On the Culture of the Plankton Diatom *Thalassiosira gravida* Cleve, in Artificial Sea-water.

By

E. J. Allen, D.Sc., F.R.S.,

Director of the Plymouth Laboratory.

In a former paper,* written in conjunction with my colleague Mr. E. W. Nelson, the conditions under which a rapid and continuous growth of marine plankton diatoms can be obtained in laboratory cultures were discussed. It was pointed out that when natural sea-water is used as the basis of the culture media we are dealing with a solution of a very complex and variable character, the exact nature of which it is extremely difficult to determine, and that the ideal to be aimed at is to find a culture medium with artificially prepared sea-water as its basis, such that the absence or diminution in quantity of any one of its constituents would have a profound effect upon the growth of diatoms in it. A reference was made (*loc. cit.*, p. 446)† to some experiments with artificial sea-water, which, whilst pointing to the probability of successful work being possible on these lines, were in themselves too uncertain to be satisfactory.

Experiments in this direction have been continued at intervals during the past three years, and although the problem has not been completely solved the results obtained seem to be of sufficient interest and importance to warrant publication in their present incomplete form, more particularly because points remaining to be cleared up probably require a knowledge of the chemistry of organic compounds to which I cannot lay claim.

Stated in general terms the most interesting result so far obtained is that in the artificial sea-water tried, made by dissolving Kahlbaum's pure chemicals in doubly distilled water, little or no growth of diatom (*Thalassiosira gravida* Cleve) takes place, but if to this artificial sea-water as little as 1 per cent of natural sea-water is added vigorous and large cultures are obtained, and with an addition of about 4 per cent of

* Allen, E. J., and Nelson, E. W. "On the Artificial Culture of Marine Plankton Organisms," *Journ. Mar. Biol. Assoc.*, VIII, 1910. Also in *Quart. Journ. Micr. Sci.*, Vol. LV, 1910. The two papers are identical.

† *Q.J.M.S.*, Vol. LV, p. 393.

natural sea-water from the Laboratory tanks better cultures result than have so far been got in any medium which has natural instead of artificial sea-water as a basis.

THE DIATOM CULTURE USED.

A culture of the diatom *Thalassiosira gravida* Cleve, isolated some years ago,* which has been kept since then by successive inoculations in fresh culture medium, has been used almost entirely for these experiments. This species is especially useful owing to the fact that in healthy cultures the cells hang together in long chains, whereas when the culture is unhealthy or becoming exhausted the chains break up. This is a most useful guide when watching the progress of an experiment.

The Purity of the Culture.—The culture contains no other diatom except *T. gravida* and no other organisms except bacteria. It would of course be preferable, if it were possible, to remove all the bacteria, so as to deal with a perfectly pure culture of the diatom. Many attempts have been made to attain this end, but so far without complete success, though it has been possible to carry the process of purification so far that only one species of bacterium capable of forming colonies on a peptone-agar plate† was at all abundant. The method adopted for purifying the culture was that of differential poisoning, a suitable poison being added to a number of culture flasks in a series of gradually diminishing strengths, in the hope that one strength might be found which would kill the bacteria without killing the diatom.

A measure of success was obtained with Copper sulphate in this way. In the most successful case a solution of the salt was added to 100 c.c. of culture medium containing *Thalassiosira gravida* in such proportion that

* Allen and Nelson, *loc. cit.*, p. 460. [*Q.J.M.S.*, p. 412.] The species was then thought to be a variety of *Thalassiosira decipiens*. Subsequent examination by Mr. Nelson has convinced him that it is really *Th. gravida*. The extreme delicacy of the siliceous skeleton of these diatoms makes the determination of species founded chiefly on valve structure very difficult. The species was formerly thought to be a variety of *Thalassiosira decipiens* Grun. since the only markings that were observed were characteristic of this species, although no markings at all could be resolved with the great majority of valves. Examination of the present cultures by Mr. Nelson with more perfect apparatus has shown the typical *Th. gravida* Cleve valve structure to which species this form is now referred. It is not unlikely that the older cultures were a mixture of *Th. decipiens* and *gravida* from which the *decipiens* have died out.

† It should be remembered that possibly the presence of some bacteria in the cultures is necessary for their success, though Miquel (*Le Diatomiste*, I, 1890-3, pp. 153–6) states definitely that he obtained cultures of fresh water diatoms which were entirely free from bacteria, and Richter (*Ber. deut. bot. Gesell*, XXI, 1903 and later papers) also succeeded in obtaining such bacteria-free cultures on solid culture media.

the 100 c.c. contained ·001 grams of $CuSO_4$ $5H_2O$. After an interval of twelve minutes a fresh flask containing 100 c.c. of culture medium was inoculated with 1 c.c. from the first one. In the second flask a very fine growth of diatoms appeared, which was much more healthy and vigorous than untreated cultures, and contained far fewer bacteria, as shown by peptone-agar plates.

Still better results were got, however, by a method which was first recommended to me by Mr. D. J. Matthews, who had made use of it for destroying bacteria in aquarium water. This consists in passing an electric current through the sea-water between carbon poles, until a considerable formation of hypochlorous acid has taken place and the water smells strongly of chlorine. The following description of an experiment will show how the method was applied in the case of the diatom cultures.

Experiment 449.—2½ litres of sea-water from the Laboratory tanks, which had been treated with animal charcoal and filtered through a Berkefeld filter, were put in a sterilized square glass jar, and an electric current varying from 1·7 to 1·5 ampères was passed through it for three minutes, two carbon plates* (sterilized by heating) being used as poles, the plates being constantly moved as the current was passing. The electrolysed water then smelt strongly of chlorine. It was allowed to stand for one hour, and then 50 c.c. of it was added to a flask (*x*), which contained 50 c.c. of unelectrolysed Berkefeld water,† to which had been added a quantity of *Thalassiosira gravida* from the culture which was to be cleansed.

Sixteen flasks (*a–q*) had previously been made ready, each containing about 75 c.c. of sterile culture medium (outside sea-water treated with Miquel's solutions and boiled). After the electrolysed water had been in contact with the *Thalassiosira* for thirty-one seconds about ½ c.c. from flask *x* was added to flask *a*, and similar amounts were added to the remaining flasks *b*, *c*, *d*, etc., at intervals of about ten seconds for the first two minutes, and then at longer intervals until the last flask *q* was inoculated after the *Thalassiosira* had been in contact with the electrolysed water for four minutes.

In this way a series of culture flasks was obtained inoculated with *Thalassiosira* which had been in contact with electrolysed water for varying times. The flasks were placed in a suitable position before a north window and the diatoms allowed to develop. At the end of a week the first flasks in the series (*a*, *b*, *c*, etc.) showed good growth, the later ones

* The size of each plate was 120 × 44 × 6 mm.

† See Allen and Nelson, *loc. cit.*, p. 432 [*Q.J.M.S.*, p. 375].

(*m*–*q*) showing little or none. At the end of three weeks the result was quite different, for whilst the early flasks showed only moderate growths and were already beginning to go off, a sure sign of contamination, two amongst the later ones (*m* and *o*) showed very fine growths of a rich brown colour and forming very long chains. The culture in flask *o* was one of the best and most vigorous that I have obtained during the whole course of my experiments, and sub-cultures from it remained excellent for many months.

The following table shows for the last few flasks of the series the times that the *Thalassiosira* remained in the electrolysed water, and the kind of growth that was obtained :—

Flask. Time during which *Thalassiosira* was in electrolysed water.	Result culture.
l. 2 min. 28 secs.	Moderate culture, not persisting very long.
m. 2 min. 43 secs.	Very good culture, with long chains, second best of series.
n. 3 min. 0 secs.	No growth of *Thalassiosira*.
o. 3 min. 22 secs.	Very fine culture, best of series, dark brown colour and very long chains. Remained good for a long time and gave a long series of good sub-cultures.
p. 3 min. 40 secs.	No growth of *Thalassiosira*.
q. 4 min. 0 secs.	No growth of *Thalassiosira*.

(Flasks *a*–*k* all gave moderate growths which did not persist, with the exception of flask *h* (1 min. 44 secs.), which had no growth.)

Peptone-agar plates inoculated with 1 c.c. from flask *o* showed bacteria of two kinds only, a few large yellow colonies, and many minute, slow-growing colonies. They were of quite a different character from plates made from ordinary cultures of *Thalassiosira*, which were always crowded with yellow colonies, mixed with a large number of large milk-white colonies which liquefied the agar, both kinds of colonies developing very rapidly.

After some experience it becomes easy to distinguish a clean culture of *T. gravida* from one which is much contaminated by bacteria, by the character and progress of the growth. In a clean culture, at any rate during the summer months when the light conditions are favourable, the growth is much more rapid and vigorous, the tendency to form long

chains is very great, especially at first, the colour is a deep rich brown, and healthy growth in a flask will go on for months. In a contaminated culture, on the other hand, growth is slower and only quite short chains are seen, the colour is a much lighter brown, and the culture does not continue to grow in a healthy way, generally forming auxospores and often dying off altogether in the course of two or three weeks.

All the main conclusions detailed in this paper have been confirmed with clean and healthy cultures. Experiments with contaminated cultures are not, however, without value, since they sometimes emphasize the differences between culture media that it is desired to compare, a contaminated culture often failing to grow at all in an unfavourable medium, whereas a clean culture might give a growth, less in amount, it is true, but not much different in character from the growth in the control culture in a favourable medium.

THE ARTIFICIAL WATER.

The artificial sea-water used in the experiments was made by dissolving Kahlbaum's pure chemicals in ordinary distilled water made in a copper still which had been redistilled in all-glass apparatus after being treated with bichromate of potash and sulphuric acid, to destroy volatile organic matter. This double distilled water contained at most 0·01 mg. of ammonia per litre.*

The composition of the water was based on the analysis of sea-water published by Dittmar in the " Challenger " Reports.† The figures given by Dittmar are :—

	Per 100 parts halogen.
Cl	99·848
Br	·3402
SO_3	11·576
CO_2	·2742
CaO	3·026
MgO	11·212
K_2O	2·405
Na_2O	74·462

Dividing these figures by the respective molecular or atomic weights, and treating those for Cl and Br together as chlorine, we get after

* In connection with the preparation of the artificial sea-water I received constant help and advice from my colleague, Mr. D. J. Matthews. Without his ready assistance in connection with all chemical questions this investigation could hardly have been carried out.

† "Challenger" Report, Chemistry, Vol. I, p. 203.

reducing Na_2O to 100, the following figures, which give the relative number of molecules or atoms :—

Na_2O	100
K_2O	2·130
MgO	23·104
CaO	4·499
CO_2	0·519
SO_3	12·048
Cl	234·54

which gives the following molecular proportions for the bases and radicals separately :—

Na	100·0
K	2·13
Mg	11·55
Ca	2·25
CO_3	0·259
SO_4	6·024
Cl	117·27

If we use solutions of salts containing a gram molecular weight per litre, since 1 c.c. of each solution contains the same number of molecules, the relative number of c.cs., keeping the proportional amounts of the bases, the CO_3 and the SO_4 as above, and making the remainder chlorine, will be :—

NaCl	99·58
KCl	2·13
$CaCl_2$	2·25
$MgCl_2$	5·53
$MgSO_4$	6·02
Na_2CO_3	0·26

Since these figures give the number of molecules of *Na* somewhat too high, it was thought better to use 0·26 c.c. of sodium bicarbonate ($NaHCO_3$) instead of the normal carbonate, and this has been done throughout.

In making up artificial sea-waters it has been found most convenient to prepare first of all gram molecular solutions of each of the above salts and then to mix these in the proportions indicated. These molecular solutions are easily prepared and the strengths of the chlorides compared and corrected by titrating them with silver nitrate.

The following details of the preparation of the molecular solutions may be of assistance to future workers :—

Msol. NaCl. Kahlbaum's "Sodium chloride for Analysis." 58·5 grams dissolved in double-distilled water, and brought to 1000 c.c. at 15°C.

Msol. KCl. Kahlbaum's "Potassium chloride." 74·5 grams dissolved in double-distilled water and brought to 1000 c.c. at 15°C.

Msol. $CaCl_2$. Kahlbaum's "Calcium chloride cryst. for Analysis." About 300 grams were dissolved in about 1 litre of double distilled water. On titration with silver nitrate solution 2 c.c. of the above $CaCl_2$ solution required 30·3 c.c. of $AgNO_3$. 2 c.c. of Msol. KCl required 8·34 c.c. $AgNO_3$, so that 2 c.c. of Msol. K_2C_2 would require 16·68 c.c. $AgNO_3$. The $CaCl_2$ solution is therefore too strong in the proportion $\frac{30·3}{16·68} = 1·8166$. In order to get the Msol. $CaCl_2$ 1000 c.c. of the strong solution prepared must be diluted to 1816·6 c.c. This was done and the final solution again titrated against the Msol. KCl.

Msol. $MgCl_2$. Kahlbaum's "Magnesium Chloride for Analysis." As in the case of $CaCl_2$ a strong solution was first prepared, titrated with $AgNO_3$ and diluted with double-distilled water to the required extent, Msol. KCl being used as standard.

Msol. $MgSO_4$. Kahlbaum's "Magnesium Sulphate for Analysis." Crystallized magnesium sulphate has the formula $MgSO_4\ 7H_2O$, the molecular weight of which is 246·4. To make the molecular solution 246·4 grams of the salt were dissolved in double-distilled water and brought to 1000 c.c. at 15°C.

Msol. $NaHCO_3$. Kahlbaum's "Sodium Bicarbonate for Analysis." 84 grams dissolved in double-distilled water and brought to 1000 c.c.

In order to prevent the growth of moulds in the stock solutions these were all brought to the boil and kept in sterilized glass-stoppered bottles, the stoppers being tied down with a cap of parchment paper which was taken directly out of boiling water. When any of the solution was used,

the parchment cap was removed and placed in boiling water, the bottle was carefully opened and the amount of solution required poured out, the stopper being quickly replaced and tied down. These precautions are important, as the growth of mould in the solutions may have an important influence on the diatom cultures.

It has generally been found most convenient to make up the sodium chloride solution, of which large quantities are required, as it is wanted, and not to store it.

In the last table above the relative amounts (c.cs. of M solutions) of the different salts required to prepare the artificial sea-water are given. There remains to consider the actual salinity of the water which we are to employ, which is generally expressed as the weight in grams of the total salts contained in 1000 grams of the water. The salinity of natural sea-water in the western portion of the English Channel generally varies from about 35·5 to 35·0 per thousand, the water being generally lower in salinity near the coast. In laboratory experiments the water in the flasks becomes progressively more concentrated owing to evaporation, and a low salinity has therefore been adopted for the artificial sea-water used, namely, 35·0 per thousand.

The following table gives the composition of an artificial sea-water having a salinity of 35 per thousand, and with the salts in the relative proportions obtained above from Dittmar's analysis. The composition is stated (1) as the number of cubic centimetres of gram molecular solution contained in 1000 c.c. of the artificial water, and (2) as the number of grams of each salt contained in 1000 c.c.

	c.cs. of M. solution contained in 1 litre.	Grams per litre.
NaCl	480·80	28·13
KCl	10·28	0·77
$CaCl_2$	10·86	1·20
$MgCl_2$	26·70	2·55
$MgSO_4$	29·06	3·50
$NaHCO_3$	1·25*	0·11

To make up a litre of artificial sea-water the simplest procedure is therefore to weigh out 28·13 grams of sodium chloride, dissolve it in about half a litre of double-distilled water placed in a 1 litre flask, add the re-

* 2·6 c.c. was the amount generally used, as the increased alkalinity is favourable to diatom growth. See below.

quisite number of cubic centimetres of M solutions of the other salts (KCl 10·28, $CaCl_2$ 10·86, etc.) and then make the whole up to exactly 1 litre by adding double-distilled water.

Water prepared according to the figures given in this table was titrated for me by Mr. Matthews against the standard water supplied by the International Council, and was found to have a salinity of 35 per thousand.

Alkalinity.—The alkalinity has also been compared with that of sea-water from outside the Plymouth Breakwater by Sorensen's method, and was found to be very close to it, the artificial water being slightly less alkaline. It was found experimentally that better growths of diatoms were obtained when the alkalinity was increased somewhat,* the best result being obtained when an extra 1·33 c.c. of Msol. $NaHCO_3$ per litre was added, making a total of 2·6 c.c. of the molecular solution of this salt.

DIATOM CULTURES IN ARTIFICIAL SEA-WATER.

As was to be expected, it is not possible to obtain cultures of diatoms in the artificial sea-water prepared as described in the last section as it stands. The water must be first treated with nutritive solutions, and for this purpose the modifications of Miquel's solutions described in our former paper† have been used. Two solutions are employed as follows :—

Solution A.

Potassium nitrate 20·2 grm.
Distilled water to 100 c.c. } =2M KNO_3.

Solution B.

Sodium phosphate ($Na_2HPO_412H_2O$)	4 grm.
Calcium chloride ($CaCl_26H_20$)	4 ,,
Ferric chloride (melted)	2 c.c.
Hydrochloric acid (pure, concentrated)	2 c.c.
Distilled water	80 c.c.

To each 1000 c.c. of artificial water add 2 c.c. solution A and 1 c.c. solution B. Sterilize by bringing to the boil. When cool decant‡ off the clear

* Cf. Allen and Nelson, *loc. cit.*, p. 452 [*Q.J.M.S.*, p. 401]. The figure here given was derived from later experiments.

† Allen and Nelson, *loc. cit.*, p. 428 [*Q.J.M.S.*, p. 370]. For details as to the preparation of Solution B that paper or Miquel's original account should be studied.

‡ Filter papers should not be used to filter off the precipitate. They appear to contain some substance which inhibits the growth of the cultures. The cultures were made in wide-mouthed spherical glass flasks covered with glass capsules. Cotton wool plugs were not used, as these were found to be injurious to the growth of the diatoms.

liquid from the precipitate which is formed on the addition of solution B. The clear liquid is referred to in what follows under the name " artificial miqueled water."

It was found, however, that even after the addition of these two solutions very slight growth, if any, took place on inoculating with a small quantity (say one drop) of healthy *Thalassiosira gravida* culture. This was the case even after the addition of potassium bromide and iodide, or of Miquel's own solution A, which contains these two salts. If, on the other hand, the artificial miqueled water was inoculated with *a considerable quantity* of a culture of *Thalassiosira* in which natural sea-water had formed the basis of the culture medium, so that a considerable quantity of this natural water was transferred to the artificial medium, then the latter would develop a fine healthy growth. Experiences of this kind led me to suspect that the irregularities which had previously been met with in trying to make cultures in artificial media* might be due to varying amounts of natural sea-water introduced when inoculating. Definite experiments were therefore undertaken in order to ascertain whether the addition of natural sea-water to the artificial miqueled water would make it effective as a culture medium, and if so what proportion of natural sea-water was essential. *In all cases the culture medium was boiled after the addition of the natural sea-water and then allowed to cool before inoculation.*

As a result of these experiments it was shown that an addition of even 1 per cent of natural sea-water to the artificial miqueled water was sufficient to give very heavy cultures after inoculation with only one drop of *Thalassiosira* culture, and that with an addition of 4 per cent of natural sea-water better cultures were obtained than in any other culture medium known to me. This result has now been obtained so many times that it is in my opinion quite definitely established. If the proportion of natural sea-water added is reduced below 1 per cent smaller growths are obtained, and it is somewhat difficult to decide whether there is a definite minimum below which no growth takes place. An addition of 0·3 per cent of natural sea-water in one satisfactory experiment produced quite a heavy growth, whereas without this addition only a small growth was obtained. It has often been observed that whilst flasks containing 75 c.c. of artificial miqueled water show distinct signs of diatom growth when inoculated with one or two drops of a culture of *Thalassiosira* in natural sea-water, such flasks inoculated with one or two drops of a culture which has artificial sea-water as the basis of the culture medium show practi-

* Allen and Nelson, *loc. cit.*, p. 447 [*Q.J.M.S.*, p. 394].

cally no signs of growth at all, and remain quite clear. Since the above conclusions were reached it has been my practice in critical experiments always to inoculate from a culture in the artificial medium, so as to reduce the amount of natural water carried over on inoculation to a minimum.

From what has been said it seems clear that there is in natural sea-water some substance (or substances) not contained in the artificial water treated with Miquel's solutions, minute traces of which are essential to the growth of *Thalassiosira*. That the quantity present in the culture flasks after the addition of even 4 per cent of natural sea-water must be extremely minute is obvious from the fact that all substances which are present in natural sea-water in quantities beyond a mere trace are contained in the artificial culture medium. It becomes a matter of great interest and perhaps also of great importance to endeavour to find out what this substance may be, of which such exceedingly minute traces make all the difference between practically no growth at all and a vigorous and continued development of the diatoms, for the growths once started may go on increasing rapidly and healthily for several months.

The addition of many substances, both organic and inorganic, to the artificial miqueled water has been tried, generally in several concentrations, but up to the present no definite chemical compound has been found which can take the place of the 1 per cent of natural sea-water.

Of inorganic substances the following have been tried in different concentrations without result: Potassium bromide, potassium iodide (alone and with bromide), gold chloride, potassium nitrite, aluminium chloride, strontium chloride, lithium chloride and lithium carbonate.

It may be suggested that silica is the missing substance, but this seems precluded from the fact that all the experiments have been carried out in glass vessels, and the amount of silica which would go into solution from the glass would certainly be greater than that contained in the added 1 per cent of natural sea-water. Richter * has shown that diatoms grown in glass vessels obtain the silica they require from the glass.

In the course of the experiments it was found that the addition to the artificial miqueled water of a small percentage of sea-water from the tanks of the Plymouth Laboratory gave distinctly better cultures than the addition of the same percentage of natural sea-water brought in from outside. This comparison has been repeated a great many times, and the difference has been so marked and constant that I am compelled to regard it as firmly established. Different samples of sea-water brought

* Richter, O., *Verh. d. Gesell. deut. Naturf. u. Ärzte.*, Breslau, II, 1904, and *S.B.K. Akad. Wiss. Wien.*, CXV, 1906.

in from outside also appear to give somewhat different effects, and, although the experiments have not given sufficiently uniform results to justify a definite statement, I am left with the impression that on the whole samples of water taken from Plymouth Sound, when added to the artificial medium, give better growths than are obtained with samples from the English Channel in the neighbourhood of the Eddystone.

Now the tanks at the Plymouth Laboratory are worked on a closed system of circulation, the same water being circulated over and over again, so that the principal difference between the water taken from them and that obtained from outside consists in the greater abundance in the tank water of organic compounds, which result from the metabolism of living organisms. Is it the presence of some organic substance that is necessary for the growth of the diatoms ? A very large number of experiments have been made with a view to obtaining some light upon this question, and some of these will now be referred to.

Ulva infusion. A small piece of green seaweed (about 1 square cm. of *Ulva latissima*) was boiled for about five minutes in a flask containing 75 c.c. of artificial miqueled sea-water, and was then removed with a sterile platinum needle. In this way a weak organic infusion was obtained. When cold the flask was inoculated with one or two drops of *Thalassiosira* from a culture in artificial water. In this organic infusion a good growth was obtained, nearly equal to that in the control in artificial miquel plus 4 per cent of tank water. This experiment was repeated a number of times with a similar result.

Though it is most probable that the result is due to some organic compound the experiment is, of course, not conclusive, as an inorganic salt may have been dissolved from the ulva. In any circumstances we obtain no hint as to the nature of the organic substance, and the result remains indefinite.

It may be pointed out that Miquel * in his account of his original experiments on diatom cultures, insists upon the value of the addition of some organic infusion or maceration to his culture solutions.

Ulva Extract. A piece of *Ulva latissima* was washed in several changes of artificial sea-water and then an extract was made in absolute alcohol at a temperature of 58° C. The alcohol was evaporated to dryness on a water-bath. 75 c.c. of artificial miqueled sea-water was then boiled in small portions at a time in the vessel containing the extract, so that all soluble parts of the extract were dissolved. The water was then returned to a culture flask, which, when cold, was inoculated with *Thalas-*

* *Le Diatomiste*, I, 1890–3, p. 95.

siosira, as described in the experiment with ulva infusion. No growth was obtained in the flask.

Ulva Ash. A piece of ulva measuring about 5 cm. by 3 cm. was washed in several changes of double-distilled water. It was then put in a porcelain crucible, dried and heated over a bunsen burner till it was reduced to a white ash. The ash was added to a flask containing 75 c.c. of artificial miqueled sea-water, which was boiled, allowed to cool and inoculated with *Thalassiosira*, as in the two previous experiments. The result of the experiment was again negative.

Experiments with Hemimysis. In order to test whether the products of animal metabolism could immediately supply the substance sought for, the following experiment was carried out with *Hemimysis lamornæ* Couch, a small crustacean which lives in numbers in the Laboratory tanks. In the first experiment (Exp. 404) four *Hemimysis* were passed through two changes of Berkefeld filtered water, the animals being placed on a piece of filter paper to remove surplus fluid before being placed in each change of water. They were then passed in a similar way through two changes of artificial miqueled sea-water (75 c.c. was used altogether, being divided into two portions), and finally placed in a fresh quantity of the artificial miqueled sea-water (75 c.c.). They remained healthy and active and deposited a considerable amount of fæces on the bottom of the vessel. After they had been in the water four hours the *Hemimysis* were taken out and the water placed in a culture flask and brought to the boil. A control experiment with 75 c.c. artificial miqueled sea-water to which 3 c.c. of tank water had been added was set up and brought to the boil in the same way. On the following day both flasks were inoculated with two drops of a *Thalassiosira* culture. During the first week there was a very small growth of diatoms in the flask with the water in which the *Hemimysis* had been, which died out during the next few days. This growth was similar to that which usually occurs in artificial miqueled water to which nothing has been added. The control experiment to which 3 c.c. tank water had been added gave a very fine growth from the first, which persisted for at least five months. The result of this experiment was therefore negative. In another experiment, carried out in other respects in practically the same way, the *Hemimysis* were allowed to remain living in the water for twenty-four hours before they were removed. The result was again negative.

In a third experiment five *Hemimysis* lived for nineteen hours in 75 c.c. artificial miqueled sea-water to which 3 c.c. of tank water had been added. The animals were removed, the water boiled, and when cold inoculated as

before with *Thalassiosira*. A good growth resulted, showing that the animals do not excrete substances which completely inhibit the growth of the diatoms.

Evaporated Tank Water. A number of experiments were made in which a quantity of sea-water from the Laboratory tanks was evaporated to dryness on a water bath, the residue heated to different degrees, treated with strong, pure hydrochloric acid and evaporated two or three times to get rid of the acid, and then redissolved to the original volume in double-distilled water. After being neutralized by the addition of $NaHCO_3$, 4 per cent of the resulting solution was added to artificial miqueled sea-water, the resulting culture medium being boiled, cooled and inoculated with *Thalassiosira* in the usual way.

The results of these experiments are set out in summary form in the annexed Table A. In each case proper control experiments were set up at the same time, generally one with artificial miqueled sea-water to which nothing was added, and one with the same water to which 4 per cent of tank water was added, and the controls were boiled at the same time as the other flasks of the experiment.

As is seen from the table, five series of experiments were made. In the first (Series A) the salts obtained by evaporating the tank water were heated in a porcelain dish over a bunsen burner, the heating being carried out carefully so that the flame did not actually touch the dish, which never became anywhere near red hot. In Series B the evaporation and heating were done in a platinum basin, which was raised to a dull red heat over a bunsen. In Series C the salts were again evaporated and heated in a porcelain basin and made as hot as they could be with a bunsen burner, the flame of which played directly on the outside of the dish, and was moved about so as to heat different portions in turn. In Series D the salts were heated in a hot-air oven, being kept at a temperature of 164° to 170° C. for an hour. In Series E the heating was again carried out in a hot-air oven, a temperature of from 200° to 237° C. being maintained for two hours.

In Series A, D and E, where the heating of the residue was not excessive, quite good cultures resulted. Although they did not quite come up to the controls in which 4 per cent of tank water was added, they were in every case altogether of a different order from what took place in the controls in artificial miqueled sea-water to which nothing had been added.

In the other two series, B and C, where the degree of heating was much greater, in most cases the culture was an entire failure, and in those

TABLE A, showing the results of experiments, in which 4 per cent of tank water, which had been evaporated, heated, and redissolved, was added to artificial miqueled sea-water. The number of the experiment and date of inoculation are given in each case. The cultures were inoculated with *Thalassiosira gravida.*

Evaporated Tank Water. Date of Preparation and Degree of Heating.								
A. Prepared July 11th, 1912. Heated carefully over bunsen in porcelain basin. It never became red hot.	399. D. 13. VII. 12. Good growth not up to controls.			431. D. 18. XI. 12. Good growth equal to or better than control.	433. A. 8. I. 13. Good growth, not up to controls.	455. A. 22. VIII. 13. Good growth, not up to control.	460. A. 9. IX. 13. Good growth, nearly up to control.
B. Prepared Aug. 29th, 1912. Heated over bunsen in a platinum basin to a dull red heat.	407. L. 31. VIII. 12. No growth.	407. M. 31. VIII. 12. (More alkaline than L.) No growth.	408. E. 4. IX. 12. No growth.	431. E. 18. XI. 12. Small growth, but some good chains.	433. B. 8. I. 13. Good growth, not up to A.	455. B. 22. VIII. 13. Below the control in which nothing was added to the artificial miquel.	460. B. 9. IX. 13. Some growth at first. It then went off entirely.
C. Prepared Sept. 26th, 1912. Heated over bunsen in porcelain basin. Made as hot as possible with the flame playing directly on the outside of the dish.			424. G. 18. X. 12. No growth.	431. F. 18. XI. 12. No growth.	433. C. 8. I. 13. Very small growth.	455 C. 22. VIII. 13. Below control, in which nothing was added to the artificial miquel.	460. C. 9. XI. 13. At first the worst of the series, then a fair growth, but below A, D, and E.
D. Prepared Jan. 2nd, 1913. Heated in an oven to 164°–170° C. for 1 hour.					433. D. 8. I. 13. Moderate, healthy growth, better than B.	455 D. 22. VIII. 13. Good growth not up to A.	460. D. 9. IX. 13. Good growth, nearly as good as control.
E. Prepared Jan. 4th, 1913. Heated in an oven to 200°–237° C. for 2 hours.					433. E. 8. I. 13. Moderate, healthy growth, as D.	455. E. Good growth not up to A.	460. E. 9. IX. 13. Good growth, nearly as good as control.

instances in which some growth was obtained it was distinctly below that of cultures of the former series made at the same time.

A study of Table A can, I think, leave no doubt that the general statement is justified that whatever the substance may be which occurs in tank water and the addition of which to artificial miqueled sea-water enables the latter to support a vigorous diatom growth, that substance may be dried and heated to a moderate degree without greatly impairing its efficacy, whilst if it is heated to too high a temperature its efficacy tends to be destroyed.

The experiments are consistent with the theory that the substance is an organic compound, but one of a very stable kind, which is only decomposed with difficulty.

Addition of organic substances to artificial water. Many experiments have been made by adding organic substances in a number of different concentrations to artificial miqueled sea-water, but by none of these has any marked or constant effect been produced upon the growth of *Thalassiosira*. It will be understood, of course, that such negative results are in no way conclusive, as in a case of this kind the attainment of an exactly correct degree of concentration may be essential, and when one is working quite without clue it is hardly possible to carry out a sufficiently extensive series of experiments with every substance, especially when two or three weeks must elapse before the result of any experiment becomes definite. The following substances have been tried : asparagin, calcium succinate, calcium malate, sodium salicylate, theobronine, leucine, tyrosine * (the three latter alone and together with atropine),† peptone, urea and uric acid. In all cases the result was negative.

Putrified Peptone. An isolated result which I have entirely failed to repeat in spite of many attempts may be worth putting on record as a hint for future work, but no other importance should be attached to it. Starting from the idea that the substance sought for might be one of the ultimate products of the breaking down of organic matter under the influence of bacteria, since it appears to be more abundant in the tank water of the Laboratory than in sea-water from outside, the following

* In consequence of the work of Thornton and Geoffrey Smith on Euglena (*Proceed. Roy. Soc.*, B., Vol. LXXXVIII, p. 151, 1914) special attention was given to tyrosine, and a arge number of different concentrations were tried. Entirely negative results were, however, obtained.

† The use of these three substances alone and with atropine was suggested by the work of H. C. Ross on "Auxetics." See H. C. Ross, *Induced Cell-Reproduction and Cancer*, London, J. Murray, 1910 ; *Further Researches into Induced Cell-Reproduction and Cancer*, I and II, London, J. Murray, 1911 and 1912.

experiment was carried out. 100 c.c. of a 1 per cent solution of peptone in artificial sea-water was sterilized by boiling on successive days. When cold it was inoculated by adding two drops of tank water. Under the influence of the bacteria of the tank water putrefaction set in and was allowed to continue for nineteen days. The solution was then again boiled. To 75 c.c. of artificial miqueled sea-water three drops of the putrified peptone solution were added, and the flask boiled, and when cold inoculated with two drops from a culture of *Thalassiosira* in artificial miqueled water plus 4 per cent of outside sea-water. At first the water in the culture flask became milky from the growth of bacteria, but this milkiness gradually disappeared and the diatoms commenced to grow, giving finally an excellent culture which was quite up to the control. I do not think there was any flaw in the actual carrying out of the experiment, but, as already mentioned, a number of attempts to repeat it all gave negative results.

A final point may be mentioned, which also seems to suggest some organic substance as the missing factor which the artificial miqueled sea-water must contain before it will sustain a vigorous growth of the diatoms. It has been noticed that artificial miqueled sea-water which has been kept for some weeks gives (without any addition of natural sea-water) more growth than does similar water used within a few days of being prepared. Plate-culture tests have shown that such water after a few days develops bacteria, and it is possible that the products of the metabolism of these bacteria are able to help the growth of the diatom.

The Omission of Miquel's Solutions. If 4 per cent of tank water (i.e. water from the Laboratory tanks, which are worked on a close system of circulation *) be added to artificial sea-water, made according to the formula already given, but to which neither of the Miquel solutions is added, a good growth will result after sterilization and inoculation with *Thalassiosira*. This growth may for the first week or two be quite as good as a similar culture to which the Miquel solutions have been added, but it will not continue healthy for as long as the latter, so that the total growth will be less. It is interesting to note that the mere dilution of the tank water with pure artificial sea-water produces an increase of growth, for the amount of growth obtained in say 100 c.c. of sterilized tank water is less than that obtained in a mixture of 96 c.c. of artificial sea-water with 4 c.c. of sterilized tank water. This is partly explained by a difference in alkalinity, but it also suggests that the tank water contains not only an abundance of the food sub-

* Cf. Allen and Nelson, *loc. cit.*, p. 430, *et seq.* [*Q.J.M.S.*, p. 373].

stances which the diatoms require, but also substances which in higher concentrations are detrimental to growth, whereas in low concentrations their inhibitory action is reduced or disappears.

CHANGES IN THE COMPOSITION OF THE ARTIFICIAL SEA-WATER.

A series of experiments was made to ascertain to what extent the composition of the artificial sea-water could be changed without affecting the growth of *Thalassiosira*, and it was found that, provided 4 per cent of natural sea-water were added, the various constituents of the artificial water might be varied to a surprising extent without in any way retarding the growth. Only those results are included here which were quite marked and definite. Other variations in composition were tried, but an account of these is reserved until the experiments have been repeated and extended.

Varying the Amount of Magnesium Sulphate. A series of flasks was set up, the basis of the culture medium in each being artificial sea-water prepared according to the table on p. 424, the quantity of magnesium sulphate being varied. The full amount of alkali favourable to diatom growth was added (i.e. 2·6 c.c. of M.$NaHCO_3$ per litre), together with the usual quantities of 20 per cent KNO_3 and Miquel's solution B (Na_2HPO_4; $CaCl_2$; $FeCl_3$; HCl) and 4 per cent of natural sea-water. The series contained (*a*) no magnesium sulphate, (*b*) $\frac{1}{4}$ the normal amount, (*c*) $\frac{1}{2}$ the normal, (*d*) $\frac{3}{4}$ normal, (*e*) the normal amount, i.e. 29·06 c.c. of M.sol. per litre, (*f*) $1\frac{1}{4}$ times the normal and (*g*) $1\frac{1}{2}$ times the normal. All the flasks were inoculated in the same way with *Thalassiosira gravida*. During the first month all the flasks gave excellent growths, and it was not possible to distinguish between them. At the end of three months (*a*) and (*b*) had gone off more than the others, and (*f*) and (*g*) were not quite up to (*c*), (*d*) and (*e*). A repetition of (*a*) to (*e*) again gave the same result, the cultures being particularly large and healthy. In speaking of this result, it must be remembered that although the only sulphur present in (*a*) was that introduced in the 4 per cent of natural sea-water a considerable amount of magnesium was present as magnesium chloride.

Varying the Amount of Calcium Chloride. Another series of experiments was made in every respect similar to the last, excepting that the calcium chloride in the artificial water was varied instead of the magnesium sulphate, which remained normal: (*a*) contained no calcium chloride,

(*b*) $\frac{1}{4}$ normal amount, (*c*) $\frac{1}{2}$ normal amount, (*d*) $\frac{3}{4}$ normal amount, (*e*) the normal amount, i.e. 10·86 c.c. M.sol. $CaCl_2$ per litre, (*f*) $1\frac{1}{4}$ times the normal amount, (*g*) $1\frac{1}{2}$ times normal.

(*a*) During the first week showed little sign of growth and was far behind the others. At the end of a month, however, there was quite a good growth, still very healthy, but the quantity was far below that in (*c*), (*d*), (*e*), (*f*) and (*g*).

(*b*) Small growth during the first week and remained always better than (*a*), but never equal to (*c*), (*d*), etc.

(*c*) Fair growth during first week and went on well, though the quantity was never up to (*d*), (*e*), etc.

(*d*) The growth was nearly equal to the normal (*e*) throughout, and at the end of a month it was not possible to distinguish between the two.

(*e*) A fine healthy growth with long chains.

(*f*) About the same as (*d*) throughout.

(*g*) About the same as (*d*) and (*f*) throughout.

A repetition of (*a*) to (*e*) gave just the same result. In connection with this series it must be noted that Miquel's B solution contains $CaCl_2$, so that the amount of Ca present in (*a*) will be that contained in the 4 per cent of natural sea-water, plus that contained in the Miquel B.

Varying the Amount of Potassium Chloride. An exactly similar series was set up in which the potassium chloride was varied from 0 to $1\frac{1}{2}$ times the normal. All these gave very fine growths, of which the last two ($1\frac{1}{4}$ and $1\frac{1}{2}$ times normal) were the best during the first week. Subsequently it was not possible to distinguish between the amounts in the different flasks. This result was also confirmed by a second experiment.

It should be remembered that potassium was added as nitrate in this as in the other experiments (2 c.c. of a 2 M.sol. KNO_3 per litre).

Variations in Salinity. It was shown in our previous paper * that in the case of *Skeletonema costatum*, *Biddulphia mobiliensis* and *Coscinodiscus excentricus*, plankton diatoms of very similar habit and distribution to the species *Thalassiosira gravida* chiefly used in the present experiments, the salinity of the culture medium could be varied within wide limits without greatly affecting the growth of the diatoms. Thus between 35 and 40 per cent of the water could be evaporated from a culture medium having natural sea-water as its basis without seriously affecting the growth of the diatoms, whilst dilution of the culture medium up to 100 per cent

* Allen and Nelson, *loc. cit.*, p. 453 [*Q.J.M.S.*, p. 402].

also made no appreciable difference. Even when the dilution was extended to 200 per cent a fair quantity of growth took place.

The following experiment was made in order to test the same point on *Thalassiosira gravida*.

Experiment 476. Artificial sea-water was made up with the normal relative proportions of salts, but of double the normal strength. A series of dilutions was then prepared, doubly distilled water being added in the proportions stated :

	Artificial sea-water, double strenth. c.c.		Doubly distilled water added. c.c.	
A	100	+	0	
B	100	+	25	
C	100	+	50	
D	100	+	75	
E	100	+	100	*Normal*
F	100	+	125	
G	100	+	150	
H	100	+	175	
J	100	+	200	

The right quantities of Miquel's solutions were added to each, and 4 per cent of sea-water from the Laboratory tanks. Flasks were then inoculated with three drops each of *Thalassiosira gravida* culture. No growth took place in A and B. Excellent, healthy growths with good chain formation took place in all the others. E and F were best, and one as good as the other. G and D were excellent growths, but the quantity at any time was less than in E and F. In C, H and J, although the growths were quite good the quantity was considerably less than in E and F, that in C also being less than in H and J.

It will thus be seen that very considerable changes in the salinity of the culture medium can be made without much effect being produced on the growth of *Thalassiosira*. Dilution of the medium is less detrimental than concentration.

The experiments described in this section show how wide the variation in the chemical composition of the culture medium may be without any very marked effect being produced on the growth of the diatoms. The difficulty in growing the diatoms in artificial sea-water is clearly not due, as at one time I thought might be the case, to the fact that a very delicate balance between the amounts of the different salts is

necessary and that this balance had not been attained sufficiently exactly in preparing the solutions. It is quite clear that the artificial sea-water lacks some substance which occurs in natural sea-water, and that a very small trace of this substance is sufficient to make the difference between a considerable and continued growth of the diatoms and practically no growth at all.

GENERAL CONSIDERATIONS.

Several instances have recently been described which seem to show that in food material used to support animal life the presence of minute traces of particular organic substances is essential, if the food material is to maintain the animal body in a healthy state.

The work of Leonard Hill, M. Flack, G. Hopkins and Casimir Funk * has shown that in the outer layers of wheat and rice there is an active principle which is of essential importance to their value as food material. Young rats and mice would not live when fed exclusively upon white flour in the preparation of which the outer layers of the wheat had been removed, whilst those fed on whole meal flour did much better. Pigeons could be successfully fed on bread made of white flour to which an extract of bran and sharps had been added, but when fed on pure white bread all died. Polished rice from which the husk has been removed in the process of polishing, when used as an exclusive diet, produces the disease known as beri-beri. Cooper and Casimir Funk † were able to isolate from rice polishings a substance to which they gave the name vitamine, which effected a rapid cure when given to pigeons suffering from beri-beri. The same substance was obtained from yeast, from milk and from bran.

Hopkins ‡ has shown that young rats do not grow on an artificial diet composed of pure protein, starch, cane sugar, lard and inorganic salts, but if quite a small quantity of natural milk is added to the diet they thrive.

Thornton and Geoffrey Smith § have shown that strong growths of *Euglena viridis* in culture media prepared according to Miquel's formula are produced when in place of the organic matter used by Miquel slight traces of amido acids are added to the solutions of inorganic salts. Tyrosin in the proportion of 1 in 24,000 gave an optimal growth. The authors

* A summary of this work, as described at the meeting of the British Association in Dundee (1912), will be found in *Science Progress*, January, 1913, pp. 423–5.

† *The Lancet*, Nov. 4th, 1911, p. 1266.

‡ *Journal of Physiology*, Vol. XLIV, 1912, p. 425.

§ *Proceed. Roy. Soc.*, B., Vol. LXXXVIII, p. 151, 1914.

suggest that the amido acid acts as an auxiliary or stimulant rather than as the main source of nutrition. This view is similar to that taken by H. C. Ross in his work on *Induced Cell-Reproduction and Cancer*, to which reference has already been made (see p. 432).

It would seem that the plankton diatoms, the culture of which has been considered in the present paper, show a phenomenon of a similar character to those just mentioned. The minute trace of substance added to the culture medium in the small percentage of natural sea-water would seem to act as a catalytic agent, initiating the processes of metabolism but not being itself used up.

The experiments may also help to throw light upon what takes place in the sea. It is well known that the waters of the open ocean far from land support a much smaller proportion of plant and animal life than is to be found in coastal waters. On the other hand, in regions where a current of coastal water meets and becomes mixed with a current of ocean water conditions are produced which are specially favourable to a luxuriant growth of animal and vegetable life. This is shown in the first place in the very rich character of the plankton, and as a consequence of the abundant plankton we find a rich fauna of bottom living organisms and of fishes of different kinds. This is in agreement with the observation recorded in the present paper that a small quantity of natural sea-water of an inshore type (tank-water) mixed with a large proportion of pure artificial sea-water gives a good culture medium for the plankton diatoms. There is reason to hope therefore that culture experiments may in time throw additional light upon the general questions relating to the production of animal life in the sea, questions which are of immediate importance to a study of the productivity of the fisheries.

SUMMARY.

1. Attempts to obtain good cultures of *Thalassiosira gravida* in a purely artificial medium, made by dissolving in doubly distilled water Kahlbaum's pure chemicals in the proportions in which the salts occur in sea-water, adding nitrates, phosphates and iron according to Miquel's method and sterilizing the medium, have not succeeded.

2. If, however, a small percentage of natural sea-water (less than 1 per cent will produce a result) be added to the artificial medium and the whole sterilized excellent cultures are obtained, which are often better than any which have been got when natural sea-water forms the foundation of the culture medium.

3. The result appears to be due to some specific substance present in minute quantity in the natural sea-water which is essential to the vigorous growth of the diatoms. The nature of this substance it has not been possible to determine, but some evidence seems to suggest that it is a somewhat stable organic compound.
4. Provided the 1 per cent of natural sea-water is added, the various constituents of the artificial sea-water forming the basis of the culture medium can be varied in amount within wide limits. The salinity of the medium can also be considerably altered without serious detriment to the cultures.
5. The experiments recorded are of interest as furnishing another instance of the importance in food substances of minute traces of particular chemical compounds. They may also eventually throw light upon the nature of the conditions in the sea which are specially favourable to the production of plant life and therefore also of the animal life which that plant life sustains.

ADDENDUM.

Since the above was printed a paper has been published by Prof. W. B. Bottomley on " Some Accessory Factors in Plant Growth and Nutrition " (*Proceed. Roy. Soc.*, B., Vol. LXXXVIII, p. 237, Sept., 1914), in which it is shown that a minute trace of an organic substance, which is formed by the action of aërobic soil bacteria upon peat, acts as a powerful stimulant to the growth of plants and of nitrogen-fixing bacteria. Following the method of Cooper and Funk for obtaining " vitamines " from rice polishings, namely, by precipitating by phosphotungstic acid from an aqueous solution of the dry residue from an alcoholic extract, Bottomley has succeeded in obtaining from the bacterized peat a substance which is quite as powerful a stimulant to plant growth as the original alcoholic extract of the bacterized peat. This substance, as in the case of Funk's vitamines, can be further purified by precipitation with silver nitrate and baryta, the resulting substance being an effective growth stimulant.