

*Limnol. Oceanogr.*, 47(5), 2002, 1514–1521  
© 2002, by the American Society of Limnology and Oceanography, Inc.

## Maximum rates of N<sub>2</sub> fixation and primary production are out of phase in a developing cyanobacterial bloom in the Baltic Sea

**Abstract**—Although N<sub>2</sub>-fixing cyanobacteria contribute significantly to oceanic sequestration of atmospheric CO<sub>2</sub>, little is known about how N<sub>2</sub> fixation and carbon fixation (primary production) interact in natural populations of marine cyanobacteria. In a developing cyanobacterial bloom in the Baltic Sea, rates of N<sub>2</sub> fixation (acetylene reduction) showed both diurnal and longer-term fluctuations. The latter reflected fluctuations in the nitrogen status of the cyanobacterial population and could be correlated with variations in the ratio of acetylene reduced to <sup>15</sup>N<sub>2</sub> assimilated. The value of this ratio may provide useful information about the release of newly fixed nitrogen by a cyanobacterial population. However, although the diurnal fluctuations in N<sub>2</sub> fixation broadly paralleled diurnal fluctuations in carbon fixation, the longer-term fluctuations in these two processes were out of phase.

Marine cyanobacteria make a major contribution to both the global carbon (Waterbury et al. 1979) and nitrogen (Capone et al. 1997; Zehr et al. 2001) cycles. Indeed, the activity of N<sub>2</sub>-fixing marine cyanobacteria may be a key factor influencing the ability of the oceans to sequester atmospheric CO<sub>2</sub> (Falkowski 1997). However, these organisms can also have a negative effect on the environment. For example, every year, massive blooms of N<sub>2</sub>-fixing cyanobacteria occur in the Baltic Sea (Kahru et al. 1994), and, when they decay, they can have a damaging effect on fish stocks and an adverse effect on the recreational use of the Baltic coastal zone (Edler et al. 1985; Sellner 1997). Herein we describe a detailed study made on a cyanobacterial bloom in the Baltic Sea during the summer of 1998 as part of the development of an integrated model that will enable us to predict how growth, primary productivity, N<sub>2</sub> fixation, and toxin production might respond to a changing environment. Our findings shed new light on how rates of N<sub>2</sub> fixation and primary production might fluctuate in a bloom of Baltic cyanobacteria, the extent to which these processes are coupled, and how these fluctuations may affect measurements of N<sub>2</sub> fixation. In addition, a rational explanation is proposed for the wide variations observed between estimates of N<sub>2</sub> fixation based on acetylene reduction and on <sup>15</sup>N<sub>2</sub> assimilation (Peterson and Burris 1976; Montoya et al. 1996).

**Methods**—Sample station and water chemistry: The experiment was carried out between 6 and 11 July 1998 by following a drifting array with an attached sediment trap at 33 m. The study site was at 56°18'N, 19°05'E, southeast of the Swedish island of Gotland. The experimental measurements were made over three 24-h periods, starting respectively at 0400 h (local time) on 6 (day 1), 8 (day 3), and 10 (day 5) July. Analysis of the water for NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, and dissolved inorganic P was performed as described by Grasshoff (1976), by use of samples collected in 5-liter bot-

tles connected to a conductivity, temperature, and depth rosette sampler (General Oceanics).

**Sampling:** During each experimental day, samples were collected by a vertical tow every 4 h from three different depths (0–7, 7–14, and 14–21 m) by use of a 100- $\mu$ m Apstein plankton net fitted with a closing device (Hydrobios). This ensured that the large aggregates of N<sub>2</sub>-fixing cyanobacteria were collected but excluded the smaller unicellular cyanobacteria. Collected samples were suspended in 2 liters of filtered (Whatman GF/F, 47 mm) seawater. Aliquots were then removed for the various analyses described below. Where analyses were not performed immediately, samples were immediately frozen and remained frozen during transportation to the laboratory.

**Analysis of cyanobacterial cells:** Particulate organic carbon (POC) and particulate organic nitrogen were measured on 50-ml samples by use of the improved methods of Sharp (1974), chlorophyll *a* as described by Barlow et al. (1997), and phycobiliproteins (PBP) as described by Bennett and Bogorad (1973). The intracellular ratio of glutamine to glutamate (GLN:GLU) was measured by analysis of 50 ml of cyanobacterial material that had been retained on a 20- $\mu$ m filter and suspended in 1 ml of deionized water (Flynn 1988). For sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting of the Fe protein of nitrogenase, 50-ml samples were collected on a 20- $\mu$ m filter and suspended in 0.5 ml of 10 mM Tris-HCl buffer (pH 8.0) that contained 1 mM ethylene diamine-tetraacetic acid and 25 g L<sup>-1</sup> SDS. The samples were then boiled for 5 min and frozen for transportation to the laboratory. After thawing, 2-mercaptoethanol was added to 50 g L<sup>-1</sup> and bromophenol blue to 0.1 mg L<sup>-1</sup>. The samples were then sonicated for 5 min (Reade et al. 1999), reboiled for 5 min, and centrifuged at 13,000  $\times$  g for 2 min. SDS/PAGE and Western blot analysis were performed on the supernatant as described by Reade et al. (1999).

**N<sub>2</sub> fixation:** Acetylene reduction was measured on board according to the method of Gallon et al. (1993) as described by Evans et al. (2000). Samples (2 ml) were incubated on deck in sealed 10-ml glass vials in an incubator that was maintained at the temperature of the surface water, either at the in situ photon flux density (achieved by use of natural illumination and neutral density filters) or under saturating illumination of 100  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> (provided by Osram white fluorescent lights) during the period of assay. Assimilation of <sup>15</sup>N<sub>2</sub> was measured under identical conditions with use of completely filled, sealed 250-ml Duran glass bottles, as described by Montoya et al. (1996). For measurement of the response of acetylene reduction to photon fluence rate, the continuous flow system described by Staal et al. (2001) was used.

**Primary production:** Samples (100 ml) were incubated for

Table 1. Physical conditions and water chemistry at the sample site in the Baltic Sea.

	Day 1 (6 July 1998)			Day 3 (8 July 1998)			Day 5 (10 July 1998)		
Total quantum flux (mol m <sup>-2</sup> )	30.8			57.4			32.8		
Average wind speed (m s <sup>-1</sup> )	9.3			3.4			4.2		
Depth (m)	4	9	20	4	9	20	4	9	20
Temperature (°C)	13.7	13.7	11.9	13.0	12.9	9.4	13.4	13.3	9.8
Nitrate (μM)	0	0	0.04	0.02	0.05	0.06	0.03	0.02	0.02
Nitrite (μM)	0.05	0.06	0.07	0.03	0.05	0.03	0.05	0.04	0.03
Ammonium (μM)	0.04	0.05	0.05	0.04	0.06	0.12	0.06	0.08	0.08
Phosphate (μM)	0.11	0.12	0.16	0.11	0.08	0.15	0.08	0.08	0.09

4 h with 0.26 MBq (0.11 μmol) NaH<sup>14</sup>CO<sub>3</sub> in sealed 250-ml acid-washed polycarbonate Erlenmeyer flasks. The conditions of incubation were as described for acetylene reduction. Primary production was then measured according to the method of Fitzwater et al. (1982).

**Results and discussion**—At the sample site, day length was ~17.5 h, whereas the weather conditions were variable, being cloudy and stormy on day 1, sunny and breezy on day 3, and cloudy and breezy on day 5 (see Table 1). Above the thermocline, which occurred between 15 and 40 m below the surface, the water column had a temperature close to 13°C throughout the experimental period. Despite the rough weather on day 1, there was no evidence that extensive mixing had occurred below 15 m. Moreover, the drift track was unaffected by surface weather conditions. The extinction coefficient of the water column was 0.353, so that at 3.5 m (the average depth for the 0–7 m fraction), the prevailing irradiance was 65% of that at the surface. For depths of 10.5 (average depth for the 7–14 m fraction) and 17.5 (average depth for the 14–21 m fraction) m, the equivalent values were 7.5% and 0.9% of surface irradiance, respectively.

Nutrient analysis of the water (Grasshoff 1976) revealed that there were no substantial fluctuations in the concentrations of NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, and NH<sub>4</sub><sup>+</sup> (Table 1). Dissolved inorganic P was significant but not high. Some fluctuations were observed, but these were not consistent. The relative constancy of these nutrients supports the view that a single body of water had been followed throughout the experiment. The ratio of dissolved inorganic N to dissolved inorganic P was consistently <2. This value is well below the Redfield ratio of 16 and implies that growth of N<sub>2</sub>-fixing cyanobacteria might be expected.

Biomass data (POC, Chl *a*, and PBP) all supported the view that most of the large cyanobacteria were in the upper 0–7 m of the water column (Table 2). There was a large

accumulation of material in the upper zone by the end of the experiment, as was confirmed visually by the accumulation of cyanobacteria at the water surface. It appeared, therefore, that the period of the experiment coincided with the formation of a surface cyanobacterial bloom. The biomass in the upper layer approximately doubled between day 1 and day 5. This increase was too great to be explained simply by upward drift of cyanobacteria from the deepest zone, which showed a decline of no more than 32% over the same period (Table 2). It seems most likely, therefore, that the increase in biomass largely reflected synthesis of new cells, although the possibility of lateral advection cannot definitely be excluded. This increase in cyanobacterial biomass was due mainly to an increase in the toxic heterocystous cyanobacterium *Nodularia spumigena* (Barker et al. 1999). Between days 1 and 5, the population of *N. spumigena* (56.6% of the cyanobacterial biovolume on day 1) and *Anabaena* sp. (1.4% of the cyanobacterial biovolume on day 1) increased by a factor of 4.7 and 3.9, respectively, whereas that of *Aphanizomenon* sp. (42.2% of the cyanobacterial biovolume on day 1) increased by a factor of only 1.5 (Congestri et al. 2000).

Under in situ conditions, the highest specific rates of N<sub>2</sub> fixation, measured by use of the acetylene reduction technique, were found in the upper 7 m of the water column, with peak activity occurring between 1200 and 2000 h each day (Fig. 1a). N<sub>2</sub> fixation was consistently low (although not absent) during the hours of darkness. In cells sampled from depths of 7–14 m, rates of N<sub>2</sub> fixation were lower, but the overall pattern of activity was similar to that in cells from the upper layer (Fig. 1c). Cells from the lowest depth (14–21 m) showed the lowest nitrogenase activity per unit of Chl *a* and smaller fluctuations in activity (Fig. 1e). The fact that Baltic cyanobacteria exhibited nitrogenase activity during the dark period and also at depths where the prevailing level of illumination was low suggests that cells can sustain N<sub>2</sub>

Table 2. Markers of cyanobacterial biomass in a cyanobacterial bloom in the Baltic Sea. Particulate organic carbon (POC), particulate organic nitrogen (PON), Chl *a* are expressed as mg m<sup>-2</sup> and phycobiliproteins (PBP) as μg m<sup>-2</sup>.

Depth (m)	Day 1				Day 3				Day 5			
	POC	PON	Chl <i>a</i>	PBP	POC	PON	Chl <i>a</i>	PBP	POC	PON	Chl <i>a</i>	PBP
0–7	11.2	1.70	1.45	36.7	14.1	2.37	1.53	51.2	22.9	3.91	2.41	88.9
7–14	8.37	1.32	0.59	16.7	6.32	1.07	0.78	31.6	8.36	1.47	0.84	22.6
14–21	5.71	0.96	0.41	16.3	3.88	0.74	0.22	14.8	3.88	0.70	0.43	14.6

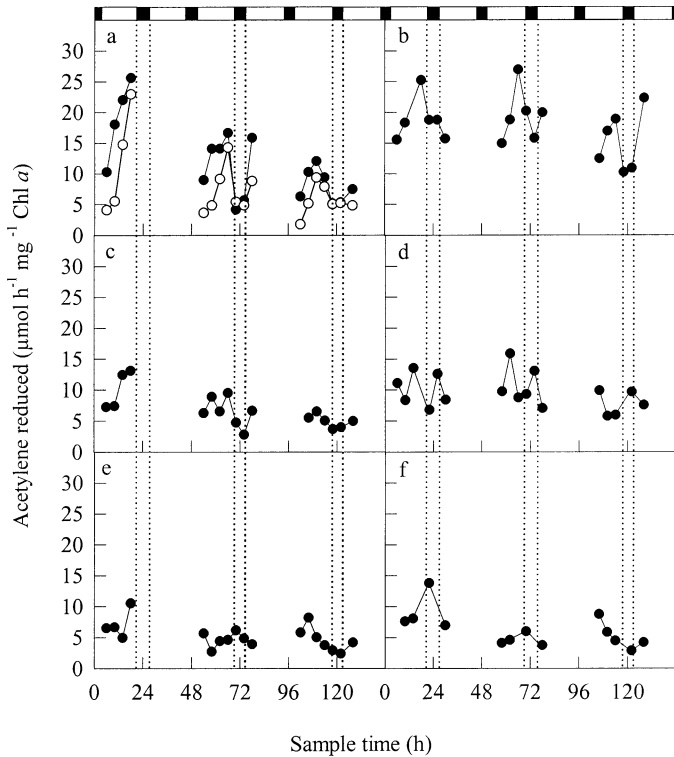


Fig. 1.  $N_2$  fixation (acetylene reduction) by cyanobacteria sampled from a bloom in the Baltic Sea between 6 and 11 July 1998 (days 1–5). Samples were collected by vertical tows between (a, b) 0–7, (c, d) 7–14, and (e, f) 14–21 m, resuspended in filtered seawater, and incubated (a, c, e) either at the in situ photon flux density or (b, d, f) under saturating illumination of  $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  during the period of assay. In panel a, solid circles show acetylene reduction in the absence of additions, and open circles show acetylene reduction in the presence of  $0.1 \text{ mg ml}^{-1}$  chloramphenicol, added at the start of each incubation. Zero time corresponds to 0000 h (local time) on 6 July 1998. The shaded areas and their associated dotted lines represent nighttime during the experimental period.

fixation, at least to a limited extent, by metabolic processes that operate in the dark.

Over and above the observed diurnal fluctuations, however, rates of acetylene reduction per unit of Chl *a* were high on day 1 but declined during days 3 and 5. This was especially obvious in cells sampled in the depth range 0–7 m.

For all sample depths, the specific rates of acetylene reduction (normalized to Chl *a*) were maximal above a photon flux density of  $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . However, the value of  $I_K$  varied with sample depth (Table 3). The maximum surface irradiance recorded during the experiment was  $2,122 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , observed between 1254 and 1300 h on day 3. This corresponds to  $1,379 \mu\text{mol m}^{-2} \text{ s}^{-1}$  at 3.5 m,  $159 \mu\text{mol m}^{-2} \text{ s}^{-1}$  at 10.5 m, and  $19 \mu\text{mol m}^{-2} \text{ s}^{-1}$  at 17.5 m, so, at least down to 10.5 m, Baltic Sea cyanobacteria would receive sufficient illumination to support maximum rates of  $N_2$  fixation for at least part of the day. Nevertheless, in samples from deeper waters, the specific rates of nitrogenase activity, measured either under saturating illumination or in the dark, were always lower than the corresponding rates seen in sam-

Table 3. Parameters relating to the response of acetylene reduction to photon fluence rate for cyanobacteria sampled from a bloom in the Baltic Sea. Samples were collected from the depth ranges indicated on 9 July 1998 at 0400 h. Acetylene reduction was measured over the irradiance range  $0\text{--}877 \mu\text{mol m}^{-2} \text{ s}^{-1}$ .  $I_K$  represents the irradiance at which rates of  $N_2$  fixation were 63.2% of maximum.

Parameter	Depth (m)		
	0–7	7–14	14–21
$I_K$ ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ )	25.7	30.2	16.4
Maximum total rate of acetylene reduction ( $\mu\text{mol h}^{-1} \text{ mg}^{-1} \text{ Chl } a$ )	22.00	12.63	4.94
Rate of acetylene reduction in dark ( $\mu\text{mol h}^{-1} \text{ mg}^{-1} \text{ Chl } a$ )	9.49	5.12	2.29

ples from surface waters (Table 3). This was particularly apparent in cells sampled below the mixed layer but was also seen in the fraction from 7 to 14 m, which implies rapid adaptation to the intensity of illumination at the depth from which they were sampled. This behavior may reflect physiological differences between cyanobacteria at different depths, or it may be that a large part of the biomass (Chl *a*) collected from deeper waters does not contribute to  $N_2$  fixation.

Even when sampled cyanobacteria were incubated under saturating illumination at  $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  during the period of assay, nitrogenase activity was often lower at night than during the day (Fig. 1b,d,f). This suggests that the amount of nitrogenase per unit of Chl *a* may vary during a 24-h cycle in these populations. In the case of the Fe protein of nitrogenase, this was demonstrated directly by SDS/PAGE coupled to immunoblotting, which showed that intracellular concentrations of this protein were highest in *N. spumigena* during the morning and lower during the afternoon and evening, although the Fe protein never completely disappeared from cells even during the dark period (Fig. 2). Furthermore,  $0.1 \text{ mg ml}^{-1}$  of chloramphenicol (an inhibitor of protein synthesis) was more inhibitory to  $N_2$  fixation during the morning than at any other time (Fig. 1a), which implies that nitrogenase was being actively synthesized during

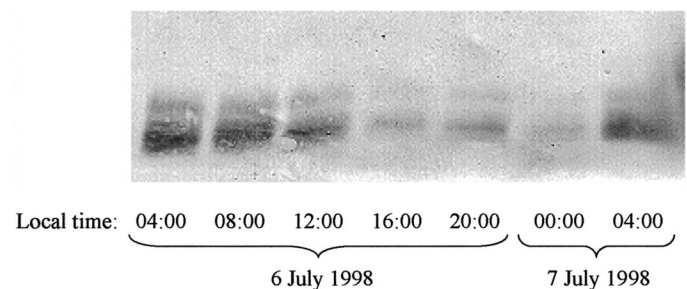


Fig. 2. Western immunoblot after SDS/PAGE of extracts of *N. spumigena* collected at 0–7 m from a bloom in the Baltic Sea on 6–7 July 1998 (day 1). Each sample contained  $25 \mu\text{g}$  of protein, and the blot was developed by use of antisera to the Fe protein of nitrogenase from *Rhodospirillum rubrum*. The two forms of the Fe protein that reacted with the antisera had approximate  $M_r$  values of 38,500 (upper band) and 36,000 (lower band).

this period. Significantly, however, the maximum rates of nitrogenase activity, seen under illumination at  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , did not appear to be much higher in samples from 0–7 m on day 1 than on days 3 and 5 (Fig. 1b). This implies that the lower activity seen during days 3 and 5 when cells were assayed under in situ conditions (Fig. 1a) reflected a relative decrease in the rate of  $\text{N}_2$  fixation independent of the amount of enzyme per cell. In contrast, however, the smaller decline in activity seen in samples from deeper waters as the experiment progressed was observed both under in situ conditions and under saturating illumination (Fig. 1c–f).

As in other cyanobacteria, the Fe protein of nitrogenase from *N. spumigena* could be resolved by SDS/PAGE into two forms of different  $M_r$  (Fig. 2). The larger form was consistently less abundant than the smaller form, and there was no evidence that the relative concentrations of the two forms varied during the 24-h period of study. It would be premature, therefore, to suggest that the observed fluctuations in nitrogenase activity were related to any shift between different forms of the Fe protein, as has been suggested for *Anabaena variabilis* (Ernst et al. 1990), *Oscillatoria limosa* (Villbrandt et al. 1992), and *Trichodesmium* (Zehr et al. 1993).

Acetylene reduction is used universally as a means of measuring  $\text{N}_2$  fixation (Turner and Gibson 1980). It relies on the fact that nitrogenase is the only enzyme that reduces acetylene to ethylene. As was discussed by Montoya et al. (1996), the theoretical ratio between the rate of acetylene reduction and that of reduction of  $\text{N}_2$  is 4 : 1. However, when rates of acetylene reduction are compared directly with rates of  $\text{N}_2$  fixation determined from incorporation of  $^{15}\text{N}_2$  into cellular material, the ratio of acetylene reduced to  $^{15}\text{N}_2$  incorporated frequently deviates from this theoretical value (Peterson and Burris 1976; Turner and Gibson 1980). In this study, a natural population of Baltic cyanobacteria gave a high value for this ratio (up to 20 : 1) during day 1, although during days 3 and 5, the ratio declined toward its theoretical value of 4 (Fig. 3).

Measurement of  $\text{N}_2$  fixation that uses  $^{15}\text{N}_2$  depends on the analysis of  $^{15}\text{N}$  incorporated into cells. Any released  $^{15}\text{N}$  would not be taken into account. Thus, if the population under investigation was releasing a portion of its newly fixed nitrogen, the  $^{15}\text{N}$  assay would underestimate the actual amount of  $\text{N}_2$  fixed. Measurement of nitrogenase activity by acetylene reduction does not suffer from this disadvantage, so, under these circumstances, the measured ratio of acetylene reduced to  $^{15}\text{N}_2$  incorporated would become greater than that theoretically expected. In support of such an explanation for the high value of this ratio during day 1 of the present study, there is evidence that, when fixing  $\text{N}_2$ , cyanobacteria, including *N. spumigena* (A. M. Evans unpubl. data), release nitrogenous material, notably as  $\text{NH}_4^+$  (Mulholland and Capone 2001) and amino acids (Flynn and Gallon 1990; Glibert and Bronk 1994). The observation that Baltic cyanobacteria fix more  $\text{N}_2$  than is needed to support their estimated rate of growth (Larsson et al. 2001) suggests that they also release newly fixed nitrogen in situ. Thus, the high rate of nitrogenase activity during day 1 (Fig. 1a) may well result in a large release of newly fixed nitrogen. In contrast, the release

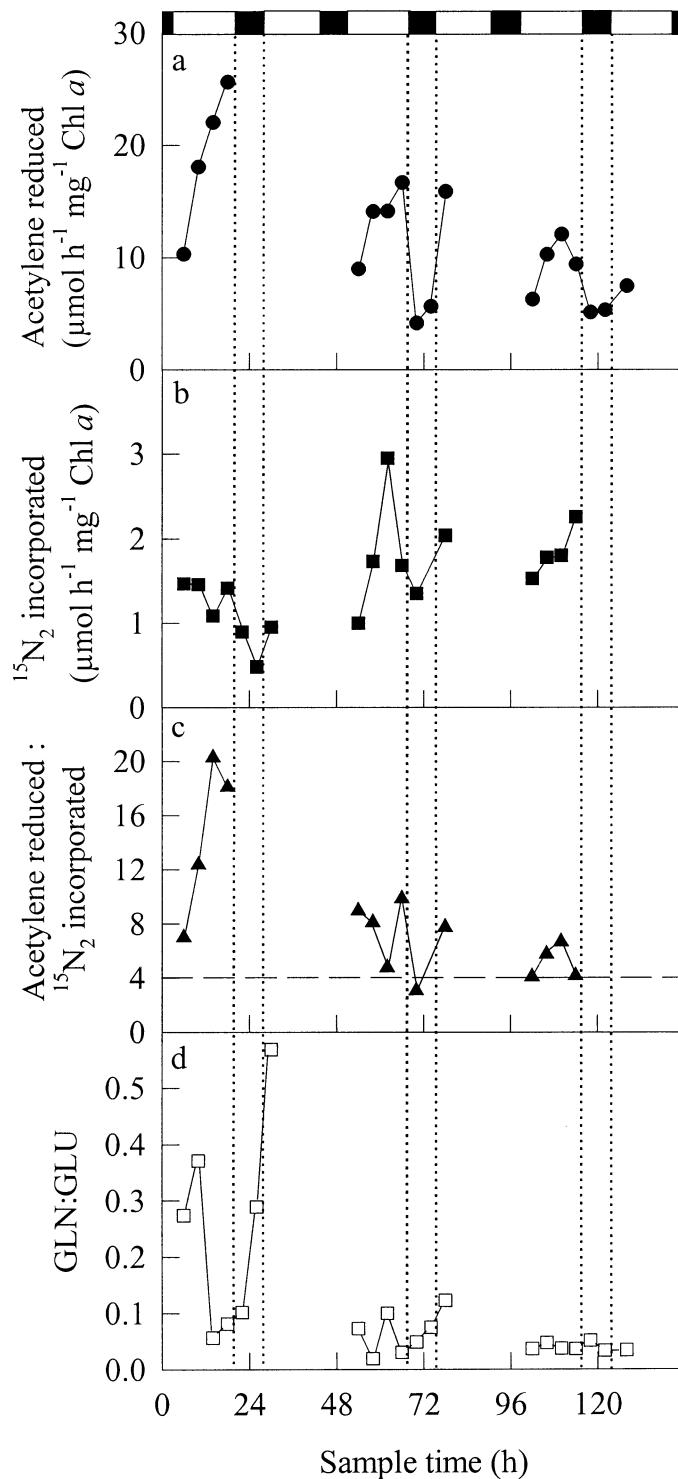


Fig. 3. Relationship between acetylene reduction and assimilation of  $^{15}\text{N}_2$  in cyanobacteria sampled from a bloom in the Baltic Sea between 6 and 11 July 1998 (days 1–5). Samples were collected at 0–7 m. Measurements of (a) acetylene reduction, (b) assimilation of  $^{15}\text{N}_2$ , (c) the ratio of acetylene reduced to  $^{15}\text{N}_2$  incorporated, and (d) intracellular GLN : GLU were all made at the in situ photon flux density. Zero time corresponds to 0000 h (local time) on 6 July 1998. The shaded areas and their associated dotted lines represent nighttime during the experimental period, whereas the horizontal dashed line in panel c represents the theoretical value (4 : 1) of the ratio between acetylene reduced and  $^{15}\text{N}_2$  incorporated.

of fixed nitrogen would be much lower during days 3 and 5. An underestimation of the true rate of  $N_2$  fixation by the  $^{15}N_2$  assay would therefore be much greater during day 1 than during days 3 and 5 and could explain the high ratios of acetylene reduced to  $^{15}N_2$  incorporated seen during day 1. To the best of our knowledge, this is the first demonstration that the ratio between acetylene reduction and  $^{15}N_2$  incorporation may vary with time in a specific natural population of cyanobacteria and also that this variation may have a physiological basis. These findings also suggest that, although  $^{15}N$  analysis accurately measures incorporation of  $^{15}N_2$  into cellular material, measurements of acetylene reduction more truly reflect the gross rate of  $N_2$  fixation.

The amino acids glutamate and glutamine constitute the primary link between N-metabolism and C-metabolism, and monitoring of the intracellular concentrations of glutamate and glutamine can provide a sensitive means of assessing the N-status of cells. The ratio of glutamine to glutamate (GLN:GLU) is higher in N-replete cells than in N-stressed cells (Flynn et al. 1989) and, in  $N_2$ -fixing cyanobacteria, responds rapidly to changes in intracellular N-status. For example, the addition of 0.1 mM  $NH_4Cl$  to  $N_2$ -fixing cultures of *Gloeothece* dramatically increased GLN:GLU within 1 h (Flynn and Gallon 1990). In the present study, the cyanobacteria sampled at 0–7 m showed a clear difference in GLN:GLU between day 1 and days 3 and 5 (Fig. 3d). By the end of day 1, the population had become relatively N-replete, probably because of the high rates of  $N_2$  fixation that were observed during that day (Fig. 3a). This would also explain how cells might be able to release amino acids at that time and thereby the high values of the ratio of acetylene reduced to  $^{15}N$  incorporated seen during day 1 (Fig. 3c). In addition, the dramatic decline in GLN:GLU that occurs between 1000 and 1400 h on day 1 (Fig. 3d) correlates well with the peak value of the ratio of acetylene reduced to  $^{15}N$  incorporated (Fig. 3c) and might therefore reflect a release of newly fixed nitrogen that results from the high rates of nitrogenase activity (acetylene reduction) observed during the preceding few hours (Fig. 3a). During days 3 and 5, however, the cells appeared to be more N-stressed than during day 1.

The change in the ratio of acetylene reduced to  $^{15}N_2$  incorporated (Fig. 3c) reflects both a decrease in the rate of acetylene reduction (Fig. 1a) and an increase in the rate of  $^{15}N_2$  incorporated into the cells (Fig. 3b). Although the former probably reflects a decline in nitrogenase activity during the course of the experiment, the latter may be explained in terms of decreased release of newly fixed nitrogen. The decline in nitrogenase activity broadly parallels the increase in nitrogen stress implied by the changes in GLN:GLU (Fig. 3d), but whether increased N-stress is caused by, or causes, a decline in nitrogenase activity is not clear.

Specific rates of primary production (incorporation of  $NaH^{14}CO_3$ ) were greatest in the upper 7 m of the water column (Fig. 4), peaked around noon, and declined to zero at night. In this respect, primary production broadly paralleled the observed 24-h fluctuations in  $N_2$  fixation. However, over the longer term, it appeared that  $^{14}C$  incorporation behaved differently from gross  $N_2$  fixation, measured as acetylene reduction. In the case of the population at 0–7 m, this differ-

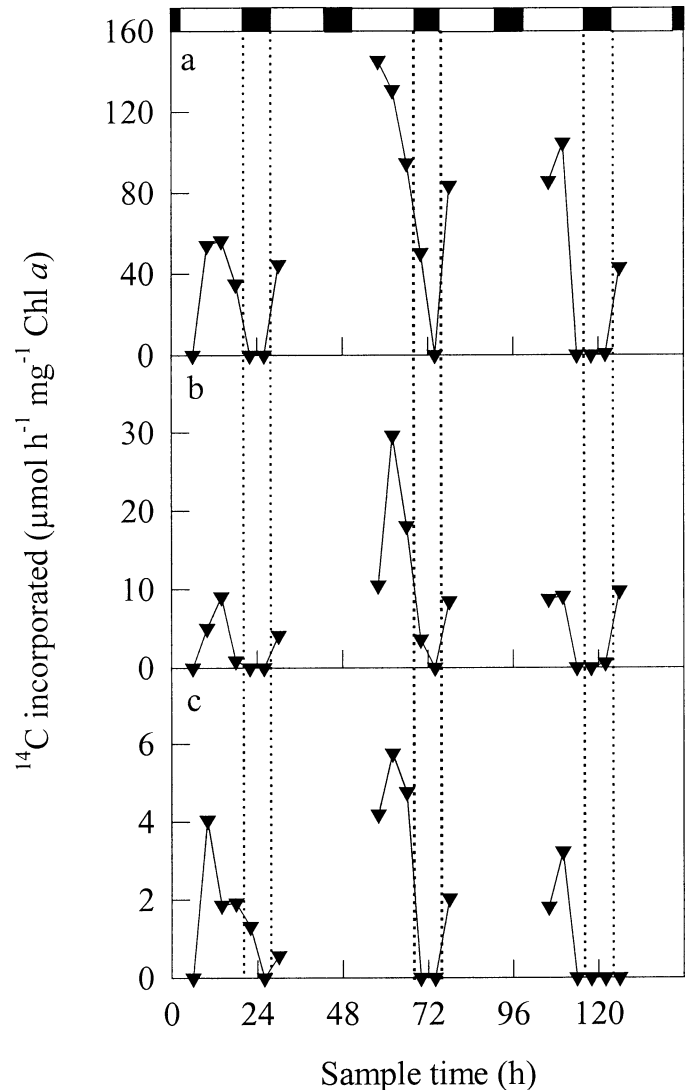


Fig. 4. Primary production by cyanobacteria sampled from a bloom in the Baltic Sea between 6 and 11 July 1998 (days 1–5). Samples were collected from (a) 0–7, (b) 7–14, and (c) 14–21 m. Zero time corresponds to 0000 h (local time) on 6 July 1998. The shaded areas and their associated dotted lines represent nighttime during the experimental period.

ence was especially apparent during day 1, when rates of acetylene reduction were high (Fig. 1a), whereas those of primary production were relatively low (Fig. 4a). This uncoupling between the two processes was much less apparent during the latter part of day 3 and during day 5, when the rates of acetylene reduction and  $^{14}C$  incorporation more closely paralleled each other. During day 1, it appears that metabolic energy was used preferentially to support  $N_2$  fixation at the expense of primary production.

It should be noted, however, that, just as incorporation of  $^{15}N_2$  into cyanobacterial biomass can underestimate gross  $N_2$  fixation, so  $^{14}C$ -incorporation can underestimate the gross rate of photosynthetic C fixation. Cyanobacteria release C-compounds such as glycolate (Renström-Kellner and Bergman 1989) as well as N-compounds, and the release of ami-

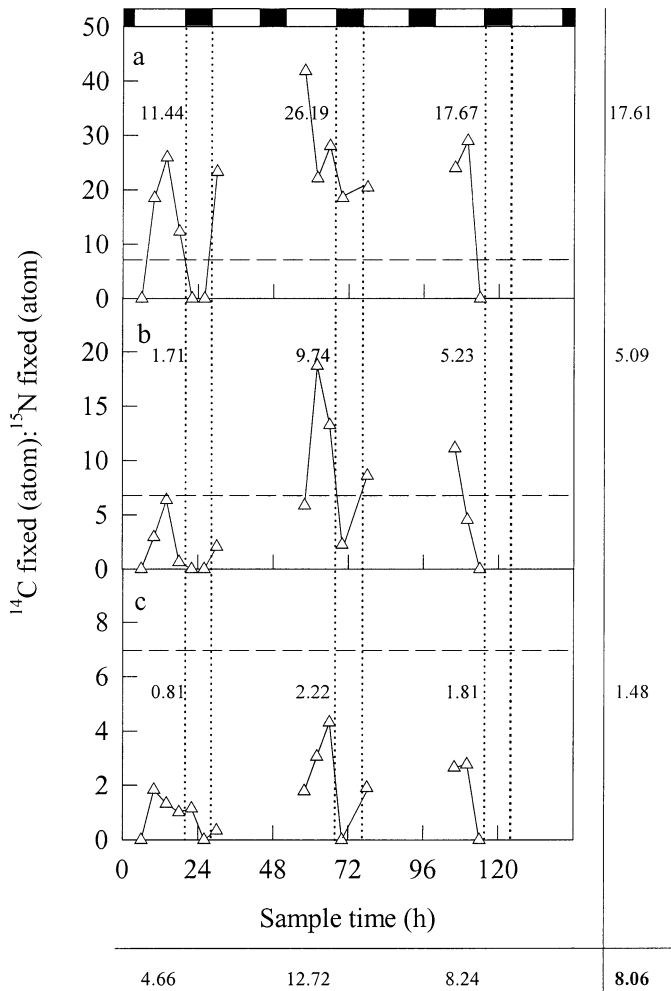


Fig. 5. Ratio of incorporation of  $^{14}\text{C}$  (primary production) to incorporation of  $^{15}\text{N}$  in cyanobacteria sampled from a bloom in the Baltic Sea between 6 and 11 July 1998 (days 1–5). Primary production and assimilation of  $^{15}\text{N}_2$  were measured in samples collected from (a) 0–7, (b) 7–14, and (c) 14–21 m (see Figs. 3 and 4 for details). Zero time corresponds to 0000 h (local time) on 6 July 1998. The shaded areas and their associated dotted lines represent nighttime during the experimental period, whereas the horizontal dashed lines represent the value of the Redfield ratio of C:N (6.9) for phytoplankton (Redfield 1958; Falkowski 2000). The numbers in the field show the average value for the ratio for that particular group of data, those to the right of the graph show the means for each sample depth, and those below the graph show the means for each experimental day. The overall mean value is shown at the bottom right corner.

no acids, for example, represents release of both C and N. Even so, however, the relative rates of C and N assimilation are not closely coupled. The ratio of incorporation of  $^{14}\text{C}$  (primary production) to that of  $^{15}\text{N}$  varied from day to day and with depth, being maximal during day 3 in the population at 0–7 m and minimal during day 1 in the population at 14–21 m (Fig. 5). At 8.06, the average value of the ratio at all sample times and at all depths throughout this experiment was reasonably close to the Redfield ratio of C:N for phytoplankton (6.9). Nevertheless, it is apparent that cyano-

bacterial blooms in the Baltic Sea oscillate between periods when  $\text{N}_2$  fixation is favored over C assimilation (for example, during day 1) and vice versa (during day 3). The high rates of  $^{14}\text{C}$  incorporation (relative to those of  $^{15}\text{N}$  incorporation) in the upper 0–7 m of the water column may, furthermore, result in an accumulation of carbon reserves that could be mobilized to support  $\text{N}_2$  fixation in deeper waters after a mixing event.

In the photic zone, nitrogenase activity in Baltic cyanobacteria is higher during the day than at night, although it never falls to zero. This 24-h fluctuation is, as might be expected, much less apparent in samples from deeper water, where the light intensity experienced during the day is much lower. These fluctuations reflect, at least in part, fluctuations in the concentration of nitrogenase in the cyanobacterial cells, which implies a regulation of nitrogenase synthesis and/or degradation. However, superimposed on these 24-h fluctuations is a variation in nitrogenase activity that takes place over several days. Cyanobacteria sampled during day 1 were more active in  $\text{N}_2$  fixation (acetylene reduction) and, relatively, more N-replete than were cells sampled on days 3 and 5. This may be only one phase of an oscillating cycle between N-stress and N-replenishment during the development of a cyanobacterial bloom, or it could simply reflect the different weather conditions prevailing during the experiment. Nevertheless, in blooms,  $\text{N}_2$  fixation may show long-term fluctuations, with cyanobacteria therefore alternating between N-replenishment (during which they may release fixed nitrogen) and N-starvation. As a consequence, the ratio of acetylene reduction to  $^{15}\text{N}_2$  incorporation may also vary, being highest when cells are releasing more of their newly fixed N. Indeed, the variable value of this ratio, far from being a problem for ecophysicologists, may provide useful information about the physiological state of the population under study.

In addition, it appears that  $\text{N}_2$  fixation and primary production are not always in phase during the formation of cyanobacterial blooms in the Baltic Sea. In turn, this implies that these two processes are neither tightly coupled nor co-regulated.

J. R. Gallon,<sup>1</sup> A. M. Evans, and D. A. Jones

Biochemistry Research Group  
School of Biological Sciences  
University of Wales Swansea  
Singleton Park, Swansea  
SA2 8PP, United Kingdom

P. Albertano and R. Congestri

Department of Biology  
University of Rome "Tor Vergata,"  
Via della Ricerca Scientifica  
00133 Rome, Italy

B. Bergman, K. Gundersen, and K. M. Orcutt

Department of Botany  
Stockholm University  
S-106 91 Stockholm, Sweden

<sup>1</sup> Corresponding author (j.r.gallon@swansea.ac.uk).

K. von Bröckel, P. Fritsche, M. Meyerhöfer, K. Nachtigall,  
and U. Ohlendieck

Institut für Meereskunde an der Universität Kiel  
Düsternbrooker Weg 20  
D-24105 Kiel, Germany

*S. te Lintel Hekkert*

Department of Molecular and Laser Physics  
University of Nijmegen  
NL-6525 ED Nijmegen, The Netherlands

*K. Sivonen and S. Repka*

University of Helsinki  
Department of Applied Chemistry and Microbiology  
P.O. Box 56  
00014 University of Helsinki, Finland

*L. J. Stal and M. Staal*

Netherlands Institute of Ecology—KNAW  
Department of Marine Microbiology  
P.O. Box 140  
NL-4400 AC Yerseke, The Netherlands

## References

- BARKER, G. L. A., P. K. HAYES, S. L. O'MAHONY, P. VACHARAPIYASOPHON, AND A. E. WALSBY. 1999. A molecular and phenotypic analysis of *Nodularia* (cyanobacteria) from the Baltic Sea. *J. Phycol.* **35**: 931–937.
- BARLOW, R. G., D. G. CUMMINGS, AND S. W. GIBB. 1997. Improved resolution of mono- and divinyl chlorophylls *a* and *b* and zeaxanthin and lutein in phytoplankton extracts using reverse phase C-8 HPLC. *Mar. Ecol. Prog. Ser.* **161**: 303–307.
- BENNETT, A., AND L. BOGORAD. 1973. Complementary chromatic adaptation in a filamentous blue-green alga. *J. Cell Biol.* **58**: 419–435.
- CAPONE, D. G., J. P. ZEHR, H. W. PAERL, B. BERGMAN, AND E. J. CARPENTER. 1997. *Trichodesmium*: A globally significant marine cyanobacterium. *Science* **276**: 1221–1229.
- CONGESTRI, R., R. FEDERICI, AND P. ALBERTANO. 2000. Evaluating biomass of Baltic filamentous cyanobacteria by image analysis. *Aquat. Microbial Ecol.* **22**: 283–290.
- EDLER, L., S. FERNO, M. G. LIND, R. LUNDBERG, AND P. O. NILSSON. 1985. Mortality of dogs associated with a bloom of the cyanobacterium *Nodularia spumigena* in the Baltic Sea. *Ophelia* **24**: 103–109.
- ERNST, A., Y.-D. LIU, S. REICH, AND P. BÖGER. 1990. Diurnal nitrogenase modification in the cyanobacterium *Anabaena variabilis*. *Bot. Acta* **103**: 183–189.
- EVANS, A. M., J. R. GALLON, A. JONES, M. STAAL, L. J. STAL, M. VILLBRANDT, AND T. J. WALTON. 2000. Nitrogen fixation by Baltic cyanobacteria is adapted to the prevailing photon flux density. *New Phytol.* **147**: 285–297.
- FALKOWSKI, P. G. 1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO<sub>2</sub> in the ocean. *Nature* **237**: 272–275.
- . 2000. Rationalizing elemental ratios in unicellular algae. *J. Phycol.* **36**: 3–6.
- FITZWATER, S. E., G. A. KRAMER, AND J. H. MARTIN. 1982. Metal contamination and its effects on primary production measurements. *Limnol. Oceanogr.* **27**: 544–551.
- FLYNN, K. J. 1988. Some practical aspects of measurements of dissolved free amino acids in natural waters and within microalgae by the use of HPLC. *Chem. Ecol.* **3**: 269–293.
- , D. M. J. DICKSON, AND O. A. AL-AMOUDI. 1989. The ratio of glutamine: glutamate in microalgae: A biomarker for N-status suitable for use at natural cell densities. *J. Plankton Res.* **11**: 165–170.
- , AND J. R. GALLON. 1990. Changes in intracellular and extracellular  $\alpha$ -amino acids in *Gloeothece* during N<sub>2</sub> fixation and following addition of ammonium. *Arch. Microbiol.* **153**: 574–579.
- GALLON, J. R., D. M. PEDERSON, AND G. D. SMITH. 1993. The effect of temperature on the sensitivity of nitrogenase to oxygen in the cyanobacteria *Anabaena cylindrica* (Lemmermann) and *Gloeothece* (Nägeli). *New Phytol.* **124**: 251–257.
- GLIBERT, P. M., AND D. A. BRONK. 1994. Release of dissolved organic nitrogen by marine diazotrophic cyanobacteria, *Trichodesmium* spp. *Appl. Environ. Microbiol.* **60**: 3996–4000.
- GRASSHOFF, K. [ED.]. 1976. *Methods of seawater analysis*. Verlag Chemie.
- KAHRU, M., U. HORSTMANN, AND O. RUD. 1994. Satellite detection of increased cyanobacterial blooms in the Baltic Sea: Natural fluctuation or ecosystem change? *Ambio* **23**: 469–472.
- LARSSON, U., S. HAJDU, J. WALVE, AND R. ELMGRAN. 2001. Baltic Sea nitrogen fixation estimated from the summer increase in upper mixed layer total nitrogen. *Limnol. Oceanogr.* **46**: 813–820.
- MONTOYA, J. P., M. VOSS, P. KAHLER, AND D. G. CAPONE. 1996. A simple, high precision, high-sensitivity tracer assay for N<sub>2</sub> fixation. *Appl. Environ. Microbiol.* **62**: 986–993.
- MULHOLLAND, M. R., AND D. G. CAPONE. 2001. Stoichiometry of nitrogen utilization in cultured populations of *Trichodesmium* IMS101: Implications for growth. *Limnol. Oceanogr.* **46**: 436–443.
- PETERSON, R. B., AND R. H. BURRIS. 1976. Conversion of acetylene reduction rates to nitrogen fixation rates in natural populations of blue-green algae. *Anal. Biochem.* **73**: 404–410.
- READE, J. P. H., L. J. DOUGHERTY, L. J. ROGERS, AND J. R. GALLON. 1999. Synthesis and proteolytic degradation of nitrogenase in cultures of the unicellular cyanobacterium *Gloeothece* strain ATCC 27152. *Microbiol. UK* **145**: 1749–1758.
- REDFIELD, A. C. 1958. The biological control of chemical factors in the environment. *Am. Sci.* **46**: 206–221.
- RENSTRÖM-KELLNER, E., AND B. BERGMAN. 1989. Glycolate metabolism in cyanobacteria. III. Nitrogen controls excretion and metabolism of glycolate in *Anabaena cylindrica*. *Physiol. Plant.* **77**: 46–51.
- SELLNER, K. G. 1997. Physiology, ecology and toxic properties of marine cyanobacteria blooms. *Limnol. Oceanogr.* **42**: 1089–1104.
- SHARP, J. H. 1974. Improved analysis for “particulate” organic carbon and nitrogen from seawater. *Limnol. Oceanogr.* **19**: 984–989.
- STAAL, M., S. T. LINTEL-HEKKERT, F. HARREN, AND L. STAL. 2001. Nitrogenase activity in cyanobacteria measured by the acetylene reduction assay: A comparison between batch incubation and on-line monitoring. *Environ. Microbiol.* **3**: 343–351.
- TURNER, G. L., AND A. H. GIBSON. 1980. Measurement of nitrogen fixation by indirect methods, p. 111–138. *In* F. J. Bergerson

## Acknowledgements

We gratefully acknowledge the crew of the R/V *Alexander von Humboldt* for their assistance at sea. This work was supported by the European Commission Environment RTD Programme DG XII (contract ENV4 CT97 0571) and forms part of the European Land-Ocean Interaction Studies (ELOISE) network. This is publication 2962 of NIOO-KNAW and ELOISE contribution 269/15.

- [ed.], Methods for evaluating biological nitrogen fixation. John Wiley & Sons.
- VILLBRANDT, M., L. J. STAL, B. BERGMAN, AND W. E. KRUMBEIN. 1992. Immunolocalization and western blot analysis of nitrogenase in *Oscillatoria limosa* during a light-dark cycle. *Bot. Acta* **105**: 90–96.
- WATERBURY, J. B., S. W. WATSON, R. R. L. GUILLARD, AND L. E. BRAND. 1979. Widespread occurrence of a unicellular marine, planktonic cyanobacterium. *Nature* **277**: 293–294.
- ZEHR, J. P., AND OTHERS. 2001. Unicellular cyanobacteria fix N<sub>2</sub> in the subtropical North Pacific Ocean. *Nature* **412**: 635–638.
- , M. WYMAN, V. MILLER, L. DUGUAY, AND D. G. CAPONE. 1993. Modification of the Fe protein of nitrogenase in natural populations of *Trichodesmium thiebautii*. *Appl. Environ. Microbiol.* **59**: 669–676.

Received: 31 August 2001

Amended: 25 April 2002

Accepted: 25 April 2002

*Limnol. Oceanogr.*, 47(5), 2002, 1521–1524  
© 2002, by the American Society of Limnology and Oceanography, Inc.

## Preservation of amino acids from in situ-produced bacterial cell wall peptidoglycans in northeastern Atlantic continental margin sediments

**Abstract**—In this study we present the results of total hydrolysable amino acids (THAA) and amino acid D/L-enantiomers in northeastern Atlantic continental margin sediments. There is increasing evidence that intrinsically labile amino acids are present in old marine sediments as part of a refractory network of peptide-like material. We used amino acid enantiomers to identify the contribution of amino acids from bacterial cell walls to THAA in organic matter ranging from relatively young to 18,000 yr old. The ratio of D/L-amino acids increased with depth in the sediment mixed layer. Application of a transport-racemization-degradation model excludes a significant production of D-amino acids by racemization and implies in situ bacterial production as the main source. Amino acids associated with a refractory pool of bacterial cell walls could account for approximately one third of the THAA deeper in the sediments. We propose that in situ bacterial production and the primary flux of labile organic matter from the water column result in a small but highly reactive pool of amino acids in the surface mixed sediment only, whereas amino acids associated with refractory cell walls persist in marine sediments.

The contribution of amino acid-nitrogen to total nitrogen (%AA-N) as well as the spectrum of individual amino acids have been used as indicators of organic matter (OM) reactivity in marine sediments (Cowie and Hedges 1992; Grutters et al. 2001). Preferential enzymatic degradation of amino acids relative to bulk OM (Harvey et al. 1995) causes the %AA-N to decline from 75% to 90% in fresh plankton to 40% to 50% in OM in sinking aggregates and further to 10%–30% in sedimentary OM (Lee 1988). However, the sorption of OM to mineral surfaces is assumed to result in the preservation of these intrinsically labile compounds (Keil et al. 1994). Amino acids are known to be a major constituent of peptidoglycans, the main structural components of bacterial cell walls (Schleifer and Kandler 1972). It has been reported that peptidoglycans contribute significantly to dissolved OM in the deep ocean (McCarthy et al. 1998). In addition, Parkes et al. (1993) proposed that with increasing depth in the sediments up to 16% of the total organic carbon (TOC) that cannot be accounted for by amino acids and car-

bohydrates (uncharacterized TOC) comes from dead bacterial biomass. They suggested that this bacterial “necromass” is relatively recalcitrant and perhaps contributes to OM preservation. Therefore, we hypothesize that the in situ production of bacterial cell walls contributes to the preservation of amino acids in marine sediments. In addition, benthic production of peptidoglycan may be a source to suspended particulate organic matter in the lower water column of the ocean (Bauer and Druffel 1998).

**Materials and methods**—Particulate matter was collected from sediment traps (Antia et al. 1999), situated 400 m above bottom, at water depths of 1,445 and 3,650 m across the Goban Spur northeastern Atlantic continental slope (49°24.89'N, 11°31.42'W to 49°05.30'N, 13°26.18'W). Sediment samples were taken from multicores and piston cores (representing ages of 7, 10, 18 kyr) at water depths of 651, 1,296, and 3,650 m (Lohse et al. 1998). Sediment trap samples (pooled averages from individual cups covering a time span of 14 months) as well as sediment samples were analyzed for total hydrolysable amino acids (THAA) and D/L enantiomers of aspartic acid, glutamic acid, serine, and alanine, the major peptidoglycan amino acids.

THAA were measured by reverse-phase high-performance liquid chromatography (HPLC) analysis, after liquid-phase hydrolysis (110°C for 24 h) and precolumn derivatization with *o*-phthalaldehyde (OPA) (Grutters et al. 2001). Amino acid enantiomers were analyzed by HPLC after vapor-phase hydrolysis at 150°C for 3 h and precolumn derivatization with OPA/*N*-acetyl-L-cysteine and ultraviolet-fluorescence detection (Glavin et al. 1999). Prior to derivatization, hydrolysates were desalted by use of cation exchange resin (AG50W-X8, Bio-Rad) and the desalted extracts were stored in borate buffer (pH 9.4). Concentrations of the THAA and enantiomers were calculated by comparison of the amino acid peak areas to those of a standard run in parallel. Very low levels of amino acids were detected in a blank carried through the same processing procedure as the sediment samples and were subtracted from the measured peak areas of the samples. The precision of the THAA analysis, expressed as the