LXV. ON THE CULTURE OF THE MARINE DIATOM *NITZSCHIA CLOSTERIUM* (F.) *MINU- TISSIMA*, IN ARTIFICIAL SEA-WATER.

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IT was shown by Miquel [1892] that the culture of diatoms, either fresh-water or marine, may be successfully carried out in the laboratory if certain nutrient elements, present in the form of salts, are added to the water, sea-water or distilled. These elements are nitrogen, phosphorus, sulphur, potassium, calcium, magnesium, iron, silicon, sodium, bromine and iodine. No particular stress was laid on the necessity for the last three though they were always added. Miquel, moreover, emphasised the necessity for the addition to the medium of some organic nutrient such as sterilised macerations of bran or straw, though this is not in accordance with the general idea of the metabolism of green plants. Allen [Allen and Nelson, 1910] stated that the omission of the addition of such infusions to natural sea-water did not affect the amount of growth obtained in the case of the plankton diatoms. He showed [1914], however, that good cultures of the marine plankton diatom *Thalassiosira gravida* Cleve, cannot be grown in an artificial sea-water even with the addition of nitrates, phosphates, and iron. The artificial sea-water was made by dissolving in doubly distilled water Kahlbaum's pure chemicals in the proportions in which the salts occur in natural sea-water. The bromine and iodine ions were, however, replaced by an equivalent amount of the chlorine ion. The addition of 1 % natural sea-water, and in some cases less, produced excellent cultures. A number of substances both organic and inorganic, the latter including potassium bromide and iodide, were added to the artificial medium but no substance was found to replace the 1 % natural sea-water. On the evidence that the active principle appeared to be destroyed by heat, Allen suggested that it was probably of an organic nature. Such a factor would appear to be comparable with the "auximones" postulated by Bottomley [1914 and later papers]. It was with the object of identifying and isolating this substance that the work reported in this paper was undertaken.

EXPERIMENTAL.

The procedure adopted in the preparation of the artificial medium, and in the culture of the diatoms, was, with a few modifications, essentially that described by Allen and Nelson [1910]. The water used in the preparation of the artificial sea-water was prepared in the following way. Distilled water from an ordinary copper-still was refluxed with alkaline potassium permanganate for 24 hours, filtered through an ordinary filter-paper to remove any manganese dioxide, and re-distilled in an all-glass distilling apparatus fitted with two Kjeldahl traps to prevent any mechanical carrying over of substances in the form of spray into the distillate. The first 100 cc. of each distillate was always rejected. Very great care was taken to protect this water from contamination by dust particles in the laboratory air. Glass or silica apparatus, freed from possible organic matter by boiling in chromic acid, was used throughout the experiments. All vessels thus cleaned were rinsed thoroughly with ordinary distilled water and finally with the water prepared as above.

The chemicals used in the preparation of the artificial sea-water were either Kahlbaum's chemicals "Pure for Analysis," or "Analytical Reagents" from the British Drug Houses.

The composition of the sea-water used by Allen was based on Dittmar's analyses in the "Challenger Reports" and was as follows:

	Cc. of molecular solution contained in one litre	G. per litre		Cc. of molecular solution contained in one litre	G. per litre
NaCl	480.80	28.13	MgCl ₂	26.70	2.55
KCl	10.28	0.77	MgSO ₄	29.06	3.50
CaCl ₂	10.86	1.20	NaHCO ₃	1.25	0.11

The artificial sea-water used by us in this work was prepared in these proportions with the exception of the sodium bicarbonate, of which 0.22 g. per litre was used in accordance with Allen's observation that increased alkalinity is favourable to diatom growth. This observation has been confirmed by us. Approximately molecular solutions of calcium and magnesium chlorides were prepared and their exact strength determined volumetrically. These solutions were kept sterile in glass-stoppered flasks. Whenever the flasks had been opened they were always re-sterilised by being heated slowly to 100°, with the stoppers loosely inserted, stoppered, and the stoppers tied down with sterilised parchment. By this means the two stock solutions were kept sterile with very little change in concentration. It had previously been observed that filamentous growths appeared in unsterilised solutions, even of molecular concentration, kept at 1-2° for some weeks. These were either of a siliceous or fungal origin [Moore, 1915]. Hence it was deemed advisable to avoid the use of stock solutions wherever possible. With the exception of the calcium and magnesium chlorides, which could not be weighed out accurately, the dry salts for each litre of artificial sea-water were weighed out as required.

To the artificial sea-water was now added Allen's modification of Miquel's two nutrient solutions.

<i>Solution A.</i>	KNO ₃	20.2 g.
	Distilled water to 100 cc.	
<i>Solution B.</i>	Na ₂ HPO ₄ · 12H ₂ O ...	4 g.
	CaCl ₂ · 6H ₂ O	4 g.
	Fe ₂ Cl ₆ · 12H ₂ O—melted ...	2 cc.
	HCl (pure, concentrated) ...	2 cc.
	Distilled water	80 cc.

These solutions also were kept sterilised. To each litre of artificial sea-water 2 cc. of solution *A* and 1 cc. of solution *B* were added, and the whole was then boiled gently for 15 minutes, when the precipitate of ferric, calcium and magnesium phosphates flocculated, and settled to the bottom of the flask. This precipitate was removed by careful decantation; we have confirmed Allen's statement that if this precipitate is removed by filtration through ordinary filter-paper the filtrate will no longer support diatom growth. No information as to the cause of this has been obtained; it is thought to be due to some toxic substance in the filter paper. The precipitate may be successfully removed by filtration through glass wool.

Following Allen's technique, the cultures were made in "carbon dioxide" flasks of 100–150 cc. capacity; the necks of the flasks were kept covered with closely fitting inverted crystallising dishes. Experiment has shown that the chance of contamination from the outside air of cultures kept under these conditions is small. About 50 cc. of liquid was placed into each sterilised flask and cover and the whole re-sterilised at 95° for 15 minutes on three successive days, with little change in concentration. Inoculation was carried out by means of a glass pipette drawn down to a fine capillary and delivering 0.01 cc. Throughout this work the usual precautions were observed to prevent contamination of cultures by bacteria and moulds.

Inoculations of *Nitzschia closterium* (*f.*) *minutissima*, made into artificial sea-water with the addition of the nutrient salts as described above, gave excellent cultures which were as good as, and, in a few cases, better than the control cultures in natural sea-water which had also been treated with Miquel's solutions *A* and *B*. The cultures in artificial sea-water contained 0.02 % of natural sea-water introduced at the inoculation, and therefore growth might have been due to Allen's factor. In order to reduce the concentration of natural sea-water to a negligible degree the following series of experiments have been repeatedly carried out.

A number of flasks containing the artificial medium were prepared and inoculated; immediately afterwards and from the same parent culture an equal number of flasks containing the nutrient medium prepared with natural sea-water were similarly inoculated. The flasks were observed daily. At the end of one week in summer definite growth had appeared in all the flasks, which

were kept on a table in front of a window facing north. Two weeks later there was heavy growth in all the flasks. Each culture was then sub-cultured into a fresh sample of its own medium and the growth again carefully watched. Similar procedure in sub-culturing was carried out a second, third, and fourth time. Although certain divergences from the average amount of growth in flasks of both series were noticed from time to time, the cultures in the artificial medium could never be distinguished from those in the medium which had for its basis natural sea-water. Good growth was always obtained in all the flasks. It is to be pointed out that inoculation with 0.01 cc. of a parent culture does not ensure the transference of a constant number of organisms. This might possibly account for slight differences in the amount of growth obtained.

We have not therefore obtained any indication of the presence in natural sea-water of a specific substance, essential to the vigorous growth of the diatom used. If there be such a compound, other than silica and the usual salts necessary for plant growth, then it must have been derived in our artificial medium either from the salts used in its preparation or from the atmosphere. This does not exclude the possibility of the "allelocatalytic effect" as designated by Brailsford Robertson [1923] being present, although Cutler and Crump [1923] failed to obtain this effect with a culture of *Colpidium colpoda*. It is to be pointed out that extreme precautions were always taken to avoid contamination of the medium by organic matter even from the dust of the air. The salts used were always the purest that could be obtained though they were not further purified by us.

One further experiment was carried out with regard to the possible existence in sea-water of such a substance specific to the growth of *Nitzschia*. As already stated, Allen, who postulates its existence for the growth of *Thalassiosira* suggests that it is destroyed by heat. To test this, 17 g. of powdered sea-salt, made by evaporating natural sea-water, was heated strongly over a blow-pipe flame in a covered silica crucible for 15 minutes. This amount of salt corresponds to half a litre of sea-water. The bulk of the fused salt was removed by means of a platinum spiral; the fused salt was dissolved as far as possible in the specially prepared water; pure concentrated hydrochloric acid was then added at intervals 0.01 cc. at a time until the solution gradually became quite clear. It was then diluted to 250 cc.; half of this volume was then titrated to neutrality with $N/50$ caustic soda made from pure caustic soda and the "organic-free" water. An equal volume of caustic soda, actually 11.0 cc. was added to the remaining 125 cc., the solution diluted to 250 cc., the nutrient solutions added, the whole boiled to flocculate the insoluble phosphates, the p_H adjusted to 8.0, and the medium sterilised. Great care was taken throughout the various operations performed to avoid contamination of the liquid. This medium gave equally good cultures of the diatom *Nitzschia* as the two control media made from natural and artificial sea-water respectively.

The culture used was a pure one in that it contained only one species of

diatom, it contained however certain common bacteria (*Staphylococcus albus*, *Staphylococcus aureus*, and *B. subtilis*) and moulds, though the latter were very rare. It is obvious that work of this nature should be undertaken with a culture containing absolutely no other organism than the one concerned. Miquel claimed to have prepared cultures of diatoms in a state of absolute purity; Allen has shown the difficulty of such a task and did not succeed in obtaining such a culture. So far our attempts have been unsuccessful though we have been able to obtain parent cultures for inoculation where the number of bacteria present per cc. has been extremely small, of the order of 200 per cc. This has been accomplished by washing the cultures with sterile sea-water in a tall narrow sterilised vessel. The diatoms, which are relatively heavy, easily sink to the bottom of the vessel. Bacterial counts performed on healthy cultures have shown us that the bacterial contamination was extremely small. The failure to prepare an absolutely pure culture of the diatom concerned invalidates the conclusion that the diatom used is definitely autotrophic, as Bottomley [1914] states that *azotobacter* has the power of elaborating products which greatly increase the growth of *Lemna* plants, which cannot thrive in a culture containing only mineral salts. It seems improbable, however, that the bacterial contamination in our cultures was ever great enough to act in this way. Further investigation is being carried out with reference to this point.

SUMMARY.

No evidence has been obtained of the existence in natural sea-water of an organic substance specific to the growth of the diatom *Nitzschia closterium* (*f.*) *minutissima*. This organism appears to be able to grow and thrive in an inorganic culture medium.

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