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THE ASSIMILATION OF AMMONIUM NITROGEN BY NITZSCHIA CLOSTERIUM AND OTHER MARINE PHYTOPLANKTON

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The origin of ammonia in the sea is a fairly well understood process but little is known concerning the formation of nitrate. It is the consensus of opinion of most oceanographers that nitrate nitrogen is essential for the production of marine vegetation, although experiments during the last two decades of many investigators (cf. reviews by Allison, 1931, and Pardo, 1935) have established that a large number of terrestrial plants assimilate ammonium nitrogen. Brandt (1929), Thompson and Robinson (1932), and others point out that oceanographic data including chemical analyses fail to indicate the utilization of ammonium nitrogen by phytoplankton. However, in discussing a more satisfactory method for the quantitative estimation of ammonia in sea water, Wattenberg (1929) suggests that it is advisable to devote more attention to this form of nitrogen as a possible nutrient for marine flora. Cooper's (1933) extensive investigations in the English channel indicate that ammonia may be directly utilized by phytoplankton. The following results furnish proof that certain bacteria-free cultures of phytoplankton do assimilate ammonium nitrogen.

Experimental.—The organisms were cultured in aged sea water to which was added 1.0 cc. per liter of a mineral solution essentially the same as that recommended by Allen (1913) plus a little silicate:

$Na_2HPO_4 \cdot 12H_2O$	5.0 gm.
$FeCl_3 \cdot 6H_2O$	2.0 gm.
$Na_2SiO_3 \cdot 9H_2O$	1.0 gm.
HCl (con.)	2.0 cc.
Distilled water	100.0 cc.

Like Harvey (1933), it was found that the silicate improved the growth of the diatoms and sometimes it became a limiting factor when sea water was not enriched with it. The medium was heated to 70°C., cooled and the clear supernatant fluid aseptically decanted into sterile Pyrex Erlenmeyer flasks. Sterile solutions of various ammonium, nitrite and nitrate salts were added in different concentrations.

Most of the results reported below were obtained with a pure bacteria-free culture of *Nitzschia closterium*. Locally isolated cultures of *Nitzschia bilobata*, *Navicula* sp., *Chlorella* sp. and certain mixed cultures were also used. The test media were inoculated with uniform suspensions of the cultures to give approximately 500 cells per cc. after which they were exposed to indirect sunlight in north windows. The comparative rates of multiplication were followed by noting the first day on which growth became macroscopically visible and estimating the relative turbidity each day thereafter. Attempts were made to count the number of cells per unit of solution but their adherence to the glass and their tendency to form tenacious clumps under certain conditions interfered with direct microscopic enumeration. Frequent quantitative determinations of the

TABLE 1

COMPARATIVE ASSIMILATION OF AMMONIUM, NITRITE AND NITRATE BY *N. closterium* AS INDICATED BY GROWTH AND THE DISAPPEARANCE OF THE NITROGEN COMPOUNDS STARTING WITH 0.20 MILLIMOL OF EACH

TIME IN DAYS	AMMONIUM GROWTH	NH_4	NITRITE GROWTH	NO_2	NITRATE GROWTH	NO_3
0	–	0.20	–	0.20	–	0.20
4	*T*	0.19	?	0.20	–	0.18
7	+	0.19	*T*	0.20	*T*	0.16
10		0.17	*T*	0.18	+	0.16
14	++	0.16	++	0.17	++	0.15
18	+++	0.12	++	0.15	+++	0.14
21	+++	0.11	++	0.15	+++	0.10
28	+++	0.06	+++	0.09	++++	0.03
35	++++	0.01	+++	0.04	++++	0.001
42	++++	0	++++	0	++++	0

– no growth, *T* trace, + to ++++ increasing amounts of growth.

nitrogen compounds furnished another accurate index to the activity of the plankton in the different media. Nitrite was determined with the sulphanilic acid dimethyl-α-naphthylamine reagent, ammonia with Wattenberg's modified Nessler's solution and nitrate with diphenylbenzidine.

Table 1 illustrates the kind of record which was kept and shows that bacteria-free cultures of *N. closterium* assimilate ammonium and in so doing multiply more rapidly at first than in corresponding media containing equi-molar concentrations of either nitrite or nitrate. Similarly *Nitzschia bilobata*, *Navicula* sp., *Chlorella* sp. and certain mixed cultures of phytoplankton flourished on ammonium nitrogen, quantitatively exhausting as much as 0.2 millimol of this nitrogen compound from the sea-water solutions. There were no macroscopic signs of multiplication in the controls to which no nitrogen was added although microscopic examinations revealed a two- to four-fold increase in the number of cells, probably attributable to a small quantity of residual available nitrogen in the sea water. Later experiments with synthetic sea-water controls demonstrated that the diatoms multiply with ammonium as the only source of nitrogen.

Provided small concentrations (0.2 millimol or less) of ammonium, nitrite and nitrate are compared, in virtually every instance multiplication and utilization first becomes manifest with ammonium. The initial superiority of ammonium vanishes in a week or two after which time there is little or no difference in the quantity of vegetation. The addition of a little ammonium to either nitrite or nitrate media accelerates multiplication. Combinations of ammonium and nitrate compounds are found to be better than either ion used separately. Ammonium nitrate in concentrations up to 0.1 millimol gave good results. The chloride, hydroxide, sulfate, phosphate or carbonate of ammonium supplied the nitrogen requirements of the diatoms.

TABLE 2

OPTIMUM AND INHIBITORY CONCENTRATIONS OF AMMONIUM, NITRITE AND NITRATE FOR THE MULTIPLICATION OF *N. closterium* AND THE CONCENTRATION OF THESE IONS REPORTED FOUND IN THE SEA

FORM OF NITROGEN	MILLIMOLS		MILLIMOLS IN SEA WATER	
	OPTIMUM	INHIBITORY	AVERAGE	RANGE
Ammonium	0.01–0.05	4	0.001–0.005	0–0.02
Nitrite	0.05–5.00	25	0.000,1	0–0.001
Nitrate	0.10–40.0	300	0.002–0.01	0–0.05

Undoubtedly some of the reported failures of phytoplankton and other plants to grow in ammonium media are due to the use of excessive quantities of ammonium. The effect of the concentration of ammonium, nitrate and nitrite nitrogen was studied by inoculating sea-water media containing the nitrogen compounds in different combinations with diatoms. The optimum concentration for multiplication was accepted as that in which in the majority of the cases vegetation first became perceptible to the naked eye and not necessarily that in which growth was best after prolonged incubation. The results for *N. closterium* are presented in table 2 which also expresses the quantity of these ions reported to be found in the sea. It will be observed that the optimum concentration of nitrate is over a hundred times greater than that for ammonium and also the optimum concentration of each nitrogen compound is greatly in excess of its average concentration found in the sea. In media which contained 0.002 millimol of either ammonium or nitrate nitrogen, approximately the average concentration which occurs in the sea, microscopic examinations revealed appreciable multiplication with ammonium being superior at first, but later there was no detectable difference in the number of cells in the two substrata.

Tiedjens and Robbins (1931) confirm the findings of several other investigators that many crop plants preferentially assimilate ammonium at pH 7.0 or above whereas in acid media nitrate is more available, and at pH 4.0 ammonium is not assimilated. Efforts were made to ascertain the effect of the hydrogen-ion concentration upon the assimilation of ammonium by the marine phytoplankton. Dilute sodium hydroxide or hydrochloric acid was added to the sea-water media to give reactions

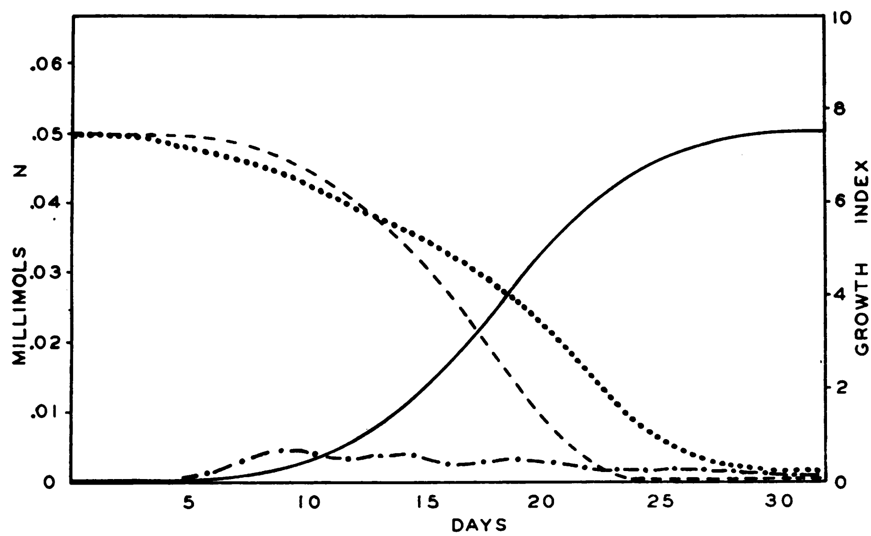

FIGURE 1

Depletion of nitrate (dotted line) and ammonium nitrogen (dashed line) accompanying the multiplication of *N. closterium* in sea-water medium which initially contained a mixture of 0.05 millimol of each. The appearance and disappearance of nitrate (dot-dash line) and the cumulation of vegetation (solid line) are also shown.

ranging by increments of 0.4 from pH 6.0 to 9.0. The most acid media in which *N. closterium* grew was pH 6.8 but the growth was so scant and abnormal that it was impossible to differentiate between the results with nitrate and ammonium. Since both forms of nitrogen supported multiplication in more alkaline media up to and including pH 8.8, these values are accepted as the limits. The optimum was found to be pH 7.6 to 8.0 although the sea-water habitat of these organisms is pH 8.0 to 8.4. There were no consistent differences in the assimilation of ammonium and nitrate at the different reactions.

Evidence for the preferential assimilation of ammonium in the presence of nitrate is presented graphically in figure 1. These data were obtained by inoculating media containing 0.05 millimol each of ammonium and nitrate with pure cultures of *N. closterium*. At various intervals the amount of each ion and of vegetation was determined. Under these conditions the ammonium was depleted from the solution several days before the nitrate. Special attention is called to the appearance of a trace of nitrites.

Nitrites have been detected in small quantities in nitrate media seeded with *Nitzschia closterium, N. bilobata, Navicula* sp., *Chlorella* sp. and several unidentified mixed cultures of phytoplankton under conditions which are unfavorable to the activity of denitrifying bacteria. Depending primarily upon the size of the inoculum, a trace of nitrite begins to appear within three to seven days. The addition of a little ammonium to nitrate media usually increases the amount of nitrite which accumulates. Inasmuch as nitrite does not occur in ammonium medium lacking nitrate and does occur in nitrate media lacking ammonium, it is assumed that the diatoms reduce the nitrate to nitrite. During the initial positive acceleration growth phase the concentration of nitrite reaches its maximum and decreases with fluctuations during the logarithmic phase of growth. Finally the nitrite is exhausted at about the same time as the nitrate but due to the difficulties of determining nitrate in the presence of nitrite, it has not yet been possible to ascertain which disappears first or if they are simultaneously depleted from solution by the diatoms. It may be significant that in nitrate media teeming with multiplying diatoms, the concentration of nitrite is usually greatest early in the morning and diminishes with exposure to light. A few million diatoms per cc. were found to reduce a little nitrate to nitrite in total darkness and it is reiterated that rigorous cultural as well as direct microscopic tests proved the absence of bacteria.

Conclusions.—It is believed that the diatoms extracellularly reduce nitrate to nitrite in the process of its assimilation and eventual conversion into organic nitrogen. Beckwith (1933) demonstrated that certain pure cultures of *Chlorella* reduced nitrate to nitrite and that they assimilated the nitrite. Eckerson (1924) and others have presented experimental evidence to show that during protein synthesis by certain higher green plants nitrate is reduced to nitrite and then to ammonia. In view of the fact that protoplasmic nitrogen is in a reduced state, theoretically nitrite or ammonium nitrogen should be more readily available than nitrate nitrogen. It does not seem logical that all plant nitrogen must go through the roundabout process of being completely oxidized to nitrate only to be reduced again for conversion into amino nitrogen of protoplasm. It is more credible that particularly in the sea where the economy of Nature is at its best there is a short-cut from this part of the nitrogen cycle with nitrification serving primarily as a regulatory or storage process. Cooper

(1933) has shown that *Biddulphia mobiliensis* assimilates ammonia from sea water. According to Schreiber (1927) more *Carteria* cells were produced from ammonium than from an equivalent of nitrate. Harvey (1933) found that *Nitzschia closterium* grew just about as well in ammonium as in nitrate. Pure cultures of *Chlamydomonas* utilized ammonium more efficiently than nitrate in experiments described by Braarud and Föyn (1931) and Bond (1933) found that ammonium nitrogen satisfied the requirements of *Portotheca zopfii, Polytoma uvella, Chlamydomonas pulvinata* and *Dunaliella salina.*

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