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Release of fixed N_2 and C as dissolved compounds by *Trichodesmium erythreum* and *Nodularia spumigena* under the influence of high light and high nutrient (P)

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ABSTRACT: Diel variations of N_2 and C fixation rates, as well as subsequent release of total dissolved N (TDN) and dissolved organic carbon (DOC) were determined for *Trichodemsium erythreum* and *Nodularia spumigena*. A circadian rhythm of N_2 and C fixation, as well as a periodicity in the calculated release of dissolved compounds, was observed. From the amounts of N and inorganic C fixed by *T. erythreum* during the light period of the experiment, 71 and 50% were released as TDN and DOC, respectively; for *N. spumigena* we found a release of 89 and 53%, respectively, during the light period. Additionally, 2 controlling factors (light and nutrient concentrations) for the release of TDN and DOC were studied. The data suggest that rapid shifts towards higher light intensity lead to a pronounced exudation of TDN and DOC. On a short-term basis (first 30 min after exposure), the exudation of NH_4^+ and DON consumed up to 52% of electrons harvested by the cells in the same time interval. Thus TDN release serves as a potential electron sink and protects cells from photodestruction. On the other hand, there was no clear effect of phosphorus concentration on the release of TDN and DOC. Our results indicate that uptake and subsequent exudation of TDN and DOC might be induced by abiotic parameters, besides being regulated endogenously by multiple feedback loops.

KEY WORDS: N_2 fixation · C fixation · DON · DOC · DFAA · Diazotroph · Cyanobacteria

INTRODUCTION

In marine waters, N availability generally controls primary production (e.g. Hecky & Kilham 1988) and is an important potential growth-limiting factor with ambient dissolved inorganic N concentrations of <1 µM (Flores & Herrero 2005). In such environments, cyanobacteria that can fix atmospheric dinitrogen (N_2) have a competitive advantage over most other photoautotrophic species. In the tropical and temperate oceans, N_2-fixing cyanobacteria can be extremely abundant and account for a considerable input of combined N into the upper mixed layer (Montoya et al. 2002, Capone et al. 2005), with a strong impact on local community production (e.g. Tseng et al. 2005). Thereby, they transiently dominate primary productivity and N cycling (e.g. Capone et al. 1998). Estimates of global biological N_2 fixation are in the range of 80 to 110 Tg N yr^{-1} (Gruber & Sarmiento 1997). Local N_2 fixation rates for the North Atlantic Ocean are in the range of 259 to 864 mmol N m^{-2} yr^{-1} (Capone et al. 2005) and mainly attributed to the activity of *Trichodesmium* spp. For the Baltic Sea, estimations of N_2 fixation are in the range of 55 to 840 mmol N m^{-2} yr^{-1} (Wasmund et al. 2005). A significant fraction of this recently fixed N can be directly released by cyanobacteria, which dispense up to 80% of the N into the surrounding environment (Bronk et al. 1994, Glibert & Bronk 1994, Ohlendieck et al. 2000, Mulholland et al. 2004). This total dissolved N (TDN) is composed of dissolved inorganic (DIN: NH_4^+, NO_2^-, NO_3^-) and organic nitrogen (DON: e.g. dissolved free amino acids [DFAA]). The latter might even be quantitatively dominating (Capone et al. 1994, Glibert & Bronk 1994, Berman & Bronk 2003). Several expla-

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nations for the active exudation of dissolved organic matter (DOM) can be found in the literature, such as the release of excess photosynthate (Fogg 1983) or DON to supply cells within the colony lacking the enzyme nitrogenase (Capone et al. 1994, Mulholland et al. 2004). Indirectly, N, incorporated into diazotrophic biomass, can be liberated by processes like sloppy feeding and excretion from zooplankton (e.g. Dagg 1974), viral lyses (Fuhrman 1999) and programmed cell death (Madeo et al. 2002, Berman-Frank et al. 2004).

To date, the knowledge about the dynamics of DOM, especially DON production in marine ecosystems, is still limited. It is not clear which factors regulate the exudation of DON in cyanobacteria. Lomas et al. (2000) postulated an internal factor being responsible for the increased release of nitrogenous compounds during periods of imbalanced cellular energy conditions in diatoms and flagellates. This effect occurred to accelerate the dissipation of excitation energy through processes other than photosynthetic C metabolism. On the other hand, nutrient supply and physiological condition may stimulate the exudation of DON as shown in batch culture studies (Watt 1969, Fogg 1983). Consequently, algae that are replete in N and whose cellular N demand is fulfilled in excess tend to release more DON, especially in the exponential growing phase (Myklestad et al. 1989). Moreover, Nagao & Miyazaki (2002) showed that DON release depends on the N source (NO_3^- vs. NH_4^+) and this release is not necessarily derived from recently assimilated N. Additionally, N_2 fixation itself can be limited by iron and phosphorus (Mills et al. 2004). Thus, any enhancement in available limiting nutrients should increase the release of TDN as soon as the cells are more N-replete.

The aim of the present study was to examine possible factors that regulate the release of TDN and dissolved organic carbon (DOC) in N_2-fixing cyanobacteria. Three questions were addressed. (1) Are DON and DOC released during the course of a diel cycle which is unaffected by any stress through high light or nutrient concentrations? (2) Which role do short-term imbalanced cellular energy conditions play? (3) Does the metabolic condition, like phosphorus availability, influence the release of TDN and DOC? Two marine cyanobacteria species were studied: the tropical, non-heterocystic *Trichodesmium erythreum* and the temperate, heterocystic *Nodularia spumigena*.

MATERIALS AND METHODS

Culture condition and survey of a diel cycle. The heterocystic cyanobacterium *Nodularia spumigena* was isolated from the Baltic Sea and maintained at the Leibniz Institute for Baltic Sea Research in batch cultures on F/2 medium free of any combined N compounds. The non-heterocystic *Trichodesmium erythreum*, strain IMS101, was originally isolated from the Atlantic Ocean and was obtained from the IFM-GEOMAR, Kiel, Germany. *T. erythreum* was grown in batch cultures on YBCII medium (Chen et al. 1996) at 30°C under an alternating cycle of 12:12 h light:dark (cool, white fluorescent lighting; normal light [NL]: 100 µmol photons m^{-2} s^{-1}). *N. spumigena* was cultured at 15°C in a walk-in incubation chamber supplied with a light cycle of 16:8 h light:dark (cool, white fluorescent lighting; NL: 60 µmol photons m^{-2} s^{-1}). Cultures of both species were axenic when starting the experiments. Overall bacterial biomass during the course of the long-term experiments (>1 d) never exceeded 1% of cyanobacterial biomass. Cultures were routinely mixed to prevent adhesion of cyanobacteria to the sides of the culture vessels. To initiate the investigation of the diel cycle, duplicate 250 ml polycarbonate incubation bottles containing N-free medium were inoculated with equal volumes of an exponentially growing *T. erythreum* or *N. spumigena* parent culture and the stable isotope tracer ($^{15}N_2$, $NaH^{13}CO_3$) at the same starting point. Trichome number of both species was identical in all setups. To follow the diel cycle of N_2 and C fixation, particulate organic N (PON) and C (POC), chlorophyll *a* (chl *a*), as well as the concentrations of DIN, TDN, DOC and DFAA were measured in duplicates by gently vacuum filtrating over GF/F (Whatman) filters every 2 h for a total of 24 to 26 h. This setup was carried out twice for both species. A summary of experimental conditions is given in Table 1.

Experimental design. ***Light shift and nutrient supply experiments:*** Light shift experiments were initiated by inoculating replicate culture vessels containing N-free medium with equal volumes of an exponentially growing parent culture. The first subset of triplicate vessels was exposed to the light intensity applied during the normal culturing process (NL: 100 and 60 µmol photons m^{-2} s^{-1} for *Trichodesmium erythreum* and *Nodularia spumigena*, respectively), while the second subset of triplicates was simultaneously exposed to high light (HL) intensities (HL: 200 and 120 µmol photons m^{-2} s^{-1} for *T. erythreum* and *N. spumigena*, respectively; see Table 1). Concentrations of DIN, DFAA, TDN, DOC and chl *a* were measured at 30, 60, 120, 180 and 360 min after exposure to the respective light regime. Rates of N_2 and C fixation, as well as PON and POC concentrations, were obtained by using particulate matter collected on pre-combusted glass fiber filters (GF/F, Whatman).

Additionally, a control set was run in *Nodularia spumigena* cultures to test for photochemically driven elevation in the concentration of dissolved compounds.

Table 1. Overview of experimental setup. Detailed information is given in 'Materials and methods'. NL: normal light; HL: high light

Treatment	*Trichodesmium erythreum*			*Nodularia spumigena*		
Diel cycle						
Growth media		YBCII			F/2	
Light:dark (h)		12:12			16:8	
Light intensity (µmol photons m^{-2} s^{-1})		100			60	
PO_4^3 (µM)		10			10	
Time of incubation (h)		24			26	
No. experimental runs		2			2	
No. replicate incubation bottles		2			2	
Light	NL		HL	NL		HL
Growth media		YBCII			F/2	
Light:dark (h)		12:12			16:8	
Light intensity (µmol photons m^{-2} s^{-1})	100		200	60		120
PO_4^3 (µM)		10			10	
Time of incubation (h)		6			6	
No. experimental runs		1			1	
No. replicate incubation bottles		3			3	
Phosphorus (P)	Low P	Mid P	High P	Low P	Mid P	High P
Growth media		YBCII			F/2	
Light:dark (h)		12:12			16:8	
Light intensity (µmol photons m^{-2} s^{-1})		100			60	
PO_4^3 (µM)	1	10	20	1	10	60
Time of incubation (d)		5			5	
No. experimental runs		1			1	
No. replicate incubation bottles		2			2	

Cyanobacteria were filtered through GF/F filters prior to light exposure. Light intensity was measured in the 400 to 700 nm range (photosynthetically available radiation [PAR]) using a spherical quantum sensor (QSL-101, Biospherical Instruments).

The cultures which were used to investigate the influence of the phosphorus concentrations were pre-incubated for 3 d in medium containing very low phosphorus concentrations (<0.1 µM) in order to empty all intracellular P-storages. Subsequently, 3 subsets of triplicates were used to study the effect of low (1 µM) medium (10 µM) and high (20 µM) phosphorus concentrations in 2.3 l polycarbonate incubation bottles. Inoculation of all replicates was started simultaneously by adding the same amount of cyanobacterial parent culture and stable isotope tracer. Subsamples were sacrificed on a daily basis for 5 consecutive days to measure rates of N_2 and C fixation and DIN, DFAA, TDN and DOC concentrations in the growth medium. Daily rate measurements were always made at 16 h.

Nutrient and chl a analysis: Subsamples of the filtrates were taken for the analysis of dissolved nutrients (NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}) and measured colorimetrically in a spectrophotometer U 2000 (Hitachi-Europe) according to Grasshoff et al. (1983) with a precision of approximately ±0.1 µM. Nitrate and nitrite concentrations remained undetectable in the course of all culture experiments. Chl *a* filters were extracted in ethanol prior to fluorometric determination of concentration.

Analysis of DFAA: DFAA were separated via HPLC (Elite LaChrom VWR) using a reversed phase column (5 h, LiChroCart 125-4, MERCK) at a temperature of 55°C. A multi-step gradient elution was used with a flow rate of 1 ml min^{-1}. Solvent A contained 50 mM formic acid and 60 mM acetic acid (pH 2.9); solvent B contained 50 mM formic acid, 60 mM acetic acid and 50% 2-propanol (pH 2.9). Prior to measurement, the amino acids were derivatised with dansyl chloride (Wiedmeier et al. 1982). The quantification of the dansyl derivatives of alanine (Ala), arginine (Arg), asparagine (Asp), glutamine (Glu), glycine (Gly), leucine (Leu), lysine (Lys), proline (Prol), serine (Ser), taurine (Tau) and valine (Val) were performed by fluorescence detection (excitation 320 nm, emission 490 nm).

TDN, DOC and DON: TDN and DOC concentrations were determined simultaneously in the filtrate by high temperature catalytic oxidation with a Shimadzu TOC-VCPN analyser. In the auto–sampler, 6 ml of sample volume (in pre-combusted vials) was acidified with 0.12 ml HCl (2 M) and sparged with O_2 (100 ml min^{-1}) for 5 min to remove inorganic C. A sample volume of 50 µl was injected directly on the catalyst (heated to 680°C). Detection of the generated CO_2 was performed with an infrared detector. Final DOC concentrations were average values of triplicate measurements. If the

standard variation or the coefficient of variation exceeded 0.1 µM or 1%, respectively, up to 2 additional analyses were performed and outliers were eliminated. Total N was quantified by a chemiluminescence detector (gas flow O_2: 0.6 l min^{-1}). After every 5 samples, 1 blank and 1 standard was measured for quality control. The concentration of DON was obtained indirectly by subtracting the measured values of TDN and DIN.

Isotopic analysis and rates measurements: Stable N and C isotope ratios (δ^{15}N-PON, δ^{13}C-POC) as well as PON and POC concentrations were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020°C in a Thermo Finnigan Delta S mass-spectrometer. Filters containing particle samples were trimmed, sectioned and then loaded into tin capsules and pelletised for isotopic analysis. The stable N and C isotope ratios measured for each sample were corrected against the values obtained from standards with defined N and C element and isotopic compositions (International Atomic Energy Agency [IAEA]: IAEA-N1, IAEA-N2, NBS 22 and IAEA-CH-6) by mass balance. Values are reported relative to atmospheric N_2 (δ^{15}N) and Vienna PeeDee Belemnite (VPDB; δ^{13}C). The analytical precision for both stable isotope ratios was ±0.2‰. The calibration material for C and N analysis was acetanilide (Merck). N_2 fixation activity was measured using the ^{15}N-N_2 assay, C fixation using ^{13}C-$NaHCO_3$. Tracer incubations were terminated by gentle vacuum filtration through pre-combusted GF/F filters; these filters were dried at 60°C and stored for isotopic analysis. Rates were calculated using the approach of Montoya et al. (1996). To compare these results to data from the literature and to relate them to biomass, rates were chl *a*-normalized. Atom percentage excess enrichment in the DON and DIN pool was not measured. Since the cultures used were axenic at the start of the experiments, release and uptake of compounds were computed by determining the difference in concentration (c) of NH_4^+, DON and DOC for each time point (t) in comparison to the previous sampling:

$$\Delta\text{cTDN/DOC} = \text{cTDN/DOC}_t - \text{cTDN/DOC}_{t-1}$$

Positive values reflect a surplus in concentrations and therefore net release, whereas negative values reflect loss in concentration due to net uptake. Release rates were chl *a*-normalized.

Statistical analysis. Statistical analysis was done using SPSS. A student's *t*-test (Tukey's multiple comparison test) was conducted to determine whether the results obtained from individual treatment incubations on the effects of light intensity were significantly different. Statistical comparisons of the effect of different phosphorus concentrations were made using either 1-way ANOVA for normally distributed data or the Kruskal-Wallis ANOVA for data sets which showed no normal distribution (Maxwell & Delaney 2003). The check for normal distribution was done using the Kolmogorov-Smirnov-test. For analysing the homogeneity of variances, Levene's test was applied (Maxwell & Delaney 2003). Mean ± SD is given throughout the text.

RESULTS

Diurnal variation in N_2- and C-fixation and release of nitrogenous compounds

The batch culture of *Trichodesmium erythreum* and *Nodularia spumigena* exhibited a characteristic diurnal pattern of N_2 and C fixation (Fig. 1). N_2 fixation started with the onset of the photoperiod (6 h). Maximum N_2 fixation rates were reached at 16 h with 6.07 ± 2.36 nmol N µg chl a^{-1} h^{-1} (n = 4) in *T. erythreum* and at 18 h in *N. spumigena* with a rate of 0.81 ± 0.12 nmol N µg chl a^{-1} h^{-1} (n = 4). The highest rates of C fixation were found at 18 h with maximum rates in *T. erythreum* of 0.19 ± 0.003 µmol C µg chl a^{-1} h^{-1} (n = 4) and 0.52 ± 0 µmol µg chl a^{-1} h^{-1} (n = 4) in *N. spumigena*. The specific growth rates for the time observed were equivalent to 0.324 d^{-1} for *T. erythreum* and 0.319 d^{-1} for *N. spumigena*. The ratios of POC:PON and C:N fixed levelled at 7.29 ± 0.7 and 10.6 ± 3.2, respectively, in *T. erythreum*. During growth in *N. spumigena*, the POC:PON ratio was 6.5 ± 0.8. The C:N uptake ratio

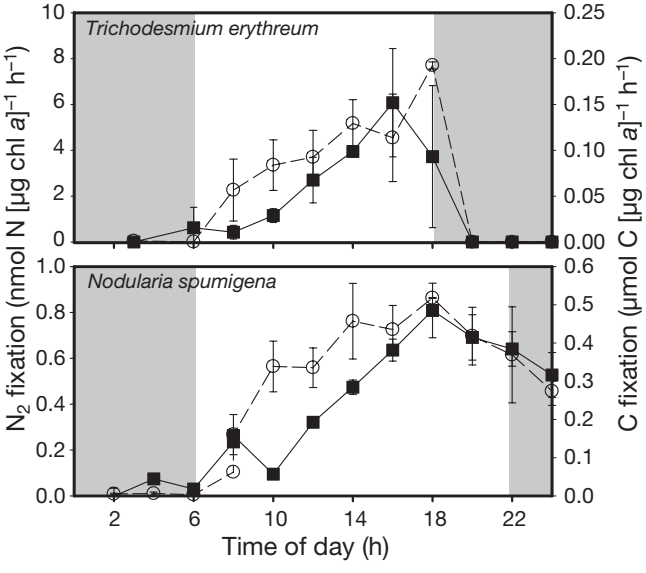

Fig. 1. *Trichodesmium erythreum* and *Nodularia spumigena*. Diel variation in N_2 (solid line, filled squares) and C fixation (dashed line, open circles) of *T. erythreum* and *N. spumigena*. Values are means ± SD of 4 replicates. Grey areas indicate dark periods

was 6.15 ± 4.8. The concentration of NH_4^+ in the batch medium of *T. erythreum* rose to a maximum at 8 h and another local maximum at 18 h, whereas *N. spumigena* showed 2 maxima during the light period at 8 and 20 h and 1 minimum at 14 h (Fig. 2a,b). The absolute concentrations were 3.7 times higher for *N. spumigena* than for *T. erythreum.* Normalised release rates of NH_4^+ in *T. erythreum* showed a maximum of 0.06 ± 0.02 µmol N µg chl a^{-1} h^{-1} (Fig. 3a). The maximum release rate in *N. spumigena* was 0.10 ± 0.067 µmol N µg chl a^{-1} h^{-1} (Fig. 3b). DON concentrations in the batch media of both species seemed to fluctuate with a distinct minimum in concentration at 14 h in *N. spumigena* and 2 minima at 8 and 18 h in *T. erythreum* (Fig. 2c,d). Absolute values of ambient concentration were lower for *T. erythreum* by a factor of 4.7 (8.4 vs. 38.5 ± 0.70 µM). The maximum DON release rate in *T. erythreum* was 0.35 ± 0.07 µmol N µg chl a^{-1} h^{-1} and in *N. spumigena* 1.35 ± 0.18 µmol N µg chl a^{-1} h^{-1} (Fig. 3c,d). DFAA contribute to the DON pool with 7.9% in *T. erythreum* and 1.3% in *N. spumigena*, with maximum concentrations of 0.48 ± 0.06 and 0.66 ± 0.06

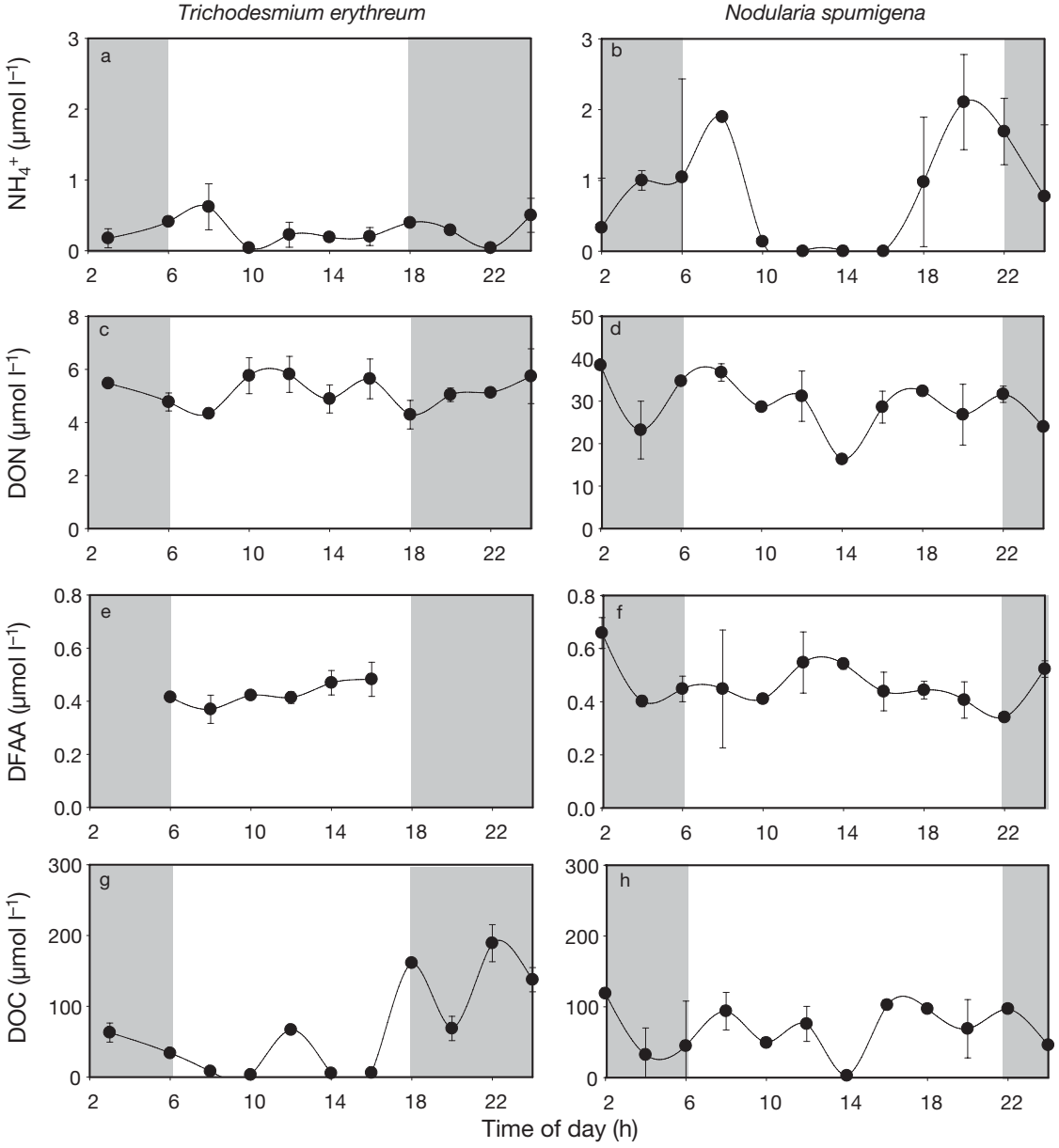

Fig. 2. Diel variation in extracellular concentrations of (a,b) NH_4^+, (c,d) dissolved organic N (DON), (e,f) dissolved free amino acids (DFAA) and (g,h) dissolved organic C (DOC) in *Trichodesmium erythreum* and *Nodularia spumigena* cultures. Values are means (±SD) of 4 replicates. Grey areas indicate dark periods. Note the different scales for DON concentrations of the 2 species

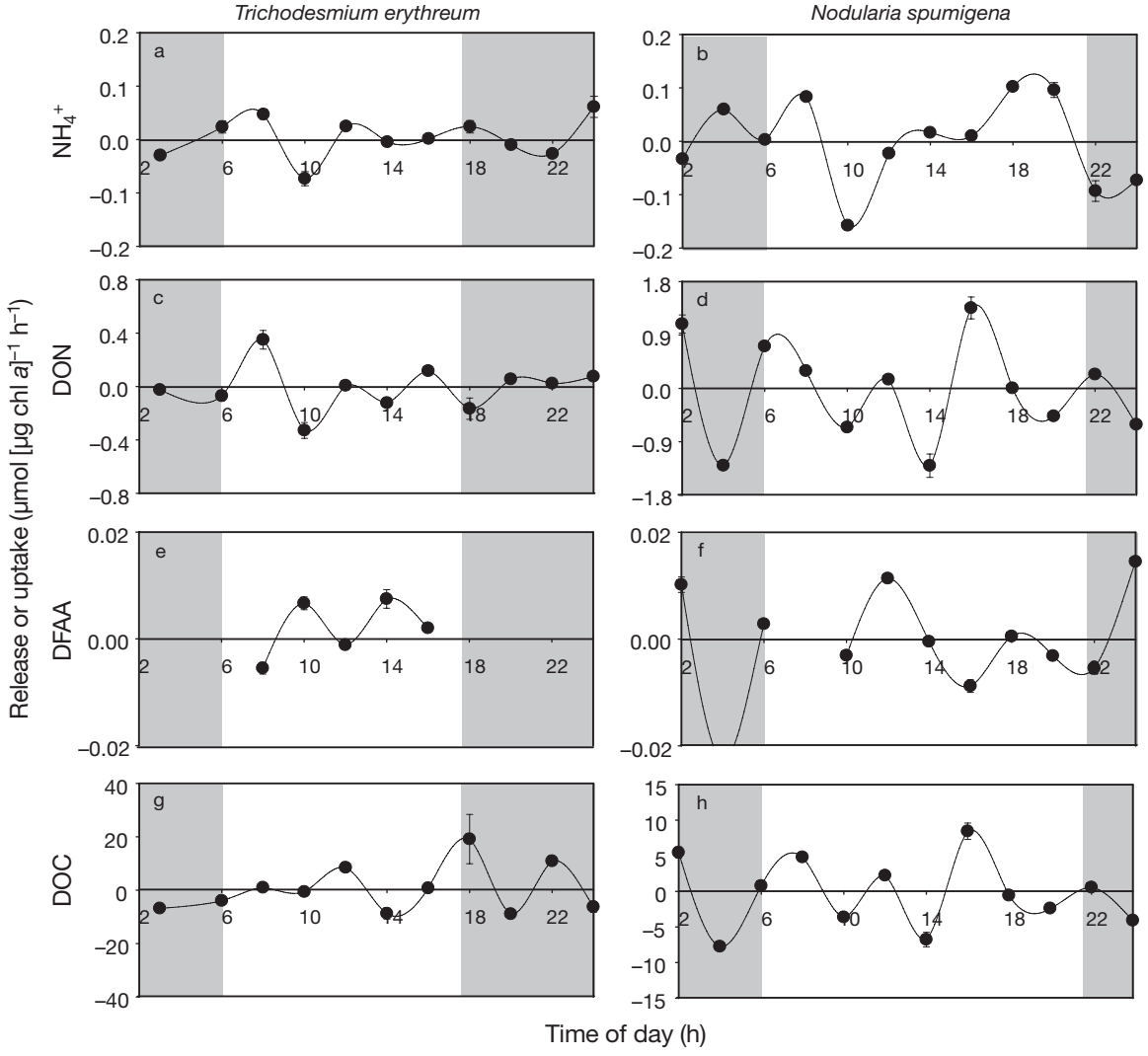

Fig. 3. *Trichodesmium erythreum* and *Nodularia spumigena*. Diel variation in release or uptake of (a,b) NH_4^+, (c,d) dissolved organic N (DON), (e,f) dissolved free amino acids (DFAA) and (g,h) dissolved organic C (DOC) for *T. erythreum* and *N. spumigena*. Values are means (±SD) of 4 replicates. Positive values indicate release, negative values uptake of compounds. Grey areas indicate dark periods

µM, respectively (Fig. 2e,f). Glu and Gly accounted for 80% of bulk DFAA, but the average percentage of the dominant amino acid differed significantly between the 2 species ($p < 0.001$, $n = 12$). In *T. erythreum*, Glu was the most abundant amino acid (Glu 47.7%, Gly 32.3%), whereas Gly dominated in *N. spumigena* (Gly 44.6%, Glu 27.4%, Fig. 4). Other detectable DFAA were Ala, Val, Prol, Asp, Arg, Tau and Lys. The maximum release rate of bulk DFAA in *N. spumigena* was 0.012 ± 0.001 µmol N µg chl a^{-1} h^{-1} (Fig. 3f); in *T. erythreum* this value was 0.008 ± 0.002 µmol N µg chl a^{-1} h^{-1} (Fig. 3e).

There was a pronounced maximum in the DOC concentration in *Trichodesmium erythreum* at 22 h of 189.2 ± 26.2 µmol l^{-1} (Fig. 2g) and the maximum release rate of DOC occurred at 18 h (10.9 ± 0.8 µmol [µg chl *a*]$^{-1}$ h^{-1}; Fig. 3g). In *Nodularia spumigena* there was a distinct minimum and fluctuation in DOC concentration visible at 14 h (Fig. 2 h). The maximum release rate of 8.4 ± 1.1 µmol µg chl a^{-1} h^{-1} occurred at 16 h (Fig. 3h).

Fixation and release of dissolved compounds in response to a shift in light intensity

Compared with the NL conditions, both species showed a significant rise in C fixation under HL ($p < 0.05$, Table 2). The mean values refer to measurements of 3 replicate incubation bottles after 180 and 360 min after the beginning of the experiment. In contrast, N_2 fixation in *Trichodesmium erythreum* cultures did not

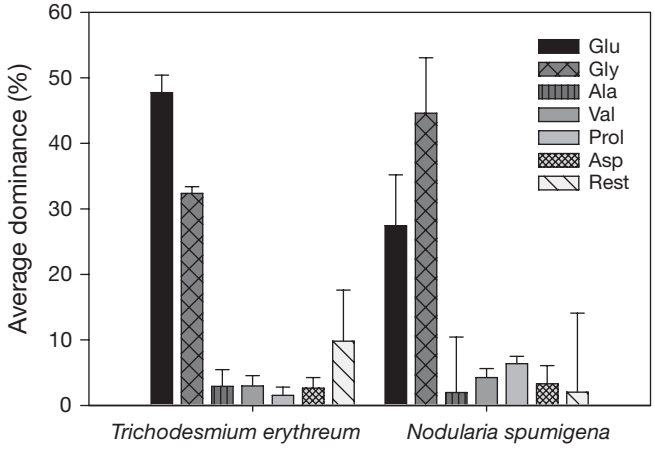

Fig. 4. Average percentage dominance of extracellular dissolved free amino acids in *Trichodesmium erythreum* and *Nodularia spumigena* cultures during the course of a day. Glu: glutamine; Gly: glycine; Ala: alanine; Val: valine; Prol: proline; Asp: asparagine; Rest: taurine, leucine and lysine combined

change significantly with doubling in light intensity (HL and NL: 1.19 ± 0.52 and 0.86 ± 0.41 nmol N µg chl a^{-1} h^{-1}, respectively, n = 6), whereas N_2 fixation in *Nodularia spumigena* was significantly higher in HL compared with NL treatments ($p < 0.05$, n = 6, Table 2). Overall rates were within the range of values obtained during the survey of the diel cycle (Fig. 1) with the exception of C fixation in *T. erythreum*, which was elevated in HL and NL light regimes by a factor of 20 and 13, respectively, compared to rate measurement during the day survey. In *T. erythreum* the ratio of C:N fixed was 463 ± 80 in HL treatments and 375 ± 94 in NL treatments; in *N. spumigena* these ratios were 113 ± 38 and 65 ± 21, respectively.

Changes in concentrations of NH_4^+, DON, DFAA and DOC in the batch medium after exposure to HL are presented in Fig. 5 and Table 2. A significant increase was measurable in the ambient concentrations of all compounds in both species within the first 30 min upon exposure to HL in comparison to NL conditions, except for DFAA in both species and DOC in *Nodularia spumigena*. After 60 min the concentrations of compounds in the HL treatments resembled those of the NL treatment. *Trichodesmium erythreum* exhibited a net release of DON, NH_4^+ and DOC, whereas *N. spumigena* showed a net release of only DON and NH_4^+ under the increased experimental irradiance within the first 30 min (Table 2). To check for photochemically derived increases in TDN and DOC concentrations, a control setup was carried out using the same *N. spumigena* parent culture and the same light treatments. Prior to the start of the experiment, algae were removed by filtering through GF/F filters (Whatman) to stop any biological activity. The control indicated no elevation in the concentration of NH_4^+ ($p > 0.5$, n = 3), DON or DOC between HL and NL treatments (Fig. 5b,d,h). The POC:PON ratios were not different in either cultures or between light treatments. In *T. erythreum*, POC:PON ratios were 8.8 ± 1.2 and 8.6 ± 1.6 in HL and NL treatments, respectively; in *N. spumigena* the ratios were 6.6 ± 0.1 and 6.6 ± 0.2, respectively).

Fixation and release of dissolved compounds under different phosphorus concentrations

The concentrations of TDN in the treatments did not differ significantly from each other and were on average 11 µmol l^{-1} in *Trichodesmium erythreum* and 30 µmol $^{l-1}$ in *Nodularia spumigena* cultures. The

Table 2. Results of the Student's *t*-test on the difference in N_2 and C fixation for the observed time interval (360 min) and the release of NH_4^+, dissolved organic N (DON) and dissolved organic C (DOC) for the first 30 min between the 2 experimental irradiance levels: high light (HL; 200 or 120 µmol photons m^{-2} s^{-1}) and normal light (NL; 120 or 60 µmol photons m^{-2} s^{-1}). Units of single parameters are N_2 fixation (nmol N µg chl a^{-1} h^{-1}), C fixation (µmol C µg chl a^{-1} h^{-1}), and release of NH_4^+, DON, dissolved free amino acids (DFAA) and DOC (µmol [µg chl *a*]$^{-1}$ h^{-1}). Negative release rates indicate uptake of N and C compounds. Values are means ± SD. *: $p < 0.05$; **: $p < 0.01$; n: number of single measurements

	Trichodesmium erythreum				*Nodularia spumigena*			
	HL	NL	p	n	HL	NL	p	n
N_2 fixation	1.19 ± 0.52	0.86 ± 0.41	0.2	6	1.71 ± 0.54	0.75 ± 0.32	0.01*	6
C fixation	4.37 ± 1.14	2.47 ± 0.51	0.001**	6	1.5 ± 0.68	0.28 ± 0.07	0.002**	6
Release of:								
NH_4^+	0.05 ± 0.03	−0.05 ± 0.05	0.02*	3	3.05 ± 0.92	0.13 ± 0.22	0.01*	3
NH_4^+ control					0.085 ± 0.104	0.059 ± 0.029	>0.5	3
DON	14.2 ± 95	−9.3 ± 1.2	0.02*	3	4.9 ± 2.1	1.2 ± 0.2	0.04*	3
DFAA	0.12 ± 0.05	0.37 ± 0.06	>0.5	3	−0.007 ± 0.004	0.02 ± 0.04	0.5	3
DOC	196.2 ± 22.4	−69 ± 88	0.01*	3	19.4 ± 6.4	−27.2 ± 35	0.1	3

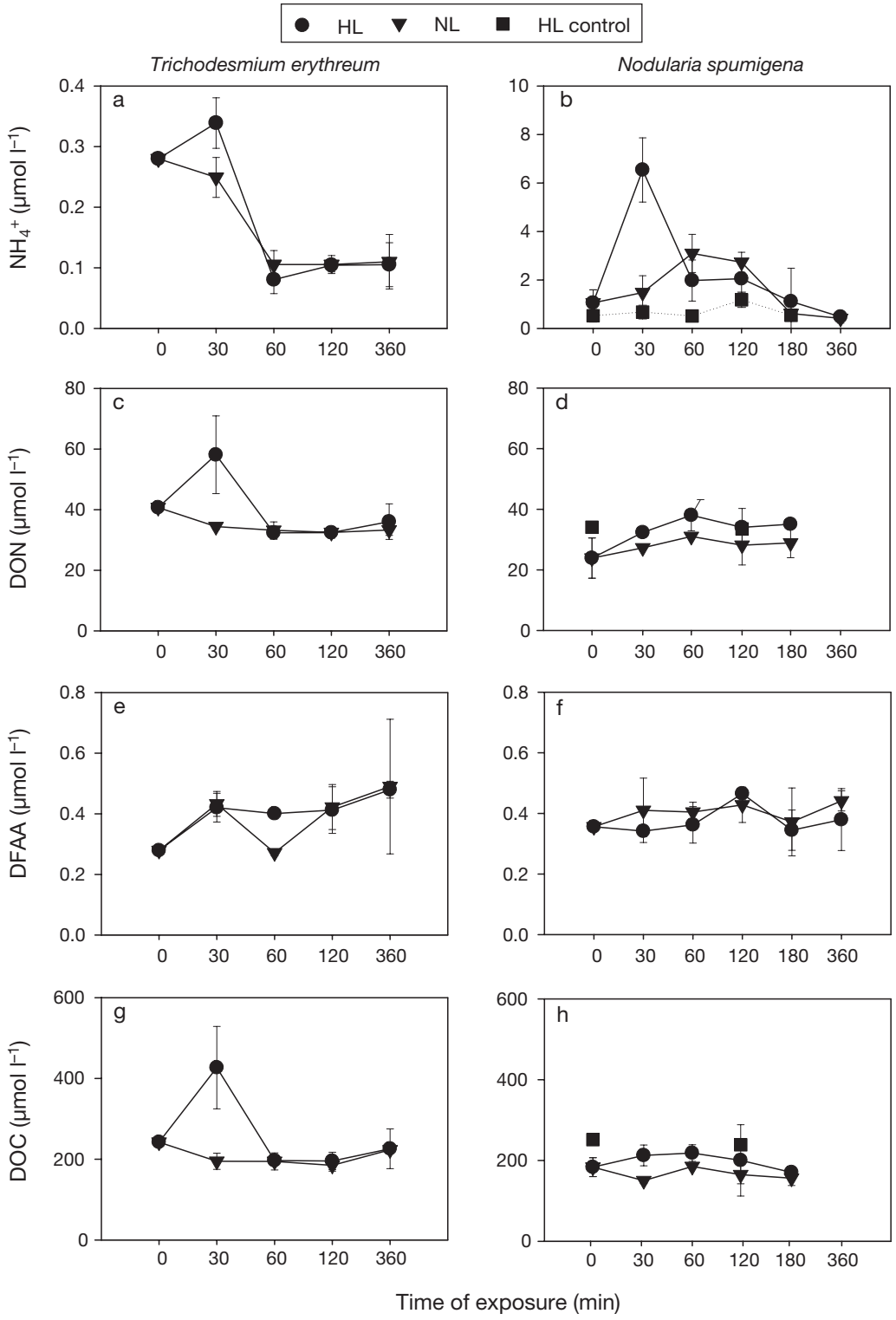

Fig. 5. Time-dependent variability of (a,b) NH_4^+, (c,d) dissolved organic N (DON), (e,f) dissolved free amino acids (DFAA) and (g,h) dissolved organic C (DOC) concentrations in *Trichodesmium erythreum* and *Nodularia spumigena* cultures and the 2 light regimes: high light (HL; 200 or 100 µmol photons m^{-2} s^{-1}) and normal light (NL; 100 or 60 µmol photons m^{-2} s^{-1}). No *N. spumigena* were present in HL control treatments (*N. spumigena* cultures only). Control measurements for DFAA (f) were not available. Values are means (±SD) of 3 replicates

resulting TDN:DIP ratios were 11, 1 and 0.6 (low, mid and high P, respectively) in *T. erythreum* and 31, 3 and 2 (low, mid and high P, respectively) in *N. spumigena*.

N_2 and C fixation rates did not show any significant trend in both species treated with the different phosphorus concentrations ($p > 0.1$, $n = 10$, Fig. 6). Moreover, no significant differences occurred in the concentration of nitrogenous compounds and DOC between the 3 applied phosphorus concentrations during the time of observation. The uptake rates of phosphorus (calculated from mass balance) for the different treatments were highly variable within the triplicates, leading to high standard deviations (low vs. mid vs. high P: *Trichodesmium erythreum*, 0.01 ± 0.04 vs. 0.01 ± 0.14 vs. 0.04 ± 0.18 µmol l^{-1} d^{-1}; *Nodularia spumigena*, 0.02 ± 0.10 vs. 0.27 ± 1.1 vs. 0.68 ± 1.63 µmol l^{-1} d^{-1}). The growth rates obtained from *T. erythreum* were not statistically different between phosphorous treatments (0.28, 0.28 and 0.35 d^{-1} for low, mid and high P, respectively; $p > 0.5$, $n = 5$). Growth in *N. spumigena* was significantly higher in the mid P than the high P treatment (0.35 d^{-1} and 0.13 d^{-1}; $p < 0.05$, $n = 5$), but not in the low P treatment (0.35 d^{-1} and 0.21 d^{-1}, $p > 0.1$, $n = 5$).

Again, there was no significant influence of ambient phosphorus concentration in the media upon the ratio of POC to PON in both species (low vs. mid vs. high P; *Trichodesmium erythreum*, 6.5 ± 0.8 vs. 6.4 ± 0.6 vs. 6.4 ± 0.5; *Nodularia spumigena*, 7.21 ± 0.4 vs. 7.58 ± 0.30 vs. 7.64 ± 0.40; $p > 0.1$, $n = 5$).

The C:N uptake ratio (C:N fixed) did not differ significantly within the applied phosphorus treatments (low vs. mid vs. high P: *Trichodesmium erythreum*, 26 ± 8 vs. 28 ± 9 vs. 24 ± 7; *Nodularia spumigena*, 28 ± 9 vs. 19 ± 13 vs. 18 ± 11; $p > 0.1$, $n = 5$).

DISCUSSION

Endogenous control and diel pattern

The present study provides fixation rate measurements and release of both N and C compounds by diazotrophic cyanobacteria over a diel cycle. The overall fixation rates are at the lower end of previous studies with maximum N_2 fixation rates below 10 nmol N µg chl a^{-1} h^{-1} and maximum C fixation below 1 µmol C µg chl a^{-1} h^{-1} (Berman-Frank et al. 2001, Mulholland et al.

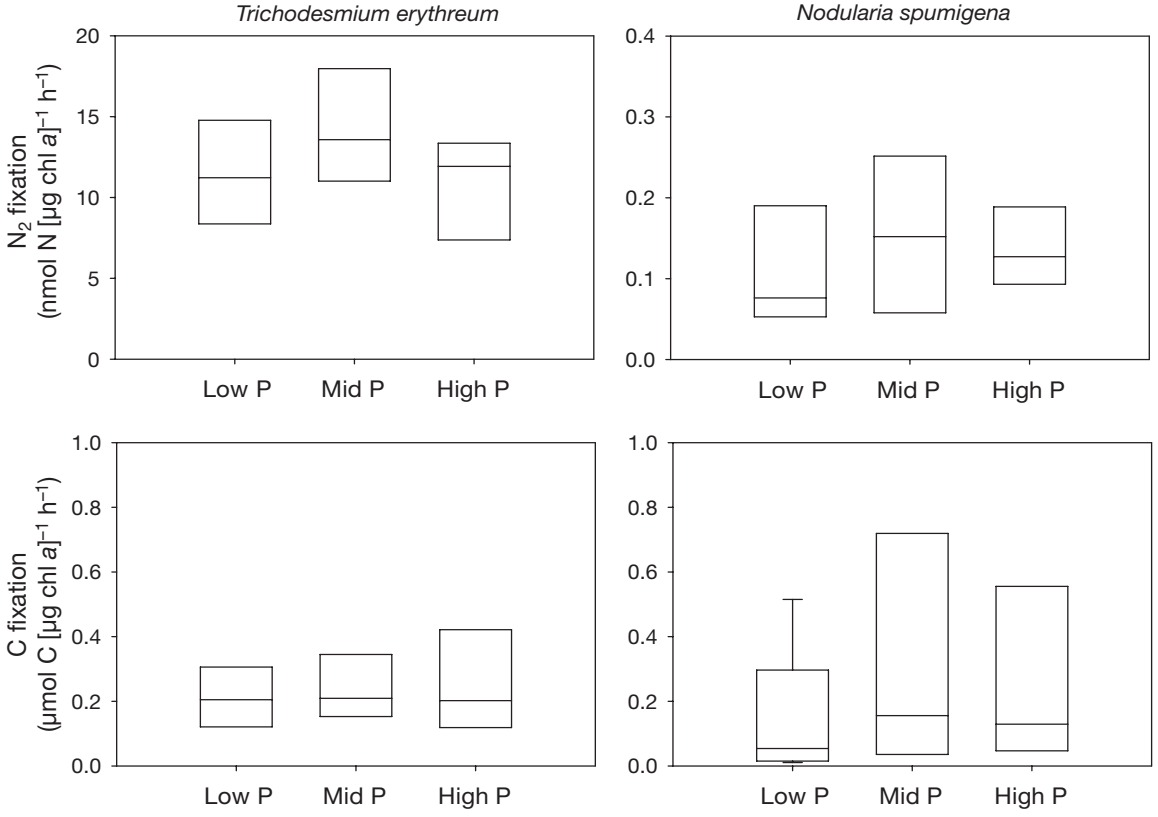

Fig. 6. *Trichodesmium erythreum* and *Nodularia spumigena*. Box plot ($n = 10$) of N_2 and C fixation for the 2 cyanobacterial species, and 3 different phosphorus concentrations: low P (1 µM), mid P (10 µM) and high P (20 µM). Lower boundary of each box indicates the 25th percentile; upper boundary is the 75th percentile. The line within each box marks the median and error bars (whiskers) indicate the 90th and 10th percentiles

2004, Mulholland & Bernhardt 2005). The diel pattern of N_2 and C fixation, with a constant rise in the late afternoon as shown here (Fig. 1), has been reported previously (Chen et al. 1998, Berman-Frank et al. 2001, Mulholland & Capone 2000). The fact that 2 independent runs using the same exponential growing parent culture resulted in a similar diel pattern confirmed its persistence under constant environmental conditions.

Initiation of C fixation occurred rapidly after initiation of the light period, reaching 40% of its maximum rate in *Trichodesmium erythreum* and 20% in *Nodularia spumigena* after 2 h and slowing down in the later photoperiod (Fig. 1). N_2 fixation started slowly and increased significantly after 4 h in the light (Fig. 1). This temporal pattern has been shown by Gallon et al. (2002) and recently by Popa et al. (2007) using the heterocystic cyanobacterium *Anabaena*.

The molecular mechanism of the circadian oscillation of N_2 and C fixation are light-dependent and based on transcription and translation processes and on the phosphorylation of enzymes to reset the inner clock (Dunlap 1999, Nishiwaki et al. 2000). In addition, N_2 fixation is dependent on energy stored by photosynthesis (ATP, $NADPH_2$), which adds to the circadian rhythm. Data collected in the present study showed an identical progression of N_2 and C fixation in *Nodularia spumigena* and no sequential down-regulation of either process, as in heterocystic cyanobacteria both processes are strictly spatially separated in order to create anaerobic conditions for the nitrogenase enzyme. The timing of N_2 and C fixation in the non-heterocystic cyanobacterium *Trichodesmium erythreum* exhibited a slight down-regulation of C fixation between 16 and 18 h, while N_2 fixation is optimal. This pattern has been observed in other investigations (e.g. Berman-Frank et al. 2001) and is caused by the need to separate N_2 and C fixation. This is necessary because N_2 fixation depends on stored energy (ATP, $NADPH_2$) and is negatively influenced by the accumulation of oxygen over the light period. Moreover, both fixation processes are harmonized in non-heterocystic cyanobacteria by allocating cells with nitrogenase activity within the trichomes and colonies. Only 12% of trichomes in *Trichodesmium thiebautii* seem to actively express nitrogenase (Bergman & Carpenter 1991). This in turn causes a supply problem within colonies in those trichomes that lack nitrogenase activity. The surrounding environment then acts as an extracellular vacuole (Flynn & Gallon 1990). The oscillation of dissolved compounds detected in the experiments is therefore not surprising (Figs. 2 & 3). However, there is no explanation for the observed oscillation in the concentrations of dissolved compounds also visible in *N. spumigena.*

The relatively high amount of extracellular NH_4^+ (~1 µM, Fig. 2a,b) and DON (10 to 40 µM, Fig. 2c,d) presumably originated directly from the reduction of newly fixed N_2. The percentage of DFAA in the DON pool was ~1 to 8%. No further identification of the remaining DON was performed in the present study, but possible compounds reported in the literature comprise dissolved combined amino acids (DCAA), urea and ribonucleic acid (Bronk 2002). The concentration of NO_3^- and NO_2^- remained below the detection limit throughout all the investigations.

In spite of NH_4^+ and DON being present, N_2 fixation continued (Figs. 1 & 2), which has been shown by other studies as well (e.g. Ohki et al. 1991, Fu & Bell 2003). Concentrations of dissolved compounds changed throughout the day in almost sine-like oscillations, although the observation period of 2 h is too coarse to fit the data points with a sine model. The observed changes in both species had different amplitudes and frequencies. In *Trichodesmium erythreum* amplitudes were smaller and the periods longer than in *Nodularia spumigena.* Only the change in the amount of DFAA (mainly Glu) in *T. erythreum* was directly and positively correlated with the rise in N_2 fixation. This was caused by differences in either the release or uptake rates, such that in *T. erythreum* these processes were quicker than the investigation time and accumulation in the external media was therefore not as pronounced as it was for *N. spumigena* (except for DOC). A surplus in concentration measured in the course of the present study indicated release and a decrease uptake of compounds (Fig. 3), on the premise that experiments were done using axenic cultures. On average, 71% of fixed N_2 was exudated by *T. erythreum* as NH_4^+ (41%) and DON (30%) and 89% in *N. spumigena* as NH_4^+ (39%) and DON (50%) during the light period. The total rates (NH_4^+ + DON) are high compared to available data from the literature: in the present study the release of both NH_4^+ and DON was considered, whereas in other studies often only one of these components was investigated. Rates of DON exudation obtained here are well within the rates published (e.g. Capone et al. 1994, Hutchins et al. 2007). Field studies using stable isotopes as a tracer yielded DON release rates up to 50% (Glibert & Bronk 1994). Investigations using pure cultures of *Trichodesmium erythreum* resulted in rates of up to 81% (Hutchins et al. 2007). The average release rates of C as DOC in the present study were ~50% in both species, which is within the range of literature data (Sellner 1997). To date, several studies have revealed that the release of both TDN and DOC is a considerable fraction of the net N and C uptake (Bronk et al. 1994, Slawyk et al. 1998, Varela et al. 2006). Overall, we suggest that the release of compounds in the present study is presumably not regulated by de novo synthesis of permease involved in the transport of compounds, but rather by the amount of

previously assimilated N and C and the regulation of permease activity, as presented by Vincent (1992) and Flores & Herrero (2005). While uptake of compounds against a concentration gradient involves a membrane potential-driven transport and ATP-costly fixation using the nitrogenase enzyme, the reverse transfer out of the cell coincides with a concentration gradient. Cells perceiving a sufficient N status upon fixing N_2 do not increase the synthesis of new permease transporter to exudate this N in excess, but rather modulate the activity of the transporter by post-translational regulation (Flores & Herrero 2005). Thus, the observed oscillation of TDN release rates reported here within the course of the day (Fig. 3) may derive from the consumption and fixation of N and C. Any overconsumption of N relative to C should lead to a release of nitrogenous compounds. The connection between C and N metabolism in cyanobacteria is the glutamine synthetase–glutamate synthase cycle (GS-GOGAT, Flores & Herrero 1994). Intracellularly produced NH_4^+ is incorporated into the C skeletons through GS-GOGAT in the form of 2-oxoglutarate, which in turn is used for biosynthesis of Glu and Glu-derived compounds. N deficiency is perceived as an increase in the intracellular 2-oxoglutarate level, and N excess as a decrease in the intracellular 2-oxoglutarate level. Therefore, 2-oxoglutarate acts as a signal by which cyanobacteria perceive the intracellular N status, leading to a feedback signal that drives N uptake in the form of N_2 or NH_4^+ or N release as NH_4^+ or DON.

The phasing of chl *a*-specific release rates in *Trichodesmium erythreum* was shorter than the changes in concentrations, suggesting again that uptake and release activity was regulated quicker and might not be fully resolved by an investigation time of 2 h. In *T. erythreum*, DOC accumulates during the day with its maximum in concentration occurring during the night (22 h). When looking at the various patterns of cycles during the day, 2 forms can be distinguished. There are long period cycles of fixation of N and C on the one hand and short period cycling (presumably <2 h) of TDN compounds on the other hand, suggesting a much faster feedback control regulated by the amount of fixed N and the N status of the individual cell itself (Lillo et al. 2001). Rapid feedback occurs within seconds, slower feedback depends on the further metabolization (Kerby et al. 1987).

It should be remembered that individual cells within a trichome in *Trichodesmium* spp. are not necessarily in the same stage of the cell cycle. Temporal separation of N_2 fixation and O_2 evolving during photosynthesis may occur, depending on the present stage of the cell cycle of individual cells within the same trichome or in trichomes in the colony (Popa et al. 2007, Ohki et al. 2008).

Exogenous control

Fluctuation in cellular light energy supply

Our experiments simulating changing light intensities support the hypothesis that an increase in cell energy may not be used completely for biosynthesis (Lomas et al. 2000). Instead, the energy is dissipated by the release of dissolved nutrients (NH_4^+, DON and DOC). We observed a rise in C fixation rates in *Trichodesmium erythreum*, whereas N_2 fixation did not increase significantly in this species under HL incubation (Table 2). It is known that *Trichodesmium* is strongly light adapted and needs higher irradiances for growth than other phytoplankton (Kana 1993). A linear rise was determined in Kana (1993) in photosynthesis up to 600 µmol photons m^{-2} s^{-1} and saturation was reached at 1600 µmol photons m^{-2} s^{-1} (the light intensity of a full bright sunny day is 1000 µmol quanta m^{-2} s^{-1}). We assume that the HL intensity in the present study (200 µmol photons m^{-2} s^{-1}) was not sufficient to significantly increase N_2 fixation in *Trichodesmium* (Table 2). However, the N_2 fixation rates of *Nodularia spumigena* increased significantly when the cells were exposed to higher light intensities in the present study (Table 2), a result in accordance with Evans et al. (2000, their Fig. 7b), who used light intensities up to 400 µmol quanta m^{-2} s^{-1}.

The ratios of fixed C:N in the light experiments were very high in both *Trichodesmium erythreum* and *Nodularia spumigena* (HL treatment 463 and 113, NL treatment 375 and 65, respectively), indicating that C and N incorporation were not balanced relative to somatic demand in light experiments (in HL and NL treatments). Studies have shown that the ratio of fixed C:N can be much higher than the Redfield ratio (Orcutt et al. 2001) and even reach up to 700 (e.g. McCarthy & Carpenter 1979). The observed periodical uncoupling of primary production and N_2 fixation might be explained by C ballasting, storage of glucose, lipids and polyhydroxybutyrates (Romans et al. 1994, Villareal & Carpenter 2003) and/or exudation of newly fixed N (Mulholland et al. 2004). Uptake of C in excess often occurs when phytoplankton photosynthesize under high light conditions (e.g. Mague et al. 1980) and dispose the surplus of fixed C as DOC. This might account for the high ratio of fixed C:N in the HL treatments compared to the ratio observed in the diel cycle experiments (10.6 ± 3.2 and 6.2 ± 4.8 in *T. erythreum* and *N. spumigena*, respectively). Nevertheless, it does not explain the high ratios of fixed C:N in the NL treatments, where identical experimental conditions were applied as in the diel cycle experiments. It should be noted that parent cultures for both experiments were taken at different stages of exponential growth. On the

other hand, fixation in the light experiments was only surveyed for 6 h (06:00 to 12:00 h), and it could be that N_2 fixation might have increased during a longer observation period. Much of the C that exceeds the demands for somatic growth may be put to various other fitness-promoting uses (Hessen & Anderson 2008) or released as DOC.

Furthermore, there was a striking short-term effect of increasing light intensity on the concentration of dissolved compounds detectable (NH_4^+, DON, DFAA and DOC; Fig. 5). Within the first 30 min after the shift from NL to HL, concentrations rose in nearly all compounds in both species (taking the concentrations of the NL treatment as a reference), except for DFAA. This effect diminished after 60 min of exposure. Therefore, only release rates for the first 30 min after exposure were considered in the following discussion and argumentation. A control setup using *Nodularia spumigena* was applied to prove that the increase in NH_4^+, DON and DOC (control measurements for DFAA were not available) concentrations solely resulted from the metabolism and physiology of the cyanobacteria and not from photochemical reactions of dissolved compounds in the extracellular media. The quantity in the control set up of dissolved compounds in the extracellular media did not change (Fig. 5b,d,h).

The release rates calculated for each compound were significantly higher in HL than in NL treatments (Table 2), except for the release of DFAA. The release of DON therefore must comprise compounds other than DFAA. DON release might be attributed to passive leakage or disrupted cells. In the latter case, the dominance of several amino acids measured in the present study should have been identical to those found intracellularly. Glu and Gly were the dominant extracellular amino acids in the present study (Fig. 4), but dominant intracellular amino acids are usually Glu, Ala and Arg (Flynn & Gallon 1990). This discrepancy in composition contradicts a passive efflux or the breakage of cells in the present study. Overall, exudation is still controversially discussed in the literature. Whether it is an overflow mechanism, where excess photosynthetic products are actively released when the fixation rates exceed the rate of macromolecular synthesis (Wood & van Valen 1990), or a passive diffusion of small metabolites through the cell membrane (Bjørnsen 1988) remains unclear. If the overflow mechanism dominates, significant DON and DOC production would preferentially occur under conditions of high irradiance and low nutrient concentration (molar N:P of 3.2 in Alcoverro et al. 2000) as a mechanism for dissipating cellular energy (Wood & van Valen 1990).

If passive diffusion is the main mechanism, DOC and DON production can take place whenever a pool of small, recently fixed metabolites is available. The findings in the present study support the opinion that exudation is an active and adaptive process reacting towards changes in the energy status of cells. To underline our hypothesis, we also tested the potential of active exudation of NH_4^+ and DON as a sink for electrons by quantifying the percentage of electrons consumed by these processes (Table 3) as has been carried out by Lomas et al. (2000) for diatoms and flagellates. In particular, the numbers of electrons that were required to yield the observed extracellular accumulation of NH_4^+ and DON in the HL treatment were calculated, relative to the number of electrons harvested during the given time interval. The chl *a*-specific release was multiplied by a total of 8 electrons required for fixation of N_2 and production and release of NH_4^+ and DON. The number of electrons harvested by the cells (per µg chl *a*) in the same time period was calculated using the formula given by Lomas et al. (2000):

$$\text{Electrons harvested} = E \times T \times a^{\star} \times 0.5$$

with E being the incident irradiance (photons m^{-2} s^{-1}), T the time interval (180 s in this case), $a^{\star}$ the chl *a*-specific absorption (m^2 mg^{-1} chl *a*) and 0.5 a constant, assuming a 50% distribution of chlorophyll between the photosystems (Falkowski & Raven 1997). Species-specific values for $a^{\star}$ used in the present study are given in Table 3. The percentages of electrons consumed supporting the observed accumulation of dis-

Table 3. Estimates of average percentage of electron consumption supporting the observed rates of NH_4^+ and dissolved organic N (DON) release under the experimental irridiance at high light (200 or 120 µmol photons m^{-2} s^{-1}) between 0 and 30 min. $a^{\star}$ (m^{-2} mg chl a^{-1}) represents the chlorophyll-specific absorption coefficient

Species	$a^{\star}$	Compound released	% electron consumption		Source
			Max	Min	
Trichodesmium erythreum	0.0187	NH_4^+	0.18	0.03	Subramaniam et al. (1999)
		DON	52.4	10.5	
Nodularia spumigena	0.024	NH_4^+	8.79	4.72	Metsamaa et al. (2006)
		DON	15.5	4.6	

solved N compounds (Table 2) under HL treatment were ~52% in *Trichodesmium erythreum* and ~16% in *Nodularia spumigena*; these are average values over the first 30 min of exposure (Table 3). The results from the concentration measurements (Fig. 5) as well as the calculated number of electrons needed for the observed accumulation of TDN in the media further support the hypothesis that release of N compounds is an active way to dissipate excess energy consumed. The differences in percentage between the 2 species might result from the differences in the overall available light intensity and resulting number of possible electrons harvested (200 µmol photons m^{-2} s^{-1} in *T. erythreum* and 120 µmol photons m^{-2} s^{-1} in *N. spumigena*). Additionally, release might be more instantaneous and pronounced in the non-heterocystic *T. erythreum* because cells within the filament fixing N_2 are not coated with a 3-layer envelope like in heterocysts, which in turn is impermeable to ions (Haselkorn 1978). N_2 fixed in heterocysts of *N. spumigena* cultures must firstly be exported via permease into adjacent vegetative cells where it is subsequently released into the extracellular environment. Overall, when light energy is available in excess, the release of NH_4^+ and DON may serve as a short-term sink for electrons, in addition to other dissipation processes being activated within minutes (e.g. Mehler reaction, heat dissipation). Adaptations on the macromolecular basis (e.g. the adaptation of abundance of the messenger RNA encoding the light-harvesting chlorophyll proteins) even take longer, from 2 h after the onset of light shifts to 12 h (Fujita et al. 1994, Falkowski & Raven 1997).

Fluctuation of nutritional status

A co-limitation of N_2 fixation by iron and phosphorus has been documented by several studies (e.g. Mills et al. 2004, Degerholm et al. 2006). Our results are insufficient to clearly verify the hypothesis that increases in the supply of limiting nutrients like phosphorus fuel N_2 fixation and thus the release of nitrogenous compounds. There were no significant differences in fixation of both compounds when comparing the applied phosphorus concentrations in the batch media (Fig. 6). Overall, the experimental setup testing the influence of PO_4^{3-} addition on N_2 and C fixation and exudation of TDN and DOC was using phosphorus concentrations which turned out to be too high to identify any significant trend. The resulting N:P ratios of dissolved compounds were 10, 1 and 0.5 (low, mid and high P), considering the ambient NH_4^+ and DON concentrations in the batch media (10 µM, data not shown). Besides the low N:P ratio, cyanobacteria were not N-limited, because they actively fixed N_2. The ratio of fixed C:N ranged between 19 and 28 and was not significantly different between phosphorus treatments. The deviation of this ratio from the Redfield ratio (~6) is still in the range of literature data (Orcutt et al. 2001). N_2 fixation rates are saturated at a P concentration of 1.2 µM in *Trichodesmium* (Fu & Bell 2003). Still, in experiments using the same species, significantly higher N_2 fixation rates were observed with extracellular P concentrations of 5 µM compared to 1 µM (Mulholland & Bernhardt 2005).

CONCLUSIONS

Cyanobacteria in natural environments are exposed to continuous changes in light intensity during passive or active movement within the upper water column. These constant shifts towards imbalanced cellular energy conditions seem to lead to peaks of TDN and DOC exudation. The diel rhythm is probably controlled both endogenously and exogenously, creating temporarily patchy nutrient-rich local habitats. Furthermore, DOM is needed in colonies of non-heterocystic cyanobacteria to supply trichomes and cells that lack nitrogenase activity with nitrogenous compounds. Moreover, exudation might support the nutrient flow within the food web (e.g. Tseng et al. 2005). Organisms like bacteria, fungi, diatoms, ciliates and juvenile decapods are found spatially and temporarily associated to cyanobacteria and benefit from higher nutrient availabilities in close connection with cyanobacterial colonies (e.g. Devassy et al. 1979), pointing to the key position of cyanobacteria in marine food webs.

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