

Nitrification Rates in the Baltic Sea: Comparison of Three Isotope Techniques

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Simultaneous measurements of nitrification in the Baltic Sea were made at 10- to 30-m intervals in the months of June and November by three isotope techniques: [¹⁵N]nitrate dilution, N-serve sensitive [¹⁴C]bicarbonate incorporation, and [¹⁵N]ammonium oxidation to nitrite and nitrate. Nitrification rates of 1 to 280 nmol liter⁻¹ day⁻¹ were recorded, and each method showed that the highest rates of nitrification occurred below the halocline. Even in the presence of ammonium, dark incubations of mixed layer (above ca. 50 m) waters never yielded nitrification rates exceeding 45 nmol liter⁻¹ day⁻¹. The rates measured by the ammonium oxidation method were two- to sevenfold greater than those obtained by ¹⁴C incorporation or ¹⁵N dilution. The merits of each technique are discussed, and it is suggested that the [¹⁵N]ammonium oxidation method should be used in conjunction with the [¹⁴C]bicarbonate incorporation method.

Reported measurements of nitrification rates in the sea are few, undoubtedly due to the lack of sensitive methods. Previously, the only isotope used in such measurements in the open sea was [¹⁵N]ammonium, by Hattori and Wada (9) and Miyazaki et al. (15, 16) in the West Pacific Ocean and Sagami Bay. Olson (17) observed the Southern California coastal waters, the central North Pacific gyre, and the Scotia Sea. The latter study showed that the addition of [¹⁵N]ammonium (1 μM) did not stimulate nitrification, and the reported rates were between 1 and 10 nmol liter⁻¹ day⁻¹ in the euphotic zone and between 2 and 50 nmol liter⁻¹ day⁻¹ below 50 m.

The present investigation is a preliminary, parallel evaluation of three isotope techniques used to measure nitrification in natural seawater. In this study nitrification is the oxidation of ammonium, with the end products being nitrate or nitrite. The [¹⁵N]ammonium oxidation method (9, 15-17) was chosen because it measures nitrification unequivocally and provides the possibility for comparing the nitrifying activity observed in the Baltic Sea with that in other areas. However, the K_s (see below) for marine nitrifiers given by Helder and de Vries (10), for example, suggests that an increase in the ammonium concentration, in most cases high above the ambient concentration, gives potential rather than actual rates. With high concentrations of added [¹⁵N]ammonium, one would expect the percentage of ¹⁵N in the ammonium pool to be unaffected by the ammonification. If, however, trace amounts of ¹⁵N could be used then the percentage of ¹⁵N in the small ammonium pool may decrease during incubation because of ammonium production, and therefore the [¹⁵N]ammonium would have to be monitored as well.

The [¹⁵N]nitrate dilution method, previously used by Koike and Hattori (14) in sediment, was chosen because the ammonium concentration is not increased. The sensitivity, however, is low and highly dependent on the pool sizes of nitrite and nitrate. Assuming a reproducibility in the determination of the percentage of ¹⁵N of 1%, then 2 days to 2 weeks would be the minimum length of incubation in the studies cited above (9, 15, 16). In the Scotia Sea, where the ambient nitrate concentration in the photic zone is about 30 μM, the ammonium oxidation rate measured by Olson (17) would not be detected by this method. The nitrite pool is low

compared with the nitrate pool, which would make the alternative determination of the [¹⁵N]nitrite dilution rate much more sensitive, but the possibility of nitrate reduction to nitrite makes it difficult to interpret these data as nitrification.

The ¹⁴C method was included because its sensitivity is compatible with reported rates with appropriate sample volumes and added amounts of [¹⁴C]bicarbonate. The [¹⁴C]bicarbonate uptake by ammonium oxidizers was measured, taking advantage of their sensitivity to N-serve (2-chloro-6-trichloromethylpyridine). Unfortunately, there are doubts about the specificity (3, 23) or efficiency of N-serve in the sea, and carbon fixation by ammonium-oxidizing bacteria may not always be quantitatively related to nitrite and nitrate production (7, 8). Billen (3) first used the ¹⁴C method in sediments, and it has been used by Somville (21) in the Scheldt Estuary waters. These measurements constitute the first for the distribution of nitrification rates with depth in the Baltic Sea.

MATERIALS AND METHODS

Sampling and analysis. The main field work was done at Stations BY31 (Landsort deep) and BY38 (Karlsö deep) (Fig. 1 and Table 1) in November 1980 and June 1982.

Water samples were taken at different depths with standard hydrographical samplers (Hydro Bios) or with a 30-liter polyvinyl chloride Niskin sampler. Routine determinations of temperature, salinity, alkalinity, oxygen, nitrite, nitrate, and ammonium were carried out on board the ship by the methods of Carlberg (5). Samples for particulate nitrogen analysis were collected from 500 ml of water on precombusted glass fiber filters (25 mm; GF/F; Whatman) and stored dry for later analysis on a Carlo Erba 1106 CHN elemental analyzer. Light penetration into the water was determined with a Secchi disk.

Table 1 gives the experimental protocol. Water samples for incubation experiments were withdrawn directly from the samplers into acid-rinsed incubation bottles, and excess aeration of the samples was avoided. Rubber stoppers were not used to seal the bottles because Hooper and Terry (11) have found that the growth of *Nitrosomonas* sp. is inhibited by rubber. Incubations were carried out in the dark at ca. 7°C. Incubated samples with added ¹⁵N were analyzed for

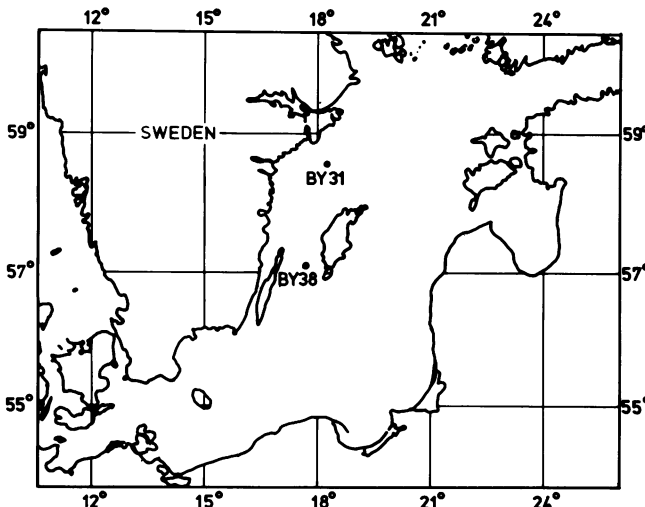


FIG. 1. The Baltic Sea. Positions of Stations BY31 and BY38, from which samples used in this study were obtained.

oxygen and ammonium, as described above, and for nitrite and nitrate with a Technicon autoanalyzer I by the method of Armstrong et al. (1).

Nitrification as determined by N-serve sensitive [^{14}C] bicarbonate incorporation. By the procedure of Somville (21), four 100-ml glass bottles were completely filled with water from each depth. In the experiment carried out in June, all the bottles were wrapped in aluminum foil to exclude light during sampling. Water from 70 m and shallower was passed through a 25- μm -mesh nylon screen to remove the larger phytoplankton. A 0.5% (wt/vol) solution of N-serve (100 μl ; 2-chloro-6-trichloromethylpyridine; Dow Chemical Co., King's Lynn, United Kingdom) in ethanol was added to two replicate samples to a final concentration of 5 mg liter $^{-1}$. The ethanol was added to the remaining replicate samples as a control. All bottles were glass stoppered and kept in a dark room at constant temperature (ca. 7°C). After 2.5 to 5 h, 1 ml of [^{14}C]bicarbonate solution (total activity, 14 to 24 μCi) was added to each sample, and the bottles were returned to the room at constant temperature. After 24 h of incubation, each sample was filtered through a 25-mm Millipore membrane filter (pore size, 0.45 μm) which was treated with HCl fumes for 20 min and dissolved in 15 ml of Aquasol (Packard Instrument Co., Inc., Rockville, Md.). The activity of the [^{14}C]bicarbonate solution was determined on 40- μl fractions in 15 ml of Oxifluor (New England Nuclear Corp., Boston, Mass.). All filters were counted in a Packard Tri Carb liquid scintillation counter. Nitrate production was calculated by multiplying the N-serve-sensitive CO_2 incorporation by 8.3, an empirical molar nitrate production- CO_2 incorporation ratio for nitrifying bacteria given by Billen (3).

[^{15}N]ammonium oxidation. Samples were collected in 2-liter amber glass bottles with glass stoppers, and 10 μmol of [^{15}N]ammonium (97%; Prochem B.O.C. Ltd., London) per bottle was added immediately. They were incubated at 7°C for 1 to 2 days, transferred to the laboratory, and incubated until they were analyzed 4 to 5 days after sampling. Determinations of [^{15}N]nitrite and [^{15}N]nitrate were made on 500-ml portions of incubated samples that were filtered through prerinsed glass fiber filters (25 mm; GF/F; Whatman). For nitrate, the filtrate was passed through the Cd-Cu column described by Strickland and Parsons (22) to reduce the nitrate to nitrite. Subsequently, the procedure of

Schell (20) was essentially used: 5 ml each of 0.5% (vol/vol) aniline in 2 M HCl, 0.75% beta-naphthol in 3 M NaOH, and 2 M phosphoric acid solution was added to the eluate. The nitrite reacted to form an azo dye in which one atom of nitrogen was derived from nitrite and one from aniline. The dye was extracted with CCl_4 , washed once with 250 ml of 0.05 M HCl and twice with 250 ml of distilled water, and then taken to ca. 5 ml by evaporation at room temperature. A fraction of the extract containing ca. 1 μmol of nitrogen was taken up in a capillary tube (3 by 30 mm) and converted to N_2 by Dumas combustion as described by Rönner et al. (19), and the ^{15}N - ^{14}N ratio was determined by emission spectrometry by the method of Fiedler and Proksch (6). The same procedure was used on the sample to determine the ^{15}N - ^{14}N ratio in nitrite by omitting the reduction of nitrate to nitrite. A ^{15}N correction curve for the azo dye was made by means of a series of [^{15}N]nitrite standards.

[^{15}N]nitrate dilution. The principle we followed was previously used by Koike and Hattori (14). The samples were treated in the same way as those in which [^{15}N]ammonium was added, except that the incubation lasted for 7 and 12 days and that 0.5-liter bottles were used in the 7 days series from Station BY38. About 4 $\mu\text{mol liter}^{-1}$ of [^{15}N]nitrate (97%; Prochem) was added. Blackburn and Henriksen (4) gave the following formula for calculating the production (Prod.) of a substance, in this case nitrite and nitrate, when the initial (i) and final (f) percentage of ^{15}N is known: $\text{Prod.} = [\ln(i - n) - \ln(f - n)] \times c \times t$, where n is the naturally occurring percentage of ^{15}N , c is the concentration of the substance, and t is the incubation time.

RESULTS

Figure 1 shows the location of the two sampling stations, and Table 1 gives the incubation conditions. Figure 2 shows the physical and chemical conditions in the profiles made in November 1980 and June 1982. In November 1980 there was a halocline in the region of 60 m at Station BY31 and 80 m at Station BY38 (Fig. 2A and B). In June 1982 it was found at 70 m at Station BY31; it was less pronounced at Station BY38 (Fig. 2C and D). The oxygen concentrations rapidly declined below the halocline, and in November the waters became virtually anoxic below 100 m (Fig. 2A and B). In June an oxygen concentration of 0.14 ml liter $^{-1}$ was found at the bottom at BY38 (Fig. 2D), and anoxia developed at 200 m at BY31 (Fig. 2C), as shown by the presence of hydrogen sulfide. The Secchi depth was 11 m at BY31 and 15 m at BY38 on both sampling occasions. The 1% level (1.5×10^{15} quanta $\text{cm}^{-2} \text{s}^{-1}$) would correspond to 25 and 35 m or shallower at BY31 and BY38, respectively (estimated by using the insolation at full sunshine in June in the Baltic Sea, 1.0×10^{17} quanta $\text{cm}^{-2} \text{s}^{-1}$; E. Sahlsten, personal communication).

Figure 2 also shows that the observed nitrification rates ranged from 1 to 280 nmol liter $^{-1} \text{day}^{-1}$, with there being an agreement between the three methods within a factor of 10. All three methods showed that the highest rates of nitrification were at or below the halocline. The highest rates of nitrification obtained in the mixed layer (above 50 m) was 45 nmol liter $^{-1} \text{day}^{-1}$ in November and 9 nmol liter $^{-1} \text{day}^{-1}$ in June at Station BY31 (Fig. 2A and C). The highest nitrification rates were obtained in the deep water samples, in which both ammonium and oxygen were present. However, there was no evident correlation between either the ambient ammonium or dissolved oxygen concentrations and the nitrification rate; the highest nitrification activities coincided with the lowest (0.4 ml liter $^{-1}$) oxygen concentrations in

TABLE 1. Experimental protocol for the comparison of nitrification rates in the Baltic Sea by three methods in samples collected at the Landsort deep (Station BY31) and the Karlsö deep (Station BY38) in 1980 and 1982

Station and position ^a	Depth to bottom (m)	Date	Method used	Additions		Incubation time (days)
				Ion	$\mu\text{mol liter}^{-1}$	
Station BY31, 58°35'/18°14'	459	11 November 1980	¹⁴ C incorporation	H ¹⁴ CO ₃ ²⁻	11	1
	459	2 June 1982	¹⁵ NO ₃ ⁻ dilution	¹⁵ NO ₃ ⁻	4	7 and 12
			¹⁵ NH ₄ ⁺ oxidation	¹⁵ NH ₄ ⁺	5	4-5
		¹⁴ C incorporation	H ¹⁴ CO ₃ ²⁻			1
Station BY38, 57°07'/17°40'	110	12 November 1980	¹⁴ C incorporation	H ¹⁴ CO ₃ ²⁻	11	1
	105	3 June 1982	¹⁵ NO ₃ ⁻ dilution	¹⁵ NO ₃ ⁻	4	7 and 12
			¹⁵ NH ₄ ⁺ oxidation	¹⁵ NH ₄ ⁺	5	5
		¹⁴ C incorporation	H ¹⁴ CO ₃ ²⁻			1

^a North latitude/east longitude.

ammonium-enriched samples from ca. 30 cm above the bottom at BY38 in June. The [¹⁵N]ammonium-enriched samples showed rates that were two- to sevenfold higher than those obtained with the other two methods below the mixed layer.

Figure 3 shows the net changes of inorganic nitrogen fractions and of particulate nitrogen at BY38 during the June incubations of samples in which [¹⁵N]ammonium (Fig. 3A) and [¹⁵N]nitrate were added (Fig. 3B). There was a net decrease in the ammonium concentration in all [¹⁵N]ammonium-enriched samples. Ammonium was nearly depleted in the [¹⁵N]nitrate-enriched samples collected below 60 m, but at 50 m there was a net ammonification. There was a peak of nitrite in the upper part of the halocline in June at BY38 at 60 m and a less pronounced peak at BY31 at 40 to 60 m (Fig. 2D and C). During incubation ([¹⁵N]nitrate or [¹⁵N]ammonium added) nitrite accumulated in samples from these depths (Fig. 3A and B), and practically all nitrite originated from the ammonium pool, as shown by ¹⁵N analysis. Below the halocline, nitrite (¹⁵N) did accumulate when [¹⁵N]ammonium was added, but not when [¹⁵N]nitrate was added. Figure 3A also shows that there was a net loss of inorganic nitrogen in the ammonium-enriched, oxygen-poor bottom water of up to 170 nmol liter⁻¹ day⁻¹. The increase of the particulate nitrogen pool did not explain the loss.

Figure 4 shows the [¹⁴C]bicarbonate incorporation rates. In 46 of 53 cases, the agreement was better than ± 1 nmol of C liter⁻¹ day⁻¹ between replicate samples and not worse than ± 2 nmol liter⁻¹ day⁻¹, except in samples with very low oxygen concentrations. The N-serve-sensitive bicarbonate uptake constituted 24 to 78% of the total uptake in the same depth range. The background uptake rate was 1 to 14 nmol of C liter⁻¹ day⁻¹, which was the highest level measured in the mixed layer in November. Additions of as much as 200 μl of ethanol alone per bottle had no effect on the total bicarbonate incorporation rate as compared with the references.

DISCUSSION

For the [¹⁵N]ammonium oxidation method, ca. 0.5 μmol of nitrite or nitrate must be present in the samples for the emission spectrometry determination. The detection limit of the emission spectrometry was ca. 0.2% of ¹⁵N enrichment. The most important and obvious objection to the method is that it is necessary to increase the ammonium concentration. In waters in which the in situ concentration of ammonium is

not limiting to the nitrifiers, the technique should measure the true nitrification rate, e.g., in the mixed layer at both stations. Olson (7) has reported that ammonium oxidation rates are independent of ammonium concentrations over a concentration range of 0.1 to 20 μM in samples from Southern California coastal waters. Helder and de Vries (10) and Knowles et al. (13) found, however, half saturation constants of 55 and 93 μM , respectively, for cultures of ammonium oxidizers at 25°C. Using the temperature dependence of K_s given by Knowles (13), the K_s at 7°C should be on the order of 10 μM . In the present study, in which the final ammonium concentration was ca. 5 μM , the rates measured below the mixed layer may have been potential rather than actual. In general, the rates (1 to 280 nmol liter⁻¹ h⁻¹) obtained by the [¹⁵N]ammonium method were within the same magnitude and showed similar depth distributions as those reported by others (9, 15, 16, 17, 24), who used the same method in different parts of the Pacific Ocean.

The N-serve-sensitive [¹⁴C]bicarbonate incorporation method has two important advantages: (i) it is a true tracer method with high sensitivity and (ii) it is the most convenient method for field studies. However, the assumption must be made that the relation between bacterial carbon fixation and nitrite and nitrate production is constant. This may not always be true because nitrifiers produced less nitrite and fixed more carbon at low oxygen concentrations than in air, according to Gundersen et al. (8). Goreau et al. (7) have reported that a larger proportion of the oxidized ammonium ended up as nitrous oxide at lower oxygen concentrations. Belser (2) showed recently, however, that the nitrification-CO₂ fixation ratio is independent of growth rate and ammonium concentration (if steady). Figures 2 and 4 show that there is no relation between the N-serve-sensitive bicarbonate incorporation and that of the background.

The [¹⁵N]nitrate dilution method has the advantage that it measures nitrifying activity at the ambient ammonium concentration (at least initially), but it is the least sensitive of the three methods. Contamination with nitrogenous compounds is always a potential error that causes overestimation of rates. The sensitivity of the determination of the ¹⁵N/¹⁴N ratio (in nitrite and nitrate) in this study was about ± 0.5 atom percent, which is often too low for use in the open sea, as discussed above, but the method should be useful for the confirmation of other methods in natural samples in which the turnover of nitrite or nitrate (sum of ambient and added)

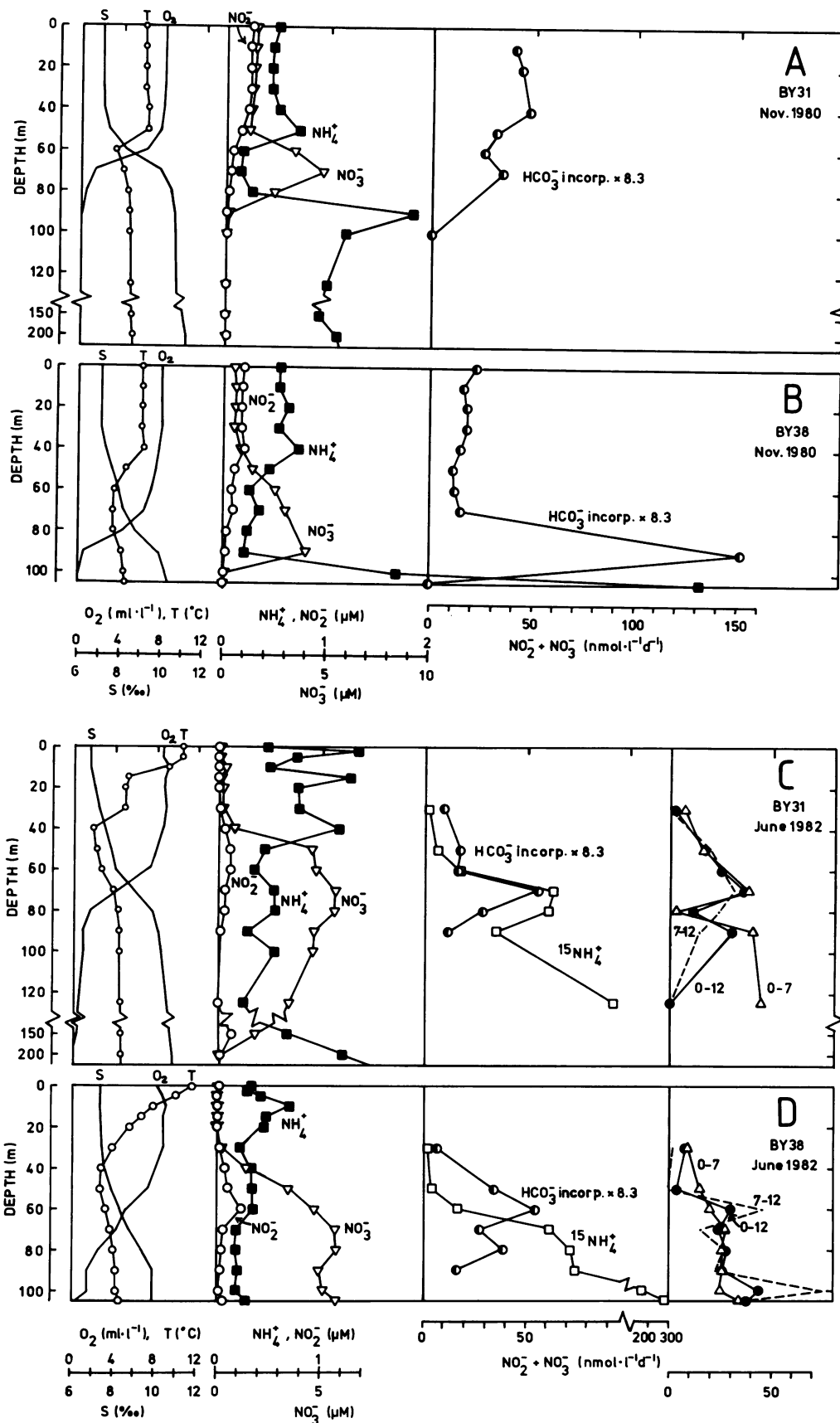


FIG. 2. Vertical distribution of ammonium (■), nitrite (○), nitrate (▽), oxygen (O₂), salinity (S), and temperature (T). (A) Station BY31, November 1980. The nitrification rate was measured as N-serve-sensitive [¹⁴C]bicarbonate incorporation (●). (B) Station BY38 in November 1980; ● is as defined for panel A. (C) Station BY31 in June 1982; nitrification rate from [¹⁵N]ammonium oxidation (□) compared with the nitrification rate obtained by the ¹⁴C incorporation technique (see panel A) and the [¹⁵N]nitrate dilution technique in samples incubated for 7 (Δ) and 12 (●) days and from days 7 to 12 (-----). (D) Station BY38 in June 1982; symbols are as described for panel C.

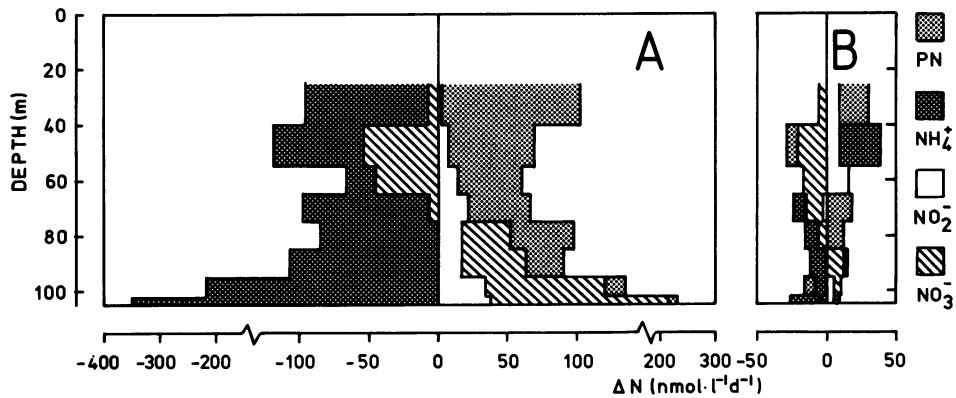


FIG. 3. Rates of net changes of particulate nitrogen (PN), ammonium, nitrite, and nitrate during dark incubations of water from station BY38 in June 1982. (A) 5 μmol of [^{15}N]ammonium per liter was added; incubation was for 5 days; (B) 4 μmol of [^{15}N]nitrate per liter was added; incubation was for 7 days.

is on the order of 5% per hour. The need to run samples at time zero makes the dilution method twice as laborious as the [^{15}N]ammonium oxidation method. In this study, freezing did not preserve the time zero samples properly, which was revealed by their lower nitrate concentrations compared with those obtained by shipboard analyses. However, the initial percentage of ^{15}N calculated from the shallowest

sample at Station BY31 differed by only 0.5% from the measured percentage of ^{15}N after incubation, and therefore, contamination was of minor consequence. In the 7- to 12-day (Table 1) time course profile (Fig. 2C and D), the 7-day samples constitute values at time zero for the 12-day samples. The similarities between the profiles imply that no systematic analytical error was made and that the prolonged

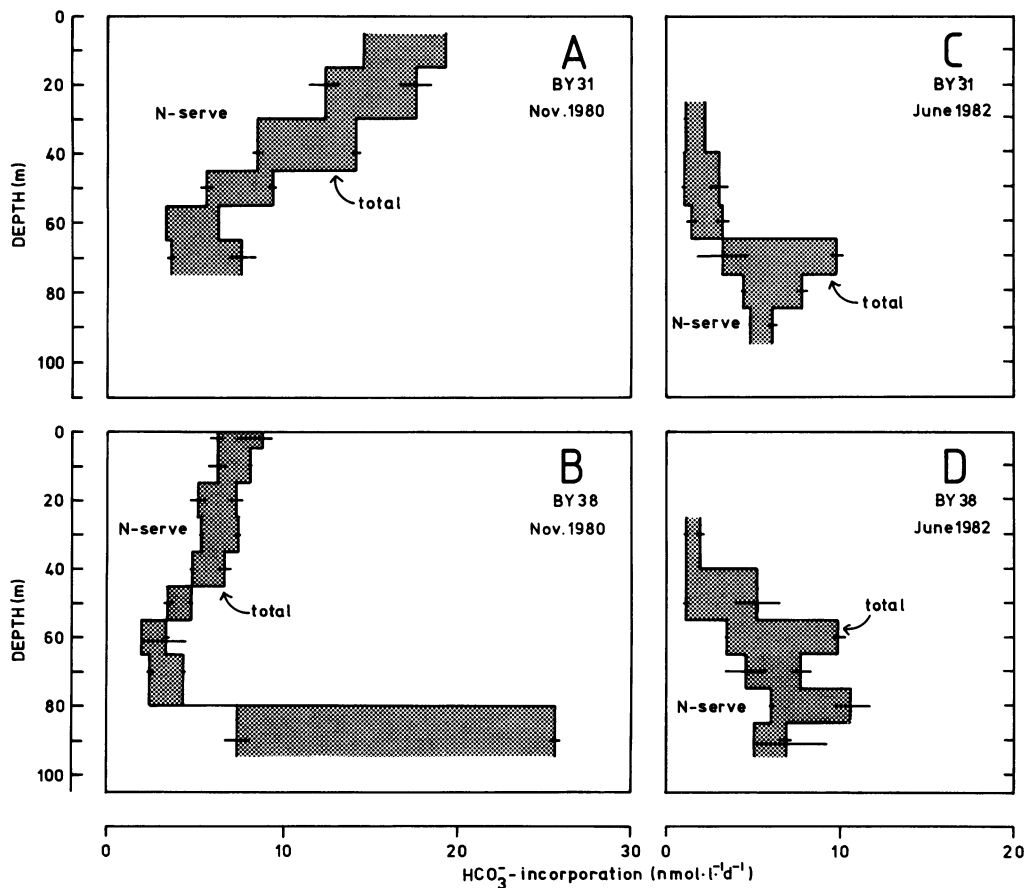


FIG. 4. Rates of [^{14}C]bicarbonate incorporation; the total rate and the rate with added nitrification inhibitor (N-serve) during incubation for 24 h are shown. Stations in panels A through D are the same as those described for panels A through D in the legend to Fig. 2.

incubation time was no serious drawback, except below the halocline at Station BY31 in June 1982 (Fig. 2C).

To conclusively evaluate the methods in waters in which ammonium limitation is suspected, the ammonium concentration must be raised to the same level in all samples, and the incubation time must be the same. In the present preliminary study, this was not done, and the cause of the two- to sevenfold spread in rates between the methods below the halocline is not known. It was either due to stimulation of nitrifiers by the addition of ammonium or it was due to ammonium depletion during incubation in which no ammonium was added or both. For example, the disappearance of ammonium during the 12-day incubation period in deep water samples from Station BY31 (Fig. 2C) implies that the measured rates might have been underestimated by the [¹⁵N]nitrate dilution method. The analytical error could account for a twofold discrepancy, at most. The ¹⁵N dilution method did yield rates (Fig. 2C and D) which were in fairly good agreement with the N-serve-sensitive [¹⁴C]bicarbonate incorporation rates. The latter method gave much higher rates above the halocline, as compared with the ammonium oxidation method, especially at Station BY38 in June (Fig. 2D). Dilution of the ¹⁵N in the ammonium pool due to ammonification, as discussed above, would only account for a minor fraction of the discrepancy, Ward et al. (24) showed by the fluorescent antibody staining technique that the number of ammonium-oxidizing bacteria is approximately constant with depth (about 5×10^4 cells per liter) in Southern California coastal waters, even though the rates of ammonium and nitrite oxidation varied with depth and was virtually zero in the euphotic zone. Ward (23) also found that the relative activity of single nitrifiers, measured as [¹⁴C]bicarbonate incorporation in combination with the fluorescent antibody staining technique, was not depressed in the shallow waters of the Northeast Pacific Ocean. The light inhibition of ammonium oxidation, as suggested by Olson (18), therefore probably did not affect the carbonate incorporation by these bacteria. Horrigan et al. (12) reported light inhibition of ammonium oxidation which lasted for several days. Therefore, incubation in complete darkness should be a safe procedure. There is, however, confusion on the effects of light on nitrifiers, and light-enhanced ammonium oxidation to nitrite was even reported by Miyazaki et al. (15).

Each of the three methods examined in this study have disadvantages, and probably none of them has been tested sufficiently to be used independently. Because the [¹⁵N]ammonium oxidation method and the [¹⁴C]bicarbonate incorporation method are the two most sensitive and least laborious, they should be used in future studies, possibly in parallel, especially in waters in which the ammonium concentration is high, to determine whether the nitrification-carbon fixation ratio is constant. The environments in which the [¹⁵N]ammonium oxidation method can be used alone with some confidence was discussed above. In those cases in which nitrification is expected to be limited by ammonium, the [¹⁴C]bicarbonate incorporation method can be used to determine carbon fixation by nitrifiers. However, the discrepancy between bicarbonate fixation and ammonium oxidation by nitrifiers (23) in the upper mixed layers needs further work.

The measured nitrification rates varied greatly with depth (Fig. 2). Nitrification was very low in the mixed layer, especially in June. The highest nitrification rates were found in and beneath the halocline. No single environmental factor explains this depth distribution. Ammonium, particulate nitrogen, and oxygen concentrations were not in correlation with the rates. The very highest nitrification rates were

measured at depths near the anoxic zone (Fig. 2B) or the bottom water (Fig. 2D), possibly because an active population of nitrifiers was maintained in situ on ammonium that was provided from below by advection or there was an inoculum from the oxic-anoxic interface. The net loss of inorganic nitrogen in the oxygen-poor bottom water at Station BY38 in June (Fig. 3A and B) suggests that there is a coupling between nitrification and denitrification.

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