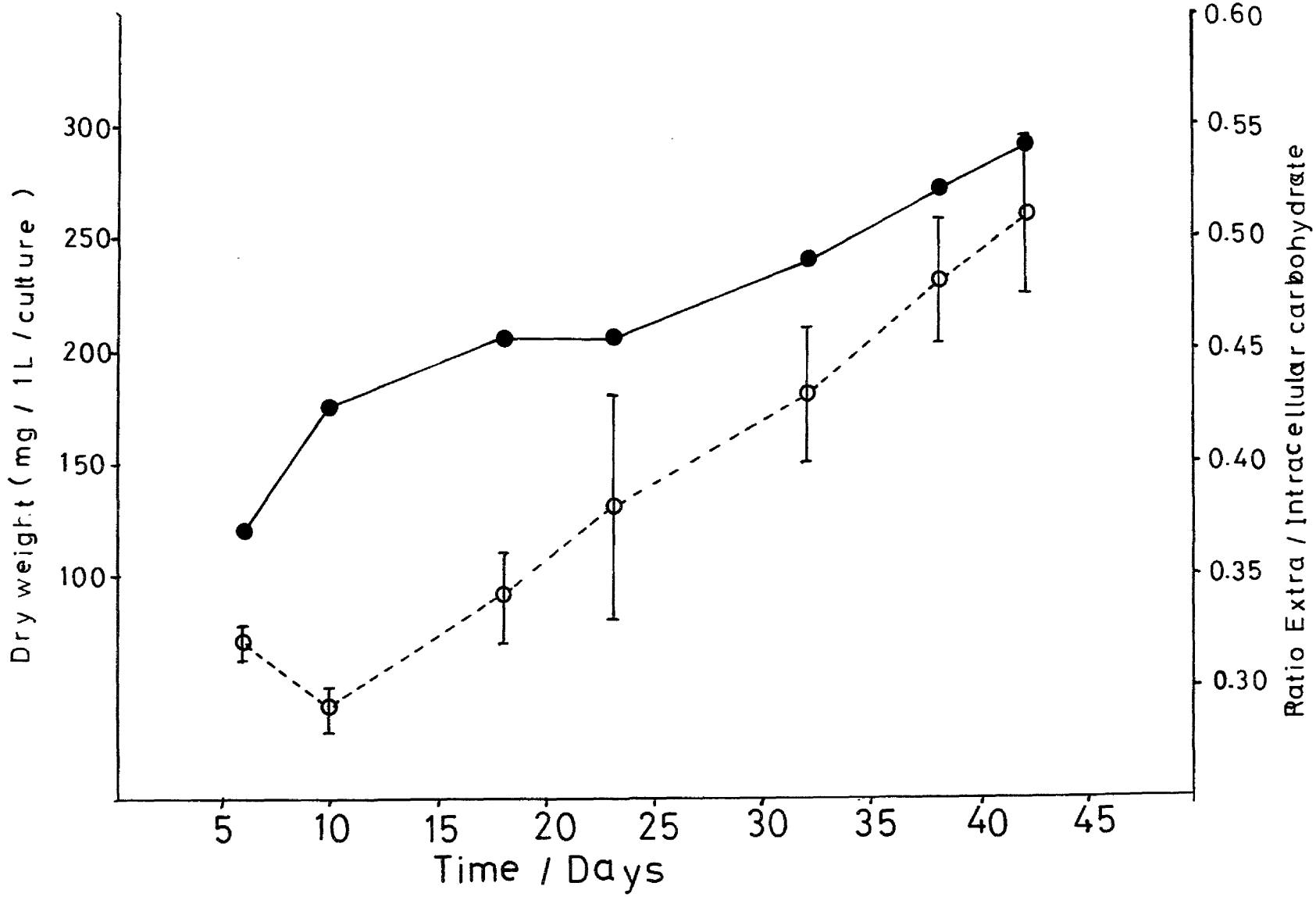

Dry weight (mg / 1L / culture)
300
250
200
150
100
50
5
10
15
20
25
30
35
40
45
Time / Days
0·60
0.55
0·50
0.45
0.40
0.35
0·30
Ratio Extra./ Intra-cellular carbohydrate

Figure 11.

Extracellular liberation of carbohydrate during the course of growth of P. marinus at increased salinity ($45^{o}/oo$). ——o——, dry weight measured as mg/1 litre culture; ---o---, the ratio extra/ intracellular carbohydrate.

$\overline{\underline{\varphi}}$ represents the standard deviation about the mean.


Dry weight (mg / 1L / culture)
300
250
200
150
100
0.60
0.55
0.50
0.45
0.40
0.35
0.30
Ratio Extra / Intracellular carbohydrate
5
10
15
20
25
30
35
40
45
Time / Days

7. Composition of the carbohydrate in P. marinus.

Extensive literature covers the extracellular production of carbohydrates, while only little work has been reported on the chemical nature of the extra-cellular products of algae (Fogg, 1966). Whether the quality of carbohydrate is affected by stress conditions of salinity was investigated in the following experiment. Cultures of P. marinus (2 litres) of salinities 15°/oo, 35°/oo and 45°/oo were prepared under aseptic conditions and incubated at a temperature of 15°C and light intensity of 2160 lux. After 2 weeks the cells were separated by centrifugation. The filtrate thus obtained was passed through a millipore filter (pore size 0.37 μ), reduced in volume and desalted. The carbohydrate in the extracellular and the intracellular fractions was hydrolysed and spot loaded on to 3MM chromatography paper (see method of analysis). The chromatography papers were run in the solvent systems for 48 hours, then air dried and the different spots were stained and identified. The identification was mainly based on the Rg values of the corresponding authentic samples. The sugars separated are listed in Table 4. Two sugars (mannitol and glucose) were detected in the intracellular fractions in the different treatments (15°/oo, 35°/oo and 45°/oo). In the extracellular fractions, on the other hand, only a faint spot was observed, which was identified as glucose.

Table 4. The chemical composition of the carbohydrates in *P. marinus*.

	Solvent		Intracellular		Extracellular
			Soluble	Insoluble	
1)	n-butanol	53	Mannitol Glucose	Mannitol Glucose	Glucose
	Ethanol	32			
	water	16			
2)	Ethylmethyl ketone	9	Mannitol Glucose	Mannitol Glucose	Glucose
	Acetic acid	1			
	water	1			

8. Uptake of minerals (nitrate and phosphate) at various salinities.

High on the list of critical elements are nitrogen and phosphorus. Their importance rests upon their role in metabolism and in nutrition. The source of nitrogen is mostly nitrate and the source of phosphorus is orthophosphate in solution. How the cells behave at various salinities, in relation to their capacity to utilize the available nitrate and phosphate, was therefore studied. By altering the concentration of the major salts in the artificial sea water medium ASP_2 proportionately, various salinities were prepared ($5^{o}/oo$, $15^{o}/oo$, $25^{o}/oo$, $35^{o}/oo$ and $45^{o}/oo$). The pH was adjusted between 7.5 - 7.8; other minerals and trace elements were kept constant. Cultures (200 ml each) were prepared by inoculation with equal inocula of P. marinus under aseptic conditions. After 2 weeks incubation at light intensity of 2160 lux and a temperature of 15°C (with gentle daily agitation) the cells were separated by centrifugation and the medium was further filtered through a millipore filter (pore size 0.37 μ). The unused nitrate-nitrogen and phosphate-phosphorus were estimated colorimetrically (see method of analysis), and then the quantities which were taken up by the cells were evaluated.

The results are summarized in Table 5. It can be seen that in the various salinities studied, the quantities of nitrate-nitrogen and phosphate-phosphorus taken up by the cells were observed to correspond to the increase of cell populations in each range. At the salinities in which a marked growth occurred ($15^{o}/oo$, $25^{o}/oo$,

35°/oo and 45°/oo) the ratio of N to P uptake was about 15 : 1. In the more reduced salinity (5°/oo), on the other hand, at which the growth was much slower, the ratio of N to P uptake was affected and shifted to 10.5 : 1. In fact, this phenomenon is not easy to understand due to the complex role of nitrogen and phosphorus which both enter various metabolic processes. But it would seem to be that some aspects of the cell metabolism were being affected by the very reduced salinity. Hence the ratio of N to P uptake was shifted to 10.5 : 1 from that of 15 : 1 (normal salinity).

Table 5. Uptake of phosphate-phosphorus and nitrate-nitrogen at various salinities. Initial inoculum equal to 5.1 mg dry weight. Each reading mean of three replicates.

Salinity range	mg dry weight	µg at -P/L uptake	µg at -N/L uptake	ratio N : P
5°/oo	5.82	3.13	33.01	10.5 : 1
15°/oo	10.87	15.95	226.95	14.2 : 1
25°/oo	12.45	18.67	259.8	13.9 : 1
35°/oo	14.49	19.98	302.13	15.12: 1
45°/oo	13.33	18.38	277.96	15.6 : 1

9. Pigments.

The effect of salinity variation on the pigment composition of P. marinus was included in the present study. Both qualitative and quantitative examination was carried out using thin layer chromatography and spectrophotometry.

Absorption spectra of the total extracts from cultures grown at salinities of 15^{o}/oo, 35^{o}/oo and 45^{o}/oo when studied using a Unicam S.P.800, were seen to exhibit no differences in the nature of the peaks (Fig. 12). The three extracts were noted to have absorption maxima of 665 nm in the red region and 452, 430 nm in the blue region.

The chromatograms loaded with pigments extracted from cultures of various salinities, when developed in a mixture of petroleum spirit, ethyl acetate and diethyl amine (Riley and Wilson, 1965), showed similar patterns of separation. The characteristics and constituents of some members of the Prasinophyceae have been reported (Jeffrey, 1961; Riley and Wilson, 1967; Hussain, 1969; Ricketts, 1966, 1967*, 1967** and 1970).

The results are summarized in Table 6 and illustrated in Figs. 13, 14 and 15. A bluish-green spot appeared at an Rf of 0.76 on the developed thin layer of silica gel. Its spectrum analysis presented the characteristics of chlorophyll a in spite of its Rf being relatively lower than the published data. The other pigments (carotenes, chlorophyll b, lutein, vialoxanthin and neoxanthin) showed a reasonable agreement with the previous published Rfs and absorption maxima.

Author: HADI, Rafia A M

Title: Studies on salinity tolerance, growt and extracellular carbohydrate yield in Prasinocladus marinus (Cienki) Waern.

Date of deposit: 3.12.74 M.Sc.

Figure 12.

Absorption maxima of the total pigments extract from *P. marinus* growing at various salinities.

a) 15°/oo

b) 35°/oo

c) 45°/oo

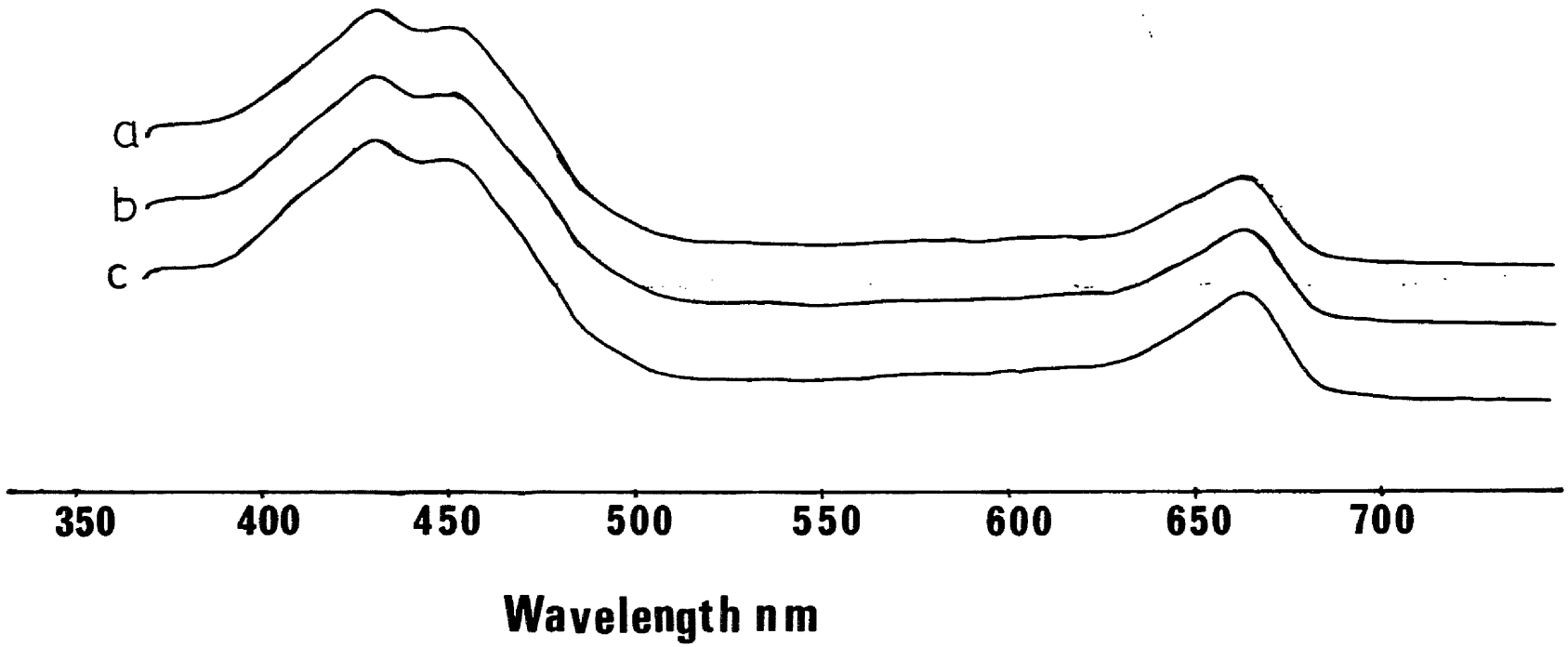

a
b
c
350
400
450
500
550
600
650
700
Wavelength nm

Table 6. Pigments of *Prasinocladus marinus*.

No.	Colour	Rf			Published absorption maxima			Elutant	Pigment
		A	B	C	Jeffrey (1961)	Hussain (1969)	Present work		
1	Orange	0.87	0.88	0.88	429, 450, 478	420, 450, 476	420, 450, 475	Hexane	Carotenes
2	Bluish-green	0.82	0.81	0.76	580, 618, 665	408, 428, 570 618, 665	408, 426, 530, 576, 618, 663	Ether	Chlorophyll *a*
3	Yellowish-green	0.60	0.62	0.65	455, 598, 645	426, 448, 596, 646	428, 452, 594, 644	Ether	Chlorophyll *b*
4	Yellowish-orange	0.42	0.41	0.41	422, 444, 476	424, 446, 473	442, 444, 472	Ether	Lutein
5	Yellow	0.37	0.30	0.31	421, 441, 471	421, 444, 472	418, 440, 468	Ether	Vialoxanthin
6	Yellowish-orange	0.23	0.18	0.16	414, 437, 466	426, 448, 465	410, 434, 463	Ether	Neoxanthin

A = Rf values on silica gel (Riley and Wilson, 1965)

B = Rf values on silica gel (Hussain, 1969)

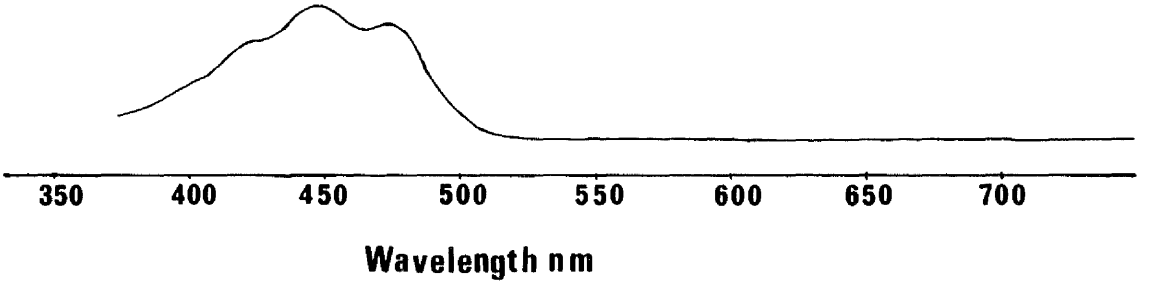
C = Rf values on silica gel (Present work)

Figure 13.

A - Absorption maxima of the carotenes from the extract of P. marinus.

B - Absorption maxima of the chlorophyll a from the extract of P. marinus.

A


350
400
450
500
550
600
650
700
Wavelength nm

B

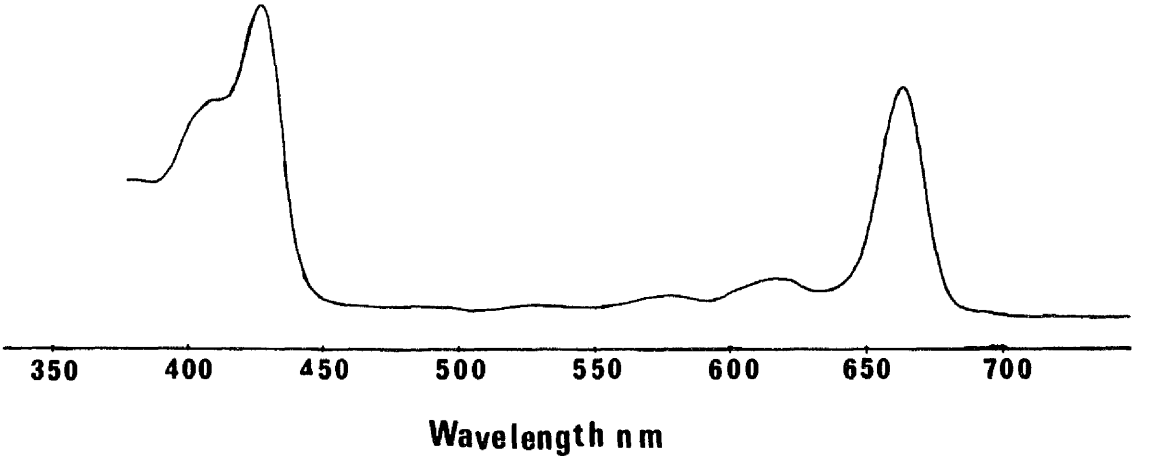
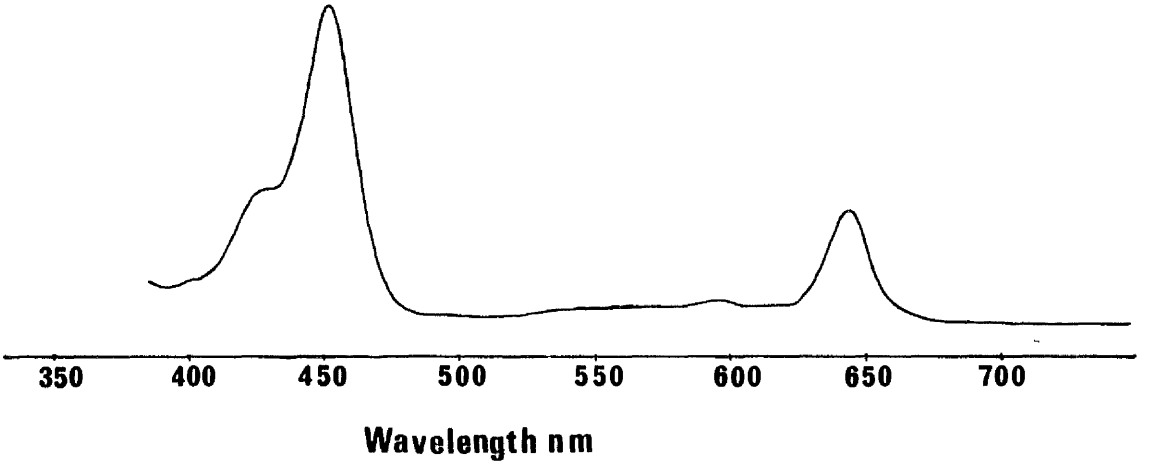

350
400
450
500
550
600
650
700
Wavelength nm

Figure 14.

A- Absorption maxima of the chlorophyll b from the extract of P. marinus.

B- Absorption maxima of the neoxanthin from the extract of P. marinus.

A


350
400
450
500
550
600
650
700
Wavelength nm

B

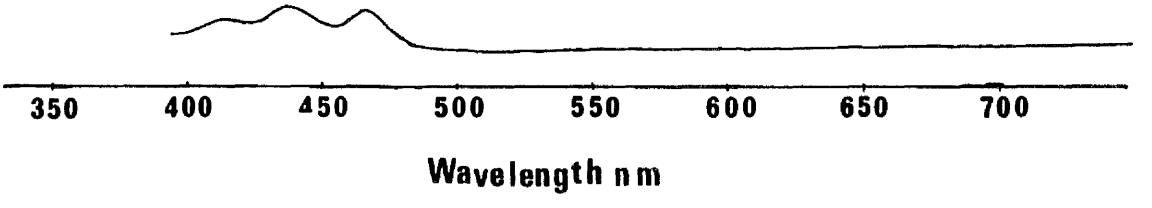
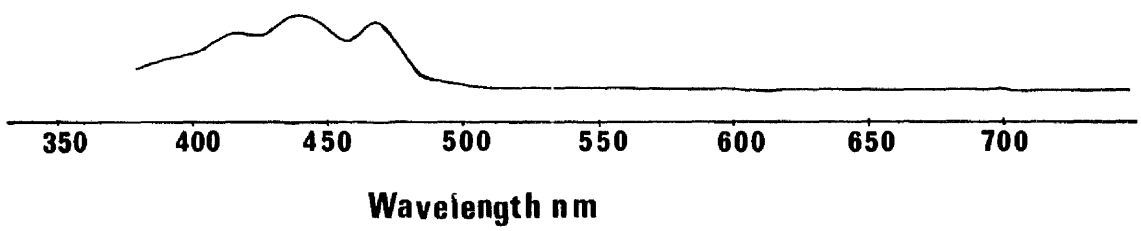

350
400
450
500
550
600
650
700
Wavelength nm

Figure 15.

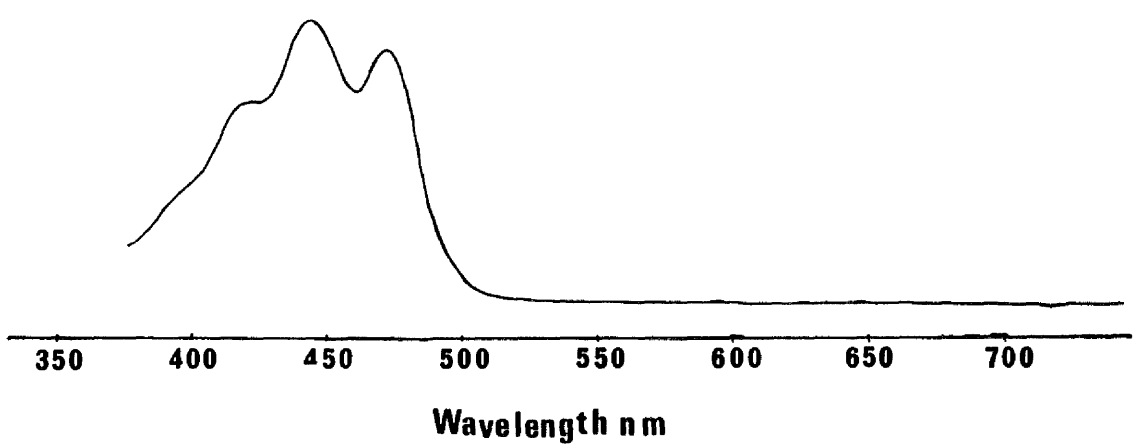
A- Absorption spectrum of the vialoxanthin from the extract of P. marinus.

B- Absorption spectrum of the leutin from the extract of P. marinus.

A


350
400
450
500
550
600
650
700
Wavelength nm

B


350
400
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700
Wavelength nm

The quantities of chlorophyll a and total carotenoids that formed in P. marinus when growing at various salinities, were further investigated by taking equal aliquots of wet packed cells (0.05 ml) from cultures aged 3 weeks, growing at 15°C and 2160 lux with salinities of 15°/oo, 35°/oo and 45°/oo. An extraction was made quantitatively by ultrasonic treatment (see method of analysis). The optical densities were determined by using a Unicam S.P.600, and the quantities of chlorophyll a and total carotenoids calculated by the equations of Strickland and Parsons (1968). The results of the different treatments are shown in Table 7, which indicates that for the known volume of packed cells, the variation in the salinity of the culture medium (within ranges at which a considerable growth takes place) has only a little effect on the quantities of the chlorophyll a and total carotenoids that are synthesized by P. marinus. The ratio chlorophyll a / total carotenoids was approximately the same in the three treatments.

Table 7. Relative quantities of chlorophyll a and total carotenoids (per 0.05 ml wet packed volume) at various salinities.

Salinity range	Chlorophyll a µg	total carotenoids µg	Ratio carotenoids/ chlorophyll a
15°/oo	4.77	4.01	0.84
35°/oo	5.357	4.221	0.79
45°/oo	5.281	4.218	0.80

Chapter 4

DISCUSSION

Prasinocladus marinus is a typical member of the class Prasinophyceae, a class which includes flagellates distinguished from the Chlorophycean monads in many characters, notably the scale-bearing flagella which arise from an apical depression, and the absence of the cell wall which is replaced by scales or a special type of theca. The multicellular (thalloid) formswhich are seen in the Chlorophyceae are not known in the Prasinophyceae. In addition to these main characters, P. marinus is distinguished by its dendroid habit, with cells occurring on a stalk-like structure of empty thecal material. The growth (which may be defined as the increase in total cellular material) takes place only in the non-motile phase of the organism and in a very unusual manner. Any cell is capable of producing a new theca within the old one at frequent intervals and with or without an intervening cell division. No previous reports are available regarding the measurement of the growth of this organism. The growth estimation technique which has been used in this study provides a direct measure of the whole increase of organic matter which is produced by the plant (increase in cells and the thecael material). Measurement of packed cell volume is a relatively straightforward operation, and measurements can be made in a short interval of time.

Prasinocladus is found at high water mark on rocky shores and in estuaries (Fritsch, 1949; Proskauer, 1950; Parke and Manton, 1965), all regions of salinity variation, and it would be expected therefore to contend with lowered and increased salinities. The external

morphology of the plant, when examined under the microscope, was not similar in cultures of various salinities. The empty thecae were usually filamentous at normal salinity (Fig. 1a), while mainly compact and irregular at the reduced salinities (Fig. 1b). An interesting finding was that the production of theca l material was closely similar in plants growing at various salinities, irrespective of the form taken by the thecae. Hence the differences due to the salinity seem to be differences in cell form but not in quantity of cells/thecael material.

The pattern of growth within the range of salinities which is expected in the natural habitat of P. marinus (5^{o}/oo - 45^{o}/oo) was studied. The optimum salinity for the growth was observed within the range 35^{o}/oo - 40^{o}/oo, which is the full strength of sea water or slightly higher. Over or below this range reduction in growth occurred. At the very reduced salinity (5^{o}/oo, Table 2) the growth ceased or was slow but the cells were still green in colour, and they were still able to produce the zooids (Boney, 1970). Hence P. marinus appears to be a marine organism but is able to adapt effectively to the osmotic stress due to the decrease of the salinity, as in estuarine situations.

Experiments have confirmed that under a wide range of salinity a considerable proportion of the carbohydrate was in the extracellular form. At both increased and reduced salinity the level of the extracellular carbohydrate rose. Boney (1970) found that P. marinus, when growing at reduced salinity, became more sensitive to the toxic substance (oil-spill emulsifier) than the normal salinity. It is probable that the less favourable conditions for growth are reflected in the cell metabolism, so causing a relatively higher release of carbohydrate from the cells; or the salinity of the medium might affect the relative importance of the metabolic pathway in the system, leading to less carbohydrate in the more brackish environments (Marker, 1965). At the

very reduced salinity (5°/oo), however, the higher level of release may be partially due to the damage of the cells.

During the course of growth, the level of the extracellular carbohydrate liberation was relatively high at the early period of growth in all salinities studied (15°/oo, 35°/oo and 45°/oo); later on, during the active phase of growth, the release became relatively less and then increased again at the late stage of growth. The higher level of release at the early stage of growth may have resulted from the low initial population density and the shock occurring on inoculation (Huntsman, 1972), or may be partially due to the break up of some cells of the inoculum (Marker, 1965). The senescence of the cells at the late stage, on the other hand, may have led to a higher level of release (Ignatiades and Fogg, 1973).

Analysis of the carbohydrate component showed that mannitol and glucose were found in the intracellular fraction; these observations are in agreement with those reported by Craigie et al. (1967). In the extracellular fraction only glucose was found. In fact, it is hard to say that mannitol is not present in the filtrate, but it could be explained that the quantity of mannitol in the medium was little and undetectable.

Absorption spectra of the total pigment extracts from cultures growing at different salinities (15°/oo, 35°/oo and 45°/oo) exhibited no differences in the nature of the peaks, and similar bands were separated on the thin layer of silica gel from these pigment extracts.

Previous work regarding the effect of salinity fluctuation on pigment synthesis is very little. McLachlan (1961) found that in some unicellular marine algae which grew well in a wide range of

salinities, there is no distinct relationship between the relative chlorophyll content per cell and growth. In the present study, the quantities of chlorophyll a and total carotenoids per equal amount of wet packed volume at various salinities (15^{o}/oo, 35^{o}/oo and 45^{o}/oo) were approximately the same (see Table 7). The higher quantities were observed at 35^{o}/oo, which is the optimum salinity for growth. But it is difficult to say that these little differences were definitely due to the salinity variation, bearing in mind that it is not possible to extract the pigments completely from the plant material. It would seem that stress salinities restrict the growth of P. marinus whereas the syntheses of chloroplast pigments are not affected to the same degree.

This work gives some insight into the growth and metabolism of this unique green alga. Further work is necessary on other features not covered because of the stress factors likely in its habitat, since the metabolic pathways in members of the class Prasinophyceae are not necessarily the same as in green algae of the Chlorophyceae.

References

BENDSCHNEIDER, K. and ROBINSON, R.J. (1952).

A new spectrophotometric determination of nitrite in sea water.

Journal of Marine Research, vol. 11, pp. 87-96.

BONEY, A.D. (1966).

A Biology of Marine Algae.

Hutchenson Educational ed. (1966).

BONEY, A.D. (1970).

Toxicity studies with an oil-spill emulsifier and the green alga Prasinocladus marinus.

Journal of the Marine Biological Association of the United Kingdom, vol. 50, pp. 461-473.

CHRISTENSEN, T. (1962).

Alger. In, Botanik Bd. 2 (Systematisk Botanik), edited by T.W. Böcher, M. Lange and T. Sørensen, Munksgaard, Copenhagen, Nr. 2, Udg. 2, 178 pp.

CHIHARA, M. (1963).

The life history of Prasinocladus ascus as found in Japan, with special reference to the systematic position of the genus.

Phycologia, vol. 3, pp. 19-28.

CRAIGIE, J.S., McLACHLAN, J., ACKMAN, R.G. and TOCHER, C.S. (1967).

Photosynthesis in Algae.

III. Distribution of soluble carbohydrates and Dimethyl-β-Proplothetin in marine unicellular chlorophyceae and Prasinophyceae.

Canadian Journal of Botany, vol. 45, pp. 1327-1334.

DRING, M.J. (1967).

Effects of daylength on growth and reproduction of the conchocelis-phase of Porphyra tenera.

Journal of the Marine Biological Association of the United Kingdom, vol. 47, pp. 501-510.

DROOP, M.R. (1967).

A procedure for routine purification of algal cultures with antibiotics.

British Phycological Bulletin, vol. 3, pp. 295-297.

FOGG, G.E. (1962).

'Extracellular products' in Physiology and Biochemistry of Algae, ed. by R.A. Lewin, Academic press. New York and London, pp. 475-489.

FOGG, G.E. (1966).

The extracellular products of algae.

Oceanography and Marine Biology Annual Review, vol. 4, pp. 195-212.

FRITSCH, F.E. (1949).

Contribution to our knowledge of British algae.

Hydrobiologia, vol. 1, pp. 115-125.

GUILLARD, R.R.L. and WANGERSKY, P.J. (1958).

The production of extracellular carbohydrates by some marine flagellates.

Limnology and Oceanography, vol. 3, pp. 449-454.

HELLEBUST, J.A. (1965).

Excretion of organic compounds by marine phytoplankton.

Limnology and Oceanography, vol. 10, pp. 192-206.

HUNTSMAN, S.A. (1972).

Organic excretion by Dunaliella tertiolecta.

Journal of Phycology, vol. 8, pp. 59-63.

HUSSAIN, A. (1969).

Ph.D. Thesis, University of Wales.

IGNATIADES, L. (1973).

Studies on the factors affecting the release of organic matter by Skeletonema costatum (Grev.) Cleve in field conditions.

Journal of the Marine Biological Association of the United Kingdom, vol. 53, pp. 929-935.

IGNATIADES, L. and FOGG, G.E. (1973).

Studies on the factors affecting the release of organic matter by Skeletonema costatum (Grev.) Cleve in culture.

Journal of the Marine Biological Association of the United Kingdom, vol. 53, pp. 937-956.

JEFFREY, S.W. (1961).

Paper chromatographic separation of chlorophylls and carotenoids from marine algae.

Biochemical Journal, vol. 80, pp. 336-342.

LAMBERT, F.D. (1930).

On the structure and development of Prasinocladus.

Zeitschrift für Botanik, vol. 23, pp. 227-244.

MARKER, A.F.H. (1965).

Extracellular carbohydrate liberation in the flagellates Isochrysis galbana and Prymnesium parvum.

Journal of Marine Biological Association of the United Kingdom, vol. 45, pp. 755-772.

McLACHLAN, J. (1961).

The effect of salinity on growth and chlorophyll content in representative classes of unicellular marine algae.

Canadian Journal of Microbiology, vol. 7, pp. 399-406.

MURPHY, J. and RILEY, J.P. (1962).

A modified single solution method for the determination of soluble phosphate in natural waters.

Analytica Chimica Acta, vol. 27, pp. 31-36.

MYKLESTAD, S., HAUG, A. and LARSEN, B. (1972).

Production of carbohydrates by the marine diatom Chaetoceros affinis var. Willei (Gran) Hustedt.

II. Preliminary investigation of extracellular polysaccharide.

Journal of Experimental Marine Biology and Ecology, vol. 9, pp. 137-144.

NELSON, D.J. (1960).

Improved chlorophyll extraction method.

Science, vol. 132, p. 351.

PARKE, M. and DIXON, P.S. (1964).

A revised check-list of British marine algae.

Journal of the Marine Biological Association of the United Kingdom, vol. 44, pp. 499-542.

PARKE, M. and MANTON, I. (1965).

Preliminary observations on the fine structure of Prasinocladus marinus.

Journal of the Marine Biological Association of the United Kingdom, vol. 45, pp. 525-536.

PROSKAUER, J. (1950).

On Prasinocladus.

American Journal of Botany, vol. 37, pp. 59-66.

PROVASOLI, L. (1958).

Nutrition and ecology of protozoa and algae.

Annual Review of Microbiology, vol. 12, pp. 279-308.

PROVASOLI, L., McLAUGHLIN, J.J.A. and DROOP, M.R. (1957).
The development of artificial media for marine algae.
Archiv für Mikrobiologie, Bd. 25, pp. 392-428.

RICKETTS, T.R. (1966).
On the chemical composition of some unicellular algae.
Phytochemistry, vol. 5, pp. 67-76.

RICKETTS, T.R. (1966).
The carotenoids of the phytoflagellate, Micromonas pusilla.
Phytochemistry, vol. 5, pp. 571-580.

RICKETTS, T.R. (1967)*
The pigment composition of some flagellates possessing scaly flagella.
Phytochemistry, vol. 6, pp. 669-676.

RICKETTS, T.R. (1967)**
Further investigations into the pigment composition of green flagellates possessing scaly flagella.
Phytochemistry, vol. 6, pp. 1375-1386.

RICKETTS, T.R. (1970).
The pigments of Prasinophyceae and related organisms.
Phytochemistry, vol. 9, pp. 1835-1842.

RILEY, J.P. and WILSON, T.R.S. (1965).
Use of thin layer chromatography for the separation and identification of plankton pigments.
Journal of the Marine Biological Association of the United Kingdom, vol. 45, pp. 583-591.

RILEY, J.P. and WILSON, T.R.S. (1967).
The pigments of some marine phytoplankton species.
Journal of the Marine Biological Association of the United Kingdom, vol. 47, pp. 351-362.

SPENCER, C.P. (1952).

On the use of antibiotics for isolating bacteria-free cultures of marine phytoplankton organisms. Journal of the Marine Biological Association of the United Kingdom, vol. 31, pp. 97-106.

STRICKLAND, J.D.H. and PARSONS, T.R. (1968).

A practical handbook of sea water analysis. Bulletin Fisheries Research Board of Canada, no. 167, 311 pp.

WAERN, M. (1952).

Rocky-shore algae in the Öregrund Archipelago. Acta phytogeographica suecica, vol. 30, 298 pp.

WOOD, E.D., ARMSTRONG, F.A.J. and RICHARD, F.A. (1967).

Determination of nitrate in sea water by cadmium-copper reduction to nitrite. Journal of the Marine Biological Association of the United Kingdom, vol. 47, pp. 23-31.

YEMM, E.W. and WILLIS, A.J. (1954).

The estimation of carbohydrates in plant extracts by anthrone. Biochemical Journal, vol. 57, pp. 508-514.