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Evidence of nitrogen fixation by non-heterocystous cyanobacteria in the Baltic Sea and re-calculation of a budget of nitrogen fixation

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ABSTRACT: Nitrogen fixation rates and related plankton parameters were determined at 8 stations in the Baltic proper and Mecklenburg Bay in 1997 and 1998. Nitrogen fixation was measured with ^{15}N-tracer method in unenriched samples. Measurable nitrogen fixation rates were found from July to October, with highest rates in August when heterocystous cyanobacteria formed blooms. Nitrogen fixation rates measured during a moderate bloom in 1998 were related to biomass of heterocystous and coccoid (non-heterocystous) cyanobacteria and primary production. The size fraction <10 µm contributed significantly to total nitrogen fixation both during day and night. It is discussed whether this may be due to small, non-heterocystous cyanobacteria, which were abundant in summer and autumn. They may separate photosynthesis and nitrogen fixation temporally (day and night) and may be especially responsible for the high nitrogen fixation rates observed in the dark. As the fraction of pico- and nanoplankton was not considered in earlier studies, a new budget of nitrogen fixation in the Baltic proper has been estimated. On average, daily nitrogen fixation rates of 2.5 mmol N m^{-2} d^{-1} (in July/August 1997/1998) and mean annual nitrogen fixation of 125 mmol N m^{-2} yr^{-1} were estimated for the Baltic proper. The high variability is discussed. For summer 1998, a budget of the nitrogen cycle at the main station in the Gotland Sea is given.

KEY WORDS: Nitrogen fixation · Primary production · Coccoid diazotrophic cyanobacteria · Diel cycle · Nitrogen budget · Baltic Sea

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INTRODUCTION

Phytoplankton development in the central Baltic Sea is primarily limited by the supply of nitrogen, as the winter concentrations in the surface water show a very low ratio of inorganic N:P (7–8:1) compared to the Redfield ratio required for the build-up of biomass (16:1) (Granéli et al. 1990, Matthäus et al. 1999). This excess of phosphate can favour the development of cyanobacteria populations able to fix elemental nitrogen (Sellner 1997). Impressive blooms of the heterocystous species *Nodularia spumigena* and *Aphanizomenon* sp. occur in the central Baltic Sea every summer (Niemistö et al. 1989, Kononen 1992, Wasmund 1997). Both species form buoyant aggregates of several millimetres length. They are regarded as the most important nitrogen fixers in the Baltic Sea.

For the central and northern Baltic proper, including the Gulf of Finland, Melvasalo et al. (1983) estimated an annual fixation of nitrogen of 100 000 t N. Leppänen et al. (1988) found the same amount through the compilation of an annual nitrogen budget for the Baltic proper, compared to 166 570 t N introduced from land run-off and 230 000 t N from atmospheric input. Rönner (1985) estimated for the Baltic proper and the Gulf of Finland an annual nitrogen input of 130 000 t N from nitrogen fixation, 573 000 t N from river load and

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252 000 t N from atmospheric input. However, these fairly consistent figures of nitrogen fixation mask some very large fluctuations. For the Baltic proper and Mecklenburg Bay, Hübel & Hübel (1995) calculated for the year 1975 an annual nitrogen fixation of 185 950 t N, but for the year 1985 only 18 200 t N. Wulff et al. (2001) estimated an external load (river loads, point sources and atmospheric deposition) of nitrogen into the Baltic proper of about 880 000 t N, of which 600 000 t N is dissolved inorganic nitrogen (DIN). The calculation of nitrogen fixation based on the surplus phosphorus supply amounted to an internal load of the Baltic proper of 30 000 to 260 000 t N yr^{-1} for different years from 1992 to 1997 (Rahm et al. 2000).

The less noticeable coccoid cyanobacteria were not considered in the investigations on nitrogen fixation in the Baltic Sea up to now, although there had been evidence for their ability for nitrogen fixation (i.e. León et al. 1986, Gallon & Chaplin 1988). The early acetylene-reduction technique required enrichment of the samples by net plankton in order to concentrate enough biomass for the measurement. For these reasons pico- and nanocyanobacteria were lost from the samples. Therefore, interest was focused on the bloom-forming heterocystous cyanobacteria and on measurements in the summer and during daytime.

This study had 2 aims: (1) to determine whether the pico- and nanoplankton contribute significantly to total nitrogen fixation in the Baltic Sea (for this, a new ^{15}N tracer method [Montoya et al. 1996] was applied which is sensitive enough for unenriched samples); (2) to estimate a new budget of nitrogen fixation and nitrogen cycle in the upper mixed layer incorporating the expected significant contribution of pico- and nanoplankton to total nitrogen fixation. The calculations included spatial (vertical and large-scale horizontal distribution) and temporal (diel and annual) variations. The relationship between nitrogen fixation rates and cyanobacteria abundance, primary production and nutrient supply were investigated.

MATERIALS AND METHODS

Sampling. Samples were mainly collected during 4 cruises of the RVs 'Petr Kottsov' and 'Alexander von Humboldt' as part of the European BASYS (Baltic Sea System Study) project (Table 1). Most of the investigation was carried out at 1 station of the Baltic Monitoring Programme in the eastern Gotland Sea (Stn: J1 Fig. 1). Additional samples were collected at a range of monitoring stations in the southern Baltic proper and Mecklenburg Bay during 7 cruises of the Baltic Monitoring Programme on the RVs 'Alexander von Humboldt' and 'Professor Albrecht Penck'. One additional experiment was conducted in 1999 (Table 1). The investigated area covered the main area of cyanobacterial blooms in the Baltic Sea (cf. Kahru et al. 1994). All samples were collected by rosette water samplers (5 l) mounted on a Seabird CTD-system .measuring profiles of temperature, conductivity, fluorescence, turbidity and oxygen. On the down cast, the physical parameters were measured and the water samples were collected at fixed depths.

Phytoplankton biomass, abundance and species composition. For determination of chlorophyll *a* (chl *a*) and pigment composition, samples were filtered through Whatmann GF/F filters and stored frozen (–20°C) until analysis. The chlorophyll filters were homogenized, extracted in 10 ml 90% acetone for 3 h in the dark at room temperature, and centrifuged at 10 000 rpm. The extracts were measured in a Turner fluorometer 10-AU-005, acidified, and re-measured for determination of phaeopigments (Edler 1979, Joint Global Ocean Flux Study 1993). Pigment analysis by HPLC measurement was carried out according to Wright et al. (1991), to estimate the occurrence of cyanobacteria via zeaxanthin.

For the determination of species composition and abundance of phytoplankton, samples taken at 1, 2.5, 5, 7.5 and 10 m water depths were mixed in equal quanti-

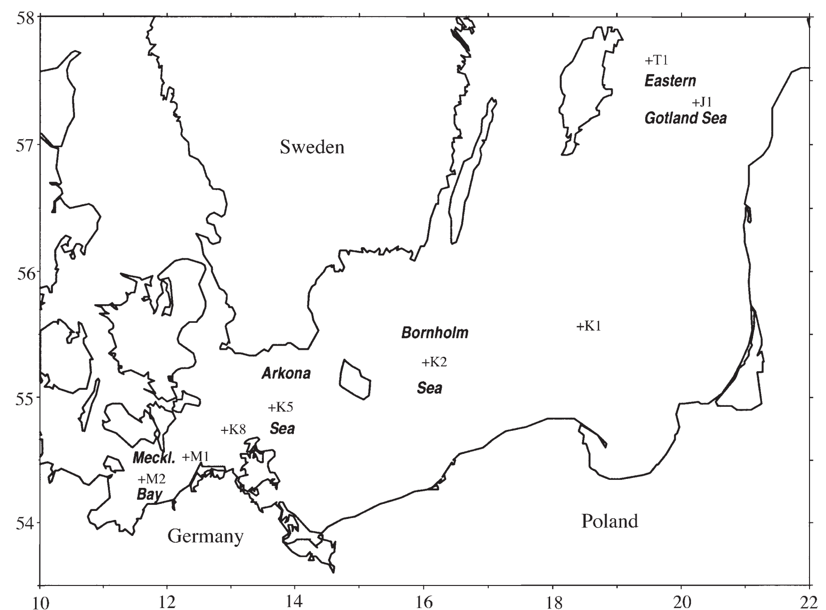

Fig. 1. Map of the southern Baltic Sea with all sampling stations (for sampling dates see Table 1)

Table 1. Overview of sampling cruises

Cruise	Ship	Dates	Stations sampled (Fig. 1)
Monitoring	'P. A. Penck'	26 Jul–13 Aug 1997	M2, K5, K2, J1
BASYS II	'Petr Kottsov'	02–12 Aug 1997	J1, repeated sampling
BASYS III	'A.von Humboldt'	06–12 Oct 1997	J1, repeated sampling
Monitoring	'A. von Humboldt'	24 Oct–13 November 1997	M1, K8, J1
Monitoring	'A. von Humboldt'	05–25 Feb 1998	M1, M2, K8, K5, K2, K1, J1
Monitoring	'A. von Humboldt'	19 Mar–1 Apr 1998	M1, M2, K8, K5, K2, K1, J1
Monitoring	'A. von Humboldt'	05–14 May 1998	M1, M2, K8, K5, K2, K1, J1
BASYS IV	'A. von Humboldt'	15–21 May 1998	J1, repeated sampling
BASYS V	'Petr Kottsov'	26 Jul–08 Aug 1998	J1, T1, repeated sampling
Monitoring	'P. A. Penck'	30 Jul–12 Aug 1998	M1, M2, K8, K5, K2, K1, J1
Monitoring	'A. von Humboldt'	27 Oct–16 Nov 1998	M1, M2, K8, K5, K2, K1, J1
Monitoring	'A. von Humboldt'	02 Aug 1999	K8

ties. Additional samples from individual depths (down to 50 m) were also taken. All samples were fixed with 1 ml Lugol's solution per 250 ml of sample and stored at room temperature until analysis. They were microscopically analysed using an inverted microscope (Leitz Fluovert) according to the Utermöhl method as described by Edler (1979). Phytoplankton wet weight was calculated according to Edler (1979).

Rates of primary production and nitrogen fixation. Primary production was determined in samples collected at 1, 2.5, 5, 10, 15 and 20 m. During cruises 'BASYS IV' and 'BASYS V' (May and July/August 1998) the 2.5 m sample was omitted, and instead a sample was taken at 50 m depth. For each determination, two 280 ml samples were placed in polycarbo-nate bottles, each amended with 148 kBq $NaH^{14}CO_3$ (Amersham); 1 or 2 of the samples from a medium depth served as dark control. All samples were incubated from sunrise to sunset on a moored or drifting rig *in situ* at the same depth from which they were collected. At the end of the incubation period the samples were filtered through Whatman GF/F filters. Inorganic carbon was removed by treatment with fuming hydrochloric acid. The samples were stored refrigerated until analysis in the laboratory. They were placed in 5 ml Ultima Gold (Packard) scintillation cocktail and measured in a liquid scintillation counter Tri-Carb 2560 TR/XL (Packard). Concentration of dissolved inorganic carbon in the samples was determined according to Grasshoff et al. (1983).

Nitrogen fixation rates were determined by ^{15}N tracer method in parallel to primary production incubations in replicate subsamples from the same depths. Measurements and calculation of nitrogen fixation rates were carried out after Montoya et al. (1996). The samples were filled without air bubbles into 250 ml glass bottles. Through a gas-tight septum, each sample received 1 ml $^{15}N_2$ (99% $^{15}N_2$, Campro Scientific, Berlin) injected by syringe to achieve a tracer addition of roughly 10%. The sample was carefully shaken to allow isotopic equilibrium between added N_2 tracer and natural N_2 dissolved in the sample. They were incubated *in situ* in the same way as the primary production samples. Incubation was from sunrise to sunset, and in some cases also during night periods. After incubation the samples were filtered through precombusted Whatman GF/F filters and stored frozen until analysis with a continuous-flow isotope ratio mass spectrometer (Thermo-Finnigan, Delta S, Bremen). The reference gas was pure N_2 from a gas cylinder calibrated against IAEA-N1, N2, N3. Data on particulate organic carbon (POC) and particulate organic nitrogen (PON) concentrations were provided by the same measurement. Acetanilid and Peptone (Merck) served as lab-internal elemental and isotope standards for daily calibration.

On monitoring cruises, primarily outside the bloom periods, deck incubations ('simulated *in situ*') were made when the cruise schedule did not allow *in situ* incubations (Table 1). In these cases, samples were collected at depths corresponding to 100, 75, 50, 25, 15 and 5% of surface light intensity. They were wrapped in neutral grey filter foil of the corresponding light transmission plus an additional dark sample and incubated for 2 h in water baths at surface-water temperatures within the daylight period 8:00 to 16:00 h. On 3 occasions (1 in August and 2 in October 1997), both incubation methods were carried out in parallel in order to test whether both sets of results are comparable. The quotient of the results of all parallel *in situ* and simulated *in situ* incubations was 1.01 ± 0.07 for nitrogen fixation and 1.08 ± 0.35 for primary production. Because of this good agreement of both incubation methods, all data were used without any correction factors.

The hourly nitrogen fixation rates, based on $^{15}N_2$ incorporation, decreased with increasing incubation time during strong cyanobacterial blooms. They became more or less constant with incubation periods of more than 6 h. This points to release or degradation of

organic nitrogen previously produced in the process of nitrogen fixation. Therefore, we chose day or night incubations of 8 to 16 h. Short-term incubations were only accepted for budget calculations in periods of very low nitrogen fixation rates, when nitrogen fixation was proportionate to incubation time. Montoya et al. (1996) found good agreement between the acetylene-reduction method and ^{15}N tracer method with a ratio of acetylene reduced to N_2 fixed of 4.68 under simulated *in situ* conditions in the Baltic Sea.

Nutrient analyses were carried out after the methods described in Rohde & Nehring (1979) and Grasshoff et al. (1983).

RESULTS

Phytoplankton species composition and biomass distribution

Cyanobacteria aggregates are highly patchy in their distribution. This was observed from aboard the cruising ship. Wind-induced strips of higher accumulation at the sea surface from Langmuir circulation and thick surface patches during calm weather were observed. On occasions we observed that even at very low wind speeds the surface accumulation of cyanobacteria aggregates disappeared through cooling and convection at night and were re-established in the morning. In August 1997 during very calm weather, high abundances of cyanobacteria concentrated at the sea surface, especially south of Gotland; this was also detected by satellite images (Wasmund et al. 1998). In August 1998, no surface bloom was visible due to a rather strong wind.

The annual variations in phytoplankton biomass (including chl *a*) and of selected taxonomic groups of algae at Stn J1 between May 1997 and November 1998 are shown in Table 2. The highest biomass of total phytoplankton occurred between May and November, while the biomass of total cyanobacteria was highest in July and August only. The compilation in Table 2 shows that at this station the spring phytoplankton bloom was dominated by dinoflagellates (Dinophyceae), the summer phytoplankton by cyanobacteria, and the autumn bloom by diatoms (Bacillariophyceae). The typical autumn bloom of *Coscinodiscus granii* was missed in 1997, but recorded in 1998. Amongst the filamentous cyanobacteria, only 2 species were of importance: *Nodularia spumigena*, which was abundant in July and August, but absent during the other months and *Aphanizomenon* sp. (a species not described yet) which was found all year round. The duration of the bloom was approximately 2 mo (Fig. 2). During that bloom, small coccoid cyanobacteria also occurred in high abundance (some million cells l^{-1}), but due to their small size (<10 µm) their biomass was relatively low. This group mainly comprised *Aphanocapsa delicatissima*, *Aphanothece minutissima*, *Chroococcus microscopicus*, *Coelosphaerium minutissimum*, *Cyanodictyon reticulatum*, *Cyanodictyon planctonicum*, *Lemmermanniella pallida* and *Synechococcus* sp. The biomass of coccoid cyanobacteria continued to increase until the end of September, as shown by Swedish data (courtesy of S. Hajdu, Stockholm University). Although the Swedish phytoplankton biomass calculation might have led to higher values than ours, we combined the 2 data sets into 1 curve. Larger coccoid colonies (*Snowella* spp., *Woronichinia* spp.) are also included in Fig. 2.

During August of 1997 and 1998, microscopic investigations showed that the heterocystous cyanobacteria species preferrentially occupied different water depths. *Nodularia spumigena* was found primarily at the sea surface if wind mixing was low, while *Aphanizomenon* sp. was more abundant at greater depth (ca 10 m,

Table 2. Monthly means of chlorophyll *a* concentration and biomass (wet wt, mg m^{-3}) of the most important phytoplankton groups at Stn J1 (0 to 10 m, mixed samples). T Phyto: total phytoplankton; T Cyano: total cyanobacteria; *N. spum*: *Nodularia spumigena*; *Aphan* sp.: *Aphanizomenon* sp.; *Anab* spp.: *Anabaena* spp.; Crypto: Cryptophyceae; Dino: Dinophyceae; Bacillario: Bacillariophyceae

Date	Chl *a* (mg m^{-3})	T Phyto	T Cyano	*N. spum*	*Aphan* sp.	*Anab* spp.	Coccoid cyanobacteria <10 µm	Coccoid cyanobacteria >10 µm	Crypto	Dino	Bacillario
May 97	2.07	3365	21	0	19	0	1	2	16	3062	161
Aug 97	2.35	630	285	96	153	4	29	2	63	192	36
Oct 97	2.23	334	39	0	36	0	0	1	60	160	13
Nov 97	2.09	194	42	0	40	0	0	1	24	83	13
Feb 98	0.31	47	17	0	12	0	1	0	15	4	1
Mar 98	1.19	220	18	0	15	0	2	1	17	87	41
May 98	1.36	884	25	0	20	0	2	2	24	836	1
July 98	3.64	607	338	208	104	2	20	4	33	92	49
Aug 98	3.57	712	414	162	144	4	29	20	88	128	8
Nov 98	4.76	1207	27	2	11	0	7	4	26	59	1034

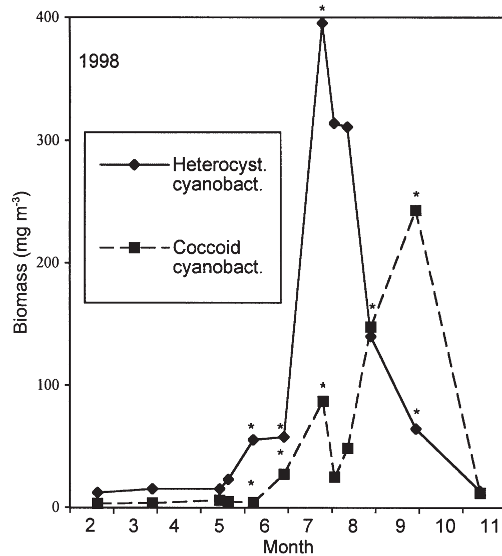

Fig. 2. Annual pattern of the biomass of heterocystous cyanobacteria (*Nodularia spumigena*, *Aphanizomenon* sp., *Anabaena* spp.) and coccoid cyanobacteria (sum of <10 and >10 µm fractions) at Stn J1 in 1998. *Data from June, July and September are monitoring data by S. Hajdu (Stockholm University), extracted from the Helsinki Commission (HELCOM) data bank

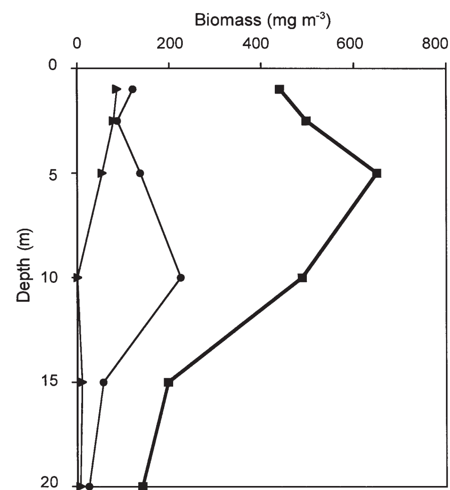

Fig. 3. *Nodularia spumigena* (▶) and *Aphanizomenon* sp. (●) depth distribution in the upper 20 m of the water column on 3 August 1997 at Stn J1. (■) total phytoplankton biomass

Fig. 3). Below the thermocline (20 m depth), almost no filaments of these species were found. The changing depth distribution of the cyanobacteria had a minor effect on our calculations, because we used integrated means from the surface to the depth of the thermocline, thus including the whole community. If sharp subsurface peaks outside our standard depths were noticed in the fluorescence profile, additional samples were taken. Sampling directly from the water surface was not possible, and this may have caused under-estimations on a few occasions when the sea was calm.

Rates of nitrogen fixation

Primary production and nitrogen fixation decreased with increasing depth due to reduced light intensity and phytoplankton biomass. The examples shown in Fig. 4 are from 3 August 1997 (calm, some clouds), from 4 August 1997 (calm and sunny; short-term incubation only, therefore excluded from the final calculations) and 5 August 1997 (windy). In contrast to primary production, nitrogen fixation occurred in samples incubated in the dark. These dark samples were from 5 m depth and had previously received light. Their nitrogen fixation rate was higher than in samples from the low-light regime at 20 m depth (<5% of surface light), but not as high as in the same sample incubated at the original light intensities of 5 m depth. In samples from 50 m water depth no nitrogen fixation was detected (data not shown). These samples were from well below the strongly developed thermocline located in summer at around 20 m depth. Thus, for vertical integration of nitrogen fixation, only the water column above the thermocline was consid-

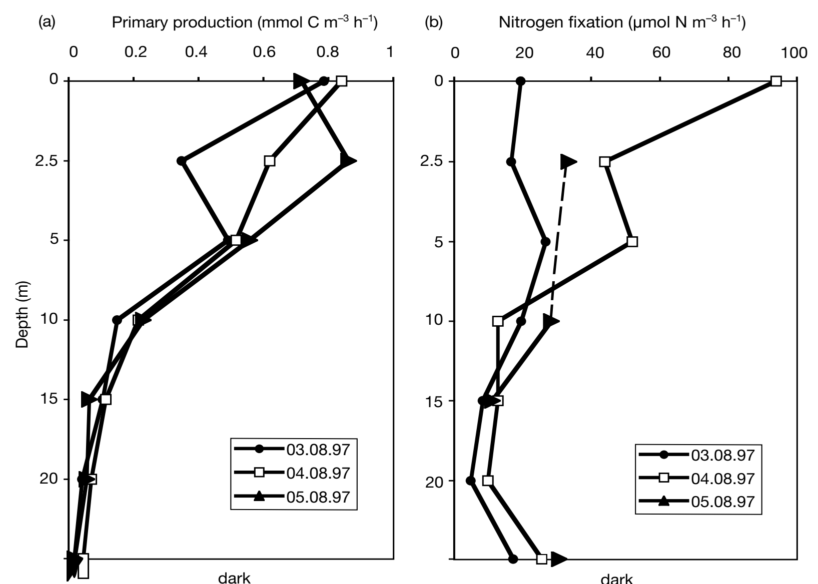

Fig. 4. Depth distribution of (a) primary production and (b) nitrogen fixation (*in situ*) in August 1997. The dark-incubated samples were from 5 m depth. On 5 August some incubation bottles were lost (dashed portion of line)

ered. Nitrogen fixation rates decreased with increasing water depth both during day and night (data not shown). This indicates that the surface-water samples, which had received more light than the deeper samples, obviously had a higher capacity for dark nitrogen fixation.

In order to determine the magnitude of dark nitrogen fixation on a diel basis, incubations were carried out during day and night (Table 3). The depth-integrated hourly fixation rates in unaltered samples during the night were higher than those measured during the day. However, in samples enriched with net plankton (>100 µm), nitrogen fixation rates, after division by an enrichment factor of 10, were higher during the day than during the night. Thus, the size-fraction <100 µm, which is lost in net samples, was more active at night. The same differences between day and night were found when nitrogen fixation rates were normalized to biomass units. The use of POC as a biomass indicator (in comparison with chl *a*) had advantages, since POC and ^{15}N were measured from the same filter, therefore avoiding uncertainties arising from differences between parallel filters. The nitrogen fixation rates normalized to POC in the net samples from 8 August 1997 (Table 3) were similar to those of isolated aggregates of *Nodularia spumigena* and *Aphanizomenon* sp. (Table 4), indicating that POC contribution by zooplankton, bacteria and detritus to the net sample was small.

In the experiment from 8 August 1997, aggregates of *Nodularia spumigena* and *Aphanizomenon* sp. were separated by removing the colonies by pipette, and were incubated for 5.5 h in a water bath on deck. Specific rates of primary production and nitrogen fixation normalized to biomass (POC) of both species did not vary significantly (Table 4). The frequency of heterocysts is, however, very different. In July and August 1997 and 1998, *N. spumigena* had on average 1.89 heterocysts 100 µm^{-1}, while *Aphanizomenon* sp. had only 0.20 heterocysts 100 µm^{-1}. One reason for the higher

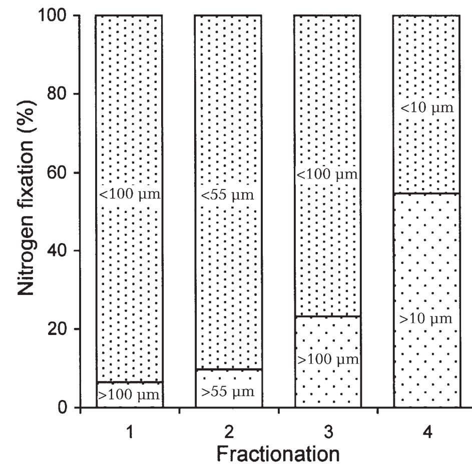

Fig. 5. Percentage of nitrogen fixation by various plankton size classes after fractionation by different nets in the eastern Gotland Sea. Fractionation Expts: 1 & 2 = 7 August 1997, 3 = 10 August 1997, 4 = 30 July, 1 August and 5 August 1998 (mean values; for details see Table 5)

frequency of heterocysts in *N. spumigena* may be that its heterocysts have to provide a much thicker filament than in *Aphanizomenon* sp. Therefore, heterocyst activity in these 2 species was not very different: 5.4 × 10^{-15} mol N (heterocyst)$^{-1}$ h^{-1} in *N. spumigena* and 7.2 × 10^{-15} mol N (heterocyst)$^{-1}$ h^{-1} in *Aphanizomenon* sp. in our experiment.

Although the aggregates of *Nodularia spumigena* and *Aphanizomenon* sp. were much larger than 100 µm, significant numbers of these species, frequently as single filaments, passed through the 100 µm net. To hold them back more efficiently, gauze of 10 µm mesh size was used. The effect of separation by different mesh sizes on nitrogen fixation rates in the respective size fractions is shown in Fig. 5. The 10 µm gauze separated the filamentous from the coccoid cyanobacteria more efficiently and more gently. How-

Table 3. Comparison of *in situ* nitrogen fixation rates during the day and during the night in the upper 10 m (8 August 1997) or 20 m (6 August 1997, 28 and 30 July 1998) at Stn J1. Additional experiment (simulated *in situ*) on 2 August 1999 at Stn K8. (a) Unamended samples; (b) samples enriched (10:1) by 100 µm net plankton; recalculated to original concentration by dividing by 10

Date	Hourly nitrogen fixation					
	µmol N m^{-3} h^{-1}		mmol N (mol POC)$^{-1}$ h^{-1}		mmol N (g chl *a*)$^{-1}$ h^{-1}	
	Day	Night	Day	Night	Day	Night
(a) Non-enriched samples						
08 Aug 97	6.14	6.50	0.108	0.158	2.29	2.52
28 Jul 98	1.14	2.14	0.020	0.042	0.32	0.63
31 Jul 98	1.71	2.57	0.038	0.067	0.50	0.85
02 Aug 99	1.29	1.32	0.025	0.026	0.98	1.00
(b) Samples enriched by net plankton (100 µm)						
06 Aug 97	1.74	1.14	0.253	0.170	4.14	3.26
08 Aug 97	1.02	0.43	0.139	0.048	2.04	1.08

Table 4. *Nodularia spumigena* and *Aphanizomenon* sp. particulate organic nitrogen (PON) and particulate organic carbon (POC) concentration, primary production and nitrogen fixation in cyanobacteria aggregates selected from 10 l surface water and incubated on deck at 100% light intensity during afternoon of 8 August 1997

Species	No. of aggregates per sample	PON (µmol)	POC (µmol)	Primary production (mmol C [mol POC]$^{-1}$ h^{-1})	Nitrogen fixation (mmol N [mol POC]$^{-1}$ h^{-1})
N. spumigena	10	1.02	9.75	0.197	0.141
Aphanizomenon sp.	30	1.60	11.48	0.279	0.125

ever, 8.0 ± 0.7% of the filamentous cyanobacteria from natural samples still passed through the 10 µm mesh.

Separation experiments were performed before or after incubation for the nitrogen fixation measurements (Table 5). The fractions were treated separately, in parallel to an unfractionated sample. The contribution of nitrogen fixation by the <10 µm fraction to the sum of the 2 fractions was 43.3 ± 16.0% and 42.8 ± 19.8% in the pre-filtration and post-filtration treatments, respectively. Despite the high variability, the similarity of these 2 values suggests that nitrogen fixation in the <10 µm fraction originated from this small size fraction and was not due to uptake of organic nitrogen excreted by the heterocystous cyanobacteria.

Nitrogen fixation measurements were carried out throughout the year in order to obtain an estimate of the annual nitrogen fixation capacity. Fixation rates were only clearly measurable between July and October (Table 6). At all stations investigated in 1998, the highest nitrogen fixation rates were found in August, ranging from 4.3 µmol N m^{-3} h^{-1} at Stn K1 to 7.8 µmol N m^{-3} h^{-1} at Stn K5 (Fig. 6). The annual pattern of nitrogen fixation was similar in all areas investigated. The high patchiness is exemplified by a series of daily measurements at the fixed Stn J1

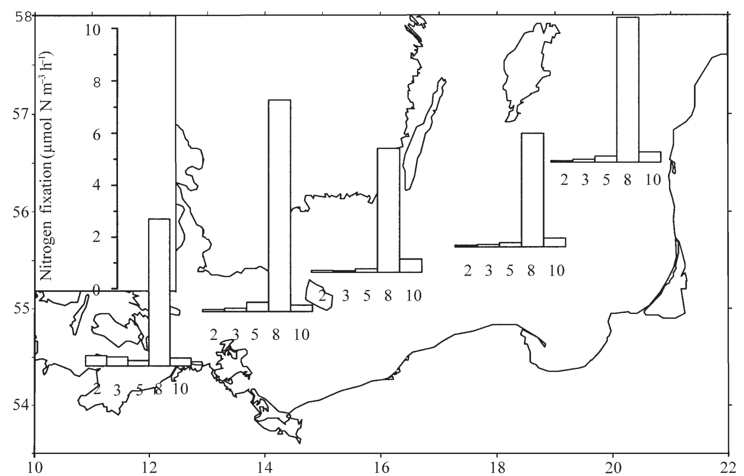

Fig. 6. Nitrogen fixation in February, March, May, August and October (2, 3, 5, 8, 10 on abscissas) 1998 at 5 stations (M2, K5, K2, K1, J1) in southern Baltic Sea and Baltic proper

Table 5. Nitrogen fixation in size fractions <10 and >10 µm, fractionated either before or after incubation (in daylight), as well as in unfractionated (Total) samples. Percentage of the nitrogen fixation contributed by the smaller fraction to sum of the 2 fractions is shown, together with primary production rates of the 2 fractions (pre-filtration only) and the biomass of the heterocystous and the coccoid (<10 µm) cyanobacteria, for the sample as a whole

Stn	Date	Nitrogen fixation (µmol N m^{-3} h^{-1}) Total	>10 µm	<10 µm	% of <10 µm fraction	Primary production (µmol C m^{-3} h^{-1}) Total	>10 µm	<10 µm	Phytoplankton biomass (mg m^{-3}) Heterocystous cyanobacteria	Cyanobacteria <10 µm
Pre-filtration										
J1	30 Jul 98	4.00	1.71	2.50	59	185	24	147	282	22
J1	01 Aug 98	3.29	1.07	0.86	44	187	23	145	189	10
T1	05 Aug 98	2.71	1.71	0.64	27	95	17	94	132	14
Post-filtration										
K8	01 Aug 98	7.57	1.86	2.93	61				3213	34
K5	02 Aug 98	8.14	7.29	2.50	26				813	33
K2	03 Aug 98	4.79	3.57	1.29	26				260	19
K1	07 Aug 98	4.43	1.79	5.64	76				158	21
J1	08 Aug 98	5.50	3.57	2.36	40				311	29
K5	10 Aug 98	9.00	3.64	3.07	46				536	34
K8	10 Aug 98	16.86	9.21	3.00	25				1092	18

Table 6. Measurements at Stn J1 on different sampling dates: average values of the upper 20 m of chlorophyll *a*, phytoplankton biomass (wet weight estimated from microscopic counts), particulate organic nitrogen (PON), particulate organic carbon (POC), primary production and nitrogen fixation rates per hour, daily primary production (estimated for the upper 20 m from dusk to dawn) and daily nitrogen fixation (estimated for the upper 20 m for 24 h). *Data from 8 August 1997 were available only from 0 to 10 m depth and were therefore excluded from further calculations

Date	Chl *a* (mg m^{-3})	Phytoplankton biomass (mg m^{-3})	PON (mmol m^{-3})	POC (mmol m^{-3})	Primary production (mmol C m^{-3} h^{-1})	Nitrogen fixation (µmol N m^{-3} h^{-1})	Primary production (mmol C m^{-2} d^{-1})	Nitrogen fixation (mmol N m^{-2}d^{-1})
03 Aug 97	2.14	880	4.63	47.2	0.253	15.50	53.7	7.44
05 Aug 97	2.17	800	4.84	55.6	0.337	23.57	73.7	11.31
08 Aug 97	2.64*	253*	6.64*	80.0*	–	6.14*	–	2.95*
09 Aug 97	2.51	337	5.26	56.8	0.304	5.43	94.3	2.59
08 Oct 97	2.36	349	2.72	19.6	0.354	5.70	79.3	2.74
09 Oct 97	2.23	359	2.13	16.3	0.114	0.48	25.6	0.23
10 Oct 97	2.10	294	2.54	25.5	0.139	0.24	31.2	0.12
03 Nov 97	2.09	194	2.42	18.9	0.098	0.21	19.7	0.10
18 Feb 98	0.31	47	1.02	8.5	0.028	0.05	3.4	0.02
27 Mar 98	1.19	220	2.36	17.4	0.089	0.11	17.1	0.05
12 May 98	1.99	1731	4.70	37.3	0.125	0.23	46.5	0.11
17 May 98	1.14	677	4.34	35.5	0.077	0.11	22.7	0.05
18 May 98	1.14	–	3.25	22.1	0.158	0.07	41.8	0.03
19 May 98	1.15	525	4.54	30.9	–	0.07	–	0.03
28 Jul 98	3.51	730	6.06	54.3	0.203	1.61	60.8	0.77
30 Jul 98	4.19	606	4.94	43.3	0.247	4.00	74.0	1.92
31 Jul 98	3.21	484	5.19	44.3	0.348	2.07	104.3	1.00
01 Aug 98	3.10	371	4.37	42.0	0.186	3.79	55.8	1.82
02 Aug 98	3.09	430	5.26	42.0	0.200	2.86	60.0	1.37
03 Aug 98	4.52	–	7.46	54.1	0.152	4.36	45.5	2.09
08 Aug 98	3.46	1055	5.70	45.5	0.173	5.52	53.5	2.65
07 Nov 98	4.76	1207	2.56	23.0	0.101	0.39	18.8	0.19

(e.g. 3 to 5 August 1997 or 28 July to 3 August 1998: Table 6).

When the biomass of heterocystous cyanobacteria was <40 mg m^{-3}, nitrogen fixation was not measurable (Fig. 7a, see also Table 2). Similarly, at concentrations of zeaxanthin (a pigment marker for cyanobacteria) of <0.28 mg m^{-3}, no nitrogen fixation occurred (Fig. 7b). Above these levels, nitrogen fixation rates were correlated with the biomass of heterocystous cyanobacteria (r = 0.89; Fig. 7a), the biomass of coccoid cyanobacteria <10 µm (r = 0.87; data not shown, as similar to that in Fig. 7a) and primary production (r = 0.67; Fig. 7c), but not with zeaxanthin concentrations (Fig. 7b) and number of heterocysts (data not shown) in July/August 1998. Because of the relatively low number of experiments during the dense surface bloom in August 1997, no correlations were found.

DISCUSSION

Heterogeneous distribution of bloom-forming cyanobacteria

The strong horizontal patchiness of cyanobacterial blooms is documented by satellite images which were used to identify cyanobacterial blooms between 1982 and 1993 (Kahru et al. 1994). However, satellite images can only detect accumulations near the sea surface, but do not allow conclusions about total cyanobacteria biomass in the upper water column or the associated physiological activity. At wind speeds exceeding 6 m s^{-1}, cyanobacteria disperse in the upper mixed layer (Wasmund 1997).

Under calm conditions, we observed the 2 bloom-forming species accumulating at different depths: *Nodularia spumigena* at the surface, and *Aphanizomenon* sp. at approx. 10 m depth. Niemistö et al. (1989) described a similar distribution pattern, with maximal abundance of *N. spumigena* at 0 to 5 m depth and of *Aphanizomenon* sp. at 10 to 15 m depth. In the Gulf of Riga, Heiskanen & Olli (1996) observed *Aphanizomenon* sp. to descend after noon in the water column and to rise again in the early morning. However, our observations of the accumulation of cyanobacteria colonies at the surface during calm days and their dispersal during the night may only partly be due to active buoyancy regulation: it may also be an effect of thermal stratification. Strong irradiance during the day builds up a shallow stratification in which rising cyanobacteria are trapped. During the night, cooling leads to deeper convection and dispersal of the plankton.

Cyanobacterial blooms in the Baltic develop only during summer, with highest abundances in July and August at high irradiances (>120 W m^{-2}) and temperatures (>16°C) (Wasmund 1997). Until May, the biomass of both heterocystous and non-heterocystous cyanobacteria remains low in the Baltic proper and increases from June to July. In this study, detectable nitrogen fixation rates were only found at biomasses of heterocystous cyanobacteria >40 mg m^{-3} or at zeaxanthin concentrations >0.28 mg m^{-3} (Fig. 7a,b). During the cyanobacterial bloom in 1998, nitrogen fixation was closely correlated with the biomass of heterocystous cyanobacteria, as reported previously by Rinne et al. (1978) and Hübel (1984). Usually, nitrogen-fixing activity by coccoid cyanobacteria does not change this pattern, since we observed covariations of heterocystous and non-heterocystous cyanobacteria in our investigation. However, during the strong surface bloom in 1997, no such correlation was found. Consequently, it is not possible to estimate the level of nitrogen fixation from the biomass of heterocystous cyanobacteria alone.

During the course of 1 yr we did not observe a correlation between heterocyst abundance and nitrogen fixation rates. Fairly constant numbers of heterocysts and abundances similar to those found in our investigation (1.89 heterocysts 100 μm^{-1} in *N. spumigena*, 0.2 heterocysts 100 μm^{-1} in *Aphanizomenon* sp.) were reported for these 2 species by Lindahl & Wallström (1985) and by Niemistö et al. (1989). Moisander et al. (1996) found a decreasing abundance of heterocycsts between 24 July and 3 August paralleling decreasing nitrogen fixation rates. On the other hand, the data of Lindahl & Wallström (1985) showed that heterocyst frequency is correlated with the biomass of heterocystous cyanobacteria but not with nitrogen fixation, indicating variations in heterocyst activity or in the contribution of non-heterocystous cyanobacteria to nitrogen fixation. In the present study, primary production was correlated with nitrogen fixation in 1998 (Fig. 7c), indicating a link between energy supply and the fixation process.

The patchy distribution of the bloom-forming cyanobacteria is reflected in samplings on consecutive days while the ship was drifting. Nitrogen fixation could change by nearly 100% from day to day (Table 6). The coefficient of variation of the 7 measurements within 12 d in summer 1998 was 40%. This high natural variability has to be kept in mind when assessing our budget data (see following subsections). Also the low precision (especially of the Utermöhl method) of the biomass determinations has to be considered. In addition to a theoretical maximum error in phytoplankton counting of ± 10% for the Utermöhl method (Edler 1979) there is a much higher potential error in cell-size determination and biomass estimation, particularly for picoplankton.

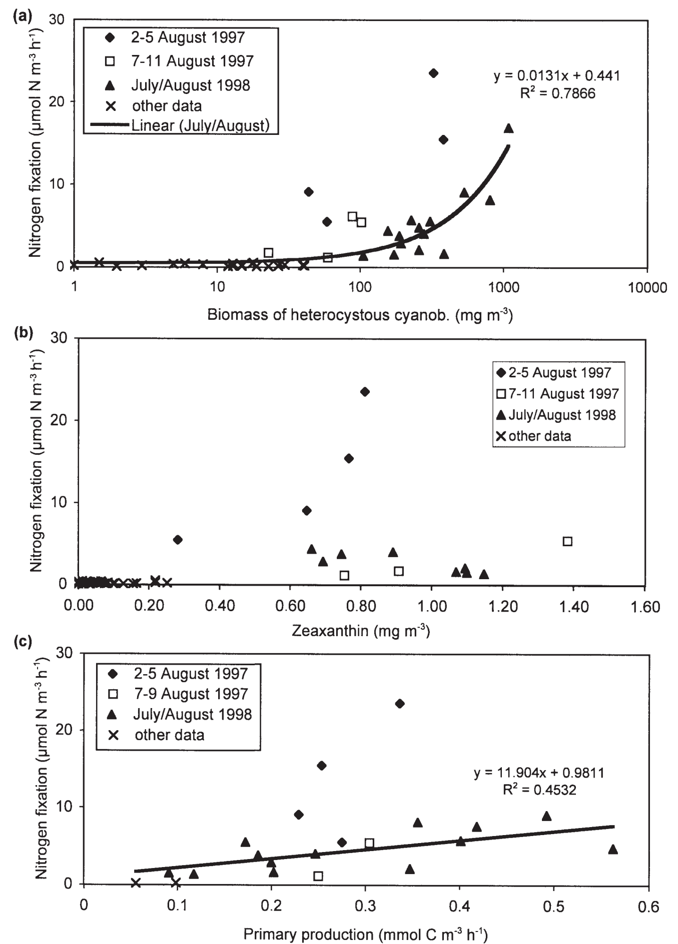

Fig. 7. Relationship between nitrogen fixation rate and biomass (wet weight) of heterocystous cyanobacteria (a), concentration of zeaxanthin (b), and primary production (c). All data are from 0 to 20 m; (a) and (b) are for all collected data, and (c) for samples of >40 mg m^{-3} heterocystous cyanobacteria

Importance of small coccoid cyanobacteria as nitrogen fixers and diel cycle of nitrogen fixation

Complete separation between filamentous (almost exclusively heterocystous) and coccoid (non-heterocystous) cyanobacteria is not possible due to the overlap of their size ranges. A 10 µm net was used to optimise separation of the 2 groups. Experiments with filtration through a 10 µm net prior to incubation in daylight revealed the largest contribution to nitrogen fixation to arise from the heterocystous cyanobacteria fraction (>10 µm). However, the contribution of coccoid organisms (<10 µm) to total nitrogen fixation was surprisingly high (43%; cf. Fig. 5 & Table 5).

When a natural phytoplankton assemblage, containing heterocystous and non-heterocystous cyanobacteria, was transferred to the dark during the daytime, the

Table 7. Overview of data from the literature on nitrogen fixation in the Baltic Sea

Area	μmol N m^{-3} h^{-1}	mmol N m^{-2} d^{-1}	mmol N m^{-2} yr^{-1}	Source
1972–1974		0.4 (up to 1.8)[a]		Brattberg (1977)
Archipelago near Stockholm				
Jul/Aug 1976			21–78[a] (x = 43)	Lindahl et al. (1978)
Askö area (Sweden),				
Aug 1974 + 1975				
Northern and central Baltic proper	0.18–9.3[a]			Rinne et al. (1978)
Aug 1978 + 1979				
Gulf of Bothnia	0.066–1.13[a]			Rinne et al. (1981)
Entrance to the Gulf of Finland	0.176–2.13[a]			
North. Baltic proper	0.30–0.50[a]			
1974–1983				
Coastal and open waters of the Arkona Sea	annual max.: 11.4 (1979) –16 464 (1982)[b]		coastal water: 15.7–100 (x = 48) Arkona Sea: 17.3–110 (x = 55)	Hübel (1984: Table 6)
1978				
Mecklenburg Bay	0.57	0.057	1.26	Hübel (1984: Table A-14)
Arkona Sea	2.86	0.286	6.28	
Bornholm Sea	2.86	0.286	6.28	
Gulf of Gdansk	8.57	0.857	18.86	
E. Gotland Sea	3.57	0.357	7.86	
1977–1978				
Öregrundsgrepen			1977: 5.0[a]	Lindahl &
SW Bothnian Sea			1978: 4.3[a]	Wallström (1985)
1980 + 1982 + 1984				
Baltic proper,		North. Baltic proper: 0.14–6.3[c]	9.3–186[a]	Niemistö et al. (1989)
Jul/Aug		Centr. Baltic proper: 0.36–4.6[c]	27.1–55.7[a]	
		South. Baltic proper: 0.41–2.1[c]	31.4 (in 1982)[a]	
Aug 1990				
E. Gotland Sea		2.44[a]		Haupt (1991)
1975–92				
Diff. areas of the Baltic Sea			1.3 (Meckl. Bay) –320 (Arkona Sea)	Hübel & Hübel (1995)
Jul/Aug 1993				
Baltic proper		0.78–1.42 (x = 1.04)[a]		Stal & Walsby (1998)
Jul/Aug 1995 + 1996				
Baltic proper	0.38–11.2			Ohlendieck et al. (2000)
1990–1997				
Baltic proper		0.21–2.6[d]	14.3–214[d]	Rahm et al. (2000)
1994–1998				
Baltic proper (without Arkona Sea)		2.3–5.9[d]	1997:113–140[d] 1998: 62–108[d]	Larsson et al. (2001)
1997–1998				
Baltic proper	x = 5.07 Max. '97: 36.0 Max. '98: 16.9	Mean in Jul/Aug.: 2.5 Summer 1997: 7.1 ± 4.4 Summer 1998: 1.7 ± 0.6	annual mean: 125 (101–263)	This paper

[a]Net samples
[b]Enriched by net plankton when necessary. Maximum found in extremely dense bloom
[c]Recalculated for daily nitrogen fixation. Net samples >25 µm
[d]Calculated from nutrient budgets

nitrogen fixation rate decreased (Fig. 4b), as also shown by Stal et al. (1999) for a *Nodularia spumigena* bloom. However, dark incubations of natural phytoplankton during the night-time led to slightly increased nitrogen fixation rates compared to daylight incubations (Table 3: non-enriched samples). In contrast, the nocturnal specific nitrogen fixation rates in net samples (100 µm in this experiment), containing primarily large aggregates of heterocystous cyanobacteria were significantly lower during the night than during the day (Table 3; see also Brattberg 1977). The difference in nitrogen fixation between the complete natural samples and the net samples is due to the contribution of the small-sized fraction (<100 µm in this experiment), which contributes much more to total nitrogen fixation during the night than during the day.

Because of the oxygen-sensitivity of nitrogenase, nitrogen fixation in non-heterocystous cyanobacteria is expected to occur mainly during the night, when oxygen production from photosynthesis ceases (e.g. Reddy et al. 1993), while heterocystous cyanobacteria are able to perform nitrogen fixation during day in their heterocysts (review: Bergman et al. 1997). Gallon & Chaplin (1988) unequivocally demonstrated nitrogenase activity in axenic clones of *Gloeocapsa alpicata*, with nitrogenase activity being temporally separated from oxygenic photosynthesis. Similarly, León et al. (1986) have shown a cyclic inverse relationship between nitrogen fixation and photosynthesis in some marine *Synechococcus* species. In contrast, Stal et al. (1999) could not find any indication of nitrogen fixation in *Synechococcus* spp. in the Baltic Sea. Nitrogen fixation of different species of coccoid cyanobacteria or heterotrophic bacteria could not be distinguished in our natural samples. Perhaps non-photosynthetic bacteria are responsible for a part of the nitrogen fixation in the light. However, Hübel (1984) could not detect nitrogen fixation by heterotrophic microorganisms in the pelagial.

Significant incorporation of DON, previously excreted by heterocystous cyanobacteria, as suggested by Ohlendieck et al. (2000), is unlikely to have occured in our experiments with the <10 µm size-fraction, since heterocystous cyanobacteria were reduced to a mere 8% of their original biomass by pre-filtration (10 µm). Also, pre- and post-filtration resulted in the same contribution to total nitrogen fixation by the <10 µm fraction, indicating that a transfer of previously fixed organic nitrogen between the 2 size fractions was of little importance in early August 1998 (Table 5). However, re-utilization of excreted nitrogen by bacteria passing the GF/F filter is possible, as indicated by decreasing nitrogen fixation rates with increasing incubation time (see last subsection of 'Materials and methods'). The high variability in the experiments is problematic, especially since the pre- and post-filtration experiments were not conducted in parallel (cf. Table 5).

Since only 4 day-and-night incubations were carried out with natural (non-enriched) samples (Table 3), the data base is too small to allow a generalization of our factors of day and night ratios of nitrogen fixation. We tentatively suggest that nitrogen fixation proceeds in unaltered (non-enriched) samples at night at the same rate as during the day.

Nitrogen budget during the summer bloom 1998

In May, the surface layer is already completely nitrate-depleted, whereas approx. 0.1 mmol m^{-3} PO_4 is still present, corresponding to 2.0 mmol m^{-2} PO_4 to the depth of the thermocline (Fig. 8). The P-surplus in the upper water layer is used up by the end of July at the latest. The corresponding nitrogen requirement (according to the Redfield ratio) would be 1.6 mmol N m^{-3} or 32 mmol N m^{-2}. This amount of nitrogen is assumed to be supplied by nitrogen fixation. The mean nitrogen fixation measured in July/August 1998 was 1.7 mmol N m^{-2} d^{-1} (cf. Table 7). This means that the

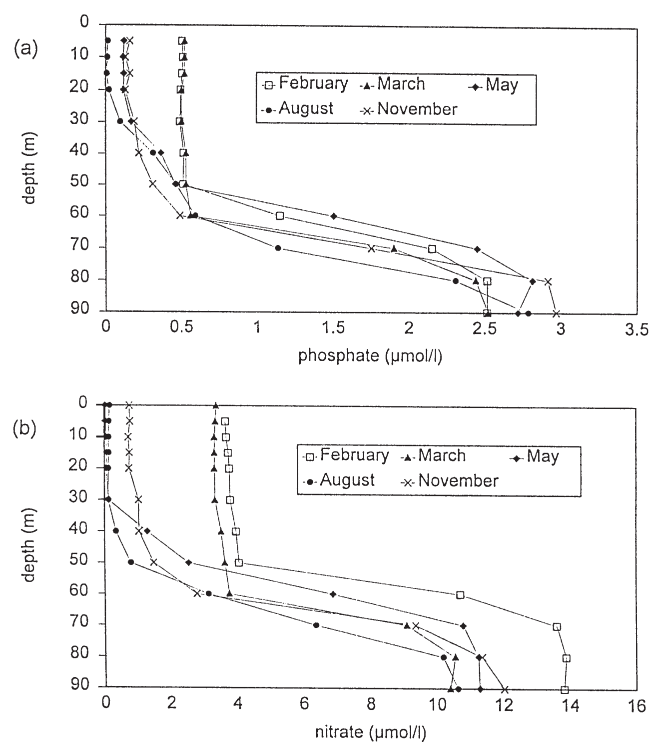

Fig. 8. Vertical profiles of reactive soluble phosphate (a) and nitrate (b) at Stn J1 in 1998 (courtesy of G. Nausch unpubl. data)

cyanobacterial bloom in 1998 could use up the surplus phosphate reserve still available in May within 19 d. However, the bloom lasted for at least 1 mo. Therefore, additional phosphorus resources, e.g. from remineralization or diapycnic input, were necessary for the continued proliferation of the bloom. In fact, the depth profiles of nutrients in the eastern Gotland Sea show that phosphate concentrations increased directly below the thermocline at 20 m, while nitrate concentrations only began to increase below 30 m (Fig. 8). This gradient implies that diffusion and mixing across the thermocline may provide phosphate but no nitrate to the upper water layer.

From primary production and nitrogen fixation measurements, the average C:N incorporation ratio was estimated to be 6.8 (3 to 5 August 1997; cf. Table 6), which is close to the Redfield ratio, indicating that nitrogen fixation satisfied the complete nitrogen requirements of phytoplankton primary production at this particular stage of a bloom. At other stages, the C:N incorporation ratio was much higher (20 to >100). Alternative sources of nitrogen are required under these conditions. M. Olesen et al. (unpubl. data) found in parallel investigations that ca 90% of the primary production must be based on recycled nutrients (Fig. 9).

During July/August 1998, an average phytoplankton primary production of 65 mmol C m^{-2} d^{-1} (Table 6) would require approx. 10 mmol N m^{-2} d^{-1} (Fig. 9). Only 1.7 mmol N m^{-2} d^{-1} (i.e. 17% of this amount) was supplied on average by nitrogen fixation (Table 6, Fig. 9). Also, Sörensson & Sahlsten (1987) found that nitrogen

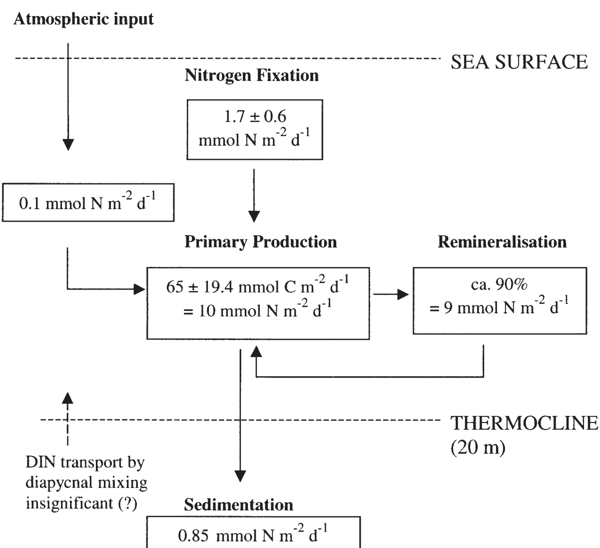

Fig. 9. Budget of nitrogen cycling in August 1998 for the Gotland Sea. Primary production and nitrogen fixation for August 1998 are from Table 5, atmospheric deposition from Schulz et al. (1999), remineralisation and settling loss from Olesen et al. (1999)

fixation contributed 16% to the nitrogen requirement for primary production in the Baltic proper in summer. Thus, we conclude that in the later stages of the cyanobacterial bloom or in weak blooms, the majority of nitrogen is supplied by internal recycling and that only a small fraction is supplemented by nitrogen fixation.

The atmospheric input of nitrogen species was estimated by M. Schulz et al. (unpubl. data) at around 0.1 mmol N m^{-2} d^{-1}; this is only a minor contribution to the nitrogen cycle in summer (Fig. 9). During the 'BASYS II' and 'BASYS V' cruises, sediment traps moored at 25 m water depth recorded average sedimentation rates of 1.4 (1997) and 0.8 (1998) mmol PON m^{-2} d^{-1} (M. Olesen et al. unpubl. data). Thus, the particulate nitrogen export from the upper mixed layer amounts to about one-fifth to one-half of the daily nitrogen fixation in these 2 yr (Fig. 9).

Estimate of annual nitrogen fixation

As we found that the small size fraction made a significant contribution to total nitrogen fixation, a contribution ignored in earlier studies, a re-calculation of the annual input of nitrogen via nitrogen fixation for the Baltic Sea was required. The present study concentrated on the period of the most obvious accumulation of cyanobacteria in the central Baltic Sea at the end of July/beginning of August. In the period from November to May, nitrogen fixation as well as cyanobacterial biomass was insignificant (Table 6, Figs. 2 & 6). We did not sample during the early phase of cyanobacterial development in June, when nitrogen fixation may be low since the cyanobacteria are still utilizing internal nitrogen reserves (Larsson et al. 2001) at this time.

Because of the limited seasonal coverage, we pooled all data for the 2 yr of investigation. The relatively low regional compared with seasonal variability (Fig. 6) allowed us to estimate a representative value for the whole Baltic proper. Based on the data from July/August 1997 and 1998, an average nitrogen fixation of 2.5 mmol N m^{-2} d^{-1} was calculated during the bloom (all stations; n = 24). The bloom lasts for approx. 1 to 2 mo (Fig. 2; cf. Wasmund 1997, Larsson et al. 2001). The active growth phase, until the strong decline in the first half of August, is assumed to be 30 d and to account for a nitrogen fixation of 75 mmol N m^{-2}. After the bloom, nitrogen fixation continues until the beginning of October. As we have no measurements for September, we had to adopt the mean of our October data for September also. For 50 d from mid-August to the first week of October, a nitrogen fixation of 50 mmol N m^{-2} was calculated. An average annual nitrogen fixation of 125 mmol N m^{-2} yr^{-1} was estimated for 1997 and 1998. Of course, this is a very

rough and also a very conservative estimate. It represents a lower limit rather than an overestimation. Also, it does not take into account the contribution made by excreted organic nitrogen previously fixed by nitrogen fixation and not re-utilized by the phytoplankton. The calculation of nitrogen fixation for the individual years is problematic because the seasonal coverage was further reduced in comparison to the combined 2 yr. Only the period of the peak of the bloom (end of July to first half of August) was well-covered and enabled us to calculate representative means for this period for the years 1997 and 1998 individually, i.e. 7.1 and 1.7 mmol N m^{-2} d^{-1}, respectively (cf. Table 7). When multiplied by 30 for the duration of the actively growing bloom with 50 mmol N m^{-2} added for the following period until October, an annual nitrogen fixation rate of 263 and 101 mmol N m^{-2} yr^{-1} was calculated for 1997 and 1998, respectively. Because of the high uncertainty of these values, they should be considered with caution. We include them in Table 7 only as upper and lower estimates of the representative mean of 125 mmol N m^{-2} yr^{-1}. A much higher nitrogen fixation in 1997 than in 1998 was also found by Larsson et al. (2001). Literature data on nitrogen fixation rates measured in the Baltic proper are summarized in Table 7. Our data are higher than earlier data obtained from net samples, and compare best with the recently published data of Hübel & Hübel (1995), Ohlendieck et al. (2000), Rahm et al. (2000) and Larsson et al. (2001) which were obtained from unenriched samples or estimated from nutrient balances.

As we included pico- and nanoplankton in our experiments, our estimate of nitrogen fixation in the whole Baltic proper are higher than previous data. We calculated an annual nitrogen fixation of 370 000 t N, which is 3 to 4 times higher than earlier estimates (Melvasalo et al. 1983, Rönner 1985, Leppänen et al. 1988). Our estimate of nitrogen fixation is almost as large as the entire riverine load (480 000 t N yr^{-1}) and twice that of the atmospheric load (196 000 t N yr^{-1}) (Elmgren & Larsson 2001).

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