FURTHER EXPERIMENTS ON THE DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER[1]

THEODOR VON BRAND, NORRIS W. RAKESTRAW AND CHARLES E. RENN

(*From the Woods Hole Oceanographic Institution, Woods Hole, Mass.*)

In an earlier paper we reported (1937) that the cycle of decomposition and regeneration of nitrogenous organic matter in sea water can be reproduced experimentally. The main stages in this cycle are: living organism—dead organism—ammonia—nitrite—nitrate—living organism. In view of the importance of nitrogenous material in the economy of the sea, it seemed worth while to carry these experiments somewhat further, and especially to consider the following questions:

1. Is it possible to reproduce more than one cycle in the same water?
2. Can the cycle be made shorter by eliminating certain stages?
3. Do successive cycles differ significantly in character or in rates of development?
4. In what ways do anaerobic and aerobic decomposition differ?
5. How completely can the changes observed in the different forms of nitrogen be accounted for in terms of each other; in other words, how constant is the quantitative balance?

Methods and Material

The plan of the experiments was the same as in our previous investigation. Organic material was suspended in sea water in large carboys and allowed to decompose in the dark, during which time chemical analyses were made periodically. Artificial cultures of *Nitzschia Closterium* were chosen as a source of organic matter, for our previous experience had shown these diatoms to be more satisfactory from an analytical standpoint than mixed plankton. Waksman, Stokes and Butler (1937) also used them successfully for a somewhat similar purpose. The diatoms were separated from the culture medium by centrifugation, washed several times with nitrate-free sea water, and finally suspended in a carboy of sea water which had been filtered through No. 4 sintered-glass. All experiments were carried out at the

[1] Contribution No. 222 from the Woods Hole Oceanographic Institution.

uncontrolled room temperature, varying from 15° C. in the winter to 25° in the summer. Before samples were removed for analysis the carboys were shaken vigorously to distribute the suspended matter evenly. When the latter showed any tendency to stick to the glass it was loosened with a rubber-tipped glass rod before shaking, after the rubber had been carefully cleaned to avoid contamination.

The methods for the determination of particulate nitrogen, ammonia, nitrite and nitrate were the same as those used in our previous experiments and have already been described in detail.

The Possibility of Consecutive Cycles

In our previous investigation we found that the nitrate resulting from plankton decomposition could be regenerated into diatom protoplasm. This raised the question of the possibility of repeating such a cycle of decomposition and regeneration more than once in the same water. When the original plankton has undergone decomposition and the nitrate stage has been reached the water is inoculated with fresh *Nitzschia*. After about a week in the light an abundant growth is always observed. The nitrate drops to a minimum and when placed in the dark this newly-developed plankton undergoes decomposition again. In Series 12 three complete, successive cycles were carried out in this way. In another similar series, for which the data are not given, two cycles were completed, and in several others a part of the second cycle.

A poor diatom growth was observed after the second cycle in Series 12 (Table I), in which unidentified algae developed, among the *Nitzschia*.

It was important to know if a regeneration of phytoplankton material is possible in parts of the decomposition cycle other than the nitrate stage. During the plankton decomposition in Series 23, when the ammonia had reached its maximum, but before nitrite or nitrate had appeared, a portion of the water was transferred to another carboy (23A). After inoculation with *Nitzschia* and a week's exposure to light the ammonia had almost entirely disappeared and a heavy diatom growth had occurred. In a similar way Series 19A was removed from No. 19 before the nitrate had reached its maximum. Here, too, diatoms developed rapidly and abundantly and the soluble nitrogen compounds disappeared almost quantitatively. At these stages, at least, no toxic substances had been formed, or deficiencies developed, which could inhibit the development of phytoplankton. "Short-cuts" in the nitrogen cycle can evidently take place, and the possibility of their occurrence in nature suggests an explanation for

TABLE I

Series 12. Woods Hole harbor water. Fresh culture of *Nitzschia Closterium*. Quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen detd.	Diatom count $\times 10^3$ per ml.
7- 5-37	256	31	0	14	14	301	174
7	225	65	0	14	14		134
11	107	215	0				12
17	92		0	19	18		3
23	78		0	11	11		<1
30	49	260	0	11	11	320	<1
8- 5	46		1	11	10		<1
13	45		5	25	19		<1
20	40	210	105	110	5	360	<1
24		70					
27	41	30	265	365	0	435	<1
9- 2	31	13	350	355	5	402	
12			390	395	5		
25		25	370	390	20		
10-28	37	61	0	305	305	413	
31	Reinoculated with diatoms and put in light						
11- 7							137
13							780
16	455	21	0	2	2	478	
16			Put in dark				
21							706
12- 1	164						40
24		59	0				
2- 1-38			1	8	7		
4		247					
24	189	246	1			(440$\pm$)	
5- 6		0	70	350	280		
29			0				
6-25	142	12	1	300	300	455	
7- 5	Reinoculated with diatoms and put in light						
12	408			80	80?	(490$\pm$)	
15				10			
18	489	0	0	40	40	529	
18			Put in dark			*	
29	304	115	0	25	25	445	
8- 5	190	175	4	18	14	387	
12	202	110	45	50	5	362	
19	196	20	150	155	4	371	
25	208	10	170	160	0	388	
31	237	0	200	(160)	0	437	
9-13	260	18	0	300?	300?	578	

* Dissolved organic nitrogen = 370.

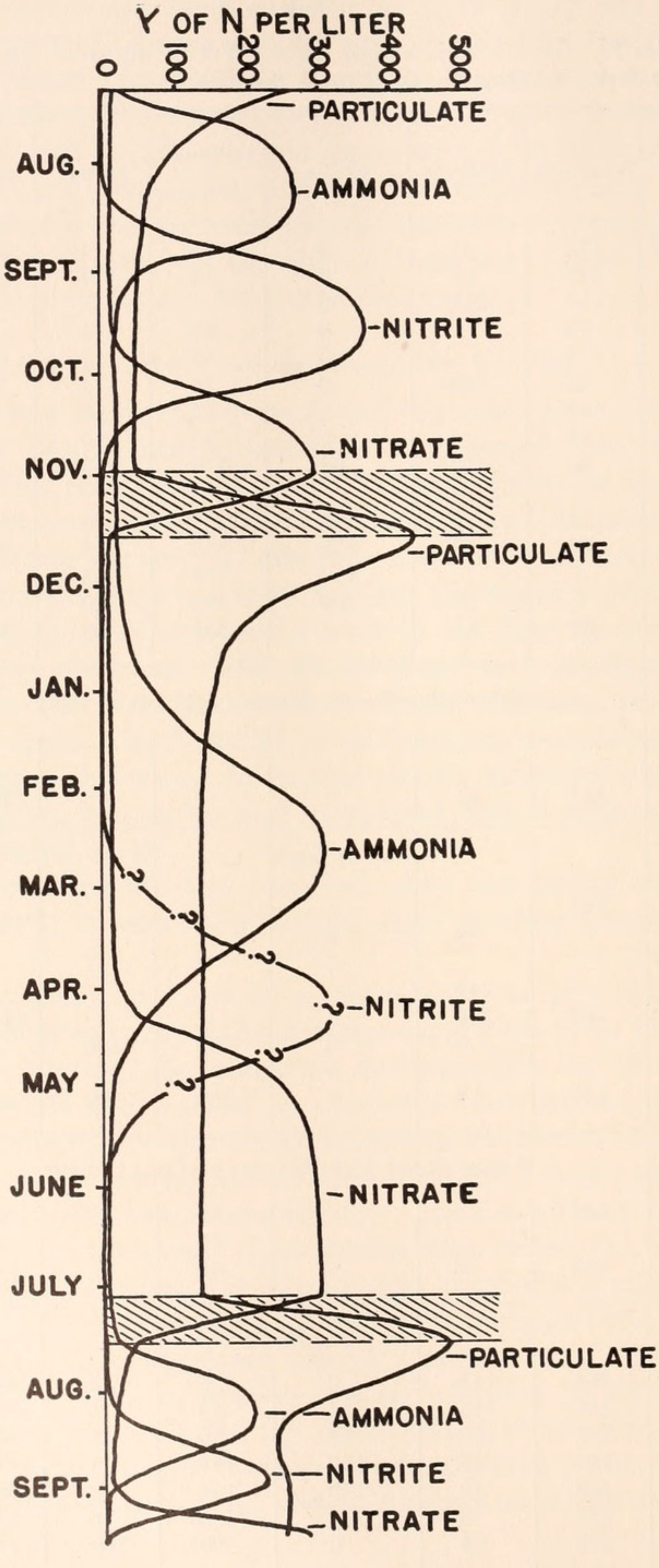


Fig. 1. Series No. 12. The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate, plotted against time. Source of organic matter, fresh culture of *Nitzschia Closterium* suspended in sea water from Woods Hole harbor. The shaded areas represent regeneration periods, with the culture in the light; the remainder, decomposition in the dark.

the rapid succession of great numbers of phytoplankton organisms belonging to different species. In nature, all stages of the cycle must be taking place simultaneously, and the momentary picture is simply one of equilibrium.

Chemical Observations

Decomposition of Particulate Nitrogen

The particulate nitrogen is that contained not only in diatoms, but also in bacteria and miscellaneous debris. This material began to decompose rapidly in all cases, as soon as the water was placed in the dark. It never disappeared entirely, however, but reached an apparently constant level after periods varying from two to six weeks. This was true not only in the first decomposition cycle but also in the subsequent ones. This residual material is very resistant to further decomposition, and consequently the level of residual particulate nitrogen is higher after each successive decomposition cycle. In the first cycle of Series 12, for example, the residual particulate nitrogen was about 40 γ per liter, in the second cycle about 150 γ, and in the third 200 γ. These amounts are about 16 per cent, 33 per cent and 41 per cent of the particulate nitrogen present at the beginning of each respective cycle. It seems not unlikely that under natural conditions in the sea the plankton is incompletely decomposed, and a large part of the particulate nitrogen found by von Brand (1938) in the deeper levels may be contained in such resistant or slowly decomposing plankton and bacterial residues. The occurrence of bacteria and debris in this resistant fraction is indicated by the fact that the quantity of particulate nitrogen does not consistently follow the diatom count. While the sum of particulate and ammonia nitrogen is fairly constant during the first part of the decomposition cycle, the diatom count falls off much more rapidly than does the particulate nitrogen. Nevertheless, in nature this refractory residue cannot be entirely resistant to decomposition, otherwise the insoluble nitrogenous material, in the water or on the bottom, would increase without limit.

A curious irregularity was observed in Series 25, which was aerated by a constant stream of pure air. After about six weeks of normal decomposition, accompanied by the appearance, first of ammonia and then of nitrite, the particulate nitrogen rose abruptly to its initial value and the ammonia and nitrite disappeared entirely. A microscopic examination, carried out for us by Dr. Lois Lillick, showed the presence of an enormous number of bacteria and a few flagellates. The diatoms had disappeared completely. This phenomenon must be attributed to the development of a peculiar bacterial flora, since we did not observe it in any other series, including No. 24, which contained the same water and plankton as No. 25 but differed only in not being agitated with an air-stream during the decomposition.

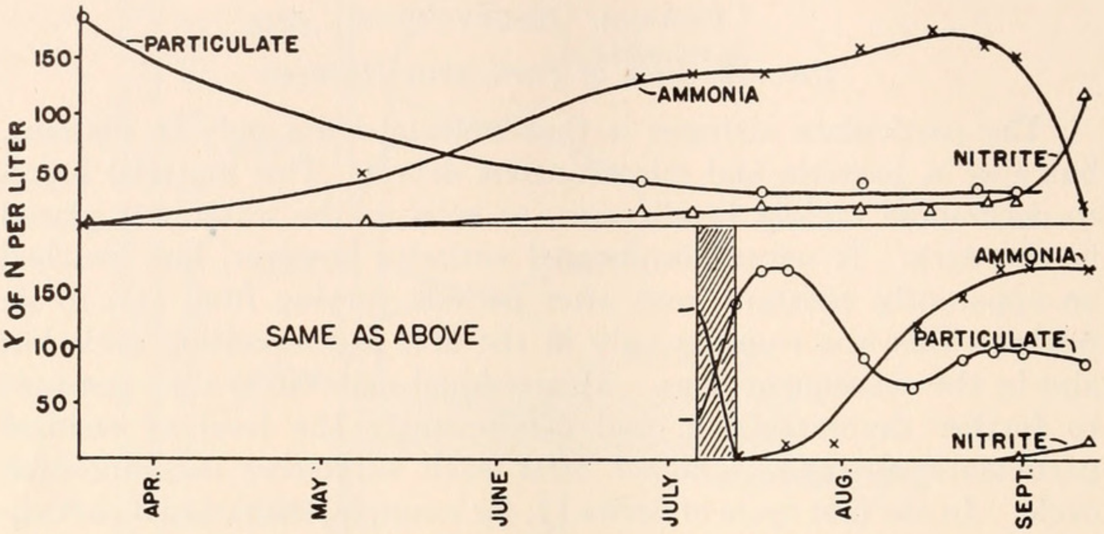
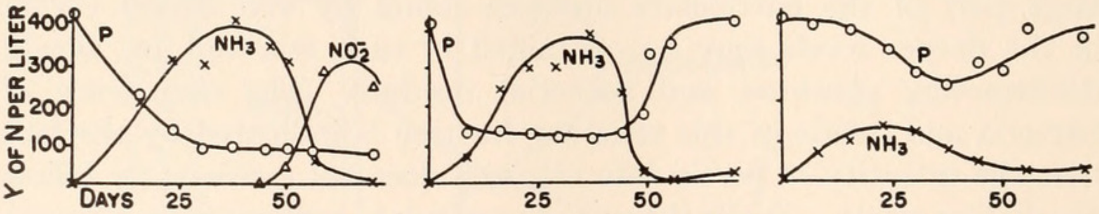


FIG. 2. Series No. 23 (above) and 23A (below). The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate, plotted against time. Source of organic matter, fresh culture of *Nitzschia Closterium* suspended in sea water from the Sargasso Sea. The shaded area represents a regeneration period, with the culture in the light; the remainder, decomposition in the dark.



Series No. 24 Series No. 25 Series No. 26

FIG. 3

The disappearance of particulate nitrogen (*P*) and the simultaneous changes in ammonia and nitrite in cultures of *Nitzschia Closterium* in sea water from Woods Hole harbor. The three cultures were identical at the start. Series No. 24 stood quietly in the dark. Through No. 25 a continuous stream of purified air was bubbled. The air was completely removed from Series No. 26 and a continuous stream of purified hydrogen bubbled through it.

Ammonia

During the first decomposition cycle, in all cases, ammonia appeared in the water rapidly and in such amounts as to exclude the possibility of soluble nitrogen compounds intermediate between particulate nitrogen and ammonia. This but confirms our previous findings. But in the second cycle, although the particulate nitrogen disappeared at about the same rate, ammonia was not formed as rapidly as in the first. In Series 19A, for example, ammonia appeared only after 30 days of decomposition in the dark, during which time the particulate nitrogen had diminished by 160 γ per liter. The same behavior is found in Series 23A and in the second cycle of No. 19, and very likely also in the second cycle of Series 12, although the data are incomplete in the latter. These cases indicate the formation of soluble nitrogen compounds of higher molecular weight, intermediate between dead protoplasm and ammonia. Although there are two or three possible explanations for this lag in ammonia formation, we are not yet inclined to urge the acceptance of any one of them. It is also to be observed that the third cycle of Series 12 resembles the first in its more rapid rate of ammonia appearance.

In the first cycle it generally required from 16 to 25 days for the ammonia to reach its maximum, where it remained for a period of from 21 to 50 days before dropping. A notable exception, however, occurred in Series 22 and 23, in which the ammonia did not appear until about the forty-eighth day, required three to four months to reach its maximum, and remained for another two months before disappearing entirely. This unusually long duration of the ammonia stage would seem to be connected with the source of the water in the experiments. That used in Series 22 came from the Caribbean, and that in Series 23 from the Sargasso Sea, while in all other cases the water was taken from near the shore. Since the diatoms in all experiments were from persistent cultures the bacterial flora introduced with them was presumably constant, but whether the difference in decomposition is due to differences in the bacterial flora of the water itself or to such properties of the water as might influence the growth of bacteria, is not yet clear.

The lag in the oxidation of ammonia to nitrite in Series 22 and 23 was apparently not due to the absence of the necessary bacteria, for portions of these cultures were inoculated with 1 ml. from Series 12, in the midst of its nitrite stage, without any resulting change in the rate of nitrite formation.

Nitrite

In the first cycle nitrite began to appear in 31 to 58 days, corresponding to the beginning of the disappearance of ammonia. It reached its maximum when the ammonia had dropped to a minimum; that is, in a period of from 46 to 74 days. The total duration of the nitrite stage of the first cycle was quite irregular: two months from beginning to end in Series 12, but more than six months in Series 13 (See Table II). The lag in the latter case may in some way be related to the continuous aeration.

Our data concerning nitrite in the second cycle are too scattered to tell whether or not the rate of nitrite formation is the same in the second cycle as in the first. In the third cycle of Series 12 nitrite, like ammonia, developed rapidly.

Table II

Series 13. Woods Hole harbor water. Fresh culture *Nitzschia Closterium* in dark, with ammonia-free air bubbling through. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen detd.	Diatom count $\times 10^3$ per ml.
7- 8-37	269	43	1	11	10		234
10	380	11	0	17	17	407	274
13		115	0				
14	280						74
19	109	240	0	11	11	360	2
24	86	300	0	11	11		<1
31	60	310	0	11	11	381	<1
8- 5	53		1	11	10		<1
13	29		0	11	11		<1
20	25	400?	0	9	9	434	<1
24		360					
27	23	330	3	10	7	365	<1
9- 2	34	350	30	35	5	419	
12			330	340	10		
25		15	330	350	20		
10-28	7	82	345	(310)	?		
11-18			321				
2- 1-38		20	272	(260)	?		
24	74	20	232				
5- 6			0	400?	400?		
6-25	120	25	1				
7-15	118	74	7	230	225	422	
7-18		Put in light, not reinoculated					
8- 2	97	20	4	200	200		<1
4	Reinoculated with diatoms						
10	389	9	0	10	10	408	*

* No diatoms present; unidentified algae.

Nitrate

Nitrate begins to appear only when nitrite disappears, and this never seems to happen as long as a significant amount of ammonia remains. Since one cannot rely upon a greater accuracy than 10 per cent in the analytical determination, any quantitative balance is uncertain when large amounts of nitrate are involved.

TABLE III

Series 19. Woods Hole harbor water, collected 2–25–38. Fresh culture *Nitzschia Closterium.* Standing quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count × 10³ per ml.
2–25–38	316						
26		12	0	10	10	338	275
5– 6		41	12	25	13		
6–25	71	50	87	285	200	406	
7– 6		5	12		(Series 19A separated)		
15	48	15	1	350	350	414	
7–18	Reinoculated with diatoms and put in light						
29				17			
8– 2	396	0	0	10	10	406	56
8– 2	Put in dark						
10	451	0	0	15	15	466	240*
16	262	10	0	20	20	292	220†
23	201	30	0	15	15	246	
30	304?	15	1	20	19	339	
9–13	193	70	0				

Series 19*A*. Portion separated from Series 19 on 7–6–38. Reinoculated with diatoms and put in light.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count × 10³ per ml.
7– 6		5	12				
12	382	12	0	15	15	409	475
12	Put in dark						
18		0	3	25	22		350
21	396	7	0	10	10	413	
29	252	5	0	15	15	272	5
8– 5	221	90	0	15	15	326	
12	141	175	0	10	10	326	
20	208	186	0	10	10	404	
25	187	250	0	15	15	452	
31	201	250	0	20	20	471	
9–13	171	250	0	10	10	430	

* Few *Nitzschia;* mostly *Skeletonema Costatum.*
† Both *Nitzschia* and *Skeletonema.*

ANAEROBIC DECOMPOSITION

In addition to the experiments already described, which were carried out under aerobic conditions, the anaerobic decomposition of diatoms was also studied in two series. The carboys containing the water and diatoms were first evacuated to remove all the air from the water and the container. Then a slow, continuous stream of purified hydrogen was bubbled through the water. After decomposition in the dark for some time a strong odor of hydrogen sulfide was observed. In both cases the particulate nitrogen diminished very slowly, but remained constant at a level very much higher than that in the aerobic decompositions. The diatom counts also remained high; in fact, when Series 26 was discontinued, after 10 weeks, living diatoms were still found, which grew when placed in fresh culture medium.

Table IV

Series 22. Water from the Caribbean Sea. (18°–35′ N; 79°–14′ W); one year old. Fresh culture *Nitzschia Closterium*. Quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count × 10^3 per ml.
3–18–38	123	0	0			125*	213
5– 6		25	0	10	10		
6–25	41	113	12	10	0	166	
7– 6		115	12				
13	22						
15		118	12	25	13		
8– 3	30	150	11	12	1	191	
13		145	12		(Series 22B separated)		
23	23	150	16	20	4	193	
30	30	125	30	30	0	185	
9–13		20	90			(150±)	

Series 22*B*. Portion separated from Series 22 on 8–13–38 and inoculated with 1 ml. from Series 12. In dark.

8–13		145	12				
20		205	22	25	3	160	
30		210	36	35	0	245	
9–13		220	50			(270±)	

* Dissolved organic nitrogen = 93.

Ammonia also increased in this series for the first three weeks, and then gradually disappeared. In the other series no appreciable amount of ammonia was formed; on the contrary, a small amount of that originally present disappeared. This ammonia was not recoverable from the effluent hydrogen, nor are we able as yet to account for the behavior of ammonia in either of these series. As might be expected, no nitrite was formed.

After about two months under anaerobic conditions a portion from one of the cultures was aerated and kept henceforth aerobically.

Two months later a large amount of ammonia had been formed, but no nitrite or nitrate had appeared by the time the experiment was stopped.

Quantitative Balance

In our previous investigation we pointed out that in nearly every case studied the total determined nitrogen in the system (that is, the sum of the particulate nitrogen, ammonia, nitrite and nitrate) increased continuously throughout the period. In the cases we are now reporting the quantitative balance is much more satisfactory. In Series 13, 22, 23, 24 and 25 the changes in total nitrogen are small and probably explainable in terms of accumulated errors. In Series 12, 19, 19A, 23A and one other, the increase observed is too large to be accounted for in this way, but is less, relatively, than the increase noted in our first experiments. Previously, we discussed three possible explanations for this increase in total nitrogen: systematic errors in the determination of particulate nitrogen, the participation of dissolved organic nitrogen in the decomposition, and nitrogen fixation. We were in no position to prefer any one of these explanations. The fact that a good nitrogen balance was observed in half of our later experiments, including one which extended over more than a year, seems definitely to eliminate the possibility of systematic errors. The difficulty of determining dissolved organic nitrogen with any accuracy makes it almost impossible to test the second hypothesis directly. (The one determination given in the data for Series 12 was made, with some difficulty, by the method of Krogh, 1934.) However, we sought to investigate this question indirectly, by trying to see whether ammonia appears, on standing, in water devoid of gross particulate matter, and if so, whether the process is related to the content of dissolved organic matter. Two filtered samples of water, one from Woods Hole harbor and one from the Sargasso Sea, were placed in the dark and the usual determinations made periodically. Previous work had shown that the harbor water had a higher organic nitrogen content than water from the open sea, but there was no difference in the behavior of the two kinds of water. A small increase in nitrogen, about 60 γ per liter, was observed in both. This could indeed be the result of the participation of dissolved organic nitrogen, but we are inclined to believe that it is due to an entirely different cause. All the stored carboys were tightly stoppered, but before the removal of samples they were vigorously shaken and opened. During this time the water comes into contact with a rather considerable quantity of air, from which it may take up ammonia. To test this possibility, clean filtered air was aspirated for 12 hours through a sample of sterilized Sargasso water. This resulted in an increase of 50 γ of ammonia-N and 20 γ of nitrate-N. Such a quantity of ammonia is not surprising, in view of the amount of decomposing organic matter in this laboratory and its vicinity. It seems quite possible, therefore, that at least a large part of the increase in total nitrogen observed in some of our experiments may be due to contamination from the air. Nevertheless, this is not conclusive, and we are still in no position to exclude the possibility of either nitrogen fixation or participation of dissolved organic nitrogen. Atmospheric contamination was ruled out in Series 13 and 25, through which purified air was aspirated, and in these cases, indeed, the total nitrogen did not increase. Still, no increase was observed in Series 22, 23 and 24, which were not aerated, but which stood side by side with carboys in which nitrogen accumulated. Further work is still being done in the effort to clear up these discrepancies.

Summary

1. Several consecutive cycles of decomposition and regeneration were carried out in the same water.

2. It is confirmed that in the first cycle the main stages of decomposition are: dead body—ammonia—nitrite—nitrate. In the second cycle there is evidence of intermediate soluble substances between dead body and ammonia.

3. Under anaerobic conditions the initial states of decomposition take place more slowly than under aerobic conditions, and no nitrite or nitrate is developed.

4. Regeneration of nitrogen into phytoplankton protoplasm is possible not only in the nitrate stage but also in the ammonia stage and before the nitrate has reached its maximum.

5. The quantitative nitrogen balance was better than that reported in previous experiments, and possible reasons for the discrepancies still present are discussed.

BIBLIOGRAPHY

von Brand, T., N. W. Rakestraw, and C. E. Renn, 1937. The experimental decomposition and regeneration of nitrogenous organic matter in sea water. *Biol. Bull.*, 72: 165–175.

von Brand, Theodor, 1938. Quantitative determination of the nitrogen in the particulate matter of the sea. *Jour. du Conseil*, **13**: 187–197.

Krogh, August, and Ancel Keys, 1934. Methods for the determination of dissolved organic carbon and nitrogen in sea water. *Biol. Bull.*, **67**: 132–144.

Waksman, S. A., J. L. Stokes, and Margaret R. Butler, 1937. The relation of bacteria to diatoms in sea water. *Jour. Mar. Biol. Ass'n.*, **22**: 359–373.