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Seasonal significance of N_2 fixation in coastal and offshore waters of the northwestern Baltic Sea

Jenny Degerholm[1,2,*], Kjell Gundersen[1,3], Birgitta Bergman[1], Erik Söderbäck[1,4]

[1]Department of Botany, Stockholm University, 106 91 Stockholm, Sweden

[2]*Present address:* Faculty of Natural Sciences, Södertörn University College, 141 89 Huddinge, Sweden

[3]*Present address:* Department of Marine Science, University of Southern Mississippi, 1020 Balch Boulevard, Stennis Space Center, Mississippi 39529, USA

[4]*Present address:* QIAGEN, Frösundaviks Allé 15, 169 70 Solna, Sweden

ABSTRACT: Annual rates of N_2 fixation were measured over 3 yr (1998–2000) at an open water station (BY31) and 2 coastal stations (H4 and X1) in the Baltic Sea. This is the first report on depth-integrated rates of N_2 fixation from more than one complete growth season in the Baltic Sea. Annual estimates of N_2 fixation ranged from 56 000 to 125 000 t N in the Baltic Proper, and 18 000 to 162 000 t N at the inshore stations (Himmerfjärden). Rates of N_2 fixation were measured *in situ* at 4 depths between 0 and 25 m using the ^{15}N tracer technique for size fractionated organisms larger and smaller than 20 µm. Maximum rates of N_2 fixation were found in surface waters (0 to 4 m depth), and a major part of this activity (80% in coastal and 89% in offshore waters) took place during daylight hours. Integrated rates of N_2 fixation in cells >20 µm followed the average abundance of filamentous cyanobacteria (primarily *Aphanizomenon* sp.) in the water column. Molar C:N mass ratios in particles >20 µm, i.e. filamentous cyanobacteria, suggest that this size fraction was N-sufficient during summer, whereas the molar C:P mass ratios indicated P-limitation during this period. A reduction in sewage discharge to the Himmerfjärden bay area during the study period appears not to have been compensated for by increased rates of N_2 fixation. The patchy distribution of cyanobacteria and the high seasonal variability in N_2 fixation rates emphasize the need for adequate spatial and temporal sampling strategies in studies of N_2 fixation in coastal and open waters of the Baltic Sea.

KEY WORDS: Baltic Sea · *Aphanizomenon* · *Nodularia* · N_2 fixation · Himmerfjärden

INTRODUCTION

On a global scale, cyanobacterial N_2 fixation contributes significantly to new production in aquatic ecosystems (Capone et al. 1997, Montoya et al. 2004). In the Baltic Sea, mass occurrences of cyanobacteria in surface waters are regular events during the summer months (Lindahl et al. 1978). Satellite imaging studies suggest that the frequency and magnitudes of the blooms have increased in the Baltic Sea (Kahru et al. 1994, Finni et al. 2001). A general increase in phytoplankton productivity in both coastal and open waters of the Baltic Sea has been attributed to anthropogenic eutrophication (Larsson et al. 1985). Estimates suggest that the total input of nitrogen (N) and phosphorus (P) to the Baltic Sea increased by approximately 4- and 8-fold, respectively, during the last century (Larsson et al. 1985).

Diazotrophic cyanobacteria with the ability to assimilate atmospheric N_2 may have a competitive advantage in waters with low ratios of dissolved inorganic nitrogen (DIN) to phosphorus (DIP) (Smith 1983). This conclusion derives from the concomitant appearance in summer of low dissolved N:P nutrient ratios in surface waters of the Baltic Sea with the annual peaks in N_2 fixation (Hübel & Hübel 1976). Growth and N_2 fixation in Baltic Sea cyanobacteria are characterised by a high spatial and temporal variability, including

*Email: jenny.degerholm@sh.se

great inter-annual fluctuations (Rahm et al. 2000, Wasmund et al. 2001). The highest recorded rates of N_2 fixation are in the southern and western parts of the Baltic Proper (Hübel & Hübel 1976, Rahm et al. 2000), which is characterised by persistently low dissolved N:P ratios. Annual estimates of N_2 fixation in the Baltic Sea are higher for open waters (60 to 263 mmol N m^{-2}; Larsson et al. 2001, Wasmund et al. 2001) than for the coastal zone (21 to 79 mmol N m^{-2}; Lindahl et al. 1978). Earlier reports estimated that N_2 fixation contributes 3 to 13% of annual input of total N to the Baltic Sea (Lindahl et al. 1978, Lindahl & Wallström 1985). High nutrient loading in coastal waters caused by river run-off and anthropogenic sources may explain why N_2 fixation appears less significant in coastal waters of the Baltic Sea.

The long history of N_2 fixation measurements in the Baltic Sea has demonstrated the importance of this biogeochemical pathway to new production. Estimated annual N input by N_2 fixation to the Baltic Proper (based on acetylene reduction (AR) measurements) are in the range of 100 000 to 167 000 t (Rinne et al. 1978, Brattberg 1980, Leppänen et al. 1988). Recent estimates, however, based on measurement of the summer increase of total N in surface waters or on sensitive ^{15}N-label assays, suggest a wider range (30 000 to 434 000 t N yr^{-1}), with even greater inter-annual variability (Rahm et al. 2000, Larsson et al. 2001, Wasmund et al. 2001, 2005, Rolff et al. 2007). For comparison, estimates of the N contributions from river run-off and coastal anthropogenic point sources amount to 390 000 t yr^{-1} (Stålnacke et al. 1999) and, between 1991 and 1995, the average loading of atmospheric N to the Baltic Sea was 185 000 t yr^{-1} (HELCOM 1997). In recent years, coastal point source discharges to the Baltic Sea have been reduced by improving N and P retention in the existing sewage treatment plants. Since 1998, the Himmerfjärden sewage treatment plant (Fig. 1) has been removing 90% of the total N load (Elmgren & Larsson 2001). However, reduced N availability may result in lower dissolved N:P ratios, and hence create an environment favourable for diazotrophic cyanobacteria. A potential increase in filamentous cyanobacteria followed by increased total N_2 fixation in inshore waters may in fact counteract the intensified sewage treatment (Savchuk & Wulff 1999).

Despite the long history of N_2 fixation measurements in the Baltic Sea, few studies have covered the entire growth season of the filamentous cyanobacteria. In Swedish coastal waters, there have been only 2 limited seasonal field studies (Lindahl et al. 1978, Lindahl & Wallström 1985), and most annual estimates of N_2 fixation in open waters are extrapolations of short-term cruises made in summer. The aim of this 3 yr study was to conduct a continuous seasonal investigation of the occurrence and rates of N_2 fixation in filamentous cyanobacteria collected in open and coastal waters of the Baltic Sea. By applying the sensitive ^{15}N tracer technique we estimated the significance of N_2 fixation as a source of N for new production in coastal and offshore phytoplankton communities.

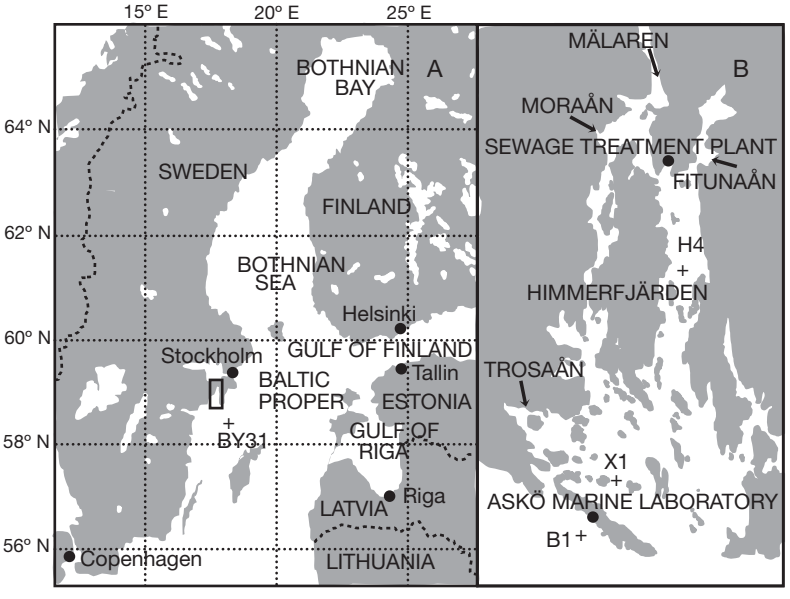

Fig. 1. (A) The Baltic Proper showing the open water reference Stn BY31, and (B) the Himmerfjärden bay area with sampling Stns X1 and H4. The sewage treatment plant is situated in the inner part of the bay and the main freshwater discharge to the area (arrows) is indicated. Askö Marine Laboratory and the adjacent Stn B1 are located in the outer part of the archipelago

MATERIALS AND METHODS

Study area and water collection. The study was conducted in the Himmerfjärden bay area and off the southeastern coast of Sweden (Fig. 1). The shallow bay area (mean depth 15 m) is sheltered, and a sewage treatment plant is located near the head of the bay. Sewage discharge and river run-off (Fig. 1B) contribute to high concentrations of dissolved inorganic nutrients in the inner parts of the bay, and a strong declining nutrient gradient is observed towards the open Baltic Sea. Two coastal stations (H4 and X1: 31 and 52 m depth, respectively) and 1 offshore station (BY31: the Landsort Deep, 459 m depth) were selected along this nutrient gradient (Fig. 1). At each station, samples were collected at the surface (nominal depth 0.25

m), 4, 10 and 20 m depth using a 5 l Hydrobios water sampler. The water collected was immediately transferred to 10 l polypropylene carboys that had been precleaned with 10% hydrochloric acid (HCl). In order to achieve homogeneity, each carboy was gently inverted twice prior to sub-sampling.

Diel (24 h) estimates and diurnal (daylight) rates of N_2 fixation. The open water Stn BY31 was only visited around noon and during daylight hours (first sample collected around 10:00, followed by 4 to 5 h incubation time), whereas Stns H4 and X1 were sampled in the morning (09:00) and incubated for 24 h. In order to make all 3 stations comparable, the data generated from Stn BY31 were converted to daily rates of N_2 fixation by the following method. Gallon et al. (2002) demonstrated a diel pattern in diazotrophic filamentous cyanobacteria in the Baltic Sea whereby the rate of N_2 fixation was driven by photosynthetic energy and diurnal solar irradiance. We investigated the diel pattern of N_2 fixation at Stn BY31 in August 2000 by performing a number of short-term acetylene reduction (AR) incubations over a 24 h period. Surface net tows (Hydrobios, 90 µm mesh, 0.5 m diameter) showed that *Aphanizomenon* and *Nodularia* were present at the time of sampling. The cyanobacterial filaments were carefully collected using the plankton net and re-suspended in an acid-cleaned polypropylene container and subsequently dispensed into glass septum bottles (35 ml) in triplicate for each time-point. The AR incubations followed the recommendations of Capone (1993). Acetylene (C_2H_2) was added to a final concentration of 14% (v/v) in the liquid phase and the samples were incubated for 30 min using an on-deck incubator. The incubator was flushed continually with ambient surface seawater, and a neutral screen provided 63% of incident photosynthetic irradiance to the bottles. Assuming an average attenuation coefficient (k) of −0.353 in surface waters of the Baltic Sea (Walsby et al. 1997), the screen reduced photosynthetic active radiation (PAR) to that occurring at 1.3 m depth. A new set of samples was incubated every 2 to 4 h through a 24 h cycle. A gas sample from the head space of each incubation bottle was withdrawn at each time-point and stored separately in 5 ml Vacutainers in darkness at 4°C. The ethylene (C_2H_4) end product concentrations were determined on a Shimadzu GC-8A gas chromatograph equipped with a 2 m long stainless steel column (Porapac, 3 mm inner diameter, N80/100 mesh) using ethylene of known concentration (100 ppm) as a gas standard.

A comparison was made between the 4 to 5 h incubations and the 24 h diel incubations at Stn BY31 to validate the conversion of short-term N_2 fixation into daily rates. These parallel incubations, which were conducted in August 1999 and again in June and July 2000, were measured by the ^{15}N-labelled dinitrogen gas incorporation technique (see below). A third diel study was performed in August 2000 at Stn BY31 and at coastal Stn B1 (Fig. 1B) when measured rates of N_2 fixation in daylight incubations were compared with similar incubations at night. Surface seawater samples were collected, size fractionated, and then incubated using ^{15}N-labelled dinitrogen gas (see below) under growth conditions as described for the on-deck incubator (above).

N_2 fixation measurements. Duplicate polycarbonate Erlenmeyer flasks (250 ml) were filled to the brim with sample water from surface, 4, 10 and 20 m depth. Great care was taken to avoid air-bubbles as the lid was gently screwed back onto each flask. Each lid was equipped with a Teflon-coated silicon membrane and 4.6 ml of ^{15}N-labelled dinitrogen gas ($^{15}N_2$; Cambridge Isotopes) were injected into each flask using a syringe. Since gas dissolution in seawater may vary as a function of the injected gas volume, salinity and temperature (Weiss 1970), the percentage $^{15}N_2$ enrichment was calculated for each station and time (range 43 to 48%).

Each pair of bottles was incubated *in situ* at its respective depth overnight (Stns H4 and X1) or for 4 to 5 h around noon (Stn BY31) using a tethered floating array. A time zero sample from each depth and station was filter-fractionated immediately after deployment and processed as described below. The samples retrieved at Stns H4 and X1 were transported in the dark in a portable insulated cooler back to the Askö Marine Laboratory (approximately 1.5 h transit time) and processed immediately upon arrival. At the offshore Stn BY31, the retrieved samples were filtered immediately onboard the ship. All samples were size fractionated by gravity filtration and collected on precombusted Whatman GF/C (cells >20 µm) and Whatman GF/F (cells <20 µm) filters. The samples were dried at 65°C, fumed by concentrated HCl, dried again and packed in tin foil capsules. The ^{15}N enrichments were analysed on a Europa Mass Spectrometer 20-20, with an ANCA-SL preparatory unit. The mass spectrometer was standardised with bovine serum albumin and the precision of the analytical procedure, as measured by the standard deviation of the nitrogen isotope, was typically 0.04%. The rate of N_2 fixation was calculated as outlined by Montoya et al. (1996) and using the gas dissolution constants of Weiss (1970).

Abundance of filamentous cyanobacteria. Plankton samples were collected twice per month from stations H4, X1 and BY31 on the day (or preceding/following days) of N_2 fixation measurements. Each station was sampled by submerging a plastic hose (inner diameter 25 mm) from the surface to 14 m (Stns H4, X1) or 20 m depth (Stn BY31). The hose

was closed off and the sample was collected in a bucket. Sample aliquots of 200 ml were preserved with 0.8 ml acidified Lugol's solution and stored at 4°C until processing. The samples were concentrated in an Utermöhl settling chamber (10 or 25 ml subsample volume) and viewed under a Nikon inverted microscope with phase contrast optics. The lengths of the cyanobacterial filaments were counted in diagonals or as half or whole chamber bottom areas at 100 or 150× magnification (HELCOM 1988). Abundance of the cyanobacteria was expressed as the total length of filaments enumerated per volume of sample water (m ml^{-1}).

Particulate organic carbon (C), nitrogen (N) and phosphorus (P). Elemental C and N samples were collected in 250 ml duplicates, filtered onto precombusted (5 h, 500°C) Whatman GF/F or GF/C filters (same sampling procedure as for N_2 fixation measurements) and stored at –20 °C. Prior to analysis, the C and N filter samples were dried at 65°C, fumigated with concentrated HCl, dried again and packed in tin capsules. Particulate organic C and N contents were analysed on an LECO CHNS-932 Analyser.

The samples used for P determinations were collected as duplicates of 5 or 10 ml water aliquots filtered onto HCl-washed Whatman GF/C filters. The P samples were analysed by the ash-hydrolysis method following Solorzano & Sharp (1980). The filters were soaked in phosphate-free, deionised water (particle free) and dried prior to use. The filter-collected samples were rinsed with 0.17 M Na_2SO_4 and stored in acid cleaned containers at –20°C. Prior to analysis, the samples were thawed, 0.017 M $MgSO_4$ was added to each, and they were left overnight at 95°C. The samples were combusted at 500°C for 3 h and the remaining P was extracted in 2 ml of 0.2 M HCl at 80°C for a minimum of 1 h. The acid extract was centrifuged (30 min, 4100 × *g*) and the supernatant was analysed for soluble reactive phosphate (SRP) following Strickland & Parsons (1972). The blue-coloured reaction complex was measured on a Jasco 7800 Spectrophotometer at 885 nm.

Physical data. Surface irradiance was measured in Visby on the island of Gotland (100 km south of Stn BY31) and provided by the Swedish Meteorological and Hydrological Institute (SMHI). Diurnal surface irradiance was reported as hourly averages of continual measurements of total irradiance (290–4000 nm) on a flat surface and recalculated as a fraction of each daily maximum. Sea surface temperature (SST), relative salinity and density (δ) data were extracted from continuous CTD profiles (Meerestechnik Electronik) provided by the Stockholm Marine Research Centre (SMF, available at: www.smf.su.se/english/). SST was calculated as 0–5 m depth averages, and the water column stability index was calculated as the difference in density between measurements at surface and 15 m depths ($\delta_{15m} - \delta_{Surface}$).

RESULTS

Diel versus diurnal rates of N_2 fixation

At Stns H4 and X1, all *in situ* incubations were done overnight, while the offshore Stn BY31 was visited only during daylight hours (4 to 5 h incubations). In order to obtain diel estimates of N_2 fixation at Stn BY31, we factor-corrected the daily estimates by an equation obtained as follows. Rates of N_2 fixation in Baltic Sea cyanobacteria change during the course of a

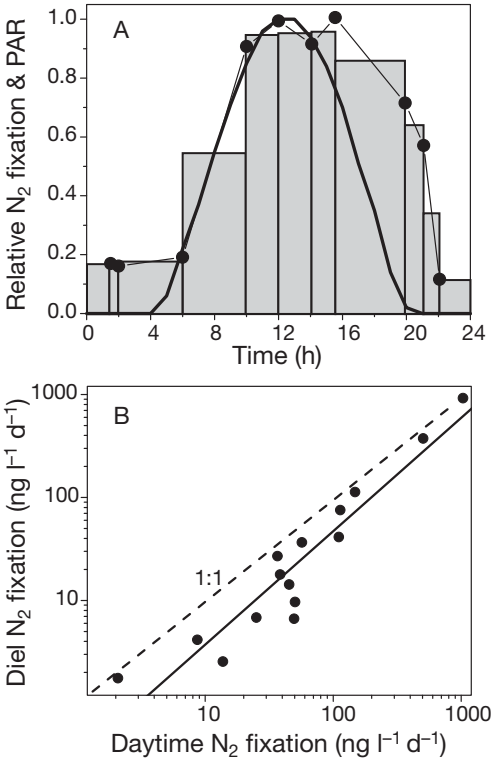

Fig. 2. (A) Diel rates of N_2 fixation at Stn BY31 in August 2000 measured in surface net-collected cyanobacteria using the acetylene reduction technique. The activities were calculated as a fraction of the maximum rate of N_2 fixation (solid circles) and the daily rate is expressed by trapezoidal integration (histograms). Diurnal sea surface irradiance (heavy solid line) was measured on the same day and is expressed as a fraction of the noon maximum. (B) Rates of N_2 fixation calculated from ^{15}N incubations over a 24 h period plotted as a linear function of daylight measurements converted to daily rates in samples collected between 0 and 20 m depth at Stn BY31. The linear function (solid line) is expressed (with SE) as: $y = 1.10(\pm 0.11)x - 0.53(\pm 0.20)$, r = 0.942, n = 15, $p < 0.0001$. Note the $\log_{10}$ scale on both axes

day (Gallon et al. 2002) and this trend appears to follow the relative distribution of PAR (Fig. 2A), with a time-lag extending into the evening. The relative distribution of PAR (PAR_A) was calculated by trapezoid area integration over a 24 h period ($PAR_A = \int_0^{24} PAR$). From the surface irradiance data, we observed a seasonal change in PAR_A as a function of the daylength, with a maximum during mid summer (authors' unpubl. data). The area of the relative distribution of N_2 fixation (Fig. 2A) was calculated by trapezoid integration ($AR_A = \int_0^{24} AR$), and we assumed a constant relationship between diel AR_A and diurnal PAR_A ($f = AR_A/PAR_A = 1.666$) for the entire year. A daily rate of N_2 fixation (mg N d^{-1}) could then be calculated from the short-term incubations (mg N h^{-1}) at Stn BY31:

$$N_2 \text{ fixation (mg d}^{-1}) = N_2 \text{ fixation(mg h}^{-1}) \times (T_1 - T_0) \times \left(\left(\int_0^{24} PAR \right) / \left(\int_{T0}^{T1} PAR \right) \right) \times f$$

where T_0 and T_1 are the time points of the short-term incubation during daylight hours (h) and $\int_{T0}^{T1} PAR$ is the area integration of PAR during the incubation period. Fig. 2B shows a comparison between diel incubations of N_2 fixation (0 to 24 h) and daytime incubations converted to daily rates using the equation above. There was no significant difference ($p < 0.4$) between the 1:1 relationship and the regression line generated from the 3 overnight visits to Stn BY31 in 2000 (Fig. 2B).

Vertical profiles of N_2 fixation

In order to assess the maximum contribution of fixed nitrogen to new production at each site, an average of the vertical profile of N_2 fixation was created from 3 selected cruises to each of the stations in each year (Fig. 3). The cruises were selected as 'prior to', 'during' and 'after' maximum rates of N_2 fixation in each year. Maximum rates of N_2 fixation were in surface waters, and rates were higher in cells >20 µm than in cells <20 µm at all stations (Fig. 3). At Stns H4 and X1, rates of N_2 fixation decreased by a factor of approximately 10 between surface waters and 10 m depth, whereas rates at these depths were in the same order of magnitude at Stn BY31.

Seasonal and annual integrations of N_2 fixation

Vertical integrations of the measured rates of N_2 fixation showed great spatial and temporal variability at all stations investigated (Figs. 4 to 6). Peaks in abundance of filamentous cyanobacteria were frequently coincident with peaks in total N_2 fixation (Fig. 7). Of the total integrated N_2 fixation, the particle fraction >20 µm contributed an average of 77% (3 yr range: 74 to 78%) at Stn BY31, 73% (range: 65 to 79%) at Stn X1 and 70% (range: 61 to 74%) at the innermost Stn H4.

The measured peak in N_2 fixation within each year occurred in highly stratified waters when sea surface temperature (SST) exceeded 15ºC. However, periods with such hydrographical conditions were not always coincident with high rates of N_2 fixation (Figs. 4 to 6). Peak activities in N_2 fixation typically appeared in July–August, with great inter-annual variability in the magnitude and timing of the event. In 1998 and 1999, N_2 fixation was, in general, higher at Stn BY31 than at the inshore stations. This pattern was reversed in 2000, when the highest N_2 fixation rates occurred at the 2 inshore Stns H4 and X1 (Figs. 4 to 6). In pre-filtered seawater collected in August 2000, rates of N_2 fixation

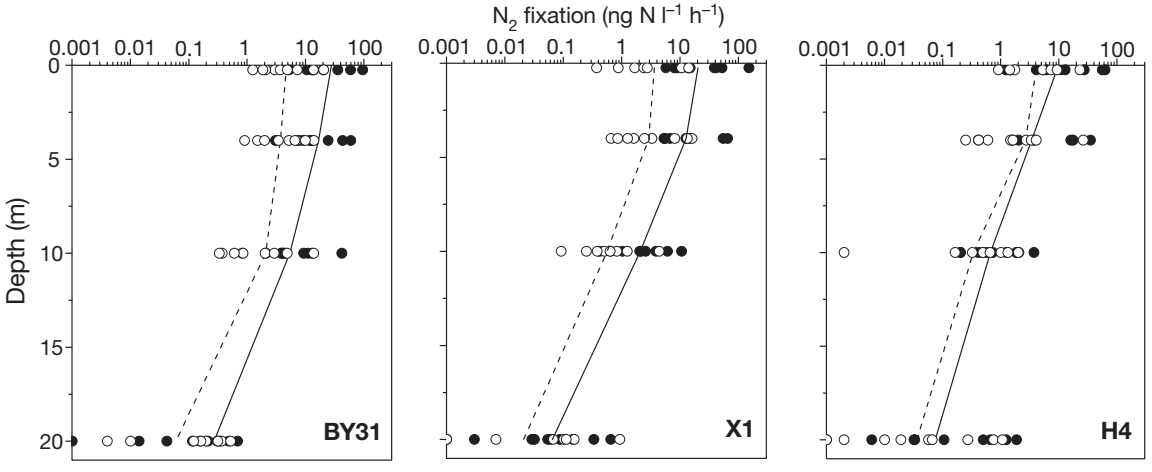

Fig. 3. Rates of N_2 fixation by depth measured in cells >20 µm (●) and in all cells <20 µm (○) at Stns BY31, X1 and H4 in 1998-2000. The 3 yr median value was calculated for each depth in cells >20 µm (solid line) and cells <20 µm (dashed line) from 3 field trips in each year selected prior to, during and immediately after maximum rates of N_2 fixation

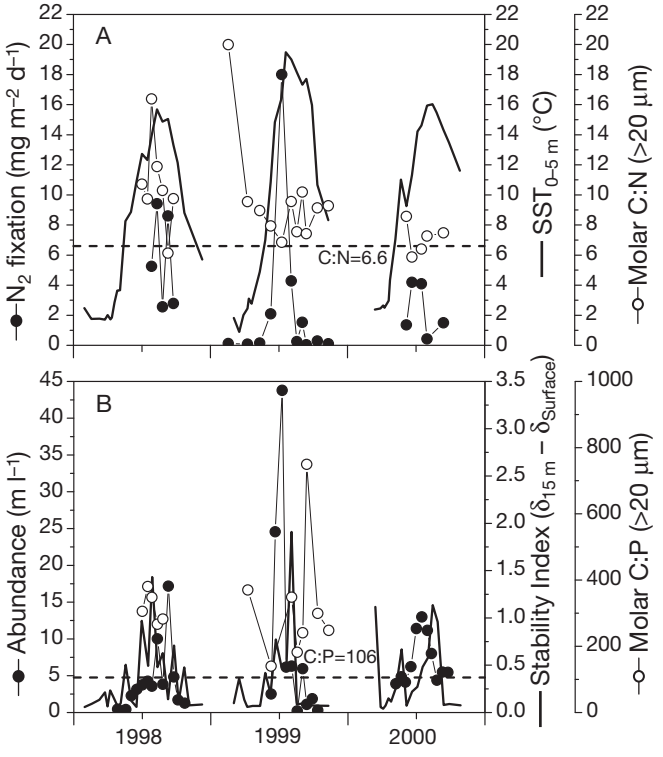

Fig. 4. Stn BY31. (A) Integrated rates of N_2 fixation in all cells (●) collected on GF/F filters from depths between 0 and 20 m in 1998–2000. Sea surface temperature (SST, solid line) was calculated as an average of the upper 0 to 5 m in the water column. The integrated molar C:N ratio was calculated from particles >20 µm (○) and the dashed line is the Redfield relationship (C:N = 6.6). (B) Abundance of cyanobacteria (●) averaged between 0 and 20 m depth in 1998–2000. Abundance expressed as the total length of filaments measured per volume of sample water (m l^{-1}). The water column stability index (solid line) was calculated as the difference in density (δ) between 15 m and surface ($\delta_{15m} - \delta_{Surface}$). The integrated molar C:P ratio was calculated from particles >20 µm (○) in 1998 and 1999, and the dashed line is the Redfield relationship (C:P = 106)

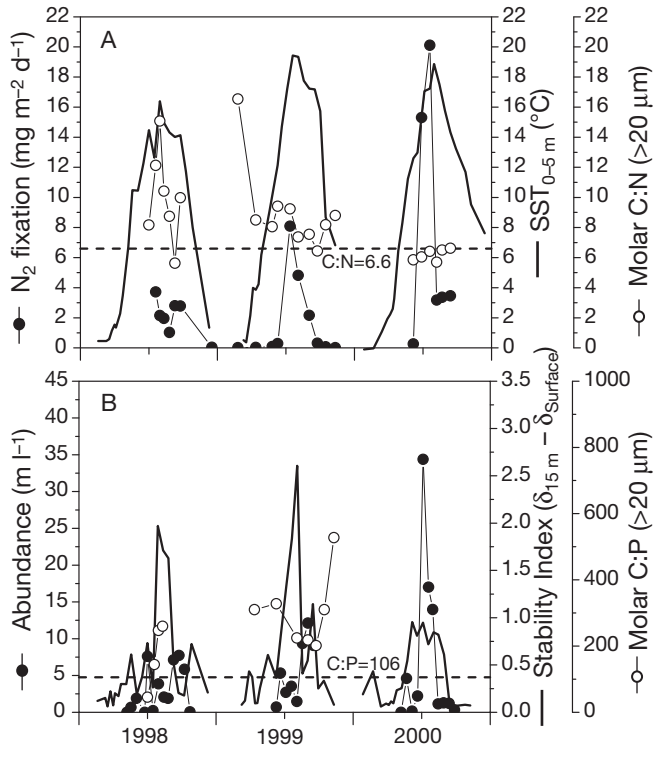

Fig. 5. Stn X1. (A) Integrated rates of N_2 fixation measured in all cells (solid circles) collected on GF/F filters from depths between 0 and 20 m in 1998–2000. Sea surface temperature (SST, solid line) was calculated as an average of the upper 0 to 5 m of the water column. The integrated molar C:N ratio was calculated from particles >20 µm (○) and the dashed line is the Redfield relationship (C:N = 6.6). (B) Abundance of cyanobacteria (●) averaged between 0 and 14 m depth in 1998–2000. Abundance expressed as the total length of filaments measured per volume of sample water (m l^{-1}). The water column stability index (solid line) was calculated as the difference in density (δ) between 15 m and surface ($\delta_{15m} - \delta_{Surface}$). The integrated molar C:P ratio was calculated from particles >20 µm (○) in 1998 and 1999, and the dashed line is the Redfield relationship (C:P = 106)

Table 1. Rates of N_2 fixation (ng N l^{-1} h^{-1}) measured in cells collected at Stn B1 and at the offshore Stn BY31 in August 2000. Cells larger and smaller than 20 µm were separated prior to the *in situ* incubations in daylight hours (Day) and after dark (Night). Total: Total rates of N_2 fixation; SD_{n-1}: standard deviations; n: number of replicate incubation bottles

	Day	SD_{n-1}	n	Night	SD_{n-1}	n	Day:Night
Stn B1							
<20 µm	14.8	2.5	2	1.2	0.1	2	12.3
>20 µm	26.7	3.8	2	4.8	0.9	2	5.6
Total	40.4	0.7		5.2	0.8		7.8
Stn BY31							
<20 µm	1.4	0.5	3	4.1	4.4	3	0.3
>20 µm	125.5	43.4	3	30.0	4.2	3	4.2
Total	126.6	43.4		31.1	0.2		4.1

in cells >20 µm were always higher during the day (Table 1). With the exception of Stn B1, this was also the case for cells <20 µm.

Molar C:N and C:P

The integrated molar C:N ratios of particulate matter >20 µm collected at 0 to 20 m depth in 1998 were high in late winter/early spring and approached, but rarely went below, the Redfield relationship (6.6) during summer at all stations (Figs. 4A, 5A, 6A). The C:N ratio was generally higher in 1998 and 1999

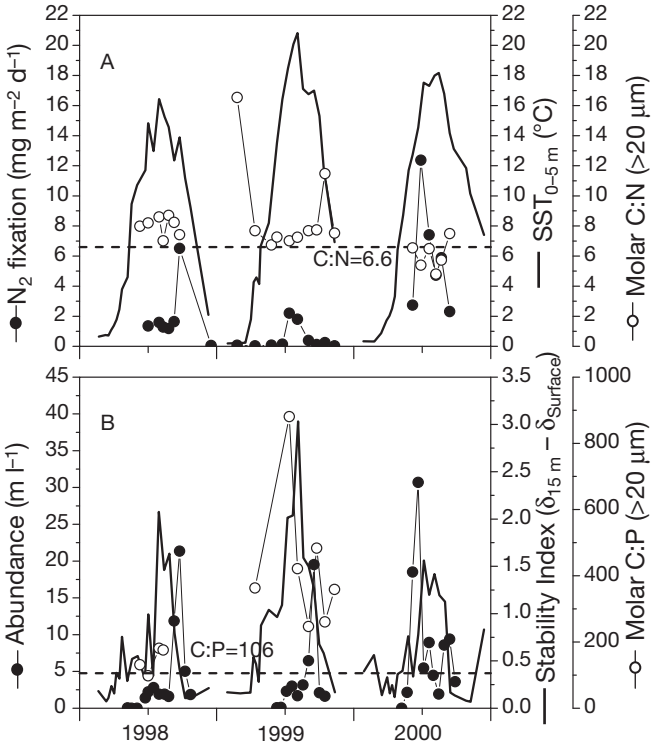

Fig. 6. Stn H4. (A) Integrated rates of N_2 fixation measured in all cells (●) collected on GF/F filters from depths between 0 and 20 m in 1998–2000. Sea surface temperature (SST, solid line) was calculated as an average of the upper 0–5 m of the water column. The integrated molar C:N ratio was calculated from particles >20 µm (○) and the dashed line is the Redfield relationship (C:N = 6.6). (B) Abundance of cyanobacteria (●) averaged between 0 and 14 m depth in 1998–2000. Abundance was expressed as the total length of filaments measured per volume of sample water (m l^{-1}). The water column stability index (solid line) was calculated as the difference in density (δ) between 15 m and surface ($\delta_{15m} - \delta_{Surface}$). The integrated molar C:P ratio was calculated from particles >20 µm (○) in 1998 and 1999, and the dashed line is the Redfield relationship (C:P = 106)

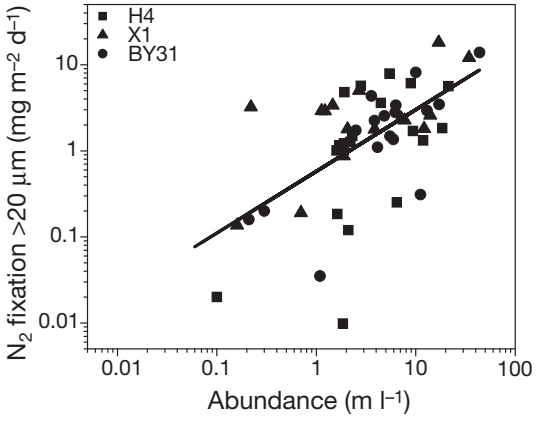

Fig. 7. Integrated rates of N_2 fixation in cells >20 µm at Stns BY31, X1 and H4 plotted as a function of the abundance of filamentous cyanobacteria. Cyanobacterial abundance was expressed as the total length of filaments measured per volume of sample water (m l^{-1}). The linear relationship is expressed (±SE) by $y = 0.72(\pm 0.13)x - 0.24(\pm 0.10)$, r = 0.602, n = 53, p < 0.0001. Note the $\log_{10}$ scale on both axes

than in 2000 at Stn BY31 (Fig. 4A). A similar trend was found at Stns X1 and H4, and in 2000, most C:N values were grouped at or just below the ratio of 6.6 (Figs. 5A, 6A). Integrated molar C:P ratios in particles >20 µm were measured only in 1998 and 1999, and were always higher than the Redfield relationship (106) (Figs. 4B, 5B, 6B). While molar C:P was similar in both years at Stn BY31 (Fig. 4B), the ratio increased well above 106 in 1999 at Stns X1 and H4 (Figs. 5B, 6B).

Cyanobacterial abundance

Three species of cyanobacteria were detected at all 3 stations during 1998–2000 (data not shown). *Aphanizomenon* sp. was the dominant taxon at Stns H4 and X1 (98%) and at Stn BY31 (95%). *Nodularia spumigena* Mertens appeared infrequently at the inshore Stns H4 and X1, but regularly during the summer months (July–September) at the offshore Stn BY31. Least frequent was *Anabaena lemmermannii* P. Richter, which appeared intermittently in minor abundances at all stations (authors' unpubl. data). Major peaks in total cyanobacterial abundance appeared during periods of maximum rates of N_2 fixation (Figs. 4 to 6). The N_2 fixation rate in cells >20 µm were plotted as a linear function of total cyanobacterial abundance (Fig. 7).

DISCUSSION

There was great temporal and spatial (depth and geography) variability in our field measurements of N_2 fixation in the Baltic Sea. The apparent patchiness and temporal fluctuations in cyanobacterial abundance and rates of N_2 fixation confirm the importance of long-term inter-annual studies over a wide arrary of sampling stations for obtaining comprehensive information required for decisions on sewage treatment and nutrient management efforts.

Diel variability and calculations of N_2 fixation in filamentous cyanobacteria

In filamentous cyanobacteria such as *Aphanizomenon*, *Nodularia* and *Anabaena*, the oxygen-sensitive enzyme nitrogenase is protected by heterocysts allowing maximal rates of N_2 fixation during daytime photo-

synthesis. This diel pattern in N_2 fixation (Fig. 2B) has been reported in several investigations of heterocystous cyanobacteria from the Baltic Sea (Brattberg 1977, Vuorio et al. 1978, Evans et al. 2000, Gallon et al. 2002). A number of diel estimates of N_2 fixation in the Baltic Sea are based on short, daytime measurements. Brattberg (1977), however, found that the average rate of N_2 fixation between 10:00 and 14:00 h comprised only 28% of the total 24 h rate, which is close to our area integration of AR during the same daytime period (34%; Fig. 2A). According to the pre-filtered ^{15}N incubations, mean nocturnal N_2 fixation rates were 13 and 25% of the average daytime fixation rates at Stns B1 and BY31, respectively (Table 1). In our diel measurements, total integrated N_2 fixation at night was 20% of total daytime fixation (06:00 to 20:00 integration, Fig. 2A). This is slightly lower than the range (23 to 50%) reported for *Aphanizomenon* colonies (Horne 1979, Storch et al. 1990, Evans et al. 2000, Gallon et al. 2002), but similar to 20% reported by Vuorio et al. (1978). Therefore, diazotrophic activities during the dark period may also contribute significantly to the total rate of N_2 fixation in aquatic environments and should not be overlooked in diel estimates. In this study we propose a way to extrapolate diel rates of N_2 fixation using measurements of short-term N_2 fixation and the daily surface irradiance curve (see equation in Results). The calculated diel rate of N_2 fixation appeared to follow actual 24 h rates measured coincidentally at Stn BY31 (Fig. 2B).

DON utilization and N_2 fixation in non-filamentous cyanobacteria

Passive leakage or active release of DON has frequently been documented in actively growing phytoplankton (Bronk et al. 1994, Glibert & Bronk 1994, Mulholland et al. 2004). However, whether or not healthy growing cells excrete DON is still under debate (Bronk & Ward 2000, Slawyk et al. 1998). Reported rates of DON release from cyanobacteria are in the range of 40 to 50% of the rate of N_2 fixation (Glibert & Bronk 1994), but the rate of excretion may depend on physiological growth conditions. The molar ratio between AR and $^{15}N_2$ incorporation varied in Baltic Sea cyanobacteria during the course of a diel cycle (Gallon et al. 2002). Therefore, the ^{15}N-enrichment method may not have reflected gross N_2 fixation as release of ^{15}N-labelled DON may have caused the high molar ratios observed in their study (Gallon et al. 2002). We are aware of no specific studies of DON excretion from Baltic Sea diazotrophic cyanobacteria. No detectable rates of N_2 fixation in cells <5 µm were observed during daytime in offshore waters of the Baltic Sea, but 5 to 10% of the total N_2 fixed was recovered in these cells and attributed to DON transfer from the larger filamentous cyanobacteria to the 'picoplankton' fraction (Ohlendieck et al. 2000). Of total N_2 fixation 43% occurred in cyanobacterial cells <10 µm (Wasmund et al. 2001). This size fraction, however, had the same level of ^{15}N-enrichment in pre-screened cells and cells separated after the incubations, suggesting that DON excretion was insignificant for cells <10 µm in their study. Higher night-time rates in natural seawater samples (up to 1.9 × the daytime rate) suggested that smaller non-filamentous diazotrophs may have been more active at night (Wasmund et al. 2001). Recent open ocean studies using the ^{15}N method have demonstrated the significance of night-time N_2 fixation by single cell diazotrophs (Montoya et al. 2004). At times, significant rates of N_2 fixation have also been found in cells other than filamentous cyanobacteria (Wasmund et al. 2001), and a variable but significant fraction of N_2 fixation has been observed in cells between 5 µm and 20 µm (Ohlendieck et al. 2007). Therefore, assuming that cells <5 µm are non-diazotrophic and incorporate excreted DON, we can deduce that cells between 5 µm and 10 µm may sometimes contribute significantly to N_2 fixation in the water column. In our study, most N_2 fixation occurred in cells >20 µm (Fig. 3). However, a considerable fraction of the ^{15}N-label in our diel incubations (31 to 44% of total N_2 fixation at H4, 15 to 24% at X1, and 18 to 28% at BY31) was also recovered in cells <20 µm. In a day-night comparison of N_2 fixation with size-fractionated samples, the coastal stations had higher rates in the smaller cell fraction in daytime rather than at night (Table 1). Only at Stn BY31 did cells <20 µm have higher rates of N_2 fixation at night than during the day. Our data suggest that non-filamentous cyanobacteria in offshore waters may at times have significant rates of N_2 fixation at night. However, the average fractions of ^{15}N enrichment in cells <20 µm in this study (Fig. 3) far exceeded the rates of N_2 fixation attributed to non-filamentous cyanobacteria in the Baltic Sea (Ohlendieck et al. 2007). Therefore, we conclude that a significant proportion of the total N_2 fixation was initially incorporated by filamentous cyanobacteria and, by way of DON excretion, may have been further assimilated by microplankton.

Cyanobacterial blooms and growth

The great variability in the abundance of filamentous cyanobacteria and hence, their potential contribution to new production by N_2 fixation may result from a multitude of physical, chemical and physiological factors. Previous studies (see overview by Leppänen et al. 1988) reported a relationship between rates of N_2 fixation, surface temperature and the stability of the water

column. Although SST and the water column stability index may appear to be important correlates of bloom formation in filamentous cyanobacteria, we did not see any direct relationship between these factors and increased cell abundance and elevated rates of N_2 fixation (Figs. 4 to 6). However, surface stability in offshore waters (Stn BY31) reverted to winter levels in the time preceding the expected bloom of cyanobacteria in the summers of 1998 and 2000, and this may explain, in part, the low rates of N_2 fixation measured in these 2 years (Fig. 4). The surface water stability index also had intermittent lapses in early summer of 1999 at Stn BY31 when cell abundance and N_2 fixation rates were high, but the lapses were less frequent and not as severe as in the other years. Surface water stability was stronger in inshore waters, and Stn X1 had a relatively high stability index through the summer of 2000, coincident with the highest abundance of filamentous cyanobacteria and rates of N_2 fixation during the 3 yr study (Fig. 5). This pattern was not as apparent at the innermost Stn H4, where water column stability was often higher than at the other stations investigated (Fig. 6). In fact, an extended period of high surface temperatures followed by high water column stability prevailed in the summer of 1999 at Stn H4 and yet, these conditions did not generate any increase in the abundance of filamentous cyanobacteria, and the rates of N_2 fixation were the lowest recorded within the study period (Fig. 6).

Relatively high dissolved N:P nutrient ratios in the inner parts of the bay may have favoured the growth of non-diazotroph phytoplankton with higher affinity for P, a circumstance previously suggested by Wallström (1988). We therefore conclude that dissolved nutrient concentrations with a low N:P ratio are important in determining the presence and extent of N_2 fixing cyanobacteria in surface waters, as suggested by Smith (1983). Moreover, the generally high N:P ratios in Bothnian Bay have been used to explain the absence of filamentous cyanobacteria in that region of the Baltic Sea (Niemi 1979, Rinne et al. 1981). In addition to a low dissolved N:P ratio, high concentrations of P may also promote and sustain blooms of cyanobacteria (Wallström et al. 1992, Wasmund 1997). High levels of available phosphate stimulate growth and rates of N_2 fixation in cyanobacteria collected from natural populations (Horstmann 1975, Kononen et al. 1998, Moisander et al. 2003), in mesocosms (Wallström et al. 1992) and in laboratory cultures (Huber 1986, Moisander & Paerl 2000). In contrast, high ambient concentrations of combined dissolved nitrogen can cause a reduction in N_2 fixation (Huber 1986). Therefore, the high rates of N_2 fixation measured at Stns H4 and X1 in the summer of 2000 (Figs. 5A, 6A) may, in part, have been stimulated by the higher concentrations of phosphate (0.05 to 0.15 µM) in the Himmerfjärden bay area during that particular year (U. Larsson pers. comm.). Our results demonstrate the multitude of parameters that may affect cyanobacterial growth and N_2 fixation.

Balanced growth of cyanobacteria (i.e. cellular C:N:P ratios approximating the Redfield relationship of 106:16:1, Redfield et al. 1963) requires a flux of dissolved N and P in a ratio that is constant to that of photosynthetically derived C (Dubinsky & Berman-Frank 2001). Our particle fractionation technique did not discriminate detrital matter from live cells. Therefore, the presence of detritus (with extremely high C:N and C:P) in particles >20 µm may have overestimated these ratios. The C:N ratios in cells >20 µm (the fraction largely dominated by filamentous cyanobacteria) were slightly higher or grouped around the Redfield relationship of 6.6 (Figs. 4 to 6) and similar to the mass ratios for *Aphanizomenon* and *Nodularia* in the same area (Larsson et al. 2001, Walve 2002). The high molar C:P ratios in particles >20 µm at Stn BY31 in summer were also similar but slightly higher than those reported by others (Larsson et al. 2001, Walve 2002) using more refined techniques to separate filamentous cyanobacteria. The high C:P ratios found in filamentous cyanobacteria in offshore waters indicated severe P-limitation in the diazotrophs in summer (Larsson et al. 2001). While C:P in the fraction >20 µm remained similar in 1998 and 1999 at Stn BY31, the elemental mass ratio increased dramatically between the years at Stns X1 and H4 (Figs. 5B, 6B). This observation, combined with the molar C:N ratio at Stns X1 and H4, suggests that cells >20 µm were initially P-replete and may have transformed into an N-replete community during summer by the end of our field study. The N-replete community >20 µm may have been supported by the high rates of N_2 fixation (relative to previous years) in the summer of 2000 at Stns X1 and H4 (Figs. 5A, 6A).

Spatial variability in rates of N_2 fixation

Highest rates of N_2 fixation were found in surface waters (Fig. 3), in good agreement with earlier studies from the Baltic Sea (Brattberg 1977, Vuorio et al. 1978, Niemistö et al. 1989, Evans et al. 2000, Wasmund et al. 2001, Gallon et al. 2002). The low rates of N_2 fixation measured at 20 m depth (Fig. 3) can be attributed to low biomass of heterocystous cyanobacteria and low light at these depths (Stal & Walsby 1998, Wasmund et al. 2001). Vertical biomass distribution in the open Baltic Sea shows that maximum abundance of *Aphanizomenon* is frequently around 10 m depth, while *Nodularia* aggregates in surface waters (Walsby et al.

1995, Heiskanen & Olli 1996, Wasmund et al. 2001, Hajdu et al. 2007). Therefore, a significant and active part of the diazotrophic community may exist in subsurface waters and must be included in order to obtain accurate estimates of total water column N_2 fixation rates (Stal & Walsby 1998).

Cyanobacterial biomass had abrupt seasonal maxima and great inter-annual differences at our stations (Figs. 4B, 5B, 6B). Although *Aphanizomenon* sp. was most frequent, surface blooms of *Nodularia* sp. often occurred during summer at the offshore Stn BY31. An overview of the abundance distribution of filamentous cyanobacteria in the Baltic Sea (Finni et al. 2001) also shows that *Aphanizomenon* sp. is the most commonly encountered species in recent history. The positive linear relationship between average abundance of filamentous cyanobacteria and the rate of N_2 fixation (Fig. 7) largely explains the rather ephemeral biomass distribution and the temporal and spatial variability in measured rates of total N_2 fixation.

We calculated annual depth-integrated estimates of N_2 fixation for each of our stations (Table 2). Missing months in some of the years were extrapolated from the adjacent months or by using averages from other years in which measurements were made. There was considerable variability in the annual rates of N_2 fixation at the coastal Stns H4, X1 and the offshore Stn BY31, but these differences appeared to even out in the 3 yr averages of total N_2 fixation (calculated from Table 2: 400 and 545 mgN m^{-2} yr^{-1} for Stns H4 and X1, respectively, and 555 mgN m^{-2} yr^{-1} for Stn BY31). The annual averages of N_2 fixation in cells >20 µm at the coastal stations (21 and 31 mmol N m^{-2}) also fell within the annual range (21 to 78 mmol N m^{-2}) reported by Lindahl et al. (1978). At offshore Stn BY31, our total annual estimates for 1998–2000 (range 22 to 51 mmol N m^{-2}) were similar to the rates measured at coastal Stns X1 and H4. Coastal waters are frequently ignored in annual estimates of N_2 fixation to the Baltic Sea. We suggest that diazotrophic cyanobacteria may contribute significantly to new primary production in both coastal and open waters of the Baltic Sea.

Table 2. Regional integrated estimates (0 to 20 m) of N_2 fixation (mg N m^{-2} yr^{-1}) measured offshore at Stn BY31 and inshore at Stns X1 and H4 in the Baltic Sea

Year	Stn BY31	Stn X1	Stn H4
Total N_2 fixation			
1998	655	242	259
1999	709	422	121
2000	301	970	821
N_2 fixation cells >20 µm			
1998	494	212	213
1999	549	283	78
2000	227	821	598

Significance of N_2 fixation in the Baltic Sea

The annual contribution of N_2 fixation to new N in the Baltic Proper was estimated from depth integrated (0 to 10 m) diel rates of N_2 fixation from the offshore Stn BY31. Assuming that 240 000 km^2 is a representative area of the Baltic Proper (Rolff et al. 2007), we calculated an annual input of fixed nitrogen to new production in the order of 56 000 to 125 000 t (Table 3). This estimate is within the range of some of the earlier studies (Rinne et al. 1978, Brattberg 1980, Niemistö et al. 1989, Hübel & Hübel 1995, Rahm et al. 2000), but significantly lower than most recent studies (Larsson et al. 2001, Wasmund et al. 2001, 2005, Rolff et al. 2007). In a recent study, Rolff et al. (2007) showed that the abundance of *Aphanizomenon* (one of the major contributors to N_2 fixation) was higher and lasted longer through the summer at all stations south of Stn BY31 in the Baltic Sea. Therefore, annual integrated rates of N_2 fixation extrapolated from regions south of BY31 might generate higher estimates than ours. Indeed, the more recent estimates of N_2 fixation (Larsson et al. 2001, Wasmund et al. 2001, 2005, Rolff et al. 2007) were all generated from locations south and east of Stn BY31 (Table 3). This observation emphasizes the need for a sampling strategy with high spatial diversity and high temporal frequency in biogeochemical studies of the Baltic Sea (Ohlendieck et al. 2007, Rolff et al. 2007). Our estimates of N_s fixation are a close third in rank behind riverine inputs (363 000 t N, Stålnacke et al. 1999) and atmospheric depositions (185 000 t N, HEL-

Table 3. Estimates of total N_2 fixation (10^3 t N yr^{-1}) in the Baltic Proper

Source	N_2 fixation
Rinne et al. (1978)	100
Brattberg (1980)	130
Leppänen et al. (1988)	167
Niemistö et al. (1989)	100[a]
Hübel & Hübel (1995)	20–190
Rahm et al. (2000)	30–260
Larsson et al. (2001)	180–430
Wasmund et al. (2001)	370
Wasmund et al. (2005)	434–792
Rolff et al. (2007)	310
This study (BY31)	56–125[b]

[a]Assuming a 3 mo growth period
[b]Assuming Baltic Sea Proper area = 244 000 km^2 (Rolff et al. 2007)

COM 1997) among the main sources of new N to offshore waters of the Baltic Sea.

The annual input of N_2 fixation to the Himmerfjärden Bay area alone (179 km^2; Stns H4 and X1) was very variable and the estimates should be regarded with caution. Therefore, we are only considering here the 3 yr annual averages of 65 t N at Stn H4 and 89 t N at Stn X1 (calculated from Table 2). The estimated annual discharge of N from the sewage treatment plant in the Himmerfjärden bay area (Fig. 1) was on average 130 t N during the period of our study (Elmgren & Larsson 2001). This is in sharp contrast to previous years (1976–1994) when the annual load ranged between 500 and 900 t N (Elmgren & Larsson 2001). Due to legislation, the sewage treatment plant today removes 90% of the annual load of total N. Prior to 1998, the sewage treatment plant was a significant point source contributing on average 40% of the annual input of total N to the bay area. Our estimates suggest that during the study period the reduction in N discharged from the sewage treatment plant was not compensated for by an increase in new N via cyanobacterial N_2 fixation. After agricultural run-off and sewage discharge, N_2 fixation was still only the third largest source of new N to the Himmerfjärden bay area. However, the large annual variability in the measured rates of N_2 fixation in the bay area demonstrates the need to investigate the multitude of factors determining cyanobacterial growth and N_2 fixation. This effort should be considered before costly nutrient waste management efforts are implemented.

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