CULTURE AND GROWTH KINETICS OF SELECTED NANOPLANKTERS

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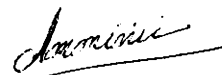OF THE
UNIVERSITY OF COCHIN

BY
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COCHIN-682018

DECEMBER 1983

Declaration

I hereby declare that this thesis entitled "CULTURE AND GROWTH KINETICS OF SELECTED NANOPLANKTERS" has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.
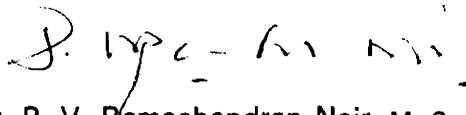
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Certificate

This is to certify that the thesis entitled "CULTURE AND GROWTH KINETICS OF SELECTED NANOPLANKTERS" is the bonafide record of the work carried out by Miss AMMINI JOSEPH under my guidance and supervision and that no part thereof has been presented for any other Degree.

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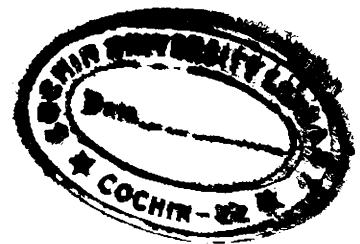
P R E F A C E

The candidate after passing M.Sc. in Genetics and
Plant Breeding from the University of Kerala joined the
Centre of Advanced Studies in Mariculture, Central Marine
Fisheries Research Institute as a Senior Research Scholar
in the first batch of the Ph.D. programme in July, 1980.
During the first semester, the candidate underwent an
intensive course work in mariculture comprising the
following aspects: Biology and culture of fishes,
crustaceans, molluscs, seaweeds and live-food organisms,
biochemistry, statistics, research methodologies, farm
engineering and aquaculture management. The optional
subject i.e. 'Structure and Reproduction of Algae' was
studied in detail during the second semester. During
the same period the candidate also passed the qualifying
examination conducted by the Cochin University in
December, 1981.

The research work for the thesis was started
during the second semester. Several field trips were
undertaken in the backwaters and inshore waters of Cochin
on board the research vessels of the Institute. During
this period she also got familiarised with various
analytical techniques connected with the marine biology

and oceanography work programmes. For certain
specialised work the candidate made trips to the
Environmental Survey Laboratory and Reactor Research
Centre, Kalpakkam. The Liquid Scintillation Counter
availaole at the ESL was made use of for certain studies
with metabolites of nanoplankters. The SEM facility at
RRC helped in understanding the nature of flagella of the
flagellates isolated by the candidate. In connection with
field trials, the candidate made trips to Mandapam to
familiarise with the higher algae and its cultivation as
a part of the course work and to do feed trials with
oysters at the newly developed hatchery at Tuticorin. Most
of the other facilities were developed by the candidate on her
own initiative and with the guidance of the scientists,
technical staff and fellow scholars. The culture tube
for continuous culture was developed locally and modified
for the particular requirements based on a photograph of
the tube used by Prof. Stemann Nielsen in the Danish
Pharmaceutical Institute, Copenhagen. The modified version
has got much more advantages in the fact that inoculation
and sampling could be done without contamination.

Similarly the water bath for carrying out
physiological study under different temperature was designed
and fabricated at the Institute. It could maintain

temperature within $\pm 0.5^\circ$C. Thus many of the facilities for the experimental work had to be developed or improvised locally and in certain cases make use of the facilities available with sister institutions elsewhere. This involved the co-operation from colleagues and scientists whose willing help enabled to complete this work in the minimum possible time. They have been individually acknowledged in the following pages.

Cochin-18,
November, 1983.

(AMMINI JOSEPH)

CULTURE AND GROWTH KINETICS OF SELECTED NANOPLANKTERS

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1. INTRODUCTION

The biomass of phytoplankton and primary productivity in the sea have always been the basis in the estimation of the relative fertility and potential fishery resources. Net sampling was one of the accepted and widely used methods for assessment of population composition and biomass. Lohmann (1903) drew attention to the fact that an unknown proportion of biomass was lost by net sampling since the cells of many organisms were too small to be retained. He applied the term 'nannoplankton' (more often referred to as the 'nanoplankton') to the autotrophic plankters that passed through the meshes of fine silk net. Holligan and Harbour (1977) restricted the term to photosynthetic organisms and used 'Nanophytoplankton' instead of nanoplankton.

Several schemes have been proposed for the size classification of the nanoplankton. The maximum size as visualized by Lohmann (1903) is 50-60μ. But later, when plankton nets of smaller mesh size became available, further fractionation was possible. 'Some authors suggest separating the very smallest cells (less than 5 microns) as 'ultra plankton' and to limit the nanoplankton to algae not exceeding 30μ' (Raymont, 1980). Dussart (1965) and

Hannah and Boney (1983) consider the maximum dimensions
to be less than 20μ.

Irrespective of size the nanoplankton includes
algae from different classes: Chrysophyceae, Haptophyceae,
Prasinophyceae, Chlorophyceae, Dinophyceae, Bacillariophyceae
and Cyanophyceae. In structural organisation they may be
naked flagellates, thick walled diatoms, dinoflagellates,
silicoflagellates or coccolithophorids and coccoid forms.
The life history and structure of many of these have been
studied by investigators such as Parke, Manton, Green,
Paasche, Dodge, Boney and Bernard.

The contribution of nanoplankton to the primary
productivity of the seas has been much investigated
(cf. Raymont, 1980). Most of these works show that the
contribution of the nanoplankton fraction in terms of cell
numbers, chlorophyll a content and carbon fixation,
whilst variable, represent sizeable proportions (50-100%
of the total) irrespective of whether these data refer to
polar, temperate or tropical seas. Some investigators
point out that nanoplankton dominate the oceanic waters
and in low latitudes while net plankton are abundant in
the neritic waters and high latitudes.

For temperate waters it has been suggested that in
many areas, nanoplankton becomes important after the spring

diatom outburst (Holligan and Harbour, 1977). Flagellates
of less than 10μ have been reported to form a stable
component of the Scotland Sea. Coccolithophorids abound
in some seas, though minute diatoms are also not excluded
(Collier and Murphy, 1962; Beers et al., 1975; Throndsen,
1976).

Nanoplankton population of warmer seas have been
found to be significantly high. Hulburt, Ryther and
Guillard (1960) estimated that in the Burmuda Region the
nanoplankton population was at least hundred times denser
than the larger phytoplankton. Further studies on the
nanoplankton of the tropical waters have been done by
Wood (1963), Tundisi (1971) and Malone (1971a,b,c) to
mention a few. Saijo (1964) did the size fractionation
of the Indian Ocean phytoplankton. He observed that the
size group between 0.8 and 90 microns contributed 50% or
more to the productivity compared to the larger net plankton.
Devassy and Bhattathiri (1981) reported that the
nanoplankton chlorophyll a averaged 0.034 mg/m^3 as against
0.043 mg/a^3 for the net plankton in the Andaman sea during
the year 1979.

Subrahmanyan (1959) and Subramanyan and Sarma (1965)
did the pioneering studies on the nanoplankton of the Indian
coast. These studies showed that in the south west coast

near Calicut, about 30-50% of the standing crop was due
to the nanoplankton fraction. They did not observe any
seasonal trends in their occurrence.

Qasim et al. (1974) reported that in the Cochin
backwaters the relative contribution of photosynthesis by
nanoplankton varied from 43-96% while that of net plankton
was only 4-53% of the total production. The amount of
nanoplankton chlorophyll registered higher values throughout
the year. However, during the monsoon and premonsoon
months, the net plankton occurred in higher numbers. In a
similar study in the coastal waters of Cochin, Vijayaraghavan
et al. (1974) estimated that nanoplankton contributed 66.4%
of the total productivity.

The dynamics of phytoplankton growth in nature
depends on the behaviour of individual species. The latter
can be understood only by laboratory studies on unialgal
cultures. Miquel (1890) was among the pioneers to initiate
the culture of microalgae. Adequate amounts of nitrate
and phosphate added to filtered sea water was used as the
basic growth medium for phytoplankton.

Pringsheim (1946) has summarised the history and
procedure of phytoplankton culture. In the 1950's,
Provasoli, Droop and others modified the culture media

by adding trace elements, chelating compounds and
vitamins (Provasoli et al., 1957; Provasoli, 1958;
Droop, 1954,1958a,1961a,b). The mineral requirements of
algae were further reviewed by Lewin (1962), O'Kelly (1968),
Stein (1973) and Stewart (1974).

Many axenic cultures of phytoplankton were
developed using such enriched media. About 50% of these
were diatoms and dinoflagellates and the rest composed of
small green flagellates (Chlorophyceae, Prasinophyceae,
Euglenophyceae), golden brown flagellates and coccolithophorids
(Chrysophyceae, Haptophyceae, Chloromonadophyceae),
cryptomonad flagellates (Cryptophyceae), red algae
(Rhodophyceae) and blue-green algae (Cyanophyceae). Some
well known species in culture are: Skeletonema costatum,
Asterionella japonica, Phaeodactylum tricornutum,
Chaetoceros calcitrans, Ceratium tripos, Amphidinium carteri,
Anabaena cylindrica, Isochrysis galbana, Monochrysis lutheri,
Chlorella pyrenoidosa and Dunaliella tertiolecta.

The cultures are raised in the laboratory in a
limited volume of the medium inoculated with a small number
of cells and exposed to suitable conditions of light,
temperature and aeration. These batch cultures are
maintained by periodic reculturing.

For experimental purposes many investigators make use of continuous cultures. These are of two types: Chemostat culture (Monod, 1950; Novick and Szilard, 1950) and 'Turbidostat' culture (Myers and Clark, 1944). Synchronous cultures are also used in experimental studies (Tamiya et al., 1953).

The culture collections of phytoplankton have been used to study many basic processes relating to photosynthesis, growth, algal phylogeny and biomass estimates. Experiments with algal cultures are providing clues to important ecological processes such as succession patterns, mechanism of phytoplankton blooms and many other phenomenon of basic importance to plant productivity in the sea.

In the 1960's the idea of single cell protein (SCP) was mooted. The International Biological Programme (IBP) clearly stated that 'Investigation should be carried out on those unused and little used plant and animal products which might be converted into nutritionally exploitable materials by bio-engineering techniques'. Microalgae was identified as a rich source of SCP and consequently large scale production of different species was undertaken to be incorporated into human food as well as cattle and poultry feed.

In nature nanoplankton is the source of food for
the minute zooplankters and the larvae of many aquatic
animals (Atkins, 1945; Thorson, 1950; Beers and Stewart,
1969; Parsons and Le Brasseur, 1970). Hence these
microalgae are used as live-food in rearing the larvae of
fish and shell fish in aquaculture practices. Though
in natural environment they feed on a variety of organisms,
in culture systems only a few species have been found
successful. Diatoms such as Skeletonema, Chaetoceros,
Thalassiosira and Nitzschia are known to be good food for
Crustacea (Kinne, 1977). May (1970) and Lasker et al. (1970)
have reported success in growing marine fish larvae on algal
diet alone. The larvae of the bivalve molluscs feed and
grow best on nanoplankters especially the naked golden
brown flagellates (Bruce et al., 1940; Guillard, 1958;
Walne, 1963,1970,1974; Ukeles, 1975).

Recently micro-particulate diets have gained some
progress in the rearing of bivalve larvae. But many authors
opine that live-food is superior to non-living diets and the
former is still being widely used in hatcheries (Jirasek et al.,
1977; Kinne, 1977). As such, the culture of nanoplankton
remains a necessary component of molluscan hatcheries.

The micro-flagellate _Isochrysis galbana_ Parke
is used to rear the larvae of molluscs in the laboratory of
CMFRI. The nutritional value of this flagellate is well
recognised (cf. Loosanoff and Davis, 1963). But, _I. galbana_
is a temperate strain the growth of which is retarded above
22°C (Ukeles, 1961). So mass cultures of _I. galbana_ is
quite unstable in the tropical climate. Hence the need to
isolate tropical strains of nanoplankters that could be of
use in mariculture.

There has been a few attempts in the past to isolate
nanoplankters from the Indian waters. Samuel (1970)
collected nine species of nanoplankton from the sea off
Cochin. Studies on the culture and growth kinetics of a few
species have been attempted by Nair (1974), Vijayaraghavan
et al. (1975) and Gopinathan (1981). But there has been no
comprehensive study on the culture of nanoplankton in
relation to its food value.

So these investigations were initiated with a view
to isolate strains of nanoplankton that could be of food
value to bivalve larvae and to study the growth requirements
of these algae in culture. This has been presented in
detail in the six chapters to follow. The introductory
chapter explains the relevance of the work together with
the literature review. Chapter 2 gives a taxonomic account

of some nanoplankters of the Indian coast including the present isolates. Chapter 3 presents an exhaustive description of the material and methods adopted for the study. The results of the various experiments on growth and other physiological activities are presented in Chapter 4. Chapter 5 deals with the mass culture and food value of the two microflagellates isolated during the period of investigation. The conclusions arrived at by the study are discussed in Chapter 6.

Growth kinetics: Literature

Literature on the growth kinetics of unicellular algae in culture is voluminous (cf. Strickland, 1960; Fogg, 1975). In batch cultures under suitable conditions, the microalgae grow and multiply rapidly in a sequential manner. Thus it is possible to observe a lag phase, exponential phase, stationary phase and death phase in such cultures. Lag phase has been detailed by Fogg (1944), Gerloff et al. (1950), Spencer (1954) and Eberly (1967). The exponential or the logarithmic phase produces the peak growth after which the growth rate decreases and stationary phase begins. The stationary phase may be maintained for several weeks before decline sets in. The duration of these phases, however, varies with the species and the prevailing environmental conditions.

There is no single parameter to measure growth of unicellular algae. When cell number is known, the rate of doublings per day is often used as a useful index of growth (Eppley and Strickland, 1968). Specific growth rate (μ) or the relative growth constant (k) and generation time (t_g) are also useful parameters for measuring growth (Fogg, 1975). The growth rate is usually calculated from cell number, cell carbon, or the amount of chlorophyll a. Values of relative growth rates for different species in culture have been summarised by Hoogenhout and Amesz (1965). Thus a study of growth kinetics envisages different aspects such as population density, the pigments and cellular composition.

In regular oceanographic analysis the phytoplankton biomass is often determined as photosynthetic pigments. Analysis of the same by spectrophotometry has been popularised by Richards and Thompson (1952) and Parsons and Strickland (1963). In the 1960's chromatography became widely used for more detailed analysis of the algal pigments (Jeffrey, 1961, 1965,1968). These studies have shown that algae contain three basic groups of pigments namely chlorophylls, carotenoids and biliproteins. These function as photoreceptors in photosynthesis. The distribution of these in the various algal taxa has been ascertained by separating the component pigments on chromatograms. At first, paper chromatography

was used to separate the chlorophylls and carotenoids of
marine algae in culture (Jeffrey, 1961,1965) and sea water
samples (Jensen and Liaaen Jensen, 1959; Jeffrey, 1974).
Further, micro-chromatographic techniques were developed
which needed only small amounts of the material and gave
rapid and good resolution of the components. Investigators
like Riley and Wilson (1965), Madgwick (1965) and Jeffrey
(1968,1974,1981) employed the thin layer technique to study
the algal pigments. Different adsorbents and solvent
systems were used by these authors so as to get maximum
resolution.

The solar energy trapped by the pigments is used to
produce organic matter which further is partitioned between
proteins, carbohydrates and lipids and within each of these
are numerous organic substances. Some data are given by
Spoehr and Milner (1949), Myers (1949), Parsons et al. (1961),
Lewin and Guillard (1963) and Myklestad (1974). All these
workers point out that the microalgae are mainly protein
synthesizers. According to Goldman (1980) a balanced
nutritional state produces about 50-60% protein, 30-40%
carbohydrate and 10-20% lipids. However, prolonged growth
under nitrogen deficiency cause synthesis of lipids or
carbohydrates at the expense of protein. The changing
protein-carbohydrate ratio during batch growth has been

studied by Fogg (1959) and Healey (1975). Reviewing the development of the work in the field Morris (1981) concludes that besides the general similarity between species in the relative amounts of crude protein, lipid and carbohydrate there exist important differences as function of species and ecological conditions.

Another integral component of algal photosynthesis is the release of organic metabolites into the surrounding medium (Fogg, 1975; Aague et al., 1980). However, there is little agreement on the relative amounts of the material released (Hellebust, 1965; Watt, 1966; Fogg, 1966; Thomas, 1971). The exponentially growing cells may release from 1% to 50% of their photoassimilated carbon while cells in lag or stationary phase release more (cf. Hellebust, 1974). The variation in the rate of excretion has been attributed to different factors - physical, chemical and biological (Guillard and Wangersky, 1958; Marker, 1965; Fogg, 1966; Samuel, Shah and Fogg, 1971; Williams and Yentsch, 1976).

The method of estimation of the extracellular products (ECP) employed by these investigators makes use of the carbon-14 incubation method. The carbon-14 incubated samples are filtered and the organic fraction in the filtrate (ECP) is determined with the aid of Geiger Counter or Scintillation Counter (cf. Vollenweider, 1974).

Apart from these central features of growth and photosynthesis the physiological ecology of microalgae have been much discussed. The algae in general show high metabolic diversity. This allows extensive variability to be superimposed on the basic processes. The nature of this variability is regulated by the environmental factors and the physiological state of the organism.

In nature phytoplankton populations are subject to a variety of factors like light, temperature and nutrient fluctuations acting simultaneously. Nature has normally established an equilibrium between all the factors governing phytoplankton production. The incidence of any one factor becoming limiting in such systems is only occasional. However, in culture systems single factors like light intensity, nitrate or phosphate concentration can become limiting resulting in growth arrest. This has been illustrated in experimental cultures (cf. Fogg, 1975; Stewart, 1974; Platt, 1981).

Solar radiation was early understood to be a prime factor in photosynthesis (cf. Rabinowitch, 1951). As such, in productivity studies the euphotic zone and light – photosynthesis relationship gained importance. Talling (1957) introduced the term I_K to describe the light intensity that marks the onset of saturation of photosynthesis. This saturation level has been defined for a number of

species (Steemann Nielsen and Jensen, 1957; Steemann Nielsen and Hansen, 1959,1961; Steemann Nielsen and Jorgensen, 1962, 1968a; Jorgensen, 1964,1969; Steemann Nielsen and Willemoes, 1971).

The algal growth and species competition in culture systems and natural ecosystems is also a function of temperature. Eppley (1972) has discussed in detail the effect of temperature on phytoplankton growth rates. He found that there is a gradual and exponential increase in the specific growth rates with increasing temperature upto $40^{\circ}C$. He quantified the temperature - growth rate relationship in terms of Q_{10}. However, this response to temperature need not be direct. In fact many authors have tried to find out the combined effect of temperature, light, salinity and nutrient status in axenic cultures (cf. Eppley and Strickland, 1968; Stewart, 1974). Goldman and Carpenter (1974) correlates the effect of temperature to nutrient concentration in many fresh water and marine algae. Redalje and Laws (1983) has illustrated in _Thalassiosira alenii_ Takano that the effect of temperature and nutrients interact to govern how a particular parameter like protein content vary with temperature while light affected the range of these values.

The kinetics of uptake of nutrients and the relation between nutrient uptake and growth of phytoplankton has

received much attention. The results of Ketchum (1939) and Harvey (1957) indicated a hyperbolic relationship between the rate of uptake of nutrients and the concentration of the same in the medium. Dugdale (1967) and Caperon (1968) established that the rate of uptake of a limiting nutrient and the specific growth rate are related to nutrient concentrations in the medium in a way similar to that of the Michaelis-Menten relation of enzyme kinetics. Further work on this aspect has been done by Eppley and Coatsworth (1968); Eppley and Thomas (1968); Eppley, Rogers and McCarthy (1969); Droop (1973); Qasim et al. (1973); Goldman (1977); Goldman and McCarthy (1978) and McCarthy (1981).

The nutrient requirement of a species is expressed in terms of half-saturation constant(K_S). It is defined as the concentration supporting half the maximum growth (K_μ) or half the maximum uptake (K_S) and is calculated using the Monod expression (Monod, 1942). Most of the research in this line has centred around the kinetics of uptake of nitrate, phosphate, ammonium, silicate and vitamin B_{12}.

Other than light, temperature and nutrients, phytoplankton of coastal waters is subject to high fluctuations of salinity, pH and many other factors due to land drainage and other human activities. The salinity and pH requirements of marine algae have received attention of

certain investigators (Droop, 1958a; McLachlan, 1961;
Soeder et al., 1966; Qasim et al., 1972; Majumder et al.,
1980). Cells of Dunaliella salina are found to respond
drastically to the changes in salinity by changing the
pigment content (Loeblich, 1982). Thus investigations on
microalgae have envisaged all aspects of growth and
assimilatory products in connection with their eco-physiology.

2. SYSTEMATICS

A taxonomic account of some nanoplankters of the coastal and estuarine waters of India suitable for development as axenic cultures for feed.

The diatoms and dinoflagellates of the Indian waters are well documented (Menon, 1945; Subrahmanyan, 1946,1958a, 1959,1968,1971; Subrahmanyan and Sarma, 1960,1965; Nair, 1959,1960; Gopinathan, 1975,1981). These works deal mostly with the net plankton. Except for a few reports information on nanoplankton is scanty. Subrahmanyan and Sarma (1965) identified 58 species of nanoplankters of which 27 are diatoms and 26 dinoflagellates. The remaining are species of Carteria, Chlamydomonas, Coccolithus pelagicus, Euglena and Merismopedia. Sarma and Khan (1980) have listed some nanoplankters reported from the Indian seas which again include mostly diatoms and dinoflagellates. Nine species of the smaller nanoplankters (less than 20 microns) have been described by Samuel (1970). A few species have been isolated from the coastal region of Cochin and is available in the culture collections of CMFRI. In order to present a complete picture of this study, the taxonomy and salient features of the smaller nanoplankters occurring in Indian waters are included in this section, so as to provide information on the species suitable for culture of live-food

in hatchery development. Only those species below 20 microns in size are listed here; all diatoms, dinoflagellates, chain forming and colonial types have been omitted.

Classification

Algal systematics has been subjected to much change since the time of publication of the exhaustive work on algae by Fritsch (1935,1945). Many taxa had been reshuffled and a few new groups created based on advanced studies. Recent revisions of algal systematics are provided by Christensen (1962), Parke and Dixon (1976), Bold and Wynne (1978) and many others.

The classification followed here is that of Parke and Dixon (1976).

CYANOPHYTA

CYANOPHYCEAE

CHROOCOCCALES

Chroococcaceae

SYNECHOCYSTIS Sauvageau, 1892

Sauvageau, C. 1892. Algues recolt. en Algerie. Bull. Soc. Bot. France, 39: 115.

Fritsch, F.E. 1935. Structure and Reproduction of the Algae. Vol. II, p.814.

Desikachary, T.V. 1959. Cyanophyta, p.144.

Spherical cells usually single; sometimes occur
in two's or rarely in colonies of a few cells. No
distinguishable mucilage envelope.

Distribution: The genus occurs as plankton in the fresh
and marine waters. The genus is also reported to be
epizoic on ascidians and within mangrove roots.

Key to the species

1) Spherical cells, solitary or in
   two's and no characteristic
   motility.   ... S. pevalekii Geitler

2) Solitary, spherical cells with
   jerky movements   ... S. salina Wislouch

Synechocystis pevalekii Geitler, 1932

Geitler, L. 1932. Kryptogamenflora, p. 269.
Desikachary, T.V. 1959. Cyanophyta, p. 145.

Spherical cells; sometimes two together and appear
hemispherical after division. The cells measure 2.5-3.5μ
in size. The cell contents is bluish green and homogeneous.

Distribution: The species is reported from the fresh waters
of Madras, Beira Lake and Ceylon by Desikachary. The strain
available in CMFRI culture collection was isolated from the
coastal region of Tuticorin.

<u>Synechocystis saline</u> Wislouch, 1924

Wislouch, S.M. 1924. <u>Acta. Soc. Bot. Pol.</u>, <u>2</u>: 99.

Geitler, L. 1942. <u>Schizophyceae in Engler-Prantl Die
     Naturlichen pflanzenfamilien</u>, II Ed. Ia.

Lund, J.W.G. 1950. <u>Naturalist</u>, London, <u>835</u>: 143-148.

Small spherical cells of diameter 3μ with bluish

green colour. Sometimes occur in two's. The species is

characterised by the jerky movements of the cells.

<u>Distribution</u>: The species was isolated from the coastal

region of Cochin.

CRYPTOPHYTA

CRYPTOPHYCEAE

CRYPTOMONADALES

Cryptomonadaceae

<u>CRYPTOCHRYSIS</u> Pascher, 1911

Pascher, A. 1911. <u>Ber. Deutch. Ges. Bod.</u>, <u>29</u>: 139-203.

Fritsch, F.E. 1935. <u>Structure and Reproduction of the Algae</u>.
     Vol.I, p.652.

Pierre P. Grasse. 1953. <u>Traite De Zoologie</u>, 285-308.

Butcher, R.W. 1952. <u>J. mar. biol. Ass. U.K.</u>, <u>31</u>: 185-187.

Samuel, S. 1970. <u>J. mar. biol. Ass. India</u>, <u>12</u>: 33-42.

Motile naked cells with slight dorso-ventral

construction. A narrow furrow extents from the slightly

constricted anterior end to the posterior part. The
flagella are of unequal length. Chromatophore single,
two or numerous with diverse shades of brown.

Distribution: Distributed in the coastal seas of both
temperate and tropical waters.

Key to the species

1) Ovoid cells with a vertical
   furrow running down to the
   basal pyrenoid   ... C. **fulva** Butcher
2) Ovoid cells with a shallow
   furrow extending upto the
   centre of the cell. Pyrenoid
   sub-median in position   ... C. **virescens** Butcher

Cryptochrysis fulva Butcher, 1952

Butcher, R.W. 1952. *J. mar. biol. Ass. U.K.*, **31:** 185-187.
Samuel, S. 1970. *J. mar. biol. Ass. India*, **12:** 35-42.

Motile naked flagellates measuring 6-8x3-4µ. The
cells appear ovoid in dorsal view. The apex is truncate
and posteriorly the cell tapers. A vertical furrow extents
from the apex to the pyrenoid which is posterior in
position.

The motile cell or the resting cell divides
longitudinally to form daughter cells. Palmelloid stages
also occur.

<u>Distribution</u>: Found in the shell fish tanks of Conway, Northwales. In India it has been reported from the west coast.

<center><u>Cryptochrysis virescens</u> Butcher, 1952</center>

Butcher, R.W. 1952. <u>J</u>. <u>mar</u>. <u>biol</u>. <u>Ass</u>. <u>U.K</u>., <u>31</u>: 187. Samuel, S. 1970. <u>J</u>. <u>mar</u>. <u>biol</u>. <u>Ass</u>. <u>India</u>. <u>12</u>: 35-42.

Cells measure 5-7x3-4μ in size; ovoid with a convex dorsal side and flattened ventral side. A shallow furrow runs down laterally from the anterior end to the centre. There are two flagella that are unequal in length. Chromatophore single, large, bluish green, smooth and almost fills the cell. There is a single large and sub-median pyrenoid.

Reproduction occurs by the longitudinal division of the motile cell into two. Palmelloid stages are also reported.

<u>Distribution</u>: The species is reported from the river Crouch, Essex. The Indian report is from the Cochin coast.

<center>HAPTOPHYTA

HAPTOPHYCEAE

ISOCHRYSIDALES

Gephyrocapsaceae</center>

DICRATERIA Parke, 1949

Parke, M. 1949. J. mar. biol. Ass. U.K., 28: 255-286.

Bourrelly, P. 1958. Review Algologique Memoir. Hors. Serie, 1. 229.

Samuel, S. 1970. J. mar. biol. Ass. India, 12: 35-42.

Green, J.C. and R.N. Pienaar. 1977. J. mar. biol. Ass. U.K., 57: 7-17.

Cells solitary, spherical and golden brown in colour. Two flagella of equal length. Asexual reproduction by the division of the motile cells. Cysts are also reported.

Distribution: Reported from the coastal waters of Britain and Europe. In India it has been collected from the Laccadive sea.

Dicrateria gilva Parke, 1949

Parke, M. 1949. J. mar. biol. Ass. U.K., 28: 255-286.

Samuel, S. 1970. J. mar. biol. Ass. India, 12: 35-42.

Biflagellate spherical cells measuring about $3.5-7\mu$ in diameter. Two flagella of equal length. There are two golden brown chromatophores that are parietal in position. The rest of the cell is occupied by the nucleus, leucosin, oil bodies and contractile vacuole.

The species is characterised by the spherical shape, slow rotatory movements and the absence of stigma. As the

cells grow old, the number of chromatophores and leucosin increase.

Reproduction takes place by the binary fission of the cells. Cysts and palmelloid phases also occur.

Distribution: The species was first reported from the plankton samples taken from the Plymouth Sound, Britain. The Indian strain was found in the Laccadive sea.

Isochrysidaceae

ISOCHRYSIS Parke, 1949

Parke, M. 1949. J. mar. biol. Ass. U.K., 28: 255-286.
Billard, C. and P. Gayral. 1972. Br. phycol. J., 7: 289-297.
Green, J.C. and R.N. Pienaar. 1977. J. mar. biol. Ass. U.K., 57: 7-17.

Slightly elongated motile cells with a capacity to change shape. Two smooth apical flagella, once to twice the length of the cell. The motile cell has haptophycean scales on the cell surface. Benthic phases occur usually.

Distribution: The genus has been reported from the fish ponds of Britain, from below the chalk-cliffs of the French and British coasts and from the shrimp culture ponds of Tahiti.

Isochrysis galbana Parke, _forma nova_

.CMFRI Culture No.12: Isolated from the inshore waters of
Cochin on the west coast of India in November, 1981.

Motile cell

Length   = 12-14μ

Breadth   = 5-6μ

Length of the flagellum = 14μ

Golden-yellow flagellate moving actively. The
cell is slightly elongated and compressed. There is
marked capacity to change shape. The cells are surrounded
by a thin mucilage layer.

Two flagella of equal length arise from the
anterior end. They are visible feebly in the light
microscope as two stiff hair like structures directed away
from the body. The two are equal in diameter throughout
their length. During movement the flagellae are directed
forwards with the body rotating and gyrating in the course.
The dry specimens observed under the electron microscope
showed a knob like haptonema in between the flagellae
(Plate I).

There is a single chromatophore that is golden-brown
in colour and parietal in position. Embedded in the base of

Figs. 1-4. *Isochrysis galbana* **forma nova**

Fig. 1. Young motile cell viewed under light microscope (x 3000).

Fig. 2. Scanning electron micrograph of young motile cell (x 6000); h, haptonema; f, (broken) flagella.

Figs. 3-4. Reproduction in the palmelloid phase (fig. 3, x 320; fig. 4, x 640).

PLATE I
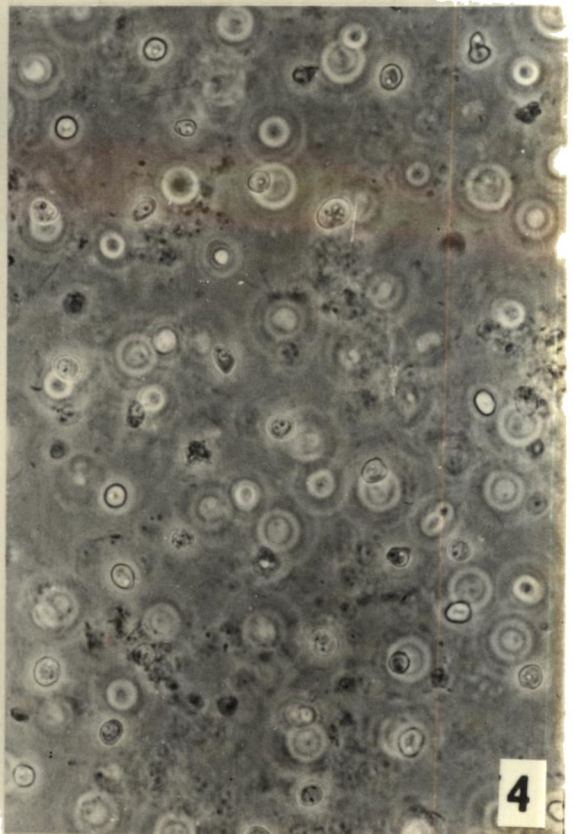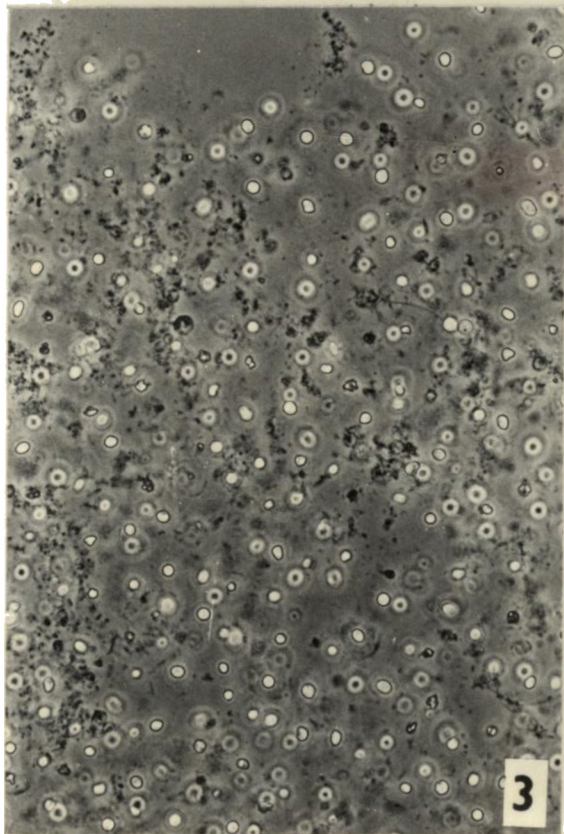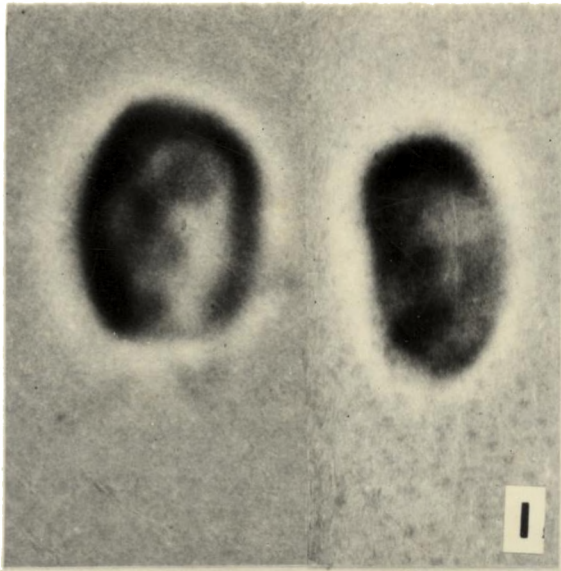
the chromatophore is the pyrenoid that appears as a
part of the chromatophore.

The young motile cells show a median red body.
In older cells three or four of such round bodies are
observed. The nucleus is median in position.

The species show phototactic response though a
clear stigma has not been found. At the posterior end of
the cell leucosin body is prominent. Small oil drops are
distributed throughout the cell (Plate III).

Asexual reproduction occurs in the young cultures
by the longitudinal division of the motile cells. When
cultures are two to three months old, the cells begin
to settle at the bottom of the culture vessel forming a
layer at the bottom. The cultures on examination was
observed to contain non-motile cells embedded in a thin
layer of mucilage (Plate I). It is inferred that this is a
form of reproduction in palmelloid phase. These spheroidal
individuals have large masses of leucosin and two to four
chromatophores. Most of these cells are observed to be
in the division phase with or without completing the
separation of the daughter cells (Plate III). On inoculating
into fresh medium, all these non-motile cells completed
division, developed flagella and became active. Division in

the non-motile phase have been reported by Parke (1949) for *I. galbana*. However, cysts as observed by Parke (1949) was not obtained even in 6 month old cultures.

The particulars of the present strain agrees with that of the genus *Isochrysis*. Three species of *Isochrysis* are reported of which *I. maritima* and *I. littoralis* are distinguished by the absence of haptonema and the dominant benthic phase (Billard and Gayral, 1972). As revealed by the SEM study, the present strain has got the much reduced haptonema similar to *I. galbana*, Parke. The new isolate has also been compared with the live cultures of *I. galbana* Parke which is available in CMFRI culture collection. The characteristic capacity to change shape, the dominance of motile phase, the single chromatophore and the absence of well defined stigma supports the assigning of the present strain to *I. galbana*.

However, the present strain differs from *I. galbana* Parke in the cell dimensions. The latter measures 5-6μ in length, 2-4μ in breadth and the flagella 7μ in length whereas the present strain is almost double the size. Moreover, *I. galbana* is purely a temperate water species while the present strain is tropical. A similar flagellate is reported from the tropical waters of Tahiti designated T-ISO (J.L. Martin, Centre Oceanologique Du Pacifique)

personal communication). The ultra-structural aspects of these tropical strains have not been carried out. So the present strain is given the designation., I. galbana forma nova* pending additional studies.

Distribution: The species is reported from the fish ponds of Port Erin, U.K. The clone T-Iso was isolated from the lagoon water of Tahiti. The present strain in the culture collection of CMFRI was isolated from the inshore region of Cochin.

CHRYSOPHYTA

CHRYSOPHYCEAE

CHROMULINALES

Chromulinaceae

CHROMULINA Cienkowski, 1870

Cienkowski, L. 1870. Arch. mikroskop. Anat., 6: 421-438.
Conrad, W. 1931. Mem. Mus. Roy. Hist. Nat. Belgique., 8: 65.
Bourrelly, P. 1968a. Les algues d'eau douce. Initiation a'la systematique. II. Les algues jaunes et brunes, Chrysophycees, Xanthophycees.
Bold, H.C. and M.J. Wynne. 1978. Introduction to the Algae, Structure and Reproduction, P.358.

Cells solitary, free swimming. Cells are naked with no well defined form. There is a single pleuronematic flagellum. One or two chloroplast present.

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*Hereafter referred to as I. galbana (C.s) meaning Isochrysis galbana (Cochin strain)

About 120 species are known. The species are distinguished by the presence or absence of stigma and pyrenoid, structure and formation of cysts or number and form of chloroplast.

Distribution: The genus occurs both in freshwater and marine habitats.

Chromulina freiburgensis Dofl. 1923

Doflein, F. 1923. Arch. Protistenk., 46: 267.
Fritsch, F.E. 1935. Structure and Reproduction of the Algae.
    Vol.I, p. 512.
C. freiburgensis (CMFRI culture No.11). Isolated from the
    inshore waters of Cochin in August, 1981.

Motile cell

Diameter   = 10µ

Length of the flagellum = 25µ

Cells spheroidal and motile with a smooth periplast that permits only slight change of shape. The cell has a thin layer of mucilage forming a halo around it. A single flagellum arises apically. As shown by the electron microscopy, the flagellum ends in a bulb like expansion (Plate II). No evidence of cilia was obtained in dry specimens. The live cells move slowly in a rotating motion with active forward movement occasionally.

EXPLANATION OF PLATE II

Figs. 1-6.   Chromulina freiburgensis

Fig. 1.   Young motile cell viewed under light
            microscope (x 1500).

Fig. 2.   Older motile stage showing the mucilage
            envelope (x 1500).

Fig. 3.   Scanning electron micrograph of young
            motile cell with stretched flagella
            (x 3500).

Fig. 4.   Scanning electron micrograph of young
            motile cell with the flagella bent upon
            itself (x 3500).

Figs. 5-6. Reproductive phase in six month old culture
            showing dividing cells and cysts (Fig. 5,
            x 320; Fig. 6, x 640).

PLATE II
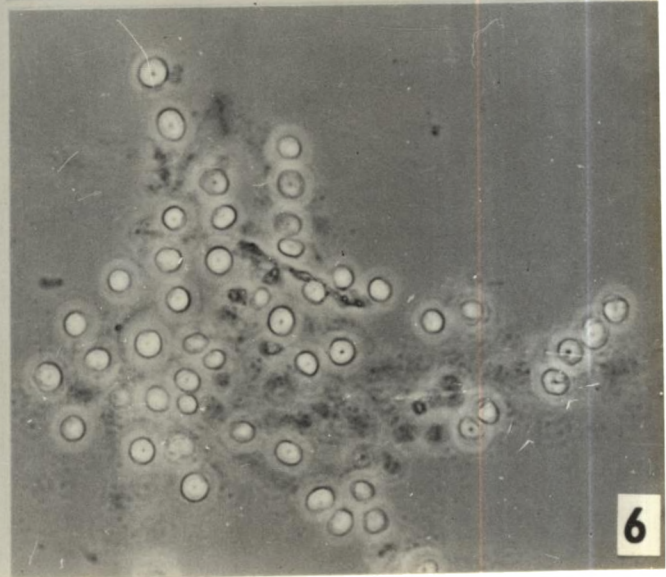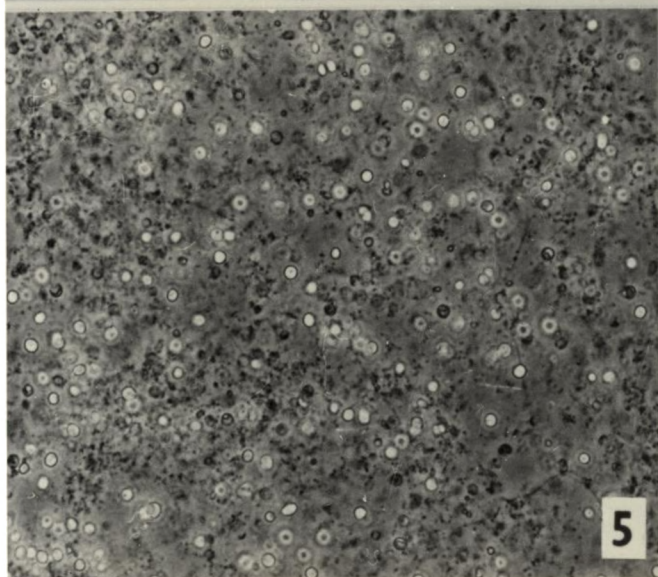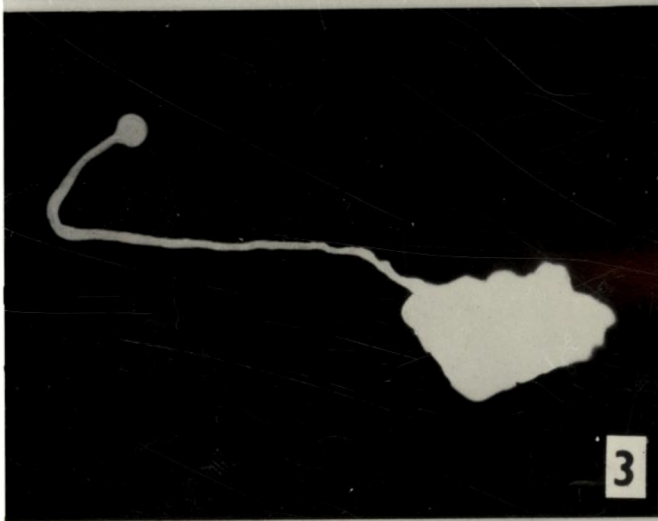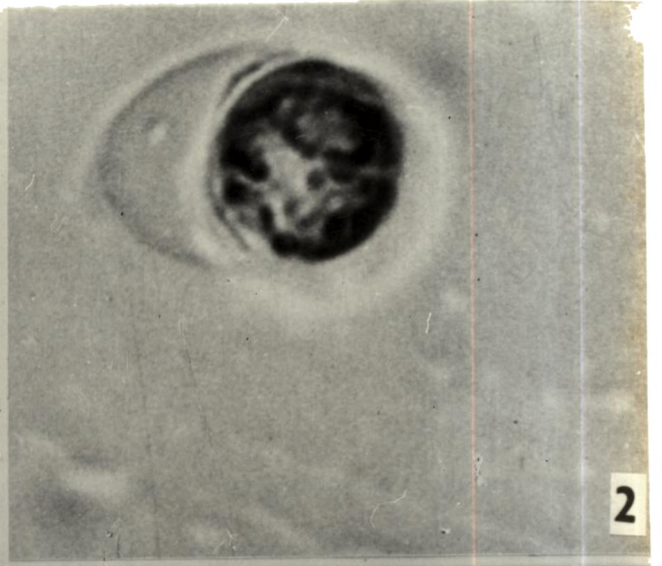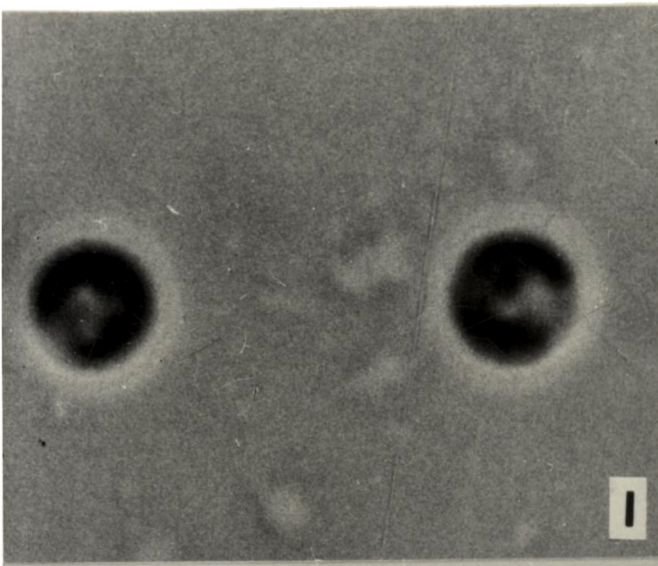
The single cup-like chromatophore is golden-brown in colour. This imparts the characteristic colour to the culture. At the posterior end of the cell, in the cup of the chromatophore lies a lump of leucosin. Pyrenoid is found as a rounded protrusion within the chromatophore. The single nucleus is displaced to the anterior end (Plate III).

The species shows phototactic response. A red coloured stigma is visible along the anterior edge of the chromatophore. There is a spherical body at the base of the flagellum that appears granular. A few membrane bound bodies occur randomly in the cytoplasm.

Asexual reproduction occurs in the young cultures by the division of the motile cells. In cultures that are over a month old, non-motile cells are found to settle down forming a mat-like layer at the bottom of the culture vessel (Plate II). These non-motile cells have been observed to divide into two or four cells that remain together before being released. These non-motile cells are sometimes surrounded by mucilage. Cultures that are still older, showed cyst formation. The large non-motile cell divides into two within the cyst. The cyst is almost ovoid in shape with smooth outer wall and the plug region raised above the surface (Plate III).

EXPLANATION OF PLATE III

(Figs.1-10, x 1500)

Figs.1-5. *Isochrysis galbana* (C.s.)

Figs.1-2. Young motile stage illustrating the
          variation in shape and position of the
          chromatophore. c, chromatophore;
          l, leucosin; n, nucleus; s, stigma;
          v, vesicles.

Figs.3-5. Reproduction in palmelloid phase.

Figs.6-10. *Chromulina freiburgensis.*

Fig.6.   Young motile cell illustrating the
          internal structure. c, chromatophore;
          l, leucosin; n, nucleus, s, stigma.

Figs.7-8. Cell division in the non-motile phase.

Figs.9-10. Cyst formation in older cultures.

PLATE III
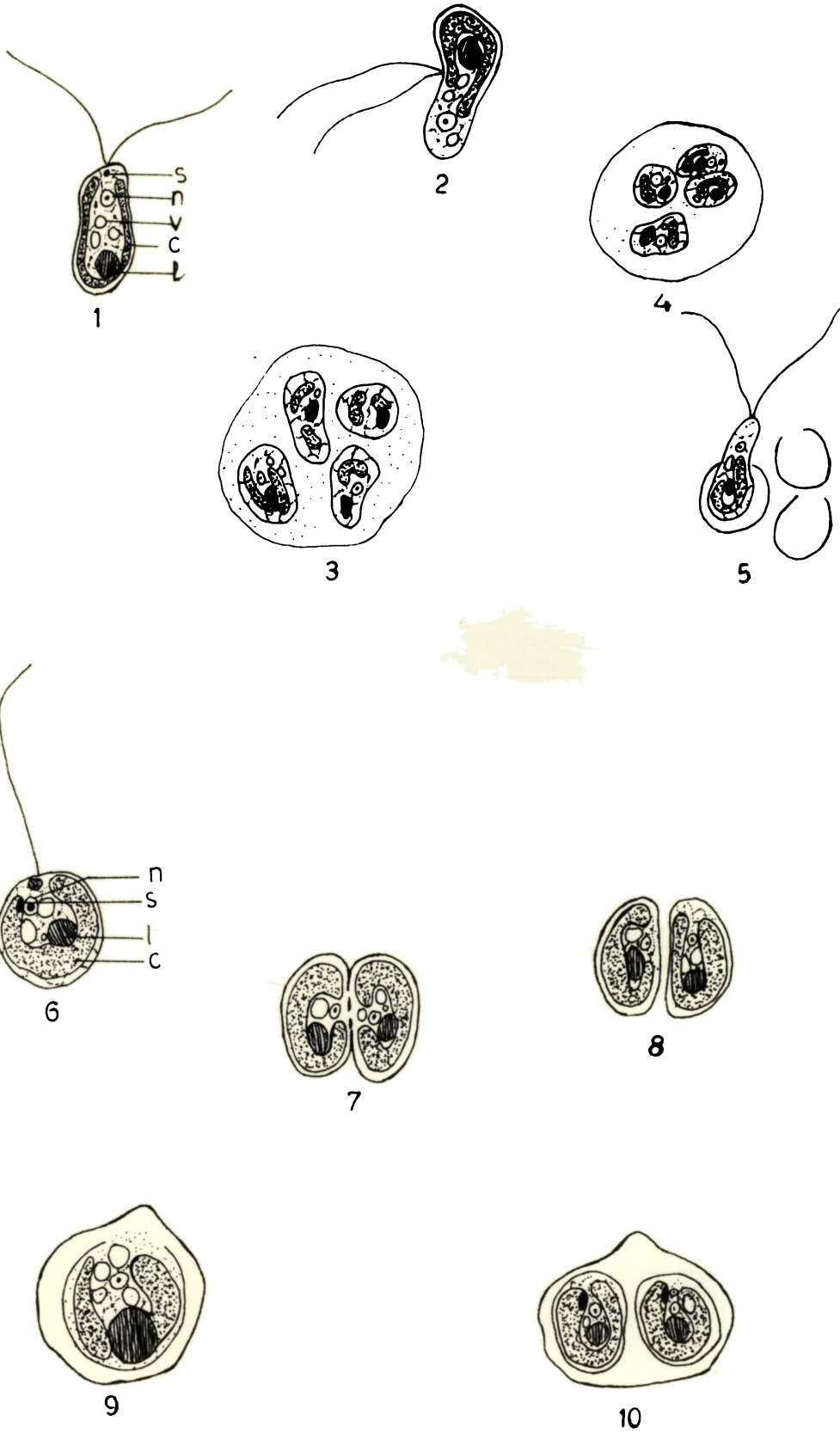
Based on the observation that the present strain has a single cup-like chromatophore that lodges the pyrenoid, a large posterior leucosin body and a single flagellum, it has been assigned to the species *C. freiburgensis.*

Distribution: The present strain was obtained from the inshore waters of Cochin.

CHLOROPHYTA

PRASINOPHYCEAE

PRASINOCLADALES

Prasinocladaceae

TETRASELMIS Stein, 1878

Stein, F. 1878. *Der Organismus der Infusions thiere,* 3: 1.
West, G.S. 1916. *J. Bot. London,* 54: 3 (Platymonas).
Kylin, H. 1935. *Kungl. Fysiogr. Salki. Lund. Forhandl.,* 5: 22.
Butcher, R.W. 1959. *Fish. Invest. Ser. IV,* 67-71.
Manton, I. and Parke, 1965. *J. mar. biol. Ass. U.K.,* 45: 753-754.
Samuel, S. 1970. *J. mar. biol. Ass. India,* 12: 35-42.
Fott, B. 1971. *Preslia,* 43: 289-303.
Parke, M. and P.S. Dixon. 1976. *J. mar. biol. Ass. U.K.,* 56: 527-594.

Actively swimming green flagellates; ellipsoid or ovoid in shape. Flagella four in number arising from an

apical furrow. Distinct eye-spot and pyrenoid present. Reproduction is by longitudinal division of the protoplast. Cyst formation also occur.

The genus resembles *Carteria* but with slight differences in the flattening of the cells, position of the eye-spot and in the nature of the cyst.

The genus has been used synonymous with *Platymonas* West. However, the evidence is inconclusive.

Distribution: Found in the inshore water of both temperate and tropical seas.

Key to the species

1) Ovoid body with reniform

   starch sheath and sub-median

   stigma   ... *I. carteriiformis* Butcher

2) Body tapering posteriorly

   with 'U' shaped starch

   sheath and median stigma   ... *I. gracilis* Kylin

I. carteriiformis Butcher, 1959

Butcher, R. W. 1959. Fish. Invest. Ser. IV, 67-71.
Samuel, S. 1970. J. mar. biol. Ass. India, 12: 35-42.
Parke, M. and P. S. Dixon. 1976. J. mar. biol. Ass. U.K.,
       56: 527-594.

Cells measure 12-16 x 8-11 x 7μ. It is broadly ovoid at the anterior end and tapers posteriorly. Four flagellae arise from the shallow furrow in front. The length of the flagella is almost equal to that of the cell.

There is a single, smooth, cup-shaped chromatophore that is pale green in colour. It becomes coarse and granular as the cell grows old. A large basal pyrenoid with reniform starch sheath is present.

Reproduction is effected by the longitudinal division of the resting cell. Thick walled cysts are observed in older cultures.

Distribution: The species is reported from the rock pools on the Isle of Cumbrae, Scotland. In India it was collected from the coastal waters of Cochin.

I. gracilis Kylin, 1935

Kylin, H. 1935. Kungl. Fysiogr. Salki. Lund. Forhandl. Bdv. Nr. 22. (P. gracilis)
Butcher, R. W. 1959. Fish. Invest. Ser. IV, 67-71.
Samuel, S. 1970. J. mar. biol. Ass. India, 12: 35-42.
Parke, M. and P.S.Dixon. 1976. J. mar. biol. Ass. J.K., 56: 527-594.

Cells cylindrical with rounded ends measuring 8-12 x 6.5-8 x 4.5μ. From the shallow apical furrow arise

four flagella of equal length. A long narrow sinus
extents from the furrow to the base of the cell.

Single pale green chromatophore. Pyrenoid with a
'U' shaped starch sheath and median stigma.

Reproduction by division of the cells or by cyst
formation.

Distribution: Reported from the ditches, salt marshes and
pools in Kent-Isle of Wight and in the west coast of Sweden
and India.

CHLOROPHYCEAE

CHLOROCOCCALES

Oocystaceae

CHLORELLA Beijerinck, 1890

Beijerinck, W. 1904. Rec. Trav. Bot. Neerl., 1: 14-27.
Fritsch, F.E. 1935. Structure and Reproduction of the Algae,
    Vol. I, p.147.
Butcher, R.W. 1952. J. mar. biol. Ass. U.K., 31: 179-181.
Samuel, S. 1970. J. mar. biol. Ass. India, 12: 33-42.
Parke, M. and P.S. Dixon. 1976. J. mar. biol. Ass. U.K.,
    56: 527-594.

Coccoid green algae with spherical or ovoidal
shape. Cells single or in groups. Reproduction is by the
division of the cells into two to eight non-motile spores.

Distribution: The genus is predominantly fresh water
in habitat. A few species are marine.

Key to the species

1) Cells oval, chromatophore
   bright green, granular,
   pyrenoid absent   ... C. marina Butcher

2) Cells oval, chromatophore
   green, smooth, pyrenoid absent ... C. ovalis Butcher

3) Cells spherical, chromatophore
   green, granular, pyrenoid
   present   ... C. salina Butcher

Chlorella marina Butcher, 1952

Butcher, R.W. 1952. J. mar. biol. Ass. U.K., 31: 179-181.
Samuel, S. 1970. J. mar. biol. Ass. India, 12: 35-42.
Parke, M. and P.S. Dixon. 1976. J. mar. biol. Ass. U.K.,
    56: 527-594.

The cells are oval measuring 8-13 x 5-8μ in size
and is limited by a thin cell wall. The single chromatophore
is bright green in colour, plate-like parietal in position
and almost fills the cell.

During reproduction the mother cell divides to
form 8-16 daughter cells.

<u>Distribution:</u> Reported from the offshore region of
Quilon, west coast of India. It has been isolated from
Port Erin, U.K.

<u>Chlorella ovalis</u> Butcher, 1952

Butcher, R.W. 1952. <u>J. mar. biol. Ass. U.K.</u>, <u>31</u>: 180-181.
Samuel, S. 1970. <u>J. mar. biol. Ass. India</u>, <u>12</u>: 35-42.
Parke, M. and P.S. Dixon, 1976. <u>J. mar. biol. Ass. U.K.</u>,
      <u>56</u>: 527-594.

The oval or ellipsoid cells measure 3-5 x 5-10u.
There is a single chloroplast that is green, parietal and
smooth filling the entire cell.

The mother cells divide asexually to produce 4-8
daughter cells.

<u>Distribution:</u> The species was first isolated from river
Crouch, Essex. In India it is reported from the water
samples collected from the sea off Cochin.

<u>Chlorella salina</u> Butcher, 1952

Butcher, R.W. 1952. <u>J. mar. biol. Ass. U.K.</u>, <u>31</u>: 174-180.
Samuel, S. 1970. <u>J. mar. biol. Ass. India</u>, <u>12</u>: 35-42.
Parke, M. and P.S. Dixon, 1976. <u>J. mar. biol. Ass. U.K.</u>,
      <u>56</u>: 527-594.

Cells 5-7μ in size; bright green and spherical in
shape. Chloroplast single, granular and saucer-shaped.

Pyrenoid is large and centrally placed. It is surrounded
by starch sheath.

Reproduction is by the successive division of the
cell contents into eight cells.

Distribution: The species was first isolated from the
Conway tanks. The Indian report is from the Laccadive sea.

Chlorococcaceae

**Tetracystis Brown and Bold, 1964

Brown, R.M. and H.C. Bold, 1964. The University of Texas
    Publication No.6417.
Bold, H.C. and M.J. Wynne, 1978. Introduction to the Algae.
    Structure and Reproduction, p. 129.

The cells are spherical occurring singly or in
groups. Chloroplast is parietal and cup-shaped. Pyrenoid
is conspicuous.

Asexual reproduction occurs by the division of the
cell into 2-8 daughter cells. Zoospore formation also does
occur. Sexual reproduction occurs by biflagellate gametes.

Tetracystis sp.

Samuel, S. 1970. J. mar. biol. Ass. India, 12: 35-42.

**The genus is included in the family due to its resemblance
    to Chlorococcum. Parke and Dixon (1976) have not dealt with
    the genus. Bold and Wynne (1978) have placed it under
    Chlorosarcinales.

The ovoid or spherical cells measure about 13-16µ in size. The cell wall is thin while young and later becomes thick and striated. The chloroplast is parietal and almost fills the cell. The median pyrenoid is surrounded by starch grains.

The species reproduces asexually by cell division. Zoospores and aplanospores are also reported. Sexual fusion of isogametes have also been noticed.

The organism resembles Chlorococcum in appearance and life cycle.

Distribution: The species is reported from the inshore marine regions of the west coast of India.

3. MATERIAL AND METHODS

3.1. Isolation of nanoplankton

Methods of isolation and maintenance of microalgae in axenic culture are based on the classical methods used in bacteriology. Despite many attempts to refine and simplify the conventional techniques, the process of isolation of unicellular algae remains lengthy and tedious. The standard methods used in the literature with necessary modifications were used to isolate nanoplankters during the present study.

The water samples for the isolation of the nanoplankton were collected from the coastal region of Cochin ($9^\circ 58'N$, $76^\circ 15'E$) near barmouth. As this group of algae is not retained by the standard phytoplankton net (No.25 bolting silk net of mesh size 64μ) no net samples were taken. Instead, surface water samples were collected. Collections were made from April to August, 1981. The water samples were stored in polythene bottles and transported to the laboratory within one to two hours of collection.

In the laboratory, the samples were filtered through Whatman No.542 filter paper to remove the larger size fraction of phytoplankters. The filtrate was

40

transferred to culture flasks; enriched with nutrients
and incubated for a few days under fluorescent lamps.
The enrichment promoted rich growth of diatoms and
dinoflagellates. The mixed cultures were then filtered
again through filter papers. Filtration was quite
effective in removing the diatoms and similar large sized
plankton. The process was repeated several times so that
only small flagellates and small coccoid algae were left
in the mixture.

Further isolation was done by agar plating and
dilution technique. 1.5% agar was prepared in Miquel's
medium and inoculated with the algal mixture. These were
incubated for a week. Certain green and brown growths of
algae were observed on the agar plates following incubation.
These were transferred to liquid medium. However, agar
plating method was not found to be effective for the
isolation of flagellates.

Dilution technique was found to be quite successful
for the isolation of flagellates. Culture medium (Miquel's
medium) was taken in a series of test tubes and each
inoculated with a drop of algal mixture. These were
examined under the microscope every fourth day and the
successful cultures were diluted. The process of successive

dilution was done repeatedly. By this method two
unialgal cultures of micro-flagellates which were not
available in the regular stock cultures of the Institute
were obtained.

The new isolates were identified to be _Chromulina_
freiburgensis Doflein and _Isochrysis_ _galbana_ Parke _forma_
nova. The morphological features and the physiological
characteristics were used for the identification of these
flagellates. Live specimens were examined under the
microscope to study the pattern of movement and the internal
organisation. Size measurements were taken from the live
specimens. The nature and number of the flagella could
not be ascertained from the light microscopic observation.
So the specimens were scanned in Scanning Electron Microscope
(PSEM 501). For SEM study the cultures were fixed in 2%
osmium tetroxide in Millonig's buffer for one hour at low
temperature. The fixed cells were washed with de-ionized
water. A drop of the concentrated culture was transferred
to a glass substratum and dried in dust free atmosphere.
The dry samples were coated with gold and examined in the
SEM.

The two new isolates were maintained in the
laboratory to study the physiological characteristics.
From the stock cultures maintained in the CIFRI culture

EXPLANATION OF PLATE IV

2. Stock cultures of C. freiburgensis, S. salina
   and I. galbana (C.s.)

1. Cultures of S. salina grown in Haffkine flasks.

PLATE IV

1
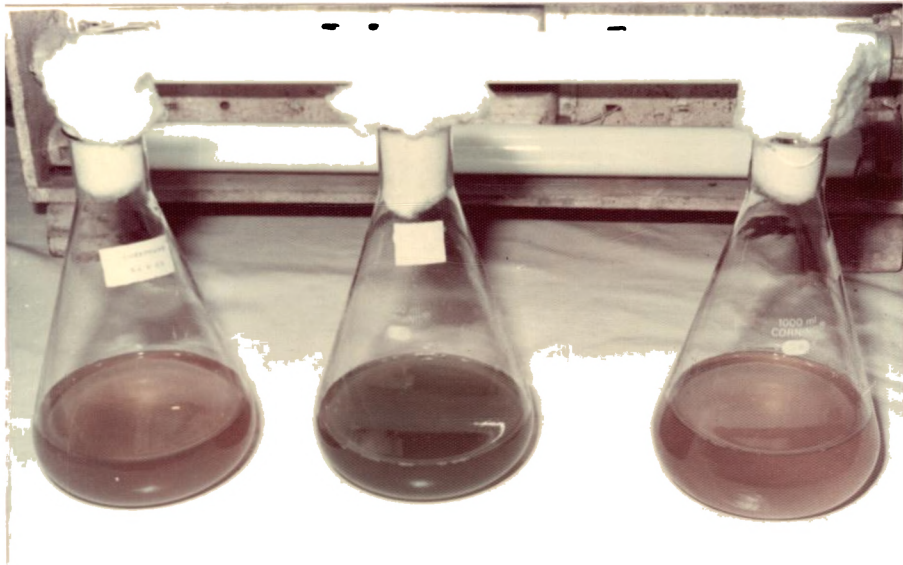
2

collection, two species: _Synechocystis_ _salina_ Wislouch and _Tetraselmis_ _gracilis_ Kylin were also used for making comparative studies (Plate IV).

3.2. Culture conditions

The nanoplankters were grown in enriched sea water as batch cultures. Sea water collected from offshore was allowed to age in carbuoys before preparing the culture medium. The sea water was filtered through Whatman GF/C filter paper; enriched with nutrients and autoclaved. The cool sterilised medium was equilibrated with filtered air for 24 hours and transferred to sterile culture flasks. Care was taken to keep the bacterial contamination low. However, the cultures were not totally bacteria free.

Different nutrient solutions like Miquel's medium (Miquel, 1391), Erd Schreiber solution (Schreiber, 1925) and Walne's medium (Walne, 1974) were used to culture the plankton. However, after a few experiments the standard media were modified based on the observations. The composition of this modified Miquel's medium is given below:

Solution A

| Potassium nitrate (KNO_3) | ... | 20 gms |
| Distilled water | ... | 100 ml |

Solution B

Sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$)	...	2.5 gms
Distilled water	...	1.0 ml

Solution C

Calcium chloride ($CaCl_2 \cdot 6H_2O$)	...	4 gs
Ferric chloride ($FeCl_3$)	...	2 gms
Con. Hydrochloric acid	...	2 ml
Distilled water	...	98 ml

Solution D

Disodium ethylene diamine tetra acetic acid (EDTA)	...	300 mgs
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	...	12 mgs
Zinc chloride ($ZnCl_2$)	...	1.5 mgs
Cobaltous chloride ($CoCl_2 \cdot 6H_2O$)	...	3 mgs
Copper sulphate ($CuSO_4 \cdot 5H_2O$)	...	0.12 mgs
Ortho Boric acid (H_3BO_3)	...	60 mgs
Distilled water	...	1 c ml

Culture medium

Solution A	...	.9 ml
Solution B	...	0.5 ml
Solution C	...	0.5 ml

Solution D   ... 0.25 ml

Sea water   ... 1000 ml

Tris buffer   ... 50 mgs

The salinity of the sea water used to prepare
the medium was 15-20%, for the culture of D. salina and
I. gracilis while C. freiburgensis and I. galbana (C.s.)
were grown at 30-35% salinity.

The stock cultures and experimental cultures were
grown in one litre conical flask or three litre Haffkine
flask made of corning glass. The flasks were plugged with
non-absorbent cotton. The cultures were placed on shelves
illuminated with fluorescent tubes giving an illuminance of
20,000 lux on a 10:14 light-dark regime. This light
condition was used for all the experiments unless specified.
The ambient temperature was 24°C at night hours to 35°C at
noon in summer months.

The cultures were not aerated. Once a day, the
flasks were swirled manually to give three to four rotations
of the suspension. The plankters grew well without
settling for about a month, but the older cultures showed
the tendency to settle down. This was avoided by using
culture tubes that could be aerated from below.

The culture tube used for the purpose was a modified version of the one used by Prof. Steemann Nielsen in his laboratory (Plate V). It consists of a tube of diameter 7.5 cm and 500 ml capacity, ending in a conical bottom which is drawn out into a narrow tube of diameter 1.- cm. The narrow tube recurves and runs parallel to the wide tube and ends in a bulb that opens out. The wide tube is provided with an outlet fitted with glass stopper. This facilitates inoculation and sampling. The upper end of the tube narrows into a curved tube. The culture tube is also provided with three supports at the base. Sterile air is let in through the narrow tube. The curved tube favours the exit of the air as well as prevents the bacterial contamination.

More controlled conditions of growth were obtained by immersing the culture tubes in a temperature bath (Plate VI). The temperature bath consisted of a Perspex tank of 10 litre capacity filled with water and heated by an immersion heater. The heater was connected to a Jumo thermometer through a thermostat unit. The water in the tank was kept in circulation so that heating was uniform. This set up could be used to attain as well as maintain temperatures at 30°, 35°, 40° and 45°C. The heating system could not be used for lower temperatures. This was accomplished by adding ice to the tank. The entire

Modified culture tubes for growing axenic cultures
of nanoplankton in the laboratory.

PLATE V

1

2
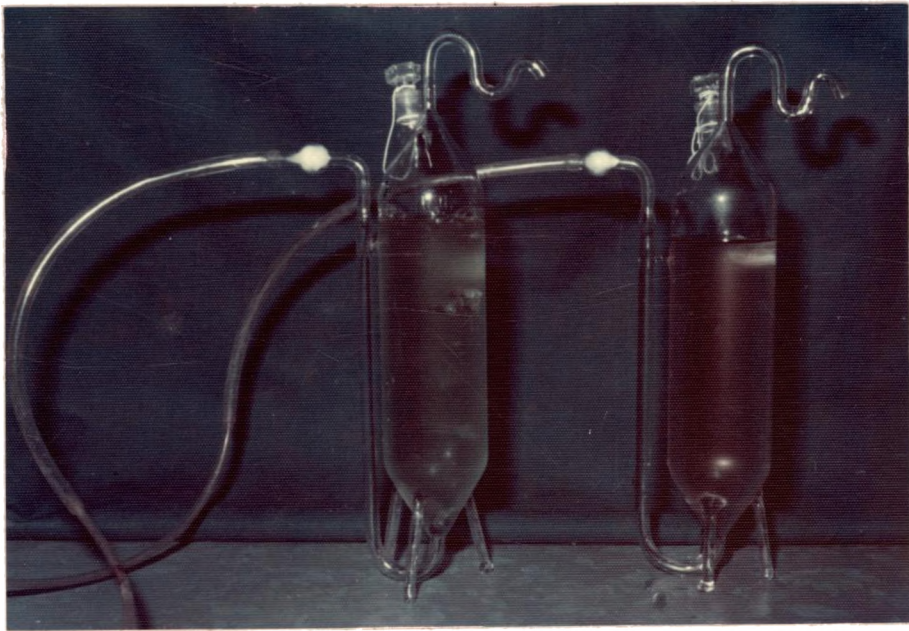
Experimental set up for controlling ambient
temperature for nanoplankton culture.

PLATE VI
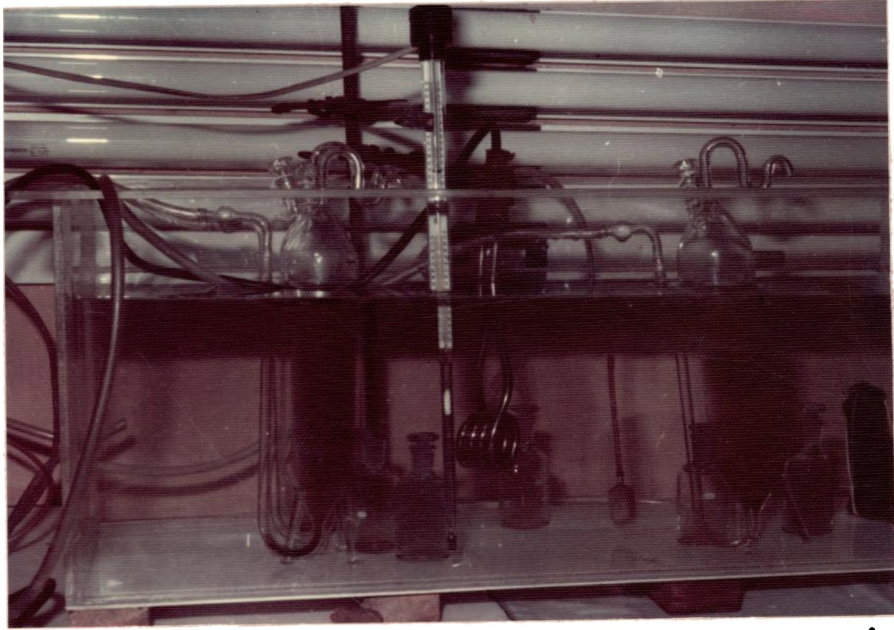
1
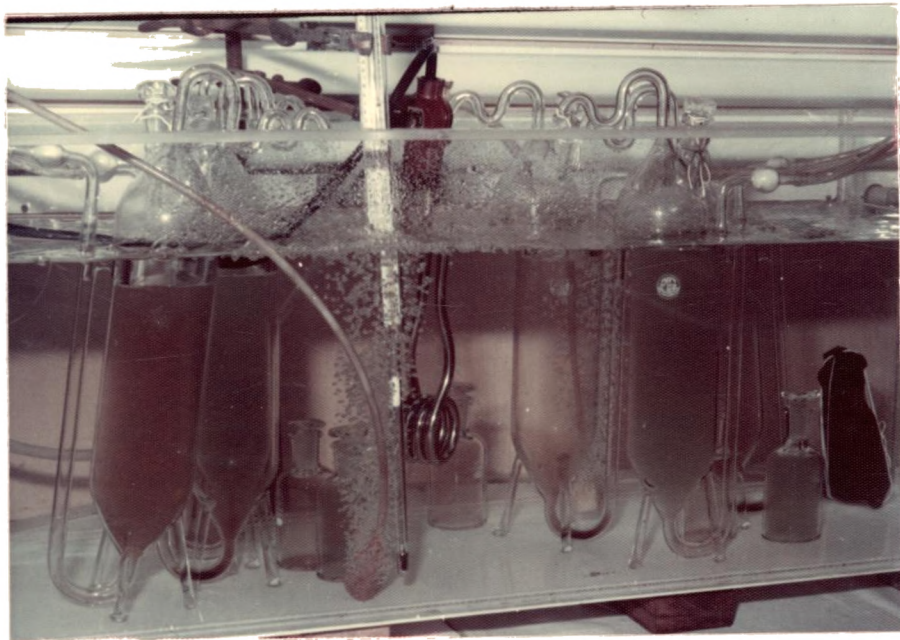
2

set up was illuminated from above as in the other experiments.

Whenever necessary the incident light also was controlled by using appropriate filters to cut off the light.

3.3. Growth measurements

3.3.1. Determination of cell number

Aliquots of the culture (5-10 ml) were taken after thorough mixing and fixed in Lugol's iodine. The cells were counted with a previously calibrated Haemocytometer.

The efficiency of growth was calculated as growth rate or doublings per day (k) given by the equation (1) (Epley and Strickland, 1968).

$$k_{(division/day)} = \frac{3.32}{t-t_o} (log_{10} n_t - log_{10} n_{to}) \quad ... \quad eq.1$$

where time is in 24 hour day and $3.32 = log_2 10$.

The instantaneous rate of growth or the specific growth rate (μ/day) was computed from 'k' following the equation given by Goldman, 1979 (eq. 2).

$$\text{doublings per day} \times 0.693 = \mu \quad ... \quad eq.2$$

The mean generation time (t_g) was calculated from eq.3 (Strickland, 1960).

$$t_g = \frac{.7}{k} \quad \text{where} \quad k = \text{relative growth constant/hr} =$$

$$\frac{1_n \cdot n_t - 1_n \cdot n_o}{t}$$

3.3.2. Determination of the physiological activity

The rate of photosynthesis and the amount of the pigments were used as indices of physiological activity.

The rate of photosynthesis was determined by the carbon-14 technique (Steemann Nielsen, 1952; Steemann Nielsen and Jensen, 1957). The cultures were diluted about ten times and taken in 60 ml bottle. Dilution was necessary to prevent any shading effect and also the self absorption of the beta rays on the filters. The ^{14}C was added in the form of sodium bicarbonate at pH 9.0. The isotope was supplied by BARC, Bombay. 1 ml of the hot solution having an activity of 5μC was added to each sample. The operations were done in subdued light. Dark bottle controls were also kept simultaneously. The hot samples were incubated for two to four hours. The incubation was done in a rotary incubator illuminated by a set of fluorescent lamps or they were left alongside the cultures for incubation. All samples had replications.

The incubated samples were filtered immediately through Millipore HA filters (0.45μ). The filter papers

were rinsed with filtered sea water to remove any
adhering inorganic ^{14}C and dried in desiccator over silica
gel.

The activity of the hot samples was determined with
the aid of Geiger Counter (GCS 16, EC) of efficiency 3.2%.
The rate of production was expressed as mg C/litre/hr.

$$\text{Rate of production} = \frac{\text{net activity x carbon dioxide}}{\text{added activity x hrs of incubation}}$$

where net activity was determined and added activity was
computed by the biological method of Steemann Nielsen, 1965
and compared with the Liquid Scintillation technique of
Jitts and Scott, 1961 (Nair, 1966).

The amount of carbon dioxide was determined by a
double titration method using phenolphalein followed by
methyl orange titration (APHA, 1980).

The light and dark bottle technique of Gaarder and
Gran (1927) was also used to determine the physiological
state of the cultures. Diluted cultures were incubated for
6-8 hours in bottles of 125 ml capacity. The oxygen content
of the initial, dark and light bottles were determined by
the Winkler titration. The oxygen values were converted to
their carbon equivalents.

$$\text{Production (mg C)} = \frac{O_2 \text{ ml x } 0.536}{P.Q.}$$

where P.Q. = Photosynthetic quotient = 1.25

The amount of chlorophylls and carotenoids were
determined by spectrophotometric analysis of the acetone
extracts applying the equations of Parsons and Strickland,
1963 and Lorenzen, 1967.

50-100 ml of the cultures were filtered through
Millipore HA filters. 1-2 drops of 1% Magnesium carbonate
was added to the samples while filtering. The pigments
were extracted by adding 10 ml of 90% acetone for each
filter. The extraction was carried out at low temperature
for about 24 hours. The extracts were centrifuged and the
extinction of the clear solution was measured by
spectrophotometer - Unicam (SP 5) and LC (GS 8630).

3.4. Organisation of individual experiments

3.4.1. Growth and activity of Chromulina freiburgensis, Isochrysis galbana (C.g.) and Synechocystis salina in batch culture.

Healthy cultures of C. freiburgensis, I. galbala (C.s.)
and S. salina were inoculated into modified Miquel's medium
taken in Haffkine flasks. The culture volume was
restricted to two litres. Duplicates were kept for each
species. The cultures were illuminated artificially at
ambient temperature.

The cultures were maintained for 30 days. Aliquots were withdrawn on alternate days to determine the cell number, pigment content and physiological activity (described in 3.3).

3.4.2. Pigment composition of C. freiburgensis, I. galbana (C.s.), S. salina and I. gracilis.

Unialgal cultures of the species were grown as in the above experiment. The cultures were harvested during the exponential phase.

Preparation of the pigment extract

The algae from 1 0-2 0 ml of cultures were harvested by filtering through Millipore HA filters and the pigments extracted with 1 ml of 9 acetone. The pigment extract was immediately transferred to an equal volume of diethyl ether. The mixture was taken in a separating flask and shaken with about ten times its volume of 10% sodium chloride. The pigments migrated to the ether layer. The ether phase was repeatedly washed with sodium chloride solution to remove any excess acetone and water soluble impurities. The ether layer was concentrated under a stream of nitrogen. Condensed water was removed from the samples by centrifugation.

Preparation of silica gel thin-layer plates

Silica gel was made into a slurry with distilled water (1:2 w/v) and coated on to chromatographic plates (20 x 20 x 0.2 cm) at a thickness of 0.25 mm using an applicator. The coated plates were air-dried. The plates were activated at 100°C for half an hour and stored in desiccator. The plates were used within a week of preparation.

The concentrated pigment extracts were applied to the TLC plates using a capillary spotting aid. Along with the samples standard chlorophyll a (supplied by Sandoz Ltd., Switzerland) was also developed. The extracts were dried over the TLC plates in a gentle stream of nitrogen. The entire process was carried out in diffused light.

Chromatographic tanks were lined with black paper and equilibrated with the appropriate solvent mixture before the plates were developed. The solvent system used was n-propanol: light petroleum (60°-80° fraction) (2.5:97.5; v/v) for the first dimension and chloroform: light petroleum: acetone (25:75:0.5; v/v/v) for the second dimension. After the first run, the plates were dried in dark and then developed in the second dimension. The first run took 60 minutes to complete and the second run, 45 minutes.

The dry plates were observed under UV light to
locate the pigments. The solvent front and the pigment
spots were marked. The colour of the pigments also was
noted. The R_F value of the pigments were computed.
Chlorophyll a was identified based on the colour of the
spot and R_F of the standard. The other pigments were
identified with the help of the guidelines given by
Jeffrey, 1968 and 1981.

3.4.3. Estimation of biochemical composition

The cultures of C. freiburgensis, I. galbana (C.s.),
D. salina and T. gracilis were grown in modified Miquel's
medium under artificial illumination with light-dark cycle
at ambient temperature. Samples were taken after five
days, ten days and twenty days of growth for analysis.
The cells were collected by either centrifugation or
filtration. The adhering NaCl crystals were removed by
washing the cells with an isotonic solution of Ammonium
formate. As the samples could not be analysed immediately
they were deep-frozen. Six replicates of each sample were
analysed. The cultures were not totally bacteria free.
So the bacterial counts also were taken during sampling.

Cell number, dry weight and moisture content

The cell number was counted by Haemocytometer.
The dry weight of the algae was determined by the standard

method of evaporating off the water content from filtered samples.

Millipore HA filters were washed with distilled water, dried and weighed. 50-100 ml of cultures and corresponding volume of media were filtered through filter papers of identical weight. After filtration the samples were rinsed with an isotonic solution of ammonium formate to remove any sodium chloride crystals retained. The papers were sucked dry, folded, blotted and weighed immediately.

The samples were dried to constant weight at $90\pm5^{\circ}C$; cooled and weighed. The weight of the filter papers were deducted to get the weight of the algae.

$$\text{Dry weight } (\%) = \frac{\text{Wt. of the dry sample}}{\text{Wt. of the wet sample}} \times 100$$

$$\text{Moisture content } (\%) = \frac{\text{Wet wt. - dry wt.}}{\text{Wet wt.}} \times 100$$

Carbohydrate

The carbohydrate content in algae is composed of cell wall polysaccharides and reserve carbohydrate in addition to intermediate sugars of the photosynthetic pathway. A good part of these algal polysaccharides break up into simple sugars on acid hydrolysis. These simple sugars can be estimated by Nelson-Somogyi method (Nelson, 1944; Somogyi, 1945).

The nanoplankton derived by the centrifugation
of 25 ml of the culture was hydrolysed with 30% sulphuric
acid in a waterbath. Hydrolysis was done for six hours to
ensure complete breakdown of the polysaccharides. The
samples were cooled and centrifuged. 1 ml of this clear
solution was pipetted into a clean test tube and the
arseno-molybdate reagent added. The absorbance of the
coloured solution was measured at 530 nm using a
spectrophotometer (Spectrome 20). Glucose was used as the
standard.

Protein

The protein content of the nanoplankton cells was
determined by Lowry's method (Lowry et al. 1951) using
Folin-Ciocalteu reagent. The algal pellet obtained by
centrifuging 250 ml of cultures was extracted with 80%
ethanol. The protein precipitated was separated by
centrifugation. The precipitate was dissolved in 1 N sodium
hydroxide. It was again centrifuged to remove any
remaining cell particles. The protein content in the
extract was estimated by the Lowry's method. The optical
density of the blue colour developed was measured at 530 nm
using spectrophotometer (Spectrome 20). The standard
graph was prepared using Bovine serum albumin.

Lipid

The lipids of algae comprise photosynthetic pigments - chlorophylls and carotenoids and other compounds like phospholipids and glycolipids. These are extractable by organic solvents. The method employed was that of Bligh and Dyer (1959).

Adequate amounts (25 ml) of the cultures were centrifuged and to the algal pellet added a mixture of chloroform and methanol (2:1 v/v). The extraction was done repeatedly with small volumes of the solvent for about 3-4 hrs. The mixture was transferred to a separating flask and shaken with distilled water. The lipid layer was stripped off; centrifuged to remove any cell particles and the solvent evaporated off in a waterbath. The weight of the lipid fraction was determined.

The level of carbohydrate, protein and lipid, in the nanoplankters was calculated as percent dry weight.

Isolation of bacteria

The standard dilution plate technique (Pramer and Schmidt, 1966) was followed for the enumeration of bacteria. Nutrient agar (Allen, 1953) prepared in filtered sea water was used as the medium for growing bacteria. The composition of the medium is given below:

Nutrient glucose agar

Glucose	...	5 gms
Lactopeptone	...	5 gms
Beef extract	...	3 gms
Agar	...	15 gms
Filtered sea water	...	1000 ml
pH	...	7.5-8.0

The bacterial plates were incubated for 96 hours and the counts were taken.

3.4.4. Estimation of the extracellular products of the nanoplankters.

The sterile media (5.0 ml modified Miquel's medium) were inoculated with approximately 10^4 cells of the unialgal cultures. The species under study were C. freiburgensis, I. galbana (C.s.), D. salina and I. gracilis. Growth conditions were the same as in the previous experiment. Sampling was done on the 4th, 8th and 16th day of growth.

The samples were diluted and incubated in triplicate with carbon-14 for two hours in the same conditions as the culture phase. After incubation the samples were filtered through Millipore HA filters. The filtrate was retained. The filters were washed with filtered sea water sucked dry and stored in desiccator. 1.0 ml of the culture filtrate

was acidified with 1 ml of N/10 HCl and the radioactive bicarbonate was removed by bubbling with air. The solution was then neutralized with 1 ml of the N/10 sodium hydroxide. The radioactivity of the samples was measured using Scintillation Counter (LSS 20, EC).

Liquid Scintillation counting

The liquid scintillation system works on the photon emission and its subsequent conversion to electronic pulse, the former being released by certain compounds when bombarded by nuclear radiation.

Procedure

1. Determination of counting efficiency

The activity of the quenched standards of ^{14}C was determined by two-channel counting and the channels ratio (AB/AC) was computed. From this the counting efficiency was worked out as presented in table 1 given below.

The counting efficiency was plotted against channels ratio to obtain the quench correction curve (Plate VII).

2. Standardization of ^{14}C ampoules

0.1 ml each of the radiocarbon was withdrawn from ampoules of 1 ml capacity and the cpm determined. The

EXPLANATION OF PLATE VII

1. Liquid Scintillation Counting System used for
   the measurement of excretory products of
   nanoplankton.

2. Channels ratio versus Detection efficiency of
   the counting system.

PLATE VII
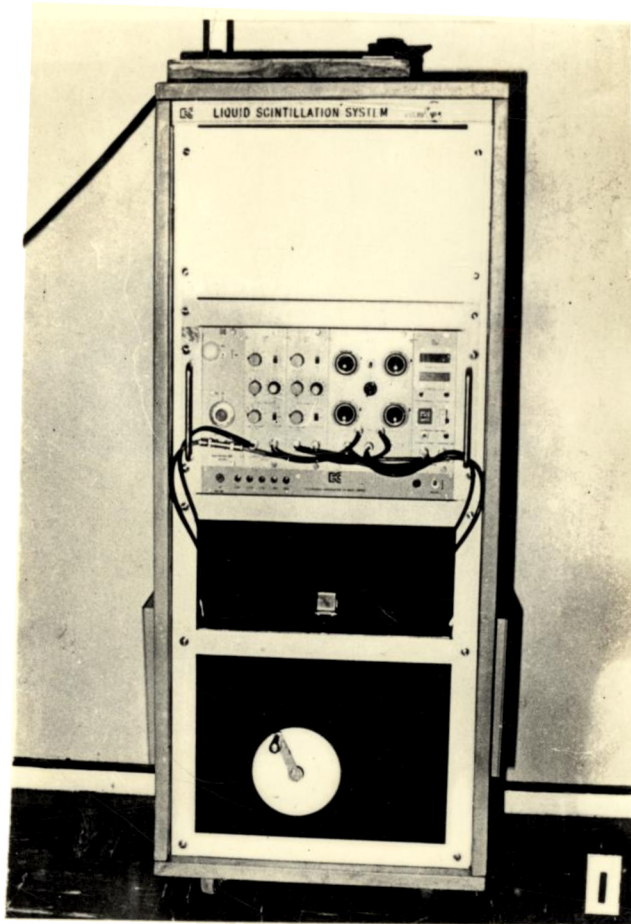
Channels ratio quench correction curve
for the liquid scintillation counting of ^{14}C

2
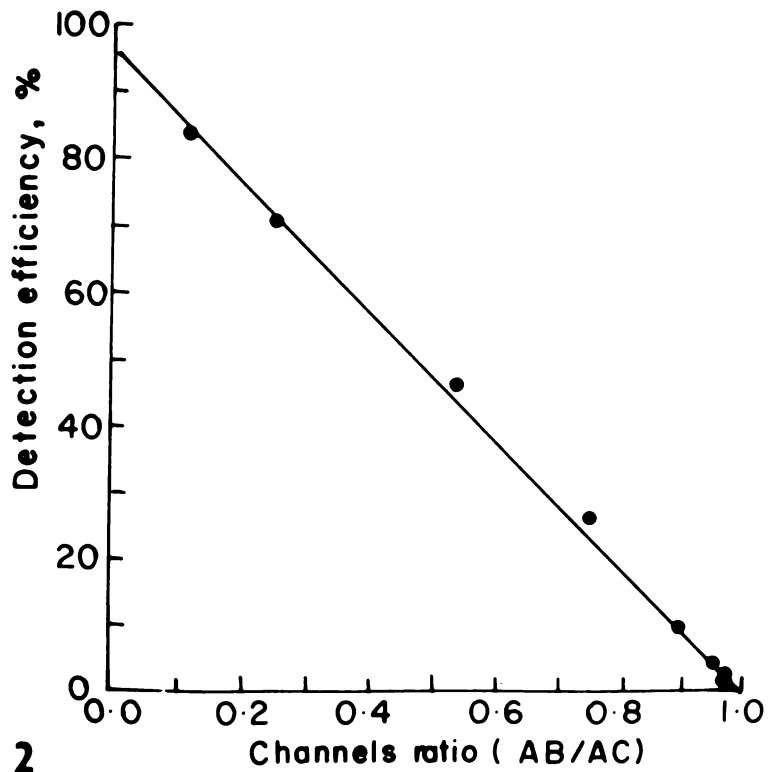
Table. The count rate and channels ratio for the
quenched standards of ^{14}C.

Quenched standard	C_1 or AC (cpm)	C_2 or AB (cpm)	C_2/C_1	Efficiency (%) $= \dfrac{C_1}{20,3\ldots}$ x1
1	1,70,973	22075	0.12	84
2	1,45,993	37559	0.25	71
3	96,867	51404	0.53	47
4	55,683	41530	0.74	27
5	21,671	19479	0.89	10
6	9,390	8968	0.95	4
7	4,398	4280	0.97	2
8	2,503	2439	0.97	1

counting efficiency of these samples were determined
from the quench correction curve and the absolute activity
was calculated as μC/ampoule.

3. The activity of the filters was determined by liquid
scintillation suspension counting (Krishnamurthy and
Viswanathan, 1963). The filter paper was dissolved in 1 ml
of acetone in a test tube, rinsed twice with dioxane and
transferred to the scintillation vial containing polystyrine
gel. The gel was prepared by dissolving 4.0 gms of
polystyrine in 10 ml of dioxane scintillator. The
composition of the dioxane fluor is given below:

PPO	:	4 gms
POPOP	:	5. mgs
Naphthalene	:	12. gms
Dioxane	:	1000 ml

The sample remained on the surface of the gel.
This was fed into the counter and the counts for hundred
seconds were taken.

Liquid scintillation solution counting was employed
to measure the activity of the filtrate. 0.5 ml of the
filtrate was taken in scintillation vial and 15 ml of
scintillator was added to it. The vial was shaken to get

a homogeneous solution. The activity of this was counted for 1ϋϋ seconds.

Jnlabelled samples served as blank in each determination and these blank values were deducted from all sample readings. The absolute activities of ϋhe samples were determined from the quenc⁁ correction curve.

3.4.5. **Influence of salinity on growth**

The nanoplankters under study were isolated from the coasϋal region of Cochin near the barmouth where the salinity fluctuates from brackish to strictly marine (Derbyshire, 1967). So the response of ϋhe nanoplankters to different salinity levels was studied in culture. The organisms used for the study were **Chromulina freiburgensis**, **Isochrysis galbana** (C.s.) and **Synechocystis salina**. The mother cultures were grown at salinity 34ϸϵ. which is often obtained in the sea waϋer off Cochin. 1 ml of these culϋures were inoculated into 5ϋϋ ml of ϋodified Giquel's medium. ϋach alga was grown in three saliniϋy levels 34ϵϵ., 24ϵ. and 14ϵϵ. Three replicates were keϋt for each ϋreatment. The nine experimental cultures of each species were randomized completely ϋo a uniform illumination of 2ϋ,ϵϵϵ lux from white fluorescent tubes at a bient

temperature (Plate VIII). The design of the
experiment is given below:

I	II	II
II	I	III
III	I	III

Treatment I   :   14‰
Treatment II   :   24‰
Treatment III :   34‰

The lower salinities of 14‰ and 24‰ were obtained
by diluting the sea water with distilled water. These
were autoclaved and aerated for 24 hours before adding
the nutrients. The pH was adjusted to 8.0 using Tris
buffer.

The growth of the culture was followed for three
weeks. The cell density and the amount of chlorophyll a
was measured regularly. After three weeks the experiment
was discontinued as it was found that the cell
multiplication and the amount of chlorophyll had decreased
considerably and the culture showed senescence.

3.4.6. Effect of pH on growth

The pH of the sea water under normal conditions
vary from 7.5 to 8.3. But factors like dilution, bacterial
action and human activities are found to change this normal

EXPLANATION OF PLATE VIII

The experimental set up of the cultures of
C. freiburgensis (1) and S. salina (2) to test
their tolerance to three different salinity levels.

PLATE VIII

1

2

state. The response of the phytoplankton also may vary to the changed pH. This was tested in cultures of nanoplankton using four different pH levels. The experiment was done on similar lines as the previous one (3.4.5) using the same organisms.

Modified Miquel's medium was prepared in sea water of salinity 34‰. and pH 8. This medium was acidified to obtain the different pH levels: 6.5, 7.0 and 7.5. 500 ml of these media taken in one litre corning flasks were inoculated with the cultures that were growing at a pH 8. Each treatment was done in triplicate. The treatments were completely randomized as shown below.

IV	III	I	III
IV	II	II	IV
I	II	III	I

Treatment I   :   6.5

Treatment II   :   7.0

Treatment III :   7.5

Treatment IV   :   8.0

The cultures were observed till they reached the stationary phase. The cell density at the peak growth

phase (4 days) and the final yield (on 16th) were compared. Simultaneously the amount of chlorophyll a was also measured.

3.4.7. Effect of cell concentration on the rate of carbon fixation.

While estimating the rate of photosynthesis many factors are reported to interfere with the accuracy of the measurements. The density of the phytoplankton is one such factor. This was tested in cultures of the nanoplankters - C. freiburgensis, I. galbana (C.s.) and S. salina so that the optimum cell density that must be used for the photosynthetic rate measurements could be fixed.

The cultures of the three organisms were grown in modified Miquel's medium. The flagellates were provided with medium of salinity about 34‰ and the blue-green algae were grown at a salinity of 15‰. The culture conditions were similar to those described in 3.2. The cultures were allowed to grow and multiply for a week so that sufficient cell population was built up. C. freiburgensis grew upto a density of 14.4 x 10^5/ml; I. galbana (C.s.) to a concentration of 6.3 x 10^5/ml and S. salina to 33 x 10^5/ml.

The samples of these cultures were withdrawn and diluted with the medium to get different densities. These were incubated in triplicate with carbon-14 for two hours in the same culture conditions as the culture. After incubation the samples were filtered through Millipore HA filters and the activity was measured using the Geiger counter.

3.4.8.   Influence of light on growth and activity of nanoplankters.

Batch cultures of the three nanoplankters were raised in modified Miquel's medium under ambient temperature and 20,000 lux light. Two sets of duplicate cultures were maintained. One set was illuminated continuously from cool-white fluorescent lamps, while the other set was subjected to light-dark cycle of 10:14 hours. The increase in the cell population was studied for a month. The final yield and growth rate were compared for both the light conditions.

During the logarithmic phase of growth of the constantly illuminated cultures aliquots were withdrawn to study their photosynthetic activity at different light intensities. The samples were diluted suitably and incubated with ^{14}C in 60 ml bottles placed on a rotary incubator.

The rotary incubator used for the purpose was
similar to that described by Steemann Nielsen and Hansen
(1961) and Jair (1974). The sample bottles were fixed on
the rotating wheel of the incubator and covered with
appropriate light filters simulating the different light
conditions. All samples had duplicates. The rotating
wheel was lowered into the tank filled with water. The
temperature of the water was $30^{\circ}C$. It was illuminated by
a bank of ten white fluorescent tubes (20 watts each)
and a battery of three incandescent bulbs (100 watts each).
These together gave an illuminance of 68 x 10^{45} quanta x
cm^{-2}_x sec^{-1} i.e. 28.80 m watt x cm^{-2} or 40,000 lux.

The samples were pre-exposed to the new light regime
for one hour before the addition of ^{14}C. The experiment
proper took two hours of incubation after which the samples
were filtered and the activity measured in Geiger counter.

3.4.9. Influence of temperature on growth and viability
of nanoplankters.

The nanoplankters were exposed to a range of
temperatures from $5^{\circ}C$ to $45^{\circ}C$ to study the effect on
motility and viability in cultures. Cultures growing at
room temperature were exposed to the low temperature of a
refrigerator $(5^{\circ}C)$. Samples from this were recultured into

fresh medium at room temperature every week. This was
done for about six months. The cultures were similarly
exposed to higher temperatures (upto 45°C) and their
motility and viability were observed.

The growth rate of these nanoplankters was
determined for the temperatures 2 ,25,30,35 and 40°C. The
cells were inoculated into about 500 ml of the culture
medium taken in the culture tubes (described in 3.2). The
culture tubes were immersed into the temperature bath
maintained at the required temperature. Each culture
had its duplicate. The cultures were aerated constantly.
The whole set up was illuminated from above with five
fluorescent tubes of 40 watts each (Plate VI). The
heating system with the immersion heater and the Jumo
thermometer functioned to acquire and maintain temperatures
above 30°C. The lower temperatures (20 and 25°C) were
obtained by adding ice to the water in the tank. The
temperature in the water bath was kept uniform by keeping
the water in circulation by means of aerators.

The growth rate of the plankters was studied for
six days taking the cell counts daily at each temperature.
The results of the duplicate experiments were averaged.

During the exponential phase of these cultures, aliquots of 1. ml were withdrawn from each; diluted and the rate of photosynthesis determined by the ^{14}C method.

3.4.1.. Kinetics of utilization of macronutrients nitrate and phosphate).

Nitrate and phosphate are the major nutrients that contribute to the culture media. So the influence of different concentrations of these nutrients on growth rate of the nanoplankters was studied in batch cultures of limited volume.

To examine growth at different nitrate levels the cultures were first depleted of nitrate. This was done by growing cells of C. freiburgensis, I. galbana (C.a.) and S. salina in modified Miquel's medium devoid of added nitrogen source for a period of 10 days. The sea water used for preparing the medium contained only 1.5 µg-at NO_3^- N/litre.

Cells from the N-depleted cultures were then inoculated into flasks of sterile culture medium and various amounts of N as Potassium nitrate was added to each flask to yield concentrations from 2.6 to 3.. µg.at. N/litre. Duplicates were maintained for each treatment. The cultures were grown for six days under artificial illumination and

ambient temperature. The cell counts were taken daily.
The amount of NO_3-N in the medium was measured at the
beginning of the experiment. The cultures were filtered
and the filtrate was used for the determination of nitrate.
The NO_3 in the medium was first reduced to NO_2 and
determined colorimetrically employing the method described
by Strickland and Parsons (1968).

A similar experiment was conducted to find out the
amount of the phosphorus required by these organisms in
culture. The source of phosphorus was $NaH_2PO_4.2H_2O$.
Phosphorus starved cultures were inoculated into medium
containing different concentrations of phosphate. The
initial levels of dissolved phosphate was determined
spectrophotometrically for each treatment following the
methodology of Murphy and Riley (1962). As in the case
of nitrate the cell counts were taken for six days. The
mean of the duplicate experiments were taken and the half
saturation constants for growth was computed using the
relation $S = \mu max (S/\mu) - K\mu.$

R E S U L T S

4.1. Chromulina freiburgensis

Growth and activity in batch culture

Cultures of Chromulina freiburgensis were grown
in modified Miquel's medium to study its growth kinetics
(3.4.1). Actively growing cells were inoculated into
fresh medium to give a concentration of 350 cells/ml.
The cell number was counted after 48 hours to find whether
there was any noticeable increase. It was found that the
cell numbers increased gradually in the first 48 hours;
between the 2nd and 4th day, cell divisions were rapid
and the cell population increased four times. In the
following days of observation, the cell concentration
increased gradually, reaching a density of 1.4 million
cells/ml in 30 days. The highest growth rate was found to
occur between the 2nd and 4th day. The phase of declining
growth rate was found to be between 4th and 12th day.
After 12 days, the stationary phase began. The cell
numbers remained stationary till the 30th day when the
experiment was discontinued (Fig.4A). The highest growth
rate recorded for the species during this study was 2.66
divisions per day corresponding to a mean generation time
of 9 hours. The relative growth constant per hour was
0.079 (Table 2).

Table 2. Cell concentration and growth rate of three
nanoplankters grown at a light intensity of
2ᴏ,ᴗᴗ lux with a light-dark cycle of 1ᴗ:14
hours at ambient temperature.

Culture	Initial inoculum	Highest density attained	Maximum doublings	Minimum generation time	Relative growth constant per/
	cells/ml	cells/ml	'k'	't_g'	
Chromulina freiburgensis	35ᴗ	14,25ᴗᴗᴗ	2.66	9	ᴗ.ᴗ79
Isochrysis galbana (C.s.)	35ᴗ	6,9ᴏᴏᴏᴏ	1.48	16	ᴗ.ᴗ43
Synechocystis salina	144ᴗ	4,75,ᴏᴏᴏᴏᴏ	2.74	9	ᴗ.ᴗ79

The live cultures were observed under the microscope to
study their motility. The motility of the cells in the aged
cultures was comparatively less. Many cells were non-motile
and sank to the bottom of the culture flask forming a thin
layer at the bottom. The colour of the young culture was
golden-yellow. As the culture grew old, the yellow colour
gave way to an orange-red tint.

The pigment content of the culture remained low
immediately after inoculation. Between the 2nd and 4th day
the amount of chlorophyll a, c and carotenoids rose up steeply.
After the 4th day the increase in the amount of the pigments
was gradual. It reached stationary phase by the 12th day

and this level was maintained till the 30th day (Fig.15).
However the amount of pigments per unit number of cells
was highest during the first week of culture (Table 3).
The maximum values for chlorophyll a, c and carotenoids was
0.243, 0.189 and 0.526 $\mu g/10^6$ cells. Chlorophyll a was
always greater than chlorophyll c while carotenoids
dominated over the chlorophylls. The amount of the
carotenoid pigments was about double that of chlorophyll a
till the 24th day of culture; but later the proportion of
carotenoids became ten times that of chlorophylla a
(Table 3).

The metabolic activity of the flagellate was measured
in terms of the oxygen exchange. The rate of production was
observed to increase during the period upto 10 days (Fig.2A);
the rate of production decreased on 12th and 14th and
remained more or less stationary for the rest of the period.
The highest production was 120 $\mu gC/10^6$ cells/hour (Gross
production) and 40 $\mu gC/10^6$ cells/hour (net production).
After the 10th day, the rate of net production decreased to
10 $\mu gC/10^6$ cells/hour.

As per the ^{14}C uptake the photosynthetic activity
increased upto 16 days and further declined (Fig. 2B). The
highest production was observed to be 60 $\mu gC/10^6$ cells/hour.

Fig. 1A.   Increase in cell number of _Chromulina_
           freiburgensis during growth in batch cultures

Fig. 1B.   Variation in the amount of photosynthetic
           pigments of the above species during growth
           in batch culture.

Fig. 1.
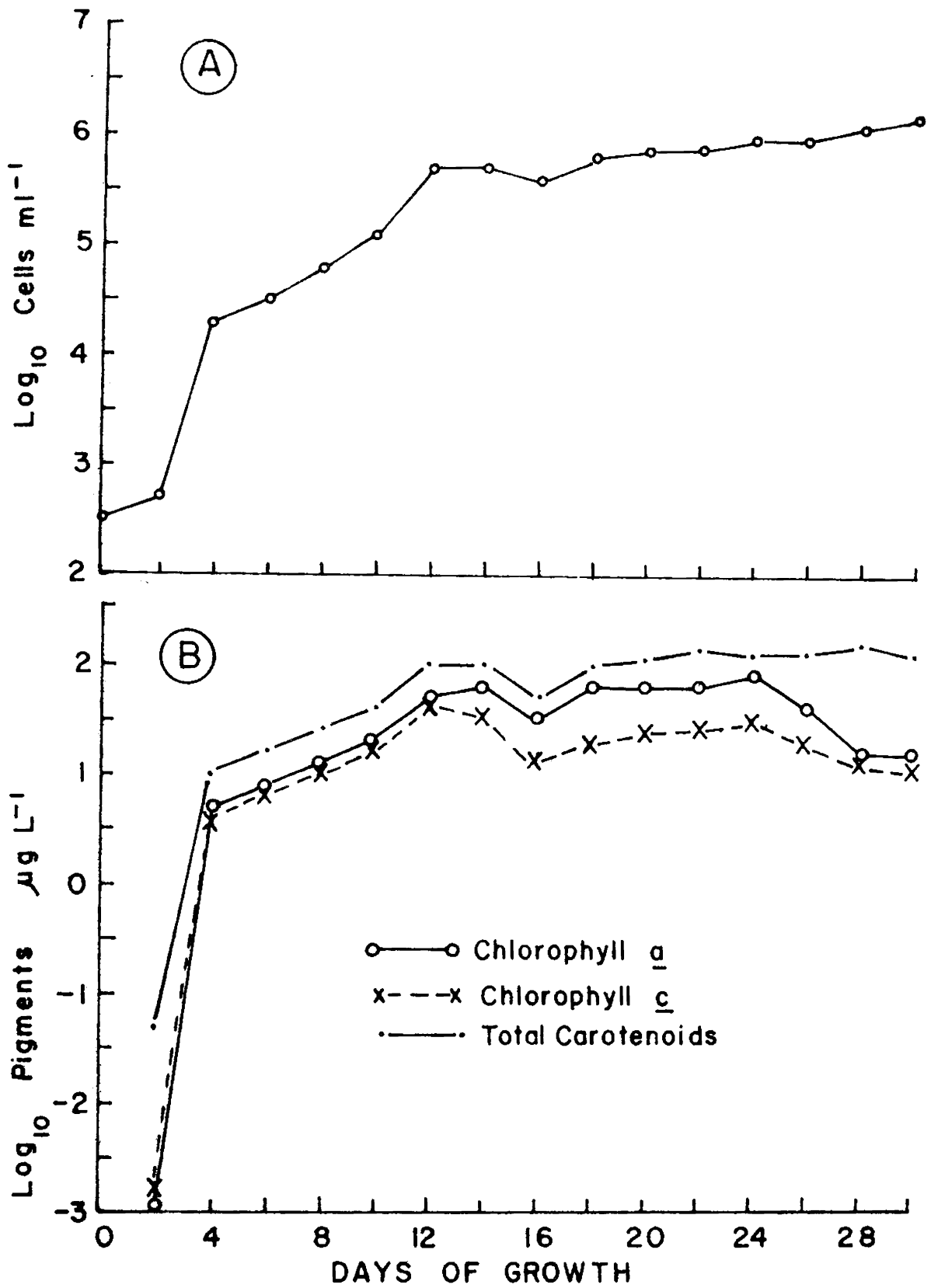
Fig.2A. Rate of production of organic carbon by
C. freiburgensis for a growth period of 30 days
in batch culture (measured by the oxygen
technique).

Fig.2B. Rate of production of organic carbon by the
above species for a growth period of 30 days
in batch culture (measured by the ^{14}C technique

Fig. 2.
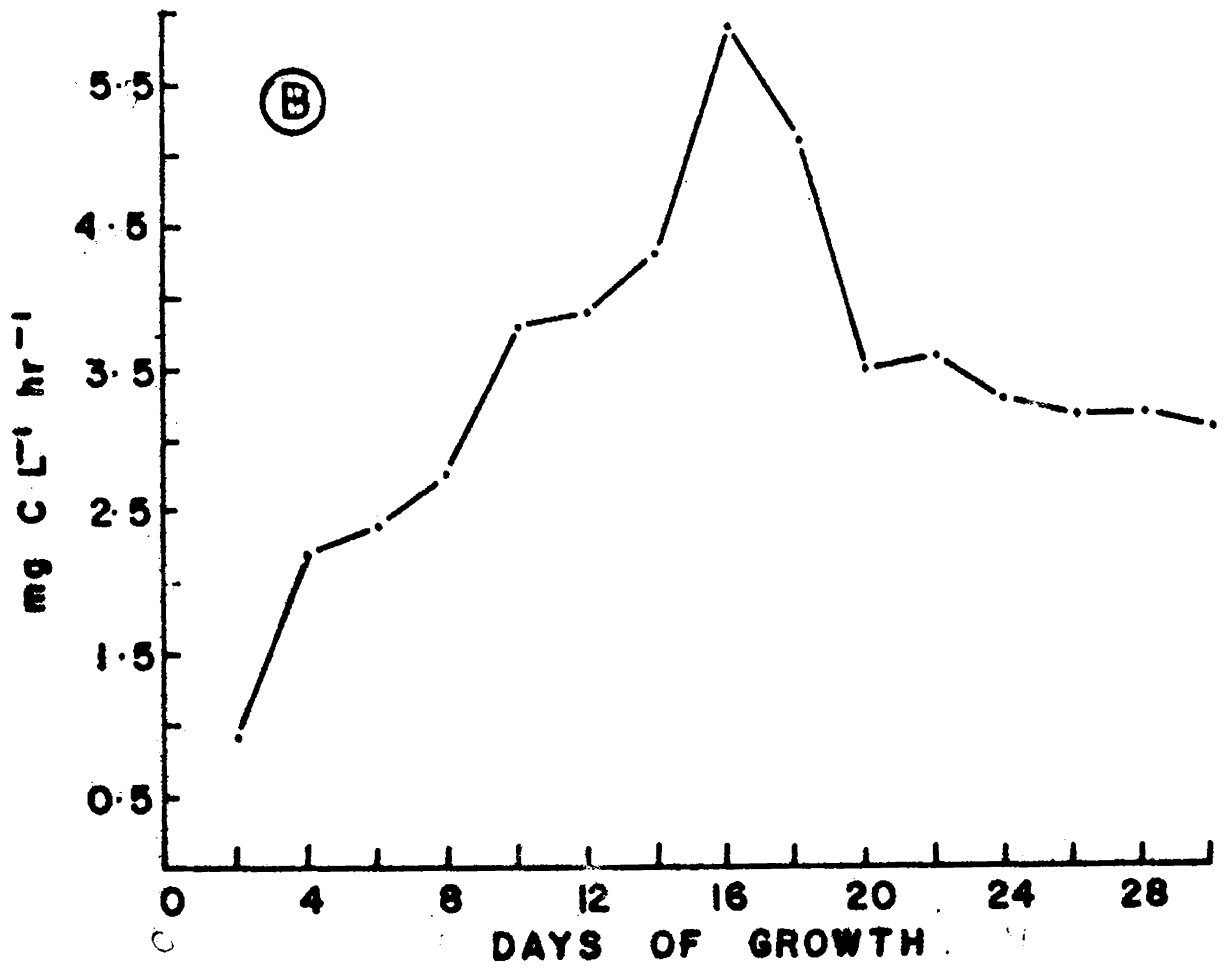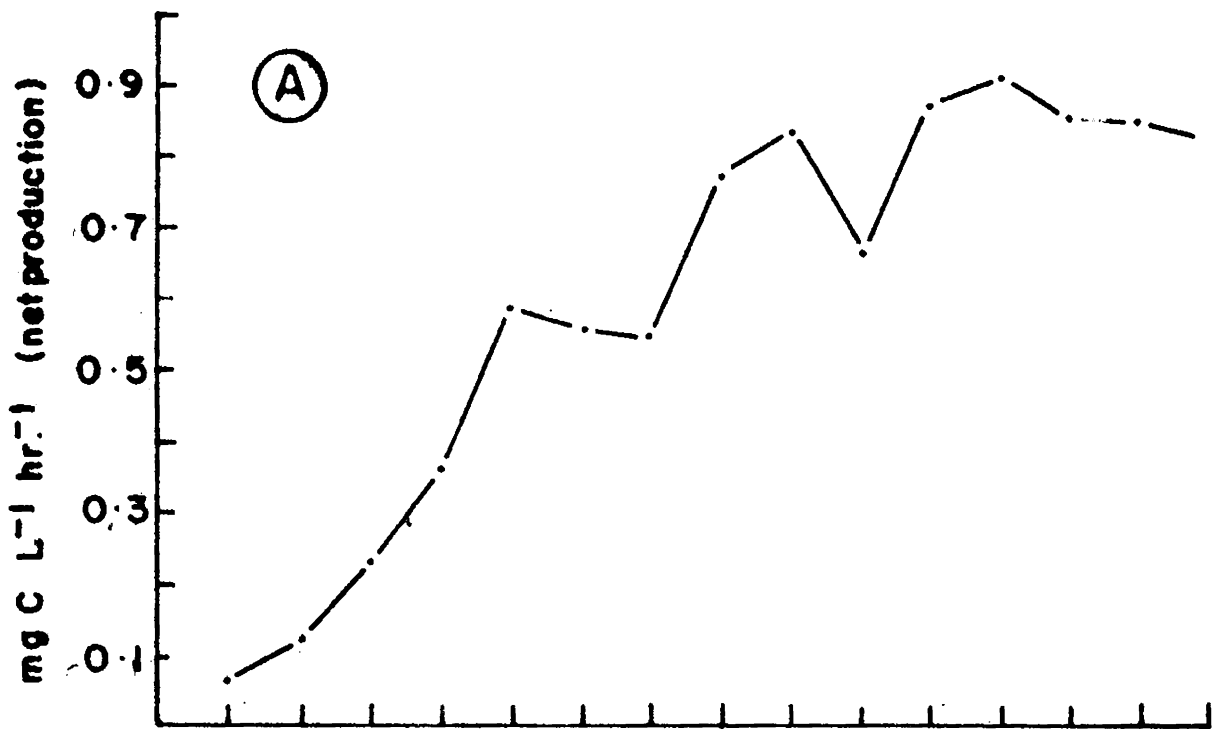
Table 3.   Variation in the amount of pigments expressed
as $\mu g/10^6$ cells of _Chromulina freiburgensis_ for
a growth period of 30 days in batch culture

Age of culture (days)	Chlorophyll _a_ $\mu g/10^6$ cells	Chlorophyll _c_ $\mu g/10^6$ cells	Total Carotenoids $\mu g/10^6$ cells	Carotenoids/ Chlorophyll _a_
2	0.198	0.150	0.405	2.05
4	0.245	0.189	0.526	2.15
6	0.236	0.178	0.453	1.92
8	0.214	0.159	0.364	1.70
10	0.182	0.134	0.312	1.71
12	0.112	0.074	0.200	1.79
14	0.110	0.058	0.182	1.65
16	0.083	0.034	0.138	1.66
18	0.085	0.030	0.143	1.68
20	0.080	0.036	0.152	1.90
22	0.078	0.030	0.148	1.90
24	0.069	0.028	0.143	2.07
26	0.040	0.018	0.125	3.13
28	0.012	0.010	0.128	10.31
30	0.010	0.008	0.105	10.49

on the 4th day. The rate of production remained at about
10 $\mu g C/10^6$ cells/hour till the 12th day and then decreased
further.

Pigment composition

The photosynthetic pigments of C. freiburgensis was analysed by TLC. The chromatogram resolved five fractions (Fig.33). The R_F value of these fractions are given in Table 4. The orange pigment, with the highest R_F value was identified as carotene. Chlorophyll a was blue-green in colour in UV light with R_F 0.34 and 0.37. The only other green pigment located was light green in colour and this was identified as chlorophyll b. The remaining two fractions were yellow in colour. These could probably be xanthophylls.

Biochemical composition

The biochemical components of the flagellate was estimated in terms of carbohydrate, protein and lipid. The water content of the flagellate varied from 91.56% in 5 day old cultures to 59.20% in 20 day old cultures. Simultaneously, the dry weight of the cells increased from 120 µg/10^6 cells to 253 µg/10^6 cells during the 20 days growth (Table 5). Protein was the major photosynthetic product during the exponential phase of culture. As the culture grew old the amount of protein decreased and that of carbohydrate increased. The ratio of protein to carbohydrate reduced by half within ten days of growth in culture. The change in the lipid content was less pronounced.

Fig.3. Two-dimensional chromatograms of pigments in nanoplankters separated on silica-gel thin layer plates. A. _S. salina_, B. _C. freiburgensis_, C. _I. galbana_ (C.s.), D. _I. gracilis_. Solvent system: first dimension, 0.8% n-propanol in light petroleum ($60-80^{o}C$); second dimension, chloroform: light petroleum:acetone (25:75:0.5); 1. carotene (orange), 2. chlorophyll _a_ (blue-green), 3. chlorophyll _b_ (olive green), 4. chlorophyll _c_ (light green), 5. fucoxanthin (orange), 6,7,8,9 unidentified pigments.

Fig.3.
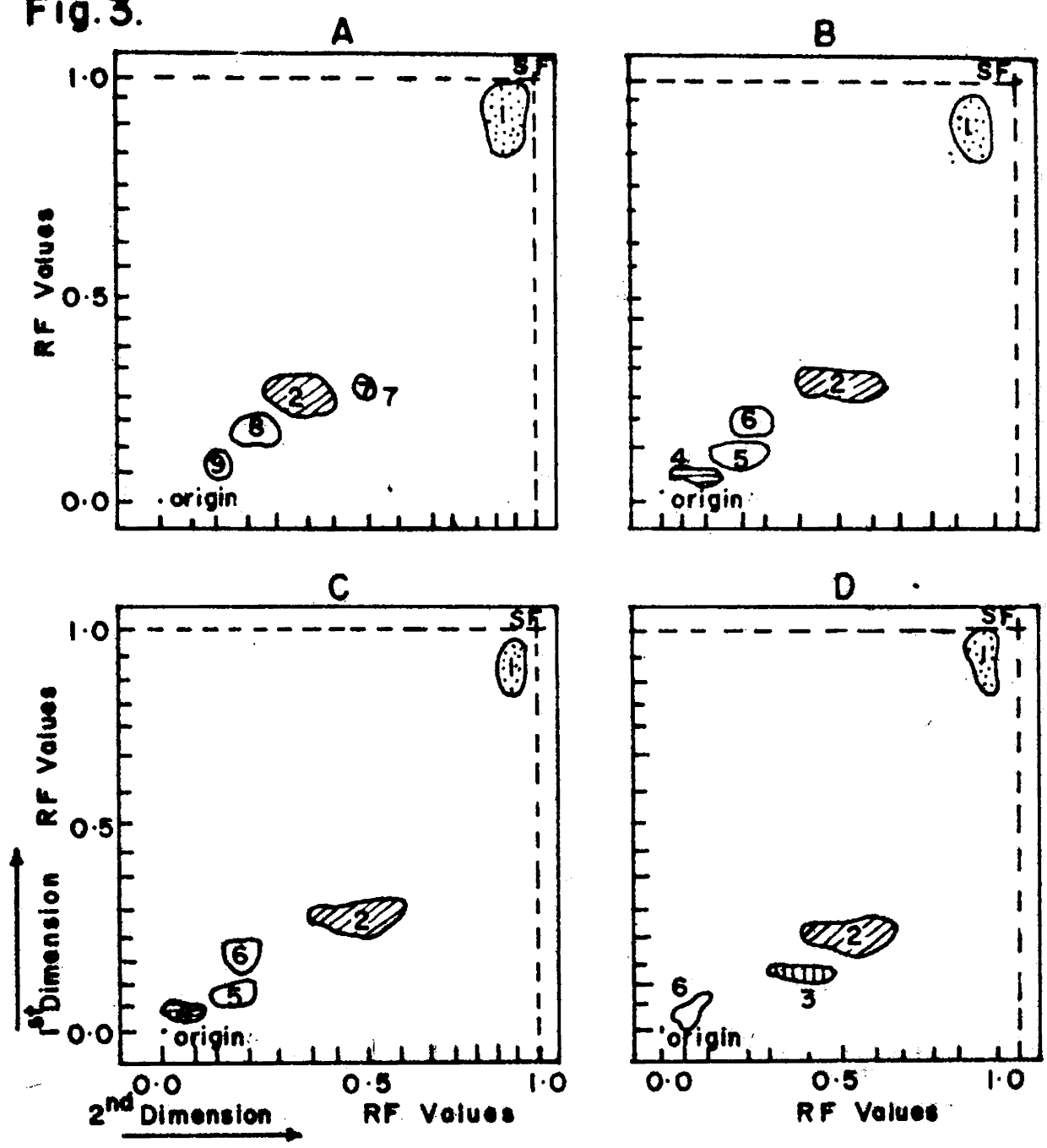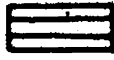
CAROTENE

CHLOROPHYLL 'a'

CHLOROPHYLL 'b'

CHLOROPHYLL 'c'

UNIDENTIFIED PIGMENTS

Table 4. R_F value of photosynthetic pigments of
Chromulina freiburgensis, _Isochrysis galbana_ (C.s.),
Synechocystis salina and _Tetraselmis gracilis_
separated on thin-layers of silica gel

	Spot number	1st Dimension: $0.3\% $ n. Propanol in light petroleum (60 - 80°C)	2nd Dimension: Chloroform: light petroleum: acetone 25: 75: 0.5
Carotenes (Orange)	1	0.93	0.93
Chlorophyll a (Blue green)	2	0.34	0.37
Chlorophyl b (Olive green)	3	0.23	0.30
Chlorophyl c (Pale green)	4	0.075	0.02
Fucoxanthin (Orange)	5	0.175	0.10
Unidentified	6	0.18	0.09
'	7	0.35	0.46
'	8	0.29	0.18
'	9	0.30	0.01

Table 5. Biochemical composition of Chromulina
freiburgensis raised in batch culture.

Fraction	Sampling interval		
	5 days	10 days	20 days
Percent moisture	91.56 ±3.68	86.0 ±4.71	59.20 ±3.16
Dry weight (µg/10⁶ cells)	120.	182.0	253.0
Protein (% dry wt.)	58.4 ±6.12	46.85 ±4.30	40.16 +11.02
Carbohydrate (% dry wt.)	26.6 ±6.48	38.5 ±7.64	43.83 ±4.98
Lipid (% dry wt.)	10.16 ±1.7	11.60 ±2.85	17.20 ±3.13
Protein: Carbohydrate	2.20	1.22	0.92
Bacterial count/ml	180	106	390

Estimation of extracellular production

The amount of extracellular products of the species
was estimated by liquid scintillation technique. The rate
of excretion was low during the phase of active growth.
During the stationary phase the amounts excreted increased
about five fold. The amounts varied from 3% to 12.57%
of the total carbon fixed (Table 6). In terms of absolute
activity, about 0.02-0.033 µC were retained in the medium
for an added activity of 5µC (Table 7).

Table 6. Percentage of extracellular products (ECP) released by C. freiburgensis during phases of growth in culture. (The % soluble represents the ECP as fraction of the total carbon fixed)

Days of growth	Cells ml^{-1}	Particulate soluble (mgC/l/hr)		% soluble
4 days	9 x 10^4	19.53	0.60	3.00
8 days	16 x 10^4	7.03	0.55	7.80
16 days	33 x 10^4	9.71	1.22	12.57

Table 7. The absolute activity in µC retained by filtrates of ^{14}C labelled nanoplankton cultures (Added activity = 5 µC).

Culture	Sampling interval		
	4 days	8 days	16 days
Chromulina freiburgensis	0.020	0.027	0.033
Isochrysis galbana (C.e.)	0.036	0.043	0.150
Synechocystis salina	0.129	0.141	0.273
Tetraselmis gracilis	0.01	0.018	0.028

Eco-physiology of Chromulina freiburgensis

Salinity: The salinity tolerance of the species was tested by growing the flagellate in culture media of salinities 14‰, 24‰ and 34‰. The rate of cell division was affected by the variation in salinity. As seen from Table 7, the cell counts showed consistently higher values for the treatment level III i.e. 34‰. Analysis of variance showed that the difference between the treatments was highly significant except on the 2nd day. The density of the culture was very low at 14‰ while at 24‰ it was higher; but the highest density was built up at the salinity level of 34‰.

The difference in the salinity of the culture medium was sharply reflected in the amount of chlorophyll a. Analysis of variance showed the treatments to be highly significant from the 4th to 24th. At the lowest salinity level the chlorophyll content was very low compared to the other two treatments. At 24‰, it was further high and the medium at 34‰ gave highest chlorophyll a value throughout the experiment. The variation in the amount of the pigment is given Table 8.

much affected in the pH rang of 6.5 to 8. But the

amount of chlorophyll a varied significantly (Table 9).

At pH 8, the amount of the pigment was about double that at

pH 6.5 both at the exponential phase and the stationary

phase. The analysis of variance showed that the

difference is significant at 1% level.

Table 9. Cell concentration and chlorophyll a content of
Chromulina freiburgensis during phases of growth
in culture media of different pH levels.

Treatment (pH level)	Logarithmic phase		Stationary phase	
	Cell density per ml	Chlorophyll a $\mu g/10^6$ cells	Cell density per ml	Chloroph a $\mu g/1^6$ ce
6.5	19,730	0.171	100,780	0.088
7.0	20,830	0.204	99,470	0.082
7.5	20,200	0.222	104,170	0.080
8.0	19,500	0.329	96,200	0.161

__Effect of cell concentration on the rate of carbon fixation__

While measuring the rate of carbon fixation the

concentration of the culture used for the experiment was

optimised so that self-shading was avoided. The cell density

and the corresponding incorporation of carbon-14 is given in

Table 10. The rate of production increased upto a cell density of 10.8 x 10^5 per ml. At the next density level tested (14.4 x 10^5 per ml) there was drastic reduction in the production. Fig.4 represents this graphically. From the available data it may be assumed that cell concentrations above 10 x 10^5 per ml was inhibitory to the rate of ^{14}C fixation.

Table 10. Carbon-14 uptake by C. freiburgensis as function of cell concentration.

Cell density per ml	Counts per minute	
12,000	143	±15.1
36,000	575	±91.0
72,000	1619	±156.0
108,000	2763	±63.2
144,000	1413	±101.0

Effect of light

The light conditions to which the species was exposed affected growth profoundly. Cultures were exposed to alternating light-dark period and also constant illumination for a month. Those exposed to constant illumination developed rapidly compared to the other. From an initial

Fig.4. Rate of carbon fixation (expressed as ^{14}C uptake in cpm) as function of different culture densities of S. freiburgensis.

Fig. 4.
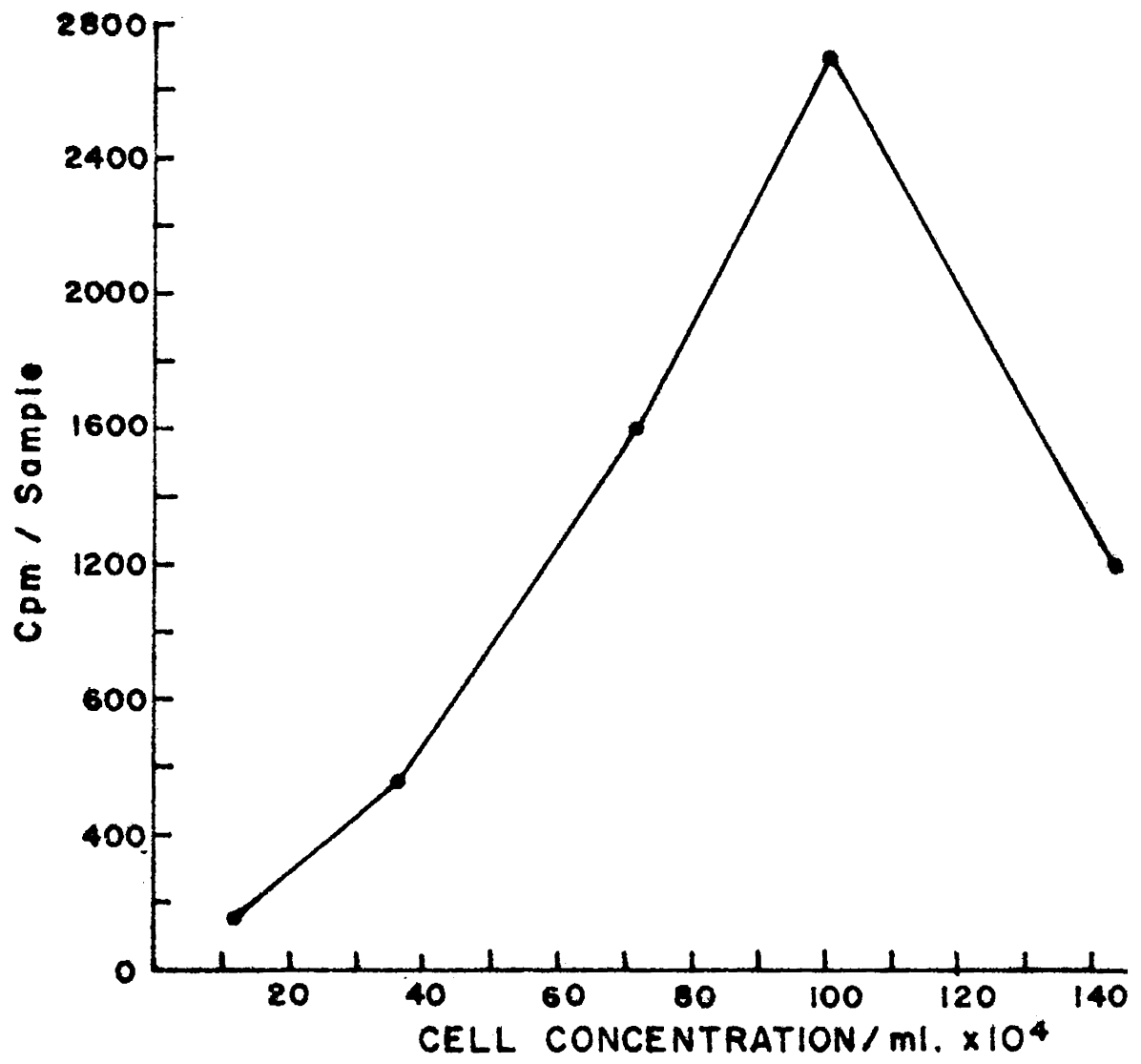
density of 4.... cells/ml, the population grew upto
12. x 10^4/ml within 26 days with light-dark cycle
while constant illumination produced a higher population
density 2.3 x 10^4 cells/ml (Table 11). As is observed,
the continuous illumination induces higher growth rate of
the flagellate.

Table 11. Effect of photoperiod on growth of
C. freiburgensis in batch culture.

Age of culture (days)	Cell density per ml	
	10:14 Light-dark cycle	Constant illumination
0	4000	4000
2	16520	20500
4	9,400	12,500
6	20,000	32,000
8	30,000	50,350
10	37,000	60,600
12	46,000	70,960
14	52,180	89,870
16	57,000	125,000
18	57,000	163,100
20	63,100	160,300
22	70,790	158,500
24	89,130	178,000
26	120,000	203,000
28	63,100	178,300
30	57,000	200,600

The metabolic activity of the flagellate was affected significantly by the intensity of illumination. The light-photosynthesis curve followed the hyperbolic relation as per the equation $Y = a+bx+cx^2$ with an I_K of $24 \times 10^{15} \times cm^{-2} \times sec^{-1}$ (or 14.1 klux) (Fig.5). The highest light intensity that supported the maximum rate of carbon fixation was 34.61×10^{15} quanta $\times cm^{-2} \times sec^{-1}$. At the lowest light intensity tested ($1 \times 10^{15} \times$ quanta $\times cm^{-2} \times sec^{-1}$) the rate of production was very low, being 15.48% of that at light saturation. A similar reduction was noticed at the highest light intensity tested i.e. 16.56% of production at 68×10^{15} quanta $\times cm^{-2} \times sec^{-1}$.

Effect of temperature

Temperature was found to be one of the important factors controlling growth and proliferation of _. freiburgensis. When exposed to low temperature ($5^\circ C$) the flagellate lost motility within five minutes. After a week when they were recultured at room temperature, the cultures were viable. The viability of the cultures stored in the refrigerator was retained for three months. After three months the cultures did not regain viability on transfer to fresh medium at room temperature.

Fig.5.   The rate of photosynthesis (expressed as ^{14}C
uptake in cpm) as function of light intensity
for C. freiburgensis.

Fig. 5.

$$Y = 48 \cdot 343 + 24 \cdot 157 \, X - 0 \cdot 349 \, X^2$$

$X_{max} = 100\% \ \text{light saturation} = 34 \cdot 61 \times 10^{15} \, \text{quanta.Cm}^{-2}$

$I_K = 240 \ \text{quanta. Cm}^{-2} \ \text{Sec}^{-1}$
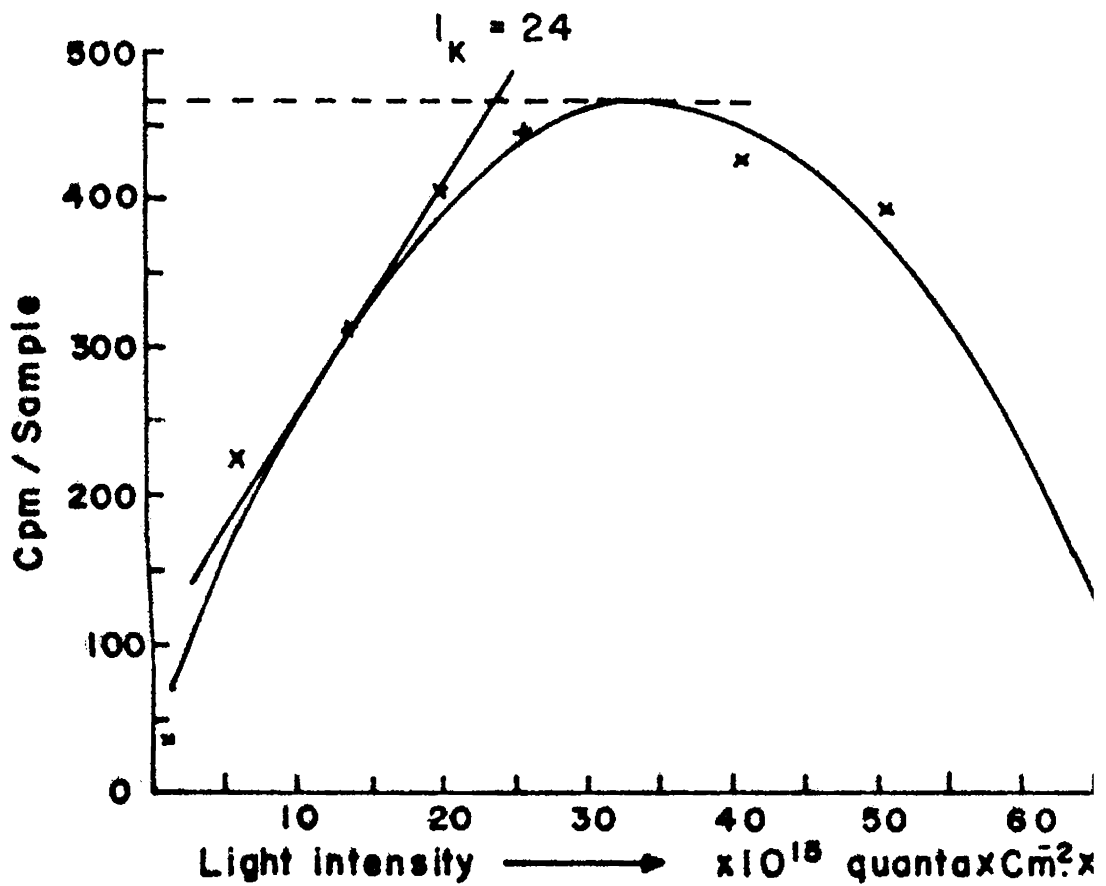
The growth of the culture was studied within the temperature range 2.-4.°C. The motility of the cells also varied within this temperature range. At 2.°C, 3.. of the cells were non-motile. At temperatures of 25, 3. and 35°C the cells behaved normally. When exposed to 4.°C all cells lost motility within 2-3 hours of exposure.

The growth of the culture within the temperature range was quantified by measuring the rate of growth. The rate of doubling did not show significant difference within the temperature range 2.-35°C. At 4.°C the rate of growth was considerably low and the culture did not survive more than five days. The mean doublings per day for the different temperatures is given in Table 12.

Table 12. Rate of growth and ^{14}C uptake (as counts per minute per sample incubated) at different temperatures in batch culture.

Temperature °C	Chromulina freiburgensis		Isochrysis galbana (C.s.)		Synechocystis salina	
	k	cpm	k	cpm	k	cpm
20	2.66	498	2.56	123	1.99	1347
25	2.76	468	2.64	342	2.26	7479
3.	3.05	4392	2.76	2559	2.13	2829
35	2.84	2334	2.66	1399	2.13	2385
4.	.18	222	.86	42	1.16	1.99

The rate of fixation of carbon was measured simultaneously. The ^{14}C incorporation by the flagellate was observed to be highly sensitive to temperature. There was significantly high production at $30^{\circ}C$ which was reduced to half at $35^{\circ}C$. At all other temperatures the rate of production was very low (Table 12).

Utilization of macro-nutrients

The nitrate and phosphate requirement of the species was also determined. The rate of cell division in the culture varied with the concentration of nitrate in the medium. Applying the linear transformation of the Monods expression, $S = \mu_{max} (S/\mu) - K_{\mu}$, the half-saturation constant for growth was obtained as 0.7 μg_at N L^{-1} with a maximum specific growth rate of 0.901 per day (Fig.6). It was also observed that at a N_3 level of more than 200 mg/litre the growth of the flagellate was highly inhibited.

Similarly, the PO_4 requirement of the species could be defined by the relation $S = 0.97$ $(S/\mu) - 1.31$ where 1.31 is the half-saturation constant for growth, and 0.97 being the maximum (unlimited) growth rate (Fig.7). It was observed that at PO_4 concentrations at and above 25 mg/litre, the growth of the flagellate is inhibited.

Fig.7. Relation between the concentration of
phosphate in the culture medium and the
specific growth rate of C. freiburgensis.

Fig.7.

$$S = \mu \max (S/\mu) - K_\mu$$

$$S = 0.97 (S/\mu) - 1.31$$

$$\mu \max = 0.97$$

$$K_\mu = 1.31 \, \mu M\text{-}P.L^{-1}$$
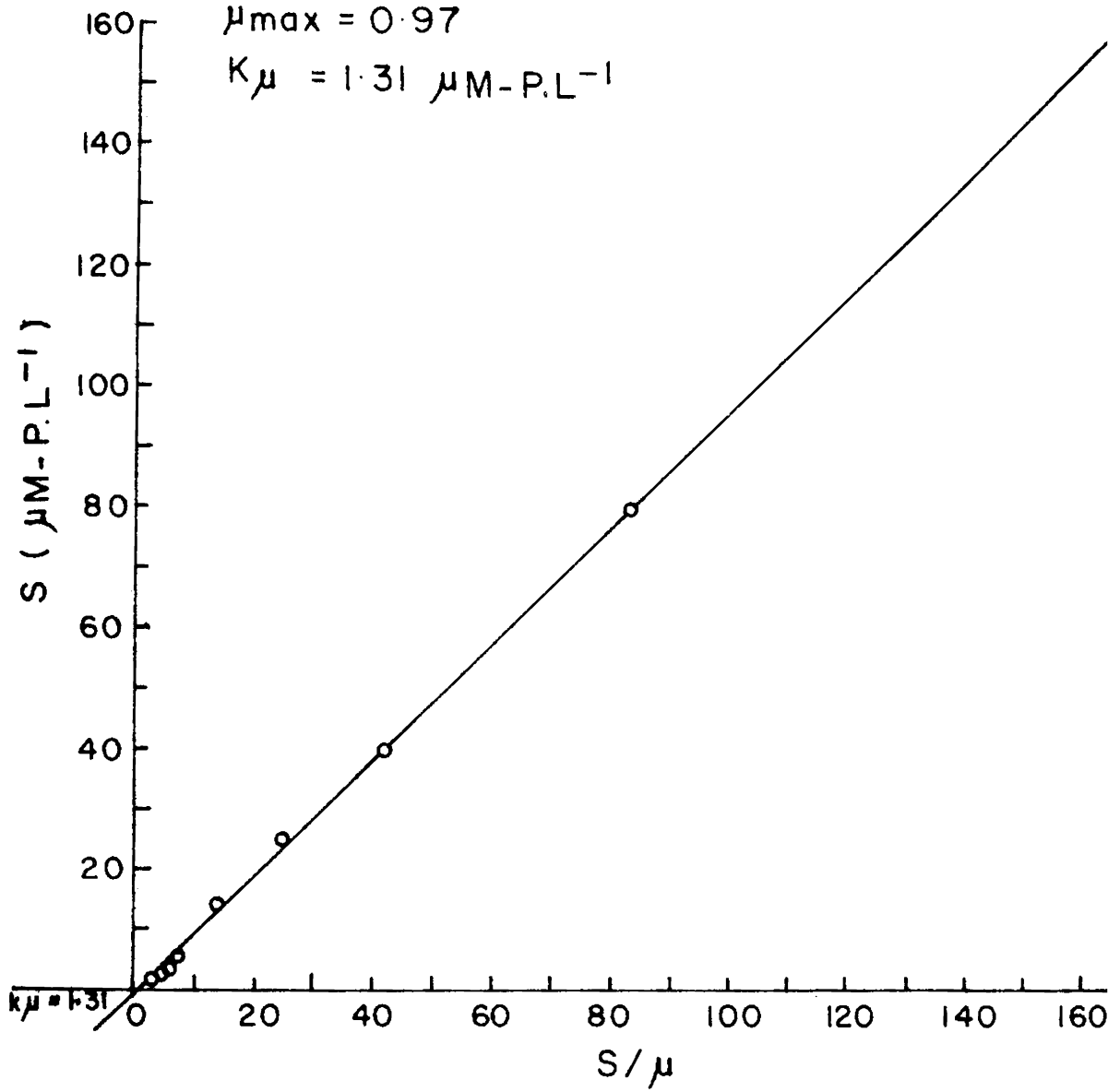
An obvious fact here was that the substrate concentration supporting, the μ_{max} (unlimited) is far lower than actually observed. Thus the observed growth rate nears the μ_{max} only at about 2$_{v}$ µg-at, $N.L^{-1}$ (Table 13) for nitrate an$_{v}$ around 7.8 µM-P L^{-1} for phosphate (Table 14)

Table 13. Specific growth rate (µ/day) during exponential phase of C. freiburgensis, I. galbana (C.s.) and S. salina as function of NO_3^- concentration in the medium.

C. freiburgensis		I. galbana (C.s.)		S. salina	
NO_3^- concentration µg-at NL^{-1}	µ/day	NO_3^- concentration µg-at NL^{-1}	µ/day	NO_3^- concentration µg-at NL^{-1}	µ/
2.6	0.56	2.6	0.46	2.6	0.5
7.0	0.81	7.0	0.60	5.0	0.5
18.0	0.90	10.0	0.73	10.0	0.6
40.0	0.90	18.0	0.83	28.0	0.8
79.0	0.89	49.0	0.94	49.0	0.8
118.0	0.89	98.0	0.938	98.0	0.9
152.0	0.90	120.0	0.944	152.0	1.1
-	-	152.0	0.920	300.0	0.9

Table 14. Specific growth rate (µ/day) during
exponential phase of C. freiburgensis, I. galbana
(.s.) and S. salina as function of PO_4
concentration.

Substrate concentration µM PL^{-1}	C. freiburgensis	I. galbana (C.s.)	S. salina
0.75	0.28	0.26	0.26
1.50	0.32	0.33	0.37
2.80	0.490	0.52	0.49
4.80	0.650	0.64	0.70
7.80	0.980	0.82	0.93
15.00	0.976	0.94	0.96
24.50	0.979	0.96	0.97
40.00	0.950	0.96	0.96
80.00	0.961	0.96	0.96
160.00	0.958	0.96	0.95

4.2. Isochrysis galbana (C.s.)

Growth and activity in batch culture

Cultures of Isochrysis galbana (C.s.) were grown in
enriched seawater to study its growth kinetics. Under the
defined conditions (3.4.1) the flagellate was found to grow
rapidly. From an initial concentration of 350 cells/ml the

cell count increased to 1.95 x 10^3/ml on the 4th day.
After the 4th day the increase in cell counts was slow
(Fig.8A). The stationary stage persisted till 20 days
after which the cell counts began to decline. The highest
growth rate was during the logarithmic growth phase with a
mean doubling rate of 1.48 divisions per day for a generation
time of 16 hours. The corresponding relative growth constant
was 0.043 per hour. The maximum density attained by the
culture was 6.9 x 10^5 per ml (Table 2).

Corresponding to the decline in cell numbers it
was also observed that the proportion of motile cells
in the culture decreased. The non-motile cells settled
to the bottom of the culture flask forming a thin layer.
The golden-brown colour of the culture changed to a reddish
brown by the 4th week.

The amount of chlorophyll a, c and carotenoids
was very low on the 2nd day; but within the next two days
there was a sudden increase of about twenty times. After
the fourth day the pigments increased gradually till the
20th. The amount of all the pigments decreased on further
aging of the culture (Fig.8B). The amount of chlorophyll a,
c and carotenoids per unit number of cells was comparatively
higher during 8-12 days (Table 15) with the maximum amounts
of 0.584, 0.418 and 0.662 respectively. The quantity of

Fig.8A. Increase in cell number of _Isochrysis galbana_ (C.s.) during growth in batch culture.

Fig.8B. Variation in the amount of photosynthetic pigments of the above species during growth in batch culture.

Fig. 8.
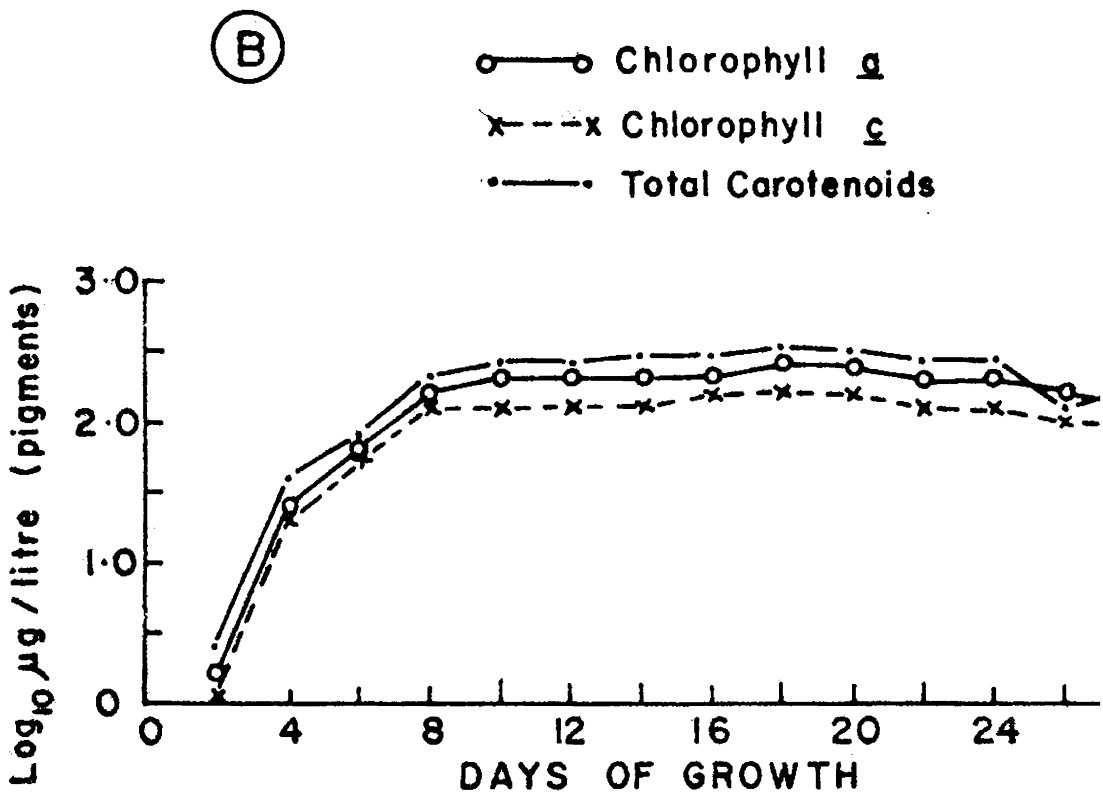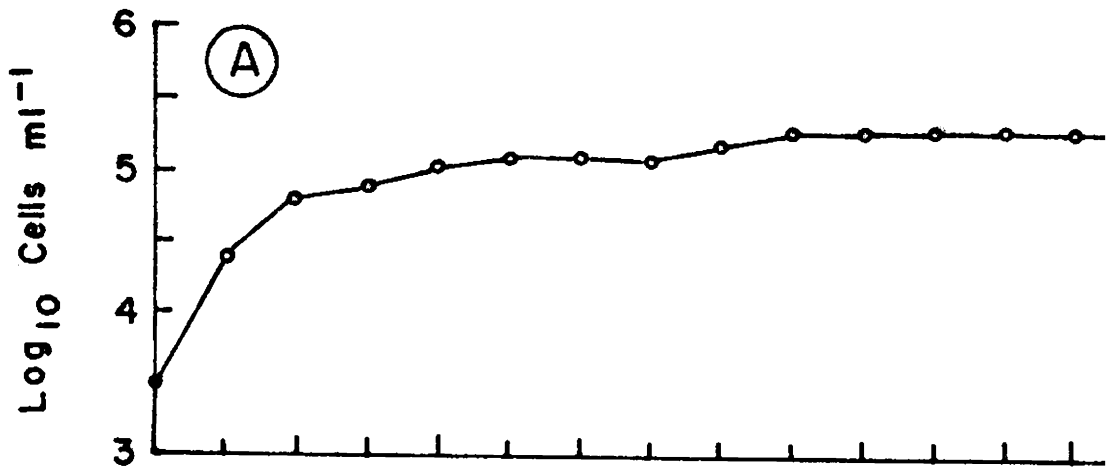
chlorophyll _a_ was less than that of carotenoids while
the latter was less in quantity compared to the total
chlorophylls. The ratio of carotenoid to chlorophyll _a_
did not show much fluctuation except for the last day of
observation.

Table 15. Variation in the amount of pigments expressed
as $\mu g/10^6$ cells of _I. galbana_ (C.s.) for a growth
period of 30 days in batch culture.

Age of culture (days)	Chlorophyll _a_ $\mu g/10^6$ cells	Chlorophyll _c_ $\mu g/10^6$ cells	Total carotenoids $\mu g/10^6$ cells	Carotenoids/ Chlorophyll _a_
2	0.068	0.042	0.100	1.47
4	0.142	0.105	0.195	1.38
6	0.265	0.202	0.364	1.37
8	0.584	0.418	0.662	1.13
10	0.502	0.380	0.612	1.22
12	0.468	0.330	0.594	1.27
14	0.430	0.316	0.570	1.33
16	0.424	0.304	0.553	1.30
18	0.420	0.300	0.550	1.31
20	0.405	0.253	0.468	1.16
22	0.365	0.230	0.460	1.26
24	0.318	0.192	0.390	1.23
26	0.226	0.165	0.280	1.24
28	0.228	0.152	0.264	1.16
30	0.108	0.101	0.256	2.30

The flagellate exhibited high metabolic activity on the 2nd and 14th day with a gross production of 40 $\mu g C/10^6$ cells/hour and a net production of 12 $\mu g C/10^6$ cells/hour as measured by the oxygen technique. From the 6th to 22nd the production remained uniform (Fig.9A) an later began to decrease.

As per the Carbon-14 experiment, the flagellate exhibited high metabolic activity during the period from 2-6 days with a maximum observed rate of $16/\mu g C/10^6$ cells/ hour. After the 6th day the rate of production decreased (Fig.9B).

Pigment composition

On chromatographic separation, the pigments of the strain resolved into five fractions (Fig.3C). The orange coloured carotene, the blue green chlorophyll a and the light green chlorophyll c were easily noticeable. The two other spots were yellow in colour and it is assumed that these may be xanthophylls. Table 4 gives the R_F values of the different pigments.

Bio-chemical composition

The water content of the cells varied from 71.4% to 61.74% from the 5th day to 2 th. There was consistent

Fig. 9A. Rate of production of organic carbon by
I. galbana (C.s.) for a growth period of
30 days in batch culture (measured by the
oxygen technique).

Fig. 9B. Rate of production of organic carbon by the
above species for a growth period of 30 days
in batch culture (measured by the ^{14}C
technique).

Fig. 9.
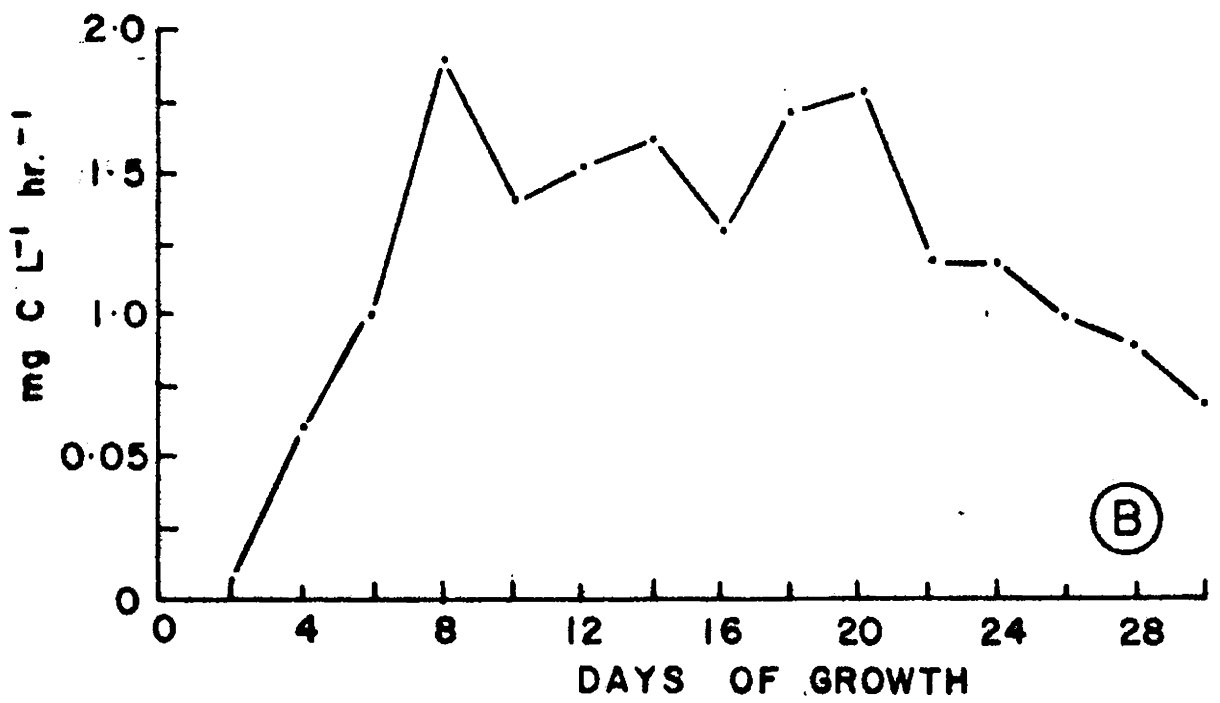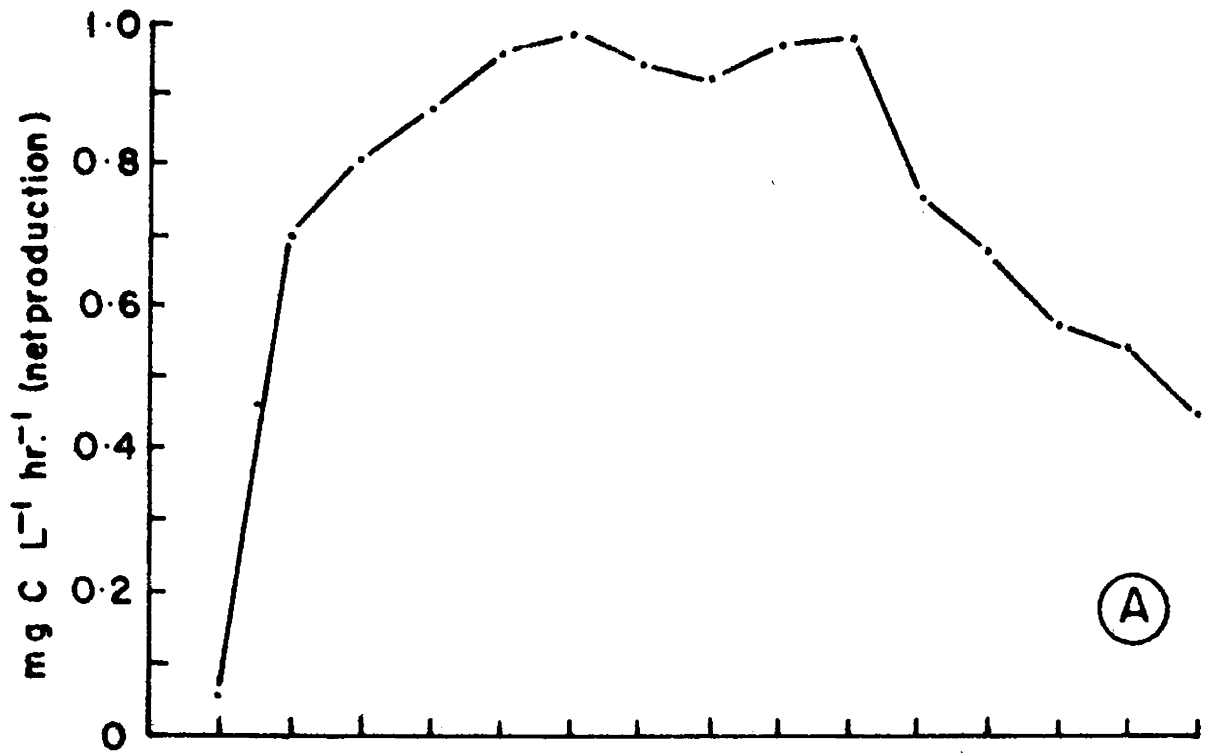
increase in the dry weight of the cells as culture aged.
The dry weight per million cells was 130 μg on the 5th day
wher as the weight increased to 373 μg and 465 μg during the
10th and 20th day respectively. The major product of
synthesis was protein during the exponential phase. As the
culture became old the amount of protein decreased while
that of carbohydrate increased. As a resul. the protein:
carbohydrate ratio decreased from 1.83 to 0.93 within
20 days (Table 16). The lipid content was comparatively low
throughout.

Table 16. Biochemical composition of Isochrysis galbana (C.s.)
raised in batch culture.

Fraction	Sampling interval		
	5 days	10 days	20 days
Percent moisture	71.4 ±6.17	66.6 ±5.62	61.74 ±4.81
Dry weight (μg/10^6 cells)	130.0	373.0	465.0
Protein (% dry wt.)	51.3 ±7.00	44.85 ±6.14	37.8 ±8.09
Carbohydrate (% dry wt.)	28.0 ±7.16	30.34 ±4.23	40.50 ±6.12
Lipid (% dry wt)	6.78 ±2.76	6.58 ±1.24	11.67 ±3.62
Protein: Carbohydrate	1.83	1.48	0.93
Bacterial count/ml	200.0	29.0	110.0

Extracellular release

Table 17 gives the relative amount of excretion of _I. galbana_ (C.s.). During the exponential phase of growth only 3.9% of the total photo-assimilate was released into the medium. In one week old cultures this increased to 13.57% and on the 16th day to 45.2%.

Table 17. Percentage of extracellular products (ECP) released by _I. galbana_ (C.s.) during phases of growth in culture. (The % soluble represents the ECP as fraction of the total carbon fixed)

Days of growth	Cells ml^{-1}	Particulate soluble		% soluble
		mgC/l/hr		
4	7 x 10^4	31.32	1.22	3.9
8	25 x 10^4	12.01	1.63	13.57
16	20 x 10^4	4.42	2.00	45.2

The amount of absolute activity retained as ECP in the medium was also determined. Out of the 5 µC added to each sample, 0.15 µC was retained as ECP in the 16 days old culture. On the 4th and 8th day this fraction was less being 0.036 and 0.043 respectively (Table 7).

Salinity:

The salinity of the culture medium affected the

growth of the flagellate. The culture density and the pigment content varied with the salinity levels 14, 24 and 34%. (Table 18). Analysis of variance showed the

Table 18. The cell concentration and chlorophyll a content of I. galbana (C.s.) grown in culture media of salinities 14%, 24%. and 34% for a period of 24 days.

Age of culture (days)	Cell concentration/ml			Chlorophyll a ($\mu g/10^6$ cells)		
	14%.	24%.	34%.	14%.	24%.	34%.
0	10000	10000	10000	-	-	-
2	19300	56200	5830	0.070	0.115	0.128
4	6580	2,9130	3,6550	0.234	0.280	0.341
6	1,7020	4,6250	5,3830	0.167	0.210	0.234
8	1,8260	5,3770	6,1300	0.174	0.188	0.195
10	3,8580	6,8080	7,1080	0.114	0.152	0.154
12	4,8250	6,2830	6,8500	0.102	0.143	0.157
14	5,1000	6,9280	7,9200	0.103	0.143	0.157
16	5,6910	8,9130	8,9130	0.106	0.132	0.144
18	5,9520	12,5900	12,5900	0.080	0.100	0.109
20	5,7020	18,5410	20,5630	0.040	0.087	0.088
22	5,4300	18,2310	17,8560	0.025	0.084	0.089
24	5,3800	18,1000	17,5340	0.020	0.078	0.079

treatment effect to be highly significant. The cell density and chlorophyll a were consistently low for the lowest salinity (14%.) tested; but the culture densities at 24 and 34%. did not differ much throughout the experiment. The

maximum cell density built up during the treatments
were 5.952 lakhs/ml (T_1), 18.541 lakhs/ml (T_2) and
20.59 lakhs/ml (T_3).

The amount of chlorophyll a was very low in
cultures grown at 14%. At 24%, the quantity was still
higher; but the highest salinity level produced the
greater quantity of pigments consistently.

Effect of pH

The pH requirement of the species was also found to
be quite specific. The range of pH from 6.5 to 8 affected
the cell multiplication and the content of chlorophyll a.
Analysis of variance showed that both at logarithmic phase
and stationary stage the treatments were highly significant.
Both the cell counts and chlorophyll a registered higher
values for the pH 8 (Table 19).

Table 19. Cell concentration and chlorophyll a content of
I. galbana (C.s.) during phases of growth in
culture media of different pH.

Treatment pH level	Logarithmic phase		stationary phase	
	Cell density per ml	Chlorophyll a µg/10^6 cells	Cell density per ml	Chlorophyll a µg/10^6 cell
6.5	101000	0.290	670300	0.168
7.0	125500	0.303	741700	0.212
7.5	121000	0.307	74530	0.217
8.0	155000	0.315	787000	0.268

Cell concentration/rate of production

The amount of carbon-14 incorporation by the
flagellate when incubated in short term experiments was
observed to increase with the concentration of the cells
in the culture (Table 2.). The increase in the rate of
production was not quite linear (Fig.1.). At about
the cell density of 5 x 10^5/ml, the rate of production
was found to saturate. The production decreased around a
density of 6 x 10^5 cells/ml.

Table 2.. Carbon-14 uptake by I. galbana (C.s.)
          as a function of cell concentration.

Cell density per ml	Counts per minute
5,0000	53 ± 9.0
16,0000	103 ±19.0
24,0000	236 ± 8.3
32,0000	309 ±18.1
47,0000	363 ± 7.6
55,0000	29. ±19.8

Influence of light

I.galbana (C.s.) was grown in constant illumination
and also subject to light-dark cycle. The cell counts

Fig.10. Rate of carbon fixation (expressed as ^{14}C
uptake in cpm) as function of different
culture densities of I. galbana (J.s.)

Fig. 10.
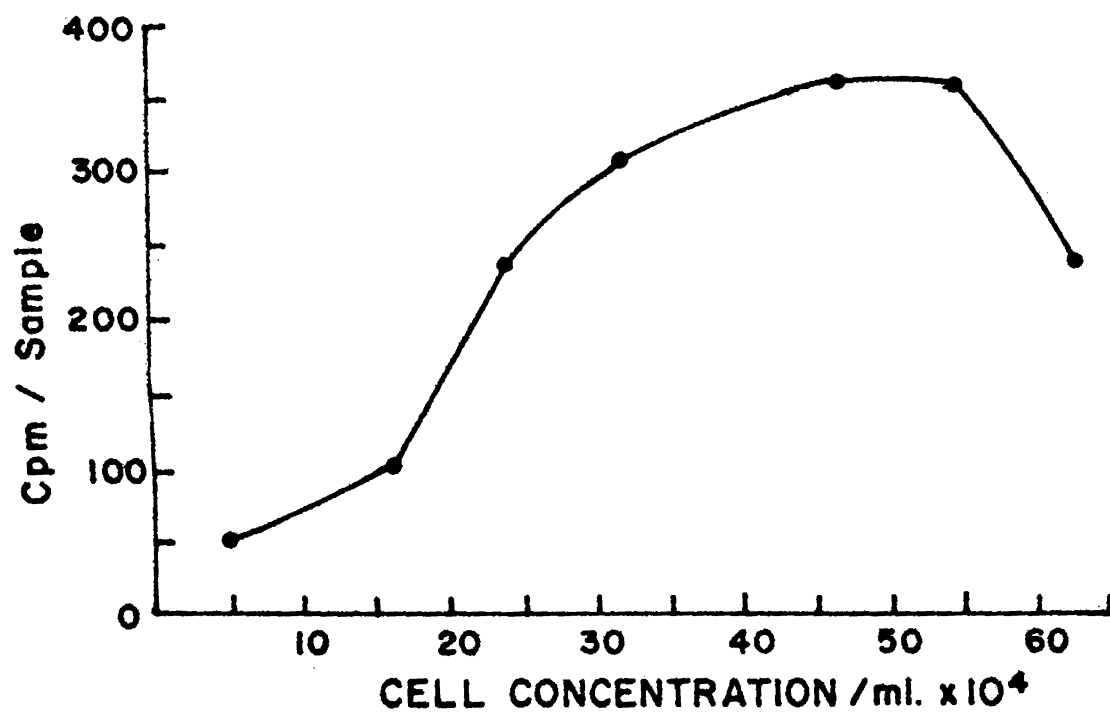
showed that the photoperiod affected the cell division rate considerably. Starting from an initial concentration of 350 cells/ml, the culture developed a density of 23 x 10^5/ml in 30 days on constant illumination while it remained at 8.2 x 10^5/ml in the cultures exposed to light-dark regime (Table 21).

Table 21. Effect of photoperiod on growth of I. galbana (C.s.) in batch culture.

Age of culture (days)	Cell density per ml	
	10:14 Light-dark cycle	Constant illumination
0	3500	3500
2	2,5000	3,330
4	19,5000	20,300
6	19,9500	25,1200
8	17,0000	36,0000
10	24,0000	40,3000
12	28,1800	39,8100
14	40,5000	45,5000
16	50,1200	63,1000
18	56,2300	79,4300
20	69,0000	124,0000
22	50,1200	125,9000
24	62,0000	150,0000
26	60,0000	125,9000
28	70,0000	158,5000
30	82,0000	230,0000

The flagellate showed different rates of activity at various light intensities. The rate of photosynthesis was plotted against the respective light intensities to get the hyperbolic relation between these (Fig.11). As the figure shows the rate of production saturated at 34.71 x 10^{15} quanta x cm^{-2} x sec^{-1} with an I_K of 24 x 10^{15} x quanta x cm^{-2} sec^{-1}. The rate of photosynthesis was considerably low at the lowest (1 x 10^{15} quanta x cm^{-2} x sec^{-1}) and highest 68 x 10^{15} quanta x cm^{-2} x sec^{-1}) light intensities tested.

Effect of temperature

The motility, viability and growth rate of the species was related to the temperature conditions. This was illustrated by the temperature controlled experiments.

The cultures were exposed to $5^{\circ}C$ to $40^{\circ}C$. At $5^{\circ}C$ the immediate response was to lose motility and the effect was complete. At $20^{\circ}C$, 50% of the cells were motile. At $25-35^{\circ}C$ all cells were active. When the cultures were exposed to $40^{\circ}C$, the cells gradually lost their active movements and within two hours 100% of the cells became non-motile.

The cultures remained viable for three months in the refrigerator ($5^{\circ}C$). At higher temperature ($40^{\circ}C$), the cells were viable for four days and did not regain viability on return to the room temperature.

Fig.11. The rate of photosynthesis (expressed as ^{14}C uptake in cpm) as function of light intensity for I. galbana (C.s.).

Fig. 11.

$$Y = 3.988 + 5.152\ X - 0.0742\ X^2$$

$X_{max} = 100\%$ Photosynthesis $= 34.71 \times 10^{15}$ quanta. Cm^{-2} Sec^{-1}

$I_K = 24 \times 10^{15}$ quanta. Cm^{-2} Sec^{-1}
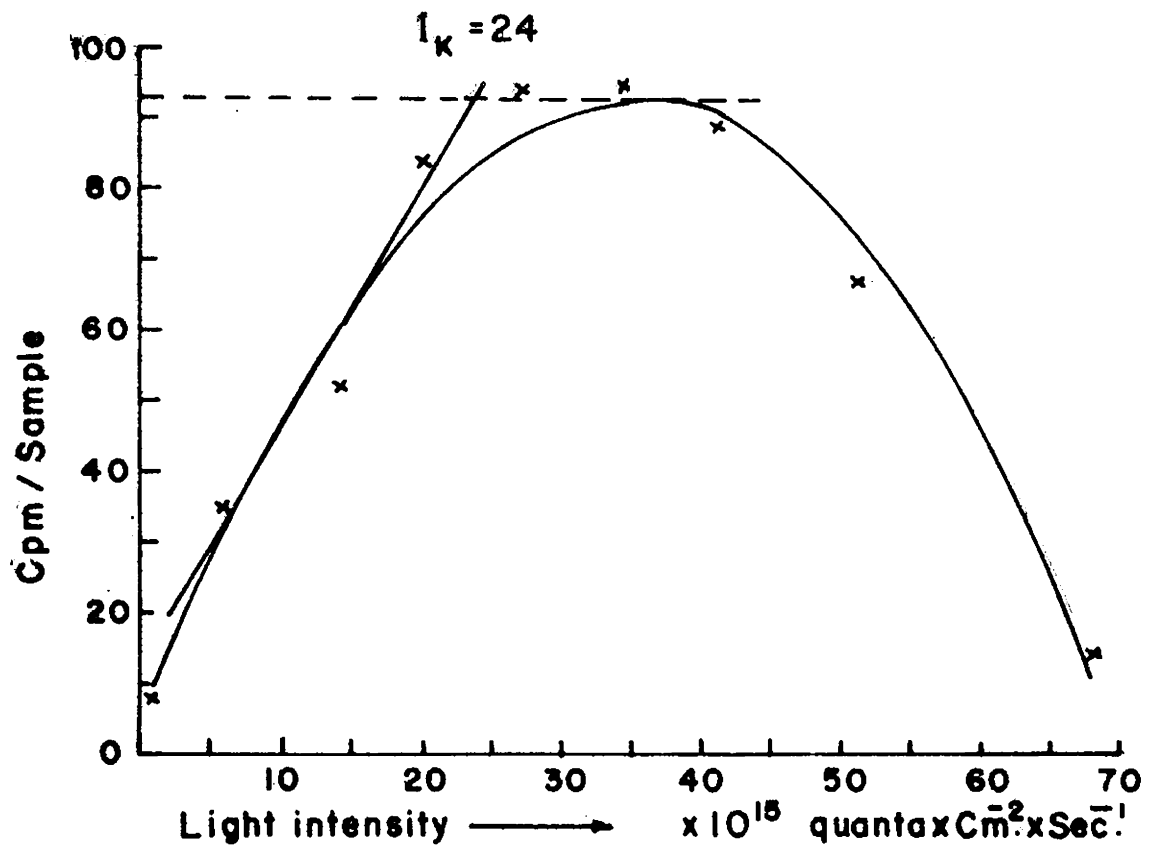
The rate of growth of the flagellate for the temperature range 20-40°C was determined. There was no significant difference in the growth rate at temperatures from 20 to 35°C; but at 40°C the growth rate was considerably low (Table 11).

Temperature influenced the physiological activity significantly. The rate of production of the flagellate was highest at 30°C. At 35°C it was reduced to half of that at 30°C. The rate of production was very low at 20, 25 and 40°C (Table 12) compared to that at 30 and 35°C.

Requirement of nitrate and phosphate

The rate of growth of I. galbana (C.e.) was dependent on the concentration of nitrate and phosphate in the medium. Applying the Monod expression, it was found that the half-saturation constant of growth for nitrate was 1.63 μg-at N L^{-1} for a μ_{max} of 0.947 (Fig.12). However, it was observed that this maximum growth rate was attained only at a relatively higher substrate concentration i.e. at about 46.23 μg-at N L^{-1} (Table 13). Clear evidence of growth inhibition was found to occur at concentrations of 400 mg/litre of Potassium nitrate.

Similarly, the half-saturation constant for PO_4 was obtained from the relation, S = 0.974 (S/μ) - 1.63

Fig.12. Relation between the concentration of
nitrate in the culture medium and the
specific growth rate of _I. galbana_ (C.s.).

Fig.12. Relation between the concentration of
nitrate in the culture medium and the
specific growth rate of _I. galbana_ (C.s.).

Fig. 12.

$S = \mu max \, (S/\mu) - K\mu$

$S = 0.947 \, (S/\mu) - 1.63$

$K\mu = 1.63 \, \mu g\text{-at N.L}^{-1}$

$\mu max = 0.947$
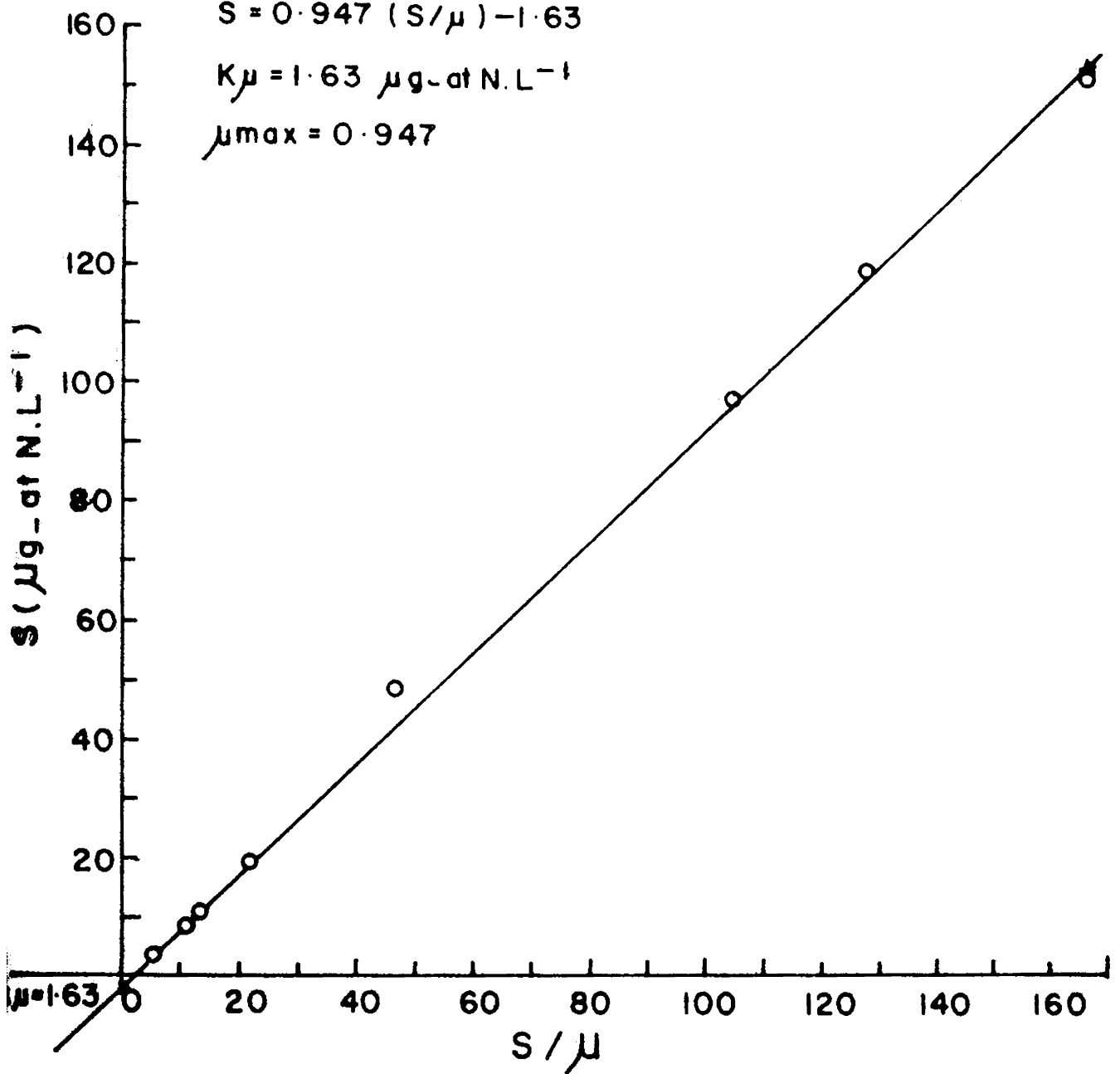
where K_μ = 1.63 $\mu M_e P$ L^{-1}. This was equivalent to a
maximum growth rate of 0.974 (Fig.13). Table 14 gives
the observed growth rate for each substrate concentration.
Accordingly, the saturation level was reached only at
a substrate concentration of about 25.42 $\mu M P$ L^{-1}.
Phosphate levels above 35 mg/litre inhibited the growth
of the organism.

4.3. Synechocystis salina

Growth rate and activity in batch culture

The culture of Synechocystis salina when grown
in batch culture clearly showed a logarithmic phase and
stationary phase which after a few days began to show
senescence. No lag phase was observed in culture. The
cell counts increased from an initial concentration of
1440 cell/ml to 9 lakhs/ml within four days. The cell
doubling continued though at a decreased pace till 18th
when the culture density was 47.5 million cells per ml.
After 18 days the cell numbers began to fall (Fig.14A).
By the 30th, the cell density came down to 30 million/ml.
During the peak growth period the rate of doubling (k)
was 2.74 for a generation time of 9 hours. The growth
constant per hour was found to be .079 (Table 2). Though
the growth rate decreased after four days it was observed

Fig.13. Relation between the concentration of
phosphate in the culture medium and the
specific growth rate of *I. galbana* (C.s.).

Fig.13. Relation between the concentration of
phosphate in the culture medium and the
specific growth rate of *I. galbana* (C.s.).

Fig.13.

$$S = \mu\text{max}\left(S/\mu\right) - K_\mu$$

$$S = 0.974\left(S/\mu\right) - 1.63$$

$$\mu\text{max} = 0.974$$

$$K_\mu = 1.63 \quad \mu\text{M P.L}^{-1}$$
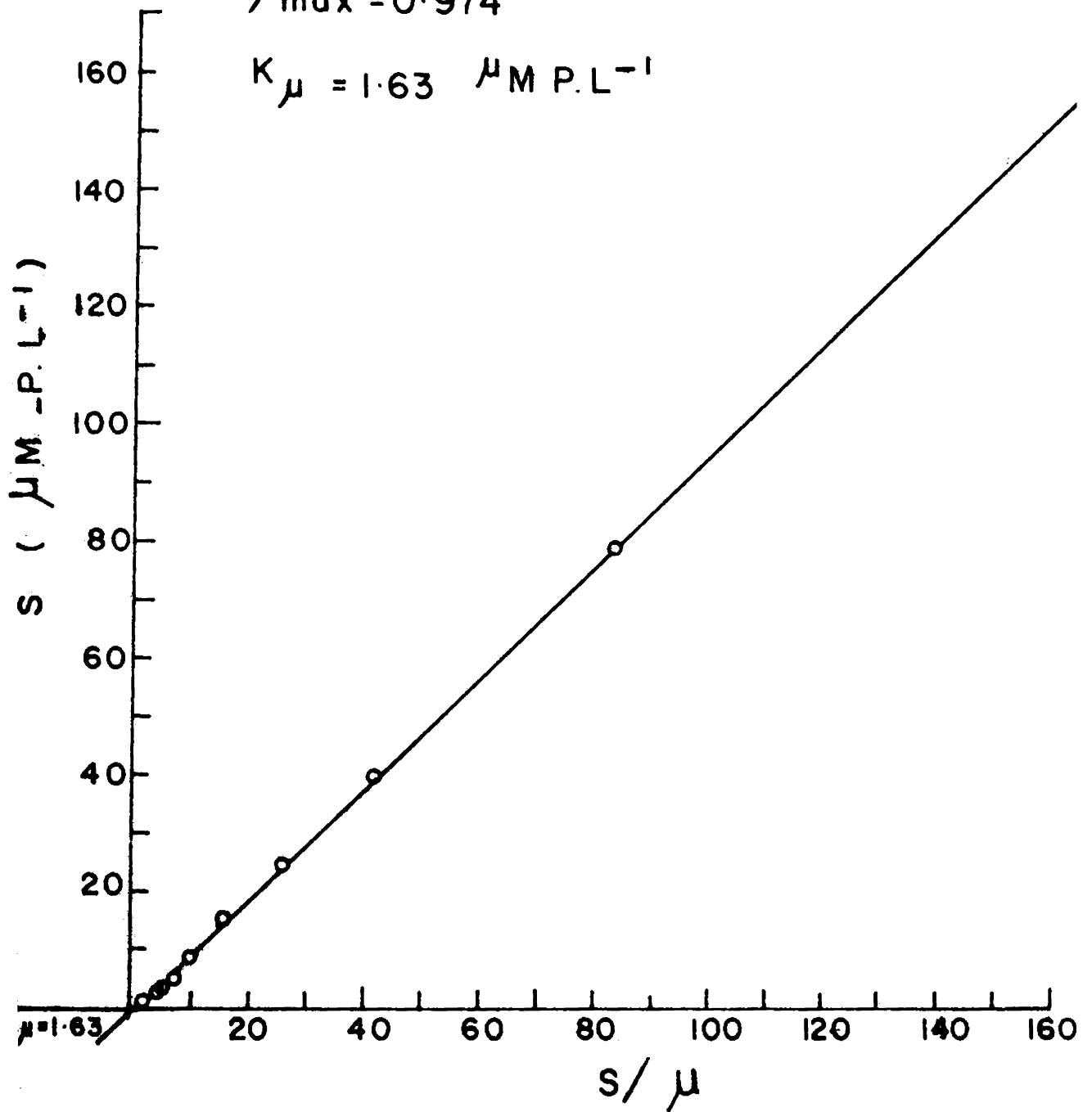
that upto 12th the population doubled itself every
48 hour thus building up a very dense culture.

The young cultures had a bluish-green colour.
The blue tint reduced as the culture aged. The declining
cultures were dull green in colour.

The amount of chlorophyll a and non-chlorophyllous
pigments also followed the same trend as the cell numbers
(Fig.14B). During the first four days the increase in
the amount of the pigments was exponential; this was
followed by a gradual rise till the 12th and later the
culture was in the stationary state. Decline in the
amount of the pigments began by 20th day. The amount
of chlorophyll a per unit number of cells was high during
8-12 days; the maximum amount being 0.019 $\mu g/10^6$ cells.
Similarly the non-chlorophyllous pigments also were
found to be maximum from 8-14 days with a concentration
of 0.012 $\mu g/10^6$ cells. On all days except the 2nd,
chlorophyll a dominated over the non-chlorophyllous
pigments. But in the declining cultures i.e. from the
20th, the amount o non-chlorophyllous pigments was
higher (Table 22).
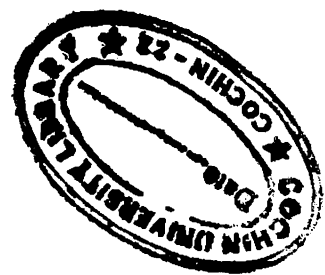
Fig.14A. Increase in cell number of _Synechocystis_
          salina during growth in batch culture.

Fig.14B. Variation in the amount of photosynthetic
          pigments of the above species during
          growth in batch culture.

Fig.14.
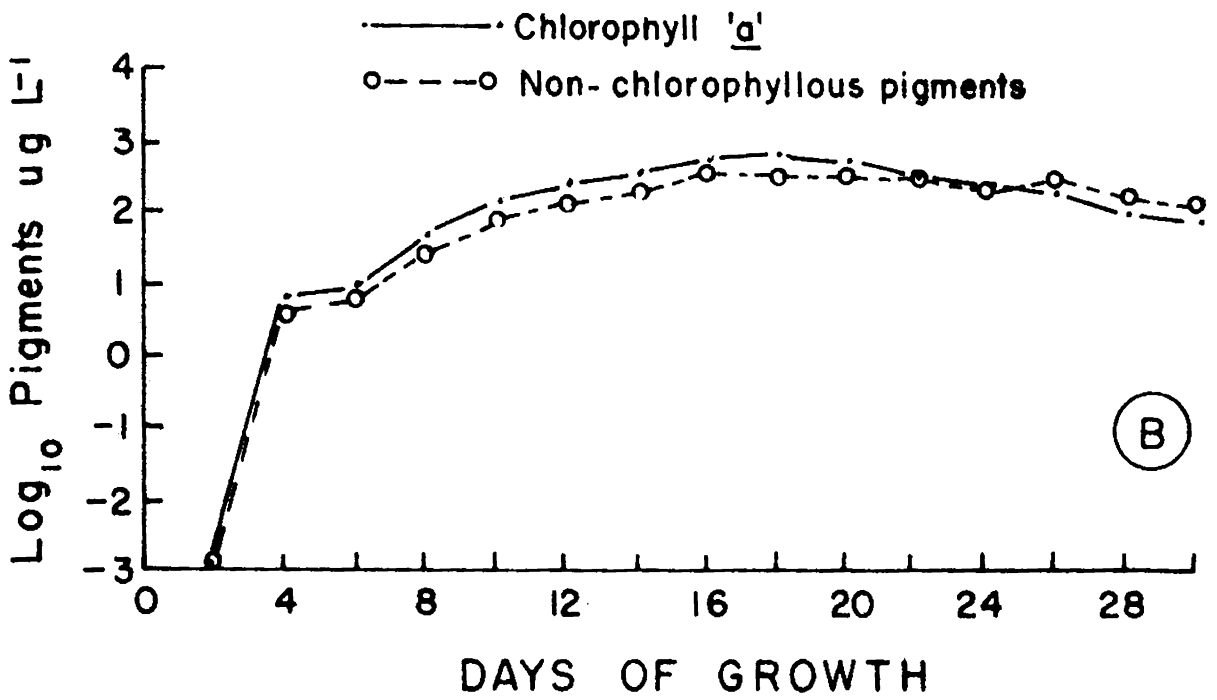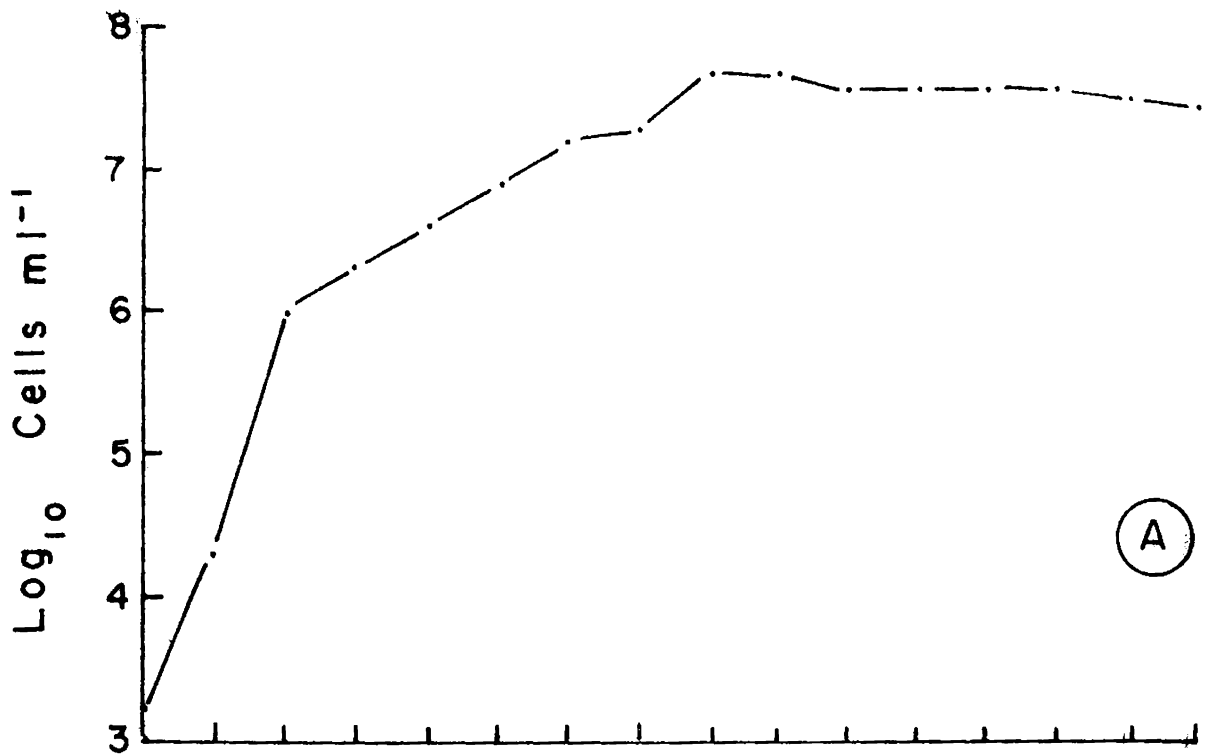
Table 22. Variation in the amount of pigments expressed as $\mu g/10^6$ cells of _Synechocystis salina_ for a growth period of 30 days in batch culture.

Age of culture (days)	Chlorophyll a $\mu g/10^6$ cells	Non Chlorophyllous pigments $\mu g/10^6$ cells	Non Chlorophyllous/chlorophyll a
2	0.003	0.004	1.33
4	0.008	0.005	0.63
6	0.009	0.006	0.66
8	0.018	0.007	0.39
10	0.018	0.010	0.56
12	0.019	0.012	0.63
14	0.018	0.011	0.61
16	0.019	0.012	0.63
18	0.016	0.009	0.51
20	0.013	0.010	0.77
22	0.009	0.009	1.00
24	0.007	0.007	1.00
26	0.006	0.008	1.35
28	0.004	0.006	1.67
30	0.003	0.005	1.67

The rate of production as shown by the oxygen and ^{14}C methods gave the highest production rate on 6 to 8 days.

After this period the rate of production reduced
considerably (Fig.13A). After ten days the activity
remained stationary with slight fluctuations.

When the experiment was discontinued after 30 days
the physiological activity was at the declining stage.
In the carbon-14 experiments the outcome was more
variable after 10 days of growth (Fig.13B). The rate
of production increased on the 12th day followed by
decrease on 14th and 16th. This fluctuation remained
till the 30th day. The highest rate of production as
measured by the oxygen experiment was 0.40 $\mu gC/10^6$ cells
(gross production) and 0.20 $\mu gC/10^6$ cells (net production).
As per the ^{14}C technique the highest rate of production
was 0.30 $\mu gC/10^6$ cells.

<u>Pigment composition</u>

The pigment system of the blue-green algae was
found to be composed of five fractions (Fig.3A) of
which carotene and chlorophyll a were dominant. The
other three fractions could not be identified. The R_F
of these are given in Table 4.

<u>Biochemical composition</u>

The biochemical composition of the species showed
definite trends in the new and aged cultures. The water

Fig.15A.   Rate of production of organic carbon by
           S. salina for a growth period of 30 days
           in batch culture (measured by the oxygen
           technique).

Fig.15B.   Rate of production of organic carbon by
           the above species for a growth period of
           30 days.

Fig.15
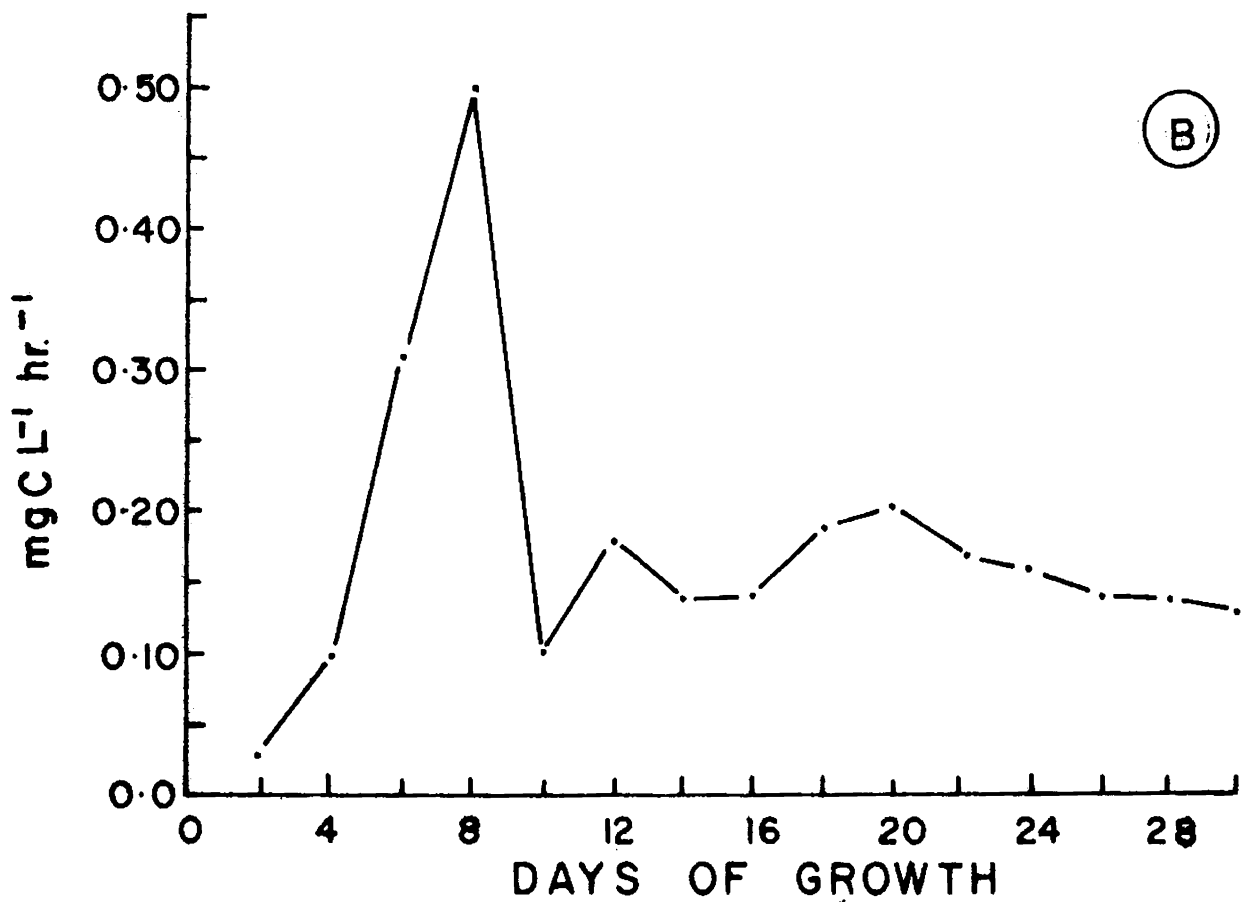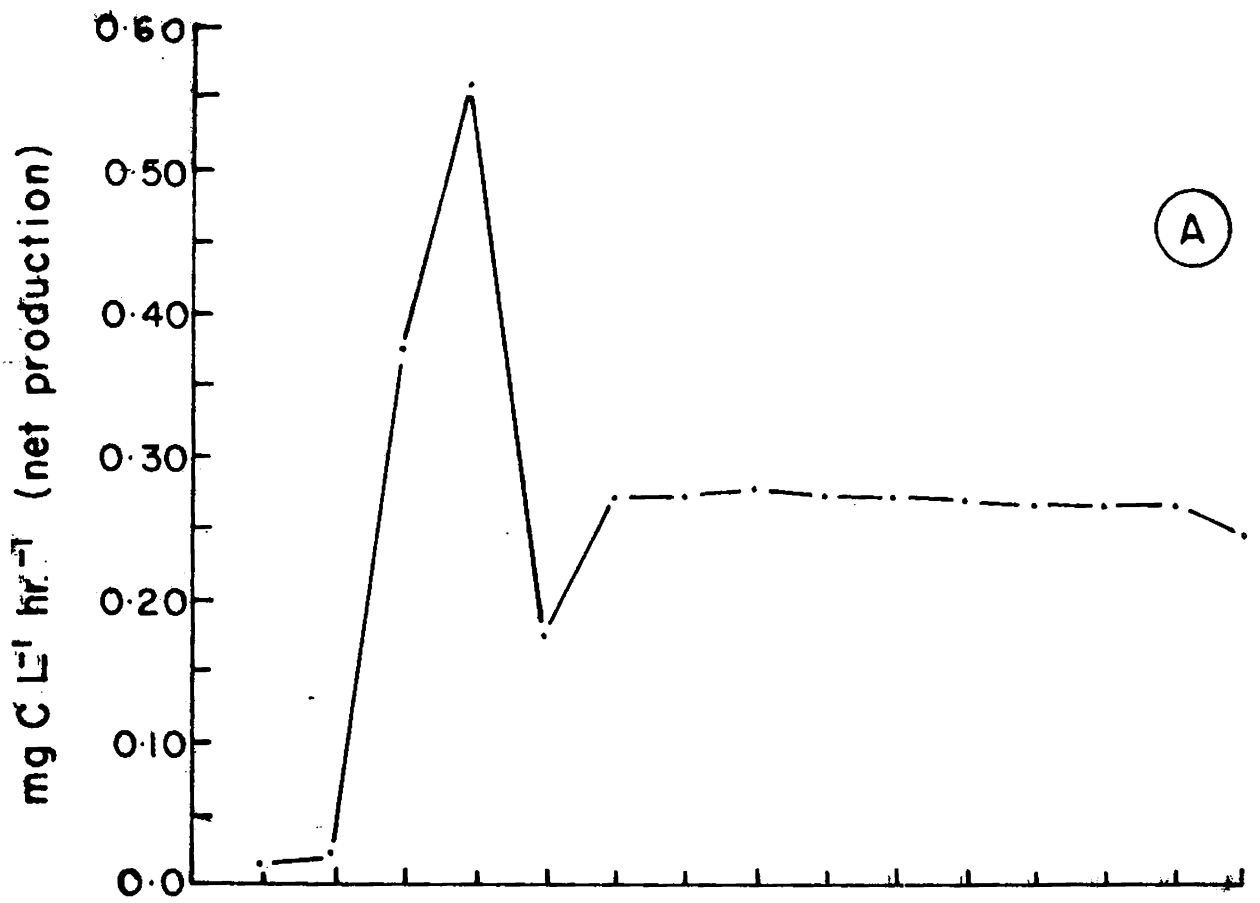
content of the cells was considerably reduced in old
cultures. There was a difference of about 35% between
the five day and twenty day old culture. Similarly
increment in the dry weight was noticed. The young culture
weighed 7 $\mu g/10^6$ cells and it doubled as the culture
became twenty days old. The orotein content was
comparatively low with an average of 33-41% . The ratio
of protein to carbohydrate decreased from 1.29 to 0.74
as the culture became old. The variation in lipid content
was relatively low. The amount of various components are
given in Table 23.

Table 23. Biochemical composition of S. salina raised in
batch culture.

Fraction	Sampling interval		
	5 days	10 days	20 days
Percent moisture	88.57 ±5.14	80.62 ±3.88	50.29 ±4.14
Dry weight ($\mu g/10^6$ cells)	7	7.9	15.0
Protein (% dry wt.)	41.0 ±9.16	34.2 ±11.32	33.12 ±6.84
Carbohydrate (% dry wt.)	31.75 ±7.55	36.22 ±8.42	44.36 ±11.19
Lipid (% dry wt.)	12.02 ±2.38	10.16 ±3.12	9.24 ±2.16
Protein: Carbohydrate	1.29	0.94	0.74
Bacteria count/ml	10	1	4

Extracellular release

The species was observed to release a part of the photoassimilates to the culture medium. The four day old cultures excreted 38.91% of the total carbon fixed while the proportion increased to 64.5% in the cultures after 16 days of growth (Table 24). In terms of the absolute activity amounts ranging from 0.129-0.273 µC were retained in the medium as ECP out of the 5 µC added to each sample (Table 7).

Table 24. Percentage of extracellular products (ECP) released by S. salina during phases of growth in culture. (The % soluble represents the ECP as fraction of the total carbon fixed).

Days of growth	Cells ml^{-1}	Particulate soluble		% Soluble
		mgC/l/hr		
4	16 x 10^4	24.62	9.58	38.91
8	360 x 10^4	18.95	7.00	36.94
16	1100 x 10^4	14.24	9.19	64.5

Eco-physiology of S. salina

Salinity:

The growth of S. salina varied with the salinity of the medium. As shown by the analysis of variance the cell

counts were significantly different till the 14th day
following inoculation with exception on the 2nd. After
14 days there was virtually very little difference between
the treatments as far as the cell counts were concerned
(Table 24). But the culture at low salinity levels were
found to be more healthy with a bluish-green tint while
that at higher salinity (34‰) was dull green in colour.

The amount of chlorophyll a was high at the
lower salinity levels (14‰ and 24‰). Analysis of
variance revealed the treatment effect to be highly
significant. Between 14‰ and 24‰ there was however,
not much difference in the amount of the pigments (Table 25).
At salinity 34‰ the amount of chlorophyll a was too low
throughout the experiment.

Effect of pH

The difference in the pH of the culture medium
was reflected gradually in the growing cultures. In the
four day old cultures the cell density did not differ
significantly in the pH range 6-5 - 8. But when the culture
became still older, there was significant difference between
the treatments (Table 26). The highest cell density was
built up at pH 7.5 to be closely followed by that at 7
and 6.5. At the stationary stage, the cell density at pH 8

Table 25. Cell concentration and chlorophyll a content of
S. salina grown in culure media of salinities
14%, 24%, and 34% for a period of 24 days.

Age of culture (days)	Cell concentration/ml			Chlorophyll a ($\mu g/10^6$ cells)		
	14%	24%	34%	14%	24%	34%
0	2400	2400	2400	-	-	-
2	20,000	19,000	28,000	0.227	0.229	0.189
4	440,000	300,000	218,000	0.158	0.139	0.100
6	1155,000	925,000	900,000	0.083	0.083	0.016
8	2270,000	2006,000	1600,000	0.032	0.036	0.002
10	3480,0000	3320,0000	2668,000	0.016	0.016	0.002
12	4320,0000	3936,000	3700,000	0.007	0.008	0.001
14	5620,000	5625,000	5654,000	0.006	0.006	0.001
16	8500,0000	8000,0000	8000,000	0.006	0.007	0.002
18	8520,0000	8515,000	8510,000	0.005	0.005	0.001
20	8500,0000	8510,000	8510,000	0.005	0.005	0.001
22	7600,0000	7600,000	7050,000	0.005	0.005	0.001
24	7534,0000	7500,000	6128,000	0.003	0.004	0.001

was too low compared to the other three treatments.
The amount of chlorophyll **a** showed highly significant
difference between the treatments with the pH levels
6.5 and 7 giving higher values. The Table 26 gives the
mean values for the cell counts and chlorophyll **a**.

Table 26. Cell concentration and chlorophyll **a** content
of _Synechocystis salina_ during phases of growth
in culture media of different pH levels.

Treatments (pH level)	Logarithmic phase		Stationary phase	
	Cell density per ml	Chlorophyll a $\mu g/10^6$ cells	Cell density per ml	Chlorophyll a $\mu g/10^6$ cel
6.5	390,6700	0.162	4506,6000	0.018
7.0	401,0000	0.147	4507,6700	0.017
7.5	411,0000	0.103	4524,3000	0.009
8.0	346,0000	0.099	4048,3000	0.006

Effect of cell concentration on the rate of carbon fixation

The amount of ^{14}C incorporation in the short term
experiment increased with the cell concentration. But the
trend did not continue indefinitely as is seen from Fig.16.
The increasing trend in the production persisted till a
concentration of 125 x 10^5 cells/ml. At higher densities,

Fig.16. Rate of carbon fixation (expressed as ^{14}C uptake in cpm) as function of different culture densities of _D. salina_.

Fig.16.

CELL CONCENTRATION /ml. x10⁴
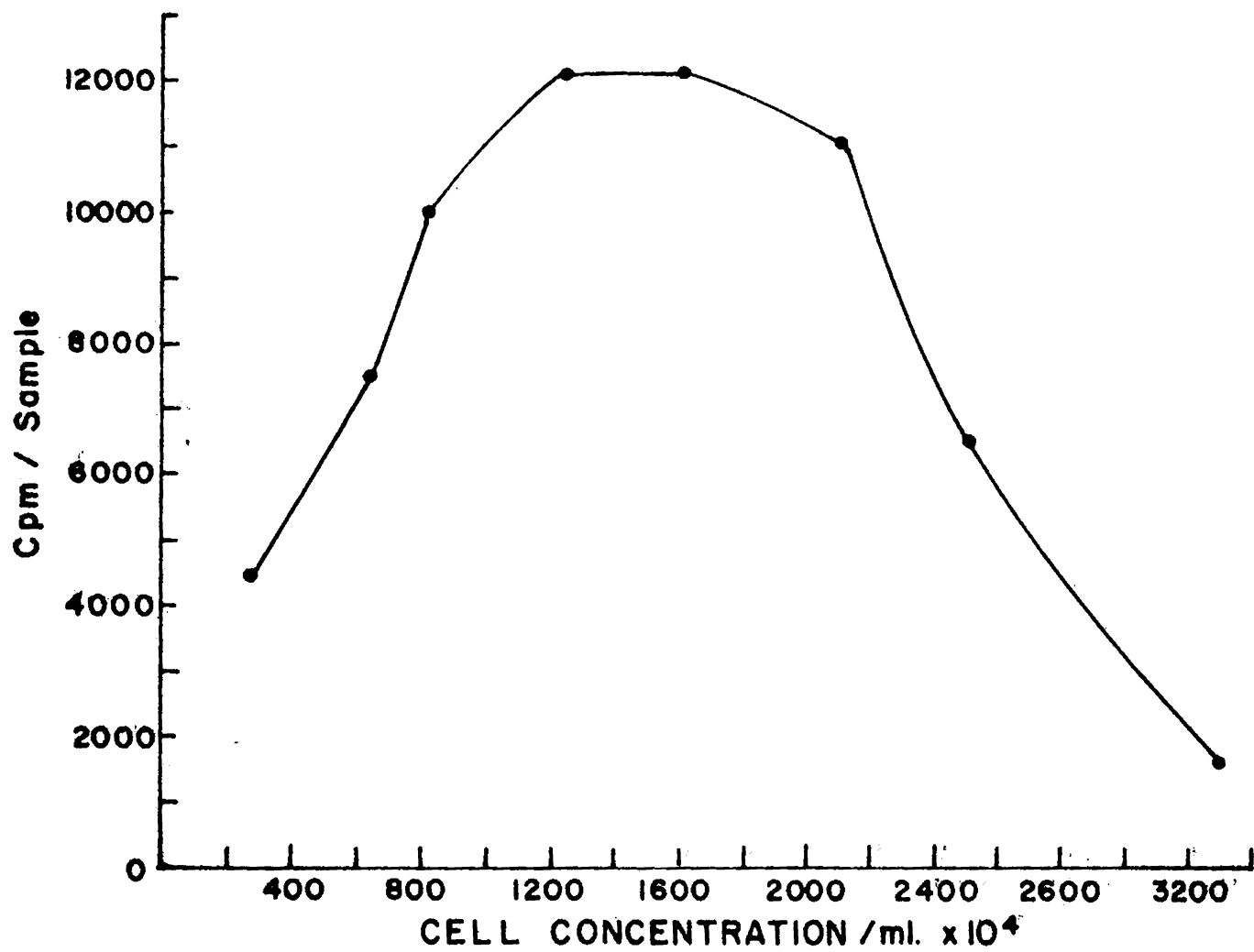
upto 175 x 10^5 cells/ml, the production remained
unaltered. Above this density, the rate of production
decreased. The cell densities and the corresponding ^{14}C
activity is given in Table 27.

Table 27. Carbon-14 uptake by Synechocystis saline as
function of cell concentration.

Cell density	Counts per minute	
275,0000	4450	± 134.0
640,0000	7506	± 98.0
825,0000	10089	± 767.0
1250,0000	12120	± 582.0
1650,0000	12156	± 74.2
2100,0000	11910	± 103.8
2500,0000	6451	± 77.0
3300,0000	1568	± 53.8

Influence of light

The response of the organism varied significantly
with the photoperiod to which the cultures were exposed.
The rate of cell division was higher in those subject to
constant illumination. From an initial density of 1440
cells/ml a density of 45 x 10^6 cells/ml was reached in
thirty days, by continuous illumination whereas the

density of the culture subject to light-dark cycle
remained at 8 x 10^6 cells/ml (Table 28).

Table 28. Effect of photoperiod on growth of S. salina
in batch culture.

Age of culture (days)	Cell density per ml	
	10:14 Light-dark cycle	Constant illumination
0	1440	1440
2	2,0000	7,0000
4	90,0000	350,0000
6	165,0000	1100,0000
8	290,0000	2000,0000
10	400,0000	2980,0000
12	500,0000	3000,0000
14	528,0000	3110,0000
16	560,0000	3650,0000
18	750,0000	3920,0000
20	730,0000	4200,0000
22	800,0000	4500,0000

The intensity of light to which the cultures
were exposed affected the rate of production. From the
hyperbolic relation between the light and photosynthesis
it was observed that the rate of carbon fixation was

saturated at 34.71 x 10^{15} quanta x cm^{-2} x sec^{-1} with an I_K of 24 x 10^{15} quanta x cm^{-2} x sec^{-1} (Fig.17). The metabolic activity was very low below 14 and above 51 x 10^{15} quanta x cm^{-2} x sec^{-1}.

Effect of temperature

The viability, growth rate and rate of production of the blue-green alga was determined at different temperatures. The cultures were kept in refrigerator ($5^{o}C$) and recultured weekly to fresh medium at room temperature. It was observed that the cultures were viable upto six months of storage under the low temperature. The cultures behaved normally from $20^{o}C$ - $40^{o}C$. The thermal death point was observed to be $45^{o}C$.

The growth rate of the cultures differed slightly at the temperature range $20-40^{o}C$. The mean doubling rate per day is given in Table 12. The highest growth rate occurred at $25^{o}C$ with slight reduction at 30 and $35^{o}C$. At $20^{o}C$, the rate of growth was less than that at 25° and it was still lower at $40^{o}C$. At this higher temperature the cells were viable though with a highly reduced growth rate.

Requirement of nitrate and phosphate

The concentration of nitrate in the culture medium and growth of S. salina in culture was related as per the

Fig. 17. The rate of photosynthesis (expressed as ^{14}C uptake in cpm) as function of light intensit for _D. salina._

Fig. 17

$Y = 33 \cdot 172 + 26 \cdot 18 \ X - 0 \cdot 4 \ X^2$

$X \max = 100 \% \ Photosynthesis = 32 \cdot 73 \times 10^{15} \ quanta. Cm^{-2}. Sec^{-1}$

$I_K = 22 \cdot 5 \times 10^{15} \ quanta. Cm^{-2}. Sec^{-1}$
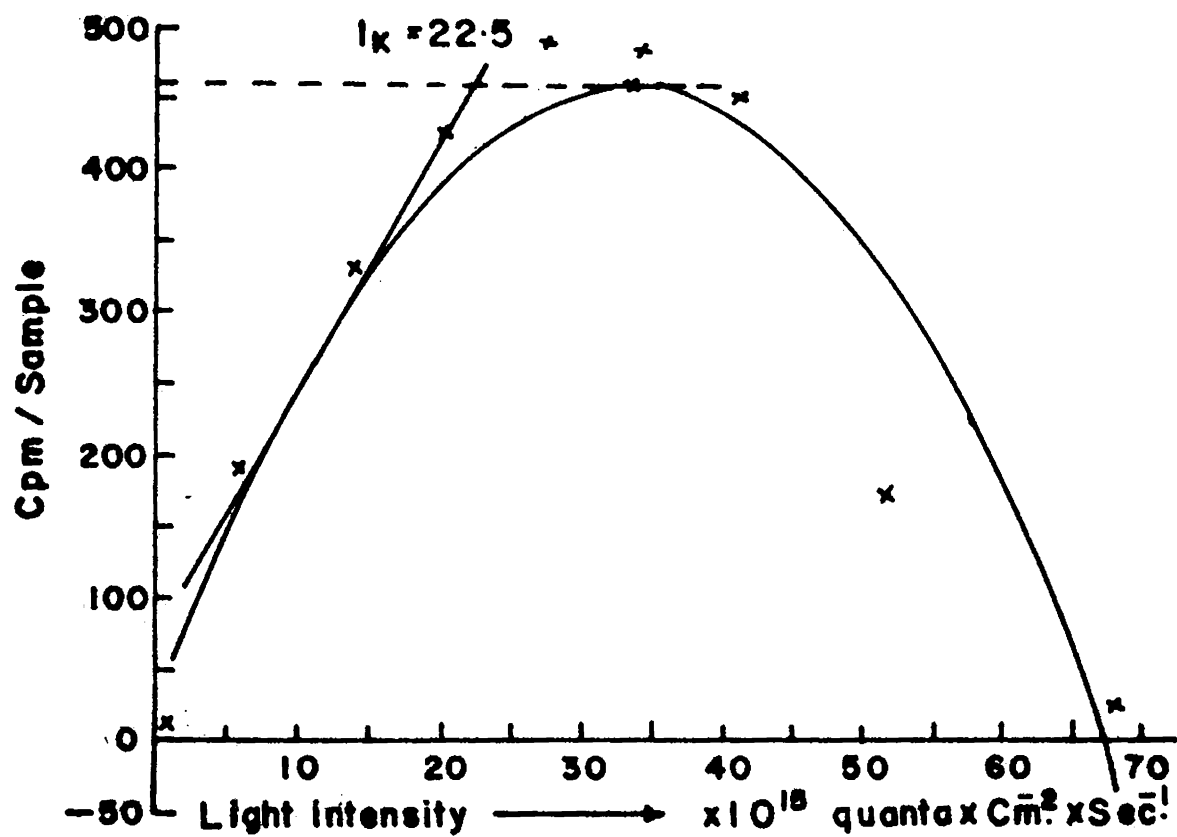
relation S = 0.983 (S/μ) - 0.81 where 0.81 = half-saturation
constant for growth and 0.983 = maximum (unlimited) growth
rate (Fig.18).

Similarly, the phosphate requirement of the
species could be defined by the relation S = 0.963 (S/μ) -
1.34 where 1.34 is the half-saturation constant for growth
and 0.963 is the maximum unlimited growth rate (Fig.19).

As in the case of flagellates, here also the
trend was that the observed rate of growth neared μ_{max} only
at very high substrate concentrations (Table 13 and 14).
It was also observed that a nitrate concentration above
600 mg/litre and a phosphate concentration of about
30 mg/litre were inhibitory to the growth of the species.

4.4. Tetraselmis gracilis

The growth kinetics of the species has been
described by Nair, 1974. I. gracilis isolated from the
inshore region of Cochin was cultured in Miquel's medium
and its growth in culture was studied for 35 days. The
highest growth rate (k) as computed from the cell counts
was 0.05 with a generation time of 14 hours. From an
initial inoculum of 200 cells/ml, the culture density
increased to 1 million by the end of the observation
period. Chlorophyll a and b were also measured

Fig.18.   The relation between the concentration o
         nitrate in the culture medium and the
         specific growth rate of _S._ _salina._

Fig.18.   The relation between the concentration o
         nitrate in the culture medium and the
         specific growth rate of _S._ _salina._

Fig.18.

$$S = \mu max\ (S/\mu) - K\mu$$

$$S = 0.983\ (S/\mu) - 0.81$$

$$K\mu = 0.81\quad \mu g\text{-}at\ N.L^{-1}$$

$$\mu max = 0.983$$
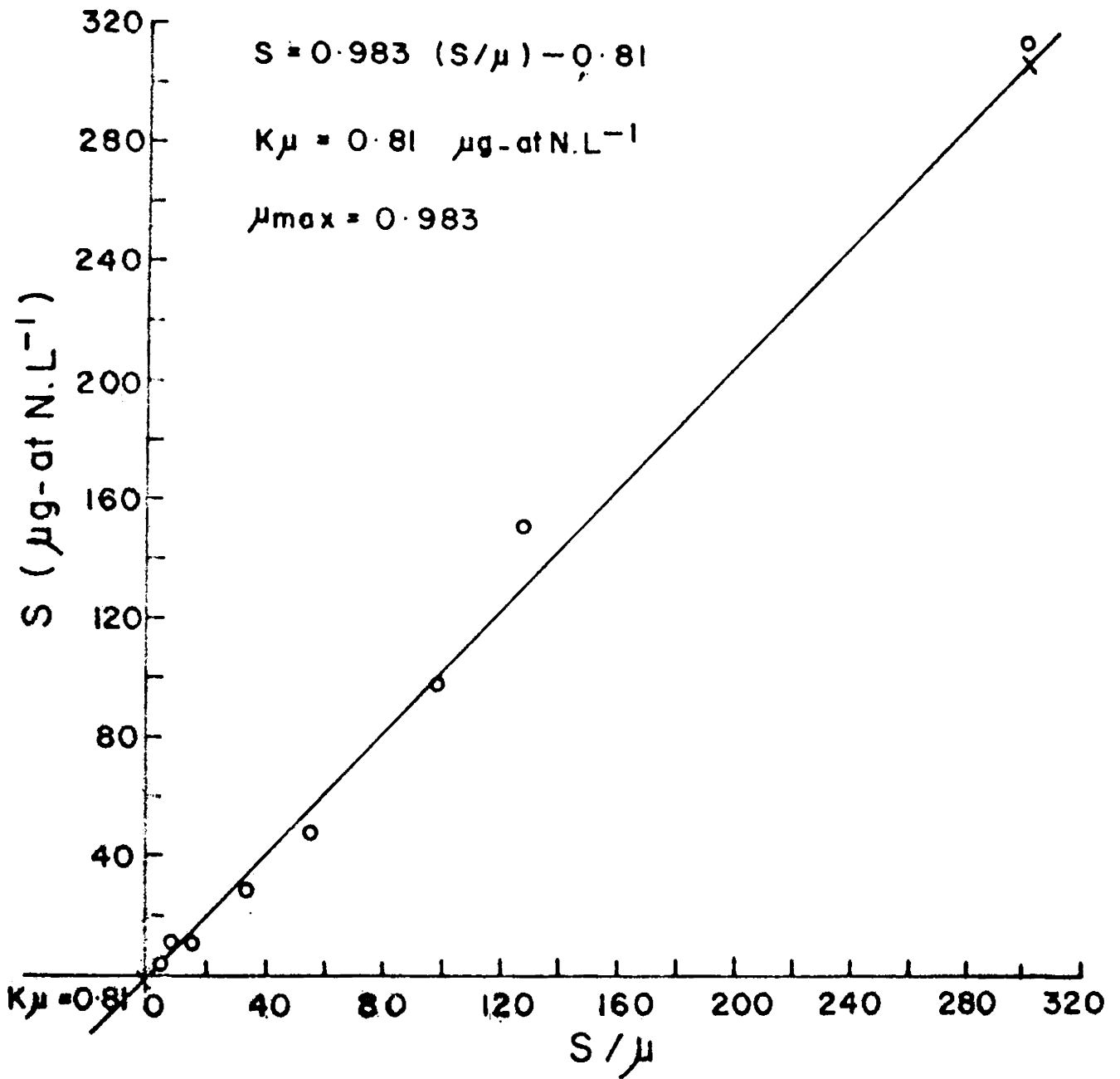
Fig.19. The relation between the concentration of
phosphate in the culture medium and the
specific growth rate of _S. saline._

Fig. 19.

$$S = \mu_{max}(S/\mu) - K_\mu$$

$$S = 0.963(S/\mu) - 1.34$$

$$\mu_{max} = 0.963$$

$$K_\mu = 1.34 \quad \mu\text{M-P.L}^{-1}$$
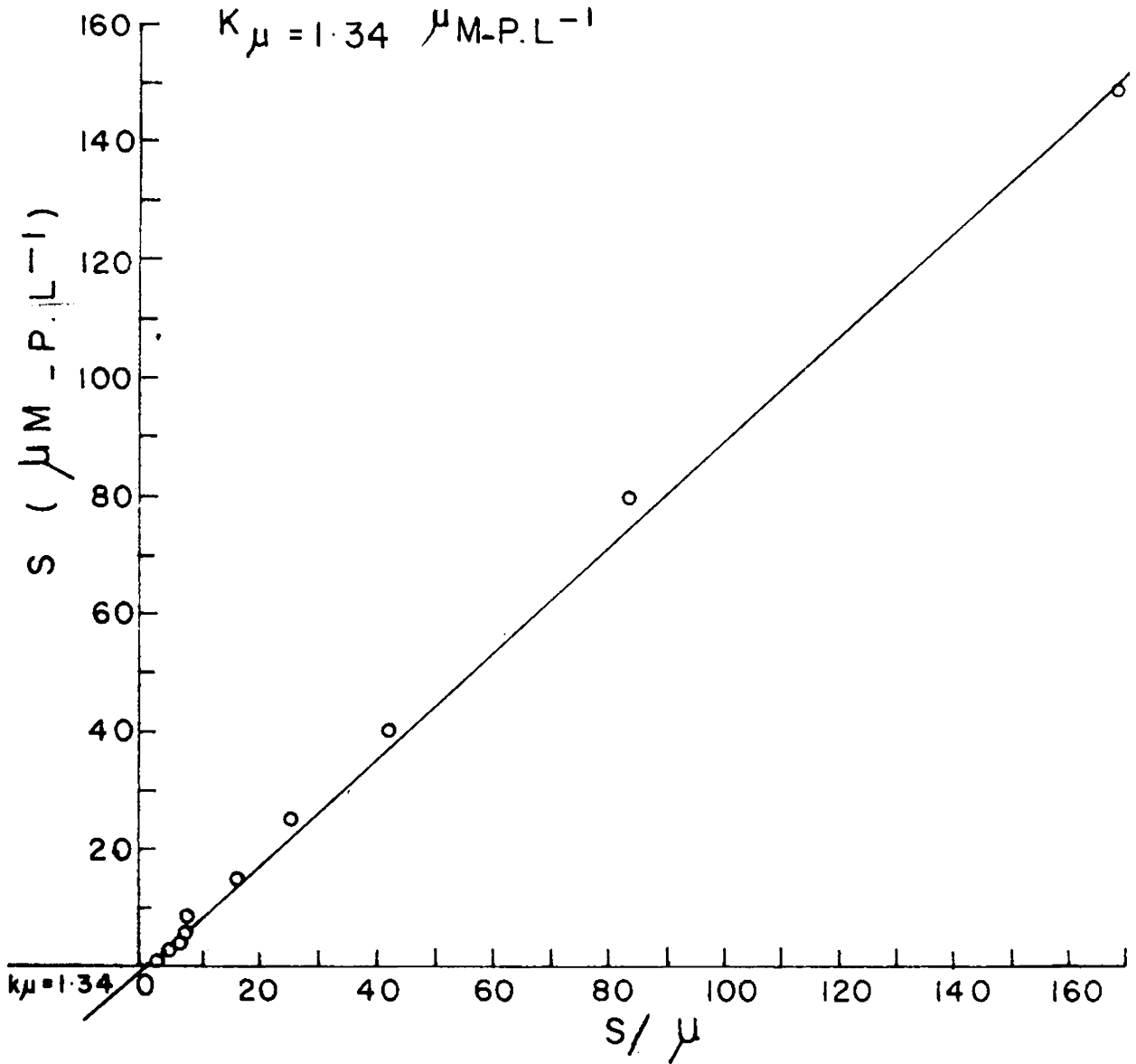
simultaneously. The gross carbon values per litre
per hour ranged from 0.07 to 0.79 mgC measured by the
oxygen technique, and from 0.03 to 0.34 mgC per hour by
the ^{14}C technique.

Certain other aspects of growth of this strain
was studied during the present investigation.

Pigment composition

The photosynthetic pigments of I. gracilis was
analysed by TLC. The chromatogram resolved 4 fractions
(Fig.3D). Chlorophyll a, Chlorophyll b and Carotene was
quite prominent. The R_F values of these are given in
Table 4. The remaining fraction was assumed to be
xanthophyll.

Biochemical composition

The biochemical components proteins, carbohydrates
and lipids of the flagellate varied in their relative
amounts. The lipid level was comparatively lower than that
of Carbohydrate and Protein. Protein and carbohydrate
contributed the major share with predominant protein
synthesis (Table 29). The ratio of protein to carbohydrate
decreased by half from five days to twenty days of growth.
Similarly there was high fluctuation in the water content
and dry weight of the cells.

Table 29. Biochemical composition of *Tetraselmis gracilis* raised in batch culture.

Fraction	Sampling interval		
	5 days	10 days	20 days
Percent moisture	86.00 ±5.14	82.00 ±3.84	52.76 ±4.81
Dry weight (μg/10^6 cells)	579	720	1249
Protein (% dry wt.)	54.00 ±12.15	46.00 ±7.68	42.00 ±8.34
Carbohydrate (% dry wt.)	25.75 ±7.35	29.69 ±8.24	42.29 ±12.13
Lipid (% dry wt.)	2.20 ±0.09	2.36 ±0.76	1.86 ±0.81
Protein: Carbohydrate	2.10	1.55	0.99
Bacterial count/ml	80	5	12

Excretion of organic matter

The extent of organic excretion also changed with the age of the culture. 2.5% of the total metabolite was released into the medium on the 4th day whereas on the 16th day this fraction increased to 22.36% (Table 30). In terms of absolute activity 0.01-0.28 μC were obtained as ECP in each sample added with a total activity of 5 μC (Table 7).

Table 30. Percentage of extracellular products (ECP) released by **Tetraselmis gracilis** during phases of growth in culture. (The % soluble represents the ECP as fraction of the total carbon fixed)

Days of growth	Cells ml^{-1}	Particulate soluble mgC/l/hr.		% Soluble
4	2.4 x 10^4	2.35	0.06	2.5
8	7 x 10^4	2.18	0.30	13.76
16	15 x 10^4	3.22	0.72	22.36

5. MASS CULTURE OF _CHLORELLA FREIBURGENSIS_ AND
   ISOCHYSIS GALBANA (C.s) AND EVALUATION OF THEIR
   FOOD VALUE IN MARICULTURE.

5.1. Mass culture

Mass production of nanoplankton has been taken up
in mariculture operations in connection with the rearing of
the filter-feeding larval stages of marine fish and other
invertebrates.

The simplest method for the mass production of
phytoplankton is by adding commercial fertilizers to sea
water collected and kept in out-door tanks. In this
system filtered sea water is let into open tanks of
2000 to 10,000 gallons capacity, fertilized with commercial
fertilizers to promote a good growth of mixed phytoplankton.
Such systems could be installed out-door or in green houses
that are artificially lit to provide an year round supply
of mixed phytoplankton culture.

Large scale culture of _Chaetoceros affinis_ is
taken up in the prawn culture laboratory of CMFRI, Cochin
on similar lines. Fibre glass tanks of capacity 1000 ml
are filled with filtered sea water and fertilized with
commercial fertilizers. These are inoculated with stock

cultures of _C. affinis_. At temperatures above 28°C
good diatom growth dominated by _C. affinis_ is obtained.

Unialgal cultures of nanoplankton of proven food
quality are raised on a large scale in controlled conditions.
Many laboratories make use of pyrex carbuoys as growth
chambers for axenic cultures. The carbuoys are maintained
at constant temperature and illuminated artificially. The
cultures are aerated by bubbling a mixture of air and CO_2.
A fixed volume of the culture is harvested daily and is
replaced by an equal volume of the medium. This is a
semi-continuous system. Similar cultures are maintained
in the molluscan hatchery of CMFRI at Tuticorin. The
culture containers used are 20 litre carbuoys or perpex
tanks that are artificially lit indoor.

Continuous culture systems have been built up
in many laboratories for mariculture operations Palmer et al.
(1975) and Trotta (1931). These laboratories use different
culture containers like spherical or cylindrical glass
bottles, perpex cylinders or plastic bags. These continuous
culture systems may function as 'turbidostat' or 'chemostat'
cultures (cf. Fogg, 1975). The continuous culture
production system usually is comprised of rows of pyrex
carbuoys or plastic bags with provision for addition of

nutrients, withdrawbl of the culture and aeration. It has also the added advantage that the culture can be maintained in steady state so that a more uniform product can be fed to the larvae continuously (Palmer et al., 1975). The present investigation pertains to mass culture of Chromulina freiburgensis and Isochrysis galbana forma nova. These were grown as discontinuous culture under controlled conditions.

The flagellates were inoculated into Walne's medium taken in 20 litre glass carbuoys. The cultures were exposed to a light-dark cycle of 12:12 hours with illumination of about 60,000 lux from fluorescent lamps at a temperature of 30±2°C (Plate IX) with constant aeration.

The growth of the culture was followed by taking the cell counts for twenty days.

Observation

Chromulina freiburgensis:

The culture was inoculated at a density of 9400 cells/ml. From this initial concentration it increased to 1.015 million cells/ml in six days. After

the 10th day the cell numbers began to decrease. However, the culture declined completely only by the 24th day. The cell counts for the various days are given in Table 31. The growth rate of the flagellate was calculated from the cell counts. The maximum growth rate was observed to be on the 2nd day being 2.1 doublings per day.

Isochrysis galbana (C.s.):

The culture had a density of 3260 cells/ml initially. During the course of growth it increased to 9.9 lakhs per ml in ten days. Later the cell numbers were found to decrease (Table 31). It was found that the growth

Table 31. Cell concentration per ml, in mass cultures of Chromulina freiburgensis and Isochrysis galbana (C.

Age of culture (days)	Chromulina cells/ml	Isochrysis cells/ml
0	9400	3260
1	1,8000	7100
2	7,7500	2,7500
3	22,5000	13,0000
4	67,0000	30,0000
5	101,5000	65,0000
6	105,0000	79,0000
8	103,0000	87,0000
10	93,0000	99,0000
12	48,0000	42,0000
14	46,0000	46,0000
16	38,0000	36,0000
18	53,0000	35,0000

EXPLANATION OF PLATE IX

Mass culture of C. freiburgensis and I. galbana (C.s.)

Mass culture of C. freiburgensis and I. galbana (C.s.)

PLATE IX

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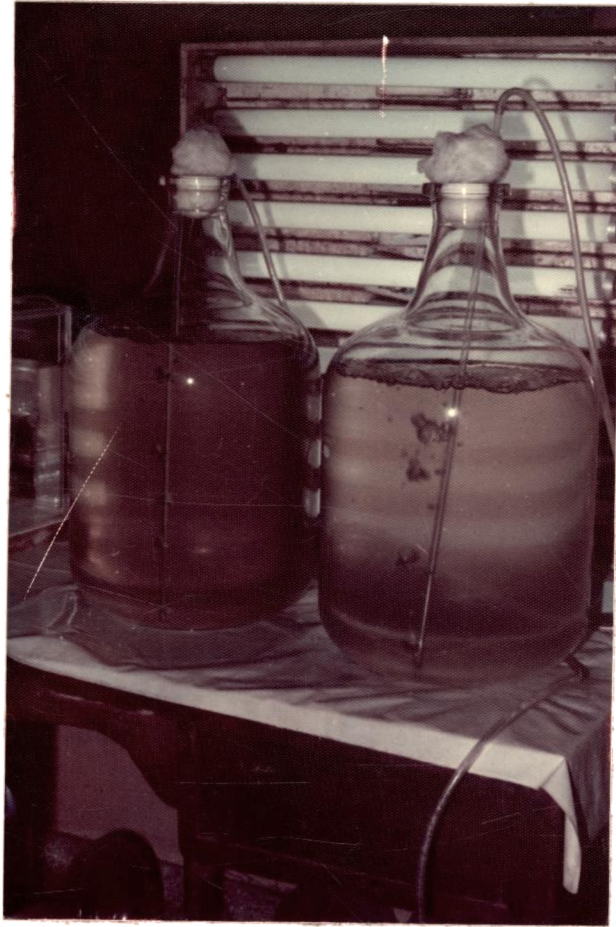
rate was maximum on the 2nd to 3rd day with a maximum value of 2.24 doublings per day. The cultures declined completely and the cells settled down by the 20th day after the initiation of the experiment.

3.2. Evaluation of the acceptability of C. freiburgensis and I. galbana (C.s.) as food for the larvae of edible oyster, Crassostrea madrasensis.

Bivalve hatcheries around the world utilise different feeds to rear the larvae from the beginning of the planktonic stage to metamorphosis. The relative efficiency of the feed is verified in terms of the larvae that survive. Loosanoff and his collaborators in the Milford laboratory and Walne, Davis, Guillard, Imai and others in Europe, America and Japan have successfully reared the larvae of several genera of bivalves(cf. Loosanoff and Davis, 1963).

It has been now well established that bivalve larvae can be reared in the laboratory on a diet of small naked flagellates. It is also observed that the phytoplankters are not equal in nutritive value. Cole (1936) suggested that the algae like Chlorella and Coccomyxa were unsuitable as food for oyster larvae probably because of the thick cellulose cell wall which the larvae may find

difficult to digest. However, Newell (1953) demonstrated
that the style enzymes of adult oyster can degrade cellulose.
Loosanoff and Marak (1951) raised the larvae of certain
bivalves on mixed _Chlorella_ culture. Davis (1953)
observed that the larvae of _Crassostrea virginica_ utilised
thick walled nanoplankton: _Chlorella_, _Platymonas_ sp.,
Chlorococcum sp. and _Phaeodactylum tricornutum_. This led to
the suggestion that certain bivalves can utilise only a few
species of microalgae while certain others have a wider
option. As Ukeles (1975) sums up, the three basic
requirements of the feed are :-

1. The feed must be non-toxic.
2. It must be easily accessible by the animals.
3. It must provide the nutrients for normal growth and
   development.

It has also been observed that a mixture of algal
species is superior for feeding compared to single species.
A mixture of _Isochrysis galbana_, _Monochrysis lutheri_,
Platymonas sp. and _Dunaliella euchlora_ induced rapid
growth of both clam and oyster larvae compared to equal
quantities of any of these fed separately (cf. Loosanoff
and Davis, 1963).

Production of toxic products at a concentration
lethal to the larvae is considered to be a drawback of

certain flagellates as _Prymnesium_ _parvum_ (Davis and
Guillard, 1958). A few studies have also been directed
to the chemical composition of the nanoplankters.

The size of the feed particle is an obvious
limitation in the development of live-food in bivalve
hatcheries. Diatoms are not usually used to rear the
oyster larvae as these are larger and difficult to digest
due to the silica walls. However, Japanese workers have
successfully reared molluscan larvae mainly that of
scallop on a diatom diet comprising _Cyclotella_ _nana_,
Skeletonema _costatum_, _Isochrysis_ _galbana_, _Chaetoceros_
gracilis, _Chaetoceros_ _simplex_ and _Chaetoceros_ _calcitrans_
(Ukeles, 1975).

Colourless flagellates have been tried by many
workers. But they have not proved to be quite successful
food for rearing oyster larvae (Walne, 1956). The
Milford laboratory team also has arrived at a similar
conclusion.

The motile green algae like _Dunaliella_ and
Pyramimonas are also considered to be satisfactory food
for rearing the larvae. However, the yellow-brown
chrysomonads in single and in combinations have proved to
be the best food for bivalve larvae. Flagellates like

Isochrysis galbana and Monochrysis lutheri were found to
be of equal food value and among the best to feed the
bivalve larvae (Loosanoff and Davis, 1963). Walne (1956)
also observed rapid growth of the larvae of Ostrea edulis
on a diet of I. galbana and another Chrysomonad
Chromulina pleiades. The significance of Isochrysis
galbana in bivalve hatcheries has further been proved by
Walne (1970) and Epifanio (1979). I. galbana is used in
molluscan hatchery of CMFRI to rear the larval stages
of pearl oyster, Pinctada fucata and edible oyster,
Crassostrea madrasensis. Recently success was achieved in
rearing the larvae of Indian mussels, Perna indica and
Perna viridis from fertilization to spat stage. A tropical
strain of I. galbana has been used with great success by
Ewart and Epifanio (1981) to rear the larvae of the American
oyster, Crassostrea virginica.

The present experiment was conducted to find out
the suitability of the tropical flagellates Chromulina
freiburgensis and Isochrysis galbana (C.a.) as food for
oyster larvae. Newly hatched larvae of edible oyster,
Crassostrea madrasensis was used for this purpose. In the
laboratory of CMFRI these larvae are usually raised on a diet
of I. galbana. The nutritional quality of the present
isolates were compared against this control feed.

Organization of the experiment

Naturally spawned larvae of _C. madrasensis_ were collected after 24 hours of fertilization. The size of the larvae was 55μ. These larvae were suspended in sea water (filtered through 1μ net and UV irradiated) of salinity 34‰ at a temperature of $28\pm2^{o}C$.

Filtered and sterilized sea water was taken in corning glass beakers of capacity 2.5 litres and equal number of larvae were suspended in them at a density of five larvae per ml. These were placed as three sets and fed on one of the three diets given below.

Diet A - _Chromulina freiburgensis_

Diet B - _Isochrysis galbana_ (C.s.)

Control - _Isochrysis galbana_

The feeding schedule was once in a day at a rate of 5000 cells per larva.

The larval density and feeding level were fixed arbitrarily. Every alternate day the sea water in which the larvae were suspended was changed. The nanoplankters used to feed the larvae were cultured in Walne's medium as described in section 5.1. These were fed to the larvae on every 5th day of inoculation.

Samples of larvae were collected from each group at 2-4 days interval and the size of the larvae was measured. The number of days taken by the larvae to settle as spat and the percent survival of the larvae were also noted.

Results

The mean size of the larvae on the various days of observation is given in Table 32. All the three diets supported the growth of the larvae to metamorphosis. But as presented in Table 32, the diets influenced the growth rate (reflected by the size of the larva) differently.

Table 32. Mean size (in microns) of the larvae of *Crassostrea madrasensis* reared on three nanoplankton diets (Initial size of the larvae - 55µ).

Diet	Sampling interval				
	5 days	7 days	11 days	14 days	19 days
Chromulina freiburgensis	93.25 ±1.08	120.0 ±4.30	160.75 ±7.24	298.0 ±8.15	573.3 ±7.91
Isochrysis galbana (C.s.)	90.00 ±2.36	91.25 ±5.80	150.25 ±3.16	261.25 ±6.74	333.20 ±5.30
Isochrysis galbana (Control)	87.25 ±1.76	106.50 ±6.81	137.25 ±3.95	201.75 ±7.18	492.50 ±8.22

The mean size of the larvae was larger in those
fed with C. freiburgensis and I. galbana (C.s.) compared
to the control. Results of the student t-test indicated
highly significant difference between the size of the spat
as measured on the 19th day of observation. The results of
t-test with the three diets is given below.

Diet A/control - P≤0.01, d.f. = 18, t = 13.32**
Diet B/control - P≤0.01, d.f. = 18, t = 5.91**
Diet A/diet B - P≤0.01, d.f. = 18, t = 5.98**

These data show that the present isolates induced
higher growth rates of the oyster larvae. It was also
observed that the larvae fed on C. freiburgensis settled
on the 17th day while in the other two the larval settlement
started only on the 19th day.

**Significant at 1% level.

The percent survival of the larvae was significantly higher in those fed with the present isolates: C. freiburgensis and I. galbana (C.s.) (Table 33).

Table 33. Percent survival of the larvae of C. madrasensis fed with three nanoplankton diets (Initial number of larvae = 10,000)

Diet	No. of spat obtained	Percent survival of larvae
Chromulina freiburgensis	279	2.79
Isochrysis galbana (C.s.)	101	1.01
Isochrysis galbana (Control)	24	0.24

Conclusion

This experiment clearly indicates that the present isolates, Chromulina freiburgensis and Isochrysis galbana (C.s.) are quite successful feeds in oyster rearing. Among these C. freiburgensis offers the greatest potential to be developed as live-food.

In the bivalve hatcheries in India and abroad greater importance has been placed on Monochrysis lutheri and Isochrysis galbana as these have been found to promote good growth of the larvae. There are some random reports of Chromulina pleiades being used successfully for rearing

the larvae of _Mercenaria mercenaria_ (Davis and Loosanoff,
1953), _Ostrea edulis_ (Walne, 1956) and _Crassostrea virginica_
(Loosanoff and team, Milford laboratory). However, these
authors consider _I. galbana_ and _M. lutheri_ superior to
C. pleiades in promoting the growth of these bivalve larvae.

Ewart and Eoifanio (1981) have presented the tropical
strain of _Isochrysis_ (T-ISO) as a suitable substitute for
Isochrysis galbana for both the larval and juvenile American
oysters, _Crassostrea virginica_. T-ISO is reported to be
suitable for development in mass cultures in green house
environment of 15-30°C under which conditions this gives
0.8 to 2 doublings per day.

The present investigation has projected to tropical
flagellates with 2.5 to 3.0 doublings per day in the
temperature range 20-35°C. It has been shown experimentally
that both these species thrive well in indoor mass culture
system. In the case of stability _C. freiburgensis_ shows
greater potential to be developed in hatcheries. The
feeding experiment also projects _Chromulina freiburgensis_
superior in food value than _Isochrysis galbana_ (C.s.).
These findings indicate the greater potential for the use of
Chromulina freiburgensis in the tropical oyster hatcheries.

DISCUSSION

The nanoplankters, *Chromulina freiburgensis*, *Isochrysis galbana* (C.s.) and *Synechocystis salina* raised in culture to study their growth rate and activity grew asymptotically building up dense populations. As seen from the results the logarithmic phase, the phase of declining growth rate and stationary phase were well marked in the course of development of the culture. In most of the cultures the lag phase was not noticed. Probably the absence of the lag phase is because the inocula were taken from exponentially growing cultures. In fact Spencer (1954) and Fogg (1944) have correlated the length of the lag phase to the age of inoculum. They report that in *Phaeodactylum tricornutum* and in *Anabaena cylindrica* the lag phase is totally absent if the inoculum had been growing exponentially.

The maximum growth rates observed (Table 1) was 2.66, 1.48 and 2.74 for *C. freiburgensis*, *I. galbana* (C.s.) and *S. salina* respectively corresponding to a relative growth constant of 0.079, 0.043 and 0.079. Previous literature shows that the relative growth constant (k) for various phytoplankters varies between 0.01 and 0.1 per hour at optimum conditions (Epoley and Strickland, 1968).

The present results are also in confirmity with this.
The growth rates observed during this study were higher
compared to that of similar strains of the temperate waters
(cf. Fogg, 1975). The temperate strain of I. galbana has a
generation time of 30.2 hours (Kain and Fogg, 1960) whereas
the present strain took only 16 hours. In mass culture
experiments, the strain was found to divide in 9 hours.
The tropical strain of the green flagellate Tetraselmis
gracilis divided once in 14 hours as reported by Jair (1974).
The increased growth rate could have been due to the
tropical conditions and the nutritional aspects of the
culture medium.

For a given species the growth rate is a function
of temperature, light intensity and nutrients. As in
this study, the culture conditions to which all the three
species were exposed were identical, any difference in the
growth rate must be species specific. The nature of the
inoculum may also have a role to play. Comparing the
flagellates with Synechocystis salina, an obvious feature
is that the latter maintained increased doublings of cell
for a longer period than the flagellates so that it could
attain very high densities (40-50 million cells per ml).
S. salina is a minute coccoid blue-green alga of volume
$14\mu^3$. The higher growth rate may be in accordance with the
fact that smaller species grow faster (cf. Raymont, 1980).

Blue-green algae in general are observed to have low generation time, the lowest t_g recorded being two hours for Anacystis nidulans (Kratz and Myers, 1955a). I. galbana (G.s.) (396 μ^3) took more time to divide than C. freiburgensis (524 μ^3). In fact in other experiments in the same series I. galbana (C.s.) showed similar growth rate as C. freiburgensis. But the higher generation time (t_g) of I. galbana (C.s.) shown in the experiment 3.4.1. may be due to some factor concerned with the inoculum. These results show that apart from culture conditions and cell volume, factors like health of the inoculum and other features characteristic of a species or taxon can affect the growth rate in culture.

The amounts of Chlorophylls a, c and total carotenoids were high during the exponential phase for the flagellates. This may be because the cells have access to abundant nutrients. The amount of chlorophyll a decreased considerably in a week's time. Similar results have been obtained in batch growth of cultures by previous workers (Morris and Glover, 1974; Vijayaraghavan et al. 1975). The relative proportion of carotenoids increased in the old cultures resulting in the change of colour of the culture from golden yellow to orange-red. Accumulation of carotenoids in ageing cultures has been frequently

encountered (Fogg, 1975). The possible reason for this
as suggested by Droop (1954) is the depletion of nitrogen
or phosphorus.

The culture of _Synechocystis salina_ behaved in a
slightly different way. In this the pigment content kept
on increasing for eight days, remained at the maximum till
14th and then began to decrease. The decrease in
chlorophyll a was reflected in change of colour of the
culture from bluish green to dull green.

The rate of production of _Chromulina freiburgensis_
and _Isochrysis galbana_ (C.s.) was at the highest level during
the first week of culture coinciding with the maximum
pigment concentration. This confirms the fact that
production is proportional to the pigment content. But in
Synechocystis salina the period of peak metabolic activity
preceded that of maximum chlorophyll a content. Probably,
other factors such as high culture concentration and its
further consequences interfered with the measurements.
The decreased rate of production in the ageing cultures
may be attributed to the decreased content of chlorophyll a
and nutrient depletion which is probable to occur in batch
cultures. Moreover many cells in the old cultures were
in the non-motile resting stage at which time metabolic
activity is normally reduced.

Chromatographic analysis of the photosynthetic
pigments of the species revealed the major fractions and
hence their taxonomic affinities. Chlorophyll a and
carotene were observed in all the four species. These
components are characteristic of all algal classes (Strain,
1951). Chlorophyll b is unique to the green algal line
and the results showed it to be present only in *Tetraselmis*
gracilis. Chlorophyll c was located in C. *freiburgensis* and
I. galbana (C.s.) indicating them to belong to the brown
algal line. No such indicative pigments could be spotted
for *Synechocystis salina*. The suggestion that the yellow
pigments of the Chrysomonads could be xanthophyll is
substantiated by the work of Jeffrey (1968) on *I. galbana*.
She fractionated the pigments of *I. galbana* into seven
fractions including chlorophyll a, c, carotene, fucoxanthin
and three other yellow xanthophylls.

The nanoplankters were observed to have predominant
protein synthesis in the exponential growth phase while
the relative proportion of protein decreased in the aged
cultures. Algae have been established as mainly protein
synthesizers (Spoehr and Milner, 1949; Lewin and Guillard,
1963). Parsons et al. (1961) observed in algae harvested
at logarithmic phase of growth, lower contents of lipids
and carbohydrate. Ricketts (1965) using stationary phase

cultures obtained relatively higher lipid and carbohydrate content. Platt and Irwin (1973) also suggest that lipids are minor components of phytoplankton. The present observation of changing ratios of protein to carbohydrate is on par with that of the results obtained by Handa (1969), Myklestad (1974) and Goldman (1980). Fogg (1956) working with the diatom, _Navicula pelliculosa_ observed that the predominant protein synthesis is replaced by lipid in nitrogen deficient algae. Nutrient depletion leading to carbohydrate accumulation in natural populations has been reported by Myklestad and Haug (1972) and Haug, _et al_. (1973).

The bacterial counts in the cultures used for the present analysis was negligible. As such the role of bacteria in the changing metabolic pattern of the algae seems to be insignificant. The variation in the chemical composition of the cell, though apparently related to the age of the culture as observed in the present study, could be due to the influence of physical and chemical factors of the environment to which the species were exposed. It was also observed that the amounts of protein, carbohydrate and lipid in the four species were not identical though they were grown under similar conditions. The difference is therefore solely a species characteristic.

The amount of organic metabolites released by
the nanoplankters C. freiburgensis, I. galbana (C.s.),
S. salina and I. gracilis varied from 2.5% to 64.5%.
Liberation of organic metabolites is shown by all
phytoplankters. However, there is little agreement on
the absolute amounts released. The consensus of opinion
is that exponentially growing cells release comparatively
less amounts of organic metabolites while the rate of
excretion increases during the stationary phase (cf.
Hellebust, 1974). The same was observed in the present
study. C. freiburgensis excreted 3% during the exponential
phase and it increased to 12.57% by the 16th day. Similarly
the other algae showed increased rates of excretion with
ageing.

Guillard and Wangersky (1958) have shown that
marine Chrysophytes release organic metabolites at increased
rates during the post exponential phase of growth.
I. galbana produced upto 25 mg/l and Prymnesium parvum,
125 mg/l of excreted material. In the present study the
Cochin strain of I. galbana (C.s.) produced upto 2.0 mg/l,
C. freiburgensis, 1.22 mg/l and I. gracilis, 0.72 mg/l.
In S. salina it was 9.58 mg/l for the given culture
densities (see Chapter 4).

The present data agree with the work of
Samuel et al., 1971 on a few tropical flagellates.
According to their observation seven day old cultures of
Tetraselmis gracilis, Tetraselmis carteriiformis,
Dicrateria sp. and Chlorella sp. excreted 3.1%, 1.43%,
4.35% and 1.65% of organic matter respectively.
Hellebust (1965) studying the rate of excretion of 22
species of phytoplankters concluded that at logarithmic
phase under similar conditions the majority of the
species excreted only 3.6% of the carbon assimilated.
A few algae excreted upto 25%. In the present experiment
the cultures of Synechocystis salina raised under the
same conditions as the flagellates exhibited a higher
percentage of excretion. During the exponential phase,
the flagellates excreted less than 4% of the ^{14}C
incorporated while S. salina showed an excretion of
38.91%. Probably this is explained by the work of
Jalewajko (1966). In dilute suspensions of microalgal
cultures excretion was less than 2% of the total carbon
fixed. Excessive population densities reduced
photosynthesis as well as increased excretion. He relates
it to the decreased carbon dioxide concentration and light
intensity that take place due to cell crowding, resulting
in higher rates of excretion. The higher excretion rate

by S. salina observed in the present study may be thus attributed to the increased population density.

The percentage of dissolved organic matter may tend to rise with decreasing physiological activity. The higher content of organic metabolites in the culture medium in ageing cultures could also be due to the passive release of organic matter from the dead and moribund cells (Fogg, 1975).

A study of the kinetics of growth of phytoplankters is never complete without an appraisal of its immediate environmental factors that control growth. An exclusive factor in the cultivation of marine microalgae is salinity of the culture medium. The response of the flagellates under study was similar to the variation in salinity of the medium while Synechocystis salina had different salinity preference. The chrysomonads grew well at higher salinities (greater than 24‰) while S. salina multiplied rapidly and had a higher pigment content at the lowest salinity level (14‰) tested. In the case of flagellates, both cell number and chlorophyll a was low at salinity of 14‰. In S. salina higher salinities affected the pigment content profoundly as shown by the chlorophyll a measurements and it could be discerned from the colour of the culture that they were not healthy. But cell counts

were nearly uniform after the culture entered stationary
phase. It has been reported that sub-optimum salinities
can affect the chlorophyll a content of the algae while
the growth may be unaffected (McLachlan, 1961). Sharp
changes in the colour of the culture is reported to occur
in cultures of Dunaliella salina grown at different
salinity levels (Loeblich, 1982). The colour of this
flagellate vary from green at lower salinity to red at
very high salinities. Obviously, at higher salinities
there is a reduction in chlorophyll synthesis and increase
in the carotenoid pigments. This particular behaviour of
D. salina is also shown to be brought about by changes in
pH, light intensity, and deficiency of nitrogen and
phosphorus. In the present study, the pigment content
was affected significantly during the exponential phase
also when nutrients could not have been limiting. So the
response of the algae is not due to nutrient deficiency.
The chance of high light intensity affecting the pigment
content of any particular treatment was also eliminated
as all the cultures were exposed to similar conditions.
As the pH was adjusted using buffer, the behaviour of
the species is attributable to salinity change alone. It
may be noticed that the rate of cell division and
synthesis of chlorophyll a was promoted by higher salinities
in the flagellates while the same salinity levels inhibited

the chlorophyll synthesis in S. salina. It may be that
salinity is interacting with intracellular features that
ultimately define the growth and activity of the organism.
Though the optimum salinity level for the species differ,
all show adaptability to the range of salinity tested and
the experiment is a good example to show how the algae can
control its metabolic activity to the requirement of the
environment,

Phytoplankton has marked capacity to adjust
osmotically. Most neritic marine flagellates can grow in
12‰ to 40‰ S with an optimum at 20‰ to 24‰ S (Provasoli,
1963a). Among the Chrysomonads, I. galbana tolerate
salinities from 15‰ to 40‰, Monochrysis lutheri also
does not show a distinct salinity optimum (Droop, 1958a).
Further, the salinity tolerance may vary significantly
with extremes of temperature, light and nutrients
Bonin et al., 1981.

Qasim et al. (1972) have attempted to define the
optimum salinity for maximum photosynthesis in some of the
tropical diatoms and dinoflagellates isolated from the
inshore region of Cochin. All the species tested showed
wide adaptability to changes in salinity and in all, the
maximum photosynthesis occurred at low salinities (less
than 20‰). They relate this to the ecological feature

of the region from which the species were isolated.
In the inshore region of Cochin, the salinity varies
from brackish (14‰) to marine (Darbyshire, 1967).
The reduction in salinity is coupled with enrichment
of nutrients enabling dense phytoplankton growth
(Subrahmanyan, 1954; Prakash and Sarma, 1964). The same,
they reason, may be responsible for accelerating
photosynthesis in the cultures at low salinities. But as
the present investigations reveal, the flagellates,
C. freiburgensis and I. galbana (C.s.) that were also
isolated from the inshore region of Cochin exhibited
better growth at higher salinities >24‰.) while S. salina
grew better at lower salinity. S. salina is a blue-green
alga, the group being mostly represented in fresh water
(Desikachary, 1959). May be it is this basic affinity to
fresh water environment, that is responsible for the better
growth of the species at the lowest salinity (14‰.) tested.

The results of the experiment with the three species
reveal that salinity tolerance is predominantly a species
characteristic though the same may by induced by an
ecological need for survival. These results lend support
to the statement of Qasim et al. (1972) that 'tropical
phytoplankton species show a wide adaptability to changes
in salinity'; but is at variance with the generalization

that 'their maximum photosynthetic rates occur at low salinities' assuming that photosynthetic rate reflects growth.

The hydrogen ion concentration of the medium affected the cell counts as well as the chlorophyll a synthesis though the effect on the latter was more pronounced. The flagellates grew best at pH 8. The acidic pH was found to be unsuitable for them. But at pH levels less than 8, $S.$ salina exhibited good growth. The marine species in culture are reported to tolerate 6.8 to 9.6 pH levels with optimum at pH 8 (Kain and Fogg, 1958b). Pigment changes are also reported to occur in relation to pH change. It is believed that pH affects the organism through the transport mechanisms of the membrane systems or it may interfere directly with the metabolic activities or indirectly by controlling the dissociation rates of the inorganic and organic compounds in the medium (cf. Soeder and Stengel, 1974).

It may be noticed that $S.$ salina has low salinity and low pH optima while the flagellates prefer higher salinity and higher pH. It may be remembered that in aquatic bodies fresh water influx that bring about reduction in salinity may lower pH. In the laboratory studies it

has been shown that the species concerned can adapt to
both these factors simultaneously.

The rate of carbon-14 uptake by the nanoplankters
was found to increase with the cell concentration. However,
the trend did not continue indefinitely. Beyond a culture
density of 6-10 x 10^5 cells/ml, the rate of photosynthesis
of the flagellates was reduced. However in S. salina,
cultures at densities exceeding 175 x 10^5 cells/ml showed
a decreasing trend. The probable reason for this inhibition
may be that the amount of light received by individual cells
was reduced due to crowding or the amount of carbondioxide
would have been limiting in high density cultures.

Light influences photosynthesis by its quality,
quantity and photoperiod. The algae used in the present
investigations were maintained in L:D cycle of 10:14 from
fluorescent lamps. However, all the three grew better at
constant illumination. It may be reasoned that longer light
period enabled light processes of photosynthesis to be
carried on further resulting in more synthesis of organic
carbon. It is generally held that blue-green algae do not
need a light-dark cycle for good growth. Reference may be
made to Fogg et al. (1973) who state that 'intermittent
illumination (16:8 L:D) does not give better yields than

continuous illumination and blue-green algae do not
appear to require a diurnal alternation of light and
dark periods'.

Response to photoperiod has been found to occur in
the green alga Dunaliella tertiolecta (Eppley and Coatsworth,
1966) and in the marine diatoms (Durbin, 1974; Holt and
Smayda, 1974). Foy et al. (1976) observed that the smaller
species of Oscillatoria namely O. redekii grew faster in
constant illumination though the larger species O. agardhii
exhibited better growth on the LtD cycle. They reason that,
in the smaller species where the surface area/volume ratio
is large the rate of excretion tend to be high in the dark.
So on constant illumination the rate of loss is minimised.
Probably the same reason may explain the better growth of
the present species studied when subject to constant
illumination.

The rate of photosynthesis in all the three cultures
increased with increasing light intensity. The increased
rate of photosynthesis with increasing light intensities
may be brought about either by an increased pigment content
or by enhancement of the photosynthetic rate itself
(Steemann Nielsen and Jorgensen, 1968a; Jorgensen, 1969).
However, all species invariably showed 100% photosynthesis
around 34 x 10^{15} quanta/cm^2/sec. with I$_K$ ranging from

$22-24 \times 10^{15}$ quanta/cm^2/sec. Light - photosynthesis relationship has been described early by Talling (1957), Steemann Nielsen (1962), Jorgensen (1964), McAllister et al. (1964) and Steemann Nielsen and Hillemoes (1971). They have recorded, differences in the light adaptation capacity (indicated by I_K) and light saturated photosynthesis, depending on the species under study and the pre-incubation light intensity. It is noticed that, in the present investigation, there is not much difference in the response of the three species concerned. It may be that all these organisms were grown under the same light condition. Jorgensen (1964) reports that Chlorella vulgaris and Cyclotella meneghiniana adapted to 35 klux and 21°C had nearly same I_K i.e. 12 klux for Cyclotella and 13 klux for Chlorella; but when grown at 3 klux and 21°C, this similarity in I_K was not obtained. This shows that both species factor and pre-incubation factor can affect the I_K not to exclude the influence of temperature. Maddux and Jones (1964) have also related the light adaptation capacity of microalgae to temperature and the chemical composition of the medium. With the available evidence it can be said that the I_K of the species tested has been influenced by the pre-incubation light intensity. Generally, the temperate species have low light saturation levels compared to tropical forms. Photosynthesis in

Phaeodactylum tricornutum is saturated at 12,000 lux
(Hayward, 1968c) while for Monochrysis lutheri it saturates
at 2000 lux (Droop, 1961c). In the present investigation
the saturating light intensities were quite high; may be
because these are tropical strains and acclimatized to
light of 20,000 lux. Qasim et al. (1972) have also reported
similar I_K values for certain tropical diatoms and
flagellates.

Within the temperature range 20-35°C the growth
rate of the three species did not differ significantly.
The growth was seriously affected at 40°C; but Synechocystis
salina continued to survive upto 45°C. However, the
temperature range for photosynthesis was found to be very
narrow. Maximum photosynthesis occurred at 30°C in the
flagellates while it was at 25°C for S. salina. The rate
of production was considerably reduced at all other
temperatures. The temperature range for survival was
however large for all species. Flagellates could survive
the low temperature storage for 3 months and S. salina for
6 months. Discrepancies in the temperature optima for
growth and photosynthesis have been reported early. It
may be that the enzymatic processes controlling the cell
division and photosynthesis are different (Innis and
Ingraham, 1978; Rhee and Gotham, 1981). Another aspect
is that the optimum temperature for growth is higher

compared to the temperate water strains, that usually prefer 15-22°C. According to Kain and Fogg (1958b) and Ukeles (1961) I. galbana does not survive above 27-30°C. But the present isolate of I. galbana grows well at 2-35°C with maximum photosynthetic activity at 30°C. In fact tropical strains are unable to grow at lower temperature. A tropical strain of Gymnodinium did not grow at 15°C, but at higher temperatures upto 37°C growth was normal. The same has been shown by certain tropical diatoms (Guillard and Ryther, 1962; Thomas, 1966). Thus the present investigation lends support to the view that different species and strains of the same species isolated from different geographical locations, may differ in their temperature responses. The temperature of acclimation has a significant role in this adaptation process. Adaptation may be brought about by many changes within the cell, like changing the pigment content or enzymes.

The thermal death point is also high for the tropical plankters. The concerned flagellates were alive only for a week when exposed to 40°C; S. salina survived at 40° though with highly reduced growth rate. However 45°C seemed to be lethal to the species. These observations are in keeping with the remark of Hall (1953) that 'active stages of 'protists' are usually killed as the temperature

approaches $45^{\circ}C'$. The thermal death may be caused by
an increase in the viscosity of the protoplasm,
denaturation of proteins or nutritional starvation
(Heilbrunn, 1952; Hutner et al., 1957).

The amount of nitrate and phosphate as indicated
by K_{μ} supporting the maximum growth rate of the flagellates
and Synechocystis salina were different. The K_{μ} indicates
the capacity of the species to adapt to low substrate
levels. A species with low K_{μ} and high growth rate can
dominate those with higher K_{μ} and low growth rate in mixed
cultures and natural populations. Comparing the two
flagellates, it is found that Chromulina freiburgensis
has a K_{μ} of 0.7 μg-at. N L^{-1} which is almost half of
that for Isochrysis galbana (C.s.). The μ_{max} of
I. galbana (C.s.) was not proportionately higher. It may
be assumed that in mixed cultures of these species
C. freiburgensis will dominate at low nitrate levels at
least during the initial phase of culture. Probably the
ratio may change as the culture ages due to the relatively
higher growth rate of I. galbana (C.s.).

The phosphate utilization capacity of the
flagellates does not differ markedly. However, there
can be marginal difference in the population density in

the mixed culture of these species at the initial
stages with a dominance of $\underline{C}$. <u>freiburgensis</u>.

$\underline{S}$. <u>salina</u> had a K_μ of 0.81 µg-at. N L^{-1} for a μ_{max}
of 0.983. This shows that this blue-green alga is better
equipped to outcompete the chrysomonads. The K_μ and μ_{max}
for phosphorus utilization of $\underline{S}$. <u>salina</u> was nearer to that
of $\underline{C}$. <u>freiburgensis</u> being 1.34 (K_μ) and 0.963 (μ_{max}) and
1.31 (K_μ) and 0.97 (μ_{max}) respectively. This would give
an almost equal chance for proliferation to the species.

It is also seen from the data that the substrate
concentration supporting maximum growth rate as per the
observation is quite high than that computed. In fact the
K_μ measures the ability of phytoplankters to utilize low
substrate concentrations. This justifies the low K_μ
values that has also been obtained for many other
phytoplankters (Thomas et al, 1968; Eppley et al, 1969).
It may be remembered that the Monod expression assumes
that 'there is only a single substrate or in the case of
multisubstrate reactions the concentration of all other
substrates are held constant'. (McCarthy, 1981). In these
experiments the nitrate and phosphate concentrations were
made to vary; but the extent of interaction of these
substrates at different concentrations to other substrates
and the algae itself was not known. Similarly the physical

factors also have a decisive role in controlling the growth rate. These factors may explain the difference in the observed and expected saturation concentrations.

At this instance it is worthwhile to note the statement of Pringsheim (1946) that 'most algae are not affected by minute changes in the composition of the medium, otherwise they could not live under natural conditions. Changes effected by algae themselves are often more decisive than the differences between various media'. To conclude it has to be remembered that metabolic activities of phytoplankters are controlled primarily by nutrients, light and temperature. The optimum amounts of these vary with the species. The nutrient sufficiency, optimum light, optimum temperature and other such factors have been, however, studied on the basis of one-species and one-factor experiments. The final result i.e. growth, is actually the combined effect of all these parameters: physical, chemical and biological. But the present investigation has revealed that factors independently can be manipulated in culture systems to modify the yield.

The growth rate obtained in the small volume laboratory cultures of the flagellates, Chromulina freiburgensis and Isochrysis ... galbana (C.e.) have been retained in mass cultures also. C. freiburgensis developed

into a more stable culture reaching a density of
6.7×10^5 cells/ml in five days time starting from
an inoculum of 9.4×10^3 cells/ml. I. galbana (C.s.)
also showed similar growth rate though the cultures were
not that stable as the former species. These results
as well as the field trials for acceptability indicate
that both these flagellates could be developed in mass
culture systems in tropical hatcheries and used
successfully as live-food in rearing the oyster larvae.

SUMMARY

The development of mass cultures of phytoplankton as live-food, forms an integral part of hatchery systems in mariculture. Among such photosynthetic microalgae nanoplankters with cell dimensions of 50-60μ and less are essential for providing the right type of food for early larval stages of bivalve molluscs. Hence the isolation and development of nanoplankters in axenic cultures in vitro conditions have been undertaken for further mass production.

The present study on nanoplankton is based on the isolation and development of unialgal cultures from the inshore waters of Cochin, characterization of their growth, assimilation products, eco-physiology and evaluation of nutritional quality. The work was carried out during the period 1980-1983. The nanoplankters were isolated and grown in the laboratory as batch cultures to study the increase in cell population, the photosynthetic pigments and physiological activity. The chemical composition of these organisms and their rate of excretion were also determined. The environmental factors - physical and chemical - that influence the growth of these cultures

were defined by conducting independent experiments.
Mass cultures of the isolated nanoplankters were raised
indoor and fed to the larvae of edible oyster to test
their suitability as live-food.

Taxonomic description of thirteen species of
nanoplankters in the Cochin estuarine and coastal regions
have been given. Of these two were isolated during the
period of this investigation. They are *Chromulina*
freiburgensis Doflein and *Isochrysis* *galbana* Parke
forma *nova* (referred as *I. galbana* (C.s.) and these are
new records for the Indian waters. The latter is
considered as a new tropical form differing from the widely
known temperate species.

The identification of *C. freiburgensis* and *I. galbana*
(C.s.) was made from their morphological and anatomical
features as obtained with light microscope and confirmed
by Scanning Electron Microscope and analysis of
photosynthetic pigments using thin-layer chromatography.

The various techniques and media employed in
isolation and development of axenic cultures of
nanoplankton and analysis of chlorophylls, chemical
composition, rate of excretion as well as the methods
employed to determine the role of various environmental

factors on growth kinetics of these nanoplankters have
been discussed under methodology.

The kinetics of growth of the present isolates
C. freiburgensis and _I. galbana_ (C.s.) were studied in
detail along with two other nanoplankters _Synechocystis_
salina and _Tetraselmis gracilis_. The growth of these
species was defined by measuring the growth rate (as
evidenced by the cell counts), the amount of chlorophyll
and carotenoid pigments and photosynthetic production
(given by the measurement of oxygen exchange and carbon-14
uptake) for a period of thirty days.

The various growth measurements of the above
species showed that they exhibited peak growth and
activity from two to six days of inoculation and then the
growth rate declined gradually. Within 12 to 16 days all
cultures attained stationary phase. As the cultures
became one month old, their growth and activity reduced
drastically with _I. galbana_ (C.s.) and _S. salina_ showing
senescence while _C. freiburgensis_ was more stable.

The amount of protein, carbohydrate and lipid of
C. freiburgensis, _I. galbana_ (C.s.), _S. salina_ and
I. gracilis was estimated at different phases of growth
in culture. The relative proportion of the protein was

high in all the species during the exponential phase
of growth—being 53.4% of the dry weight in C. freiburgensis
and 51.3% in I. galbana (C.s.). In S. salina and I. gracilis
it was 41% and 54% respectively. The relative amount of
protein decreased in the ageing cultures while that of
carbohydrates increased. Lipids were comparatively less.

The amount of extracellular products released by
the above nanoplankters during their phases of growth in
culture was estimated by the carbon-14 method using a
Liquid Scintillation Counter. It was observed that the
rate of excretion increased with the age of cultures.
During exponential phase, cultures of C. freiburgensis
released 3% of the total carbon fixed to the medium
while this fraction increased to 12.57% in stationary
phase cultures. In the cultures of I. galbana (C.s.)
there was an increase from 3.9% to 45.2% and in S. salina
the range was from 38.91% to 64.5% . In I. gracilis the
amount of excretion varied from 2.5% to 22.36% from the
5th to 16th day.

Salinity tolerance studies conducted with media
of 14%, 24% and 34% salinity showed that the flagellates,
C. freiburgensis and I. galbana (C.s.) grew best at 34%
while S. salina showed better growth at 14%.

The optimum pH for the species varied with
C. freiburgensis and I. galbana (C.s.) growing best
at a pH of 8 while S. salina proliferated better at
a pH of 7 to 7.5.

The rate of carbon fixation by the nanoplankters
was found to be affected by the density of culture. The
flagellates had a lower optimal saturation density i.e.
6-10 x 10^5 cells/ml compared to cultures of Synechocystis
salina for which the rate of ^{14}C uptake increased with
cell concentration upto 175 x 10^5 cells/ml after which
there was decline. This difference could be related to
the relative volume of the two species.

Cultures maintained on a light-dark cycle of 10:14
hours exhibited higher production rate when exposed to
constant illumination. Similarly the light intensity
supporting the maximum production of these cultures also
did not differ significantly. The light adaptation of the
species as defined by the constant I_K was found to be
22-24 x 10^{15} quanta x cm^{-2} x sec^{-1} for C. freiburgensis,
I. galbana (C.s.) and S. salina.

The influence of temperature on the growth and
activity of the nanoplankters was studied by growing them
in thermostatically controlled water baths. The

flagellates, *C. freiburgensis* and *I. galbana* (C.s.) exhibited maximum productivity at 30°C while *S. salina* was more active at 25°C. But the optimum range of temperature for growth was wider (20-35°C) for all the three species.

The thermal death point was 40°C for *C. freiburgensis* and *I. galbana* (C.s.) and 45°C for *S. salina*. *S. salina* survived longer periods (six months) of exposure to low temperature (ca. 5°C) than the flagellates (3 months).

The rate of growth of *C. freiburgensis*, *I. galbana* (C.s.) and *S. salina* was studied with respect to varying concentrations of nitrate and phosphate in the culture medium and the nutrient requirement of these species were defined in terms of half-saturation constants for growth. Among the chrysomonads, *C. freiburgensis* was found to have a lower half-saturation constant and higher growth compared to *I. galbana* (C.s.) giving the former better chances for nitrate utilization. *S. salina* was better equipped to compete with the flagellates at low nitrate levels.

The phosphate utilization capacity of *C. freiburgensis* was higher than that of *I. galbana* (C.s.) while *S. salina* was more or less at par with *C. freiburgensis*.

From the nutrient kinetic studies of the
flagellates it was evident that *C. freiburgensis* has
better chances of survival than *I. galbana* (C.s.)
in culture.

Mass cultures of the present isolates *C. freiburgensis*
and *I. galbana* (C.s.) were raised in 20 litre carbuoys. The
flagellates grew and multiplied rapidly. In these mass
cultures also *C. freiburgensis* showed more stability.

The acceptability of the two species *C. freiburgensis*
Doflein and *I. galbana* (C.s.) to the molluscan larvae was
tested. Newly hatched larvae of *Crassostrea madrasensis*
fed separately with *C. freiburgensis* and *I. galbana* (C.s.)
settled as spat in 17 and 19 days respectively. The
results of the experiment showed that these flagellates
were not only acceptable to the larvae but also induced
higher growth rate and larval survival compared to control
feed i.e. *I. galbana* Parke, the temperate water strain
that is widely in use in hatcheries. *C. freiburgensis*
seems to be the more potent species for development as
live-food in oyster hatcheries.

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