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No sign of denitrification in a Baltic Sea cyanobacterial bloom

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ABSTRACT: Denitrification in sediments is of major importance to the Baltic Sea nitrogen (N) budget. However, little is known about denitrification in the water column in the Baltic. We tested whether denitrification could be active in pelagic cyanobacterial aggregates, commonly found in the Baltic Sea during warm summer months. In these aggregates, anoxic microniches may form. Such microzones suggest a possibility for anaerobic processes, such as denitrification by the heterotrophic bacteria associated with the cyanobacteria. Denitrification and nitrogen (N_2) fixation in a cyanobacterial bloom were measured on a 3 wk cruise in the Baltic Sea in order to determine whether the Baltic Sea cyanobacterial blooms act as sources or sinks of N. Experimental conditions analogous to formation of anoxic microniches within cyanobacterial aggregates did not activate the denitrification process, even when anoxic conditions prevailed for several hours. Only in 3 cases was denitrification, measured using the ^{15}N-isotope pairing method, detectable, giving rates of 0.8 to 1.8 nmol N_2 l^{-1} h^{-1}. Nitrogen fixation, determined using the acetylene reduction assay, varied from 0.03 to 1.85 µmol N_2 l^{-1} h^{-1}. According to this study, the blooms of N_2-fixing cyanobacteria in the Baltic Sea must be seen solely as sources, not sinks for N in the marine pelagic environment.

KEY WORDS: Denitrification · Cyanobacteria · Nitrogen fixation · Baltic Sea

INTRODUCTION

Cyanobacterial blooms are a common phenomenon in the Baltic Sea during warm summer months. The dominating bloom-forming species are the N_2-fixing *Aphanizomenon flos-aquae* and *Nodularia spumigena*. Nitrogen fixation of the cyanobacteria blooms in the Baltic has been studied intensively (Lindahl et al. 1980, Lindahl & Wallström 1985, Moisander et al. 1996, Ohlendieck et al. 2000, Wasmund et al. 2001), while few attempts have been made to follow and quantify the flux of the fixed N. Heiskanen & Kononen (1994) found no evidence of sedimentation of the gas vacuolated cyanobacterial cells. They concluded that most of the cyanobacteria were decomposed within the surface layer, therefore releasing the fixed N in surface waters. In the central Baltic Sea, 5 to 10% of the newly fixed N during active growth of the filamentous cyanobacteria was incorporated into the picoplanktonic size fraction via exudates (Ohlendieck et al. 2000). The main N release mechanism was, however, lysis of the cyanobacterial cells as the bloom decayed (Ohlendieck et al. 2000). Grazing on Baltic Sea cyanobacteria has been shown to be of minor importance (e.g. Sellner et al. 1994) and therefore, direct transfer of fixed N to higher trophic levels is inefficient. In contrast, previous studies have indicated that cyanobacterial blooms support an active microbial community over the whole lifetime of the blooms (Bursa 1968, Hoppe 1981, Heinänen et al. 1995, Worm & Søndergaard 1998). These microbial interactions with cyanobacteria are not nec-

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essarily signs of decay, since cyanobacteria may provide heterotrophic bacteria a source of labile substrates, while the heterotrophic bacteria provide cyanobacteria a source of remineralised nutrients.

Different types of aggregates in various marine environments have been shown to harbour microenvironments with steep gas and chemical gradients (Paerl 1985, Paerl & Prufert 1987, Shanks & Reeder 1993, Ploug et al. 1997). In 1999, we measured respiration (Winkler titration method) in spherical *Nodularia spumigena*-aggregates (2 to 4 mm in diameter) in the Gulf of Finland and found respiration rates high enough to create anoxia in the centre of the aggregates during darkness (Hietanen et al. unpubl. data, cf. Ploug et al. 1997). In addition, anoxia inside cyanobacterial aggregates, prevailing up to 12 h, has been directly measured (using microelectrodes) in aggregates of 2 to 5 mm of diameter in a Baltic Sea bloom (H. Ploug pers. comm.). Such microzones may facilitate localised specialisation of facultatively anaerobic bacteria in anaerobic biogeochemical processes, such as denitrification. In 1999, the presence of bacterial (*nirS/K*) sequences encoding nitrite reductase, verifying presence of bacteria potentially capable for denitrification, was confirmed both from single aggregates and from cyanobacterial surface scum (J. M. Tuomainen unpubl. data).

The need for substrates formed in oxic conditions and the O_2 sensitivity of denitrification enzymes limits the occurrence of denitrification to milieus with steep O_2 gradients, such as sediment-water or oxic-anoxic water layer interfaces or, as mentioned above, inside aggregates. Cyanobacteria capable of fixing N_2 regularly aggregate during intensive blooms. Nitrogen fixation and productivity in filamentous cyanobacteria appear to benefit from low turbulence conditions, which also enhance aggregation (Paerl 1985, Moisander et al. 2002). Aggregates, like sediments, provide both surfaces to colonise and a concentrated source of organic matter to metabolise. Microbial remineralisation of organic matter results in O_2-poor microzones around and within particles. Anoxic patches remain even in well-oxygenated surroundings, such as productive surface scums of cyanobacteria, because the physical structure (e.g. mucus) provided by the aggregate slows gas diffusion in and out of the microzone (Shanks & Reeder 1993). Paerl (1985) and Ploug et al. (1997) also point out the importance of low environmental turbulence in order to establish anoxic conditions inside an aggregate.

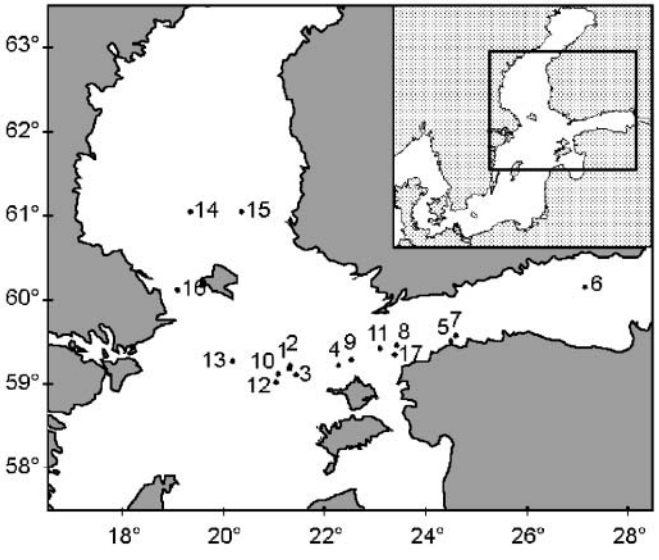

Fig. 1. Sampling stations in the northern Baltic Sea. See Table 1 for further details

From July to August 2000, we followed the N transformation processes in a cyanobacterial bloom in the northern Baltic Sea. The aim of the study was to quantify both N_2 fixation and the possible removal of N from the pelagic ecosystem by denitrification in order to calculate a N budget for the cyanobacterial blooms in the Baltic Sea in terms of inputs and losses of N. The ^{15}N-isotope pairing method (Nielsen 1992) has proven to be an excellent tool in measuring denitrification in sediments (e.g. Lohse et al. 1996, Tuominen et al. 1998). Here, we tested whether it could also be used in measuring potential denitrification in a cyanobacterial bloom. We created incubation conditions in which the O_2 concentrations decreased steadily, and monitored the onset of denitrification by following the emergence of $^{29}N_2$ and $^{30}N_2$. As denitrification sometimes does not proceed to the end (N_2) but ceases after reduction of nitrite (NO_2^-) to nitrous oxide (N_2O) (e.g. Patureau et al. 1994, Frette et al. 1997, Gejlsbjerg et al. 1998), production of N_2O was also measured. In addition to measuring N_2 fixation and denitrification, we also measured bacterial numbers and production to verify that the cyanobacterial bloom indeed supported a high level of heterotrophic microbial activity.

MATERIALS AND METHODS

Sample preparation. Samples were collected from July to August 2000 onboard the RV 'Aranda' (Finnish Institute of Marine Research) in the northern Baltic Sea area (Fig. 1, Table 1). Wind kept the upper surface layer well mixed to a depth of approximately 20 m; therefore, no visible surface accumulations (cyanobacterial scums) were detected in the study area during the study period despite high chlorophyll *a* (chl *a*) values (up to 9 µg l^{-1}). To simulate a heavy surface bloom, a zooplankton net (100 µm mesh size) was used to collect cyanobacteria from the mixed layer. The resulting

concentrated phytoplankton suspension was diluted with surface water (1:1) to better reflect the natural cyanobacterial concentration in a surface scum. This 'artificial bloom sample' was then used in the measurements. For chl *a* measurement, triplicate subsamples of 20 to 50 ml of concentrate were filtered on GF/F filters. Chl *a* was extracted in 94% ethanol for 24 h in room temperature in the dark and then measured fluorometrically (excitation at 580–680 nm, emission at 672 nm), 672 nm (Perkin Elmer LS-2) (HELCOM 1988). Averages of the upper mixed layer temperature and salinity were calculated from CTD-casts. Samples for nutrient (PO_4^{3-}, NO_3^-, NO_2^-, NH_4^+) measurements were taken from 1 m depth and analysed with a Lachat QC8000 autoanalyser using methods based on Grasshoff et al. (1999). NH_4^+ was analysed manually according to Koroleff (1983).

Denitrification. The isotope pairing method is based on adding ^{15}N-isotope in a form of $^{15}NO_3^-$ and following the emergence of N_2 molecules labelled partly ($^{14}N^{15}N$) or completely ($^{15}N^{15}N$) with the added heavy isotope. Calculating the actual denitrification occurring in nature is based on statistics of the relative abundance of the different fractions (Nielsen 1992).

Glass vials (23 ml) equipped with diagonally cut conical glass stoppers were used, enabling incubations in gas-tight, bubble-free conditions. The experiments were performed using 2 replicates for each 3 time points (oxic, hypoxic and anoxic conditions, see below). $K^{15}NO_3$ solution (99 atom%, Europa Scientific) was added to the vials (100 µM final incubation concentration) that were immediately closed and covered in aluminium foil. During incubation at *in situ* temperature, the samples were slowly rotated in a plankton wheel in order to keep the phytoplankton suspension well mixed (Ploug & Grossart 1999). The time points at which incubations were terminated were decided based on the respiration measurements done from the parallel samples (see below). The first 2 replicate vials were removed from incubation usually after 2 to 3 h when the O_2 concentration was still high (~100 µM O_2). The next 2 replicate vials were removed when the conditions in vials were hypoxic (~40 µM O_2) and the last 2 were incubated until anoxic conditions had prevailed for several hours (Joye & Paerl 1993, Frette et al. 1997). This design allowed us to follow the onset of denitrification activity as a function of O_2 availability. The activity in samples and in 1 blank at the beginning of each incubation was

Table 1. Temperature, salinity and nutrients in the upper mixed layer at the sampling stations. Chlorophyll *a* (chl *a*), bacterial carbon production (BCP) and nitrogen fixation of the phytoplankton concentrate used in the experiments. Nitrogen fixation is expressed as µmol N_2 m^{-2} h^{-1}, calculated using the conversion factor 4:1 for moles of ethylene to moles of N_2 and phytoplankton concentration factor (see text for details). The samples processed to further detail are shown in bold (see 'Results' for details). nd = not determined

Date	Station index	Map code	Temp. (°C)	Salinity	NO_3^- (nM)	NO_2^- (nM)	NH_4^+ (nM)	PO_4^{3-} (nM)	DIN:DIP (molar)	Chl *a* (µg l^{-1})	BCP (µg l^{-1} h^{-1})	N_2 fixation (µmol m^{-2} h^{-1})
20 Jul	**321**	**1**	**13.3**	**6.45**	**48**	**30**	**162**	**46**	**5.2**	**466**	**nd**	**13.95**
21 Jul	322	1	13.7	6.61	34	120	172	12	27.2	610	0.31	15.42
	324	2	13.5	6.46	0	60	192	92	2.7	2303	1.61	24.58
22 Jul	325	1	13.7	6.68	68	42	220	70	4.7	578	0.17	14.73
23 Jul	326	3	14.6	6.80	10	72	118	44	4.5	525	1.26	19.55
24 Jul	328	3	14.5	6.81	84	80	446	80	7.6	716	1.41	nd
	329	3	15.1	6.83	22	68	170	54	4.8	580	0.47	35.67
26 July	330	3	15.0	6.80	24	72	158	36	7.1	686	0.87	11.99
27 Jul	**331**	**3**	**14.6**	**6.80**	**106**	**6**	**124**	**98**	**2.4**	**657**	**0.99**	**8.93**
	332	4	15.4	6.54	97	nd	157	73	nd	824	1.21	nd
7 Aug	333	5	16.1	4.60	98	20	248	13	28.2	274	0.36	2.36
8 Aug	334	6	17.5	4.15	113	53	297	13	35.6	277	0.39	3.81
9 Aug	336	7	15.0	5.21	230	65	380	63	10.7	237	0.43	2.24
	337	8	15.2	6.03	448	112	1934	248	10.1	143	0.23	0.30
10 Aug	338	9	16.1	5.65	88	45	175	20	15.4	363	0.94	4.23
	339	10	16.0	6.70	70	nd	nd	nd	nd	305	0.73	5.94
11 Aug	**340**	**11**	**16.1**	**5.87**	**115**	**48**	**113**	**33**	**8.4**	**197**	**0.30**	**2.05**
12 Aug	341	12	15.7	6.69	18	40	140	36	5.5	226	0.56	2.73
	342	13	14.8	6.27	210	nd	nd	nd	nd	325	1.26	4.77
13 Aug	343	14	12.9	5.56	34	74	72	18	10.0	262	0.21	2.26
	344	15	14.0	5.52	240	nd	nd	nd	nd	196	nd	nd
16 Aug	347	16	14.6	4.79	92	24	520	30	21.2	374	0.17	0.98
17 Aug	348	17	16.6	5.64	110	nd	nd	nd	nd	302	0.70	4.10

terminated by carefully adding 2 ml of $ZnCl_2$ solution (500 µg ml^{-1}) under the surface, avoiding release of gas from the samples. The samples were then transferred to 10 ml gas-tight Exetainers (Labco) containing 500 µl of $ZnCl_2$ solution. The mass ratios of N_2 ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) formed during incubations and those of the original phytoplankton suspension (for background subtraction) were later analysed using a mass spectrometer by the National Environmental Research Institute in Silkeborg, Denmark. The significance of the changes in mass ratios was evaluated using a *t*-test. Denitrification was only considered to occur in samples in which both ^{15}N-labelled fractions ($^{29}N_2$ and $^{30}N_2$) increased significantly ($p < 0.05$). Denitrification (as nmol N_2 formed) was calculated from the differences in isotopic compositions at the end and the beginning of the incubation. The denitrification rate per h (nmol N_2 l^{-1} h^{-1}), however, was calculated using only the incubation time when anoxic conditions prevailed, as the denitrifying bacteria are facultative anaerobes that switch to denitrification as a form of anaerobic metabolisms when O_2 concentration decreases (Tiedje 1988).

N_2O production measurements. Samples (no replicates) were incubated following the same incubation design as in the denitrification measurements, using 3 time points (sampling at times when oxic, hypoxic and anoxic conditions prevailed). To measure both natural N_2O production and N_2O production potential, 2 sample sets were prepared. The 8 ampoules (23 ml) were filled with sample suspension and 4 of the ampoules were then enriched with KNO_3 solution to the final concentration of 100 µM while the remaining 4 remained at natural nutrient concentrations. Background subtraction blanks (1 with and 1 without KNO_3 addition) were prepared by adding 250 µl formalin before sealing all the ampoules using gas-tight rubber septums and aluminium seal rings (ScherfCroma). During incubation at *in situ* temperature, the samples were slowly rotated in a plankton wheel. Incubations were terminated at the same time points as the denitrification measurements by injecting 250 µl formalin through the septum with an overflow needle inserted. Samples were later analysed for N_2O at 35°C using a Hewlett Packard 5890 series II gas chromatograph equipped with an HP 3396 A integrator, electron capture detector and a Porapak Q column, with an argon-methane (95:5%) mixture as a carrier gas. The concentration of N_2O in the samples was calculated using the gas equation ($P \times V = n \times R \times T$, where P is pressure, V is volume, n is the number of moles of gas, R is the ideal gas constant, and T is temperature) and the Ostwald gas absorption coefficient (Weiss & Price 1980).

Bacterial production and cell numbers. The ^3H-thymidine incorporation method (Fuhrman & Azam 1980) was used to measure the heterotrophic bacterial production of the artificial bloom sample. Samples of the concentrate (5 ml; 3 replicates, 1 formalin-killed blank) were incubated at *in situ* temperatures in the dark using the tested saturating concentration (ca. 100 nM, data not shown) of ^3H-thymidine (Amersham TRK-637) and incubation time of 20 to 60 min (tested to be linear, data not shown). Incubations were terminated with formalin and samples extracted on cellulose-nitrate filters (∅ 25 mm, pore size 0.2 µm, Sartorius) (Riemann 1984, Børsheim 1990). Filters were dissolved in scintillation cocktail (Insta-Gel Plus, Packard), shaken vigorously and the radioactivity was counted in a scintillation counter (Wallac Rackbeta 1217). The ratio between incorporated thymidine and cell production varies geographically and among growth conditions. Therefore, a separate experiment was carried out in which the conversion factor for the local microbial community was determined according to Bjørnsen & Kuparinen (1991) from the slope of regression between cumulative incorporated thymidine and cell number in a batch culture. Bacterial carbon production was calculated using the conversion factor determined (3.5×10^{17} cells per mol thymidine incorporated, $r^2 = 0.90$), an average cell volume for the study area and season (0.077 $µm^3$, Tuomi 1997) and C content measured using X-ray microanalysis for the study area (0.10 pg C $µm^{-3}$, Fagerbakke et al. 1996).

Formalin-fixed heterotrophic bacteria in the concentrate were counted using DAPI staining and epifluorescence microscopy (Porter & Feig 1980). Samples were treated with 10 nM pyrophosphate (final concentration) and sonicated in an ultrasonic bath (Bransonic 3200, Branson Ultrasonics) for 20 min in order to detach the attached bacteria from cyanobacterial filaments (Velji & Albright 1986). Samples were then diluted 125- to 250-fold, stained at 5 µg ml^{-1} DAPI final concentration (Schallenberg et al. 1989) and filtered on black polycarbonate filters (∅ 25 mm, pore size 0.2 µm, Poretics). Cells were counted using a Leitz Aristoplan epifluorescence microscope equipped with a UV filter.

Community respiration. Decline of O_2 concentration in the artificial bloom sample during the incubation was measured using the Winkler titration method (Winkler 1888). Four replicate samples were used for the start point concentration measurement and 4 replicates were incubated at *in situ* temperature in the dark for the measurement of the community respiration. Samples in volume calibrated, approximately 11 ml vials were slowly rotated in a plankton wheel during the incubation (90 to 150 min). The linearity of O_2 decrease was tested twice during the cruise and the respiration was found to be linear for at least 6 h, down to 80 µM O_2 (data not shown). Results from respiration measurements were used to adjust the incubation times for denitrification and N_2O production samples (see above).

Nitrogen fixation. Nitrogen fixation was measured using the acetylene reduction assay (Burris 1972). Quadruplicate 90 ml 'artificial bloom' samples were incubated under 9 ml acetylene in 117 ml serum vials sealed with red rubber stoppers. Triplicate blanks (deionised water) were included for each measurement. Incubation was carried out under slow rotating motion under a constant low light level (approximately 50 µmol m^{-2} s^{-1}) at *in situ* temperature. At the end of the incubation, air phase from each serum vial was sampled into Vacutainer (Beckton Dickinson) tubes. Ethylene measurements on the Vacutainer contents were made at 200°C using a Shimadzu GC-9A gas chromatograph equipped with a flame ionisation detector and at 80°C using a Porapak T stainless steel column with N_2 as a carrier gas. Moles of ethylene produced were divided by 4 to convert to moles of N_2 fixed (Schwintzer & Tjepkema 1994). To compare the N_2 fixation rates with earlier measurements from the Baltic Sea, the rates per l were converted to rates per m^2 of unconcentrated surface water. The conversion was made based on the net opening, tow depth, number of tows and upper mixed layer depths estimated from CTD casts made at each sampling site. Relatedness of N_2 fixation (values per l) with chl *a* and DIN:DIP ratio was examined by using a Pearson correlation analysis (SPSS for Windows 9.0). Data were $\log_{10}$ (1 + value) transformed before analyses to satisfy the normality assumption.

RESULTS

Denitrification and N_2O production

In the 23 samples studied, both increase and decrease of the heavier isotopes were detected. In some samples, increase in one fraction and decrease in the other were measured, showing the limits of the method in such a low activity environment. Only in 3 cases could a significant increase (*t*-test, $p < 0.05$) be detected at the same time in both isotopic fractions

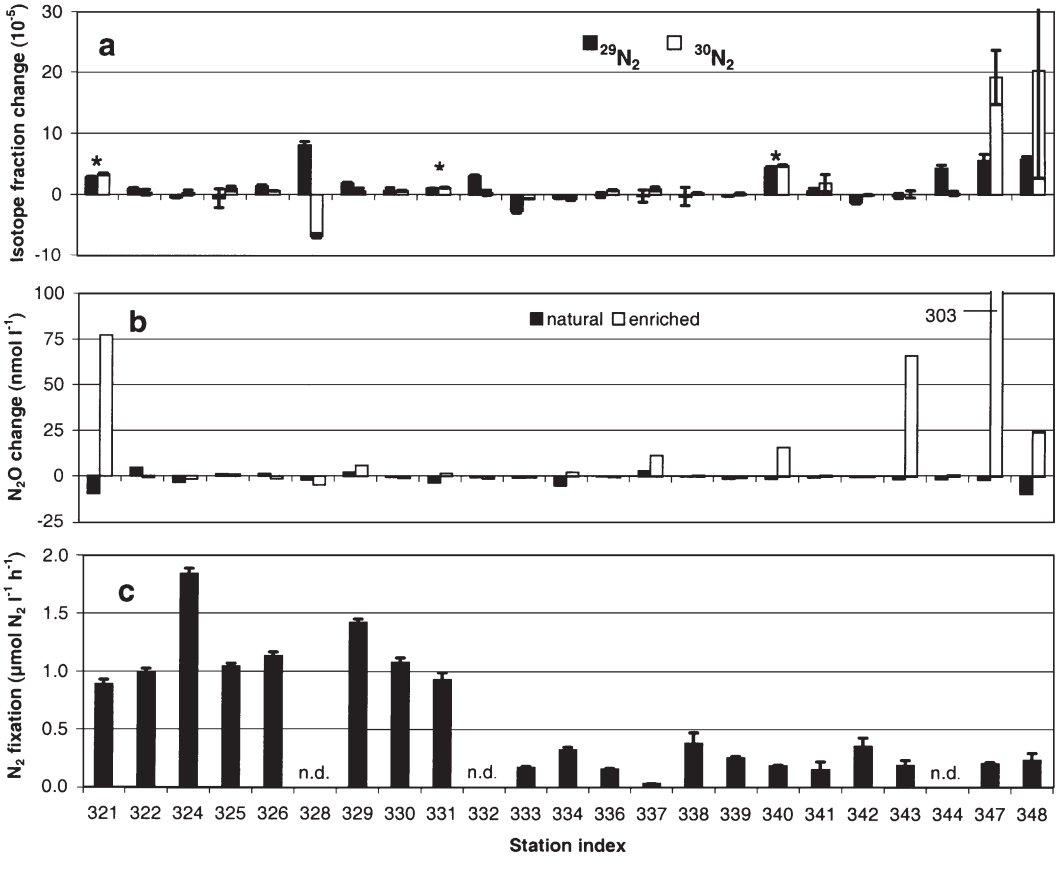

Fig. 2. (a) Changes in the relative abundance of heavier isotopes of the total N_2 in the samples. Significant increases compared to the initial situation (*t*-test, $p < 0.05$) are marked with an asterisk. Error bars show range of values (n = 2). (b) Changes in N_2O concentration (nmol l^{-1}) compared to the initial situation. Enriched samples, incubation concentration 100 µM NO_3^-; natural samples, incubation concentration 0.1 µM NO_3^-. (c) N_2 fixation (µmol N_2 l^{-1} h^{-1}). Error bars show standard deviation (n = 3)

(Fig. 2a) and the results of these 3 samples were calculated further. On the 3 occasions out of 23 (Indices 321, 331, 340) when calculating denitrification was meaningful, the rate based on the added $^{15}NO_3^-$ varied between 1.3 and 3.9 nmol N_2 l^{-1} h^{-1}, whereas the rate based on naturally occurring $^{14}NO_3^-$ varied between 0.8 and 1.8 nmol N_2 l^{-1} h^{-1} (Fig. 3).

In addition to formation of N_2 as the end product of the denitrification reaction series, the changes in N_2O concentration, a side product of nitrification and an intermediate product of denitrification, were measured. The amount of N_2O slightly decreased during incubation in most cases, but a clear increase was noticed in 6 out of 23 measurements when the sample was enriched with NO_3^- (Fig. 2b). The N_2O production rates varied between –0.55 and 0.39 nmol l^{-1} h^{-1} in the natural samples and between –0.41 and 12.48 nmol l^{-1} h^{-1} in the enriched samples. In the 3 above-mentioned samples the natural N_2O production rate varied from –0.55 to –0.05 nmol l^{-1} h^{-1} and in the enriched samples from 0.13 to 4.40 nmol l^{-1} h^{-1}.

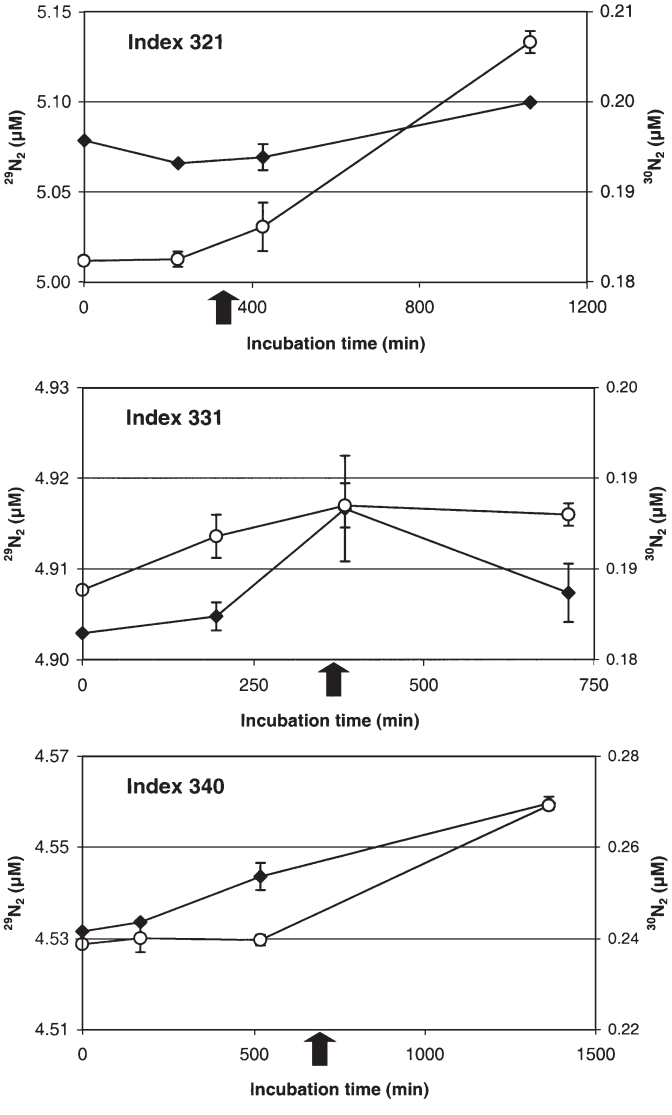

Fig. 3. The average $^{29}N_2$ and $^{30}N_2$ (µmol l^{-1}) of the 2 replicate measurements in the samples showing significant change (*t*-test, $p < 0.05$) during incubations. (◆) $^{29}N_2$; (○) $^{30}N_2$. Error bars show range of values (n = 2). The arrows show beginning of anoxia

Bacterial production and cell numbers

Thymidine incorporation rates varied from 61 to 597 pmol l^{-1} h^{-1} resulting in heterotrophic C production of 0.17 to 1.61 µg C l^{-1} h^{-1} (Table 1). Cell numbers varied from 5.4×10^7 to 3.6×10^8 ml^{-1}.

Nitrogen fixation and phytoplankton

Nitrogen fixation varied from 0.03 to 1.84 µmol N_2 l^{-1} h^{-1} in the phytoplankton concentrate (Fig. 2). The coefficient of variation (CV%) for the measurements from replicate flasks was from 2.2 to 44%, but remained <10% most of the time. Nitrogen fixation rates were the highest (from 0.89 to 1.84 µmol N_2 l^{-1} h^{-1}) during the first part of the study period (until July 27) and were reduced to a lower level (from 0.03 to 0.36 µmol N_2 l^{-1} h^{-1}) during the second half. Nitrogen fixation had a strong positive relationship with chl *a* ($r = 0.908$, $p = 0.000$; Pearson correlation) and a negative relationship with the ratio of dissolved inorganic nutrients (DIN:DIP, Table 1) ($r = -0.524$, $p = 0.031$; Pearson correlation). N_2 fixation rates per m^2 varied from 0.3 to 35.7 µmol N_2 m^{-2} h^{-1} (Table 1). Microscopical analyses (data not shown) showed that the dominant phytoplankton in the samples were the N_2-fixing cyanobacteria *Aphanizomenon* sp. and *Nodularia* sp. (present in lower densities than *Aphanizomenon*). *Anabaena* spp. were present only in a few samples. The densities of all of these cyanobacteria decreased towards the end of the study period. Other phytoplankton in the samples formed minor proportions of the total biomass.

DISCUSSION

Denitrification and N_2O production

In the experiments, it was necessary to use net-concentrated cyanobacterial biomass instead of natural aggregates as the sampling period was windy and therefore no aggregates formed despite high cyanobacterial biomass. The chl *a* content of the concentrate ranged from 143 to 2300 µg l^{-1} (Table 1), which is sev-

eral orders of magnitude higher than naturally occurring concentrations during cyanobacterial blooms in the Baltic. Although even up to 18 µg l^{-1} chl *a* concentrations have been reported (Heinänen et al. 1995), a more usual value is between 2 and 7 µg l^{-1} (Heinänen & Kuparinen 1992, Heinänen et al. 1995, Wasmund et al. 2001). Usually the reported values reflect the integrated chl *a* values in the whole mixed surface layer. Here, we wanted to simulate the conditions in the uppermost surface layer, a cyanobacterial surface 'scum' in calm conditions; more specifically, the conditions experienced by attached bacteria inside cyanobacterial aggregates. The zooplankton community in the concentrated sample was likely to be different from a natural community because the 100 µm mesh selectively collected large zooplankton, allowing smaller grazers to pass through. Herbivore-enhanced nutrient and C release along with reduced grazing pressure on heterotrophic bacteria most likely favoured the growth of bacteria in the concentrated samples. However, for purposes of measuring activities of cyanobacteria (N_2 fixation) and attached bacteria (N transformations, bacterial production), the concentrate gave a reasonable simulation of a cyanobacterial aggregate. Dissolved O_2 decreased linearly to at least 80 µM over a period of time ranging from 5 to 14 h within different experiments. This allowed us to monitor the onset of anaerobic processes in a system analogous to natural formation of anoxic microniches.

In environments with rapidly changing O_2 conditions, such as intertidal microbial mats and wastewater treatment plants, denitrification has been shown to begin within hours of the system becoming anoxic (Joye & Paerl 1993, Frette et al. 1997). However, even though we incubated our samples on average for 10 h after reaching anaerobic conditions, we did not find any sign of denitrification, with the exception of extremely low rates in 3 samples out of the 23 studied. In the 3 cases where denitrification could be calculated, the rate was approximately 1.3 nmol N_2 l^{-1} h^{-1}. Denitrification at the oxic-anoxic interface of the Baltic Sea deep waters has been estimated to be within the same range (2.1 nmol N_2 l^{-1} h^{-1}: Rönner & Sörensson 1985; 0.9 nmol N_2 l^{-1} h^{-1}: Brettar & Rheinheimer 1992), whereas the activity in sediments in the area varies from 3 to 13.5 µmol N_2 m^{-2} h^{-1} (Tuominen et al. 1998). We did not observe any increase in the N_2O concentration, which could have indicated incomplete denitrification (except in 6 enriched samples). Comparison of the measured denitrification rates with the simultaneously measured N_2 fixation rates, 0.03 to 1.84 µmol N_2 l^{-1} h^{-1}, demonstrates that even with unnaturally long anoxic conditions prevailing for the denitrification, cyanobacterial blooms acted as a source, not a sink for N in the aquatic ecosystem.

Denitrification is controlled by several factors, the most important of which are the availability of NO_3^- and organic C, and the absence of O_2. Many studies have confirmed that the substrate (NO_3 and C) availability plays a more important role than the O_2 status. It has also been suggested that denitrification in natural marine environments such as in sediments and oxic-anoxic interfaces of deep waters may be limited by C availability (Tiedje et al. 1982, Rönner & Sörensson 1985, Brettar & Rheinheimer 1992, Joye & Paerl 1993). In our samples, NO_3^- limitation can be ruled out because we added NO_3^- at the level of 100 µM (natural NO_3^- concentrations were around 0.1 µM). The possible denitrifiers were also not likely to be limited by C availability, because the fresh and decaying phytoplankton provided a rich source of dissolved organic compounds. The aggregate-attached bacteria were shown to harbour genetic potential for denitrification (J. M. Tuomainen unpubl. data) and the slow emergence of activity in our samples during extended anoxia shows that the bacteria were capable of switching to denitrification as a form of anaerobic metabolism. However, even though optimal conditions prevailed in the incubations, denitrification could only be detected in 3 samples at very low rates after prolonged anoxia, a condition not likely to occur in aggregates in nature (Ploug et al. 1997). Presumably the energy demanding synthesis of denitrification enzymes is not induced in aggregate-attached bacteria in nature because the frequency and magnitude of favourable conditions (anoxia only during the night, in limited microniches) is low compared to the occurrence of oxic conditions.

Bacterial production and cell numbers

Active metabolism of the aggregate-colonising bacteria is a prerequisite both for formation of anoxic microniches in natural aggregates, and for denitrification. The net-haul sampling, using a 100 µm mesh, was not likely to enrich the free-living bacteria in the samples and it is logical to assume that the high number of bacteria in the samples is a result of them being attached to the collected cyanobacteria. The measured bacterial activity, consequently, relates mainly to the attached instead of the free-living community. According to several studies, the attached bacteria, on a cellular basis, are metabolically more active than the free-living bacteria (Alldredge & Gotschalk 1990, Smith et al. 1992, Grossart et al. 1998, Worm & Søndergaard 1998). However, some results suggest that bacteria attached to marine snow do not grow any faster than the free-living bacteria (Alldredge et al. 1986, Simon et al. 1990, Worm et al. 2001). In our sam-

ples, both the thymidine incorporation and the cell numbers were 10 to 20 times higher than measured for the free-living bacterioplankton in the same area (Heinänen 1991, Heinänen & Kuparinen 1991, Tuomi 1997). Hence, although the filaments were heavily colonised by bacteria, on the cellular basis, the activity of the attached bacteria did not differ from the free-living bacterioplankton activity.

The thymidine incorporation was positively correlated with chl *a* concentration, which might be a sign of the cyanobacteria dominating the incorporation. However, no thymidine incorporation was detected in axenic cultures of *Nodularia* spp. and *Aphanizomenon* spp., the dominating species in our samples (Lehtimäki et al. 1997, Hietanen et al. 2002). The correlation more likely indicated close coupling between autotrophic and heterotrophic production in the samples. In ageing aggregates, senescent algae might not alone be able to provide a sufficient amount of degradable material for the maintenance of high standing stock of attached bacteria. Therefore, the role of 'recycling' of organic matter from microzooplankton (as excretion, dead cells, fecal pellets, sloppy feeding) to bacteria is emphasised as the aggregates get older (Hoppe 1981, Taylor et al. 1986, Biddanda & Pomeroy 1988). Cyanobacterial aggregates can stay in the upper water layer for weeks because of their buoyancy and reach a steady state in which in- and output of organic matter is balanced (Hoppe 1981).

Nitrogen fixation

The goal of the N_2 fixation measurements in this study was to compare the changes and relative magnitude in N_2 fixation (N inputs) with N losses through denitrification in the cyanobacterial bloom. The result of these comparisons was clear: the N_2 fixation rates were 3 to 4 orders of magnitude higher than the highest denitrification rates. Temporal comparisons of rates were not possible due to overall low or absent denitrification. The N_2 fixation rates measured were within the range of previously published rates from the Baltic Sea (Lindahl et al. 1980, Lindahl & Wallström 1985, Niemistö et al. 1989, Kononen et al. 1999). Maximum N_2 fixation rates of approximately 5-fold (Niemistö et al. 1989) as well as half the ones measured here (Kononen et al. 1999) have been reported from the Baltic Sea. When comparing these results to previous studies, one has to bear in mind that the conversion factor from ethylene to N_2 (4:1 mol used in this study) can vary in natural conditions (Moisander et al. 1996, Kononen et al. 1999). The values presented here are likely to represent an underestimate of the highest potential N_2 fixation rates in the system. This is because (1) because some of the cyanobacteria pass the 100 µm mesh net used in sample collection; (2) lower light levels were used during the incubations than the cyanobacteria would experience in the natural conditions; and (3) the artificial concentration of cyanobacteria using a plankton net may have negative effects (Leonardson 1983, Moisander et al. 1996).

Nitrogen fixation per volume was strongly coupled with chl *a* concentration, which is expected if N_2-fixing cyanobacteria are dominating the community. This was confirmed with microscopy and therefore, the chl *a* values gave a good estimate of densities of N_2-fixing cyanobacteria. Microscopical analyses showed that densities of N_2-fixers decreased towards the end of the study period, causing the reduction in N_2 fixation. The negative relationship between DIN:DIP and N_2 fixation suggests that the change in nutrient availabilities towards the end of the study period may have led to reduced growth of the N_2-fixers (Niemi 1979).

CONCLUSIONS

These experiments confirmed that the cyanobacterial filaments were colonised by heterotrophic bacteria and that these bacteria were actively growing. Conditions analogous to formation of anoxic microniches within cyanobacterial aggregates did not trigger a switch to denitrification as a form of anaerobic metabolism, even when anoxic conditions prevailed for several hours. Therefore, it is unlikely that denitrification plays any significant role in Baltic Sea N_2-fixing cyanobacterial blooms in which anoxic microzones may be present only fleetingly (Ploug et al. 1997). According to these results, the filamentous N_2-fixing cyanobacteria must be seen solely as a source and not as a sink for N in the Baltic Sea.

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