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Microbiology of Nutrient Regeneration: Part III—Experimental Studies on Marine Nitrification

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Nitrification process was investigated in the tropical Vellar estuary, by *in situ* experiments. Variation in nitrifying capacity in both estuarine and sea water was measured. Biological potential of estuarine water in the nitrification was greater than that of neritic water. Oxidation of ammonia to nitrate occurred without accumulation of nitrite. Marine nitrifiers (both ammonium oxidizers and nitrite oxidizers) were active in the photic zone of the coastal waters. *In situ* experiments revealed that nitrate reduction and ammonia oxidation were significant processes in the estuarine and coastal waters. Ammonia production, nitrite formation and nitrite oxidation took place simultaneously. At lower temperatures, decomposition was not retarded as much as the mineralization and hence dissolved organic nitrogen accumulated. Seasonal variations were also found in some of the biological processes. Concentration and distribution of nitrite (a transitory product formed from ammonia and nitrate) were influenced both by physical and biological processes.

IN the marine environment chemoautotrophic bacteria play vital role in nitrification which constitutes an essential part in the nitrogen cycle. Since nitrifying microorganisms are very important in agriculture, detailed studies have been largely carried out in terrestrial environments. Comparatively very few studies have been made on these bacteria of marine and estuarine environments.

Earlier studies[1,2] indicated that the surface sea water had only very few of these organisms, while the sea bottom contained a large population of nitrifiers. Zo Bell[3] studied the effect of redox potentials on the activity of nitrifying bacteria. Von Brand *et al.*[4] from their *in vitro* experiments established the conversion of ammonia to nitrite. However, early attempts to isolate the organisms responsible for the *in situ* oxidation of nitrogen, attained only limited success. The first known ammonia oxidizing bacterium was isolated by Watson[5] from the North Atlantic deep water. Later, enrichement cultures of ammonia and nitrite oxidizing bacteria were prepared from sea water or marine muds and tested for their activities by several workers[6-11]. Carlucci and Strickland[10] estimated the number of nitrifying bacteria to be less than one cell per ml in the open ocean. Mountain[12] dealt with the vertical distribution of the nitrification potential and the associated environmental parameters in the tropical Pacific Ocean.

Generally the activity of nitrifying microorganisms are understood by estimating the products of their activity, viz. nitrate and nitrite. However, such a study would not be of use in estuaries where the physical processes such as estuarine circulation and freshwater runoff would obscure variations due to biological processes. But from the simultaneous chemical and microbiological analysis of water samples, it would be possible to assess the biological activity. When water samples are incubated either enriched with a particular substrate or unenriched, the changes in the concentrations of inorganic nitrogen compounds would give a clear picture of the microbiological transformation. Gundersen *et al.*[13] have pointed out that the enriched culture experiments usually give biased results because an increased substrate concentration induces the hitiherto thermodynamically disadvantaged microorganisms to show greater activity and ultimately mask other normal biological processes. It has been suggested by Gundersen[14] that unenriched *in situ* experiments are much more realistic than laboratory enrichment and cultivation at high substrate levels and maximum aeration. This paper deals with the *in situ* experiments on the production of nitrite.

Materials and Methods

Estuarine water was collected at a fixed station opposite to the Marine Biological Station in Vellar estuary (2·5 km from the mouth) from a depth of 1 m. Sea water sample was collected from 5 m depth in the Bay of Bengal at 10 m line.

Water samples for both chemical and microbiological analysis were collected using Von Dorn sampler and samples for microbial *in situ* experiments were retrieved in sterile dark glass bottles (300 ml). Subsamples were drawn off into polyethylene bottles for immediate chemical analysis or kept frozen if analysed later.

The procedures given by Mountain[12] were followed to carry out the *in situ* experiments. For these

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experiments samples of water were collected aseptically, transferred immediately to sterile bottles and after sealing they were incubated in the environment itself (26-28°C). A second batch was incubated at a lower temperature (10-14°C) in the laboratory. Initial concentrations of ammonia, nitrite and nitrate were estimated immediately after the collection of samples. One experimental bottle from each batch was opened once a week and all the inorganic nitrogenous nutrients, oxygen and *p*H were estimated immediately. Oxygen was estimated by the Winkler titration method given by Strickland and Parsons[15]. *p*H was determined using a Philips *p*H meter. Nitrite was estimated using the procedure of Bendschneider and Robinson[16]. The method of Morris and Riley[17] using ammonium chloride as buffer was used for the estimation of nitrate. Ammonia was measured using the phenol hypochlorite method of Solorzano[18]. To estimate the dissolved organic nitrogen (DON) the method described by Strickland and Parsons[15] was followed.

Results and Discussion

The results of *in situ* experiments with estuarine water samples are shown in Table 1. In the first week, both ammonification and nitrite formation took place. The fact that nitrite formation was not due to oxidation of ammonia, but due to reduction of nitrate would be evident from the decrease in nitrate concentration during this period. (Table 1). Facultatively anaerobic microorganisms capable of reducing nitrate to nitrite occur in most habitats and they also occur in waters rich in dissolved oxygen. In some waters denitrification can be demonstrated although oxygen is still present. These waters are however rich in organic detritus and the bacterial flora growing on the surface of the organic debris particles can create microzones free of oxygen where denitrification proceed[19]. In the present study also, regeneration of nutrients from organic matter, suspended in sea water was determined wherein, one cannot rule out the above possibility. Further, microorganisms that are capable of reducing nitrate only to the level of nitrite have been reported to occur in water and sediments[20]. One of the conditions which determine the vigorous activity of denitrifiers is reduced oxygen content. Although denitrification proceeds when the medium is fully aerated, and although reduced oxygen content in the water is not mandatory for initiation of the process nevertheless if oxygen is present in the water in great amounts, the rate of denitrification is retarded. An indispensable condition is the presence of nitrates[21]. Ammonia production, nitrite formation and nitrite oxidation took place simultaneously up to 6 weeks.

Total inorganic nitrogen steadily increased up to the VIII week. But in the IX week, it decreased to half the amount of the previous week and then increased slowly (Table 1). However, after the XI week there was not much change in any of the processes and they remained the same even after 25 weeks. In the XI week there remained only a non-measureable amount of nitrite and it seemed that nitrite was completely oxidized to nitrate.

Ammonia oxidation commenced in the I week itself and it attained a high level in the II week. Although there was a continuous formation of

TABLE 1 — (i) *In situ* EXPERIMENT ON NITRIFICATION IN ESTUARINE WATER AT WATER TEMPERATURE IN THE ENVIRONMENT (PREMONSOON SEASON) AND (ii) LABORATORY EXPERIMENT AT LOW TEMPERATURE (10-14°C)

Days	Week	NH_4 µg at/ litre	NO_2 µg at/ litre	NO_3 µg at/ litre	Total inorganic nitrogen µg at/litre	O_2 ml/litre	*p*H	O_2 consumption ml/litre	Variation in		
									NH_4	NO_2	NO_3
					(i) *In situ* EXPERIMENT						
Initial	—	2·23	0·1	8·25	10·59	6·0	8·0	—	—	—	—
8	I	12·04	1·38	2·28	15·69	4·6	7·9	1·4	+9·8	+1·27	+5·97
16	II	5·38	13·57	3·29	22·25	3·7	8·2	0·9	−6·66	+12·19	+1·01
23	III	6·00	14·95	4·19	25·14	2·0	8·2	1·7	+0·63	+1·37	+0·90
37	V	6·96	22·68	5·72	35·36	1·5	8·0	0·5	+0·96	+7·73	+1·53
46	VI	10·96	20·69	6·28	37·93	1·0	8·5	0·5	+4·00	−1·99	+0·56
57	VIII	5·22	23·46	12·2	40·88	0·8	8·5	0·2	−5·74	+2·77	+5·92
65	IX	3·72	0·25	18·09	22·06	0·8	8·8	0·0	−1·5	−23·21	+5·89
72	X	4·16	1·58	19·26	25·00	0·8	8·5	0·0	+0·44	+1·33	+1·17
78	XI	4·80	nil	24·08	28·88	0·5	8·2	0·3	+0·64	−1·58	+4·82
184	XXVI	3·72	nil	25·29	29·01	—	—	—	−1·08	—	+1·21
					(ii) LABORATORY EXPERIMENT						
Initial	—	2·24	0·1	8·24	10·59	6·0	7·9	—	—	—	—
8	I	3·26	0·22	3·07	6·55	5·5	7·9	0·5	+1·02	+0·11	−5·17
16	II	4·94	0·24	1·96	7·15	5·2	8·2	0·3	+1·68	+0·02	−1·11
23	III	10·00	0·28	2·61	12·97	4·9	8·2	0·3	+5·14	+0·04	+0·65
37	V	12·44	0·3	2·75	15·49	4·7	7·3	0·2	+2·36	+0·03	+0·14
46	VI	13·72	0·33	3·43	17·49	3·4	7·9	1·3	+1·28	+0·03	+0·68
57	VIII	15·42	0·35	3·8	19·57	3·3	8·2	0·1	+1·7	+0·02	+0·37
65	IX	16·92	0·36	4·00	21·28	2·9	8·2	0·4	+1·5	+0·01	+0·20
72	X	16·44	0·49	4·94	21·88	2·5	8·5	0·4	−0·48	+0·13	+0·94
78	XI	13·80	4·5	6·55	24·83	2·0	8·1	0·5	−2·64	+4·01	+1·61

ammonia it was simultaneously oxidized to nitrite and hence it did not accumulate. Nitrite formation was predominant during the first 5 weeks and afterwards irrespective of nitrite formation, its oxidation was significant. After 6 weeks no ammonification was observed. There was no significant nitrite formation also after 8 weeks.

The same type of laboratory experiment conducted at a low temperature (10-14°C), however, showed some interesting results (Table 1). Ammonification, though very slow, did not stop.

Since the oxidation of ammonia and nitrite were not carried out at any accelerated rate at this lower temperature, ammonium was found to accumulate. Though there was a slight increase in nitrite content initially, it could not be due to ammonium oxidation but due to nitrate reduction since a significant decrease in nitrate concentration was noticed during the first 2 weeks. Oxidation of ammonia to nitrite was considerable only in the XI week.

Experiments conducted at low temperature also showed an increase in total inorganic nitrogen, except during the I week. However, the increase was only half of that observed in *in situ* experiments. It seemed that though nitrate reduction was less affected by lower temperature, ammonification at this temperature was relatively slower than that observed at ambient temperature. Von Brand and Rakeshtraw[22] reported that the rate of decomposition was more than doubled by an increase of 6° to 8°C in temperature. Von Brand *et al.*[23] found that low temperature might either inhibit nitrite formation completely or retard it, depending on the organisms present. They also observed that the oxidation of nitrite to nitrate was less affected by low temperature than the formation of nitrite from ammonia.

In the present study, during the 1st week of incubation at 10-14°C the dissolved organic nitrogen (DON), not shown in the Table, increased by 0·5 µg at/litre (initial value of DON was 5·63 µg at/litre. But, DON in *in situ* experiments, slightly decreased (by 0·08 µg at/litre). However, in the II week DON increased by 1·88 µg at/litre and at low temperature the increase was 3·38 µg at/litre, i.e. almost double the quantity observed at higher temperatures. This indicated that at lower temperatures the decomposition (conversion of particulate organic matter) was not retarded as much as the mineralization (conversion of organic form of nutrients into inorganic form) and so there was an accumulation of DON. But in experiments *in situ* (26-28°C) decomposition and mineralization took place simultaneously with the result that DON did not accumulate.

At the commencement of the experiment the dissolved oxygen content (30°C) was 6·0 ml/litre which depleted to a very low level in 8 weeks (0·8 ml/litre and below). Oxygen consumption was higher in the III (1·7 ml/litre) and I weeks (1·4 ml/litre). At low temperature however, oxygen depletion (Table 1) was very gradual and there was sufficient oxygen even in the VIII week. The last 3 weeks experienced an oxygen concentration below 3 ml/litre. The maximum oxygen consumption was observed during the VI week.

The above *in situ* experiments were carried out with estuarine water collected in July (premonsoon season). Another series of experiments were performed with estuarine water collected in January, i.e. after the monsoon (Table 2). Though significant variation was not observed, the rate of biological processes was considerably different. Ammonium oxidation was normally dominant in the II week, but simultaneous nitrite oxidation also was evident, and it started even in the II week itself. However, nitrate concentration decreased in the IV and V weeks. By comparison ammonium production was slower in this series of experiments but the rate of production of nitrate was more.

To understand the biological potential of the inshore waters, *in situ* experiments on nitrification using sea water collected from 10 fm line at a depth of 5 m were conducted and the results are shown in Table 3 (samples were incubated in the estuary and not in the sea).

More or less the same trend of events was observed as for the estuarine water. Initial nitrate reduction was slightly more during the first 2 weeks, while ammonium production was also in progress. After a lag period of 2 weeks, nitrite formation from ammonium oxidation commenced, but the rate of nitrite production was less than that for estuarine water. Nitrite oxidation was observed only in the X week and that too was not significant. Difference in the biological capacity for nitrification between estuarine and sea water was well manifested in these experiments.

TABLE 2 — *In situ* EXPERIMENT ON NITRIFICATION IN ESTUARINE WATER AT WATER TEMPERATURE IN THE ENVIRONMENT (POSTMONSOON SEASON)

Days	Week	NH_4 µg at/litre	NO_2 µg at/litre	NO_3 µg at/litre	Total inorganic nitrogen µg at/litre	O_2 ml/litre	*p*H	O_2 consumption ml/litre
0	—	2·7	0·21	6·24	9·16	5·7	7·9	—
8	I	4·64	0·895	5·06	10·60	5·3	8·2	0·4
15	II	1·5	5·35	8·22	15·16	5	8·2	0·3
23	III	4·96	0·965	14·1	20·03	4·8	8·5	0·2
21	IV	5·86	1·857	8·44	16·14	4·3	8·5	0·5
36	V	6·78	2·257	4·26	13·301	4	8·5	0·3
43	VI	6·4	0·95	11·82	19·17	4	8·5	0·0
50	VII	7·07	0·65	14·76	22·48	3·3	—	0·7

TABLE 3 — *In situ* EXPERIMENT ON NITRIFICATION IN SEA WATER COLLECTED FROM 10 FM LINE AT WATER TEMPERATURE

[The bottles were incubated at 1 m depth in the estuary. Values expressed in μg at/litre]

Days	Week	NH_4	NO_2	NO_3
Initial	—	1·57	0·25	12·79
8	I	5·76	0·58	7·81
16	II	6·85	1·38	5·16
23	III	7·32	2·32	5·27
37	V	15·74	3·73	4·73
46	VI	8·79	7·32	5·44
57	VIII	7·33	8·42	5·71
65	IX	6·94	8·79	6·23
72	X	6·32	7·57	7·89

The present study showed that the estuarine environment offers some interesting insight with respect to the nitrogen cycle. Rajendran[24] showed that in the Vellar estuary, though phototrophic microorganisms assimilated, recycled and as a result lowered the ammonium concentration, ammonium was added almost continuously to the system by way of land drainage, atmospheric dissolution during precipitation and through decomposition so that its concentration showed fluctuation with time. There was every possibility of this substrate being oxidized by chemoautotrophs, although, the concentration of the microbes was low. The number of nitrifying bacteria per litre in the sea is not known exactly, but Carlucci and Strickland[10] in view of the slowness of nitrifying processes, and difficulty of isolation of nitrifying bacteria concluded that the number do not exceed 1000/litre. Density of the nitrifiers was found to be higher in the estuarine water than in the open ocean water. The estuarine and coastal water contain a large amount of particulate matter which harbour a very high bacterial population. Carlucci and Strickland[10] found that substrate and microbial concentrations were considerably higher in microzones near the particles of detritus undergoing decomposition than the mean concentrations found by direct analysis. Ammonium oxidation in the estuary might be favoured by the very shallow photic zone (about 1 m in the Vellar estuary). Decomposition and ultimate ammonia production might also be increased in the bottom sediments by heterotropic bacteria. Ammonium and nitrite were the final energy sources and nitrate accumulation would be maximum. But nitrate is assimilated and reduced to amino nitrogen which is subsequently decomposed and recycled through NH_4^+, NO_2 and NO_3.

The samples of *in situ* experiments which were incubated for 3 months or more, showed an increase in nitrate content with no accumulation of nitrite. The experiemental conditions favoured nitrate production, but it would be unlikely in the natural environment because (i) nitrite concentration was always very low, (ii) nitrite formed might be recycled or assimilated and (iii) nitrite oxidizers were either absent or present in very low numbers. Nitrite is only an intermediate product formed from ammonia and nitrate. The change in the concentration and distribution of nitrite in a body of water alone could not explain the activity of chemoautotrophic nitrifiers especially in the photic zone because it would be masked by biological processes (for example phototrophic assimilation) and physical processes in the environment.

The *in situ* experiments showed that before ammonia was oxidized, nitrate decreased rapidly. This observation was consistent with the view that nitrate reduction could also be a significant process in the production of nitrite. This could possibly be more in bottom water and in the upper most surface of the bottom sediments. Mountain[12] observed that the relative amounts of ammonium oxidized and nitrate reduced might be influenced by the concentration of organic matter in the original sample and the C/N ratio. Considering the substrate concentrations for ammonia oxidation and nitrate reduction and the number of chemoautotrophs with the heterotrophs, it seemed that both nitrate reduction and ammonium oxidation were significant processes in estuarine and coastal environments. Using stable ^{15}N, Miyazaki *et al.*[52] also showed that the potential activities for nitrite production from nitrate and ammonia in the shallow waters of Sagami Bay were of the same order of magnitude. They also pointed out that the capacities for assimilation of inorganic nitrogen in the upper layers (0·5) were several times higher than those for the oxidation of ammonia and the reduction of nitrate, and that a reverse relation was observed below the euphotic zone. However, the biological activity, whether heterotrophic or autotrophic, dominates the other, when the substrate suitable for it exceed the other compounds. As pointed out by Gundersen *et al.*[13] the biological potential of a particular process, if it was thermodynamically advantageous, dominated other activities in a body of water.

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