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STUDIES ON THE GROWTH OF MARINE PHYTOPLANKTON

I. *ASTERIONELLA JAPONICA* GRAN

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(Text-figs. 1–14)

Although the basic role of planktonic algae in the economy of the seas is well recognized, knowledge of their requirements for growth is still meagre. Besides work in which certain of these organisms are used as convenient objects for specialized physiological study there is need for more general investigations on the effects of different chemical and physical factors on the growth of species representative of the main groups of phytoplankton. Apart from their value for the understanding of the growth of natural populations, investigations of this latter type are essential for assessing the possibilities of the mass culture of planktonic algae for economic purposes. The object of the investigations to be described in this and subsequent papers was to obtain general information on the growth of single representatives of each of the three main classes of marine phytoplankton. The first of these to be considered is the diatom *Asterionella japonica* Cleve & Müller ex Gran.

Previous work on the growth of marine diatoms has been summarized by Harvey (1955), and most of the important references on their nutrition are given by Provasoli, McLaughlin & Droop (1957). Among studies particularly concerned with the growth rates of these organisms that of Braarud (1937) is noteworthy as having been the first to have been made with controlled temperature and illumination. In a subsequent paper Braarud (1945) has recorded the relative growth constants of many species grown in unialgal culture under such conditions. Recent studies on the effects of a factor of particular importance for diatom growth, viz. silica supply, have been made with marine and freshwater diatoms (Jørgensen, 1953, 1955, 1957; Lewin, 1957). The photometric technique for measuring growth has been used by Gross & Koczy (1946) and by Spencer (1954).

DEFINITIONS

In this work 'growth' has been taken primarily to mean increase in cell numbers. However, because it is more conveniently determined and leaves the culture intact, optical density rather than cell density of algal suspensions

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has usually been used as the direct measure of growth. Optical density depends on cell size, pigment content and other factors as well as on cell numbers, but a means of obtaining at least approximate values for relative growth constants from optical density determinations has been devised.

The term 'optimum' as applied to nutrient concentrations and the level of physical factors has often been used loosely. Rodhe (1948) distinguished between a potential optimum level realized only under ideal conditions and an actual optimum depending on other factors. The latter can be found not only for the rate of growth but also for the final population. The two may not be identical under certain conditions, that is the range of concentration of a nutrient allowing the maximum growth rate may not extend to concentrations high enough for the attainment of maximum cell crop. As the magnitude of the latter is dependent on many factors it seems convenient to determine and express nutrient levels in terms of requirement per cell so that limitations by each factor can be readily calculated. The number of cells produced in response to the addition of a particular amount of a nutrient has been termed the 'cell crop'. This has been taken as the difference between the final cell concentration in the control cultures without the addition and that in cultures to which the nutrient was added.

METHODS

Glassware was cleaned by soaking in chromic acid followed by thirty complete rinses with tap water and three with distilled water. Steaming was found unnecessary. Pyrex glass culture vessels were used, either 50 ml. boiling tubes or 100 ml. conical flasks, plugged with non-absorbent cotton wool. They were maintained in a constant-temperature water bath, usually at 20° C, with underwater fluorescent lighting providing 5000–9000 lux.

The system used for aerating boiling-tube cultures was a modified form of that used by Spencer (1954). Compressed air from a diaphragm pump passed through the sintered-glass discs of three wash bottles of distilled water to a manifold of twelve needle valves. Through each valve it passed to a cotton-wool filter which was heated to prevent condensation (Fogg, Smith & Miller, in the press). This was attached by rubber tubing to the arm of a culture tube. This consisted of a normal boiling tube with a fine-bore glass tube sealed into the side, about 3 cm from the top, and passing down the inside against the wall to end in a jet at the bottom. This culture vessel was plugged with cotton wool and placed in the constant temperature tank.

The three media used for experiments on *Asterionella* are shown in Table 1. To avoid precipitation phosphate was added after autoclaving. Aseptic techniques were always employed. Media were sterilized by autoclaving at 15 lb. for 15 min. The media used for testing for contamination were those of Spencer (1952) made up with undiluted natural sea water.

When necessary, inocula were washed by aseptic centrifuging. The culture used was pipetted into a sterile centrifuge tube and a cotton-wool plug tied on. After centrifuging the supernatant was drawn off with a sterile pipette and replaced with sterile medium. After repeating the process, if necessary, the cell suspension was used as an inoculum.

TABLE 1. THE COMPOSITIONS OF THE MEDIA USED FOR *ASTERIONELLA*

	AK In sea water	AR_N In sea water	AR_A In distilled water
KNO_3	2·0 mM	2·0 mM	2·0 mM
K_2HPO_4	0·2 mM	0·2 mM	0·2 mM
$FeCl_3$	10 μM	5·5 μM	5·5 μM
$MnCl_2$	1·0 μM	—	—
Na_2SiO_3	20 μM	100 μM	100 μM
$ZnSO_4$	—	2·5 μM	2·5 μM
$MnSO_4$	—	20 μM	20 μM
$CoSO_4$	—	0·05 μM	0·05 μM
$CuSO_4$	—	0·02 μM	0·02 μM
H_3BO_4	—	550 μM	970 μM
NaCl	—	—	401·5 mM
$MgCl_2.6H_2O$	—	—	50·2 mM
Na_2SO_4	—	—	27·6 mM
$CaCl_2$	—	—	10·9 mM
KCl	—	—	8·91 mM
$NaHCO_3$	—	—	2·28 mM
KBr	—	—	0·806 mM
$SrCl_2.6H_2O$	—	—	0·150 mM
NaF	—	—	0·0714 mM
EDTA	—	0·342 mM	0·342 mM
Tris	—	8·25 mM	8·25 mM
Soil extract	20 ml./l.	—	

Growth was estimated by cell counts alone or in combination with optical density measurements. For cell counts a 1 ml. sample was removed from each culture and kept in a stoppered tube with solid iodine which killed and stained the cells and increased their specific gravity. The sample was shaken vigorously, which broke up the colonies to some extent, and the cells counted with a haemacytometer. Between 800 and 1000 cells were counted from each sample. Counts on twelve groups of 800 cells from the same sample gave a standard deviation of ±6·0% of the mean. For optical density (OD) determinations a Unicam absorptiometer UIC no. 6117 was used. Culture tubes were selected to fit the compartment and marked so that they were always placed in the same way round. Appropriate blanks were used. Absorption was measured at the chlorophyll absorption peak, 680 mμ. The OD was found to be directly proportional to cell concentration up to 2500 cells/mm³. The units used for OD were arbitrary.

For comparison between cultures it was necessary to express growth rates in terms of cell numbers. It was observed that the OD/cell varied according to the conditions under which the cells were grown and according to their ages and could therefore change during the course of an experiment. The initial cell concentration and also that at or near the end of the logarithmic

growth period was determined by direct counting. The logarithmic rate of increase in OD was known, but if the OD/cell had changed this would not be equivalent to the logarithmic rate of increase in cell concentration. If it is assumed that the change in OD/cell occurs progressively during division an approximate way of calculating the relative growth constant k for cell numbers is to extrapolate the straight line on the graph of $\log_{10}$ OD to the levels of OD at the time the cell counts were made, and take the corresponding time as that needed to produce the observed change in cell concentration by exponential growth. This is shown diagrammatically in Fig. 1. The relative growth constant k is then given by the usual formula:

$$k = \frac{\log_e N_T - \log_e N_0}{t} = \frac{2{\cdot}3}{t}(\log_{10} N_T - \log_{10} N_0).$$

Where N_T = the cell concentration at time T; N_0 = the cell concentration at time 0; and t = the time in days.

There was sometimes considerable variation of the relative growth constant from experiment to experiment due to variations in the age of the inoculum (p. 409). This did not affect the results within each experiment as controls were always included. Comparisons between separate experiments have been made only when the inocula were similar.

Cultures were invariably prepared in duplicate or triplicate, Student's t-test being used to test the statistical significance of the differences in growth observed in the experiments.

EXPERIMENTS

Isolation

Asterionella japonica was isolated into unialgal culture from a tow-net sample from the Hamble River, Hampshire, in October 1953.

Attempts to free this diatom of bacteria were unsuccessful. It failed to grow on a solid sea-water agar medium, and could thus not be separated from the bacteria by plating techniques. The use of antibiotics also proved unsatisfactory. The diatom was grown in concentration ranges of penicillin and streptomycin similar to those used by Spencer (1952) and also in a range of solutions of chloramphenicol. The cell concentrations after about 10 days' growth are shown in Fig. 2. Before testing for contamination the algae were subcultured to ensure sufficient dilution of the antibiotic for the development of any bacteria present. The results of the tests showed that certain colonies of bacteria were unaffected by concentrations of any of the antibiotics tolerated by the diatom. It is possible that further exposure or a mixture of the substances might have been effective. The curious increase in cell concentration with the rise of penicillin from 60 to 3000 μg/ml. will be discussed later. Finally, small colonies were washed by the method described by Pringsheim (1946). As it seemed possible that the diatom was dependent on bacteria for

organic growth substances, the washed cells were inoculated into a variety of organic media which contained small quantities of soil extract, peptone, liver extract, amino acids and vitamins (including cobalamin). Growth of *Asterionella* took place only in medium AR without organic addition and then there was bacterial contamination. Most of the remaining media were contaminated. It seems likely that this was due to adherence of bacteria to the diatom frustules.

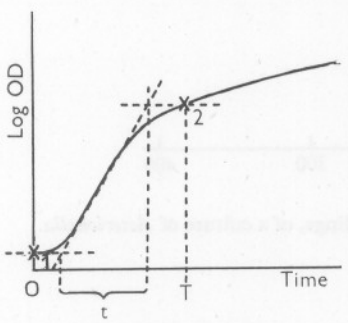

Fig. 1

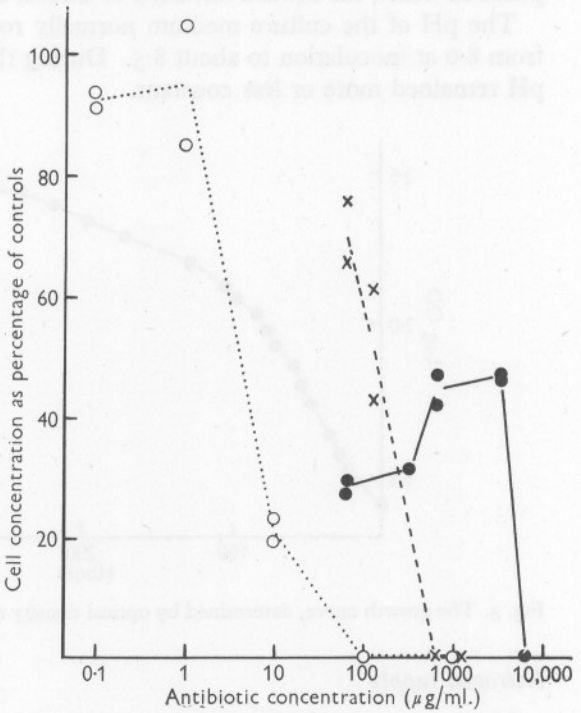

Fig. 2

Fig. 1. Diagram of the method of calculating the relative growth constant. The cell counts were made at times o and T, when the optical density readings were X_1 and X_2, respectively. The time interval t was used in the usual formula for the calculation of the relative growth constant k.

Fig. 2. Cell concentrations of *Asterionella* after 10 days' exposure to various antibiotic concentrations, expressed as percentages of those in controls without antibiotic additions. —●—●—, penicillin; –×– – –×–, streptomycin; ··○···○··, chloramphenicol.

With the failure to obtain a bacteria-free culture of *Asterionella*, studies were made of the nutritional requirements of the alga in unialgal but not pure culture. Most of the studies were on mineral nutrition and the effects of physical factors, so that complications due to the presence of bacteria were probably minimal, and identical in all the cultures of a single experiment with equal inocula.

Growth

An example of the growth curve of *Asterionella* in stagnant medium in boiling tubes is shown in Fig. 3. There was a lag period of 0–20 h followed by an exponential phase with a relative growth constant usually of $k = 0{\cdot}7–1{\cdot}2$. This gave way, after less than 100 h from inoculation, to a more or less linear phase in which the inward diffusion of carbon dioxide was evidently limiting.

The pH of the culture medium normally rose during logarithmic growth from 8·0 at inoculation to about 8·5. During the phase of slower growth the pH remained more or less constant.

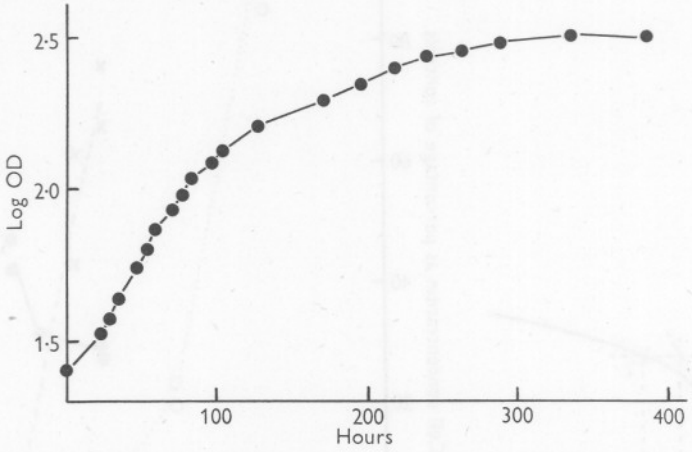

Fig. 3. The growth curve, determined by optical density readings, of a culture of *Asterionella*.

Nitrogen supply

Media were prepared containing added concentrations of potassium nitrate in artificial sea water of 0, 0·1, 0·3, 1·0, 3·0 and 10·0 mM. They were inoculated with cells that had been washed in nitrate-free artificial sea water. The cultures were aerated. The growth was followed by means of OD measurements and cell counts made when it had ceased.

Variation in nitrate concentration from 1 to 10 mM had no statistically significant effect on the relative growth constant. There was a marked difference in the OD/cell, however, that at 10 mM being three times that at 0·1 mM.

The cell crop is shown plotted against nitrate concentration in Fig. 4. The figure also shows the quantity of nitrate added to the medium divided by the crop obtained from it. This should be constant for nitrate-limited cultures. It is apparent that the two lowest concentrations were limiting. The mean quantity of nitrogen in these was 0·255 $\mu\mu$g atom N/cell. This can be regarded as the minimum required for these particular cells, since the method of

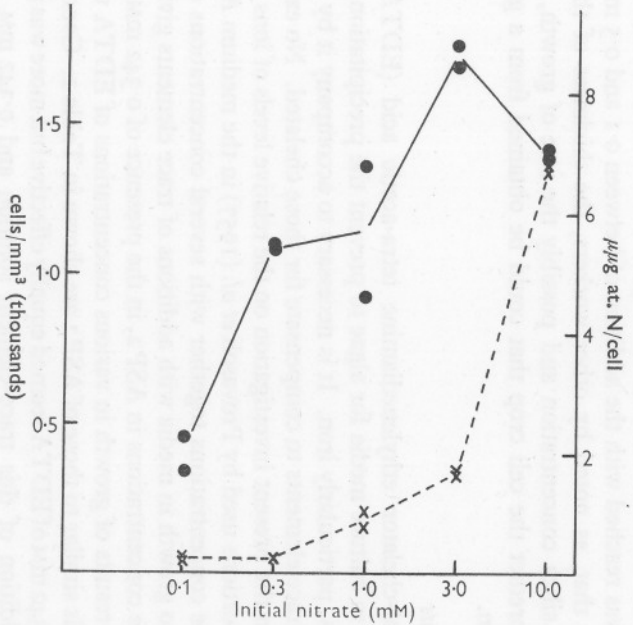

Fig. 4. The cell crop and nitrogen per cell of *Asterionella* in relation to concentration of potassium nitrate. —●—●—, cell crop; –×–––×–, nitrogen/cell.

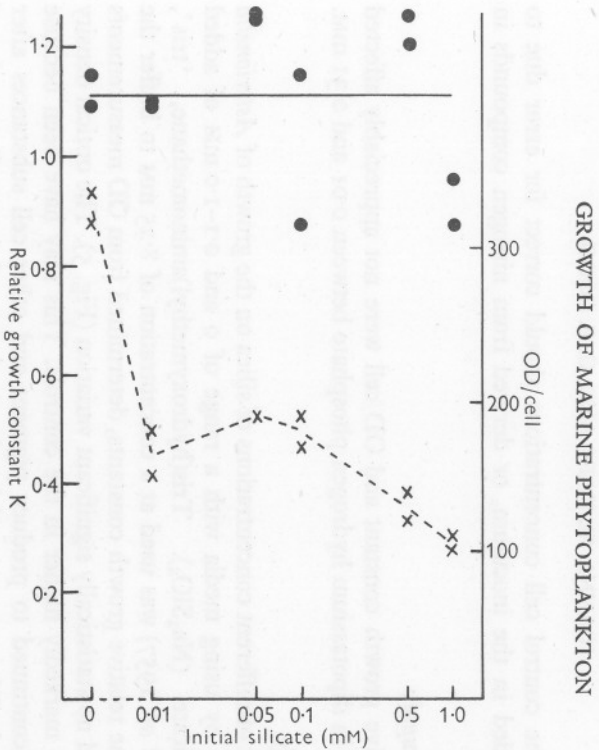

Fig. 5. The relative growth constant and final optical density per cell of *Asterionella* in relation to concentration of sodium silicate. —●—●—, k; –×–––×–, OD/cell.

deducting the control cell concentrations should correct for error due to nitrogen added in the inoculum, or derived from nitrogen compounds in the air.

Phosphorus supply

The relative growth constant and OD/cell were not appreciably affected by variation in dipotassium hydrogen phosphate between 0·01 and 0·31 mM.

Silicon supply

The effect of different concentrations of silica on the growth of *Asterionella* was studied by using media with a range of 0 and 0·1–1·0 mM of added sodium silicate (Na_2SiO_3). Tris(hydroxymethyl)aminomethane, 'tris', (Provasoli *et al.* 1957) was used at a concentration of 8·25 mM to buffer the medium. The relative growth constants, determined from OD measurements only, showed no statistically significant variation (Fig. 5). The optical density per cell was markedly higher in the controls. This may have been because the diatom continued to produce pigment and other cell substances after cell division had been prevented by the depletion of the small quantity of silica from the glass and the inoculum.

The final cell concentrations in this experiment showed that the maximum population was reached with the addition of between 0·1 and 0·5 mM silicate. It was clear that, as noted by other workers, the thickness of the frustule varied with silica concentration and possibly the rate of growth, making it difficult to predict the cell crop that could be obtained from a given silica concentration.

Trace elements

The metal-chelator ethylenediamine tetra-acetic acid (EDTA) is now widely used in culture media for algae to prevent the precipitation of certain essential ions, particularly iron. It is necessary to accompany it by additional amounts of trace elements to compensate for those chelated. No experiments were made in the present investigation on the relative levels of ions necessary, but the proportions used by Provasoli *et al.* (1957) in the medium ASP 2 were tried in three concentrations together with several concentrations of EDTA. There was no growth in media with additions of trace elements giving 10 and 100 times the concentrations in ASP 2, in the presence of 0·342 mM of EDTA or less. The results of growth in various concentrations of EDTA with trace-element levels similar to those of ASP 2 are shown in Table 2. Concentrations of 0·0342–0·342 mM of EDTA seemed equally effective but more was inhibitory.

As the addition of this trace-element solution and 0·342 mM of EDTA proved successful it was considered a convenient way of providing minor elements and was used thereafter, in media AR_N and AR_A.

TABLE 2. GROWTH OF *ASTERIONELLA* IN MEDIA WITH VARIOUS EDTA ADDITIONS

	EDTA				
	0·0342 mM	0·171 mM	0·342 mM	1·71 mM	3·42 mM
	First experiment				
Cells/mm³ at 485 h	2660	2110	3320	—	—
	2820	2400	1180	—	—
	Second experiment				
Cells/mm³ at 340 h	—	—	1067	657	363
Relative growth constant *k*	—	—	0·967	0·801	0·773
	—	—	0·912	0·801	0·690

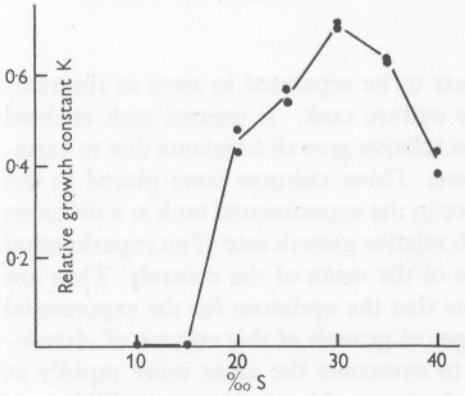

Fig. 6

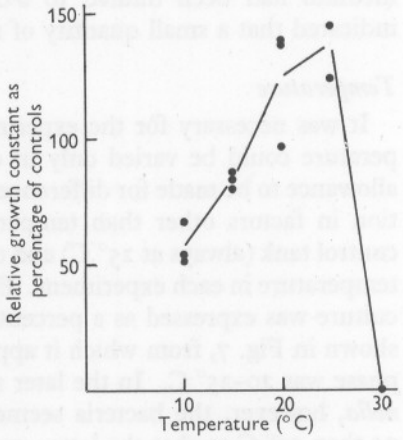

Fig. 7

Fig. 6. The relative growth constant of *Asterionella* in media based on sea water of various salinities.

Fig. 7. The relative growth constant of *Asterionella* at different temperatures (see text for further explanation).

Salinity

Asterionella was grown in natural sea water of various salinities produced by appropriate dilution or evaporation, with the usual nutrient additions. The relative growth constants, derived from cell counts only, are shown in Fig. 6. The optimum was between 30 and 35‰ S. The only salinities in which no growth took place were 10 and 15‰ S. It appears that *Asterionella* is fairly tolerant of salinities of 20 to 40‰ S or more. The relative growth constants at the extremes could possibly be increased by acclimatizing the cells to the different osmotic pressure.

Artificial sea water

Most of the experiments were made with natural sea water as a base. Although the principal constituents are remarkably constant there are several

components of biological importance which may vary. It was therefore desirable to use artificial sea water if the medium was to be defined as closely as possible. A solution of the principal constituents found by Lyman and Fleming (Harvey, 1955, p. 137) in their analysis was used in place of natural sea water. When this, with the addition of nitrate, phosphate, silicate, EDTA and trace elements (i.e. medium AR_A) was compared as a medium with natural sea water with the same additions (i.e. medium AR_N), growth was identical. This result is equivocal, however, as the inoculum introduced 5% of natural sea water. In a later experiment normal growth was obtained in artificial sea water after the inoculum had been washed in such a way that the original medium had been diluted to 0·02%. Nevertheless, further subculturing indicated that a small quantity of natural sea water might be necessary.

Temperature

It was necessary for the experiments to be separated in time as the temperature could be varied only in one culture tank. A control tank enabled allowance to be made for differences in relative growth constants due to variation in factors other than temperature. Three cultures were placed in the control tank (always at 25° C) and three in the experimental tank at a different temperature in each experiment. Each relative growth rate of an experimental culture was expressed as a percentage of the mean of the controls. They are shown in Fig. 7, from which it appears that the optimum for the exponential phase was 20–25° C. In the later stages of growth of this culture of *Asterionella*, however, the bacteria seemed to overcome the algae more rapidly at 25 than 20° C so that the latter was a better working temperature. This may have been associated with progressive inhibition with time, as noted by Rodhe (1948). After 7 days at 30° C the diatom cells did not recover when returned to 20° C.

Light

The relative growth constant and optical density per cell was determined in various intensities of fluorescent ('white') light. Light intensity was measured with an Everett Edgcumbe autophotometer. The relative growth constants in three experiments under different conditions of temperature and aeration are shown in Fig. 8. The results were variable but it appears that there was saturation at 4000 lux and no inhibition at nearly 10,000 lux. The final OD/cell was almost unaffected by light intensity.

Aeration

As the length of the exponential growth phase of *Asterionella* seemed to be limited by the partial depletion of the carbon dioxide in the medium, an attempt was made to prolong the initial growth rate by artificial aeration. The result of this is illustrated in Fig. 9, in which the means of OD readings of

aerated and stagnant cultures over the first 100 h are shown. Not only was the level to which exponential growth continued raised but the initial relative growth constant was increased. This latter could be due to keeping the cells in suspension or to the provision of carbon dioxide at a faster rate. These effects will be discussed in a later section.

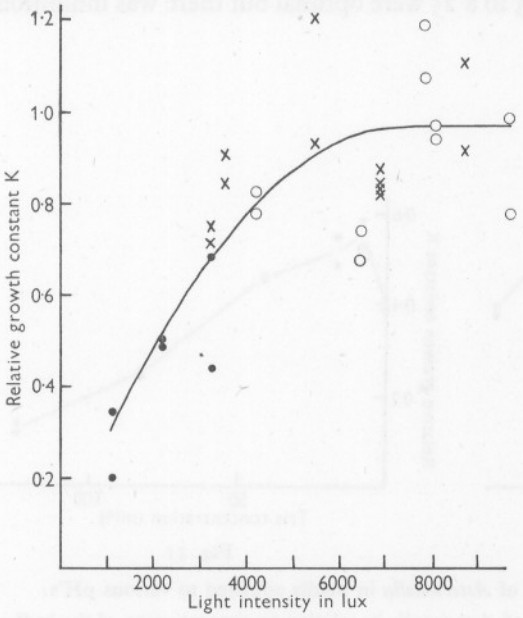

Fig. 8

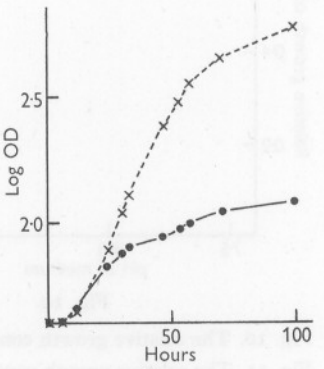

Fig. 9

Fig. 8. The relative growth constant of *Asterionella* grown under various conditions in different intensities of fluorescent light. ●, stagnant at 18° C; ×, aerated at 18° C; ○, aerated at 25° C.

Fig. 9. The growth curves, determined from optical density readings, of *Asterionella* in stagnant and aerated culture. —●———●—, stagnant; –×– – –×–, aerated.

Hydrogen-ion concentration

The apparent importance of the effect of pH on the growth of *Asterionella* necessitated a more detailed study of this factor. In the first place a method was devised to control the pH of the medium during the growth of the diatom. Two series of vessels were prepared, an 'experimental' and a 'dummy' series. Each of these consisted of duplicate cultures at six different pH's. The two series were treated in the same way except that no attempt was made to keep the dummies sterile. After inoculation and at intervals during growth the dummy tubes were opened and the pH of each medium adjusted. The quantity of acid or alkali needed for this adjustment was then added aseptically in a sterile form to the experimental series. Growth determinations were made on the latter.

In the first experiment of this kind no buffer was included in the sea-water medium. It was found that the removal of carbon dioxide altered the adjusted pH so rapidly that it was impossible to control it by this method. The buffer tris was therefore added to the medium at a concentration of 41·2 mM. The relative growth constants at the different adjusted pH's are shown in Fig. 10. It is apparent that pH's from 7·5 to 8·25 were optimal but there was inhibition at 8·5 and 8·75.

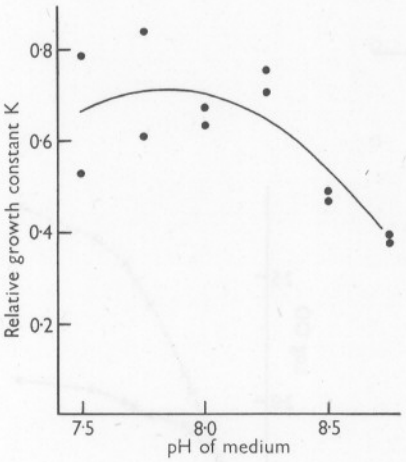

Fig. 10

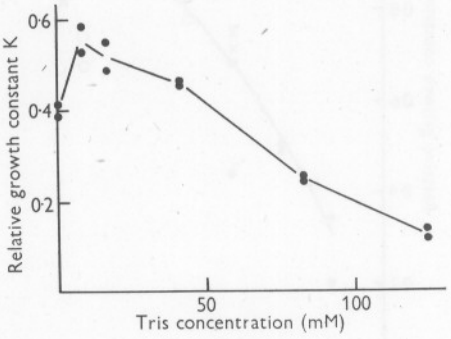

Fig. 11

Fig. 10. The relative growth constant of *Asterionella* in media adjusted to various pH's.

Fig. 11. The relative growth constant of *Asterionella* in relation to concentration of the buffer tris.

TABLE 3. GROWTH OF *ASTERIONELLA* IN MEDIA WITH AND WITHOUT TRIS

(Relative growth constant k (in triplicate).)

Without tris	0·380	0·437	0·366
With 8·25 mM tris	0·658	0·564	0·564

In using this buffer it was considered desirable to find the highest concentration that the diatom would tolerate. The relative growth constants were therefore determined in media with various additions of tris. These are shown in Fig. 11. There was stimulation at 8–16 mM, followed by inhibition with increasing concentration. Tris of greater purity caused a similar stimulation, as is shown in Table 3, the difference being statistically significant at the 1% level. The possibility that tris stimulated growth through its buffering action seemed the most likely and was studied further.

The effect of the growth of *Asterionella* on the pH of stagnant medium has already been mentioned. In order to follow the rise under different conditions the growth of two cultures was studied in detail in each case, while further

cultures under the same conditions were used successively for pH determinations. In this way the change in the pH of the medium was followed in the presence and absence of 8·25 mM of tris in stagnant culture and also in the absence of the buffer in aerated culture. The pH rose steeply during exponential growth in stagnant culture, but remained constant once this phase had ended. In the absence of tris it rose from pH 8·0 to 8·55 but in its presence only to 8·35–8·40. In aerated culture although the growth was much greater there was a negligible rise in pH. Thus, although the buffer was partly effective in preventing the pH change, it was not as effective as aeration.

TABLE 4. GROWTH OF *ASTERIONELLA* IN STAGNANT AND AERATED CULTURE WITH AND WITHOUT TRIS

(Relative growth constant k (in triplicate).)

	Stagnant			Aerated		
Without tris	0·695	0·619	0·663	1·18	1·16	1·17
With 8·25 mM tris	0·906	1·16	0·983	1·06	1·05	1·02

Arrows indicate a statistically significant difference at the 1% level.

If tris were stimulating growth solely because of its buffering action its effect would be masked under aerated conditions where little rise in pH took place in any case. An experiment was therefore made comparing the relative growth constants in media with and without tris, in stagnant and aerated culture. The results are shown in Table 4. It is apparent that instead of stimulating growth when the culture was aerated tris inhibited it, though only to the rate of stimulated stagnant growth. Aeration and tris thus had a similar action in accelerating growth. As in stagnant culture the cells settled to the bottom of the vessel, it would seem likely that this action was to prevent the pH in the vicinity of the cells from becoming inhibitory.

Unidentified inhibitory factor

The variation between relative growth constants from experiment to experiment was observed to be large, and it was therefore important always to have controls. In order to investigate the possibility that this variation might be associated with the size or age of the inoculum, identical culture media were inoculated with different amounts of inoculum from cultures of three different ages. The relative growth constants were calculated in the usual way and are shown plotted against the size of the inoculum in Fig. 12. There was evidence of a decrease in the constant both with increase in age and in increase of size of the inoculum. The extent of the variation with age was more than sufficient to account for the differences between the relative growth constants in different experiments. The decrease with increasing age may have been due to an increasing proportion of non-viable cells in the inoculum. The effect of size cannot be explained in this way. The relative growth constant should be the

same for a given proportion of viable cells with any inoculated number of these. A possible explanation might be that some inhibitory substance was carried over in the medium with the inoculum.

In an earlier experiment cultures were prepared with only half freshly autoclaved medium. The other half was Seitz-filtered sea water in three of the cultures and Seitz-filtered old *Asterionella* culture medium in another three. The relative growth constants (from OD measurements only) are given

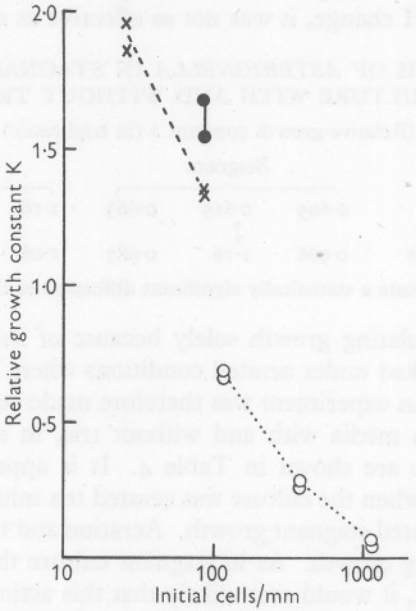

Fig. 12. The relative growth constant of *Asterionella* in cultures inoculated with different amounts of inoculum from cultures of three different ages. —●——●—, inoculum 37 h old; –×– – –×–, 190 h old; ··○····○··, 890 h old.

TABLE 5. GROWTH OF *ASTERIONELLA* IN PARTLY SEITZ-FILTERED MEDIUM

(Relative growth constant k (in triplicate).)

Half medium of sea water	0·442	0·529	0·465
Half medium of aged culture medium	0·331	0·412	0·343

in Table 5 and were significantly lower at the 2·5 % level in the cultures with the old medium. This seemed to indicate the presence of an inhibitory substance in the old culture.

Further experiments on this factor were inconclusive. It is possible that if an inhibitor was produced at all, it was produced only under certain conditions.

Unidentified stimulatory factors

It has already been suggested that *Asterionella* may require organic substances for growth and that it normally obtains these from bacteria in crude culture.

In the experiment on the range of penicillin tolerated by the diatom it was found that there was a stimulation of growth with increasing concentration of antibiotic within the range of 60–3000 μg/ml., although in general penicillin was inhibitory (Fig. 2, p. 401). It is likely that the inhibition at 60 μg/ml. was due to the effect of the antibiotic on the bacteria, inactivating them and thus slowing down the production of a substance necessary for diatom growth. Increasing quantities of penicillin caused stimulation either by providing the required substance or substances as chemical impurities, or by releasing them into solution from the bacterial cells as a result of autolysis. This might be taken as an indication that one or more organic substances stimulated the growth of *Asterionella*.

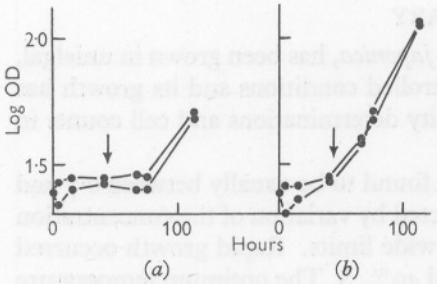

Fig. 13

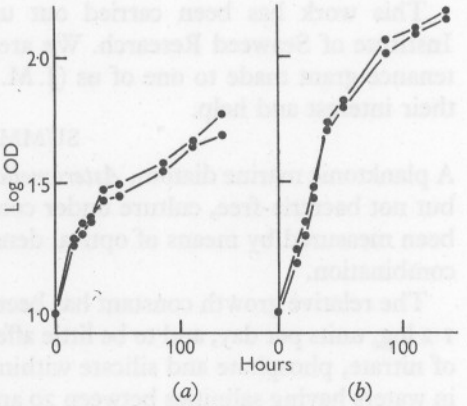

Fig. 14

Fig. 13. The growth curves of *Asterionella* in a synthetic medium, (*a*) without the addition of old medium, and (*b*) with the addition of 1 ml. of the supernatant of an aged culture. The arrow indicates the addition to each of 1 ml. of natural sea water.

Fig. 14. The growth curves of *Asterionella* in a natural sea water medium, (*a*) without addition, and (*b*) with the addition of 1 ml. of the supernatant of an aged culture.

Further evidence for this came from an experiment in which the inoculum was washed in artificial sea water for two successive subcultures in synthetic medium. The washing involved aseptic centrifuging and many of the bacteria would have remained in the supernatant and been discarded. Thus the proportion of bacterial to algal cells would have been reduced. Four of these cultures were grown, two as controls and two with the addition of 1 ml. of the supernatant of an aged medium. The growth curves are shown in Fig. 13. Growth was clearly stimulated by the presence of the aged medium, but only after the addition of 1 ml. of natural sea water to each culture. This may have been coincidental, though a lag of that length had not previously been observed.

In the next experiment the medium was based on natural sea water and an addition made from the same aged medium. The growth curves are shown in Fig. 14. Growth was immediate in all the cultures, but considerably better in those with the aged medium addition than in the controls. The absence of lag suggested that some substance, possibly cobalamin, present in natural sea water had to be present before growth could take place in the previous experiment. But it is clear that some further substance was derived from the aged medium. In a further experiment an addition of the same aged medium was made, in both autoclaved and unsterilized forms. The unsterilized addition was clearly more effective than the sterilized. Thus, some thermolabile factor is stimulatory to the diatom, but it cannot be said whether this is produced by *Asterionella* itself or by bacteria.

These results will be discussed together with those for other species on a later occasion.

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SUMMARY

A planktonic marine diatom, *Asterionella japonica*, has been grown in unialgal, but not bacteria-free, culture under controlled conditions and its growth has been measured by means of optical density determinations and cell counts in combination.

The relative growth constant has been found to be usually between 0·7 and 1·2 $\log_e$ units per day, and to be little affected by variation of the concentration of nitrate, phosphate and silicate within wide limits. Rapid growth occurred in waters having salinities between 20 and 40‰ S. The optimum temperature for growth was 20–25° C and the optimum light intensity from 4000 to 10,000 lux. The relative growth rate was affected most markedly by variation in hydrogen-ion concentration. This was manifest in stagnant cultures in which the rise of pH above 8·3, caused by absorption of carbon dioxide, resulted in the inhibition of growth. This could be countered by increased aeration or by the addition of the buffer tris(hydroxymethyl)aminomethane.

Evidence has been obtained which suggests that *A. japonica* requires a thermostable substance present in natural sea water and that a thermolabile substance stimulating its growth is produced in culture.

Final yields of the order of 4000 cells per mm^3 were obtained and, within limits, were dependent on the amounts of nitrate and silicate supplied. The nitrogen requirements per cell was determined as 0·255 $\mu\mu$g atom. Indications were obtained of the presence of a growth-inhibiting substance in the filtrates from old cultures.

The optical density of the cells was increased at high nitrate concentrations and when exhaustion of silicate prevented cell division.

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