

Dissolved organic nitrogen and carbon release by a marine unicellular diazotrophic cyanobacterium

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ABSTRACT: Dinitrogen (N₂) fixation rates may be underestimated when recently fixed N₂ is released as dissolved organic nitrogen (DON). DON release (DONr) is substantial in the filamentous cyanobacterium *Trichodesmium* but has never been reported in unicellular diazotrophic cyanobacteria. We used axenic cultures of the marine unicellular diazotroph *Cyanothece* sp. Miami BG 043511 to measure dissolved organic matter release under N₂-fixing conditions. DONr was measured as the transfer of ¹⁵N₂ from the culture medium to the extracellular DON pool. On average, the DON released represented ~1% of the total N₂ fixed. The average release of dissolved organic carbon, as determined by ¹⁴C, represented ~2% of the total carbon fixed. These results suggest that cultured populations of unicellular diazotrophs do not release much dissolved organic matter, but it cannot be excluded that DONr is important in the field when grazers and bacteria are present, or when the organism is exposed to environmental stresses such as turbulence, excess light, temperature changes, or nutrient limitation.

KEY WORDS: N₂ fixation · DON release · Unicellular cyanobacteria · *Cyanothece*

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INTRODUCTION

The release of fixed dissolved organic carbon (DOC) by healthy marine plankton has been studied since the 1960s (e.g. Fogg 1962). Release of organic matter in an oligotrophic environment might seem disadvantageous for phytoplankton as it represents a loss of energy and promotes growth of heterotrophic bacteria that compete for nutrients. Hence, these organisms may be outcompeted by those that do not release DOC. The organic carbon may serve as a substrate for autotrophic and heterotrophic plankton (Wood & Van Valen 1990). Therefore, the release of organic compounds by healthy cells has often been regarded as a paradox in biological oceanography, although it may also have advantages. For example, organic compounds help chelating elements with low

availability and solubility such as trace metals (Barbeau 2006). The release of dissolved organic compounds also helps to protect the photosynthetic apparatus in high-light regimes by dissipating excess energy, and shortens the phytoplankton's lag-phase after nutrient-impooverished periods (Wood & Van Valen 1990, Wannicke et al. 2009).

DOC release (DOCr) by phytoplankton has been widely studied during the past decades (Fogg 1962, Sharp 1977, Wood & Van Valen 1990). Diazotrophic plankton such as blooming filamentous cyanobacteria are also known to release high amounts of DOC when the bloom decays, feeding grazers and stimulating bacterial production (Sellner 1997). DOCr has also been observed in unicellular cyanobacteria (Bertilsson et al. 2005). However, little is known about the magnitude and ecological signifi-

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cance of the release of dissolved organic nitrogen (DONr) by oceanic diazotrophic cyanobacteria. Most marine dinitrogen (N_2) fixation field studies have been conducted by measuring the ^{15}N enrichment of particulate nitrogen (PN) after a certain time of incubation. The filtrates are usually discarded and, therefore, the N_2 fixed and subsequently released as dissolved organic or inorganic nitrogen (DON or DIN) is not taken into account. Thus, the amount of N_2 that has been fixed is potentially underestimated (Bronk et al. 1994, Gallon et al. 2002, Mulholland 2007).

Global oceanic denitrification exceeds N_2 fixation by ~ 200 Tg N yr^{-1} (Mahaffey et al. 2005, Codispoti 2007). Recent research indicates that this disequilibrium may be less severe when N_2 fixation measurements are expanded to higher latitudes and greater depths (e.g. Moisander et al. 2010), and that these measurements are indeed accurate, which has been questioned (Mohr et al. 2010). Less attention has been paid to the potential underestimation of global oceanic N_2 fixation rates caused by not taking into account DONr.

Estimating DONr is also important because it is a source of new nitrogen for autotrophic and heterotrophic plankton (Berman & Bronk 2003, Bronk et al. 2007). N_2 fixed by diazotrophic cyanobacteria can be an important source of nitrogen for other plankton. For instance, transfer of nitrogen from large diazotrophic cyanobacteria to picoplankton has been described for the Baltic Sea (Ohlendieck et al. 2000) and for the Southwest Pacific (Garcia et al. 2007). N_2 fixed by the filamentous cyanobacterium *Trichodesmium* can also sustain the growth of larger autotrophic plankton, such as diatoms (Lee Chen et al. 2011), or even promote harmful algal blooms, as for example those of *Karenia brevis* off the Florida shelf (Bronk et al. 2004). Agawin et al. (2007) studied interactions between N_2 -fixing and non- N_2 -fixing cyanobacteria in a coupled modeling-chemostat culture experiment and found that the DON released by the N_2 -fixing unicellular cyanobacterium *Cyanothece* induced a 4-fold increase in the abundance of the non-diazotrophic *Synechococcus* than it would have achieved in monoculture. This suggests that organic compounds exuded by diazotrophs can fuel primary production and may exert considerable control over the composition of the plankton community in the oligotrophic ocean.

Among marine diazotrophs, *Trichodesmium* was long thought to be the principal N_2 fixer in the oceans (e.g. Capone et al. 1997). However, in the last decade

molecular techniques revealed that unicellular diazotrophic cyanobacteria are more abundant and more widely distributed than *Trichodesmium* (Luo et al. 2012), and their N_2 fixation rates are often higher (Falcón et al. 2004, Garcia et al. 2007, Moisander et al. 2010). Field populations of *Trichodesmium* are known to release up to $\sim 50\%$ of the recently fixed N_2 as DON (Glibert & Bronk 1994), but in cultures the release is much less (Mulholland et al. 2004). Due to the wider geographical distribution of unicellular diazotrophs, it is necessary to study their DONr activity in order to estimate the potential underestimation of N_2 fixation rates. However, direct estimates of DONr by unicellular diazotrophs have not been reported (Mulholland 2007).

DONr rates can be estimated as the difference between gross and net N_2 fixation rates, measured by the acetylene reduction assay (ARA) and $^{15}N_2$ assimilation into biomass, respectively (Gallon et al. 2002, Mulholland et al. 2004). Alternatively, one can measure the ^{15}N atom % enrichment of the DON pool and use common tracer equations to estimate DONr rates (e.g. Glibert & Bronk 1994, Mulholland et al. 2004). Both approaches have been used to indirectly estimate DONr by unicellular diazotrophs. Benavides et al. (2011) performed size-fractionated paired ARA and $^{15}N_2$ assimilation measurements in the eastern North Atlantic and estimated that the <10 μm diazotrophs potentially released up to 67% of their recently fixed N_2 . These facts suggest that unicellular diazotrophs in the field release DON in a manner similar to *Trichodesmium*. The present study used cultures of *Cyanothece* sp. Miami BG 043511 (hereafter *Cyanothece*) as a model organism to test whether the release of dissolved organic matter in unicellular diazotrophic cyanobacteria may be an important process.

MATERIALS AND METHODS

Culture conditions and experimental setup

N_2 fixation and DONr (Expt 1), and carbon fixation and DOCr (Expt 2) were examined in the present study. Cultures of *Cyanothece* (formerly classified as *Synechococcus*) were obtained from the Culture Collection Yerseke (strain CCY 0408). Cultures were grown in 250 ml transparent polypropylene tissue flasks with filter screw caps (VWR) in an illuminated incubator (Snijders ECD01E) at 27°C under a 12:12 h light:dark cycle and a light irradiance of ~ 50 μmol photons $m^{-2} s^{-2}$. Standard YBCII medium devoid of a

source of combined nitrogen (Chen et al. 1996) was used in Expts 1 and 2. In both experiments, 2 replicate cultures were inoculated with 1% of an exponentially growing stock culture. Expt 1 lasted 10 d and Expt 2 lasted 8 d. Therefore, 20 and 16 culture replicates were prepared for Expts 1 and 2, respectively. On every experiment day, 2 replicates were sacrificed for the assays and analyses described below.

Chlorophyll, biomass, cell abundance, and cell size

During both experiments, chlorophyll *a* (chl *a*) concentrations, cell abundance, and cell size were monitored daily. For chl *a* analysis, culture aliquots were filtered onto 25 mm GF/F filters. Chl *a* was extracted with 90% cold acetone for 24 h and subsequently analyzed by means of a Cary Eclipse Fluorescence Spectrophotometer, previously calibrated with pure chl *a* (Sigma), and concentrations were calculated using the equations of Ritchie (2006). Cell counts and cell size measurements were performed daily in triplicate vortexed fresh samples using a Multisizer 3 Coulter Counter (Becton Dickinson). Bacterial contaminant numbers were checked daily by phase contrast microscopy and did not exceed 3% of the cell number of the cyanobacteria.

N₂ fixation

Net N₂ fixation was assayed once a day in the middle of the dark period using the stable isotope tracer method of Montoya et al. (1996). For this technique, 125 ml glass vials were filled with culture to overflow, closed with rubber stoppers (allowing the excess culture to escape by a sterile syringe tip piercing the septum) and crimp-sealed with aluminum caps. Trace additions of ¹⁵N₂ (500 µl; 98.3 atom % ¹⁵N, Euriso-top) were made using a gas-tight syringe (Hamilton), and the samples were incubated for 3 h. The ¹⁵N₂ incubated samples were filtered onto pre-combusted GF/F filters (6 h, 450°C) to obtain the ¹⁵N enrichment and PN concentration.

DONr and intracellular DON production

In Expt 1, DONr was estimated as the transfer of ¹⁵N from the culture medium to the extracellular DON pool, using the ammonium diffusion technique

of Slawyk & Raimbault (1995) to isolate the labeled DON, and equations reported by Slawyk et al. (1998). In this experiment, we also investigated the N₂ fixed as DON but not released during the incubation period (i.e. intracellular DON production, inDONp), computed as $\text{inDONp} = [(R_{\text{inDONf}} / R_{\text{N}_2} \times t)] \times \text{inDONf}$, where R_{inDONf} represents the excess ¹⁵N enrichment of the intracellular DON pool at the end of the incubation period, R_{N_2} represents the initial ¹⁵N₂ enrichment of the culture medium, t is the incubation time, and inDONf is the final intracellular DON concentration (i.e. after the 3 h incubation period).

After incubation and filtration, the filtrate was recovered and stored frozen for extracellular DON analysis. The intracellular DON content of cells was obtained by gently filtering (vacuum pressure <100 mm Hg) culture aliquots through 3 µm pore size, 25 mm polycarbonate filters, adding 15 ml of boiling Milli-Q water and recovering the filtrate (combined thermal and osmotic shock; Thoresen et al. 1982). For the analysis of ¹⁵N-labeled DON, aliquots of the extracellular and intracellular filtrates were poisoned with 1% HgCl₂ after 3 h of incubation with ¹⁵N₂. These filtrates were then stored in sterile 50 ml polyethylene tubes at room temperature and in the dark until analysis. To separate DIN (i.e. nitrate + nitrite + ammonium) from DON and analyze the ¹⁵N content of the latter, we followed the 3-step ammonium diffusion methodology proposed by Slawyk & Raimbault (1995). In Step 1, filtrate subsamples were covered with a screw cap provided with a needle tip and half a GF/F filter humidified with 0.25 N H₂SO₄. MgO and DeVarda alloy were added to the samples and were subsequently incubated at 50°C for 1 wk in order to strip off the DIN as (NH₄)₂SO₄. In Step 2, the remaining nitrogen in the subsamples was only DON, which was oxidized to DIN by adding a solution composed of K₂S₂O₈, NaOH, and H₃BO₃. Finally, in Step 3, the remaining nitrogen was removed by repeating Step 1.

PN, particulate carbon (PC), and ¹⁵N enrichments were analyzed by means of a Thermo Flash EA 1112 elemental analyzer connected to a Thermo Delta Advantage isotope ratio mass spectrometer (IRMS). DON concentrations were computed by subtracting DIN from total dissolved nitrogen (TDN). The concentrations of TDN were determined by autoclave destruction and the persulfate oxidation method (Valderrama 1981). DIN was analyzed with a SEAL Technicon Autoanalyzer II, following recommendations by Kirkwood (1995). Detection limits were 0.1, 0.03, and 0.16 µM for nitrate, nitrite, and ammonium, respectively.

Potential underestimation of N₂ fixation rates

Due to the slow dissolution of gaseous ¹⁵N₂ in water, N₂ fixation rates are underestimated to a variable extent when ¹⁵N₂ is added to the sample as a bubble (Mohr et al. 2010), indicating that previously published N₂ fixation rates are likely underestimated. This important observation was published while the experiments reported in the present study were in progress. For consistency, we decided not to apply the new method provided by Mohr et al. (2010), which consists of adding ¹⁵N₂ dissolved in the culture medium or seawater sample. Alternatively, we provide a second set of N₂ fixation, DON_r, and inDON_p rates estimated using the % dissolution of a ¹⁵N₂ bubble after our period of incubation. After 3 h, the % dissolution of a ¹⁵N₂ bubble into YBCII medium at 27°C is 28.8% (W. Mohr pers. comm.).

Carbon fixation and DOCr

In Expt 2, we tested carbon fixation, DOC_r, and intracellular DOC production (inDOC_p) by *Cyanothece*. We incubated 50 ml of culture for 3 h during the middle of the light period with 100 µl of 1057 kBq ml⁻¹ NaH¹⁴CO₃ (American Radiolabeled Chemicals). The initial CO₂ concentration was measured by automated titration with a Metrohm Dosimat 765 and a Metrohm 780 pH Meter. GF/F and 3 µm polycarbonate filters were used as described above for Expt 1.

After incubation and filtration, filters were transferred to polyethylene scintillation vials (Perkin Elmer Pico Prias) and exposed to HCl fumes in a desiccator in order to remove the non-incorporated ¹⁴C. Ultima Gold MV (Perkin Elmer) counting cocktail was added. Non-incorporated ¹⁴C in the filtrate was removed by adding 100 µl concentrated HCl to 50 ml samples. The next day, samples were counted using Insta-Gel (Perkin Elmer) counting cocktail. The scintillation counter used was a Packard TRI-CARB 2300. To avoid radioactive contamination of analysis equipment, parallel culture replicates not incubated with ¹⁴C were used to measure final PC concentrations. However, the replicate culture flask used for ¹⁴C measurements was split into 2 parts and analyzed separately in order to estimate analytical variability.

Samples for the analysis of the intracellular DOC and extracellular DOC concentrations were taken at the end of incubations and analyzed with a Skalar Formacs TOC continuous flow analyzer, previously calibrated with potassium hydrogen phthalate.

RESULTS

Growth and biomass

Cell number and the biomass indices chl *a*, PN, and PC were used to estimate cell division rate and growth rate of the *Cyanothece* cultures. These variables showed similar patterns in all experiments (Fig. 1). Based on the increase in cell number, the average division rate was 0.64 d⁻¹ and the average growth rates were 0.27, 0.22, and 0.23 d⁻¹, in Expt 1

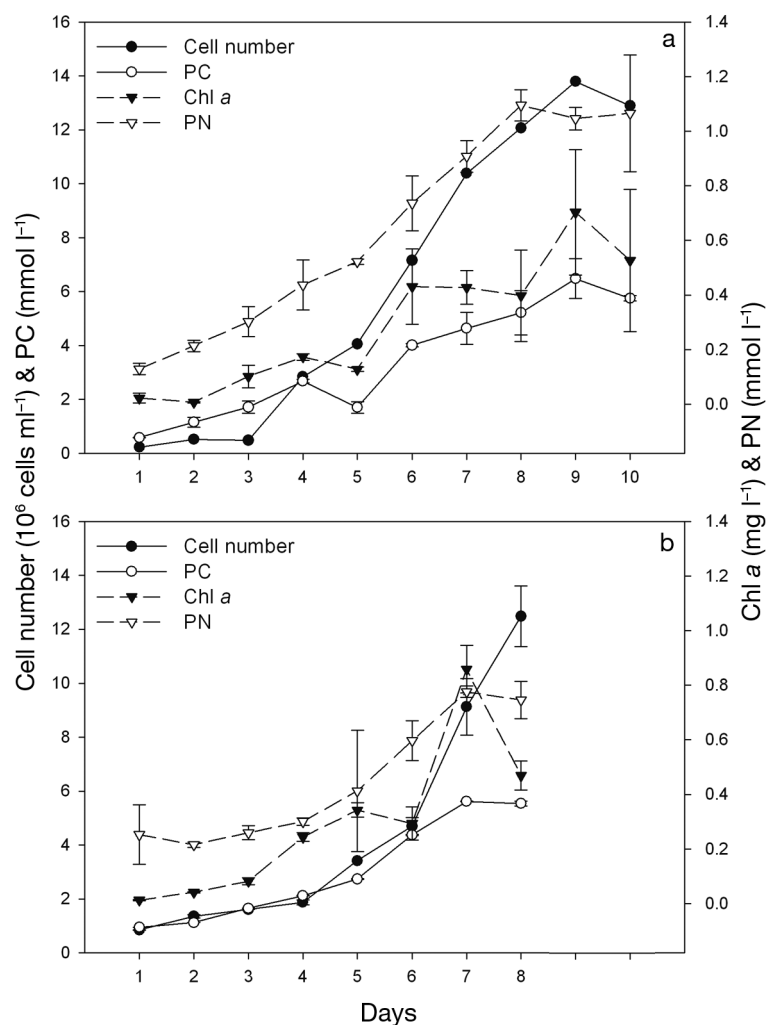


Fig. 1. Cell number, particulate carbon (PC), chlorophyll *a* (chl *a*), and particulate nitrogen (PN) in (a) Expt 1 and (b) Expt 2

as based on chl *a*, PN, and PC, respectively. In Expt 2, these values were 0.41, 0.35, 0.24, and 0.21 d⁻¹, respectively.

Particulate and dissolved nitrogen pools

The initial and final (before and after the 3 h incubation period with ¹⁵N₂) concentrations of PN, extracellular and intracellular DON and DIN are shown in Table 1 (Expt 1). The initial concentration of the PN pool ranged from 129 to 1095 μmol N l⁻¹. Except on Days 1 and 2, the final amount of PN was always lower than the initial level. However, differences between initial and final PN concentrations were statistically significant (*t*-test, *p* = 0.028). The initial concentration of extracellular DON and DIN was always smaller than the final concentration, except for extracellular DON on Days 4, 8, and 10. Initial and final extracellular DIN concentrations were statistically different (*p* = 0.017), while differences between extracellular DON concentrations were not (*p* = 0.585). Extracellular DON ranged from 10 to 97 μmol N l⁻¹, while DIN values were 1 or 2 orders of magnitude lower (<1 to 4.5 μmol N l⁻¹). In contrast, the initial concentrations of intracellular DON and DIN were always higher than the final concentrations, except intracellular DON on Day 5, and intracellular DIN on Days 2, 3, and 10. Initial and final intracellular DON concentrations were significantly different (*p* = 0.027), while intracellular DIN concentrations were not (*p* = 0.859).

While PN and extracellular DON and DIN concentrations increased as the culture grew (during the 10 d experiment), the intracellular DON and DIN pools showed a different pattern. The intracellular DON pools measured up to ~5–14 μmol N l⁻¹ during the first 2 d and last 2 d of the experiment, while between Days 3 and 8, the concentrations were generally twice as high. Intracellular DIN remained more or less constant, with concentrations between <1 and ~2 μmol N l⁻¹, with one peak of 3.14 μmol N l⁻¹ observed on Day 3. The concentration of ¹⁵N₂ added to the samples (0.16 μmol N l⁻¹) was taken into account when comparing the initial and final (pre- and post-incubation with ¹⁵N₂) total nitrogen concentrations in all pools. The final total nitrogen concentration (sum of all pools) was lower than the initial one except on Days 1 and 2. Initial total nitrogen concentrations were on average ~15% higher than final values. This is probably a result of adding the injected ¹⁵N₂ in the mass balance. Some of this ¹⁵N₂ is fixed and thus transferred to the particulate and dissolved nitrogen pools, but some

Table 1. Mean (SD in parentheses) concentrations of nitrogen in the particulate nitrogen (PN) pool and extracellular and intracellular dissolved organic (DON) and inorganic nitrogen (DIN) pools at the start and at the end of the incubations performed with additions of ¹⁵N₂ to *Cyanothece* cultures (Expt 1). The sum of all pools at the start and at the end of the incubations is given. All values are given in μmol N l⁻¹. For initial values, the concentration of ¹⁵N₂ added to the samples is included in the sum as well (0.16 μmol N l⁻¹)

Day	Initial				Final			
	PN	Extra-cellular DON	Intra-cellular DON	Total (+ ¹⁵ N ₂ added)	PN	Extra-cellular DON	Intra-cellular DIN	Total
1	129.3 (21.88)	10.23 (0.14)	9.89 (0.73)	151.46	137.47 (16.21)	11.46 (0.72)	1.35 (0.49)	156.51
2	214.16 (27.83)	10.66 (0.68)	11.78 (12.65)	238.93	245.27 (45.36)	44.91 (46.8)	1.39 (0.23)	303.47
3	302.25 (50.49)	15.81 (1.27)	37.21 (12.26)	360.56	274.72 (29.65)	26.16 (5.96)	1.59 (0.69)	331.33
4	436.81 (92.53)	49.61 (2.47)	34.74 (4.07)	524.54	388.59 (20.88)	36.37 (2.82)	1.86 (0.99)	460.49
5	522.07 (10.3)	33.97 (9.42)	25.40 (23.29)	584.93	388.55 (38.89)	60.84 (38.55)	1.8 (0.62)	483.26
6	736.19 (101.56)	60.79 (8.52)	38.88 (11.16)	838.41	720.52 (17.20)	71.4 (25.56)	1.42 (0.03)	826.01
7	908.25 (58.59)	58.54 (19.28)	45.58 (5.19)	1018.68	817.93 (106.90)	67.23 (5.78)	1.37 (0.03)	926.10
8	1095.11 (57.91)	73.49 (14.7)	42.95 (8.09)	1215.68	874.93 (96.95)	61.05 (2.78)	2.54 (1.9)	963.81
9	1046.15 (41.66)	56.16 (12.79)	14.31 (1.89)	1120.11	1028.53 (55.99)	61.17 (5.85)	2.09 (0.9)	1104.53
10	1065.71 (214.14)	96.86 (18.79)	9.20	1174.46	883.81 (3.91)	61.62 (5.74)	0.98	958.03

is probably unused or not dissolved into the culture medium during the incubation period (Mohr et al. 2010), and thus not accounted for in the final sum.

The initial and final concentrations of ^{15}N in the particulate and dissolved pools of *Cyanothece* cultures during Expt 1 are considered separately in Table 2. Unfortunately, the ^{15}N enrichment of the DIN pools was not measured and therefore these data are lacking from Table 2. Final concentrations of ^{15}N in the PN pool were larger than the initial ones, except on Days 5, 8, and 10. Differences between initial and final ^{15}N concentrations of the PN pool were not statistically significant (t -test, $p = 0.117$). However, Montoya et al. (1996) considered a minimum acceptable change of $\delta^{15}\text{N}$ between the initial and the final PN sample of 4‰. The difference between the final and initial $\delta^{15}\text{N}$ values of PN in our experiments ranged between 66 and ~ 1400 (data not shown); hence, we are convinced that the N_2 fixation rates presented here are accurate.

The final concentration of ^{15}N (sum of all pools) was higher than the initial sum on Days 1 to 3, indicating an excess recovery of ^{15}N . This imbalance is probably due to the low intracellular and extracellular DON concentrations measured on these days. However, the mean \pm SD percentage recovery for the rest of the experiment (Days 4 to 10) was $99.08 \pm 10.41\%$.

N_2 fixation, DONr, and inDONp

When considering the complete dissolution of the $^{15}\text{N}_2$ bubble (tracer method of Montoya et al. 1996), net N_2 fixation rates (assimilation of $^{15}\text{N}_2$ into bio-

mass) started at $3.66 \mu\text{mol N l}^{-1} \text{h}^{-1}$ on Day 1, peaked at $7.36 \mu\text{mol N l}^{-1} \text{h}^{-1}$ on Day 2, and then decreased to $\sim 2 \mu\text{mol N l}^{-1} \text{h}^{-1}$ at the end of Expt 1 (Table 3). When only 28.8% dissolution of the $^{15}\text{N}_2$ bubble was considered, all rates increased ~ 2.5 -fold and followed the same temporal pattern.

DONr peaked on Day 2 with rates of 0.07 and $0.19 \mu\text{mol N l}^{-1} \text{h}^{-1}$, and on Day 8 with rates of 0.06 and $0.14 \mu\text{mol N l}^{-1} \text{h}^{-1}$, when considering 100% dissolution and 28.8% dissolution of the $^{15}\text{N}_2$ bubble, respectively. In the middle of the experiment, DONr rates remained close to 0.03 and $0.08 \mu\text{mol N l}^{-1} \text{h}^{-1}$ (100 and 28.8% $^{15}\text{N}_2$ dissolution, respectively), and were lower on Days 1 and 10. On average, DONr represented $\sim 1\%$ of the total nitrogen fixed (net N_2 fixed + DON released + inDON produced). It should be noted that the 'total N_2 fixed' includes recently fixed N_2 released as DIN. Unfortunately, the ^{15}N enrichment in DIN was not measured.

The inDONp rates (transfer of $^{15}\text{N}_2$ to the intracellular DON pool) were generally 1 order of magnitude higher than DONr, with values ranging from 0.003 to $0.2 \mu\text{mol N l}^{-1} \text{h}^{-1}$ when 100% dissolution of the $^{15}\text{N}_2$ bubble was considered, and 0.008 to $\sim 0.5 \mu\text{mol N l}^{-1} \text{h}^{-1}$ when only 28.8% dissolution was considered. The overall tendency of inDONp rates was to decrease during the experiment, and the lowest rates were observed on the last 3 d of Expt 1 (Days 7 to 10).

Carbon pools, carbon fixation, DOCr, and inDOCp

Rates of net carbon fixation, DOCr, and inDOCp, and the concentration of carbon in the particulate

Table 2. Mean (SD in parentheses) concentrations of ^{15}N in the particulate nitrogen (PN) and extracellular and intracellular dissolved organic nitrogen (DON) pools, at the start and at the end of the incubations performed with additions of $^{15}\text{N}_2$ to *Cyanothece* cultures (Expt 1). The sum of ^{15}N in all pools at the start and at the end of the incubations is given. All values are given in $\mu\text{mol N l}^{-1}$. For initial values, the concentration of $^{15}\text{N}_2$ added to the samples is included in the sum as well ($0.16 \mu\text{mol N l}^{-1}$). A percentage of ^{15}N recovery is given based on the amount of ^{15}N in the samples at the start of the incubations compared to the amount of ^{15}N recovered at the end

Day	Initial				Final				% recovery
	PN	Extracellular DON	Intracellular DON	Total (+ $^{15}\text{N}_2$ added)	PN	Extracellular DON	Intracellular DON	Total	
1	0.53 (0.09)	0.04 (0.00)	0.02	0.75	1.06 (0.02)	0.04 (0.0)	0.05 (0.01)	1.15	153.33
2	0.82 (0.1)	0.04	0.02 (2.59)	1.04	2.02 (0.63)	0.17 (0.19)	0.06 (0.07)	2.25	216.35
3	1.15 (0.19)	0.05 (0.01)	0.03 (0.01)	1.39	1.73 (0.03)	0.08 (0.02)	0.12 (0.04)	1.93	138.85
4	1.65 (0.34)	0.15 (0.06)	0.08 (0.02)	2.04	2.06 (0.06)	0.16 (0.04)	0.13 (0.02)	2.35	115.19
5	1.95 (0.04)	0.12 (0.03)	0.1 (0.01)	2.33	1.95 (0.32)	0.14 (0.02)	0.1 (0.09)	2.19	93.99
6	2.74 (0.38)	0.29 (0.06)	0.09 (0.00)	3.28	3.17 (0.01)	0.2 (0.01)	0.12 (0.04)	3.49	106.40
7	3.38 (0.21)	0.25 (0.12)	0.09 (0.04)	3.88	3.42 (0.55)	0.25 (0.02)	0.15 (0.04)	3.82	98.45
8	4.08 (0.22)	0.29 (0.03)	0.12	4.65	3.68 (0.38)	0.24 (0.0)	0.15	4.07	87.53
9	3.87 (0.15)	0.2 (0.03)	0.08	4.31	4.22 (0.19)	0.24 (0.0)	0.06 (0.01)	4.52	104.87
10	3.96 (0.79)	0.26 (0.07)	0.04	4.42	3.51 (0.05)	0.31 (0.14)	0.03 (0.01)	3.85	87.10

Table 3. Mean (SD in parentheses) rates of net N₂ fixation (A), dissolved organic nitrogen (DON) released (B), intracellular DON produced (inDONp) (C), and total nitrogen fixed (A+B+C) measured considering 100% dissolution of the ¹⁵N₂ bubble, and, alternatively, considering 28.8% dissolution of the ¹⁵N₂ bubble after a 3 h incubation period. All rates are given in $\mu\text{mol N l}^{-1} \text{h}^{-1}$. The last column includes the percentage of DON released (DONr) compared to the total N₂ fixed (A+B+C), which is equal for both ¹⁵N₂ bubble % dissolutions. Note that the total N₂ fixed column does not include any N₂ fixed and released as DIN

Day	100% dissolution				28.8% dissolution				% DONr
	Net N ₂ fixation (A)	DONr (B)	inDONp (C)	Total N ₂ fixed (A+B+C)	Net N ₂ fixation (A)	DONr (B)	inDONp (C)	Total N ₂ fixed (A+B+C)	
1	3.66 (0.27)	0.00 (0.00)	0.06 (0.04)	3.72	9.46 (0.68)	0.01 (0.002)	0.16 (0.11)	9.62	0.08
2	7.36 (3.06)	0.07 (0.10)	0.15 (0.10)	7.59	18.9 (7.80)	0.19 (0.2)	0.39 (0.25)	19.25	0.98
3	4.85 (0.52)	0.04 (0.02)	0.10 (0.02)	4.99	12.3 (1.36)	0.09 (0.04)	0.26 (0.04)	12.51	0.72
4	4.28 (0.11)	0.03 (0.03)	0.20 (0.03)	4.51	10.8 (0.31)	0.08 (0.07)	0.49 (0.07)	10.95	0.69
5	3.44 (1.18)	0.03 (0.01)	0.18 (0.01)	3.65	8.62 (2.99)	0.07 (0.02)	0.45 (0.02)	8.75	0.76
6	3.47 (0.38)	0.03 (0.01)	0.06 (0.01)	3.56	8.60 (0.97)	0.08 (0.03)	0.15 (0.03)	8.77	0.95
7	2.76 (1.01)	0.04 (0.01)	0.13 (0.01)	2.93	6.78 (2.47)	0.09 (0.01)	0.33 (0.01)	6.97	1.35
8	3.08 (0.19)	0.06 (0.02)	0.01 (0.02)	3.15	7.51 (0.48)	0.14 (0.05)	0.02 (0.05)	7.79	1.78
9	2.90 (0.08)	0.03 (0.01)	0.00 (0.01)	2.94	7.02 (0.16)	0.08 (0.03)	0.01 (0.03)	7.18	1.09
10	1.83 (0.21)	0.02 (0.01)	0.00 (0.01)	1.85	4.4 (0.52)	0.05 (0.02)	0.01 (0.01)	4.49	1.01

and dissolved pools of *Cyanothece* cultures during Expt 2 are given in Table 4. Carbon fixation rates are given either as net carbon fixed (carbon fixed into biomass), or as 'total carbon fixed' (net carbon fixed + DOC released + inDOC produced). Total carbon fixation ranged from 54 to 151 $\mu\text{mol C l}^{-1} \text{h}^{-1}$, close to net carbon fixation, which ranged from 37 to 101 $\mu\text{mol C l}^{-1} \text{h}^{-1}$. The rate of DOCr rates ranged from <1 to 3 $\mu\text{mol C l}^{-1} \text{h}^{-1}$, while the rate of inDOCp was 1 to 2 orders of magnitude higher, ranging from 15 to 55 $\mu\text{mol C l}^{-1} \text{h}^{-1}$. DOCr rates represented only 1 to 4% of the total carbon fixed (average ~2%). Particulate carbon (PC) ranged from 950 to 5600 $\mu\text{mol C l}^{-1}$. Extracellular and DOC concentrations ranged from ~70 to 600 $\mu\text{mol C l}^{-1}$. Intracellular DOC concentrations were generally higher than extracellular

DOC concentrations, except on the last 3 d of Expt 2 (Days 6 to 8).

C:N fixation ratios

The ratios provided here come from 2 different experiments (Expts 1 and 2, see 'Materials and methods'). C:N fixation ratios would be more accurate if both carbon and nitrogen fixation rates had been measured simultaneously in the same culture. However, Expt 2 was done independently because samples treated with radioactivity (¹⁴C) need to be manipulated in a separate laboratory and could not be further processed by elemental analyzer or IRMS.

Table 4. Mean (SD in parentheses) rates of net carbon fixation (A), rate of release of dissolved organic carbon (DOCr) (B), and rate of production of intracellular DOC (inDOCp) (C), as well as the rate of the total carbon fixed (A+B+C) measured using ¹⁴C. All rates are given in $\mu\text{mol C l}^{-1} \text{h}^{-1}$. The percentage of carbon fixed which is released as DOC is given (% DOCr). The 3 final columns represent the concentration of carbon in the particulate carbon (PC) and extracellular and intracellular DOC pools at the end of the incubation period. Concentrations are expressed in $\mu\text{mol C l}^{-1}$

Day	Net C fixed (A)	DOCr (B)	inDOCp (C)	Total C fixed (A+B+C)	% DOCr	PC	Extracellular DOC	Intracellular DOC
1	37.47 (4.1)	2.41 (0.12)	14.55 (3.6)	54.43 (5.8)	4.43	948.92 (0.4)	71.39 (17.7)	299.97 (62.9)
2	61.38 (7.7)	0.70 (0.4)	37.44 (7.6)	99.52 (12.9)	0.70	1119.28 (0.03)	75.42 (1.7)	108.84 (39.3)
3	89.31 (7)	0.96 (0.3)	54.80 (6.32)	145.07 (11.8)	0.66	1651.62 (0.17)	111.60 (6.7)	172.30 (1.9)
4	82.62 (10.3)	1.88 (0.2)	52.50 (1.2)	137 (16.9)	1.37	2117.82 (0.13)	160 (2)	306.50 (8.7)
5	97.6 (4.5)	3.11 (0.8)	46.10 (2)	146.81 (7.8)	2.11	2735.87 (1.41)	274.77 (11.3)	559.24 (203.7)
6	84.7 (1.9)	2.54 (0.2)	46.04 (2.8)	133.28 (3.4)	1.91	4373.13 (0.24)	324 (28.2)	282.50 (172.6)
7	101 (2.7)	2.70 (0.1)	47.52 (12.2)	151.22 (4.1)	1.79	5615.35 (0.1)	459.10 (45)	360.90 (40.4)
8	98.59 (9.01)	2.43 (0.2)	49.34 (14.5)	150.36 (13.8)	1.62	5543 (0.9)	599.24 (36.9)	297.75 (17.1)

Carbon was fixed at higher rates than nitrogen, as reflected by the high molar ratios of net carbon fixation: net N₂ fixation (mol C: mol N; Table 5). C:N ratios increased throughout the experiment and exceeded Redfield on almost all experiment days. When net N₂ fixation rates were calculated considering 100% dissolution of the ¹⁵N₂ bubble, C:N ranged from 8 to ~40. When 28.8% dissolution of the ¹⁵N₂ bubble was assumed, net N₂ fixation rates were ~2.5-fold higher. Consequently, when 28.8% dissolution of the ¹⁵N₂ bubble was applied, C:N ratios were lower and did not exceed Redfield, except on Days 1 and 2. From Day 3 on, the C:N ratios ranged from 3 to ~15.

DOCr:DONr ratios give an estimation of the C:N ratio of the dissolved organic matter released. These ratios were also high, ranging from ~10 to 116.3 when 100% dissolution of the ¹⁵N₂ bubble was considered for the calculation of N₂ fixation rates, and from ~4 to 46.5 when 28.8% dissolution was assumed. On Day 1, the DONr rates were close to 0 (Table 3), inflating the DOCr:DONr ratios (Table 5).

DISCUSSION

Measuring N₂ fixation and DONr: methodological issues

There are a number of methodological issues which affect N₂ fixation and DONr rates: (1) potential underestimation of N₂ fixation rates when the ¹⁵N₂ bubble method is used, (2) unsuccessful recovery of all ¹⁵N added at the end of an incubation period, (3) methodological problems related to DON isolation

Table 5. Net carbon fixation:net N₂ fixation and dissolved organic carbon release (DOCr):dissolved organic nitrogen release (DONr) ratios (mol C: mol N). N₂ fixation rates were calculated assuming 100% dissolution of the ¹⁵N₂ bubble or, alternatively, assuming 28.8% dissolution of the ¹⁵N₂ bubble after a 3 h incubation period

Day	100% dissolution		28.8% dissolution	
	Net carbon fixation:net N ₂ fixation	DOCr:DONr	Net carbon fixation:net N ₂ fixation	DOCr:DONr
1	10.24	830.1	3.89	321.3
2	8.33	9.5	3.19	3.7
3	18.42	27.0	7.11	10.6
4	19.29	62.7	7.50	24.8
5	28.34	116.3	11.11	46.5
6	24.44	75.6	9.66	30.5
7	36.62	70.6	14.61	28.7
8	32.02	42.6	12.87	17.5

for IRMS analysis, (4) artifacts associated with sample manipulation, and (5) intracellular isotopic dilution. Each will be discussed in detail below.

(1) Recently, Mohr et al. (2010) demonstrated that the routinely used ¹⁵N₂ bubble tracer method underestimates N₂ fixation rates due to the slow dissolution of the gas into the medium or the sample seawater. These authors recommended adding ¹⁵N₂ already dissolved in the water instead of as a bubble, as previously done by Glibert & Bronk (1994), in order to provide a constant ¹⁵N enrichment of the source N₂ pool. The study of Mohr et al. (2010) was published while the experiments reported in the present study were being carried out. As the experiments had already started, we chose not to change our methodology, in order to maintain consistency. To give an approximation of what the rates would have been like using a more realistic dissolution of ¹⁵N₂ after 3 h incubation, we recalculated net N₂ fixation, DONr, and inDONp rates assuming 28.8% dissolution of the ¹⁵N₂ (Table 3), which is the dissolution of ¹⁵N₂ observed after 3 h of incubation in YBCII medium at 27°C (W. Mohr pers. comm.). When 28.8% dissolution is applied, net N₂ fixation rates explain better the daily PN increase observed (Tables 1 & 3), corroborating that the ¹⁵N₂ bubble method underestimates N₂ fixation rates.

(2) Another problem often encountered during ¹⁵N experiments is that the label is not fully recovered. Bronk & Glibert (1994) demonstrated that the fate of this 'missing' ¹⁵N differs between marine systems. In particular, ¹⁵N transfer from the source pool to the DON pool seems to be more important in oligotrophic systems, which supports the need to include DONr measurements in routine nitrogen uptake experiments in order to avoid underestimations (Bronk et al. 1994).

(3) Measuring the ¹⁵N enrichment of the DON pool is prone to difficulties. Extracting DON for isotope analysis is difficult and has prompted intensive discussion in the past (Slawyk et al. 1998, 2000, Bronk & Ward 2000). The protocol for extracting DON used here (Slawyk & Raimbault 1995) (see 'Materials and methods') could underestimate DONr because the alkaline hot experimental conditions (60°C) of the extraction process may hydrolyze some fraction of the DON as ammonium (McCarthy & Bronk 2008). The alternative DON

isolation method is the ion retardation column (Bronk & Glibert 1991); however, this is no longer commercially available in its previous quality and now retains variable amounts of DON, and therefore is not reliable (McCarthy & Bronk 2008). Hence, consistent DON extraction protocols await development.

(4) Other methodological problems may affect the measurement of DON_r, such as the breakage of cells during vacuum filtration and exposure to light and temperature changes during sample manipulation (Feuillade et al. 1990, Wannicke et al. 2009). Vacuum pressure was kept low throughout our experiments (<100 mm Hg) and care was taken to avoid temperature and light changes. Light was kept constant in our experiments ($\sim 50 \mu\text{mol photons m}^{-2} \text{ s}^{-2}$), and was well below natural intensities in tropical and subtropical waters where *Cyanothece* occurs (Langlois et al. 2008). We therefore think that culture settings or sample manipulation did not affect DON_r analysis.

(5) The intracellular isotopic dilution in cells with high DON intracellular pools might have affected the measurements of the DON_r rates. When the intracellular DON pool and its turnover time are high, isotopic equilibrium between the intracellular pool and the extracellular medium is slow. This causes artificially low DON_r rates, as the DON recovered in the extracellular pool is ^{14}N -rich material, which was inside the cell prior to $^{15}\text{N}_2$ addition (Mulholland et al. 2004).

Nitrogen versus carbon metabolism in *Cyanothece*

Diazotrophs are known to fix carbon at rates exceeding the C:N Redfield ratio (Mulholland et al. 2006, Mulholland 2007). Reasons why this phenomenon occurs in natural populations include (1) substantial nitrogen and/or carbon release due to methodological problems associated with cell manipulation, (2) excess carbon fixation (this ballast may cause sinking of the cells), and (3) excess carbon fixation to decrease cellular oxygen concentrations which may inhibit the nitrogenase (Mehler reactions), or for ATP production (Mulholland 2007). Errors in global estimates of N_2 fixation would be lessened if accurate C:N fixation ratios of marine diazotrophs were used instead of theoretical elemental stoichiometries (Mulholland 2007).

The DON released during our experiments was similar to the DOC released when expressed as a percentage of the total nitrogen or carbon fixed. On average, *Cyanothece* released $\sim 1\%$ of the recently fixed gross N_2 as DON, while $\sim 2\%$ of the gross car-

bon fixed was released as DOC. In a series of culture experiments, Wannicke et al. (2009) found that cultured *Trichodesmium* released 71% of the fixed N_2 as DON and ammonium and 50% of the carbon fixed as DOC. In our study, DOC_r:DON_r C:N ratios were high and always exceeded Redfield stoichiometry, with the only exception being Day 2, when N_2 fixation rates were calculated using 28.8% dissolution of the $^{15}\text{N}_2$ bubble (Table 5). These high ratios suggest that the dissolved organic matter released by *Cyanothece* is enriched in carbon.

It should be noted that DOC and DON were treated as independent pools, but the exudate material of natural populations of diazotrophic organisms is normally composed of both DOC and DON. For example, this is the case for amino acids (Gallon et al. 2002). Whereas eukaryotic phytoplankton organisms are known to exude dissolved organic compounds lacking nitrogen such as carbohydrates (Newell et al. 1972), diazotrophic cyanobacterial exudates are commonly rich in nitrogen (Capone et al. 1994, Mulholland 2007).

DON_r by unicellular diazotrophic cyanobacteria

Trichodesmium is thought to release fixed N_2 to provide other trichomes in the colony with nitrogen (Mulholland & Capone 2000), because not every cell in a trichome and not all trichomes express nitrogenase (Bergman & Carpenter 1991), or are in the same phase of the cell cycle (Wannicke et al. 2009). Some unicellular cyanobacteria release organic compounds to feed neighboring cells. For example, *Gloeotheca* cells live embedded in a mucilaginous sheath that acts as an 'extracellular vacuole' (Flynn & Gallon 1990). The advantage, if any, of releasing DON by free-living unicellular diazotrophs is not clear. Possibly free-living unicellular diazotrophs release DON as a response to abiotic factors such as light stress (Wood & Van Valen 1990). In our study, the DON released represented on average $\sim 1\%$ of the total N_2 fixed by *Cyanothece* during Expt 1 (Table 3). Given that optimum culture conditions were used, the growth of the cells was balanced and DON_r was negligible.

Cultured nitrogen-starved eukaryotic phytoplankton have been reported to release dissolved organic compounds when reaching stationary phase (Newell et al. 1972). In contrast, in our study similar levels of DON and DOC release were observed throughout the experiment (Tables 3 & 4). A decrease in DON_r rates was observed during the last 3 d of Expt 1,

when the cells were reaching the stationary phase (Fig. 1). We hypothesize that this decrease in DONr rates might have been caused by the increasing availability of DIN and DON in the medium during the last days of Expt 1, and/or the simultaneous uptake of DON by *Cyanothece* during the experiment (Bronk & Glibert 1993). Similarly, Bronk (1999) observed a decrease in DONr rates in *Synechococcus* cultures when the cells became nutrient limited. *Cyanothece* cells were incubated under a 12:12 h light:dark regime, and DONr rates were measured during the dark phase. However, we were unable to quantify how much of the DON released during the dark phase was taken up during the next light period.

The percentages of fixed N_2 released as DON obtained in this study are low (~1% of total N_2 fixation), even lower than those obtained with cultures of *Trichodesmium* IMS101 (~8%; Mulholland et al. 2004), and much lower than in field studies of *Trichodesmium* (~50%; Glibert & Bronk 1994). To our knowledge, there are no reports on DONr by unicellular diazotrophs (Mulholland 2007). Recently, we measured DONr by the <10 μm planktonic fraction in surface waters of the North Atlantic Ocean and estimated that on average ~20% of the recently fixed N_2 was released extracellularly as DON (M. Benavides unpubl.). The <10 μm fraction in natural waters may contain a variety of different diazotrophs, probably mostly UCYN-A and *Crocospaera*, which are dominant in this area of the Atlantic (Langlois et al. 2008). Unfortunately, we cannot discern which diazotrophs release DON and which do not from a <10 μm planktonic sample. However, this difference in DONr dynamics between natural and cultured unicellular diazotrophs is comparable to that observed by Glibert & Bronk (1994) and Mulholland et al. (2004) in natural and cultured populations of *Trichodesmium*, respectively.

The constantly growing evidence of the diversity and wide distribution of oceanic unicellular diazotrophs emphasizes the need for accurate N_2 fixation rates and to provide better estimates of the release of DON by these organisms. The lack of carbon fixation activity in UCYN-A makes them depend on compounds produced by other organisms (Tripp et al. 2010), so it is unlikely that they release any of their recently fixed N_2 unless they are under some sort of environmental or predatory stress. Recent research on extracellular polysaccharide production by *Crocospaera* (Sohm et al. 2011) suggests that their DONr dynamics in the ocean could be important.

We chose *Cyanothece* as a model organism because it is easier to grow in culture, facilitating experiments. This diazotroph is similar to *Crocospaera* in size (~2 to 6 μm) and in nitrogenase activity patterns. Thus, their organic matter excretion metabolism could indeed be comparable. However, these organisms belong to different UCYN groups. *Crocospaera* belongs to UCYN-B (Zehr et al. 2001), while *Cyanothece* is a polyphyletic group, and currently we do not know with certainty whether strain Miami BG 043511 belongs to group C (Taniuchi et al. 2012). UCYN-A and *Crocospaera* are much more abundant in the open ocean than *Cyanothece* (Luo et al. 2012). Therefore, DONr should be measured in these other species before we draw any conclusion of the importance of DONr in underestimating global N_2 fixation rates.

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