EXPERIMENTAL STUDIES ON THE GENUS CHRYSOTILA

(ORDER ISOCHRYSIDALES, CLASS HAPTOPHYCEAE)

Thesis presented by

JOHN R. STANIER, B.Sc.

for the degree of

Master of Science in the Faculty of Science

in the

UNIVERSITY of GLASGOW

DECEMBER 1979

CONTENTS

ACKNOWLEDGEMENTS

I am indebted to Professor A. D. Boney for his advice, help, guidance and encouragement throughout this work.

I wish to thank Professor M. B. Wilkins for providing the facilities necessary for carrying out this work in the Department of Botany.

I am grateful to my mother Mrs E. Stanier for the time and effort which she spent in the typing of this thesis.

The work in this thesis was partly financed by a grant from Lanarkshire Division Education Authority, and I am grateful for this support, and for the support from Calderhead High School during the final year of study.

I wish, finally, to express my thanks to my wife Marjory, and to both our families for their constant help, encouragement, and considerable patience during this work.

SUMMARY

Chrysotila lamellosa Anand. has been isolated from both fresh and marine habitats, and shows the ability to grow in salinities from 2°/oo to 32°/oo. The growth of the alga was measured within this range of salinities, and it was found that the growth rate at low salinities was greater than at high salinities. This was related to the production of zoospores in the cultures. The content of chlorophylls and carotenoids was also measured within this range of salinities and it was shown that the pigment concentration tended to rise with increasing salinity. The release of zoospores was also investigated and was shown to be moderated by salinity, temperature and aeration. These results were compared and indicate that the alga is under environmental stress at low salinities, and better adapted to growth at salinities above 16°/oo.

FIGURES

* indicates facing page

TABLES

* indicates facing page

CHAPTER ONE

INTRODUCTION

1.1 The Taxonomy of Chrysotila lamellosa Anand

Chrysotila lamellosa Anand was discovered during a study of the algal flora of British chalk cliffs (Anand, 1937). It was placed in the class Chrysophyceae family Chrysocapsaceae. The organism was subsequently placed in the Haptophyceae by Parke and Dixon (1964) and was first placed in the order Prymnesiales (class Chrysotilaceae).

The same organism was also discovered by Geitler, but from a terrestrial habitat in 1942 (Geitler, 1942). This form, which was discovered as golden yellow mucilage covered masses of cells growing on damp rocks and paths in Vienna, was named Ruttnera spectabilis, and it is under this name that most of the taxonomic studies have been made.

Geitler noted that the vegetative phase of the organism was morphologically similar to that of members of the genus Chrysocapsa and placed R. spectabilis in the family Chrysocapsaceae, class Chrysophyceae. Later, a new family, Ruttneraceae, was erected for the genus (Geitler 1943). This was based on the observation that the zoids possess two equal to sub-equal flagella, in contrast to the uniflagellate structure of the swarmers in Chrysocapsa. However Geitler was unable to state with certainty whether the

flagella of the zoids were heterokont or isokont, and this left some doubt as to the systematic position of the species. In fact having observed them to be heterodynamic, he believed them to be heterokont.

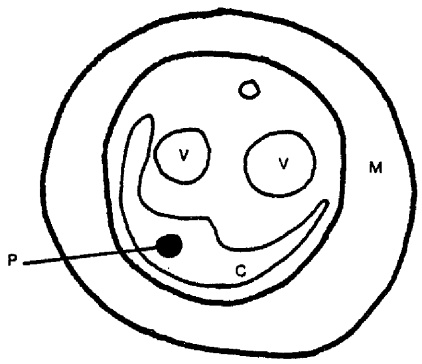
Bourelly (1968) included Ruttnera in the Ochromonadales (class Chrysophyceae) on the basis of Geitler's belief that the flagella of the zoids are truly heterokont.

The type material of Ruttnera was thus isolated from a freshwater habitat, whereas that of Chrysotila came from a marine habitat, and there is little doubt that this fact has been responsible for keeping the information on these two taxa separated. More recently, however, isolates of Ruttnera have been grown in seawater, and although the type species has since been lost (Billard and Gayral, 1972) the genus has been described from marine habitats.

Green and Parke (1974) investigated the fine structure of a strain of R. spectabilis obtained from the locus classicus. This work has shown that the flagella of the zoids are equal to sub-equal, and that there is a much reduced haptonema of a very unusual type. This haptonema is so small that it is undetectable by light microscopy, and is only visible under the electron microscope when the cell is sectioned in certain ways. Local accumulations of material within the endoplasmic reticulum cisternae were also discovered in the cells. These appeared as conspicuous spherical vesicles, usually more numerous in the region immediately external to the Golgi apparatus. Green and Parke have therefore placed Ruttnera in the Haptophyceae on the basis of these features. Such

FIGURE 1.1

Chrysotila lamellosa



Key

C - Chloroplast

M - Mucilage

P - Pyrenoid

V - Vesicle

vesicles have been found in other members of the Haptophyceae since their first description by Manton (1966, 1967).

Green and Parke also noted the similarity between C. lamellosa and R. spectabilis and have subsequently made more detailed comparisons between these two species and C. stipitata (Green and Parke 1975).

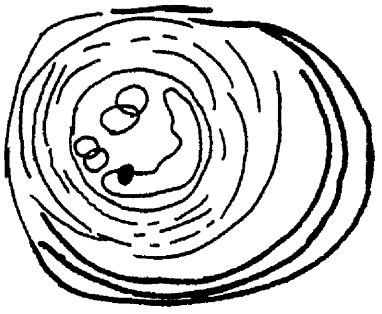
C. stipitata, like C. lamellosa, was isolated from a marine habitat. The zoids of C. stipitata have smaller scales than those of C. lamellosa. In the vegetative stage C. stipitata has a long mucilage stalk containing a variable number of ovate to elongate cells, and no obvious lamellations. C. lamellosa has a short mucilage stalk with obvious lamellations, and only one or two spherical cells situated distally. These differences confirm that C. lamellosa and C. stipitata are separate species. This is not so, however, with C. lamellosa and R. spectabilis. Their studies have shown that although there is some variation in the numbers of microtubules in the haptonema of the two forms, C. lamellosa and R. spectabilis are the same species. Such variation in the numbers of microtubules has been found within other species (Manton and Leedale, 1961, 1963) and is therefore not sufficient to keep the two forms as separate species. No conclusion was reached as to which name should have priority, although it has been suggested that Chrysotila be retained for marine isolates, and Ruttnera for terrestrial isolates but from the sources quoted Chrysotila lamellosa should have priority.

The systematics of the Haptophyceae are still in a state of flux,

FIGURE 1.2

Chrysotila lamellosa

Older Cell



and at present two orders are recognised; the Isochrysidales and Prymnesiales. The Prymnesiales are characterised by the possession of an obvious haptonema in the motile phase, whereas in the Isochrysidales this haptonema is not obvious. Green and Parke (1975) have suggested that these orders may need revision and have put forward the following characteristics for the Isochrysidales: organisms in which the rudimentary haptonema may or not be present, but if present, then not detectable with the optical microscope. Chrysotila lamellosa would therefore be placed within this order.

More recently Hibberd (1976) has proposed the class Prymnesiophyceae to replace the name Haptophyceae, with some changes in the classification of certain species. At present, however Chrysotila lamellosa is placed in the class Haptophyceae, order Isochrysidales family Chrysotilaceae in the most recent check list of the British marine algae (Park and Dixon, 1976).

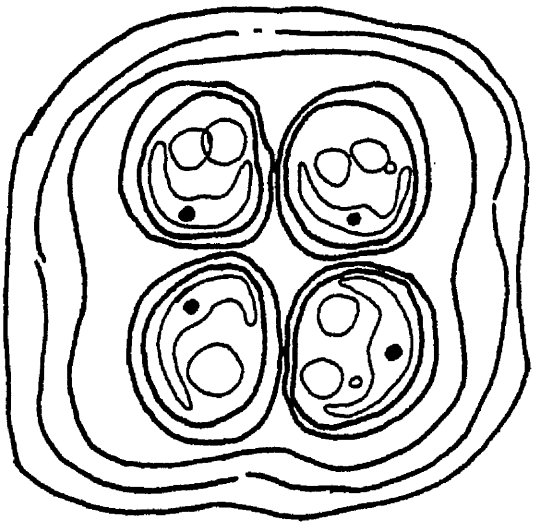
1.2 The Structure of Ruttnera phase of Chrysotila lamellosa

The cells of the vegetative phase are approximately 4.5 μm in diameter, surrounded by a layer of mucilage, of variable size (Fig 1.1). The mucilage layer stains red-purple with toluidine blue, and in young cultures is comparatively thin. With increasing age the mucilage becomes thicker and can be seen to be composed of several layers (Fig. 1.2). As ageing proceeds the cells appear to grow on a "stalk" of mucilage, assuming the Chrysotila - like form. Green and Parke (1974) have shown that in this stage, mineralized elements are present in the mucilage.

FIGURE 1.3

Chrysotila lamellosa

Ruttnera spectabilis phase



Within the cell there is a single yellow-green chloroplast, a large chrysolaminarin vesicle, and a small orange-red carotenoid body.

The mucilage sheath is absent in the motile phase. The cells, pyriform in outline and flattened dorso-ventrally, are 6-8 μm long by 3-4 μm broad. As in the non-motile phase, there is a single, parietal, golden-yellow chloroplast, a large basal chrysolaminarin vesicle, several lipid droplets, and a small orange red carotenoid body.

Two equal, or sub-equal flagella are inserted sub apically at the anterior end of the cell. The flagella are heterodynamic, the anteriorly directed one beating with an undulating motion, the posteriorly directed one having a stiff vibratory action. Green and Parke (1974) first described the reduced haptonema located between the two flagella. As previously mentioned (Chapter 1.1) this haptonema is undetectable by light microscopy.

The biology of this species poses a number of questions as a result of its two separate descriptions from widely differing habitats. Most obvious is the apparent ability to survive and grow in both marine and freshwater habitats, and it is the answers to these questions which are the main objects of the present study.

Table 2.1

BONEY'S GROWTH MEDIUM

Solution A	I	50ml 0.4% $Na_2 NO_3$	
	II	2.0ml of each of	
		$Mn SO_4$	1.47g/l
		$Cu SO_4$	0.023g/l
		$Co Cl_2$	0.064g/l
		$Li Cl_2$	0.005g/l
		$Zn SO_4$	4.98g/l
	$Na_2 MoO_4$		0.23g/l
Total 62ml.	Solutions added and autoclaved to make Solution A		
Solution B	0.012g $Fe SO_4$ dissolved in 100ml of 0.26% EDTA solution. Autoclaved		
Solution C	0.15% solution of $Na_2 HPO_4$ Autoclaved		
Final mixture	1 L aged filtered seawater + Solution A, + 15ml solution B, + 15ml Solution C, and the solution pasteurised.		

CHAPTER TWO

MATERIALS AND METHODS

2.1 Supply and Maintenance of Cultures

One culture of the "Ruttnera spectabilis" and two different strains of the "Chrysotila lamellosa" form of the species were obtained from the Plymouth laboratory. Sub-cultures of each were immediately transferred to solutions of Boney's Culture medium (Table 2.1). These cultures were kept in a regime of 12 hours light: 12 hours darkness, at 2760 lux, at a temperature of 12^{o}C. From these cultures, further sub-cultures were set up in ASP6 solution (Chapter 2.2), and maintained in the conditions described above. These cultures were used as the "stock" cultures for all subsequent experiments, and were regularly infused with fresh ASP6 medium in order to maintain them, as far as possible, in an identical condition. Although these cultures were unialgal, and were regularly inspected in order to confirm this, they were not axenic. Bacterial contamination was, however, kept to a minimum. Axenic cultures were not required for the majority of the experiments.

2.2 ASP6 Culture Medium

The cultures were originally transferred to Boney's Culture medium, as it is a good, general purpose culture medium, which is known to support the growth of a wide range of unicellular marine algae. The salinity of this medium cannot be varied easily, nor

Table 2.2

A S P 6 CULTURE MEDIUM

Na Cl	411 mg M
K Cl	9.4 mg M
Mg SO_4	32.5 mg M
Ca Cl_2	3.75 mg M
Na NO_3	3.53 mg M
Na_2 GLYCEROPHOSPHATE	317 µg M
Na_2 SiO_3	246 µg M
TRACE METAL SOLUTION	P8
VITAMIN SOLUTION	8A
TRIS	8.25 mg M
pH	7.4 - 7.6
Water to	1000 ml

P8

Zn SO_4	7.7 µg M
Mn sO_4	18 µg M
Na_2 MoO_4	5.2 µg M
Co Cl_2	0.17 µg M
Cu SO_4	0.32 µg M
Fe EDTA	35.8 µg M
VERSONAL	87.2 µg M
CHELATE : METAL	1.3 : 1
H_3BO_3	185 µg M

Table 2.2 (continued)

8A Vitamin Solution

	Concentration/L of Medium
Thiamine HCl	2.0 mg
Nicotinic acid	1.0 mg
Ca Pantothenate	1.0 mg
p Aminobenzoic acid	0.1 µg
Biotin	5.0 µg
i Inositol	10 µg
Cyanocobalamine	0.5 µg
Thymin	8.0 mg
Pyridoxine HCl	0.4 mg
Pyridoxamine 2HCl	0.2 mg
Putrescine 2HCl	0.4 mg
Riboflavin	50 µg
Choline H_2Citrate	5.0 mg
Orotic acid	2.6 mg
Folinic acid	2.0 µg

can its composition be easily altered, as it is based on aged pasteurised seawater (Table 2.1).

A culture medium suitable for this project had to satisfy the following requirements in addition to sustaining the growth of the organism in good condition:

a) to be easily varied in total salinity
b) to be totally synthetic
c) to alter the main nutrient balance as little as possible.

These requirements were met by ASP6 culture medium (Provasoli, McLaughlin and Droop 1957, Provasoli 1963, McLachlan 1973), and its composition is given in Table 2.2. This is a completely synthetic medium. Initial experiments showed that the alga would grow favourably in the full strength medium, which has a total salinity of $32^{o}/oo$.

The salinity of ASP6 medium is determined by the quantities of the major salt components - Na Cl, Mg SO_4, and Ca Cl_2. The adjustment, in proportion, of these four major salts allowed the exact determination of the salinity of the culture medium. It was noted that the pH of the medium was relatively stable over the range of salinities chosen, and from this it was assumed that the overall character of the medium was relatively constant throughout the range.

In preparation of a range of salinities, a basic stock culture was made up from the four major salts. Each individual salinity

was then obtained by dilution from the stock solution. In this way the medium was kept as constant as possible. The micronutrients were then added to the major salt solution, and the medium was autoclaved. Initially, problems were experienced due to the precipitation of some salts. This was believed to be caused by the sodium silicate forming insoluble compounds with some of the heavy metals required as micronutrients. Precipitation occurred most commonly when the sodium silicate was added as one of the first micronutrients, but not if added as the last of the solutions. This also occurred after autoclaving, and to avoid precipitation, the media were well agitated to assist complexing of all heavy metals, before the addition of the sodium silicate solution. After autoclaving and cooling, all culture solutions were carefully inspected for the presence of precipitates, and any solutions which were in any way suspect, were discarded. McLachlan (1973) has suggested that in most cases, the complete vitamin structure is not necessary, and the moieties which are present after autoclaving are equally effective. Cultures of Chrysotila were grown in solutions which had been autoclaved after the addition of the 8A vitamin solution, and in solutions which had been autoclaved, and subsequently had pasteurised 8A vitamin solution added. There was no observable difference in the growth of alga in these two solutions, however, as little work has been reported on this matter, the decision was taken to use the accepted method of adding pasteurised vitamin solution to previously autoclaved, and cooled medium.

2.3 Culture Methods

Two different culture methods were used. For small volume cultures, including the maintenance of stocks and the acclimatisation to different salinities, the algae were grown in 250ml Erlenmeyer flasks. The flasks were placed on glass shelves and illuminated from below by a single 60 watt Atlas "Daylight" fluorescent tube, giving 2160 lux at the flask level. The flasks were given 12 hours light: 12 hours darkness. The temperature of the cold room was $12^{o}C \pm 2^{o}C$.

Large volume cultures, for the experiments on growth rates, pigment analyses, DNA content, and zoospore release, were kept in more closely controlled conditions. The algae were grown in 0.5L of culture medium, in 1L Roux flasks (Fig 2.1). Each flask was aerated through a tube reaching to within 5 cm of the bottom of the flask, and this provided agitation to the culture. The offset adapter was plugged with non absorbent cotton wool to permit the escape of air, and prevent the entry of contaminants. The ventilation rate was controlled by a screw clamp on the air line, allowing independent adjustment to the rate of flow in each flask. A single high-flow pump provided sufficient aeration for twelve flasks. The air pressure was kept constant by allowing the excess to bubble off against a 150 cm column of water (Fig 2.2). The air was then passed through a silica gel column to remove moisture, and then through an aspirator filled with cotton wool to remove any remaining contaminants and airborn organisms. This aspirator, and all the silicone rubber tubing was regularly

FIGURE 2.1

Roux culture flask

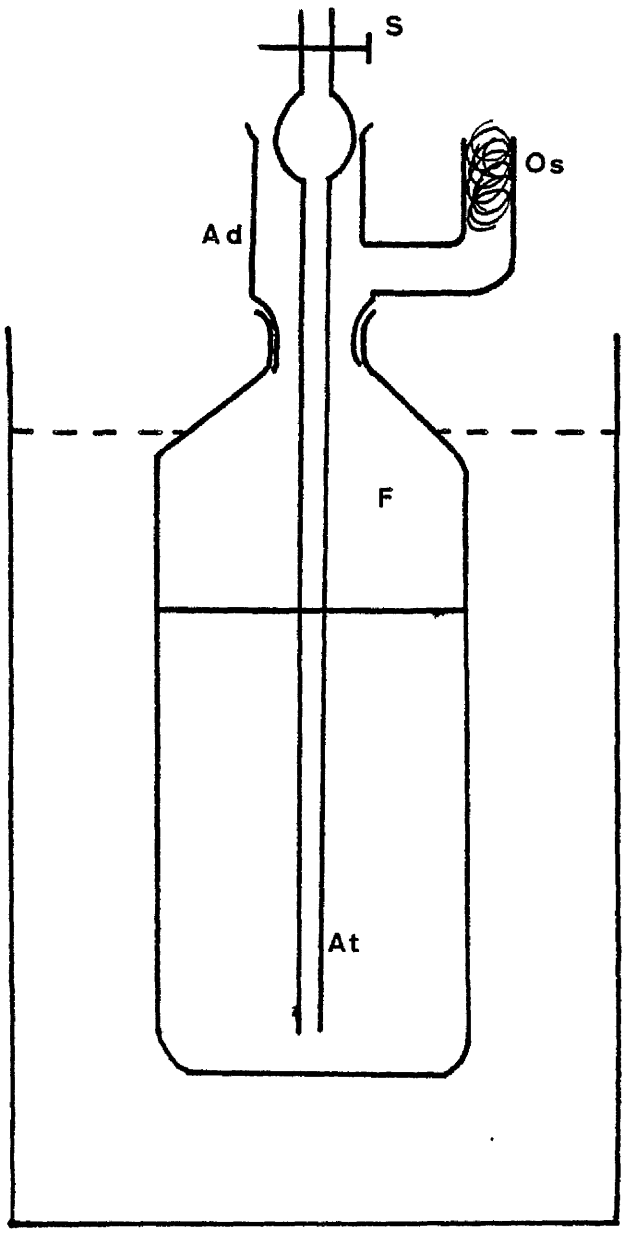
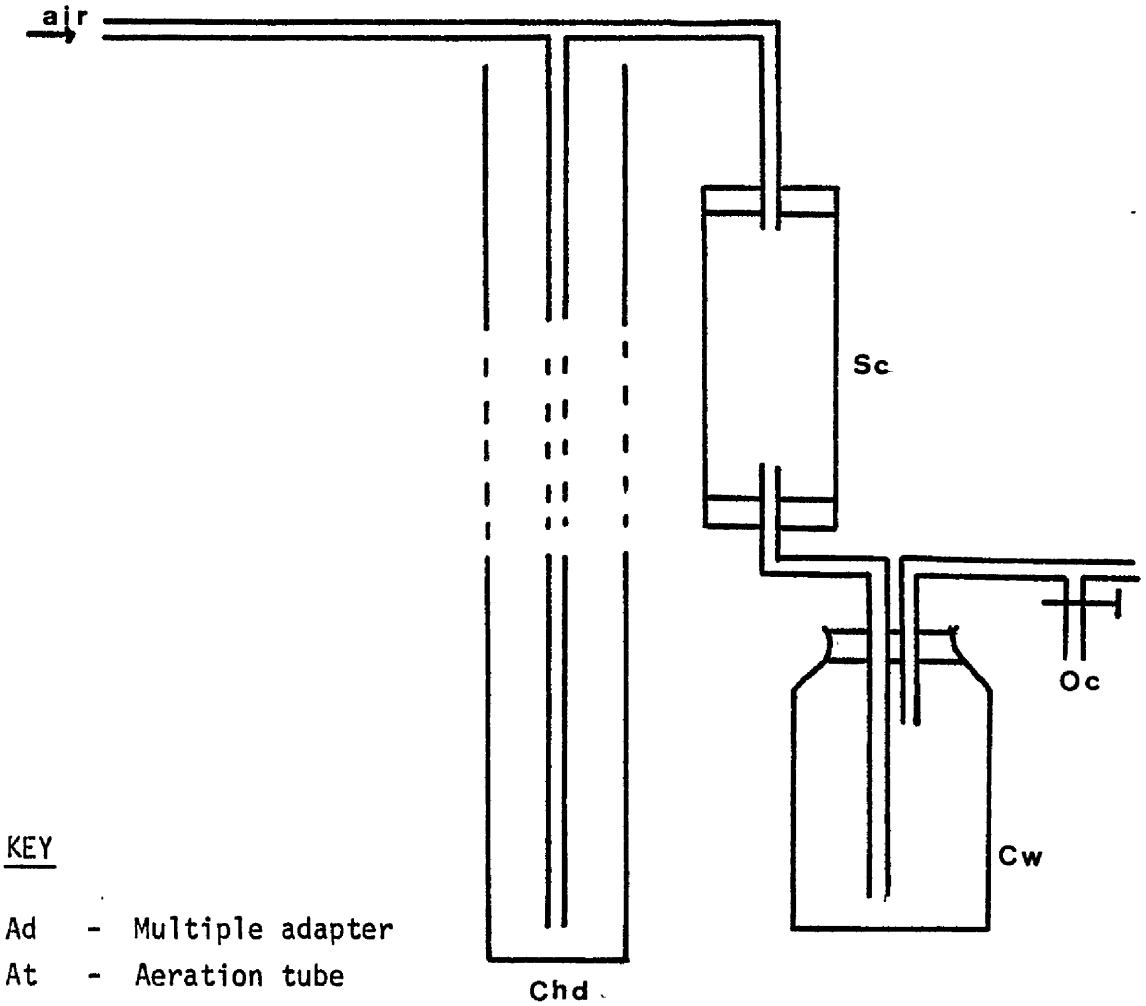


FIGURE 2.2

Aeration system



KEY

Ad - Multiple adapter
At - Aeration tube
Chd - Constant head device
Cw - Aspirator with cotton wool
F - Roux culture flask
Oc - Air outlet to flask
Os - Off-set adapter
S - Screw clip
Sc - Silica gel column

Table 2.3

CELL PACKING BY CENTRIFUGATION

SPEED	TIME	AVERAGE PACKED CELL VOLUME AS % OF TUBE VOLUME	CELL DAMAGE
	2 min	14.5%	
	4 min	14.0%	
1,000 RPM	6 min	13.9%	Negligible
	8 min	13.9%	
	10 min	13.9%	
	2 min	11.5%	
	4 min	11.1%	
2,000 RPM	6 min	11.1%	Negligible
	8 min	11.1%	
	10 min	11.1%	
	2 min	11.1%	
	4 min	11.1%	
3,000 RPM	6 min	11.1%	Negligible
	8 min	11.1%	
	10 min	11.1%	
	2 min	9.0%	
	4 min	9.0%	
4,000 RPM	6 min	9.0%	Approx 10% of cells
	8 min	9.0%	
	10 min	9.0%	

autoclaved, in order to maintain a sterile flow.

The flasks were immersed in a glass tank (122 cm x 46 cm x 46 cm) filled with water to a level above that of the culture medium in the flasks. The temperature was held at $15^{o}C \pm 0.1^{o}C$ by two standard immersion heaters placed at opposite ends of the tank, the water being circulated by a small pump. Illumination was provided by four Atlas 60 watt "daylight" tubes (2 on either side of the tank), giving a total illumination in the centre of the tank of 2160 lux. The tubes were inspected regularly, and replaced when emission was found to be falling. The cultures were maintained in 12 hours light: 12 hours darkness.

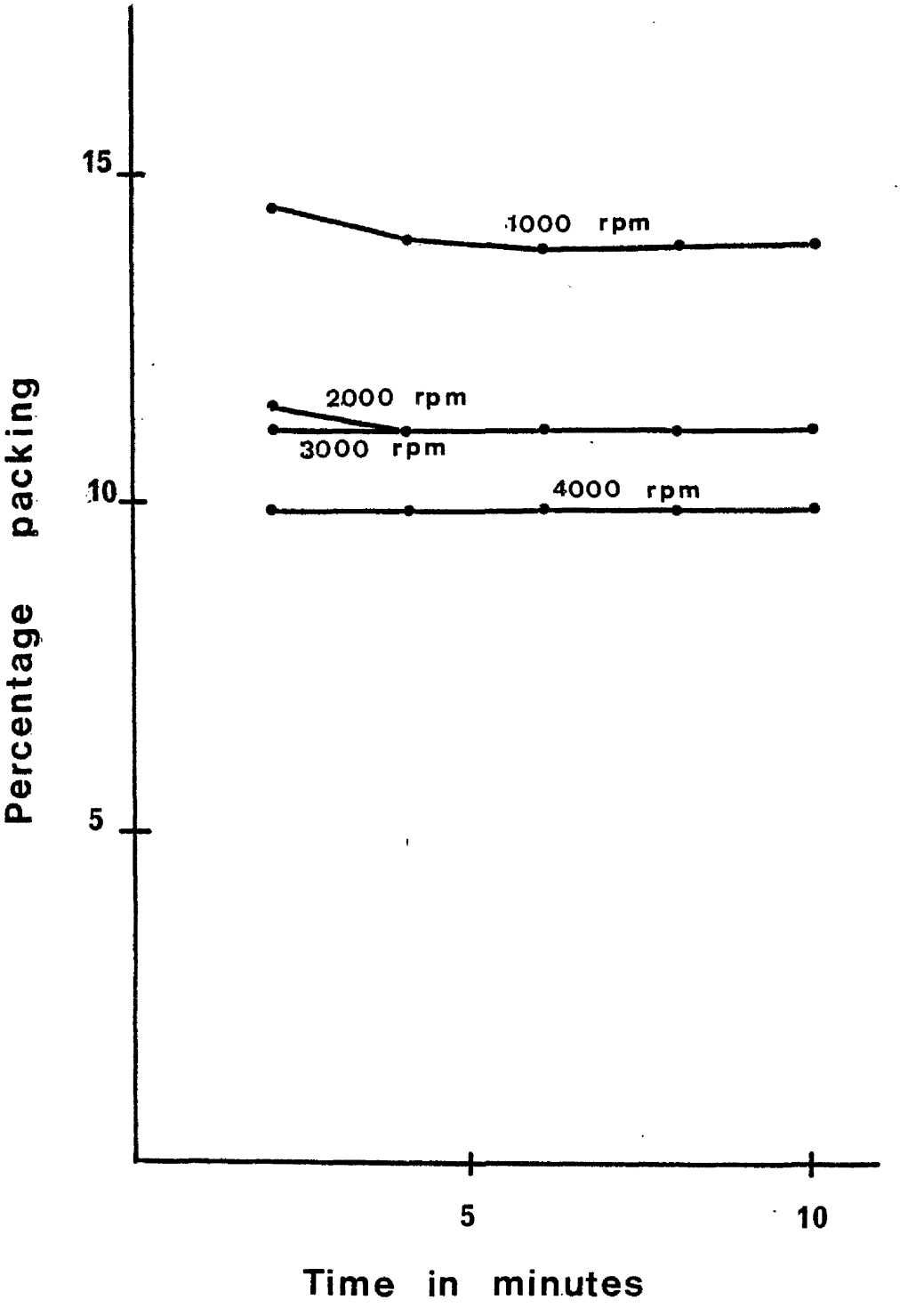
2.4 Determination of Growth in Culture by Packed Cell Volume

The measurement of the growth of Chrysotila in culture by biomass estimates is made difficult by the presence of the thick mucilage sheath. Further problems are introduced by the "Sarcinochrysis" - like growth habits, and also by the variable release of zoids.

Cell counting techniques are rendered almost useless by the variable size of the cell"blocks". The cells in agitated cultures are usually found in "blocks" consisting of between four and eight cells (Chapter 3.1). Attempts were made to estimate the number of cells in a culture using a Coulter Counter. However this apparatus counts only particles within a preselected size range, and is incapable of accounting for the variation in the number of cells which constitute a "particle". The results for different samples from the same culture were found to be so

FIGURE 2.3

Effect of Centrifge Speed on P.C.V.



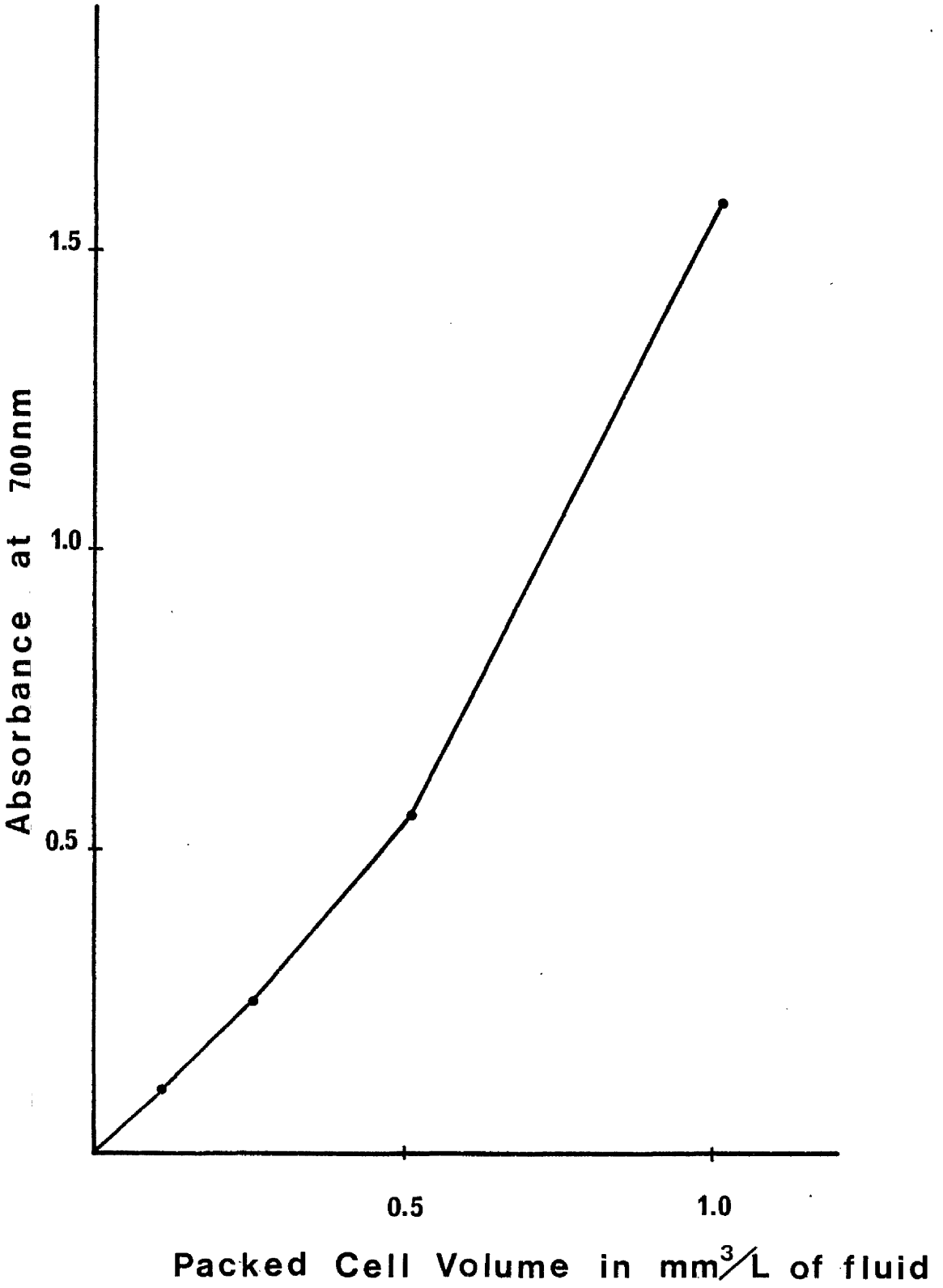
variable that even averaging several samples gave an unacceptably high level of uncertainty.

Agitation of the culture, to reduce the "blocks" to single cells was also unsuccessful. Although the large "blocks" readily fragment; as noted elsewhere, blocks of between four and eight cells seem to be relatively stable, and a large amount of agitation is required before a culture contains a majority of single cells. This may be acceptable when counting is the final stage, but when the culture is required for further experiments, the risk of damage is too great, and so this method of estimating cell numbers was rejected.

As a measure of the total growth of a culture, the determination of wet packed cell volume using calibrated Wintrobe (blood sedimentation) tubes, as used in studies with other algae (Dring 1967, Hadi 1974) was found to be the most accurate and convenient method. Preliminary experiments were undertaken to find the best centrifuge speed, with regard to cell damage, and replicable results. It was found that at speeds below 2,000 rpm the packing is uneven, and the results are somewhat variable, whereas at speeds of over 4,000 rpm there is obvious cell damage. The results of these experiments are shown in Table 2.3 and Fig 2.3. Following centrifugation, the packed cells were resuspended in culture medium, and tested for viability. These tests confirmed that some cell damage had occurred at 4,000 rpm, but at all other speeds, damage was so low as to be undetectable. From the results of these experiments it was decided to take all measurements at a

FIGURE 2.4

Turbidity at 700nm v P. C. V.



speed of 3,000 rpm for six minutes.

It is possible that the mucilage sheath acts as a cushion for the cells protecting them against some of the force of centrifugation. At low speeds, due to this mucilage, the packing is uneven, leading to variability in the results. At high speeds, the cushioning effect is insufficient to prevent disruption of the cells. It is possible that, if the mucilage is compacted during centrifugation, it may expand when the treatment stops. No data is available on this, but to minimise any such effects, all readings were taken immediately on removal of the tubes from the centrifuge.

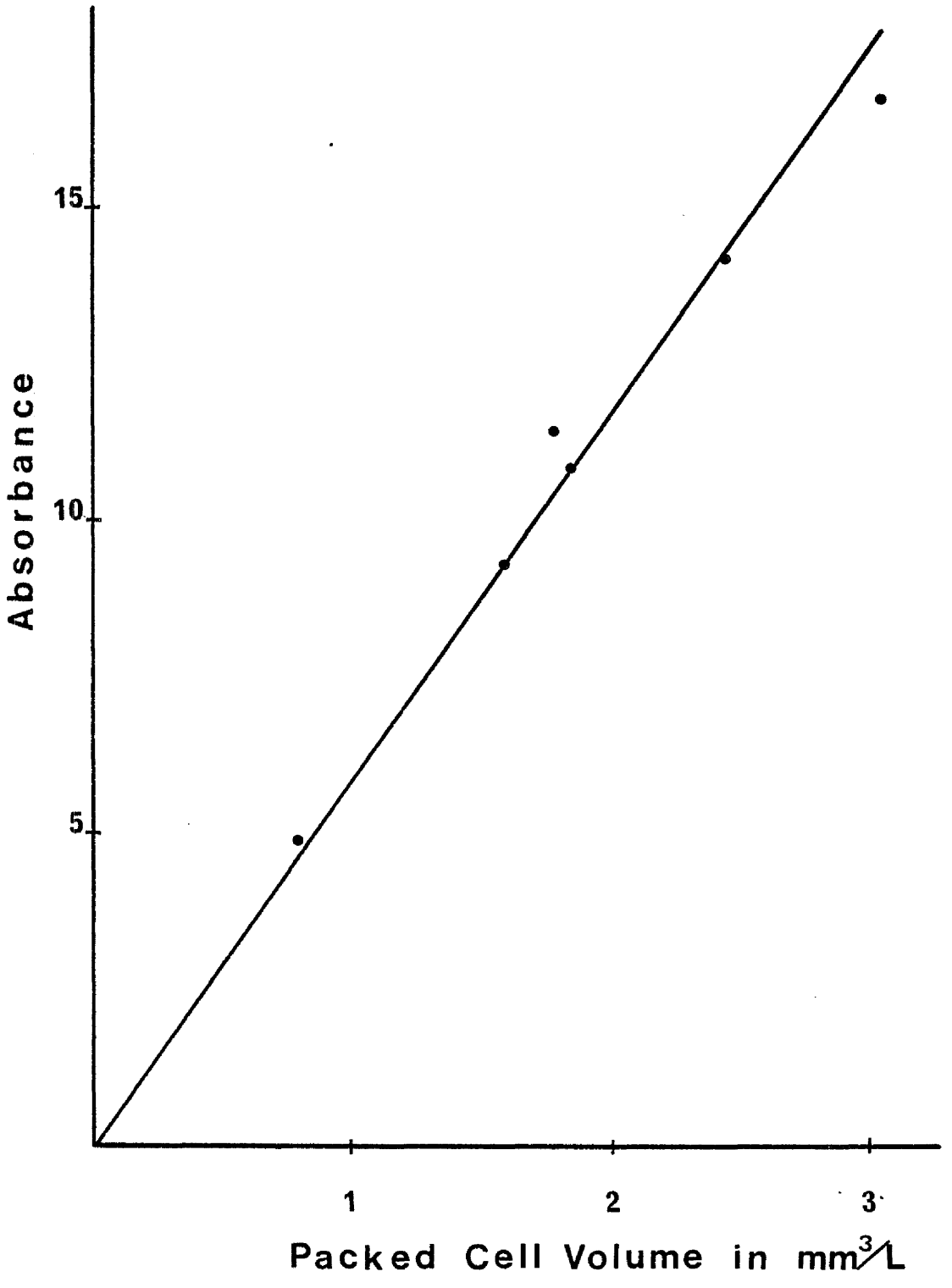
2.5 Determination of Growth by Spectrophotometric Methods

The use of Wintrobe tubes for determination of the volume of cells in culture was successful when growth was being measured at the end of an experiment. However, to obtain a sufficiency of relevant data daily measurements are required. It was felt that the interference caused to a culture by subjection to repeated centrifugation could seriously affect the growth of the organism and bias the results, and so an alternative method of estimation was required. The requirement for minimum interference could be met by using only a small sample from the culture, and estimating the cell content by spectrophotometry.

A Pye Unicam SP600 spectrophotometer was used initially, and calibrated against cultures of known wet packed cell volume. Readings were taken at 700 nm, to give a measure of the turbidity

FIGURE 2.5

Calibration of Nephelometer



of the culture. It was hoped that this would account for the variability in particle size, but the results were highly variable, and the use of 5 cm cells instead of 1 cm cells gave no noticeable improvement. The readings also changed gradually, and although this did not seriously affect the readings, it did add to the doubts as to the suitability of this method.

The results were less variable at 550 nm, and a calibration curve was drawn (Fig 2.4). Although this method could be adapted it was rejected in favour of the Nephelometer measurements. One disadvantage of the SP600 is the precision of the wavelength setting. The absorption could therefore be affected by the pigment concentration within the cells, which was later shown to vary according to the salinity of the medium. An extra variable is therefore introduced into the measurements, which cannot easily be taken into account.

The Eel Nephelometer was finally selected as it gives a more general reading of particle density, by reflection, and not by absorption. This reading is also taken using a wide, rather than a narrow frequency band, thus helping to eliminate inaccuracies due to differences in pigment concentrations between cultures grown at different salinities. The effects of settlement are considerably reduced in the larger volume of the sample, in this case 20 ml, and to the way in which the light is shone through the culture. A calibration curve was drawn (Fig 2.5) and it can be seen that there is a direct relationship between the turbidity of the sample and the wet packed cell volume. As

outlined above, the use of this method involves little interference with the culture of the organisms, and has the advantage that it is a very sensitive method, and can give accurate results for low density cultures.

2.6 Determination of Pigments

The method follows that outlined by Parsons and Strickland (1963, 1968). A known volume of cells was suspended in 10 ml of 90% acetone, and placed in complete darkness for 20 hours in a refrigerator, to allow the pigments to be extracted. The tubes were then allowed to reach room temperature, in darkness, and centrifuged at high speed. It was noted that this method gave incomplete extraction, as the residue in the centrifuge tubes was darker in colour than the supernatant. This is probably due to the mucilage sheath, which slows down the rate of extraction of the pigments by the acetone.

Allowing extraction to take place over a longer period of time could ensure more efficient removal of the pigments, but could also result in their degradation, and so this method was rejected.

Before extraction with acetone, the cells were first ground in a mortar with a measured quantity of washed silver sand. This was completed in subdued light, and the mixture was transferred to centrifuge tubes in 10 ml of acetone. The tubes were placed in darkness in a refrigerator, and treated as detailed above. To hasten extraction the tubes were agitated after one hour, and again

after two hours.

Before centrifugation, the volume was checked, and made up to 10 ml with 90% acetone, to compensate for evaporation.

All readings were taken using 1 cm cells in a Pye-Unicam SP600 spectrophotometer. Extinction values were noted at 480 nm, 630 nm, 645 nm, 665 nm and 700 nm.

2.7 Determination of DNA

A known packed cell volume of axenic culture was refluxed with 80% redistilled methanol to aid bleaching. The cells were centrifuged at high speed and the residue was treated with 10% ice cold trichloroacetic acid, for one hour at τ 15°C. The precipitate was centrifuged at 5000g and extracted with a solvent of 2 ether: 2 ethanol: 1 chloroform, to remove fat and moisture. The residue was centrifuged at 5000g and then dried in vacuo. The pellet was hydrolysed with 5 ml potassium hydroxide (0.5M) for one hour at 57°C, and then centrifuged at 0°C.

The residue was mixed with a known volume of 10% perchloric acid and incubated at 90°C for 15 minutes. The supernatant, after centrifugation, was transferred to 1 cm cells and the extinction at 260 nm was measured on a Pye-Unicam SP800 spectrophotometer (Schneider, 1946).

2.8 Production of Axenic Cultures

For most of the experiments, the presence of bacteria was of no consequence, and although all apparatus and solutions were sterilised before use, this was to ensure that the cultures remained unialgal. This procedure did, however, ensure that other microorganisms were kept within acceptable limits, and in this respect, the algae were sometimes washed in sterile solutions, to cut down the transfer of microorgansims between cultures.

In producing axenic cultures it is important to inflict as little damage as possible on the algae, consistent with ridding the culture of all other microorganisms.

The algae were first treated by vigorous washing in sterile solutions, with subsequent transfer to sterile medium. The cultures were grown in Erlenmeyer flasks for one week, and then tested for microorganisms using a solution of bactopeptone and glucose (Hoshaw and Rosowski, 1973). Two solutions were used.

I	1g	Bactopeptone
	1g	Glucose
	100 ml	Distilled water.
II	1g	Bactopeptone
	1g	Glucose
	100 ml	ASP6 solution, full strength.

Both solutions were autoclaved before use. The two solutions were used in order to cover all possible contaminating microorganisms, and were incubated at $20^{o}C$ for four days. In all cases,

microorganisms were found to be present. In order to check the method, a "control" experiment was run at the same time using sterile solution, and this procedure was continued in all further experiments. In this case, no contamination entered the control experiment.

In the second method, cultures of the algae were streaked on sterile ASP6 agar plates, and grown in the coldroom in the same conditions as the stock cultures. After several days, each culture was examined, and any which appeared clear of bacteria, or which showed reduced bacterial growth, were streaked again. The streaking is limited by the growth of Chrysotila, which was found to be very slow on agar, and it was not possible to carry this beyond three treatments. Bacterial contamination was considerably reduced by this method, and as such, it could be recommended for clearing cultures which have a high bacterial population, but in no case was it a successful method for the production of axenic cultures. Antibiotics have been successfully used (Droop 1967) to produce axenic cultures of algae, but because of the unknown effects on growth and development, this method was not adopted at first. The failure of other mechanical means however, left this method as the only acceptable alternative. Droop's method of successive dilutions from a single concentrated solution was used, each dilution producing a concentration of half the strength of the previous one, viz:-

Table 2.4

ANTIBIOTIC MIX No 1

Benzyl Penicillin	4,000 µg
Streptomycin SO_4	720 µg
Chloramphenicol	640 µg
	5,360 µg

Table 2.5

SURVIVAL OF ALGAE AND BACTERIA

	Conc in µg/ml of culture				
24 hrs	5,360	2,680	1,340	670	335
Algae	+	+	+	+	+
Bacteria	+	+	+	+	+
48 hrs					
Algae	-	+	+	+	+
Bacteria	-	-	+	+	+

Table 2.6

Conc in μg/ml of culture 72 hrs exposure

	5,360	2,680	1,300	670	335
Algae	-	-	+	+	+
Bacteria	-	-	±	+	+

Table 2.7

Survival of Algae and Bacteria with Penicillin (μg/ml)

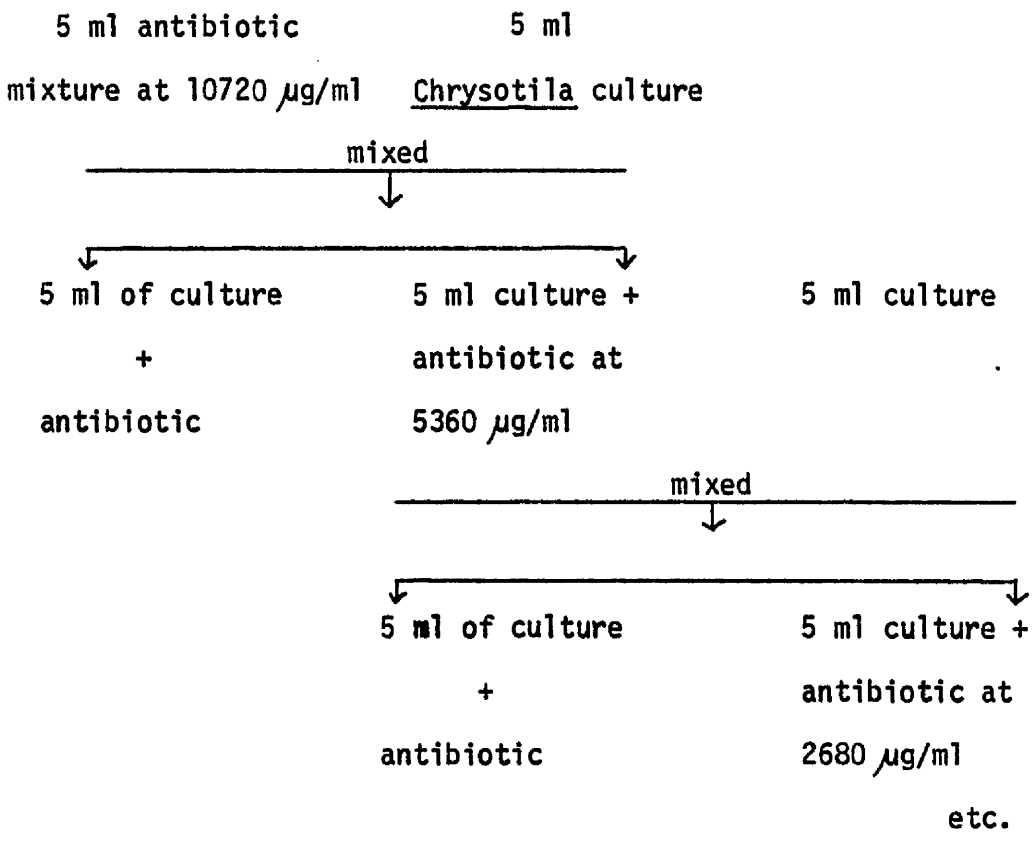
	10,000	5,000	2,500	1,250	625
Algae	+	+	+	+	+
Bacteria	±	±	+	+	+

Table 2.8

Streptomycin (μg/ml)

	5,000	2,500	1,250	625	312.5
Algae	+	+	+	+	+
Bacteria	+/−	±	+	+	+

(± : see text)



The initial mixture was made up as shown in the Table 2.4. The first attempt over 24 hours exposure gave both viable algae and bacteria, and so further exposures were tried over 48 hours, and 72 hours (Tables 2.5 and 2.6). This gave more encouraging results, in that the algae appeared to be viable at 2680 µg/ml whereas the bacteria were killed. The cultures of algae were maintained in axenic conditions, but soon began to deteriorate. The first evidence was a gradual change in the colour of the culture, and within about four weeks, the majority of cells in the culture were dead. This did not happen with cultures exposed for 24 hours to the antibiotic mix, but was evident in cultures exposed for longer periods. The failure of this technique is possibly due to the presence of the mucilage sheath around the alga. In this mucilage,

Table 2.9

Chloramphenicol (μg/ml)				
2,500	1,250	625	312	156
+	+	+	+	+
±	+	+	+	+

Table 2.10

Neomycin (μg/ml)				
2,500	1,250	625	312	156
+	+	+	+	+
±	±	+	+	+

Table 2.11

Antibiotic Mix prepared

Penicillin	5,000 μg
Streptomycin	5,000 μg
Neomycin	5,000 μg
Chloramphenicol	2,500 μg
	17,500 μg

Table 2.12

Results with Antibiotic mix (µg/ml) 24 hour exposure

	70,000	35,000	17,500	8,750	4,375
Algae	-	±	+	+	+
Bacteria	-	-	-	-	+

Table 2.13

Subcultures from above after 7 days (µg/ml)

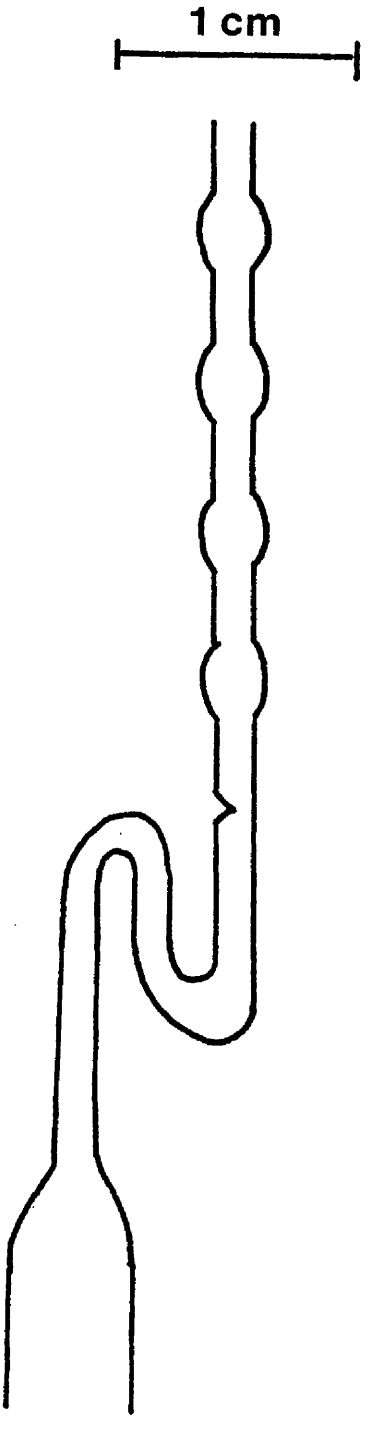
	70,000	35,000	17,500	8,750	4,375
Algae	-	-	±	+	+
Bacteria	-	-	-	-	+

bacteria are protected to some extent from the effects of the antibiotic. Longer exposures are therefore required to give the antibiotic more time to penetrate through the mucilage layer. This layer also serves to protect the algae, explaining the high levels of antibiotic which they can tolerate over a short period of time, and the delayed effect noticed at the 2680 µg/ml level, where the algae were initially viable, but only later began to show adverse effects.

It is probable that the mucilage retains the antibiotic, after transfer into fresh medium, and so the effective exposure is considerably longer than the time during which the algae are actually in the solution. It was noted, on several occasions (denoted in the Tables as ± results) that the cultures were initially axenic. This condition usually existed for one to three weeks, and subsequently showed the presence of viable bacteria. This effect can also be related to the mucilage layer. It is probable that the bacteria are inactivated, but not killed inside the mucilage. After a short period of time they begin to reproduce, first within the mucilage, and then spread into the culture, thus giving a positive test only after several days. In all cases the control experiments confirmed that the technique used, was sufficient to produce and maintain sterile culture medium.

From the first results it was obvious that the antibiotic mix was not very well suited to Chrysotila and so several individual antibiotics were tested, to assess their toxicity. These results are shown in Tables 2.7 to 2.10. From these results a new "mix"

FIGURE 2.6



was devised with the proportions as shown in Table 2.11. This proved satisfactory (Tables 2.12 and 2.13), and illustrates the results of both Chrysotila and the contaminating bacteria, to short term exposures of high antibiotic concentrations. Once obtained, the axenic cultures were subcultured, and regularly tested for contaminating bacteria.

One further method was investigated later, when a reliable method for the production of zoospores had been developed (Chapter 2.9). The zoospores of Chrysotila are strongly phototactic. In this condition they have shed the mucilage sheath, and one major source of contaminating bacteria is therefore eliminated. Using the apparatus shown (Fig 2.6),(Guillard, 1973), several attempts were made to obtain axenic cultures, but although bacterial contamination was considerably reduced, no axenic cultures were obtained. The most difficult stage is the transfer of the motile spores, in sterile conditions, once they are separate from the bacteria. The use of control experiments confirmed this observation. This method has the obvious advantage that it utilises the normal behaviour and life cycle of the organism and it eliminates the possibility of altering the culture or the organism with antibiotics.

2.9 Production of Zoospores

Green and Parke (1974) reported the release of zoospores from cultures, when exposed to osmotic shock, or to a combination of osmotic and temperature shock, but their results were inconsistent.

Some considerable time was spent investigating the correlations between light, osmotic and temperature shock, and the release of zoospores in a culture. As noted in Chapter 3, zoospores are normally present in a very low concentration in cultures of all salinities. The production of a synchronised release of zoospores was found to be dependent upon aeration and osmotic shock only, moderated by temperature.

CHAPTER THREE

RESULTS

3.1 Growth Forms of *Chrysotila lamellosa*

Chrysotila shows two distinct forms of growth in the non-motile phase, which appear to be related to the age of the cells, and the levels of nutrients in the culture.

In the first form, the cells grow in "packets" enclosed in a common mucilage sheath (Figure 1.3); the "*Sarcinochrysis*" condition (Green and Parke 1974).

If the cultures are allowed to age, the cells separate and grow singly in a manner similar to the green alga *Prasinocladus* (order Prosinocladales, class Prasinophyceae) (Figure 1.2). This form only occurs if the culture remains undisturbed for a considerable length of time. If the cultures are maintained with fresh medium, the cells remain in the juvenile "*Sarcinochrysis*" condition, and this is the form in which all experimental cultures were maintained.

Detailed observations on the juvenile condition indicate that the physical structure of the packets depends on the condition in the culture medium.

When grown in small Erlenmeyer flasks, without agitation, the cells form a mat on the base and sides of the flask. This is very unstable, and the slightest agitation results in the mat breaking

up into irregular masses of cells. If the culture is then left undisturbed, the cells soon revert to growing in a single mat. In addition, a heavy growth of cells forms around the sides of the flasks in contact with the surface of the culture medium. This growth of cells, which is identical to the cells elsewhere in the culture, is the result of settlement of zoospores. On release, the zoospores swim upwards, and settle on the side walls of the flask. Cell mats are formed by cell division from these settled zoospores.

In cultures which are agitated, the cell "blocks" are much smaller and more regular in size. Most cell "blocks" were found to be in the range of four to sixteen cells in size. This size of cell "block" is the most stable, and occurs in cultures where agitation is regular, such as by air bubbling through the medium. Increasing the agitation has little effect on the average "block" size, and vigorous shaking is required to produce single cells. No attempt was made to grow cells singly in culture, as it was felt that the level of agitation could result in cell damage. The agitation produced by air bubbling through the culture, as found when growing the algae in Roux flasks, is also sufficient to prevent the build up of tide lines, formed by the zoospores.

When investigating the growth rate of the algae in culture, agitation is an obvious advantage in that it maintains the algae in more homogeneous and in physically better controlled conditions than non-agitated cultures. The culture method used (Chapter 2.3) employing air bubbled through Roux flasks fulfills these conditions.

FIGURE 3.1

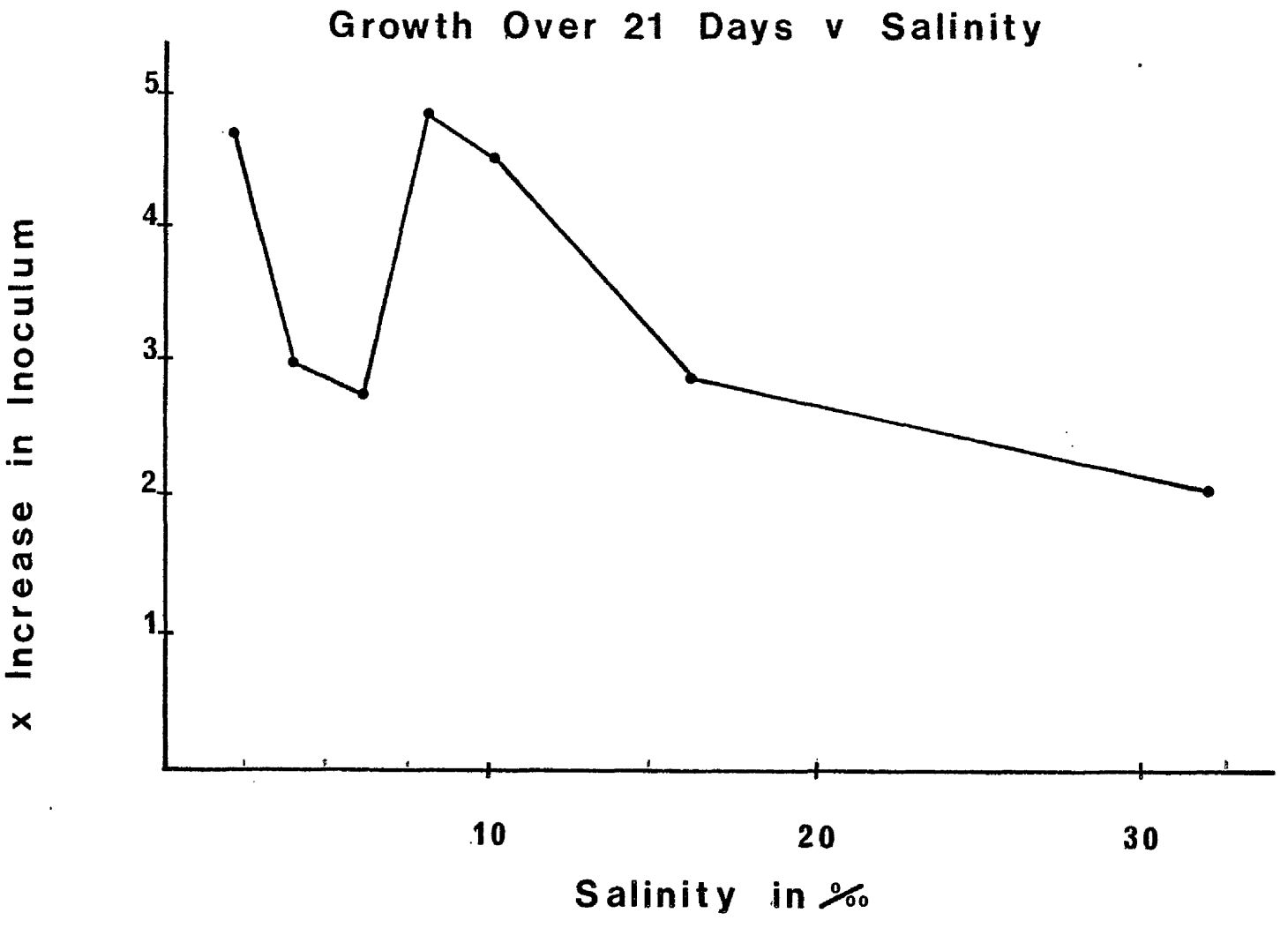

Growth Over 21 Days v Salinity
x Increase in Inoculum
5
4
3
2
1
10
20
30
Salinity in ‰

Table 3.1

Salinity and Growth over 21 days

Wet packed cell volume (mm^3)

Salinity	Initial Inoculum	Resultant Inoculum	Increase	X Increase
	0.0501	0.3004	0.2503	5.00
$2^o/oo$	0.0501	0.3270	0.2769	5.53
	0.0548	0.2493	0.1945	3.55
	0.542	0.2001	0.1459	2.69
$4^o/oo$	0.542	0.2554	0.2012	3.71
	0.0692	0.2528	0.1836	2.65
	0.0639	0.2128	0.1389	2.17
$6^o/oo$	0.0639	0.2166	0.1521	2.38
	0.0564	0.2690	0.2126	3.77
	0.0513	0.2565	0.2502	4.00
$8^o/oo$	0.0513	0.2303	0.1790	3.49
	0.0568	0.4512	0.3944	6.49
	0.0493	0.2443	0.1950	3.96
$10^o/oo$	0.493	0.2494	0.2001	4.06
	0.0506	0.3310	0.2804	5.54
	0.0928	0.2972	0.2044	2.20
$16^o/oo$	0.0928	0.3970	0.3042	3.28
	0.0956	0.3971	0.3015	3.15
	0.1003	0.2618	0.1615	1.61
$32^o/oo$	0.0883	0.2739	0.1856	2.10
	0.0717	0.2531	0.1814	2.53

From observations made, the rate of bubbling is not critical, provided a certain minimum amount of agitation is achieved, and so during culture, the air flow was regularly checked, although continuous monitoring was not deemed necessary.

As the culture ages, the mucilage layers become thicker, and the cell blocks break up. The mucilage build up at this stage also tends to become asymmetric, and with increasing age, the cell forms a stalk of mucilage (Figure 1.2). The colour of the culture changes from dull green to orange, partly due to the increase in oil reserves which form within the cells (Chapter 4). The pigment composition of the cells also changes, but no data is available on this.

3.2 The Effect of Salinity on Growth

The growth of the alga at different salinities was first measured over 21 days and estimated as a packed cell volume. This period of time was chosen after initial observations suggested that the algae would be in the exponential growth phase (Fogg 1965).

The algae were grown in 0.5L of ASP6 medium (Chapter 2.4) and the salinity of the medium was varied between 2$^{o}/_{oo}$ and 32$^{o}/_{oo}$. The results are shown in Table 3.1 and Figure 3.1. These results are summarised in Table 3.2, and a comparison by variance ratio (significance, p=0.2) is shown in Table 3.3.

Several attempts were made to grow the algae in freshwater, with all salts in micronutrient concentrations, but these were unsuccessful.

Table 3.2

Salinity	Average increase in Inoculum	Standard deviation	
		S^2	S
2%	4.69	0.70	0.84
4%	3.02	0.24	0.49
6%	2.77	0.50	0.71
8%	4.81	2.31	1.52
10%	4.52	0.52	0.72
16%	2.88	0.23	0.48
32%	2.08	0.14	0.37

Table 3.3

Salinity and Growth Rate - Significance

	2	4	6	8	10	16	32
2		+	-	+	-	+	+
4	2.91		-	+	-	-	-
6	1.40	2.80		+	-	-	-
8	3.30	9.63	4.62		+	+	+
10	1.35	2.17	1.04	4.44		-	+
16	3.04	1.04	2.17	10.04	2.26		-
32	5.0	0.58	3.57	16.50	3.71	1.64	

FIGURE 3.2

Growth at 2%

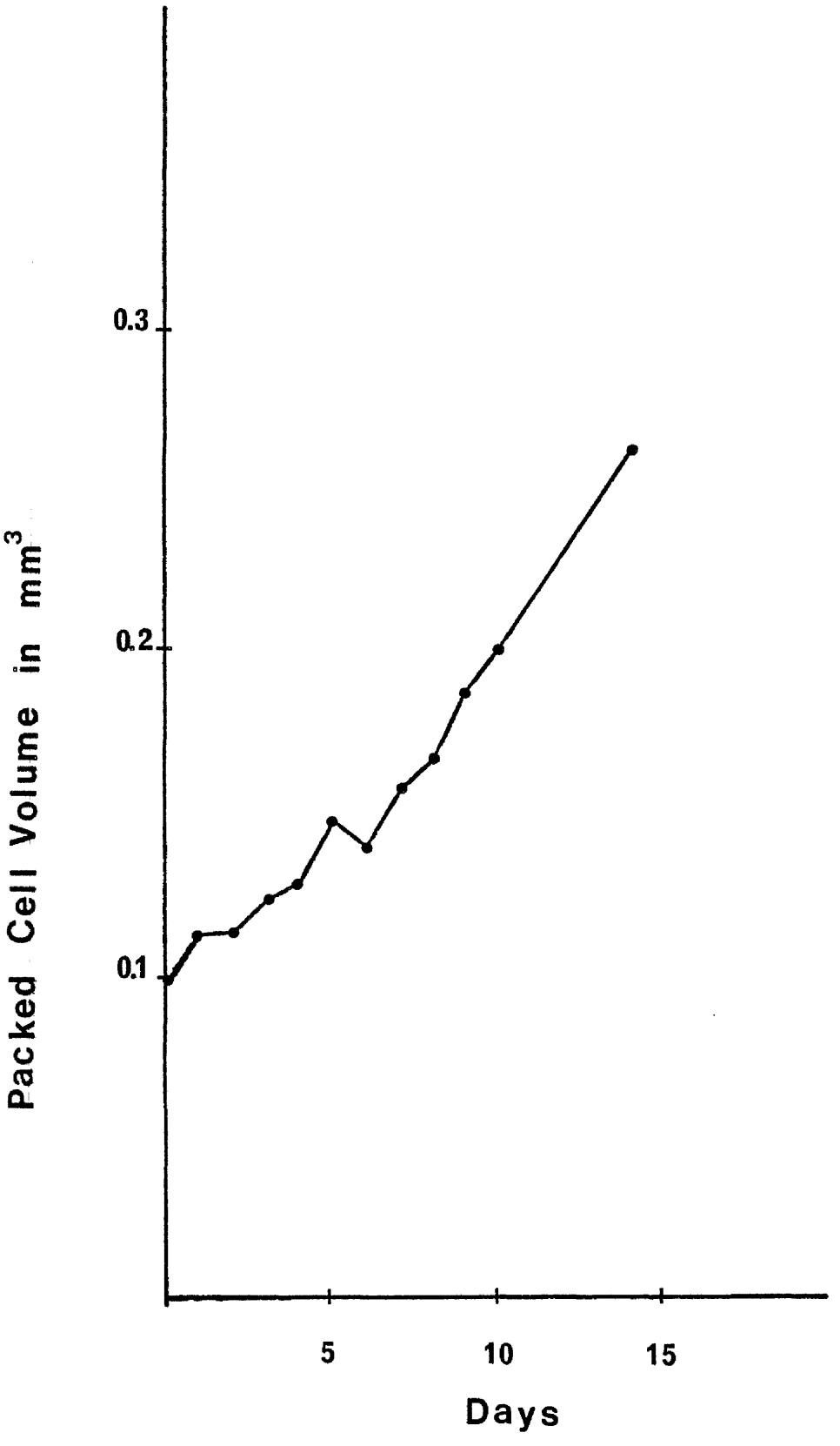


FIGURE 3.3

Growth at 4‰

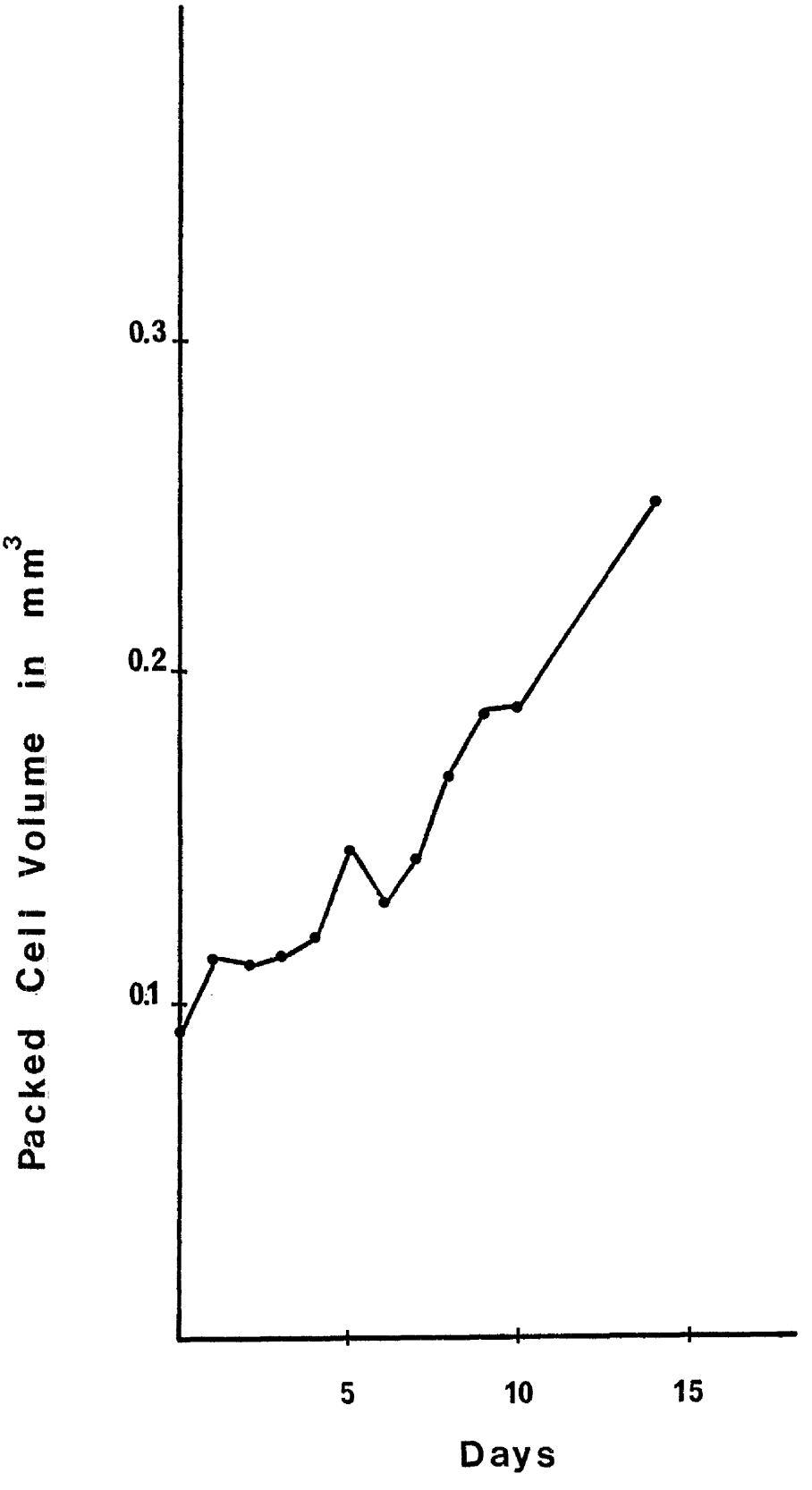


FIGURE 3.4

Growth at 8‰

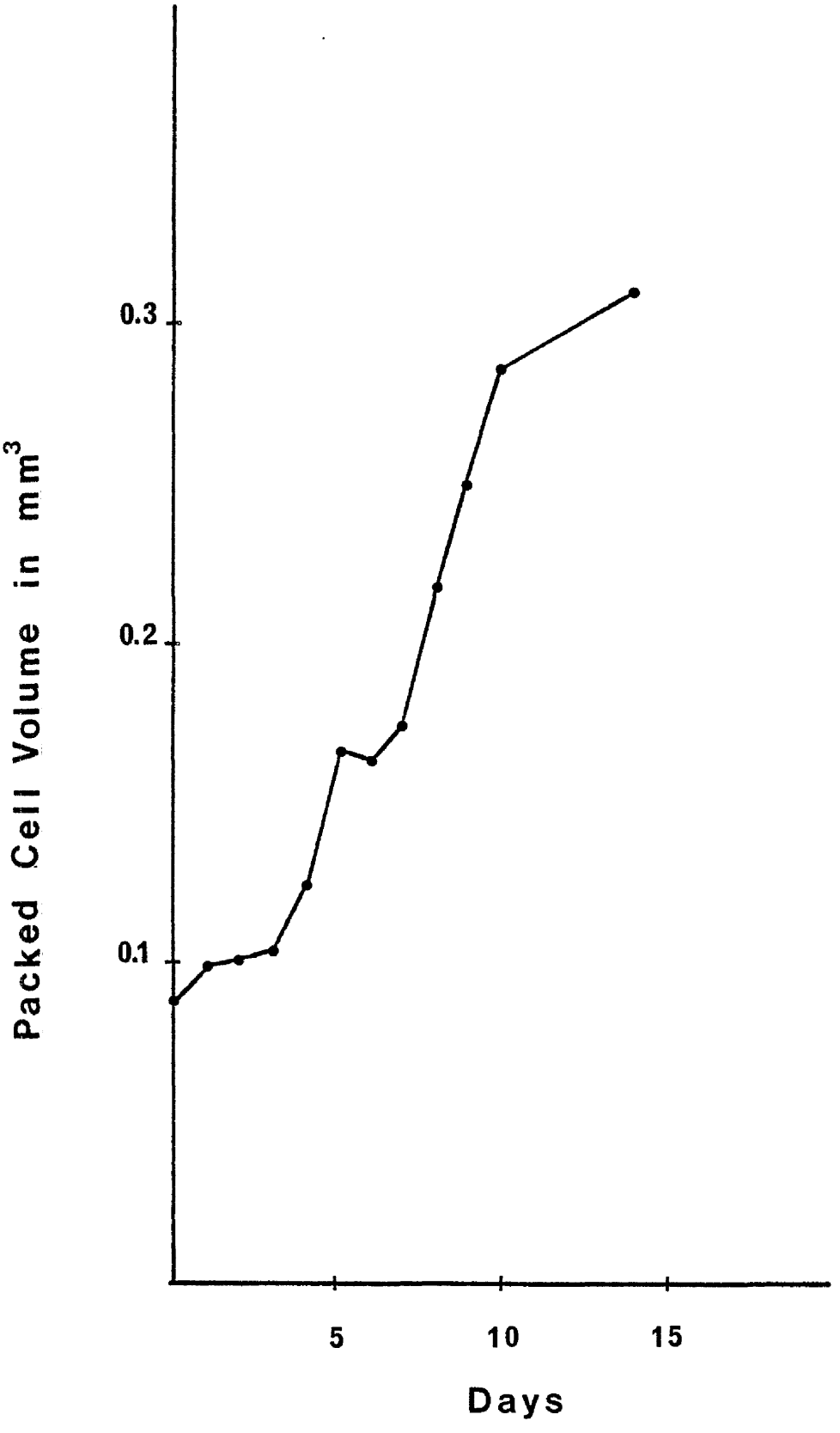


FIGURE 3.5

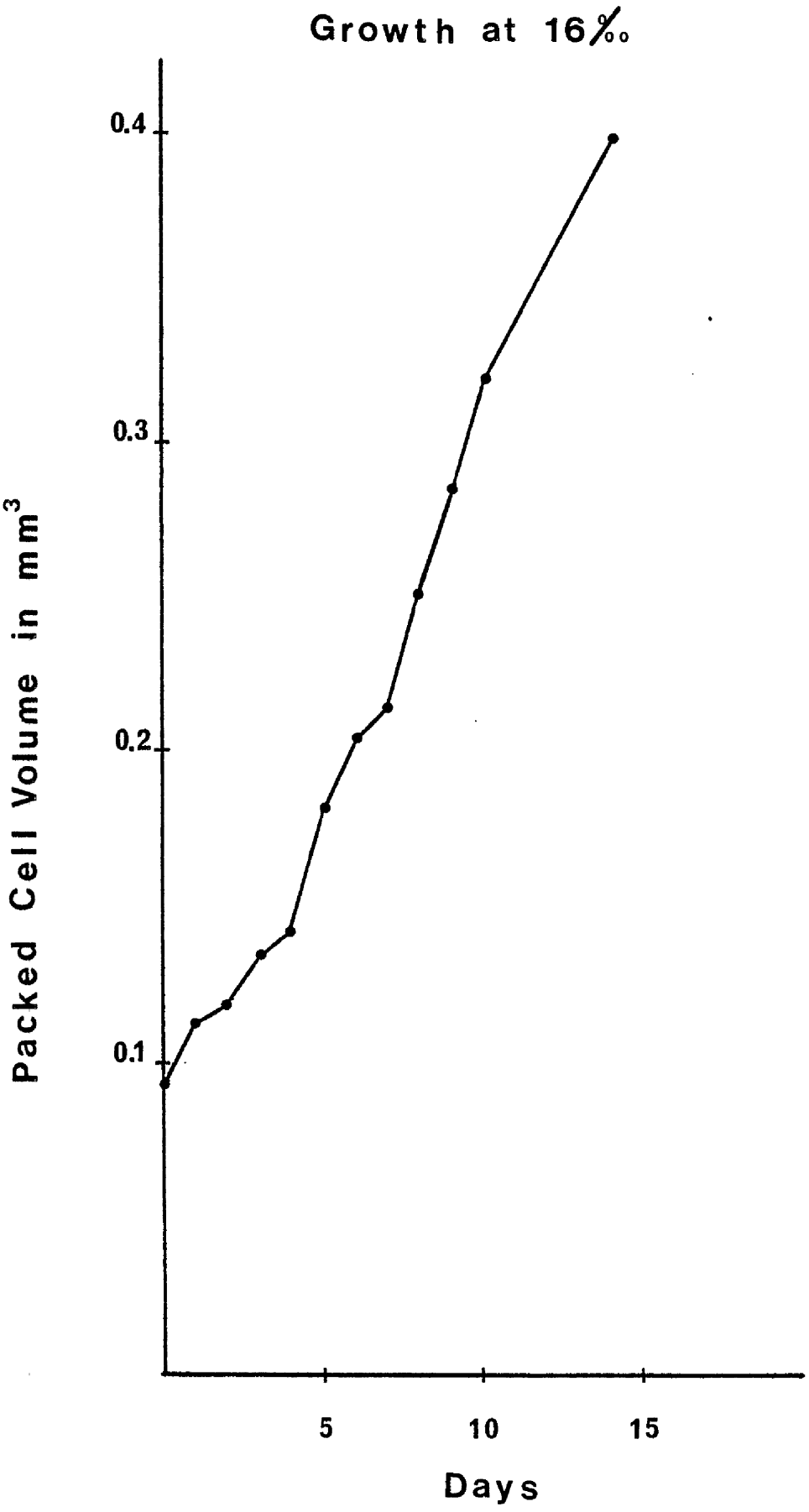
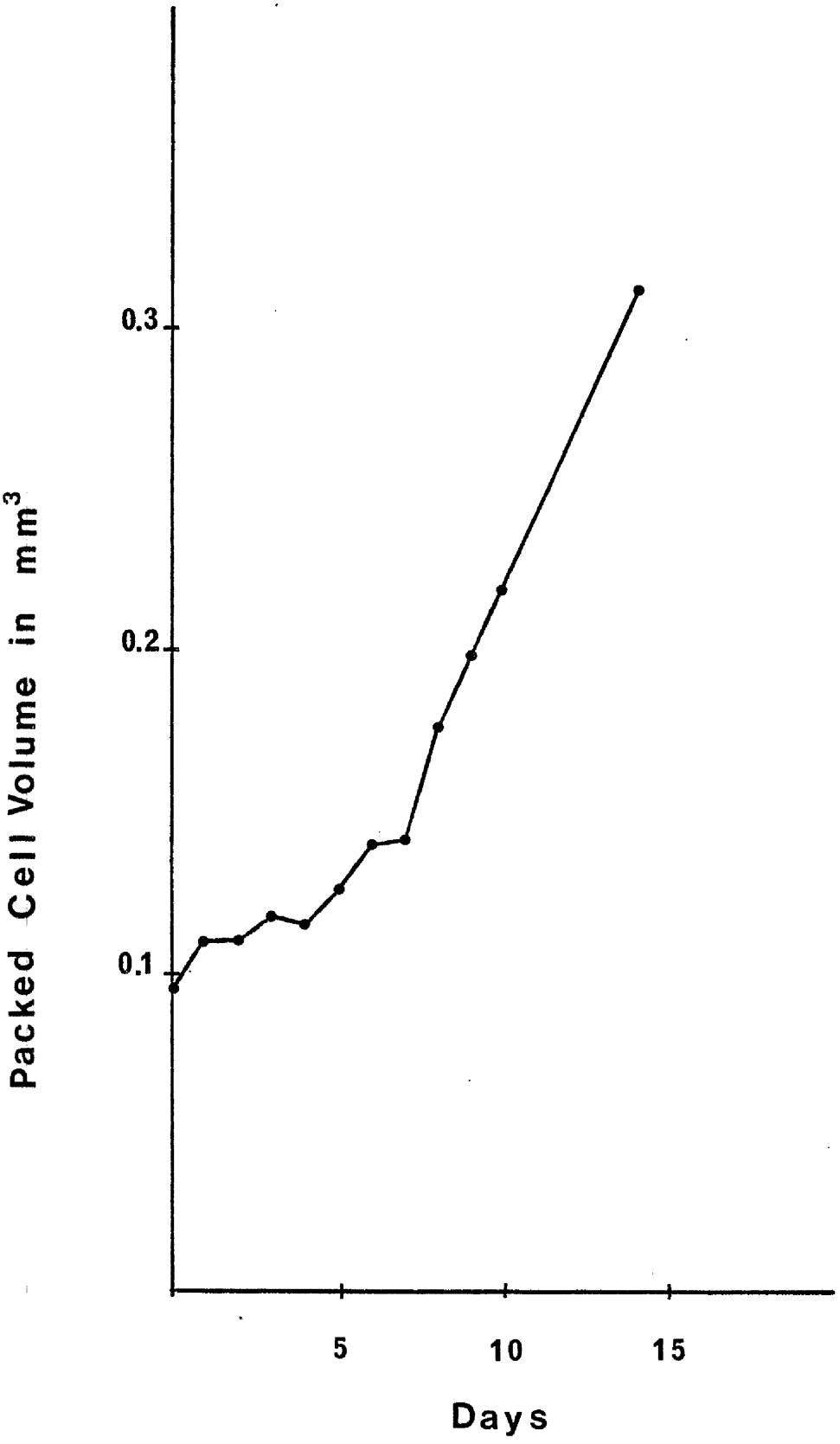


FIGURE 3.6

Growth at 32‰



Although these results are important, they give no indication of the progress of the growth of the species.

Cultures were set up, as above, at salinities from $2^{o}/oo$ to $32^{o}/oo$, and the packed cell volume was estimated by use of the nephelometer. Readings were taken daily for 14 days, and results are shown in Table 3.4. The cultures were also examined microscopically to ensure that they remained unialgal and that the bacterial comtamination remained within acceptable limits. The results of these experiments are summarised in Figures 3.2 to 3.6.

3.3 The Effect of Salinity on Pigment Concentration

The algae were grown in 0.5L of medium, as previously described, in salinities from $2^{o}/oo$ to $32^{o}/oo$. The period of growth in all cases was 21 days.

An extract was made in 90% acetone, and an absorption spectrum taken. This is shown in Figure 3.7.

The results of the pigment concentration analyses are shown in Figures 3.8 to 3.11. These were compared using the variance ratio test, and are shown in Tables 3.5 to 3.8. The significance is shown for $p=0.2$.

3.4 The Effect of Salinity on DNA Content

The algae were grown, as described previously, and the results of the DNA analyses are shown on Figure 3.12.

Table 3.4

Packed Cell Volume at Different Salinities

	Days after Inoculation											
Salinity $^{o}/oo$	0	1	2	3	4	5	6	7	8	9	10	14
$2^{o}/oo$	0.099	0.112	0.113	0.123	0.128	0.148	0.140	0.158	0.168	0.188	0.201	0.264
$4^{o}/oo$	0.091	0.114	0.112	0.114	0.120	0.145	0.129	0.143	0.168	0.188	0.189	0.249
$8^{o}/oo$	0.089	0.100	0.102	0.101	0.125	0.167	0.164	0.175	0.218	0.230	0.268	0.312
$16^{o}/oo$	0.093	0.113	0.119	0.135	0.142	0.184	0.205	0.215	0.252	0.285	0.322	0.397
$32^{o}/oo$	0.086	0.109	0.110	0.118	0.115	0.127	0.139	0.141	0.176	0.199	0.220	0.313

Average of two duplicates.

No two individual results differ by more than $\pm$ 10%.

FIGURE 3.7

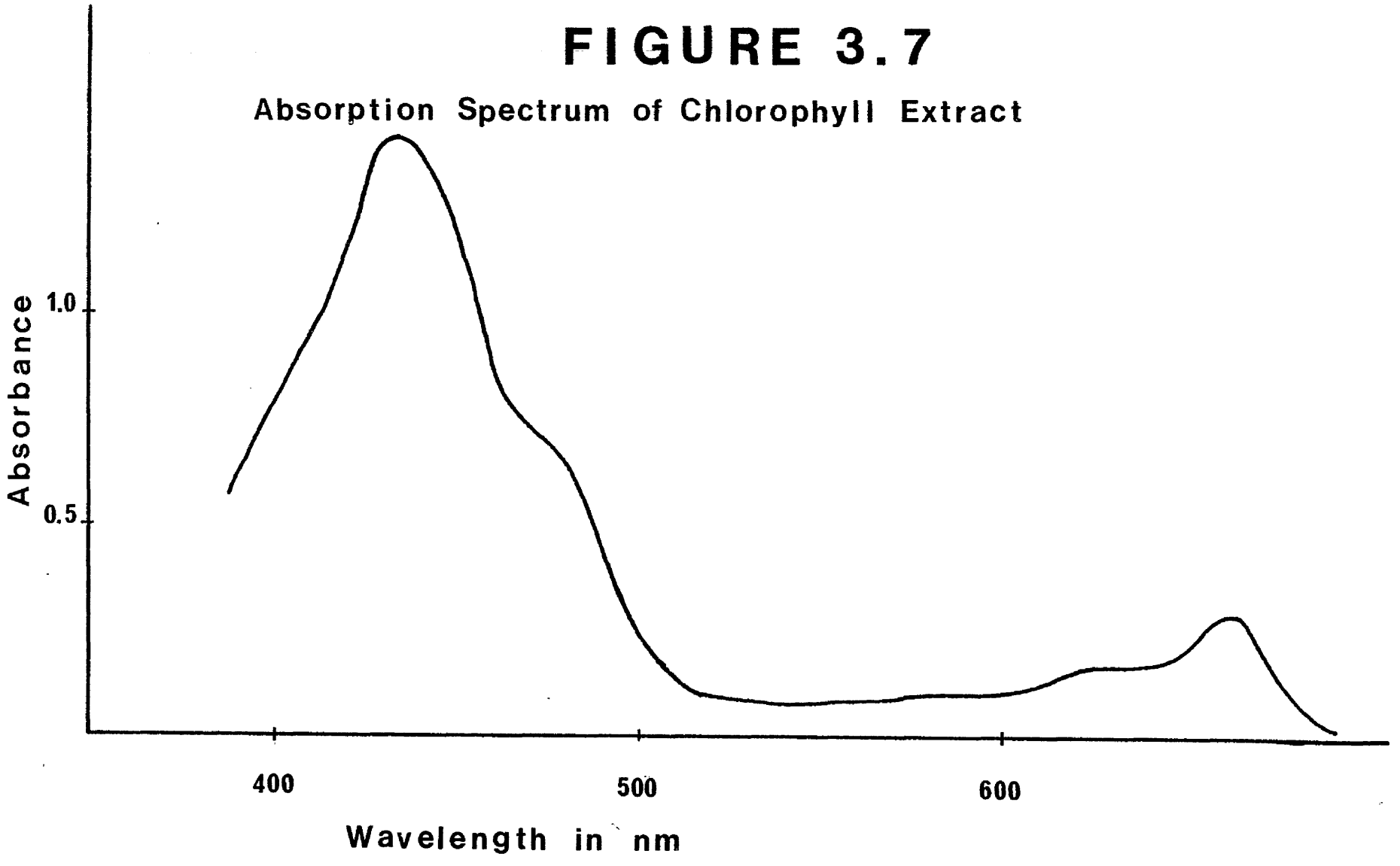

Absorption Spectrum of Chlorophyll Extract
Absorbance
1.0
0.5
400
500
600
Wavelength in nm

FIGURE 3.8

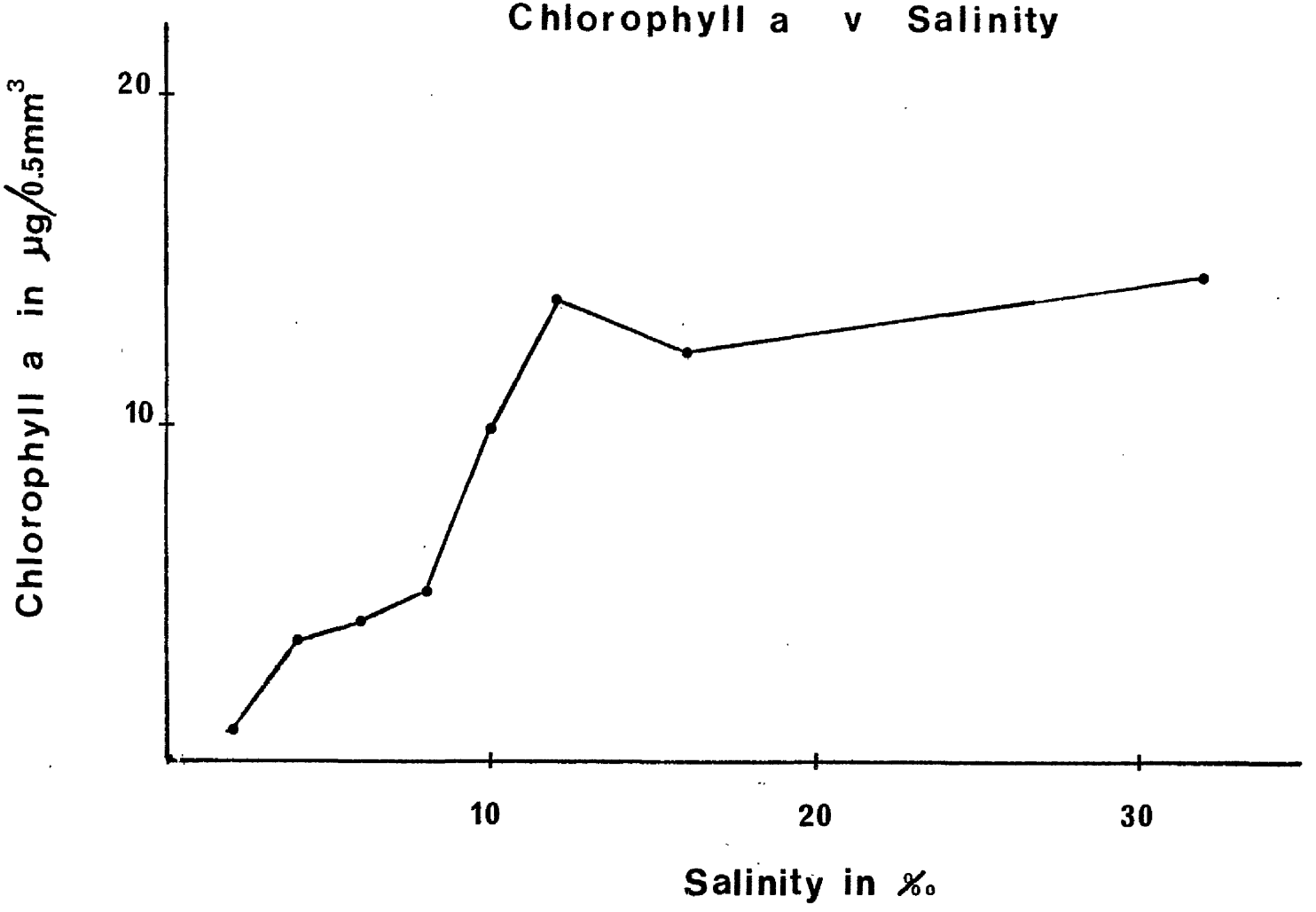

Chlorophyll a v Salinity
Chlorophyll a in µg/0.5mm³
20
10
10
20
30
Salinity in ‰

Table 3.5

Variance Ratios - Chlorophyll a v Salinity

	2	4	6	8	10	12	16	32
2		+	+	+	+	+	+	+
4	5162		+	-	-	-	+	+
6	2073	2.49		-	+	+	+	+
8	4360	1.18	2.10		-	-	+	+
10	4834	1.06	2.33	1.11		-	-	-
12	8258	1.60	3.98	1.89	1.71		-	-
16	44589	8.63	21.51	10.22	9.22	5.40		-
32	25060	4.85	12.09	5.75	5.18	3.03	1.78	

FIGURE 3.9

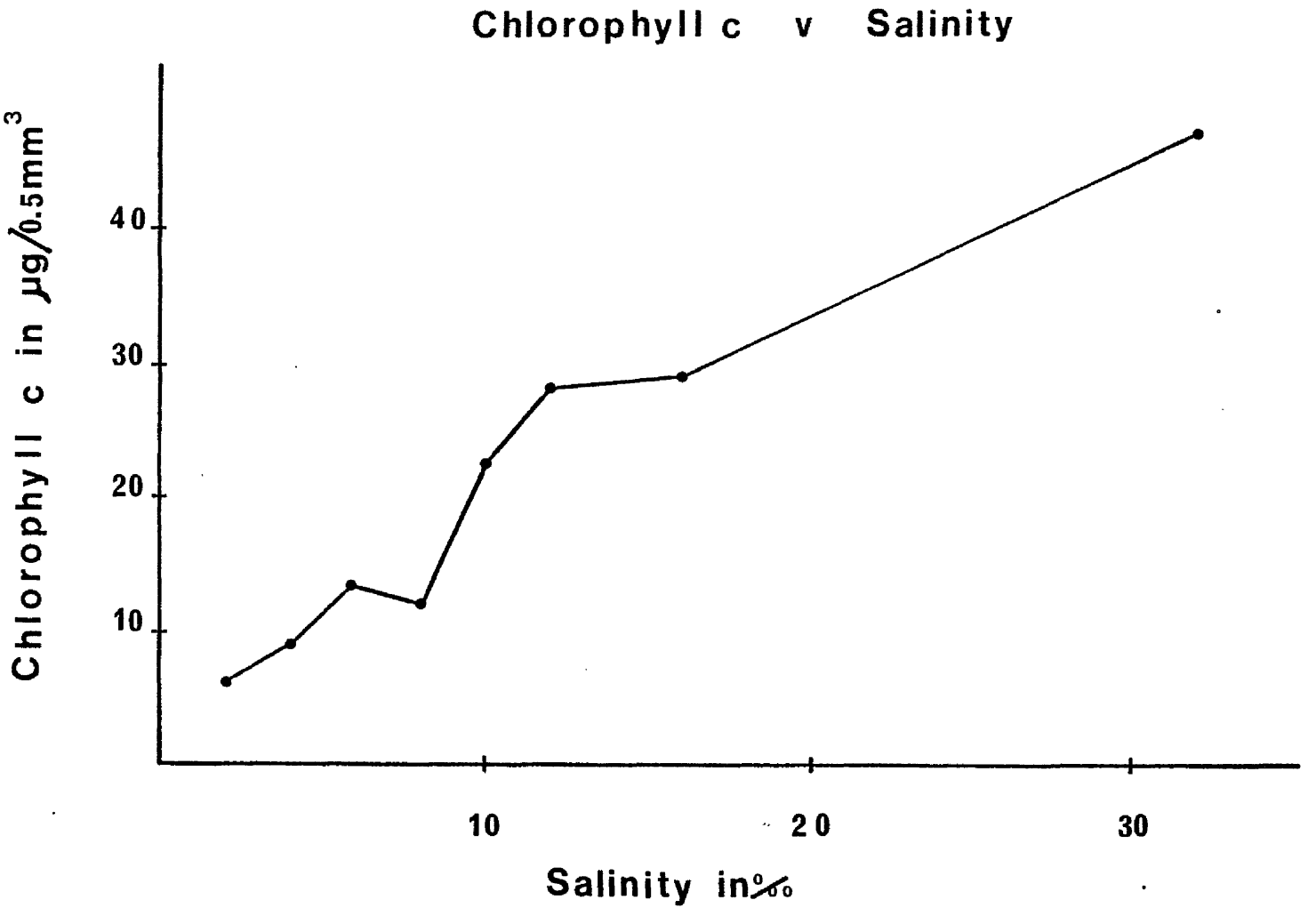

Chlorophyll c v Salinity
Chlorophyll c in µg/0.5mm³
40
30
20
10
10
20
30
Salinity in‰

Table 3.6

Variance Ratios Chlorophyll c v Salinity

	2	4	6	8	10	12	16	32
2		-	+	+	+	+	+	+
4	12.82		+	+	+	+	+	+
6	36.67	2.86		-	-	-	+	+
8	26.99	2.11	1.36		-	-	+	+
10	38.59	3.01	1.05	1.43		-	-	-
12	50.26	3.92	1.37	1.86	1.30		-	-
16	380.2	29.66	10.37	14.09	9.85	7.56		-
32	138.5	10.80	3.78	5.13	3.59	2.76	2.75	

FIGURE 3.10

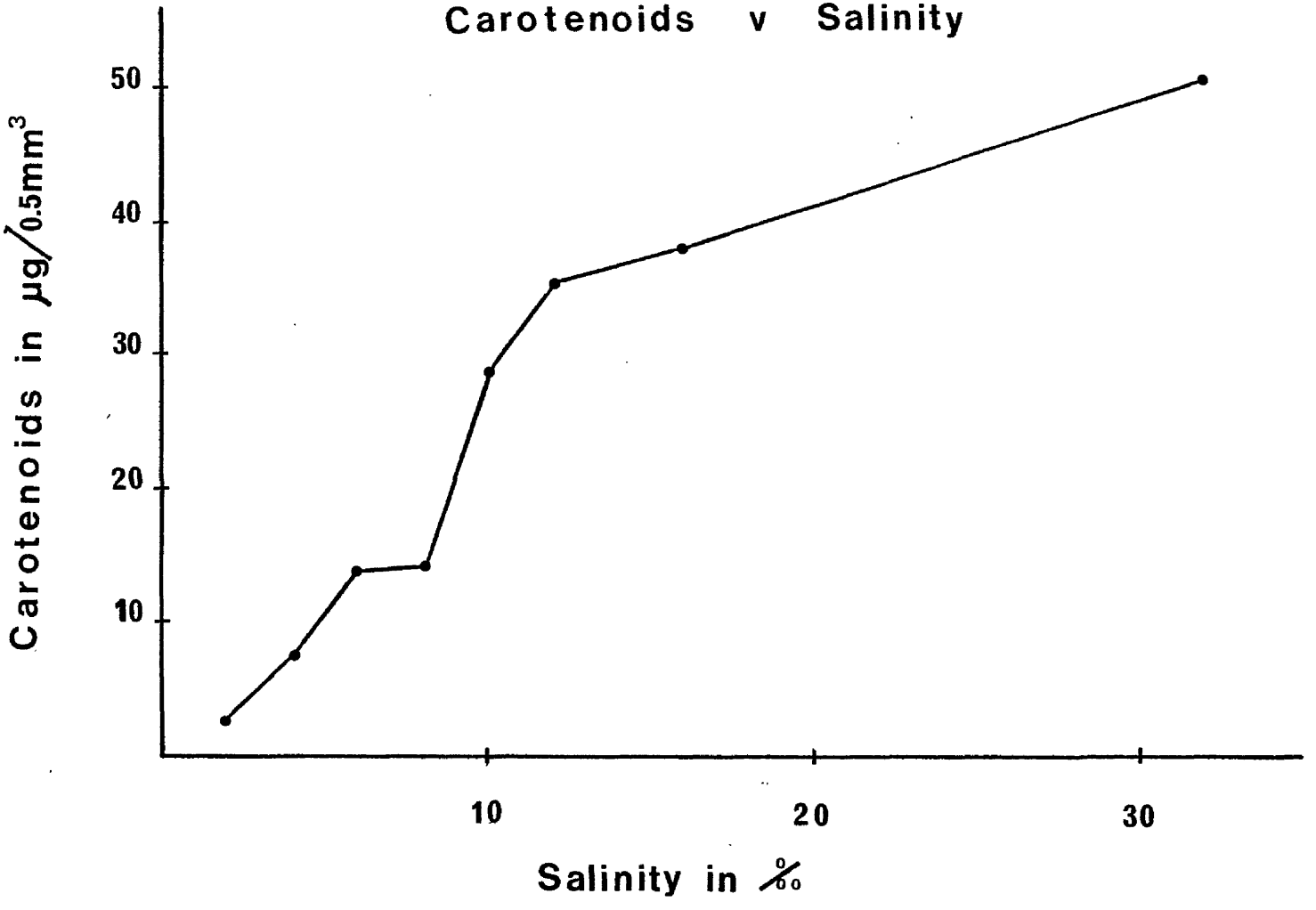

Carotenoids v Salinity
Carotenoids in µg/0.5mm³
50
40
30
20
10
10
20
30
Salinity in ‰

Table 3.7

Variance Ratios Carotenoids v Salinity

	2	4	6	8	10	12	16	32
2		+	+	+	+	+	+	+
4	335.9		+	+	+	+	+	+
6	1207	3.59		-	-	-	+	-
8	1515	4.51	1.25		-	-	+	-
10	1552	4.62	1.29	1.02		-	-	-
12	2465	7.34	2.04	1.63	1.59		-	+
16	11850	35.27	9.82	7.82	7.63	4.81		+
32	764	2.27	1.58	1.98	2.03	3.22	15.5	

FIGURE 3.11

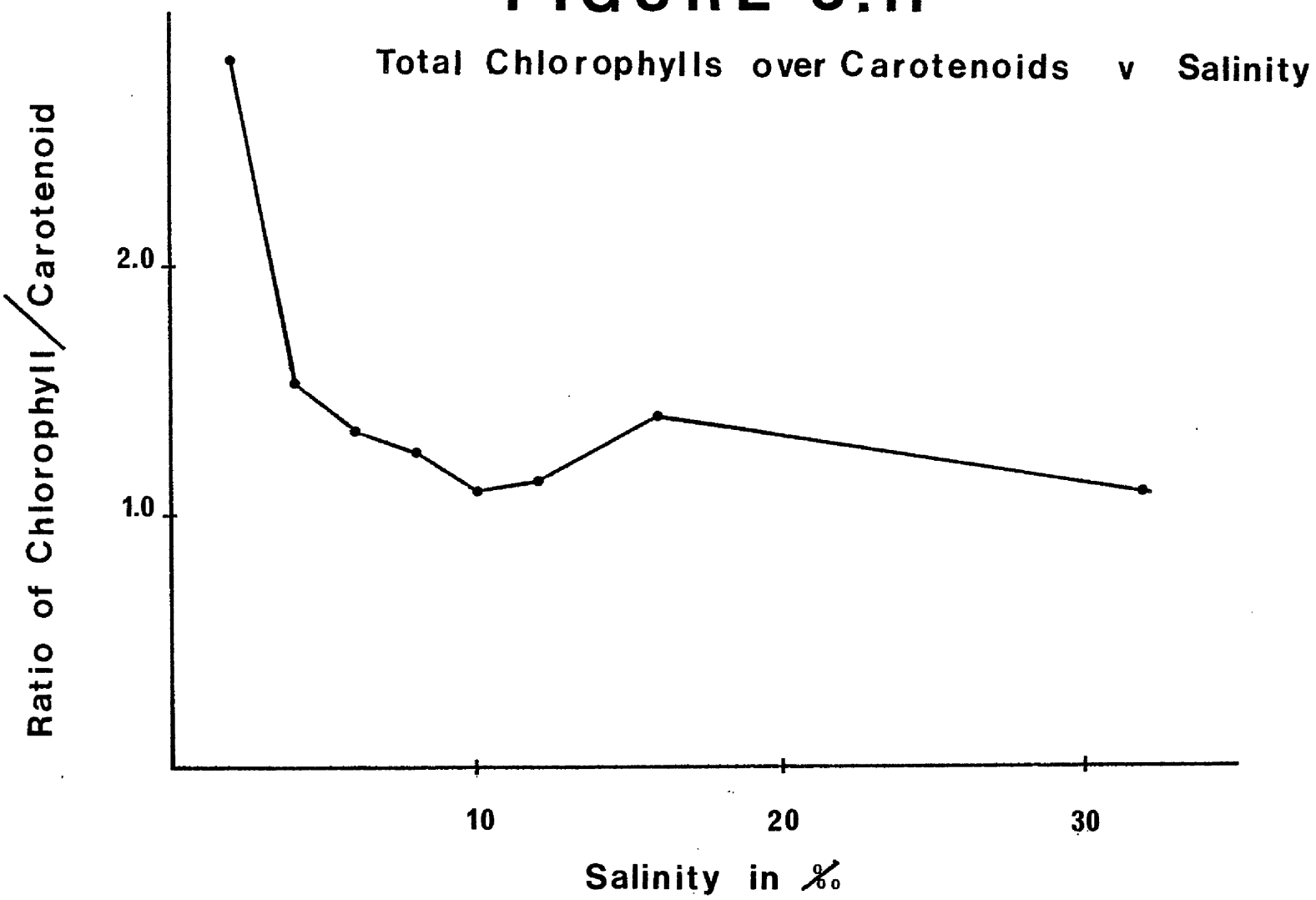
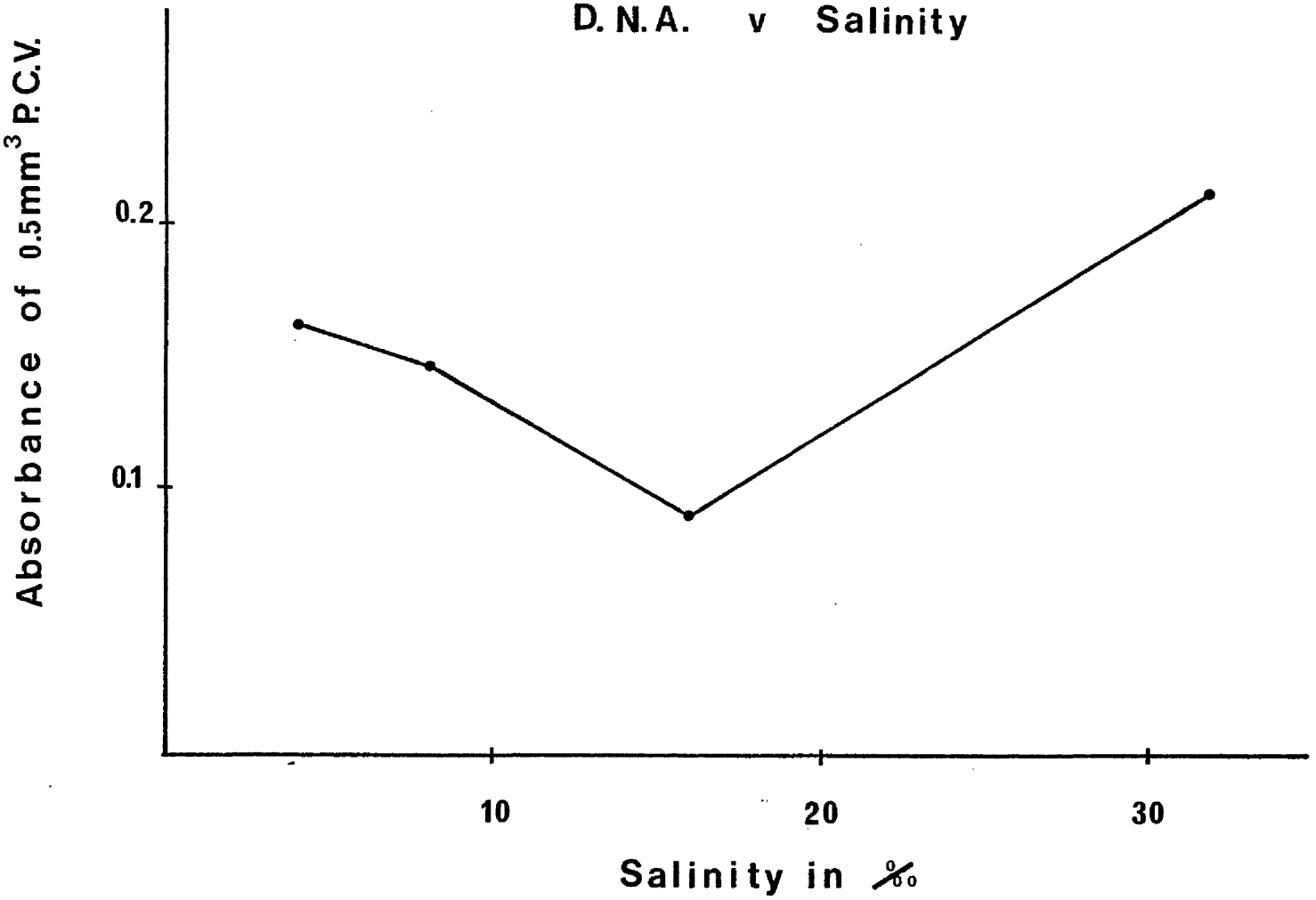

Total Chlorophylls over Carotenoids v Salinity
Ratio of Chlorophyll/Carotenoid
2.0
1.0
10
20
30
Salinity in ‰

Table 3.8

Variance Ratio Total Chlorophylls/Carotenoids v Salinity

	2	4	6	8	10	12	16	32
2		-	+	+	+	+	-	+
4	1.10		+	+	+	+	-	+
6	2.73	3.01		+	+	+	-	-
8	9.83	10.85	3.60		+	+	+	+
10	271.3	299.5	99.38	27.60		+	+	+
12	5292	5843	1938	538.4	19.51		+	+
16	1.95	2.16	1.40	5.03	138.97	2710		-
32	2.93	3.23	1.07	3.36	92.67	1807	1.50	

FIGURE 3.12


D. N. A. v Salinity
Absorbance of 0.5mm³ P.C.V.
0.2
0.1
10
20
30
Salinity in ‰

Due to the difficulty experienced in obtaining axenic cultures, and also to the fact that these were only obtained at the end of the project, the results are only available for salinities of $4^{o}/oo$ to $32^{o}/oo$.

3.5 Results of Long Term Growth at Different Salinities

The algae were grown in 100 ml of culture medium, at salinities of $2^{o}/oo$, $4^{o}/oo$, $8^{o}/oo$, $16^{o}/oo$ and $32^{o}/oo$, in 250 ml Erlenmeyer flasks, and were regularly examined over a period of six months.

In gross morphology, no significant differences could be seen between algae grown at the different salinities. Considerable attention was paid to the thickness of the mucilage layer, but at first there were no measurable differences. The growth habit of the cultures also remained the same. After about two months, differences began to become apparent. In general appearance, the cultures at the lower salinities, especially at $2^{o}/oo$ and $8^{o}/oo$, appeared to be ageing faster. Although the dimensions of the cells, and the thickness of the mucilage sheath, remained very similar, the mucilage in the lower salinity cultures showed much more obvious layering, and it was possible to see the layering in these cultures without using stains.

As ageing proceeded, all cells accumulated oil vesicles, confirmed by their positive reaction to Sudan Black, and this led to a gradual change in the appearance of the culture, from the normal green-brown, to an orange-green colour.

The experiment ran for six months, but during this period the cells did not adopt the senescent form of growth habit described previously (Chapter 3.1) although some of the signs of this, especially the presence of oil vesicles and layering of the mucilage had become apparent.

3.6 Results of Observations on Growth at Different Temperatures

Cultures of the algae were grown in 100 ml of medium in 250 ml Erlenmeyer flasks. The algae were grown at salinities from $2^{o}/oo$ to $32^{o}/oo$ at temperatures of $2^{o}C$, $10^{o}C$, $20^{o}C$ and $30^{o}C$, for 14 days. Observations were made on a regular basis during this time. All cultures were set up from the same stock culture, and the inoculum was the same for each.

At $30^{o}C$ the mucilage layer had increased in size within 24 hours and vesicles began to develop in the cells. Some of the vesicles stained positively with Sudan Black, showing an increase in the number of oil vesicles, but others showed no such positive reaction. After 48 hours the condition of the cells began to deteriorate and after four days the cultures contained a high proportion of dead cells.

At $20^{o}C$ the cells remained more normal in appearance at the high salinities. At $2^{o}/oo$ and $4^{o}/oo$ there was an increase in the number of vesicles present in the cell, some staining positively with Sudan Black.

The results at $10^{o}C$ were very similar to those at $20^{o}C$. Vesicles,

some proving to contain oil, were found to be present at $2^{o}/oo$ and $4^{o}/oo$, and were more abundant than at the higher salinities. At the higher salinities of $16^{o}/oo$ and $32^{o}/oo$ the overall appearance of the cells remained unchanged, and there was no increase in the number of vesicles present within the cells. The mucilage layers of the cells remained constant in size at all salinities.

A similar pattern was shown by the results at $2^{o}C$. The number of vesicles increased in cells grown at the lower salinities, whereas no change was apparent in cells grown at the higher salinities. The cells showed no change in the mucilage layers when grown at $16^{o}/oo$ and $32^{o}/oo$, but salinities of $4^{o}/oo$ and below, there was an increase in the thickness of the layer.

3.7 Results of Exposures to Drying

The algae were placed on glass slides, and allowed to dry out at temperatures from $2^{o}C$ to $30^{o}C$. One group was similarly treated, and frozen. At intervals of 24 hours, one slide was placed in a dish of culture medium, and the cultures were examined after three days for living cells. The test was made using Lissamine Green, which is taken up only by dead cells.

At both $20^{o}C$ and $30^{o}C$ the cells all died within 48 hours, as did the cells which were frozen.

Those at $10^{o}C$ showed some survival after six days, but the number of dead cells far outnumbered the living cells. At $2^{o}C$ survival was high after six days.

It was noted that the mucilage layer had become thicker in all living cells, especially in those cells which had survived for six days at 10°C.

3.8 Observations on Cultures in Different Media

Some cultures of Chrysotila were available which were in the senescent type of growth form. Samples were taken from this culture and transferred to ASP6 medium.

The cultures were examined daily and within seven days, there were signs of fresh growth round the top of the flask. Zoospores were found only once during examination of the cultures, but the growth of the "Sarcinochrysis" type of cells was typical of that of settled zoospores. Within three weeks the culture had changed to the "Sarcinochrysis" growth form, and all signs of the senescent type of cells had disappeared.

A second trial was set up using cell-free old media* One sample, in which Chrysotila had been grown, and one in which Prasinocladus marinus had been grown, was inoculated with "Sarcinochrysis"-type cells. The cells began to show changes in mucilage thickness after several days. Within two weeks the mucilage layer had increased in thickness and in many cells there were obvious signs of lamellations. The number of vesicles in the cells increased, and the pigmentation of the cultures began to change, becoming more brown in appearance. After one month the cells showed features of the senescent growth habit. No zoospores were found in these cultures, and there was no

* six months old

obvious tide line growth, as would be expected if zoospores had been released.

3.9 Experiments on Zoospores Release

Green and Parke (1974) reported some success in producing zoospore release in Chrysotila cultures, but were unable to do so consistently. Their method relied on osmotic and temperature shocks, either singly or combined, with a variety of culture media. In cultures which were used for other experiments, zoospores were found to be present, but in very low concentrations. The method used by Green and Parke was tried, and found to give inconsistent results.

The factors initially considered as affecting zoospores release were light, temperature, and osmotic shock.

A series of experiments was set up in which a sample of algae grown at $32^{o}/oo$ were inoculated into fresh media of salinities from $2^{o}/oo$ to $32^{o}/oo$, and maintained in 250 ml Erlenmeyer flasks at temperatures from $2^{o}C$ to $30^{o}C$.

First results were inconsistent. Investigation showed that aeration of the stock culture, as employed for cultures grown in Roux flasks, for 24 hours prior to inoculation affected the zoospore release. In all cultures in which aeration followed this sequence, the subsequent zoospore release followed a consistent pattern. In cases where the stock culture was not aerated, the results were inconsistent, and adhered to no obvious pattern. Further cultures were set up, following the sequence detailed above, but kept in

darkness. These showed no deviation from the pattern of release showed by cultures maintained in the normal light: dark regime.

The number of zoospores could not conveniently be estimated by either packed cell volume, or by absorption techniques. The results were obtained by microscopic examination of cultures, and the number of zoospores was related to the number of non-motile cells in the culture. The results are shown as the ratio of motile to non motile cells, and are summarised in Table 3.9 and Figures 3.13 to 3.17.

Table 3.9

ZOOSPORE RELEASE

Time In Hours After Inoculation

Salinity in °/oo	24				48				72				96				120			
	2°	10°	20°	30°	2°	10°	20°	30°	2°	10°	20°	30°	2°	10°	20°	30°	2°	10°	20°	30°
2°/oo	4	4	3	0	5	5	0	0	2	2	1	0	1	1	0	0	1	1	0	0
4°/oo	4	4	5	0	5	4	0	0	3	2	1	0	1	1	0	0	1	1	0	0
8°/oo	5	5	5	0	4	4	1	0	3	3	0	0	1	1	0	0	1	1	0	0
16°/oo	4	3	4	0	4	2	1	0	1	1	0	0	1	1	0	0	1	1	0	0
32°/oo	2	3	2	0	2	2	1	0	1	1	0	0	1	1	0	0	1	1	0	0

Temperature in °C

Relative Number of Zoospores

Key

0 = No Zoospores found

1 = Less than 1% of cells as zoospores

2 = 1 - 10% of cells as zoospores

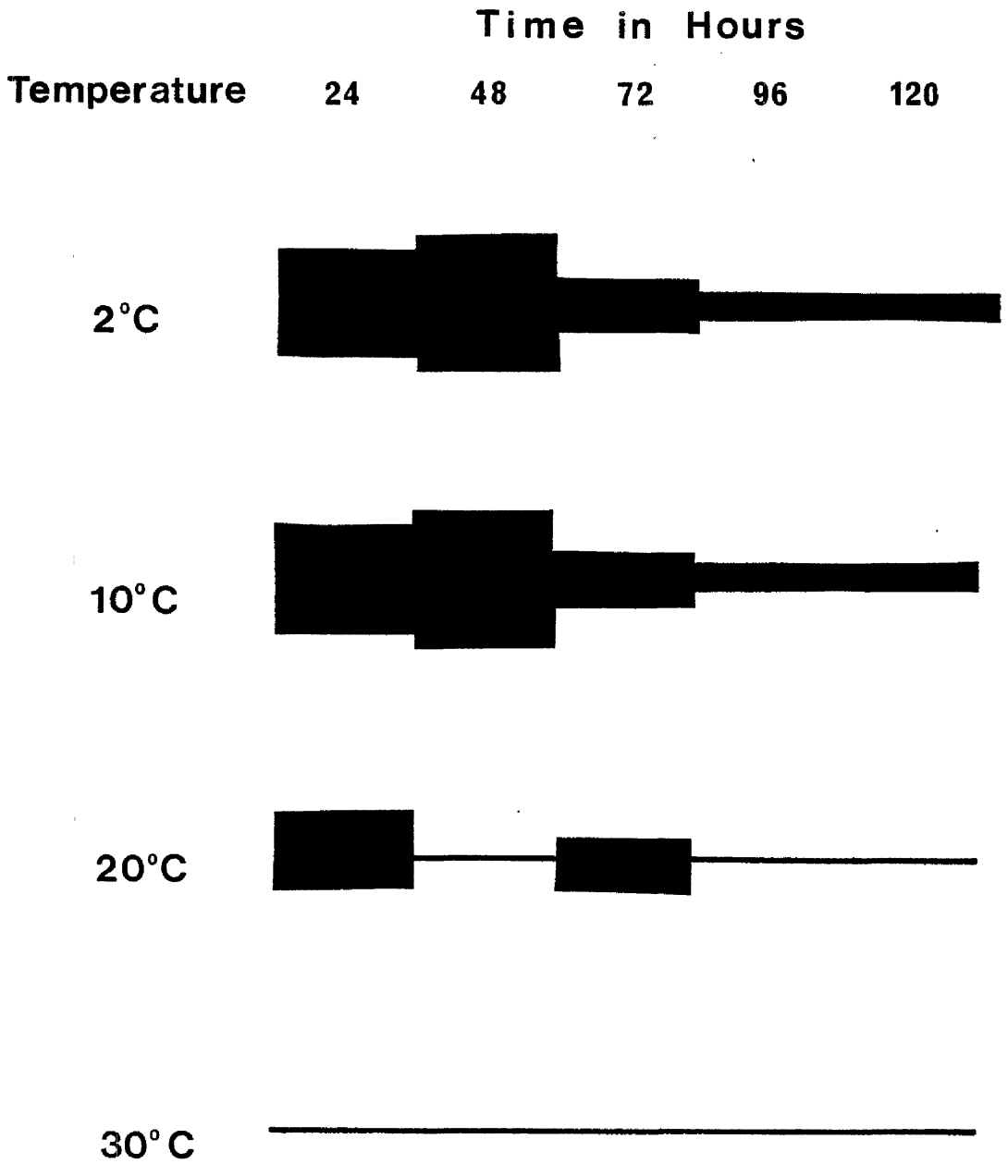
3 = 10% - 20% of cells as zoospores

4 = 20% - 50% of cells as zoospores

5 = Greater than 50%

FIGURE 3.13

Zoospore Release at 2‰



Key to Figures 3.13 to 3.17

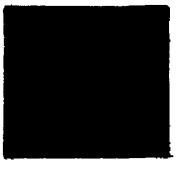
No Zoospores found

Less than 1% of cells as zoospores

1 - 10% of cells as zoospores

10% - 20% of cells as zoospores

20% - 50% of cells as zoospores



Greater than 50%

FIGURE 3.14

Zoospore Release at 4‰

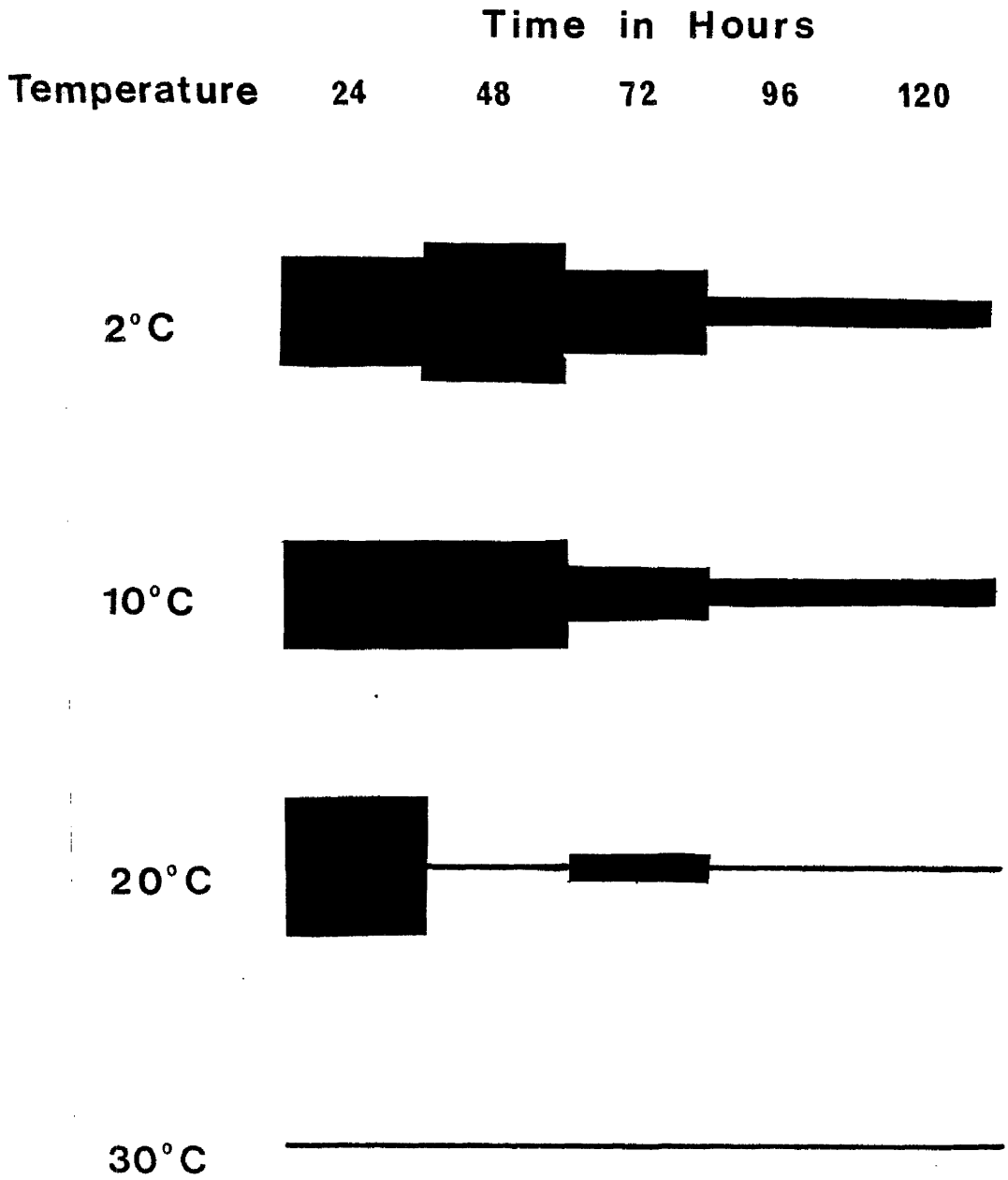


FIGURE 3.15

Zoospore Release at 8‰

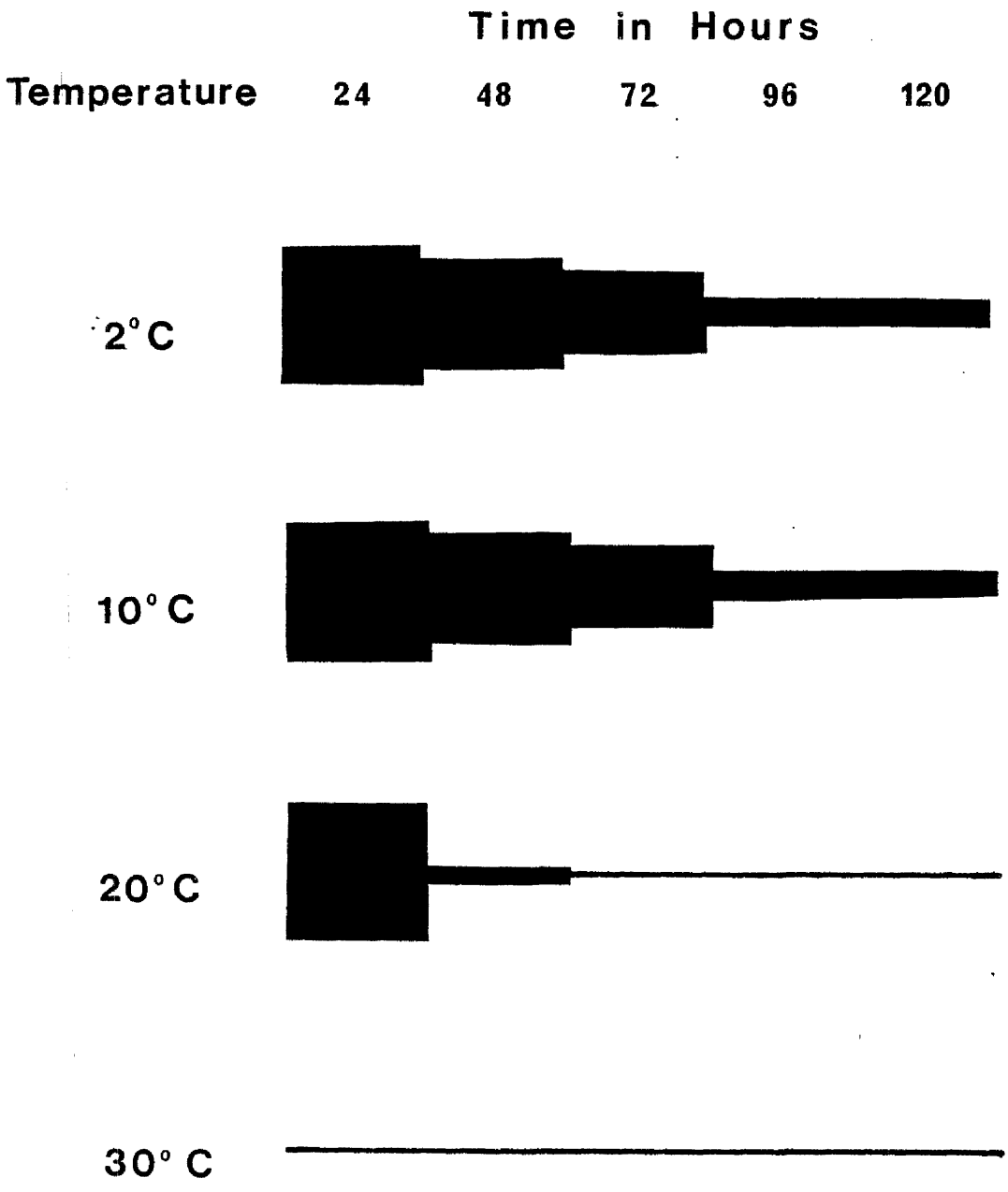


FIGURE 3.16

Zoospore Release at 16‰

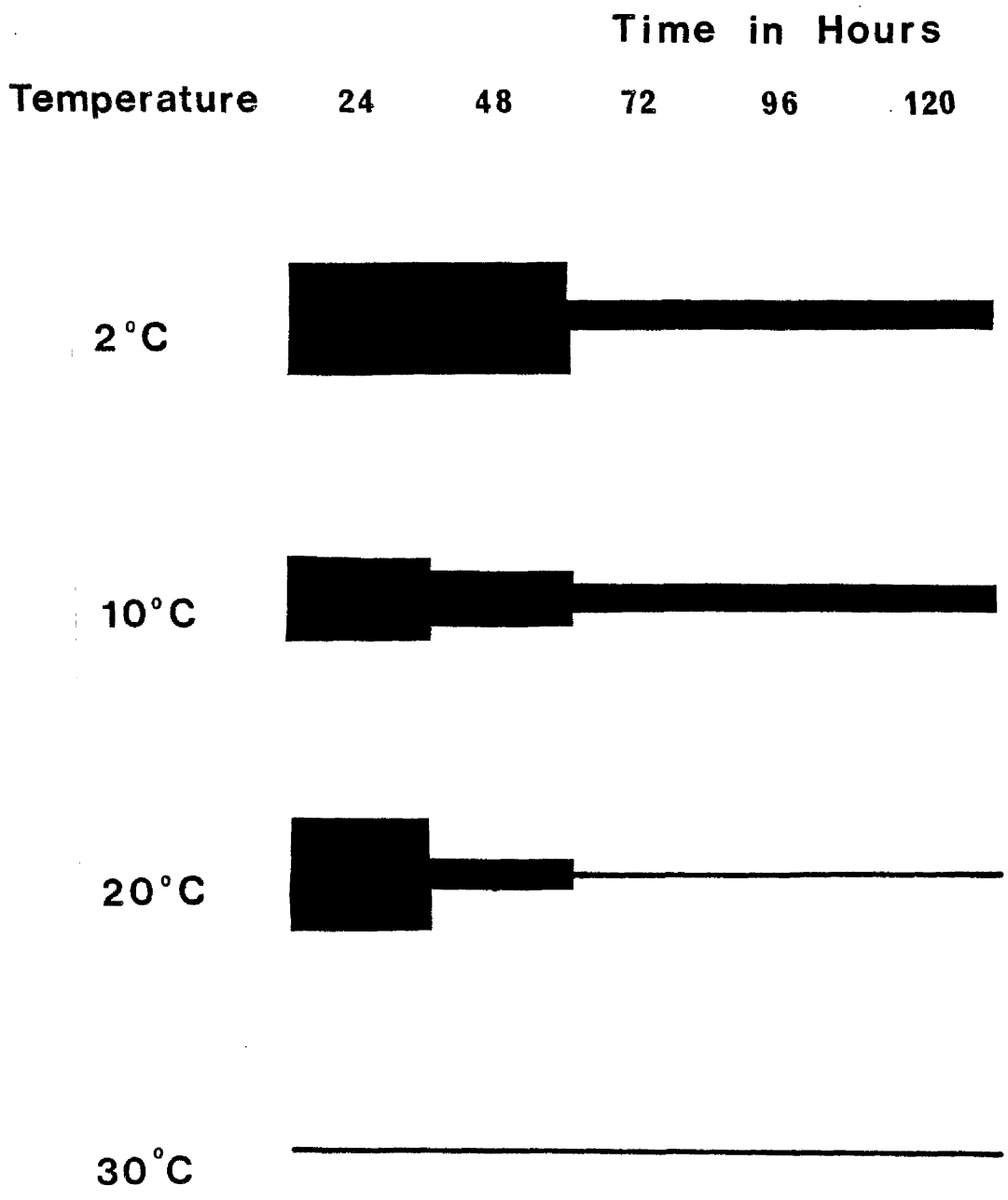
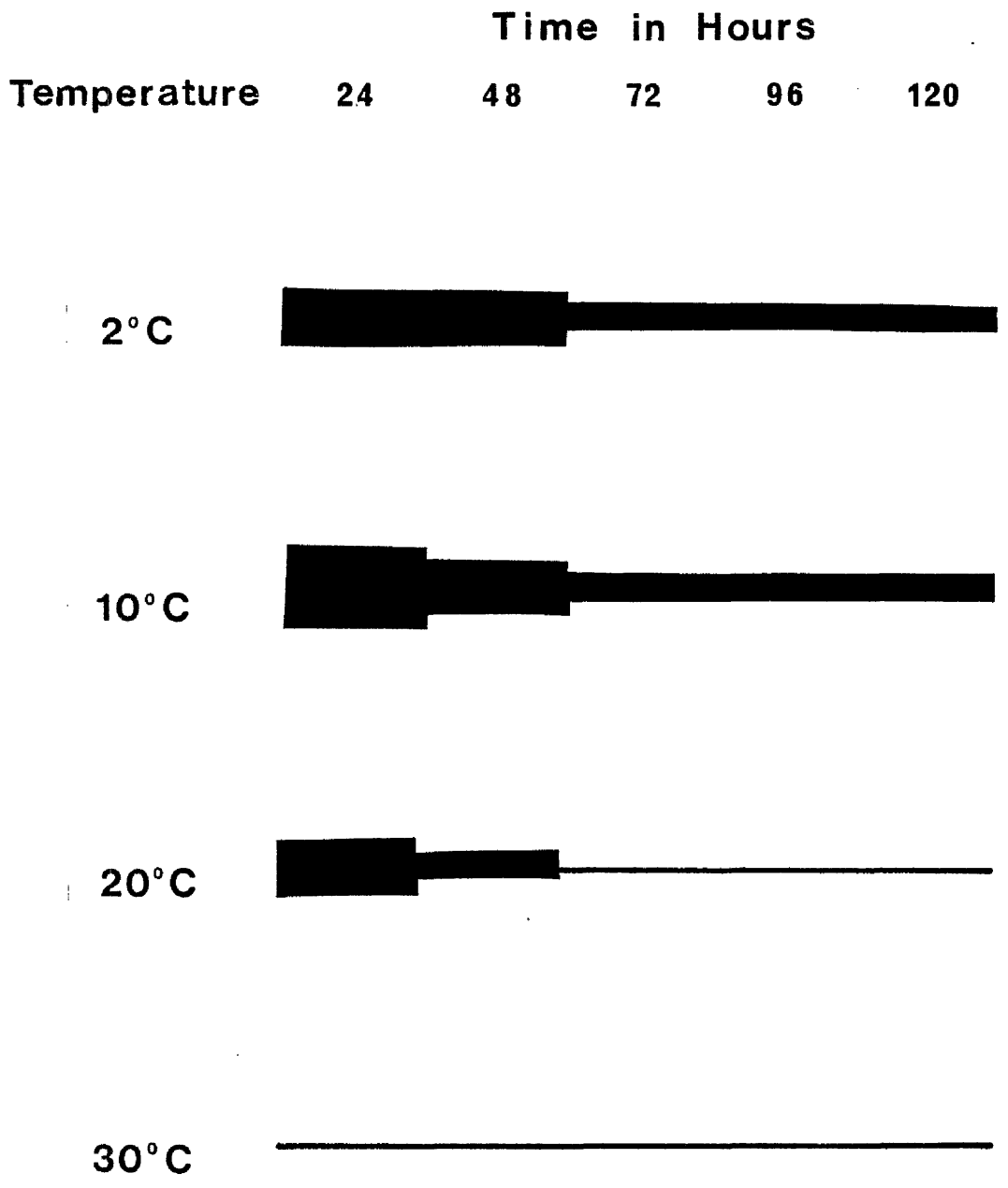


FIGURE 3.17

Zoospore Release at 32‰



CHAPTER FOUR

DISCUSSION

The principal aim of the project was to investigate the effects of salinity stress on the growth of Chrysotila lamellosa.

Until recently this genus has remained almost completely ignored, and at present interest is confined to its fine structure. These studies (Green and Parke, 1974, 1975) have shown that fundamental problems exist with respect to the classification of the alga, and its affinities with closely related species (Chapter 1.1). Scattered observations (Billard and Gayral, 1972; Dop and Vroman, 1977) have produced conflicting evidence on the habitat of this organism.

Ruttnera spectabilis was first isolated from a fresh water habitat (Geitler, 1942). It was then rediscovered in a marine habitat (Billard and Gayral, 1972) and subsequently shown to be conspecific with Chrysotila lamellosa (Green and Parke, 1975), which was also isolated from a marine habitat. The latter name has priority, as shown in Chapter 1.1. Recently the alga, described as Ruttnera spectabilis, has been found in a fresh water habitat by Dop and Vroman (1977).

Since morphologically identical forms of this organism have been found in both saline and fresh water habitats, there is a possibility that distinct physiological strains exist. This is borne out by

the findings of Green and Parke (1975) on the conspecificity of the two species mentioned above. It shows that this entity was once regarded as two separate organisms, simply on the basis of its habitat. This has resulted in the separation of the few observations available from past work.

The cultures used in the present work were initially transferred to ASP6 medium (Chapter 2.2), at $32^{o}/oo$ salinity, and all stocks were maintained in this medium. This was similar to the medium in which the cultures were supplied. In addition, this original isolate had been from seawater, and all other isolates had been maintained at this salinity.

The locus classicus of Ruttnera spectabilis is not marine, whereas that of Chrysotila lamellosa is, however there is some similarity between the two locations. No data exists for the salinity levels at the locus from which Ruttnera was isolated, but it will hardly be that of sea water. It is probable that the salinity is very low and variable at this site. The alga at this type of locality was found growing on damp rocks, and would be subject to flooding with fresh water, and to drying. This will cause a considerable variation in environmental conditions which the algae will have to tolerate. Similar variations in conditions exist at the site from which Chrysotila was isolated. The alga at this type locality was found growing on chalk cliffs exposed to the sea, and would be subject to spray from the sea, to fresh water from rain, and drainage from the cliff face. Since this is a marine site, the salinity variations will be much greater. What seems surprising is not its

ability to survive this variation, but its apparent ability to grow in such a large range of salinities.

The first group of experiments was designed to investigate the growth of the alga over 21 days. This figure was not chosen at random, but was a compromise between convenience and the results of the initial observations on growth rate. Since the project was undertaken on a part-time basis, observations could only be made at week-ends.

The results of this group of experiments confirmed that Chrysotila will grow at salinities from $2^{o}/oo$ to $32^{o}/oo$. They also show that this strain of the alga cannot grow in fresh water. In every case the alga died in culture solutions containing salts in only micro-nutrient concentrations.

The sampling and estimation of growth was made during the exponential phase. Microscopic examination was made on all cultures, when growth was being measured, and no dead or senescent cells were found. The inocula were of similar size for these experiments, and were selected to be large enough to compensate for any cell death, but not so large as to cause significant changes in the composition of the media. The light, temperature, and aeration rate were also kept constant between cultures.

The results were compared statistically and the significance at $p=0.2$ is shown in Table 3.3.

Figure 3.1 summarises these results, and certain features of this

graph are particularly striking. Growth in general seems to be greater at the lower salinities of $2^{o}/oo$ to $10^{o}/oo$. Within this range, two salinities stand out, $2^{o}/oo$ and $8^{o}/oo$. At these salinities growth is at its peak. This feature is confirmed statistically in Table 3.3. The growth at these two salinities is shown to be significantly different from growth at all other salinities. In general, growth at other salinities is fairly similar, with no significant differences.

One explanation for these apparent anomalies could be the choice of a 21 day growth period. It is possible that, due to a combination of the length of the lag phase, and other factors, the two salinities of $2^{o}/oo$ and $8^{o}/oo$ became particularly favourable to the growth of this strain of the alga.

The second group of experiments was therefore designed to give more information on the growth of the alga, and in particular to allow growth curves to be drawn for each salinity.

The estimation of packed cell volume by removal of cells was considered unsuitable and the estimation of growth by optical methods was adopted (Chapter 2.5). In this way daily estimations of growth were obtained with minimum disturbance of the culture. Fourteen days however, proved to be the maximum time over which the results could be taken. This was due to the build up of bacteria in the cultures. This build up was monitored microscopically when the samples were taken. After fourteen days the level of bacterial contamination was beginning to increase, and although still very low

Table 4.1

Growth Constants for Cultures

Salinity (%)	Growth Constants (all x 10^{-3}) 21 day Expt.	14 day Expt.
2	3.32	2.93
4	2.75	3.01
6	2.64	-
8	3.43	3.78
10	3.37	-
16	2.67	4.33
32	2.22	3.87

$$\text{Growth Constant } K = \frac{\ln N - \ln No}{t}$$

at this time compared to the algal population, the experiment was stopped. This decision was taken because at this stage the bacteria were still not detectable with the nephelometer, but it was felt that they would soon begin to affect the optical reading. In addition, a high bacterial population would begin to affect the constitution of the growth medium, and the experiment was stopped before this effect became noticeable.

The results, summarised graphically in Figures 3.2 to 3.6 conflict with those of the 21 day growth period. It can be seen clearly from these graphs that growth is most rapid at the higher salinities of $8^{o}/oo$, $16^{o}/oo$, and $32^{o}/oo$.

The best estimation of growth at different salinities is the growth constant of the culture during the exponential phase (Fogg, 1965). This has been calculated for both the 21 day growth experiments, and the 14 day growth curve experiments. These are shown in Table 4.1. In comparing these, the assumption is made that both cultures are in the exponential phase of growth, and from previous observations, this is shown to be so.

The results do not match. The second group are however, more those which would have been expected. This strain of the alga has been maintained at high salinities for a considerable length of time. It would therefore be expected to grow more quickly at higher salinities, and this is confirmed by these results.

One strain of this alga, the "Ruttnera spectabilis" form is adapted to the low salinity conditions present at the locus classicus,

whereas the "Chrysotila lamellosa" form is adapted to high salinities. Since these two forms are conspecific, and the strain used in this work has been maintained at high salinities for some considerable length of time, it would be expected that the ability to grow in low salinities might be lost, or at least, selected against.

The length of the lag phase tends to confirm this. As can be seen from the growth curves, the lag phase is very short at high salinities, suggesting that the alga is well adapted to growth at these levels. However, below $8^{o}/oo$, the length of the lag phase increases. This suggests that the algae require some time to adapt to these lower salinities, before growth can take place.

These two factors, the higher growth constant, and the shorter lag phase at high salinities are such obvious features that the first set of results; growth over 21 days, would appear wrong. Since the results were carefully measured, and several replicates were taken, then some doubt may be cast on the validity of applying these methods to studies on this organism. However these techniques are known to be reliable, and have been used successfully (Hadi, 1974).

If the results are to be accepted as valid, then some other feature must be responsible for the discrepancy between these methods. The explanation may well be in the behaviour of zoospores in the cultures.

The release of zoospores by cultures of the alga was studied in some detail, and the results show a distinct and repeatable pattern. Green and Parke (1974) reported inconsistent results for zoospore

release, and believed that osmotic shock was the most important factor.

Consistent, and synchronised release in fact appears to be related to a number of factors, the most important of which are aeration and temperature. The salinity of the medium may enhance the effect, especially at low salinities, but it is not the most important factor. The actual transfer to fresh medium is more important than the effect of osmotic shock. This is shown by the fact that Chrysotila cells at $32^{o}/oo$, transferred to fresh medium at the same salinity, gave a good release of zoospores. If this transfer is coupled with osmotic shock, the release is increased, but follows the same general pattern. Light has no effect, as several experiments were set up in total darkness, and zoospore release followed the same pattern. Below $20^{o}C$ temperature has no marked effect on the release of zoospores, but above this temperature release is inhibited. At $20^{o}C$ the release at first is comparable to that at lower temperatures, but it is not maintained, and within 48 hours it has virtually ceased. At $30^{o}C$ there is no release of zoospores. This is to be expected, as shown previously (Chapter 3.6) this temperature has adverse effect on growth.

Aeration is a vital factor in release. If the culture is not aerated, both before transfer, and after, the zoospore release is low, and erratic. The consistent release of zoospores, adhering to the pattern already described, only occurs when cultures are aerated.

These results explain the differences in the growth experiments. Both sets of experiments were taken from stocks maintained in as near identical conditions as possible. These stocks were also aerated in medium which was changed at regular intervals. During growth, the experimental cultures were aerated and kept at 12°C, and so zoospore release would have occurred in each culture, following the pattern already established. The difference in results can therefore be related to the difference in times, measuring techniques, and the effects of zoospore release.

In the 21 day cultures, the initial zoospore release will have run its course, within about four days, and then settled to become part of the non-motile culture. When these cultures were harvested, the entire contents were centrifuged, and the packed cell volume measured. In these cultures the growth of the non motile cells, plus the growth of the settled zoospores will have been measured, and since there is an enhanced release of zoospores at lower salinities these will have changed the expected result, resulting in more growth at lower salinities. This release will also have occurred in the 14 day growth cultures, but two factors affect the result. First, the time of the experiment is shorter, giving less time for the zoospores to settle, and begin growth and multiplication. The second factor is the method of measurement. The cultures were agitated, then transferred to small tubes for optical measurement. Since the zoospores are negatively geotactic, during measurement they would move upwards, and out of the measuring area. In the 14 day experiments therefore, the measurement is

Table 4.2

Summary of Pigment Analysis

Salinity ($^{o}/_{oo}$)	chlorophyll a	chlorophyll c (in $\mu g/0.5\ cm^3$)	carotenoids
2	1.02	5.92	2.48
4	3.50	8.28	7.70
6	4.30	13.78	13.85
8	5.05	12.09	13.94
10	9.98	22.71	28.56
12	13.83	28.05	35.36
16	11.17	28.59	37.39
32	14.56	47.64	50.07

primarily that of the growth of the non-motile cells. The apparent enhancement of growth at low salinity may be interpreted as a stress reaction, in which a forced release of zoospores subsequently leads to a larger biomass of the culture. The stress effect may not however lead to continued healthy growth of the organism. The pigment analysis shows that chlorophyll c and carotenoids are the most abundant pigments. Chlorophyll a is also present, but at considerably lower concentrations. A summary of the results is shown in Table 4.2.

A general trend is obvious in these pigment concentrations (Figures 3.8 to 3.10) as all three pigments are low, at low salinities, and increase with increasing salinity. The increase is greatest from $2^{o}/oo$ to $12^{o}/oo$ and subsequently becomes less pronounced.

It was possible when examining pigment concentrations, to make extra observations, and this is why results are available for the salinities at $6^{o}/oo$, to $10^{o}/oo$, and $12^{o}/oo$.

From these results, variance ratios were calculated, to assess the significance of the increase in concentration of pigments with increasing salinity. These results, with $p=0.2$, are shown in Tables 3.5 to 3.8. The differences between concentrations at $2^{o}/oo$ and $4^{o}/oo$ are significant, and in most cases, the differences are also significant at $6^{o}/oo$. This confirms the observation that the amount of pigment is low at these salinities, but that it increases more rapidly between $2^{o}/oo$ and $6^{o}/oo$, than it does between $8^{o}/oo$ and $32^{o}/oo$.

The ratio of total chlorophylls to carotenoids shows a general decrease with increasing salinity (Figure 3.11) and a comparison of variance ratios indicates that these results are significantly different for p=0.2 (Table 3.8). As the salinity increases, therefore, although all pigments show an increase in concentration, the concentration of carotenoids increases significantly more than that of the total chlorophylls. Table 4.2 shows that at $32^{o}/oo$ the carotenoids are the most abundant pigments. This can be observed in cultures of Chrysotila.

Although there is little difference, visually, between cultures grown at similar salinities, such as at $2^{o}/oo$ and $4^{o}/oo$, or $16^{o}/oo$ and $32^{o}/oo$, the cultures grown at low salinities are obviously different from those grown at high salinities. The cultures grown at high salinities are a distinct golden green, whereas those at the lower salinities are a darker, grey-green colour.

It was believed that the amount of pigment would give some indication of the reaction of the alga to the various salinities, and this has been confirmed by the results. The carotenoid concentrations, and the ratios, show how this alga is reacting to its conditions. It would be expected that this alga would contain a high proportion of carotenoids, and it does so at high salinities. However, at low salinities, not only are all pigments low in concentration, but particularly the carotenoids, indicating that this organism is under some stress at these low salinities.

The concentration of DNA at different salinities must be treated

with some caution. These results were obtained at the end of the project, and more work is required before conclusions can be drawn. The DNA content apparently falls with increasing salinity, except for the result at $32^{o}/oo$, suggesting that as salinity increases there is a smaller number of larger cells.

As stated in Chapter 3, the algae proved very difficult to handle in axenic conditions. The growth was slower than normal, and the isolation of axenic cultures was extremely difficult. No cultures were obtained at $2^{o}/oo$, and this result, in consideration with the general trends of this organism at low salinities, is significant. The fact that at such low salinities the alga is already under stress, is probably the cause of this failure. In normal cultures, the bacterial population may release nutrients useful to the alga, and possibly essential to the organism when under stress. When this factor is not present, and the alga is already under osmotic stress, it is unable to survive. It is possible that the use of a different medium could prove successful for isolation of axenic cultures.

In certain cases, environmental stress leads to the formation of increased mucilage thickness around the cell, or to the build up of vesicles within the cell.

When the algae are grown at high temperatures, they will be under considerable stress. At $30^{o}C$ it was found that the thickness of the mucilage layer increased very rapidly (Chapter 3.6). Vesicles also began to appear, and although these initially, were shown to be

oil vesicles, confirmed by their reaction to Sudan Black, other vesicles began to appear within 48 hours. It was believed that these non-staining vesicles were breakdown products of the cell. Within four days, many of the cells were dead.

At $20^{o}C$ the cells grown at high salinities, of $16^{o}/oo$ and $32^{o}/oo$, contained a few oil vesicles, but it was found that the cells at lower salinities generally contained more vesicles, especially at $2^{o}/oo$ and $4^{o}/oo$.

It was also found that at $2^{o}C$, cells grown at $2^{o}/oo$ and $4^{o}/oo$ showed a distinctly thicker mucilage layer than those at higher salinities.

The increase in the number of oil vesicles can be interpreted as a reaction to stress conditions. Messer and Ben-Shaul (1970) showed that enforced senescence on the dinoflagellate Peridinium westii caused a considerable increase in lipid material in the cell, and also caused the replacement of the theca by a thick rigid envelope. A similar study by Palisano and Walne (1972) showed that when cells of Euglena granata were "aged" they accumulated numerous cytoplasmic granules and lysosome-like structures. In Chrysotila, it is significant that the increase in mucilage thickness, and in the number of cytoplasmic vesicles, is most pronounced at salinities of $2^{o}/oo$ and $4^{o}/oo$.

When the cells were subjected to drying, it was found that where cells had survived, their mucilage layer had become thicker (Chapter 3.7).

These experiments were designed to elucidate the reactions of the algae to conditions of extreme stress. One important factor, which appears in all these results, is the effect of salinity. In all cases the reaction is more extreme at low salinities. This suggests that, for this strain of the alga, low salinities, especially of $2^{o}/oo$ and $4^{o}/oo$, are stressful, whereas high salinities are more favourable.

In these experiments, zoospores were not found. This is probably an indication of extreme stress. Slight environmental stress produces an enhanced release of zoospores, but conditions of extreme stress inhibit production.

The results of this work suggest that this strain of the alga is adapted for growth in high salinities, growing best at salinities of $16^{o}/oo$ and $32^{o}/oo$. Growth at low salinities is partly due to the effects of the salinity on zoospores release, as the low salinities enhance the production of zoospores. The alga is protected to some extent from environmental stress by the mucilage layer which surrounds it.

This layer causes many problems in investigating the organism. It acts as a reservoir of bacteria, making the isolation of the alga in axenic conditions very difficult. It also affects the determination of packed cell volume. Biochemical investigations are made more difficult as the mucilage protects the cell from chemicals, and from mechanical shock. Some of these difficulties have been overcome, but others remain to be solved.

In nature, Chrysotila lamellosa is found in the supralittoral fringe, and the "Ruttnera spectabilis" form is found on damp rocks in a terrestrial environment. In both these habitats, the organisms are subjected to considerable environmental stress.

In the "Ruttnera" form the mucilage layer, which presented so many unforseen problems in experimental manipulations, may well act as a buffer between the cell and its environment. In the "Chrysotila" form the lamellated wall layers probably fulfil the same function.

These two forms of this organism show similar abilities to withstand conditions of salinity stress. This may be due to the length of time in which both have been maintained in culture. It would, therefore, be of considerable interest to investigate new isolates obtained directly from the respective loci classici.

REFERENCES

Anand, P. L. (1973)
A taxonomic study of the algae of British chalk-cliffs
Journal of Botany vol. 7, supplement 2, pp 1 - 51

Billard, C. and Gayral, P. (1972)
Two new species of Isochrysis with remarks on the genus Ruttnera
British Phycological Journal vol. 7 pp 289 - 297.

Bourelly, P. (1968)
Les algues jaunes et brunes, Chrysophycees, Pheophycees, Xanthophycees, et Diatomees.
Les algues d'eau douce: initiation a la systematique
vol. 2 438pp N. Boubee et Cie

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.

Dop, A. J. and Vroman, M. (1977)
Observations on some interesting freshwater algae from the Netherlands.
Acta Botanica Neerlandica vol. 25 pp 321 - 328.

Fogg, G. E. (1965)
Algal Cultures and Phytoplankton Ecology
Athlone Press University of London 126 pp.

Geitler, L. (1942)
Neue luftlebige Algen aus Wien.
Osterreichische botanische Zeitschrift vol. 91 pp 49 - 51.

Geitler. L. (1943)
Eine neue atmophytische Chrysophycee, Ruttnera spectabilis, nov. gen., nov. spec.
Internationale Revue der gesamten Hydrobiologie u Hydrographie vol. 43 pp 100 - 109.

Green, J. C. and Parke, M. (1974)
A reinvestigation by light and electron microscopy of Ruttnera spectabilis Geitler (Haptophyceae) with special reference to the structure of the zoids.
Journal of the Marine Biological Association of the United Kingdom vol. 54 pp 539 - 550.

Green, J. C. and Parke, M. (1975)
New observations on the genus Chrysotila Anand, with remarks on their relationship within the Haptophyceae.
Journal of the Marine Biological Association of the United Kingdom vol. 55 pp 109 - 121.

Guillard, R. R. L. (1973)
in Stein, J. R. ed.,
Handbook of Phycological Methods
Cambridge University Press 488 pp.

Hadi, R. A. (1974)
M.Sc. Thesis. University of Glasgow.

Hoshaw, W. H. and Rosowski, J. R. (1973)
Methods for microscopic algae
in Stein, J. R. ed.,
Handbook of Phycological Methods
Cambridge University Press 488 pp.

Hibberd, D.J. (1976)
The ultrastructure and taxonomy of the Chrysophyceae and Prymnesiophyceae (Haptophyceae): a survey with some new observations on the ultrastructure of the Chrysophyceae
Botanical Journal of the Linnean Society vol. 72 pp 55-80.

McLachlan, J. (1973)
Growth media - marine
in Stein, J. R. ed.,
Handbook of Phycological Methods
Cambridge University Press 488pp.

Manton, I. (1967)
Further observations on the fine structure of Chrysochromulina chiton with special reference to the haptonema, 'peculiar' Golgi structure and scale production.
Journal of Cell Science vol. 2 pp 265 - 272.

Manton, I. and Leedale, G. F. (1961)
Further observations on the fine structure of Chrysochromulina kappa with specific reference to the pyrenoids.
Journal of the Marine Biological Association of the United Kingdom vol. 41 pp 519 - 526.

Manton, I. and Leedale, G. F. (1963)
Observations on the micro-anatomy of Crystallolithus hyalinus Gaarder and Markale.
Archiv fur Microbiologie vol. 47 pp 115 - 136.

Messer, G. and Ben-Shaul, (1970)
Fine structure of Peridinium westii Lemm., a freshwater dinoflagellate.
Journal of Protozoology vol. 16(2) pp 272 - 280.

Palisano, J. R. and Walne, P. L. (1972)
Acid phosphatase activity and ultrastructure of aged cells of Euglena granulata
Journal of Phycology vol. 8 pp 81 - 88.

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 Dixon, P. S. (1976)
Check-list of British marine algae - Third revision.
Journal of the Marine Biological Association of the United Kingdom vol. 56 pp 527 - 594.

Parsons, T. R. and Strickland, J. D. H. (1963)
Discussion of spectophotometric determination of marine plant pigments, with revised equations for ascertaining chlorophylls and carotenoids.
Journal of Marine Research vol. 21 pp 155 - 163.

Provasoli, L. (1963)
Growing marine seaweeds in
De Virville, D. and Feldmann, J., eds.,
Proceedings of the International Seaweed Symposium
vol. 4 pp 9 - 17.

Provasoli, L., McLaughlin, J. J. A., and Droop, M. R. (1957)
The development of artificial media for marine algae
Archiv fur Microbiologie vol. 25 pp 392 - 428.

Schneider, W. C. (1946)
Phosphorus compounds in animal tissues I. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid
Journal of Biological Chemistry vol. 161 pp 293 - 303.

Strickland, J. D. H. and Parsons, T. R. (1968
A practical handbook of seawater analysis
Bulletin Fisheries Research Board of Canada
No. 167 311pp.