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of the horizontal part, or in other words solid mixed crystals were obtained, the *E M F* of which was different from that of an amalgam represented by the horizontal line and which reached equilibrium much less rapidly than the semi-liquid amalgams.

Microbiology. — *"Further Researches concerning Oligonitrophilous Microbes."* By Prof. M. W. BEIJERINCK.

In my first paper on oligonitrophilous microbes [1]) I still left the question unanswered after the forms which develop in the light, in nutrient liquids, which only contain traces of nitrogen compounds, and whose nutrition with carbon can only be effected from the carbonic acid of the air.

The experiments to answer this question were made as follows. Large flasks were plugged with cotton wool or filtering paper, so that the air has free access, or closed in such a way that the air could be renewed, and that, at each renewing, it must pass through strong sulphuric acid in order to be deprived of the nitrogen-compounds. These flasks had been half filled with

100 Tap- or distilled water
0.02 $K^2 H PO^4$

and infected with a not too slight quantity of garden-soil, e. g. 1 to 2 grs. per liter [2]).

They were placed in winter at a window on the south, in spring and in summer on the north-west, and in the beginning they were now and then shaken, in order to sink the floating film of calcium-phosphate, which forms at the surface.

As the rate of nitrogen and carbon compounds is too slight to cause any appreciable development of colourless microbes, no further cloudiness results, but that of the easily precipitating phosphate. But in winter after six to eight, in summer after four to five weeks, *a characteristic flora develops consisting of some species*

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[1]) These Proceedings of March 30, 1901.

[2]) The Delft tap-water contains at present 0.42 mG. nitrogen per L., the garden-soil used 0.56 pCt. nitrogen (analyses of Mr. A. v. DELDEN); but this nitrogen can only for a minimal portion (as ammonia and nitrate-nitrogen) be assimilated by microbes. The oligonitrophili themselves possess the specific faculty of feeding on the nitrogen from the atmosphere.

of Cyanophyceae, which, once become visible, can promptly give rise to a deep bluish-green colouring of the liquid. In the beginning these Cyanophyceae are seen to develop as free colonies at the sides of the flask, later there also appear floating films, which latter consist chiefly of *Anabaena*, while among the colonies growing on the glass-wall, not only the large flat colonies of *Anabaena*, but likewise the characteristic, but rarer bluish-grey slimy lumps of *Nostoc paludosum* are most striking. A third, very intensely coloured species, which is nearly as common, I determined as *Nostoc sphaericum* [1]).

Motile Cyanophyceae, such as *Oscillaria*, do not result under these conditions, or only in much smaller numbers than those mentioned; probably for them the proportion of organic substances in the said nutrient liquids is still too large and that of nitrogen compounds perhaps too slight. I have also found that *Oscillaria* is microaërophilous [2]) in the dark, so that, at the places fit for its development, at least temporary anaërobiosis should be possible, which is not the case in my experiment.

Chlorophyceae, especially *Chlorococcum* and *Chlorella* are, as might be expected, not wholly absent in these cultures; but their number is so small that they are without any influence on their external character. This fact is the more remarkable because, if to the culture fluid is added

0,02 pCt. $NH^4 NO^3$

already after a shorter time than the above mentioned, a dense film of Chlorophyceae, in which *Chlorococcum infusionum* is the principal species, grows rapidly on the surface. Only when the nitrogen-compounds added to it have been quite consumed, the green film grows darker, as then again flakes of *Cyanophyceae*, in particular of *Anabaena*, begin to form.

The experiments have essentially the same course when the tap-water-phosphate flasks are not infected with garden-soil, but with a small flake taken from a previous culture of Cyanophyceae. Here I saw however, in some cases appear *Anabaena* only, which under these conditions of culture evidently supplanted the other Cyanophyceae.

If in my experiments I use Delft canal-water, instead of tap-water, and omit the infection with garden-soil, the process is some-

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[1]) Not all the species of Cyanophyceae obtained could be determined. Some of them I think have not been described.

[2]) It is macroaërophilous in the light.

what different. First a rich light-brown culture of *Diatomaceae* takes rise, in which here and there colonies are seen of Chlorophyceae belonging to the genera *Raphidium*, *Scenedesmus*, *Chlorella* and *Chlorococcum*, but without their multiplying sufficiently to alter the brown colour of the culture. After 8 to 9 weeks however, the colour at once grows darker by the then occurring increase of the Cyanophyceae, which increase continues a long time, evidently as long as there is a sufficient quantity of kalium phosphate and other mineral food.

I think the result of this last experiment should be explained as follows. Canal-water contains a greater amount of organic substances than the tap-water cultures; as long as these substances are present the Diatoms are prevailing; they use these substances for their carbon-nutrition, together with the carbonic acid from the air, and at the same time assimilate the nitrogen-compounds. When these are consumed the Cyanophyceae appear.

That the Diatoms can in fact utilise a fairly high rate of organic substances, is well known to the students of that group. The following experiment which, to my knowledge, has not yet been described, proves that the Diatoms are the very coloured microbes, which can, if not assimilate, at least tolerate without injury the full rate of organic matter and of nitrate- and ammonia-nitrogen of fertile garden-soil.

A high glasscylinder is filled for one half with garden-soil, for the other with pure water. After shaking the thus obtained mud is allowed to stand at a sunny window. After some days or weeks, according to season and temperature, one sees at the illumined side of the glass a deep brown film appear, consisting of the Diatoms present in the garden-soil, which slowly creep towards the light. This film increases some months by the multiplication of the Diatoms, but finally there appear in it large green spots of various lower Chlorophyceae, whose propagation becomes only vigorous, when the Diatoms and other microbes (such as bacteria and monads) have for the greater part used the assimilable organic substances and converted them into unassimilable material. Cyanophyceae do not grow under these circumstances, this being prevented by the abundance of nitrogen-compounds in the garden-soil.

Though it is certain, that the flora of Cyanophyceae in my tap- and canal-water experiments only develops with an extremely small proportion of organic matter in the food, I still consider this proportion to be of an essential signification for the experiment. I have already convinced myself that at as complete an absence as possible of organic substances, the development of the flora follows quite a

different course, but I am as yet unable thereabout to impart any decisive results.

The experiment now described, is not quite new as to its principle. In another form it was already performed in 1892 by SCHLÖSING fils and LAURENT [1]), not however with a culture liquid, but with a solid sand-soil and under conditions much more complicated than mine. Noteworthy is that also these investigators, cultivating in the light under the exclusion of all compounds of nitrogen, obtained Cyanophyceae belonging to the same or almost the same genera as those resulting from my experiments. They have moreover come to the result that by these Cyanophyceae free nitrogen was assimilated in a slight but distinctly observable quantity, and though they have not completely proved this assertion, as their cultures must have contained other organisms too, e.g. many bacteria, basing also on my own experiences I take their view to be correct.

My experiment throws some light on the two following observations. GRAEBNER [2]) observed that fresh sandy grounds, which are changing into moors, cover in the beginning with a flora of Cyanophyceae; and TREUB [3]), when visiting the isle of Krakatau after its destruction, found that the new flora which first developed on the volcanic ashes, likewise consisted of Cyanophyceae, of which he in particular mentions *Lingbya verbeekiana* and *L. minutissima*. Both, the said heathsand and the ashes of Krakatau, have no doubt been extremely poor in nitrogen-compounds.

If absolutely rejecting the theory of spontaneous generation, it might be assumed that certain Cyanophyceae, carried over from the universe by meteorites, have been the first organisms which peopled the earth, as no other living beings are known which, like the Cyanophyceae, are able to build up their organic constituents from carbonic acid and atmospheric nitrogen.

Once acquainted with the culture conditions of the Cyanophyceae I could easily obtain pure cultures on a solid medium. I therefore used as well silica as agar which by long washing with tap-water had been freed from the soluble organic substances, but saturated with the constituents of the tap-water. Plates of this agar, to

1) Fixation de l'Azote libre par les plantes. Ann. de l'Institut Pasteur T. 6 pag. 832, 1892. The authors make special mention of *Nostoc punctiforme*, *N. minutum* and *Cylindrospermum majus*.

2) Studien über die norddeutsche Heide. Bot. Jahrbücher. Bd. 20, 1891.

3) Notice sur la nouvelle flore de Krakatau. Ann. d. Jard. Bot. de Buitenzorg. T. 7, 1888.

which nothing else had been added but 0.02 pCt. $K^2 H P O^4$, and on which tap-water cultures of *Anabaena* had been sown out, were placed in the light of a window on the north, and after 10 to 14 days already produced extensive *Anabaena*-colonies free from bacteria. If the plates are not thoroughly washed *Anabaena* does not grow at all on them.

With plates prepared of silica instead of agar I obtained the same results.

The washing of the plates is effected by placing them, after solidification in the glass-box, into a large beaker with water, which is continually renewed during a few days by a current from the tap.

Then kalium phosphate is introduced into the plates by pouring over them a solution of this salt in distilled or tap-water, which solution is renewed a few times. Finally the superfluous water adhering to the plates is removed by heating the glass-box for a short time over a BUNSEN-flame.

Oscillaria and allied species do not grow on the thus prepared media, they even die on it already after some days. Mr. A. VAN DELDEN, however, has succeeded in my laboratory to obtain a pure culture on a solid medium of such a motile form related to *Oscillaria*.

This culture necessitated two other precautions. *First* the organic substance had to be removed from the agar more completely than is wanted for *Anabaena*, and therefore it proved necessary to wash with a current of distilled water. *Second*, the addition of a little of a nitrogen compound, e.g. a trace of ammonium-nitrate proved necessary, or at least favorable. On such agar the growth of the organism remains however very scanty, and, as besides many species of chlorophyceae can develop under these circumstances, we leave herewith the group of oligonitrophili, whose specific faculty consists in their being able to live on the nitrogen from the air, in opposition to the Diatoms and the Chlorophyceae. Hence this faculty seems also peculiar to a part only of the Cyanophyceae.

The question put at the head of this paper should thus be answered as follows.

In culture liquids, containing besides the mineral constituents of the food, a slight quantity of garden-soil, but to which no other nitrogen-compounds have been added, develop, under the influence of the light and the carbonic acid from the air, various species of *Cyanophyceae*, chiefly belonging to the genera *Nostoc* and *Anabaena*. Germs of these are very numerous in garden-soil. The presence of nitrogen-compounds prevents the development of the *Cyanophyceae*, but furthers that of certain *Chlorophyceae* and *Diatomaceae*.