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Variations of nitrate reductase activity in marine phytoplankton*

by

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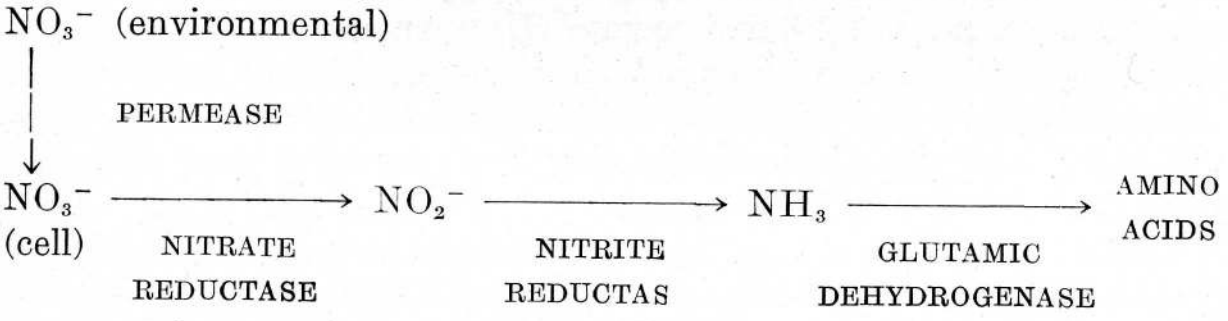
INTRODUCTION

The study of biochemical processes in the ocean can be facilitated by analysis of the enzyme systems that control them. Such analysis may provide explanations for oceanographic phenomena by facilitating the location of the sites and the determination of the rates of nitrate reduction, sulfate reduction, ammonia oxidation, photosynthesis, and respiration. Nitrate reduction is catalyzed by nitrate reductase, an enzyme widely distributed in plants and bacteria. This enzyme operates in conjunction with permeases, nitrite reductase, and glutamic dehydrogenase to remove nitrate from the environment and incorporate it into protein. It reduces the nitrate to nitrite ; nitrite reductase reduces the nitrite to ammonia, and then glutamic dehydrogenase fixes the ammonia into glutamic acid, the α-amino currency of amino acid synthesis.



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The rate limiting step in this process is thought to be that catalized by nitrate reductase (BEEVERS and HAGEMAN, 1969), a fact which partially explains the current interest in this enzyme. This paper presents observations on the variation of nitrate reductase (NR) activity with time, temperature, depth, and ammonia concentration in natural marine phytoplankton populations and in phytoplankton cultures.

METHODS

Ammonia was measured by the pyrazolone method automated by MACISAAC and OLUND (1971).

Chlorophyll a was measured by the method of LORENZEN (1966). Seawater was filtered through a 42.5 mm glass fiber filter [1] to which 1 ml of a 1 % suspension of $MgCO_3$ was added as filtration neared completion. The filters were stored (at 3°C) for less than 24 hours and ground in a teflon-glass tissue grinder with cold 90 % acetone. Chlorophyll was corrected for pheophytin formation using the equations of LORENZEN 1966) and a factor of 1.7.

Nitrate reductase (NR) was measured by the method of EPPLEY, COATSWORTH, and SOLORZANO (1969). Seawater was filtered through a 47 mm glass fiber filter [2] and ground immediately in 3 ml of cold (0-4°C) 0.2M phosphate buffer (pH 7.9) containing 1.0 mM threo, 2, 3 dihydroxy 1, 4 dithiobutane [3] (dithiothreitol or Cleland's reagent) and 10 mg polyvinylpyrrolidone [3] (PVP). The grinding time was 2 minutes. The storage time between sampling and filtering was kept below a half hour. NR activity was assayed by adding 1 ml of the crude uncentrifuged homogenate to 0.8 ml of a solution of 225 μM $NADH_2$, 175 μM $MgSO_4$ and 8 mM KNO_3 and incubating the mixture at 15°C (unless otherwise stated) for 30 minutes. The reaction was stopped by adding 5 ml 95 % ethanol and 0.2 ml of 1 M zinc acetate (HEWITT and NICHOLAS, 1964). The nitrite produced was measured after centrifugation by the method of BENDSCHNEIDER and ROBINSON (1952). Care must be taken to keep the $NADH_2$ and the dithiothreitol cold and dry until use, otherwise auto-oxidation decreases their reactivity.

Nitrate uptake was measured by the method of DUGDALE and GOERING (1967). Seawater was incubated with $^{15}NO_3$ at surface seawater temperatures for 24 hours under simulated natural illumination. Afterward the water was filtered through 42.5 mm glass fiber filters,[4] and

1 Whatman GF/C.
2 Gelman (Type A), Gelman Instrument Company, Ann Arbor, Michigan.
3 Nutritional Biochemical Corporation, 26201 Miles Road, Cleveland, Ohio 44128.
4 Reeve Angel (9844), 9 Bridewell Place, Clifton, New Jersey.

the filters were dried at 70°C for 6 hours and stored dry until the isotope ratios could be measured.

Particulate nitrogen (PN) was measured in a Coleman Nitrogen Analyzer.[5] Seawater was filtered through a glass fiber filter,[1] the filters were dried at 70°C for 6 hours and stored dry until analysis. Afterwards the filters were ground in a mortar and their nitrogen content determined by the micro-Dumas technique. Details of the method and the calculations may be found in the instruction manual for the Coleman Nitrogen Analyzer.

RESULTS

Temperature variations

EPPLEY, COATSWORTH, and SOLORZANO (1969) recommend an incubation temperature of 20-24°C for the nitrate reductase assay. In an attempt to confirm this optimum temperature, the NR activity in natural phytoplankton populations and in cultures was determined at different incubation temperatures. Both the homogenates and substrate solution were separately preincubated for 5 minutes at the test temperature. The results of these studies are shown in figure 1. In all 4 experiments (A, B, C, and D) the optimum occurs around 15°C. The Q_{10} values are shown in table 1; they vary from 1.6 to 3.3. In experiment C an

TABLE I

Increase in nitrate reductase activity with a 10 °C rise in temperature (Q_{10}).

Experiment (see Figure 1)	Q_{10}	Temperature Range
A	2.4	10.0-15.0
B	1.6	6.5-15.0
C	2.4	6.0-15.0
D	3.3	10.0-15.0

apparent energy of activation (BRANDTS, 1967) of 15 Kcal/mole was found from an Arrhenius plot of the data.

Cyclic activity

The cyclic variation of NR activity in a continuous culture of *Skeletonema costatum* exposed to natural illumination is shown in figure 2. A maximum in the NR activity occurs in the morning, but the

5 Coleman Instruments, Maywood, Illinois 60153.

time of occurrence varies. The maximum cannot be explained by an increase in biomass, because both PN and chlorophyll decreased to minimum values during the morning hours. The ratios of maximum to minimum in the NR, PN, and chlorophyll are 4, 2, and 17. These variations may explain many of the inconsistencies found in natural observations.

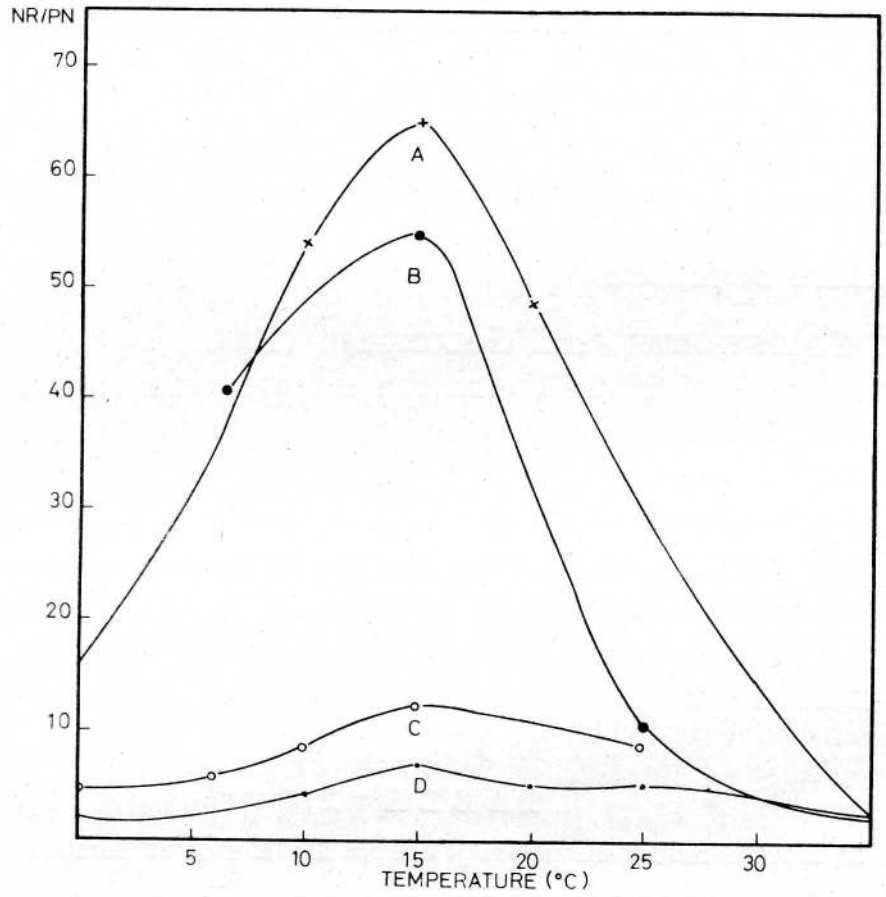

FIG. 1. — Temperature dependence of nitrate reductase (NR) activity normalized for particulate nitrogen (PN) in: A) a mixed culture of *Amphora* sp., *Skeletonema costatum*, and *Platymoras* sp. maintained at 17-20°C; B) a culture of *Skeletonema costatum* maintained at 16-19°C; C) a natural Peru current phytoplankton population consisting mainly of *Corethron histrix*, *Rhizosolenia fragilissima*, and *Skeletonema costatum* drawn from 19°C seawater; D) a culture of *Amphora* sp. maintained at 30°C. The NR/PN units are mM NO_3/μg-at N/hr.

The effect of light on the NR activity of a natural Peru Current phytoplankton population is shown in figure 3. Surface water from the Peru Current was kept in identical carboys at 18°C for 20 hours. One carboy was placed in the dark, and the other was exposed to natural illumination. The population in the dark did not show any increase in NR activity during the morning hours whereas the population exposed to light did. However, in the evening both were exposed to weak artificial illumination, and the population which had previously been in the dark displayed a slight increase in activity.

Depth variations

The vertical distribution of NO_3^- uptake and NR activity is shown in table 2. The ratio NO_3^- uptake/NR activity varies from 0.1 to 3 and appears to decrease with depth. Ratios lower than 1 were unexpected

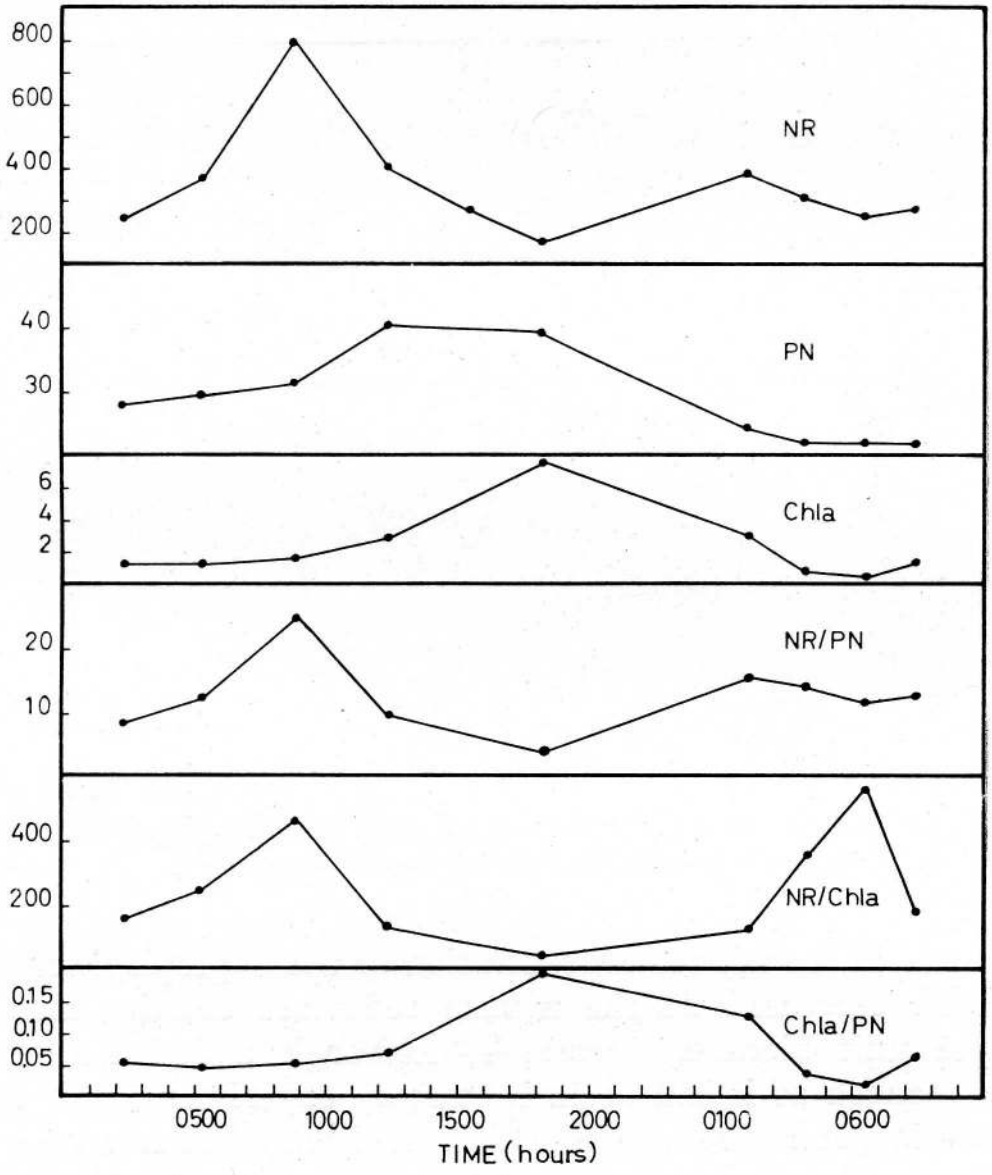

FIG. 2. — Diurnal variations in nitrate reductase activity (NR) in units of mM NO_3/liter/hr, particulate nitrogen (PN) in units of μg-at/liter, chlorophyll *a* (chl *a*) in units of μg/liter, NR per unit PN in units of mM NO_3/μg-at N/hr, NR per unit chl *a* in units of mM NO_3/μg/hr, and chl *a* per unit PN in units of μg/μg-at N in a continuous culture of *Skeletonema costatum*.

because previous results showed that NR activity ranged from 15 to 24 % of the NO^-_3 uptake (EPPLEY, COATSWORTH, and SOLORZANO, 1964; EPPLEY, PACKARD, and MACISAAC, 1970).

Ammonia inhibition

In a transition zone between high ammonia seawater and low ammonia seawater in the euphotic zone of Piraeus Harbor, Greece, both NR activity and NO_3^- uptake of the phytoplankton increased as the ammonia concentration decreased (figure 4). This increase cannot be explained

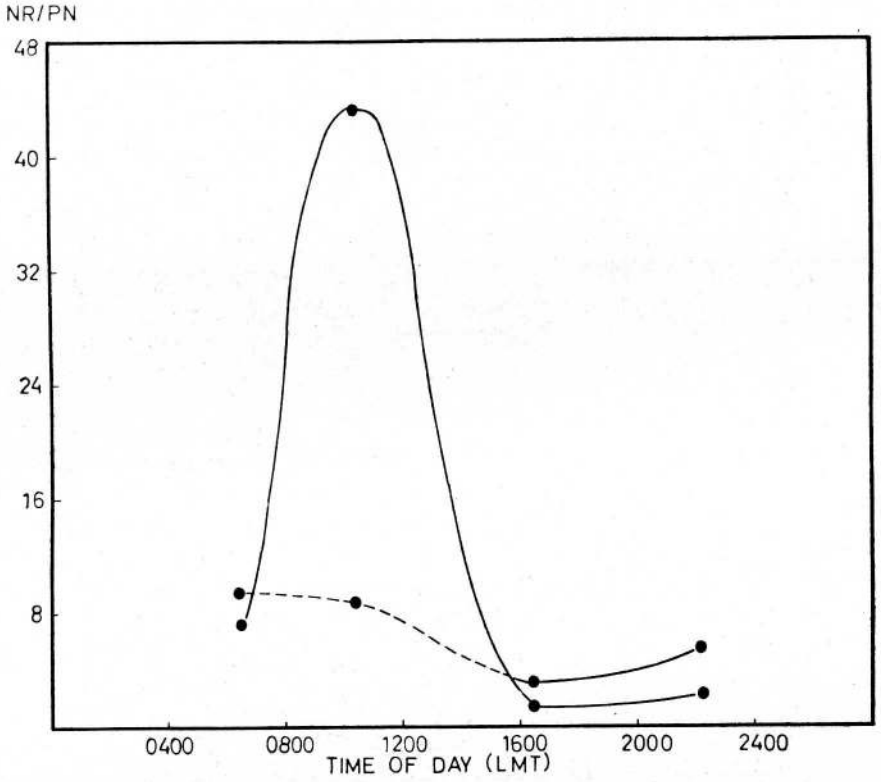

FIG. 3. — Variations of NR activity in natural Peru Current phytoplankton exposed to light (——) and exposed to darkness (- - -).

by an increase in the nitrate concentration or the phytoplankton biomass because both decreased as the nitrate reductase activity and nitrate uptake (per unit biomass) increased. Although a definite conclusion cannot be reached as it can be in laboratory experiments, we interpret this observation as a case of ammonia inhibition, similar to the phenomenon observed in cultures by SYRETT and MORRIS (1963) and EPPLEY, COATSWORTH, and SOLORZANO (1969) and by CONWAY and GALLIS (personal communication).

DISCUSSION

Nitrate reductase assays are useful because detection of activity indicates the occurrence of nitrate reduction. They would be even more useful if they could provide an estimate for the rate of NO_3 assimilation. This

use of the assays may be possible because NR is reported to be the rate limiting step in the assimilation process (BEEVERS and HAGEMAN, 1969). In this case, the optimal NR activity would represent the maximum velocity (V) of the process. The *in situ* NO_3 uptake velocity (v) could be estimated from the K_M of the enzyme system and the ambient NO_3 concentration (NO_3), under conditions where the Michaelis-Menten kinetic expression applies (DUGDALE, 1967).

$$v = \frac{V \cdot (NO_3)}{K_M + (NO_3)} \qquad (1)$$

Equation 1 predicts $v \leqq V$. Thus NO_3 uptake should never exceed NR activity. Earlier results (EPPLEY, COATSWORTH, and SOLORZANO, 1969; EPPLEY, PACKARD, and MACISAAC, 1970) and some data from station 59 (Table 2) show $v > V$. These results can only be explained if NR activity

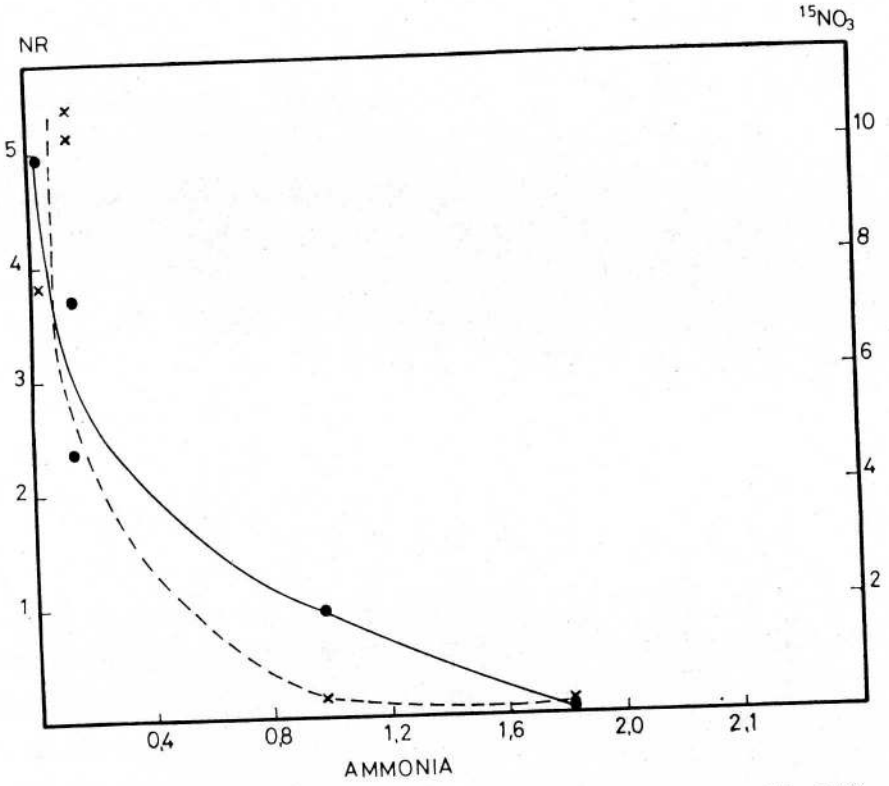

FIG. 4.—NR activity (×) and NO_3 uptake (●) from seawater with different ammonia concentrations in Piraeus Harbor, Greece.

underestimates V or if NO_3^- uptake overestimates v. The low value of V was originally interpreted as evidence that $NADH_2$-NR activity represented only one of the reduction systems (GRANT, 1968) and thus, underestimated V. Recent evidence, however, suggests that although there may be two reduction sites, one in the chloroplast and one in the cytoplasm, the enzymatic reaction at both sites may be the same (BEEVERS and HAGEMAN, 1969) and both would be measured by the

TABLE II

Vertical distribution of nitrate reductase (NR), nitrate uptake, chlorophyll *a*, and particulate nitrogen in Peru Current phytoplankton.

Depth (m)	Illumination (% of surface)	Chlorophyll *a* (μg/liter)	Particulate Nitrogen (μg-at/liter)	NO_3 Uptake (μg-at/liter/day)	NR (μg-at/liter/day)	NO_3 Uptake/NR
			STATION 59 (14° 58.3′S, 75° 35.0′W)			
0	100	6.78	11.86		0.833	
2	50	5.40	12.21		0.822	
5	25	6.61	8.33	1.799	0.754	2.38
8	10	5.92	7.37	1.430	0.516	2.77
16	1	5.07	6.89	0.212	0.440	0.48
			STATION 62 (15° 1.2S, 75° 41.3°W)			
0	100	4.93	7.12	1.878	2.010	0.93
2	50	3.30	8.34	2.234	2.770	0.83
5	25	5.12	8.64	1.134	3.660	0.31
8	10	4.47	8.28	1.058	3.310	0.32
16	1	4.88	7.45	0.265	2.910	0.09

same NR assay. Further evidence suggests another interpretation. NR appears to respond rapidly to changes in the NO_3 concentration (BEEVERS and HAGEMAN, 1969). Thus in NO_3 uptake measurements, the addition of NO_3 may induce NR and cause an overestimate in the rate of NO_3 uptake.

The low NO_3^- uptake/NR ratios at depth (table 2) may be explained by a reduction in photosynthesis resulting in substrate starvation of glutamate dehydrogenase. Under these conditions, NO_3 uptake would tend to decrease even though NR was present.

The temperature dependence of biological rates is well known (SIZER, 1942 and ROSE, 1967), and although it has been studied in connection with marine processes (RILEY, STOMMEL, and BUMPUS, 1949 ; RYTHER and GUILLARD, 1962), it is not routinely considered in the measurement of rate processes. This practice is justifiable when temperature variations are small ($\pm$ 2°C), but under conditions of thermal stratification, in the water column errors larger than 100 % may occur if corrections are not made to rates estimated by surface temperature incubation methods. The Q_{10} of the rate change can because, but the Arrhenius equation, $V_T = A\, e^{-E_a/RT}$, is more accurate, since the rate dependence on temperature is nonlinear. In this equation the absolute temperature (T) and the apparent activation energy (E_a) are used to calculate the rate of reaction (V_T). The frequency factor, A (EGGERS *et al.*, 1964) is difficult to calculate, but in estimating the rate of reaction at the *in situ* temperature (V_2) from the rate of reaction at a known temperature, V_1, the Arrhenius equation becomes :

$$V_2 = \frac{V_1 A_2}{A_1}\, e^{E_a\left(\frac{1}{T_1}-\frac{1}{T_2}\right)/R}$$

and the ratio, A_2/A_1 may be approximated by T_2/T_1 (PACKARD, 1969). E_a/R is easily calculated because it equals the slope of the line when ln V is plotted against 1/T. Experimental data from ^{14}C, ^{15}N, respiration, and enzyme experiments may be used in the calculation. This technique could be used routinely to correct rates measured in deck incubators to rates at *in situ* temperatures.

The cyclic activity of NR is not unique. Many other cycles have been observed in phytoplankton (MARGALEF, 1967 ; SWEENEY and HASTINGS, 1962). The observation is interesting because it helps to explain the cyclic activity of NO_3^- uptake (GOERING, DUGDALE, and MENZEL, 1964) and provides evidence that NR is dependent upon both respiration and photosynthesis (GRANT, 1968). It also emphasizes the importance of syncronous sampling and incubation in productivity surveys.

RESUMEN

VARIACIONES EN LA ACTIVIDAD DE REDUCTASA DE NITRATO EN EL FITOPLANCTON MARINO. — Uno de los procesos más importantes en el mar es la asimilación del nitrato por el fitoplancton. El análisis de la actividad de las enzimas que reducen el nitrato en el fitoplancton puede permitir conocer su capacidad para asimilar nitrato y la velocidad de esta asimilación. En este trabajo se estudian las variaciones diurnas en la actividad de las reductasas de nitrato y la relación de estas enzimas con diversos factores: temperatura, profundidad y presencia de amonio en el medio. El método de análisis utilizado es el de EPPLEY, COATSWORTH y SOLÓRZANO (1969). El fitoplancton fue concentrado filtrando agua con un filtro de fibra de vidrio. En los diversos experimentos realizados se ha encontrado que la actividad óptima tiene lugar a una temperatura de 15 °C y que esta actividad queda inhibida si hay amonio en el agua. En experiencias realizadas durante 48 horas se encontró un máximo de actividad en las primeras horas de la mañana. En el estudio de varias estaciones se ha visto que la actividad enzimática decrece con la profundidad. En conjunto, los resultados obtenidos concuerdan con las medidas de asimilación de nitrato que se hicieron simultáneamente con el método del ^{15}N ($NO_3^=$) (DUGDALE y GOERING, 1967). Esperamos que la aplicación de estos nuevos métodos en oceanografía permitirá conocer, de una manera rápida y cuantitativa, los mecanismos de asimilación del nitrógeno y sugerirá otras posibilidades de estudio de diferentes procesos biológicos que se realizan en el ambiente marino.

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