J. Mar. biol. Ass. U.K. (1961) 41, 287–304
Printed in Great Britain

STUDIES ON *ECTOCARPUS* IN CULTURE

II. GROWTH AND NUTRITION OF A BACTERIA-FREE CULTURE

By G. T. BOALCH*

Department of Botany University College, London

(Text-figs. 1–7)

Part I of this series (Boalch, 1961) described how bacteria-free cultures of two species of *Ectocarpus* were obtained. Here experiments on the growth and nutrition of a bacteria-free culture of *E. confervoides* (Kütz.) Batt. are described.

The usual methods of estimating growth in laboratory cultures of algae are: (i) by counting the number of cells per unit volume, (ii) from optical density—of the culture or a pigment extract, (iii) from total dry weight, (iv) from total cell volume calculated from cell measurements or packed cell volume.

Cell counting is most suitable for unicellular algae, but it has been used for early stages of filamentous algae (Boney, 1960), and also for a blue-green alga after the filaments have been broken into short lengths (Fogg, 1944). Measurement of optical density is again most suitable for unicellular algae. The optical density of extracted pigments has been used, but this assumes that the pigment content per cell remains constant throughout the growth cycle. Increases in dry weight may be due to thickening of cell walls or accumulation of storage products and may occur without any increase in cell numbers. The total cell volume method is again best applied to unicellular algae, when volume can be calculated from cell measurements and cell numbers. Whenever possible more than one method of estimating growth should be used. Pearsall & Loose (1937) in their study of *Chlorella* estimated growth by cell counts, dry weight and cell volume. Cell counts and optical density have been used by Spencer (1954), Droop (1957*a*), and Kain & Fogg (1958*a*, *b*) to study the growth of marine algae. A full discussion of the growth of a unicellular alga in pure culture has been given by Myers (1953).

* At present International Paints Research Fellow at the Plymouth Laboratory.

METHODS

Estimation of growth

Estimation of the growth of *Ectocarpus* by cell-counting was not possible, and as the filaments were not easily fragmented the filament length method of Fogg (1944) could not be used. Determination of the optical density of the culture was also impracticable. Measurement of extracted pigments was attempted, but it was found that extraction was incomplete and the percentage of the total pigment that was extracted depended upon the age of the culture. At first dry weights were not reliable. If the algal material was not washed large amounts of salt were left in the material and rendered it hygroscopic. When the algal material was washed with distilled water care had to be taken to prevent too much leaching. The method finally adopted and found quite reliable was as follows. Using a vacuum pump algal material from one culture was filtered off into a sintered glass crucible of porosity 40–90 μ. Still using the vacuum pump the material in the crucible was rapidly washed with distilled water and the crucible quickly transferred to the oven at 100° C. After 24 h in the oven the crucibles were allowed to cool in a desiccator for 1 h and then weighed as rapidly as possible.

As a culture was used up every time a sample was taken, the number of cultures needed to study the growth of *Ectocarpus* was large. It was found impracticable to take more than two replicate samples every time a dry-weight determination was required. These duplicates sometimes showed considerable divergences, especially in the early stages of growth when the amounts of algal material being weighed were small. With only two samples it is difficult to apply any statistical analysis to the results and therefore no such application has been attempted. The usual method adopted when studying the effect of a series of conditions on the growth of *Ectocarpus* was to take five samples, each in duplicate, during the growth cycle under each of the conditions investigated. The period of growth usually extended over 6–8 weeks.

Inoculation of cultures

The problem of introducing equal amounts of a filamentous alga into each of a large number of culture vessels is a difficult one to overcome. When this investigation was begun it was thought that it would be possible to use a suspension of zoospores as inoculum and carry out inoculations in the way normally used for unicellular algae. However, the transfer of zoospores of *Ectocarpus* proved difficult (Boalch, 1961) and a method had to be devised in which uniform inoculation could be made with the filamentous algal material itself. When large numbers of tubes were to be inoculated stock material was grown up in 1 l. of medium in a 'penicillin' flask. Material was then transferred aseptically to a boiling tube of sterile sea water and cut up into uniform pieces with a sterile Borradaile needle and these pieces used to inoculate the experimental tubes. In addition to the experimental tubes a number of test-tubes of sea water were inoculated in a similar way and the contents of these tubes then filtered off for estimation of the initial dry weight. An inoculating chamber, previously sterilized with an ultraviolet lamp, was used for all subculturing.

Media

All culture media were enriched with potassium nitrate and potassium phosphate at the levels given for medium A in the previous paper in this series (Boalch, 1961). Iron and manganese, unless added in trace element mixtures, were also added at the levels used for medium A. Full details of the chemical composition of all media are given in Table 1.

TABLE 1. COMPOSITION OF CULTURE MEDIA

Unless otherwise stated all units in the table are weights in mg. Medium D_1 is medium S20 of Droop 1955 and medium F is the 12S50 of Droop, 1958 but with some of the organic additions omitted.

Medium no.	A	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	C_1	C_2	D_1	E_1	E_2	E_3	F_1	F_2	G_1
Aged natural sea water (l.)	1	1	1	1	1	1	1	1	1	1	—	—	—	—	—	—	—	—	—
Glass-distilled water (l.)	—	—	—	—	—	—	—	—	—	—	1	1	1	1	1	1	1	1	1
KNO_3	202	202	202	202	202	202	202	202	202	202	202	202	202	202	202	202	202	202	202
K_2HPO_4	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8	34·8
$FeCl.6H_2O$	2·7	2·7	2·7	2·7	2·7	2·7	2·7	—	—	—	—	—	—	—	—	—	—	—	—
$MnCl.4H_2O$	0·2	0·2	0·2	0·2	0·2	0·2	0·2	—	0·2	—	—	0·2	—	0·2	—	—	—	—	—
NaCl	—	—	—	—	—	—	—	—	—	—	23,477	23,477	15,000	15,000	30,000	15,000	15,000	30,000	24,500
$MgCl_2.6H_2O$	—	—	—	—	—	—	—	—	—	—	10,634	10,634	2,500	2,500	5,000	2,500	2,500	5,000	9,800
$Na_2SO_4.10H_2O$	—	—	—	—	—	—	—	—	—	—	8,884	—	—	—	—	—	—	—	7,300
$CaCl_2$	—	—	—	—	—	—	—	—	—	—	1,102	—	—	—	—	—	—	—	400
$CaSO_4.2H_2O$	—	—	—	—	—	—	—	—	—	—	—	—	500	500	1,000	1,000	500	1,000	—
K_2SO_4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	850
Na_2 E.D.T.A.	—	—	—	—	—	—	—	—	—	20	20	—	20	—	20	40	50	50	20
$NaHCO_3$	—	—	—	—	—	—	—	—	—	—	192	—	—	—	—	—	—	—	—
KCl	—	—	—	—	—	—	—	—	—	—	664	—	375	375	750	750	375	750	—
KBr	—	—	—	—	—	—	—	—	—	—	96	—	15	15	15	30	32·5	32·5	32·5
KI	—	—	—	—	—	—	—	—	0·06	—	—	0·06	—	0·06	—	—	0·025	0·025	0·025
NaF	—	—	—	—	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—
H_3BO_3	—	—	—	—	—	—	—	—	—	26	—	—	—	—	—	—	—	—	—
$FeSO_4.7H_2O$	—	—	—	—	—	—	—	—	—	0·7	1·28	—	0·7	—	0·7	1·4	2·5	2·5	0·7
$FeC_6H_5O_7$	—	—	—	—	—	—	—	—	0·54	—	—	0·54	—	0·54	—	—	—	—	—
$AlCl_3.6H_2O$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·15	0·15	0·15
$Al(SO_4)_3$	—	—	—	—	—	—	—	—	3·0	—	0·3	3·0	0·25	3·0	0·25	5·0	—	—	—
As_2O_3	—	—	—	—	—	—	—	—	0·03	—	—	0·03	—	0·03	—	—	—	—	—
$BaCl_2.2H_2O$	—	—	—	—	—	—	—	—	0·09	—	—	0·09	—	0·09	—	—	—	—	—
$CoSO_4.9H_2O$	—	—	—	—	—	—	—	—	—	0·03	0·03	—	0·03	—	0·03	0·06	0·002	0·002	0·03
$CuSO_4.5H_2O$	—	—	—	—	—	—	—	—	0·04	0·005	0·014	0·04	0·005	0·04	0·005	0·01	0·019	0·019	0·005
$LiCl.H_2O$	—	—	—	—	—	—	—	—	1·0	—	0·1	1·0	0·05	1·0	0·05	0·1	0·05	0·05	0·05
$MnSO_4.H_2O$	—	—	—	—	—	—	—	—	—	2·0	2·24	—	2·0	—	2·0	4·0	0·155	0·155	2·0
$NaMoO_4.2H_2O$	—	—	—	—	—	—	—	—	—	0·5	0·6	—	0·5	—	0·5	1·0	0·001	0·001	0·5
$NaSiO_3.9H_2O$	—	—	—	—	—	—	—	—	10	—	—	10	—	10	—	20	—	—	—
RbCl	—	—	—	—	—	—	—	—	0·28	—	0·1	0·28	0·05	0·30	0·05	0·1	0·1	0·1	0·1
$SrCl_2.6H_2O$	—	—	—	—	—	—	—	—	—	—	40	—	5·0	5·0	5·0	10	6·5	6·5	6·5
$ZnSO_4.7H_2O$	—	—	—	—	—	—	—	—	0·01	10	8·9	0·01	10	0·01	10	20	0·022	0·022	10
Soil extract	—	20 ml	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
'Oxoid' liver extract	—	—	100	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—	—
'Oxoid' yeast extract	—	—	—	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—
'Difco' Bacto peptone	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
'Difco' Bacto tryptone	—	—	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—	—
Vitamin B_{12} (μg)	—	—	—	0·1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Agar (%)	—	—	—	—	1·5	—	—	—	—	—	—	—	—	—	—	—	—	—	—

EXPERIMENTS

GROWTH AND VIABILITY OF CULTURES

Growth curves from two experiments in which cultures were grown in medium A in unshaken boiling tubes are illustrated in Fig. 1 A, B. Duplicate samples were taken for each reading and the limits of the duplicates are indicated on the dry weight graph, but only the means are plotted on the log. graph. The lag phase is of 5–7 days duration and although during this period there is no increase in dry weight, there is considerable activity, for during this period numerous plurilocular sporangia are formed. The zoospores

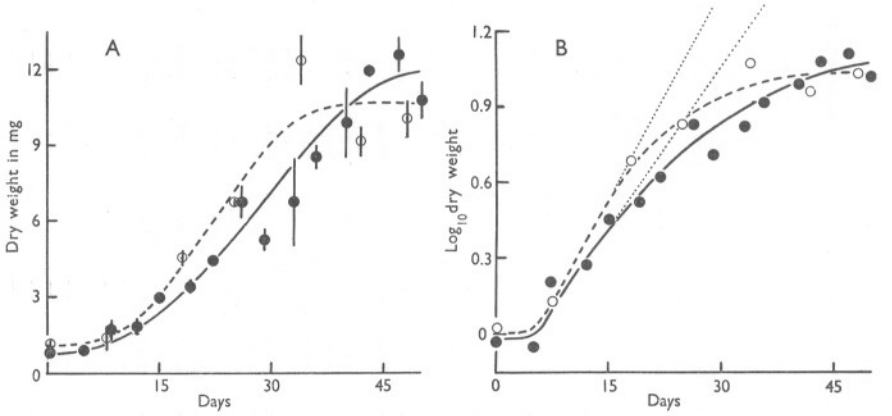

Fig. 1. Growth curves (medium A). (A) Growth of bacteria-free cultures in two experiments (○—○ and ●—●). (B) Logarithmic growth curves for the same two experiments (dotted lines are extrapolations of the early part of logarithmic growth from which k was calculated.

formed in these sporangia either are released into the medium and settle on the walls of the culture vessel and germinate, or more usually they germinate *in situ*. In some experiments a slight decrease in dry weight has been noted towards the end of the lag phase. This is probably due to the zoospores having recently been released and thus incompletely sampled. The second phase of growth is not truly exponential as with most micro-organisms. Instead of the slope of the graph being constant over a considerable period it falls off gradually. Tentative lines have been dotted in for the slope of the early part of the logarithmic curves to allow the relative growth constant k to be calculated. During the logarithmic phase growth is expressed by the following equation

$$n_t = n_0 e^{kt}.$$

When n_t is the cell number (or some other measure of the algal material in the culture) at the time 0 and n_t is the cell number at time t, k is the relative

growth constant and e the base of natural logarithms. This equation may also be expressed as

$$\log n_t = \log n_0 + kt.$$

The relative growth constant k is usually expressed as $\log_{10}$ units/day. The values of k for the two experiments are 0·04 and 0·05 giving an average value for k of 0·045. This is equivalent to a doubling time of 6·5 days, i.e. during the early stages of rapid growth *E. confervoides* doubles its dry weight every 6–7 days.

Viability of cultures

Cultures over 1 year old proved to be still viable when subcultured. *Ectocarpus* inoculated into medium A and kept in the dark did not grow but was still viable after 150 days. Cultures on medium A solidified with agar showed considerable growth in the light and were still viable after 100 days, but soon after this the cultures dried up and were dead by 130 days. Similar cultures in the dark did not grow but remained viable for at least 100 days.

INORGANIC NUTRITION

Nitrate requirements

A number of experiments was carried out using medium A in which the amount of added nitrate was varied. Levels ranging from 0·1 to 2·0 mM (10–202 mg KNO_3/l.) were used and a typical example of the results is given in Fig. 2A. Both the rate of growth and the final yield increased as nitrate was increased up to a level of 0·5 mM. However, when the nitrate was increased to 1·0 mM, or more, the rate of growth was rather less for the first 35 days but after about 50 days the final yield was the same. A further experiment was carried out in which the potassium nitrate was replaced by similar levels of sodium nitrate. The results were similar to those for potassium nitrate and it therefore seems unlikely that the reduced growth rate was due to an excess of potassium ions.

Phosphate requirements

Similar experiments were carried out with concentrations of potassium phosphate ranging from 0·025 to 0·2 mM (4·4–34·8 mg K_2HPO_4/l.) (Fig. 2B). The growth rate and final yield were increased by additions of up to 0·1 mM. phosphate but increases above this show a fall in growth rate for the first 35 days and after 50 days the final yields were less. The pattern was thus not quite the same as that for nitrate. Although the phosphate was autoclaved separately and added to the culture medium just prior to inoculation there was some precipitation at levels of 0·1 mM and above and this may have been responsible for the inhibition at these levels. This precipitate disappeared during growth but it was possible that some other essential elements were

brought out of solution by this precipitation and did not redissolve with the phosphate.

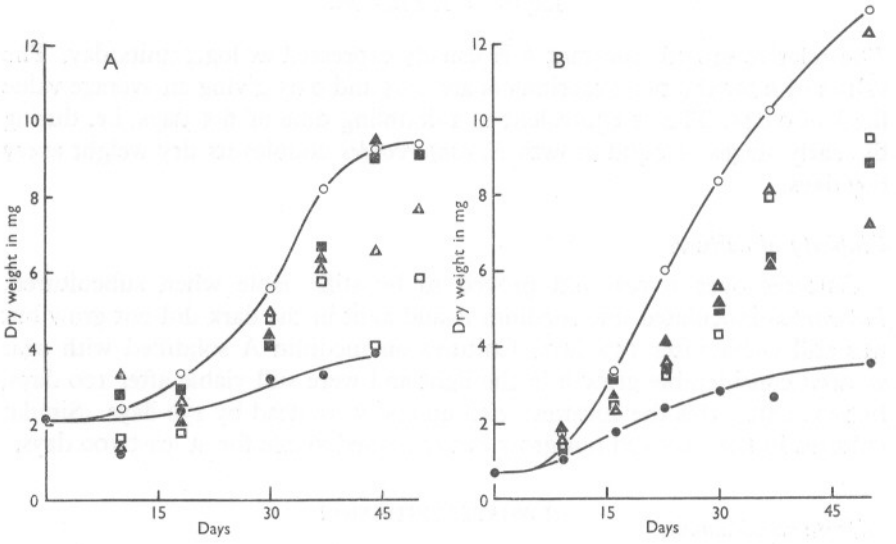

Fig. 2. Nitrate and phosphate. (A) Effects on growth of additions of potassium nitrate (KNO_3): ●, no nitrate; □, 0·1 mM nitrate; △, 0·25 mM; ○, 0·5 mM; ▲, 1·0 mM; ■, 2·0 mM. (B) Effect on growth of additions of potassium phosphate (K_2HPO_4): ●, no phosphate; □, 0·25 mM phosphate; △, 0·05 mM; ○, 0·1 mM; ▲, 0·15 mM; ■, 0·2 mM.

Iron requirements

The additions of iron, added as a freshly made solution of ferric chloride at concentrations of from 0·01 to 0·04 mM (2·7–10·8 mg $FeCl_3.6H_2O$/l.) had no effect upon the rate of growth or the final yield. The growth with these additions of iron was the same as in the control to which no iron was added. Of course at the pH of these experiments most of the inorganic iron would be in an insoluble form.

Manganese requirements

Manganese was added as a freshly prepared solution of manganese chloride at levels of 0·001–0·004 mM (0·2–0·8 mg $MnCl_2.4H_2O$/l.) and produced increased growth and final yield at all levels within this range (Fig. 3A).

ORGANIC NUTRITION

Additions of complex organic mixtures such as yeast extract and liver extract at 0·1 g/l. and soil extract at 20 ml./l. to the basic medium A gave increases in final dry weight of from 10 to 25% but peptone at 0·1 g/l. considerably reduced the final yield. Growth in medium A enriched with vita-

min B_{12} (0·1 μg/l.) or soil extract (20 ml./l.) was slightly better than in the unenriched medium (Fig. 3B). It has been shown that some algae will grow in the dark and utilize organic compounds as carbon sources. The following organic carbon sources were added to medium A at levels of 1·0 g/l. prior to autoclaving: sodium glycerophosphate, hexose 1·6 diphosphate (calcium salt), maltose, mannitol, D-mannose, lactose, dextrose, D-leavulose and sodium acetate. After inoculation some cultures of each media were placed

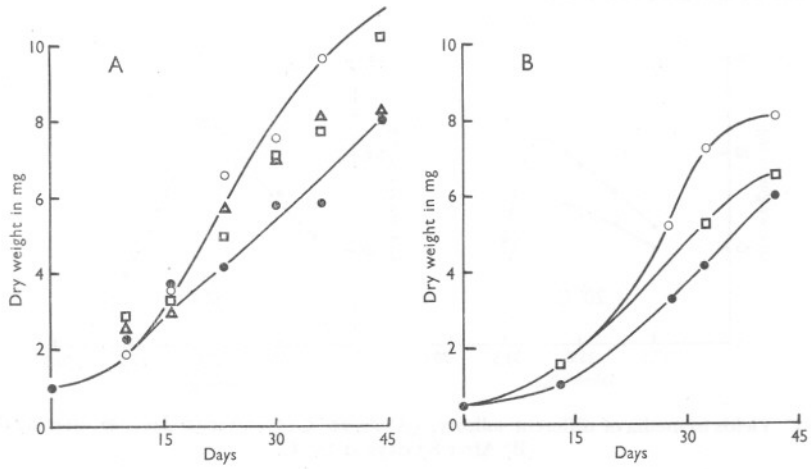

Fig. 3. Manganese and organic additions. (A) Effect on growth of additions of manganese chloride (MnCl): ●, no manganese; □, 0·001 mM manganese; △, 0·002 mM; ○, 0·004 mM. (B) Growth with additions of organic compounds: ●, normal medium A; □, medium A with vitamin B_{12} at 0·1 μg/l.; ○, medium A with soil extract at 20 ml./l.

in a dark cupboard and others were kept in continuous illumination. All cultures were at room temperature. The cultures in the light grew well but none of those in the dark showed any growth. These organic compounds clearly had no inhibitory effect in the light but they could not be utilized for chemotrophic growth.

PHYSICAL CONDITIONS

Salinity

In order to investigate the growth of *Ectocarpus* in media of different salinities some natural sea water, of approximate salinity 35 ‰, was concentrated to half its original volume by vacuum distillation. By diluting with distilled water this concentrate was used to make up salinities of 2, $1\frac{1}{2}$, $1\frac{1}{4}$, 1, $\frac{3}{4}$, $\frac{1}{2}$, and $\frac{1}{4}$ that of natural sea water. These, together with some distilled water, to give a salinity of zero, were enriched at the levels normally used for medium A. Cultures were grown in these media at 20 °C and samples taken after 50 and 67 days (Fig. 4A). There was no growth at salinities of 2,

$\frac{1}{4}$, and 0 times that of natural sea water. In a further experiment salinities of $1\frac{3}{4}$, $1\frac{1}{2}$, $1\frac{1}{3}$, 1, $\frac{3}{4}$, $\frac{1}{2}$ and $\frac{1}{4}$ those of natural sea water were used. Larger culture vessels were used for this experiment and the cultures were shaken in apparatus 3 (Boalch, 1961) at 15° C for 87 days (Fig. 4B). Although the results of the two experiments cannot strictly be compared it should be noted that at 20° C the maximum dry weight was obtained at a salinity higher than that of natural sea water, whereas at 15° C the maximum yield was at a salinity close to that of natural sea water.

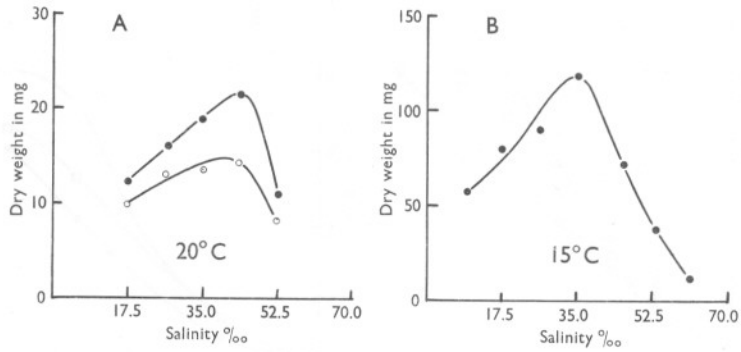

Fig. 4. Yields in media of different salinity. (A) At 20° C: ○, after 50 days; ●, after 67 days. (B) After 87 days at 15° C.

Optimum pH

In these experiments the buffer 'tris', (hydroxymethyl)aminomethane, was added to medium A at 500 mg/l. Cultures were grown at 20 °C at initial pH's of 9·0, 8·5, 8·0, 7·5, and 7·0. The results are illustrated graphically in Fig. 5 where both the initial and final pH are given. The optimum initial pH was 8·0 and during the course of the experiment the pH of these cultures rose to 8·8.

Light intensity

To study the effect of a range of light intensities on the growth of *Ectocarpus*, cultures were grown in 100 ml. conical flasks in culture apparatus 3 at 20° C. The light intensity was varied by fixing layers of black nylon net under the culture vessels, which were shaken at 88 oscillations per minute (Fogg, Smith & Miller, 1959). The investigation was carried out in two stages with a different maximum light intensity in each experiment. Unfortunately, due to differences in the condition of the inoculum, the two parts of the experiment (Fig. 6C, D) cannot be compared directly. However, in each experiment the relative growth constants at the three light intensities were similar and there were only small differences in the final yield, but there were marked differences

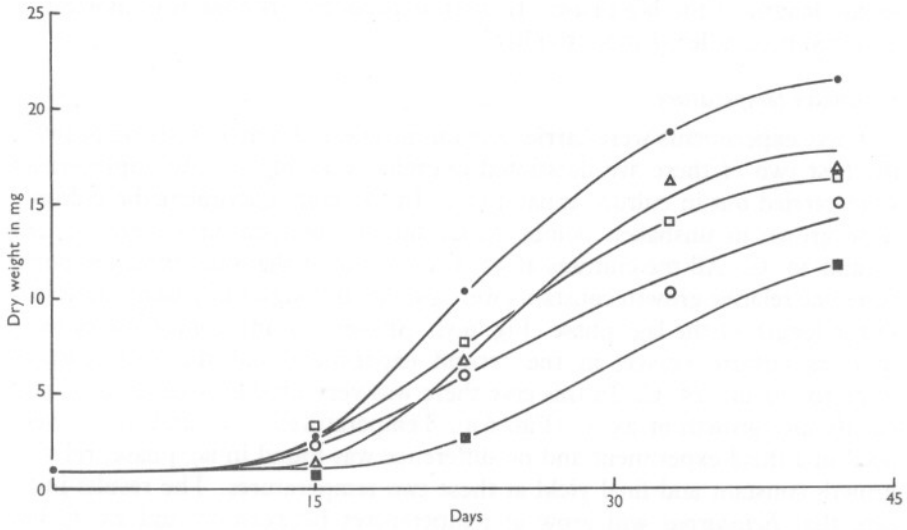

Fig. 5. Growth in media of different pH. Both initial and final pH values are given: ■, 7·0–8·0; □, 7·5–8·1; ●, 8·0–8·8; ○, 8·5–9·1; △, 9·0–9·4.

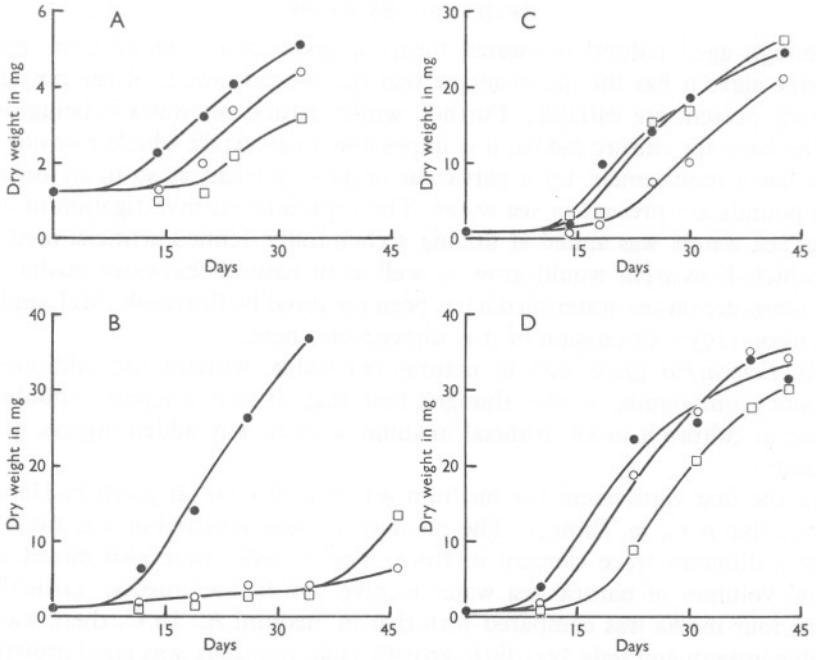

Fig. 6. Light and temperature; (A) Growth at: ●, 20° C; ○, 25° C; □, 10° C in boiling tube cultures. (B) Growth at: ●, 20° C; ○, 25° C; □, 10° C in shaken conical flasks. (C) growth at: □, 1500 ft. candles; ●, 600 ft. candles; ○, 250 ft. candles in shaken conical flasks. (D) Growth at: ●, 700 ft. candles; ○, 250 ft. candles; □, 125 ft. candles in shaken conical flasks.

in the length of the lag phase. In both experiments the lag was shortest at 600–700 ft. candles (6500–7500 lux.).

Optimum temperature

Three experiments were carried out on the effect of temperature on growth, the first two of these are illustrated graphically in Fig. 6. All experiments were carried out in culture apparatus 3. In the first experiment the cultures were grown in unshaken boiling tubes and the temperatures were 10, 20, 25 and 30° C. All the cultures at 30° C died, but at the other three temperatures the relative growth constants were similar although there were variations in the length of the lag phase (Fig. 6A). Shaken 100 ml. conical flasks were used as culture vessels in the second experiment and the temperatures were 10, 20 and 25° C. In this case there was very little growth at 10° C and hardly any growth at 25° C (Fig. 6B). Temperatures of 15 and 20° C were used in a third experiment and no difference was found in lag phase, relative growth constant and final yield at these two temperatures. The results indicate that *Ectocarpus* will grow at temperatures between 10 and 25° C but will not grow at 30° C. The optimum temperature for the growth of *Ectocarpus* under bacteria-free conditions is between 15 and 20° C.

ARTIFICIAL SEA WATER

Although aged natural sea-water forms a good basic medium for many marine algae it has the disadvantage that the concentrations of the nutrients already present are variable. Further, whilst natural sea water is being used as the basis for culture media, it is impossible to ascertain whether or not the alga has a requirement for a particular organic nutrient as so many organic compounds are present in sea water. The experimental investigation of artificial sea waters was aimed at finding a chemically defined artificial medium in which *Ectocarpus* would grow as well as in natural sea-water media. As the literature on sea-water media has been reviewed by Provasoli, McLaughlin & Droop (1957) discussion of it is unnecessary here.

As *Ectocarpus* grew well in natural sea water, without the addition of organic compounds, it was thought best that at first attempts should be made to culture it in an artificial medium without any added organic compounds.

In the first experiment the medium was that of Chu (as given in Harvey, 1955), this is C_2 in Table 1. The medium C_1 was similar but was made up with a different trace element mixture. These media were also mixed with equal volumes of natural sea water to give two further media. Growth in these four media was compared with that in medium A. In C_1 there was no visible growth and only very little growth in C_2 but there was good growth in all media containing natural sea water. The next experiment used media based on those of Droop (1955). In addition medium A was enriched with two

different trace element mixtures (A_8, A_9) to test if these mixtures were in any way inhibitory. The six media used were A, A_8, A_9, C_2, D_1 and E_1. Again there was good growth in media containing natural sea water and little growth in medium C_2. There was some growth in media D_1 and E_1 but not as good as that in natural sea water and on subculturing into the same medium again the culture died. These media of Droop have a salinity of about 16 ‰ and it has been shown previously in this paper that the optimum salinity for *Ectocarpus* at 20° C is above that of the natural sea water, i.e. above 35 ‰. In the next experiment the salinity of some media was doubled (E_2, E_3) and growth compared with that in media A and D_1. In all the artificial media there was a long lag phase, no growth being visible until after 30 days.

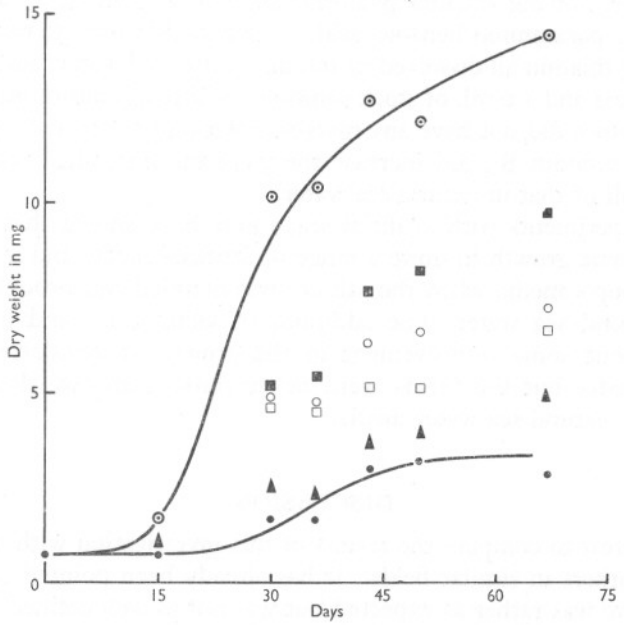

Fig. 7. Artificial media. ⊙, Natural sea-water medium A, ●, D_1; ■, E_2; ▲, F_1; □, F_2; ○, F_3. For details of media see Table 1.

However, there was growth in the two media with the higher salinity but the final yield was only about half of that in medium A. These media were then tried with additions of organic compounds. Media D_1 and E_2 were made up and each was divided into three portions. One portion was used direct, one was enriched with liver extract at 0·1 g/l. and the third portion was enriched with vitamin B_{12} at 0·1 μg/l. and the final yields in the six media compared. Once again the higher salinity media showed much better growth than those at the lower salinity. At the low salinity the addition of the nutrients reduced

the yield, but at the higher salinity there was no marked difference between the three media. The addition of further trace elements to medium E_2 enriched with liver extract had no effect on growth. A further medium of Droop (1958) was then investigated. This was prepared as described by Droop, (F_1), and also with the salinity doubled (F_2, F_3). Growth in these media was compared with growth in A, D_1 and E_2, the results being illustrated in Fig. 7. Once again growth in the low-salinity media, D_1 and F_1, was poor but even when the salinities were doubled the yields were at the maximum only two-thirds of that in the natural sea-water medium. In one further experiment vitamins were added to F_2, in one case vitamin B_{12} was added at 0·1 μg./l. and a complex vitamin mixture was added to some more medium at two different levels. This mixture consisted of 200 mg chlorine, 200 mg nicotinamide, 200 mg calcium pantothenate, 0·4 mg folic acid, 400 mg inositol, 50 mg para-amino-benzoic acid, 50 mg pyridosine, 50 mg riboflavin and 100 mg thiamin all dissolved in 100 ml. of distilled water, and was added at levels of 0·4 and 1·0 ml. of stock solution per litre of culture medium. The vitamin mixture did not have any marked effect on growth and although the addition of vitamin B_{12} did increase the yield the final yield was still only just over half of that in natural sea water.

These experiments with artificial sea waters have shown that *Ectocarpus* will show some growth in quite a range of artificial media and it grew quite well in Droop's media when the salinity was doubled and brought nearer to that of natural sea water. The additions of vitamin B_{12} and liver extract brought about some improvement in the growth of *Ectocarpus* in these artificial media but the yields were never more than two-thirds of that obtained in natural sea water media.

DISCUSSION

It is of interest to compare the results of this investigation with the findings of other workers in similar fields. It has already been pointed out that the growth curve was rather as expected but was not as well defined as for most micro-organisms. This is probably in part due to the nature of the alga itself. The growth of *Ectocarpus* is not simply a matter of each cell dividing into two. In the erect system of *Ectocarpus* cell division is limited to certain meristematic zones which are usually near the base of branches. If the number of meristematic zones remained constant, growth would be linear and not logarithmic. However, the number of meristematic zones increases as new branches are formed and new sporelings develop. The growth of an *Ectocarpus* culture is thus intermediate between linear and logarithmic growth. Further as the meristematic zones become hidden at the base of the tangled filaments the amount of light and nutrients they receive is reduced and the rate of cell division is probably reduced. In shaken cultures the algal material

tends to roll into a ball so that the effect of shaking is not as advantageous as might be expected.

The best growth of *Ectocarpus* was brought about by the addition of 0·5 mM potassium nitrate to natural sea water. In 20 ml. of culture medium this gave an increase, over the control, of 5 mg dry weight. This is an increase of 250 mg dry weight/l. for the addition of 7 mg of nitrogen (0·5 mM). Assuming complete utilization of the nitrogen, this would correspond to a nitrogen content of 2·8% on a total dry-weight basis. An estimation of the nitrogen content of a culture of *Ectocarpus* grown in a medium enriched with 2·0 mM nitrate (Fogg & Boalch, 1958) gave 7 mg of cellular nitrogen in a culture of 1 l. The dry weight of this culture was approximately 300 mg and the nitrogen content of the algal material was thus 2·3% of the dry weight. When 0·25 mM nitrate was added the calculated nitrogen content of the algal material would be 2·3% of the dry weight. At 0·1 mM the nitrogen content could not have been greater than 1·5% and it must be assumed that at this level the culture was growing under conditions of acute nitrogen deficiency. Black (1948) gives the total nitrogen content of *Laminaria* spp. as 2·5–0·7% of the dry weight depending on the time of year and conditions of growth. Milner (1953) found the nitrogen content of *Macrocystis* as 6·6% of the ash-free dry weight, or 4·2% of the total dry weight. The minimum nitrate needed for maximum crop of *Asterionella japonica* and *Isochrysis galbana* (Kain & Fogg, 1959*a*, *b*) was of the order of 1·0 mM but neither of these planktonic algae was bacteria-free.

Maximum growth of *Ectocarpus* was obtained with 0·1 mM phosphate and although the cultures had not reached the stationary phase after 50 days by this time addition of phosphate had increased the dry weight by 500 mg/l. The yields obtained with the various levels of phosphate give phosphorus figures of 0·62% of the dry weight at 0·1 mM phosphate, 0·45% at 0·05 and 0·32% at 0·025 mM. These figures all assume complete utilization of the available phosphorus, and as already noted there was some precipitation at the highest level of phosphate, but as no estimates of the phosphorus content of the algal material were made these values cannot be checked. Kain & Fogg (1958*b*) found the minimum need of phosphate for *Isochrysis* was of the order of 0·03 mM.

These experiments show that the requirements for the best growth of *Ectocarpus* were 0·5 mM nitrogen and 0·1 mM phosphorus giving a ratio for N/P of 5/1 and not the 20/1 often quoted for marine algae. Cooper (1937) found a ratio of 16·3/1 in marine phytoplankton and 20/1 in the sea, while Redfield (1934) gave a ratio of 18/1 in plankton.

It was surprising that the addition of ferric chloride to natural sea water had no effect on growth. Harvey (1937) showed that the iron requirements of a marine diatom were more than was directly available to it in a soluble form in the sea water. He also showed that the diatom could take up iron that was

only present in a precipitated form and that the removal of this precipitate limited growth. However, the precipitate could be replaced by ferric citrate. Goldberg (1952) studied the iron requirements of *Asterionella japonica* and found no matter how he added the iron to sea water there was a considerable amount of hydrolysis. It appeared that *Asterionella* needed particulate iron and could not utilize iron that was only present in the soluble form. As the addition of freshly prepared ferric chloride solution to sea water had no effect on the growth of *Ectocarpus* it must be assumed that, either the sea water contained sufficient iron for the growth of this alga under the experimental conditions of the investigation, or that the alga was unable to utilize iron that was present in an insoluble form.

The addition of manganese to sea water stimulated the growth of *Ectocarpus* up to the highest concentration used, 0·004 mM manganese. Harvey (1947) found additions as low as 0·5–2·0 mg of manganese per cubic metre (0·00001–0·00004 mM) gave increases in the growth of marine algae.

The range of salinities that *Ectocarpus* would tolerate was quite wide, being from $\frac{1}{2}$ to $1\frac{1}{2}$ times the salinity of natural sea water at 20 °C and $\frac{1}{4}$ to $1\frac{3}{4}$ times the salinity of sea water at 15° C. The results of the two experiments on salinity were not completely comparable as the experiment at 20° C was carried out with unshaken boiling tubes, whereas shaken conical flasks were used for the experiment at 15° C. It is, however, interesting that the optimum salinity differed at the two temperatures. At 20° C the greatest yield would have been given by a medium with a salinity slightly higher than that of natural sea water, but at 15° C the highest yield would have been given at a salinity less than that of natural sea water. Ritchie (1957) found the optimum salinity for the growth of two marine fungi decreased with decreasing temperature and experiments indicate that this is also the case with *Ectocarpus*. Biebl (1952) asserted that intertidal algae would tolerate salinities of from 10 to 300% that of natural sea water, and that species that occur at or near low water will tolerate 40–200% natural sea water. Although *Ectocarpus* is an intertidal species it would not grow in such a wide range of salinities. Whitaker & Clancy (1937) found that the eggs of a species of *Fucus* would germinate in salinities ranging from 30 to 180% sea water, with a marked optimum at 90–100%. Their experiments were carried out at 15° C and agree quite well with the salinity toleration of *Ectocarpus* at this temperature. It is of interest to note that *Ectocarpus* will tolerate lower salinities at low temperatures and this probably accounts for the growth of species of *Ectocarpus* in salinities of 5·5 ‰ in the Baltic near Helsinki. The findings that the optimum salinity for the growth of *Ectocarpus* at 20° C was slightly above the salinity of natural sea water probably, in part, explains the poor growth of this alga in the low salinity media of Droop. As was shown experimentally, when the salinity of this medium was doubled there was a considerable improvement in growth.

The optimum pH for the growth of *Ectocarpus* was 8·0, but it grew quite well in pH's ranging from 9·0 to 7·0.

Light intensity did not have any marked effect on the growth of *Ectocarpus*. This probably indicated that the growth process of the alga was light saturated at intensities as low as 125 ft. candles (1350 lux) and that light intensities as high as 1500 ft. candles (16000 lux) did not have any inhibitory effect. However, the length of the lag phase appeared to be very markedly affected by light intensity and there was a marked optimum at 600–700 ft. candles (6500–7500 lux). The only observable change during the lag phase was the production of plurilocular sporangia followed by the release and germination of the spores. It therefore appears that light intensity has a marked effect on the production of the plurilocular sporangia or on the subsequent germination of the spores.

Temperature had much the same effect as light intensity on the growth of *Ectocarpus* in that the main effect was on the length of the lag phase and there was no marked effect on the rate of growth. *Ectocarpus* would grow at temperatures between 10 and 25° C but not at 30° C. The optimum temperature was between 15 and 20° C. In rock pools *Ectocarpus* must be subjected to temperatures much higher than this and at first it appeared strange that it would not grow at 30° C. However, it must be remembered that in rock pools the alga is not likely to be subjected to these high temperatures for long, for even if the pool temperature rises considerably during low water it will be cooled again by the incoming tide in a few hours. Ryther (1954) found that marine phytoplankton will tolerate temperatures between 10 and 30° C and the optimum was 15–25° C. Similar temperature ranges have been found for planktonic algae by Kain & Fogg (1958*a*, *b*). Hyde (1938) found that the assimilation rate of *Fucus serratus* increased with increasing temperature up to 25° C and then fell off sharply at temperatures higher than this.

In common with the findings of many other workers with a wide range of algae, the growth of *Ectocarpus* in natural sea water was stimulated by the addition of soil extract (Harvey, 1939; Pringsheim, 1946, 1951; Provasoli *et al.* 1957). Vitamin B_{12} also showed some stimulation and similar effects have been shown for a number of marine algae (Droop, 1957*b*). Although *Ectocarpus* remained viable for long periods in the dark it would not grow on organic carbon sources in the dark and is therefore probably an obligate phototroph (Fogg, 1953) although experiments have not yet been exhaustive enough to prove this.

Ectocarpus grew in several artificial sea waters but in no case was the yield greater than two-thirds that in natural sea water. Probably the addition of soil extract would have increased the yield but as the aim was to use a medium of known chemical composition this was not desirable. It was interesting to find that the low-salinity media which have been widely used for culturing

many unicellular algae were unsuitable for *Ectocarpus*. The artificial media did not appear to suffer from an excess or deficiency of trace elements and although the addition of vitamin B_{12} and liver extract showed some improvement this was not very marked. The pH of the artificial media was always adjusted to 7·5–8·0 and it is difficult to see why growth was not as good in the artificial media as in natural sea water. This is a problem that remains for further investigation.

I wish to thank Prof. G. E. Fogg for his guidance and encouragement during all stages of this investigation. This work was carried out during the tenure of a Fishery Research Training Grant for which I am indebted to the Development Commission.

SUMMARY

The growth and nutrition of *Ectocarpus confervoides* has been studied in bacteria-free cultures. Growth was estimated by dry weights determined under controlled conditions. A method for inoculation of cultures of filamentous algae is described and a table of culture media is given. Following a lag of 5–7 days there was a period of growth, which was not truly exponential, extending over a period of 35 days. Calculation of the relative growth constant for the early stages of growth indicated that the alga doubled its dry weight every 6–7 days. Cultures of *Ectocarpus* remained viable for over 1 year in the light and for over 100 days in the dark. In natural sea-water media the maximum growth was brought about by the addition of 0·5 mM potassium nitrate and 0·1 mM potassium phosphate. Ferric chloride had no effect on growth but additions of manganese chloride did cause some stimulation. Additions of some complex organic mixtures slightly increased growth but *Ectocarpus* was unable to grow on a range of organic carbon source in the dark. The optimum salinity at 20° C was somewhat higher than that of natural sea water but was apparently lower at 15° C. The optimum pH was 8·0. Light intensities between 1350 and 16,000 lux had no marked effects on the growth rate but did effect the lag, the optimum for this being 7000 lux. Temperature also had no effect on the growth rate but did effect the lag. The optimum was 15–20° C but there was growth between 10 and 25° C. A considerable range of artificial sea waters, with and without organic additions was investigated, in no case did they give a yield greater than two-thirds that in natural sea-water media. These results are discussed in relation to other workers findings.

REFERENCES

BIEBEL, R., 1952. Ecological and non-environmental constitutional resistance of the protoplasm of marine algae. *J. mar. biol. Ass. U.K.*, Vol. 31, pp. 307–15.

BLACK, W. A. P., 1948. The seasonal variation in chemical constitution of some sublittoral seaweeds common to Scotland. *J. Soc. chem. Ind., Lond.*, Vol. 67, pp. 165–76.

BOALCH, G. T., 1961. Studies on *Ectocarpus* in culture. I. Introduction and methods of obtaining uni-algal and bacteria-free cultures. *J. mar. biol. Ass. U.K.*, Vol. 41, pp. 279–86.

BONEY, A. D., 1960. Nurture of a fruiting *Antithamnion* tuft and the physiological condition of the liberated spores. *Brit. phycol. Bull.*, Vol. 2, pp. 38–9.

COOPER, L. H. N., 1937. On the ratio of nitrogen to phosphorus in the sea. *J. mar. biol. Ass. U.K.*, Vol. 22, pp. 177–82.

DROOP, M. R., 1955. Some new supra-littoral Protista. *J. mar. biol. Ass. U.K.*, Vol. 34, pp. 233–45.

—— 1957*a*. Vitamin B_{12} in marine ecology. *Nature, Lond.*, Vol. 180, pp. 1041–2.

—— 1957*b*. Auxotrophy and organic compounds in the nutrition of marine phytoplankton. *J. gen. Microbiol.*, Vol. 16, pp. 286–93.

—— 1958. Requirement for thiamine among some marine and supra-littoral Protista. *J. mar. biol. Ass. U.K.*, Vol. 37, pp. 323–30.

FOGG, G. E., 1944. Growth and heterocyst production in *Anabaena cylindrica*, Lemm. *New Phytol.*, Vol. 43, pp. 164–75.

—— 1953. *The Metabolism of Algae.* London: Methuen.

FOGG, G. E. & BOALCH, G. T., 1958. Extracellular products in pure cultures of a brown alga. *Nature, Lond.*, Vol. 181, pp. 789–90.

FOGG, G. E., SMITH, W. E. E. & MILLER, J. D. A., 1959. An apparatus for the culture of algae under controlled conditions. *J. biochem. microbiol. Technol. Engng*, Vol. 1, pp. 59–76.

GOLDBERG, E. D., 1952. Iron assimilation by marine diatoms. *Biol. Bull., Woods Hole*, Vol. 102, pp. 243–8.

HARVEY, H. W., 1937. The supply of iron to diatoms. *J. mar. biol. Ass. U.K.*, Vol. 22, pp. 205–19.

HARVEY, H. W., 1939. Substances controlling the growth of a diatom. *J. mar. biol. Ass. U.K.*, Vol. 23, pp. 499–520.

—— 1947. Manganese and the growth of phytoplankton. *J. mar. biol. Ass. U.K.*, Vol. 26, pp. 562–79.

—— 1955. *The Chemistry and Fertility of Sea Waters.* Cambridge University Press.

HYDE, M. B., 1938. The effect of temperature and light intensity on the rate of apparent assimilation in *Fucus serratus*, L. *J. Physiol.*, Vol. 26, pp. 118–43.

KAIN, J. M. & FOGG, G. E., 1938*a*. Studies on the growth of marine phytoplankton. I. *Asterionella japonica*, Gran. *J. mar. biol. Ass. U.K.*, Vol. 37, pp. 397–413.

—— 1958*b*. Studies on the growth of marine phytoplankton. II. *Isochrysis galbana*, Parke. *J. mar. biol. Ass. U.K.*, Vol. 37, pp. 781–8.

MILNER, H. W., 1953. The chemical composition of algae. Algal culture from laboratory to pilot plant. *Publ. Carneg. Instn*, No. 600, pp. 285–302.

MYERS, J., 1953. Growth characteristics of algae in relation to the problem of mass culture. Algal culture from laboratory to pilot plant. *Publ. Carneg. Instn*, No. 600, pp. 37–54.

PEARSALL, W. H. & LOOSE, L., 1937. The growth of *Chlorella vulgaris* in pure culture. *Proc. roy Soc.* B, Vol. 121, pp. 451–501.

PRINGSHEIM, E. G., 1946. *Pure Cultures of Algae.* Cambridge University Press.

—— 1951 Methods for the cultivation of algae. In *Manual of Phycology*, pp. 347–58. Chronica Botanica, U.S.A.

PROVASOLI, L., MCLAUGHLIN, J. J. A. & DROOP, M. R., 1957. The development of artificial media for marine algae. *Arch. Mikrobiol.*, Vol. 25, pp. 392–428.

REDFIELD, A. C., 1934. On the proportions of organic derivatives in sea water and their relation to the composition of the plankton. *James Johnstone Memorial Volume*, pp. 176–92. Liverpool University Press.

RITCHIE, D., 1957. Salinity optima for marine fungi affected by temperature. *Amer. J. Bot.*, Vol. 44, pp. 870–4.

RYTHER, J. H., 1954. The ecology of phytoplankton blooms in Moriches Bay and Great South Bay, Long Island, New York. *Biol. Bull., Woods Hole*, Vol. 106, pp. 198–209.

SPENCER, C. P., 1954. Studies on the culture of a marine diatom. *J. mar. biol. Ass. U.K.*, Vol. 33, pp. 265–90.

WHITAKER, D. M. & CLANCY, C. W., 1937. The effect of salinity upon the growth of eggs of *Fucus furcatus*. *Biol. Bull., Woods Hole*, Vol. 73, pp. 552–6.