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Assessment of the dinitrogen released as ammonium and dissolved organic nitrogen by unicellular and filamentous marine diazotrophic cyanobacteria grown in culture

Hugo Berthelot[1]*, *Sophie Bonnet*[1,2], *Mercedes Camps*[1,2], *Olivier Grosso*[1] and *Thierry Moutin*[1]

[1] Aix Marseille Université, Centre National de la Recherche Scientifique/INSU, Université de Toulon, IRD, Mediterranean Institute of Oceanography, Marseille, France, [2] Mediterranean Institute of Oceanography – IRD/Centre National de la Recherche Scientifique/Aix-Marseille University, IRD, Noumea, New Caledonia

The dinitrogen (N_2) fixed by diazotrophs and released as dissolved nitrogen (DN) has been compared in batch cultures of four marine diazotrophic cyanobacteria: the colony forming *Trichodesmium* IMS101 and the unicellular strains *Cyanothece* ATCC51142, *Crocosphaera watsonii* WH8501 and WH0003. Two approaches were conducted for this purpose. The first approach consisted in the comparison of the total accumulation of fixed N_2 in the culture medium (both in the dissolved and particulate pools) with the net N_2 fixation rates (i.e., the fixed N_2 incorporated only in the particulate fraction after $^{15}N_2$ incubation). The difference between the two measures accounted for the fixed N_2 released as DN. The second approach consisted in the direct measure of the ^{15}N-enrichment of ammonium (NH_4^+) and dissolved organic N (DON) following $^{15}N_2$ incubations. The fixed N_2 released as DN accounted for ~0–20% and ~1% of the fixed N_2 after 24 h in the first and second approach, respectively. We show that the recent methodological improvements in the net N_2 fixation determination applied in this study tend to reconcile the two approaches that formerly led to contrasted values. However, the large analytical uncertainties of the first approach limit its reliability. Thus, the direct determination of the ^{15}N-enrichment of the dissolved pool remains the best tool to assess the fixed N_2 released in the DN pool, in particular as it allows shorter incubation times. There were no clear patterns detected between the filamentous *Trichodesmium* and unicellular strains, neither in terms of the amount of fixed N_2 released as DN nor in terms of the proportion of NH_4^+ relative to DON. This suggests that the release of fixed N_2 is a process shared among the filamentous and free living diazotrophs.

Keywords: N_2 fixation, excretion, dissolved organic nitrogen, *Trichodesmium, Cyanothece, Crocosphaera*

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***Correspondence:**
Hugo Berthelot, Aix Marseille Université, Centre National de la Recherche Scientifique/INSU, Université de Toulon, IRD, Mediterranean Institute of Oceanography, UM 110, Campus de Luminy, Bâtiment Oceanomed, 13288 Marseille, France
hugo.berthelot@mio.osupytheas.fr

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Introduction

The biological conversion of dinitrogen (N_2) to ammonium (NH_4^+) -referred to as N_2 fixation- is performed by organisms called diazotrophs, and represents the main external source of bioavailable nitrogen (N) to the global ocean (Deutsch et al., 2007; Gruber, 2008). This new N is thought to fuel up to 50% of new primary production in the tropical North Atlantic (Capone et al., 2005), North Pacific (Karl et al., 1997), and South Pacific (Moutin et al., 2008; Raimbault and Garcia, 2008) Oceans. The filamentous diazotrophic cyanobacterium *Trichodesmium* sp. has been the focus of most of the research on N_2 fixation until the 2000s, as it is conspicuous and easy to collect (Capone et al., 1997). However, molecular inquiries have revealed a wider diversity of marine diazotrophs (Zehr et al., 1998, 2001, 2003; Moisander et al., 2010). In particular, unicellular cyanobacterial diazotrophs (UCYN) are more abundant than *Trichodesmium* sp. at the global scale (Luo et al., 2012) and contribute at least as much as *Trichodesmium* sp. to N_2 fixation in several oceanic basins (Montoya et al., 2004; Bonnet et al., 2009; Benavides et al., 2011).

While important progress has been made over the last decades on determining the biogeographical distribution and controlling factors of diazotrophs in the global ocean, little is known about the fate of the recently fixed N_2 in marine ecosystems, its release into the dissolved pool, and its potential transfer to the pelagic food web. Most of the N_2 fixation estimates have been performed by measuring the ^{15}N-enrichment of the particulate organic N (PON) pool after incubations with $^{15}N_2$ (hereafter referred to as "net" N_2 fixation rates) according to the protocols described in Montoya et al. (1996), or more recently in Mohr et al. (2010) and Großkopf et al. (2012). In these methods, the filtrate is discarded, and the $^{15}N_2$ fixed and released in the dissolved pool as dissolved N (DN) is not taken into account. Previous field studies where ^{15}N-enrichments were measured both in the PON and DN pools (leading to "gross" N_2 fixation rates) indicate that the amount of fixed N_2 released in the DN pool at the end of the incubation accounts for 10% to more than 50% of gross N_2 fixation (Glibert and Bronk, 1994; Konno et al., 2010; Benavides et al., 2013b). This N release directly affects the ecosystems as the surrounding planktonic community can access to the DN released as ammonium (NH_4^+) or dissolved organic N (DON). As an example, in a semi-controlled competition experiment, *Cyanothece* sp. Miami BG 043511 transferred up to ∼90% of the recently fixed N_2 toward a non-N_2 fixing cyanobacteria (Agawin et al., 2007). The transfer of recently fixed N_2 by *Trichodesmium* sp. has also been evidenced in natural planktonic assemblage (Lee Chen et al., 2011; Bonnet et al., under revision) and is thought to support recurrent blooms of the harmful dinoflagellate *Karenia brevis* (Lenes and Heil, 2010; Mulholland et al., 2014). Ultimately, ^{15}N isotopic signature reveals the presence of diazotrophs derived N in DON (Meador et al., 2007), zooplankton (Montoya et al., 2002; McClelland et al., 2003; Mompeán et al., 2013) and in sediment traps of the oligotrophic open ocean (Karl et al., 1997), demonstrating the potential biogeochemical importance of diazotrophs in these vast oceanic regions.

The recently fixed N_2 released as DN reported in field studies has been related to dying diazotrophic cells, mainly through viral lysis (Fuhrman, 1999), sloppy feeding (O'Neil and Roman, 1992), and programmed cell death on decaying blooms of *Trichodesmium* sp. (Berman-Frank et al., 2004). However, studies performed on unialgal exponentially growing cultured diazotrophs reported that *Trichodesmium* sp. releases up to 80% of gross N_2 fixation, suggesting an endogenous active release or excretion mechanisms (Mulholland et al., 2004; Mulholland and Bernhardt, 2005; Benavides et al., 2013a). This active release of N appears to be counterintuitive due to the high energetic cost of N_2 fixation as compared to nitrate (NO_3^-) assimilation (Falkowski, 1983). Several explanations are detailed in the literature, such as the supply of N toward cells lacking the nitrogenase enzyme in the *Trichodesmium* sp. colonies through NH_4^+ (Mulholland and Capone, 2000; Mulholland et al., 2004) or amino acids excretion (Carpenter et al., 1992; Capone et al., 1994; Mulholland and Capone, 1999), or an extracellular N storage in the mucilage of the small colonies of *Gloeothece* (Flynn and Gallon, 1990). While these processes appear valuable for colonial diazotrophs, they are counterintuitive for the free living UCYN, as large N release would represent a net loss of N. Thus, contrasting patterns may be expected between UCYN and colony forming diazotrophs regarding the amount and dynamics of N released as DN. However, comparative studies are still lacking.

Interestingly, the literature reports a wide range of fixed N_2 released by diazotrophs, i.e., from ∼0% to more than 80% of the gross N_2 fixation. The lowest values are generally obtained by the direct measure of the ^{15}N-enrichment of both particulate and dissolved pools, while the highest values are generally obtained by an alternative indirect method consisting in comparing net and gross N_2 fixation rates, where the difference accounts for the fixed N_2 released in the DN. However, it has been recently shown that the method used to estimate net N_2 fixation rates were underestimated by a factor of 2–6 due to an incomplete and non-instantaneous dissolution of the $^{15}N_2$ gas used as a tracer (Mohr et al., 2010; Großkopf et al., 2012; Wilson et al., 2012). As a result, the proportion of the fixed N_2 released in the DN pool compared to the gross N_2 fixation is probably overestimated by the same extent. New experiments using actualized methods are thus needed to reassess the magnitude of the fixed N_2 released as DN and to reconcile the different approaches.

Here, the fixed N_2 released as DN has been measured and compared in four strains of unicellular and filamentous marine diazotrophs representing some of the major contributors to global N_2 fixation (Luo et al., 2012) grown in batch cultures. Two independent methodological approaches were used. In the first experiment the accumulation of total N (TN) in the culture medium over a period of 6 days, accounting for the gross N_2 fixation, was compared with net N_2 fixation using the actualized $^{15}N_2$ dissolution method. In the second experiment, the fixed N_2 released along a diel cycle was directly quantified in the filtrate by measuring the ^{15}N-enrichment of the DON and NH_4^+ pools after $^{15}N_2$-incubation. The two methods are compared and discussed together with the differences and similarities observed between the strains.

Materials and Methods

Culture Conditions and Experimental Setup

Four unialgal strains were studied: *Trichodesmium erythraeum* IMS101 (hereafter referred to as *Trichodesmium*), isolated in the North Tropical Atlantic (Prufert-Bebout et al., 1993), *Cyanothece* sp. ATCC51142 (hereafter referred to as *Cyanothece*), isolated in an intertidal marsh (Reddy et al., 1993), and two strains of *Crocosphaera watsonii*: WH8501, isolated in the South Atlantic Ocean (Waterbury and Willey, 1988), and WH0003, isolated in the North Pacific Ocean (Webb et al., 2009). The culture medium was composed of natural seawater collected in the oligotrophic South West Pacific Ocean and characterized by low dissolved inorganic N (DIN) and DON concentrations (lower than 0.1 and 4 μmol N L^{-1}, respectively). After collection, seawater was filtered (0.2 μm), autoclaved and amended with nutrients in the same proportion as for the YBC II medium (Chen et al., 1996), except for phosphate (PO_4^{3-}), which concentration was set at 10 μmol P L^{-1} instead of 50 μmol P L^{-1} in the original YBCII. Cultures were maintained in this medium in exponential growth at 27°C and 120 μmol photon m^{-2} s^{-1} on a 12 h light: 12 h dark cycle for at least 10 generations. Cultures were not axenic but standard sterilization procedures of all laboratory material and systematic manipulation under a laminar flow hood were done in order to keep a minimum level of bacterial contamination. This contamination was checked using flow cytometry three times during the experiment (days 3, 6, and 11) for each strain. Heterotrophic bacterial populations were generally more than 3 orders of magnitude less abundant than the diazotrophs populations and did not accumulate during the experiment. Assuming a bacterial cell content of 5.8 fg N $cell^{-1}$ (Fukuda et al., 1998), bacterial N biomass represented on average less than 0.05% ($n = 12$, range 0.01–0.5%) of the cultivated strains N biomass.

At the start of the monitoring (day 0), each of the four strains was distributed in three sterile polycarbonate 4.5 L bottles leading to a culture volume of 2.4 L in each bottle. *In vivo* chlorophyll *a* fluorescence was monitored every day at 2:30 pm local time for 13 days using a Trilogy fluorometer (Turner Designs) to determine the growth rates of each strain. Cells were gently mixed every day in order to avoid their adhesion to the sides of the bottles. Two different experiments (hereafter referred to as exp 1 and exp 2) were undertaken in order to quantify the recently fixed N_2 released in the DN pool. Exp 1 consisted in the comparison of the cumulative net N_2 fixation (using 24 h based $^{15}N_2$ incubations) with the total N (TN) accumulation (accounting for the cumulative gross N_2 fixation) where TN is defined as the sum of PON, DON, NH_4^+, and NO_x (nitrate + nitrite). Exp 1 lasted for 6 days (from day 0 to day 6) in order to be able to measure significant deviation between net and gross N_2 fixation. Every day, 110 mL of culture from each of the 4.5 L bottle were collected and apportioned as follows: 60 mL were devoted to the measure of N_2 and carbon (C) fixation rates and the concentration of particulate organic C (POC) and PON, 20 mL were devoted to the measure of DON, 10 mL to the measure of NO_x and PO_4^{3-} and 20 mL to the measure of NH_4^+.

Exp 2 consisted in measuring the ^{15}N-enrichment of the NH_4^+ and DON pools following $^{15}N_2$ incubations over a diel cycle. It was performed in the middle of the exponential growth phase, i.e., at day 6 for *Cyanothece* and *C. watsonii* WH0003 and at day 11 for *Trichodesmium* and *C. watsonii* WH8501 (**Figure 1**). The start of the monitoring was performed at the beginning of the N_2 fixing period (light period for *Trichodesmium* and dark period for UCYN strains). Just before the start of the N_2 fixing period, about 1.3 L from each triplicate culture vessel was distributed in nine sterile polycarbonate culture flasks (160 mL) resulting in 27 flasks for each strain. Each flask was $^{15}N_2$-labeled according to the method described below and incubated under the same conditions as the parent culture. Every 3 h, a set of triplicate flasks was sacrificied for the following measurements: *in vivo* chlorophyll *a* fluorescence, inorganic nutrients, PON, DON, N_2, and C fixation rates, and ^{15}N-enrichment of the NH_4^+ and DON pools.

It has to be noticed that these experiments quantify the amount of N_2 that has been fixed and then released as DN within the time of incubation (<24 h), such that DN accumulation resulting from this recent flux could be discriminated from bulk DN. Thus, the measure has to be discriminated from the total DN accumulation in the culture medium.

Dissolved Organic and Inorganic Nutrients Measurements

Samples for nitrite (NO_2^-), NO_3^-, and PO_4^{3-} determination were collected in HCl-washed 20 mL vials, poisoned with $HgCl_2$ to a final concentration of 10 μg L^{-1}, and stored at 4°C until analyses performed on a segmented flow auto-analyzer (Aminot and Kérouel, 2007). The detection limit was 0.05 μmol N L^{-1} for NO_x and 0.01 μmol P L^{-1} for PO_4^{3-}. Samples for NH_4^+ determination were collected in 40 mL glass vials and directly analyzed according to Holmes et al. (1999) on a Trilogy fluorometer (Turner Designs). The detection limit was 0.01 μmol N L^{-1}. Samples for DON were collected in 40 mL glass vials,

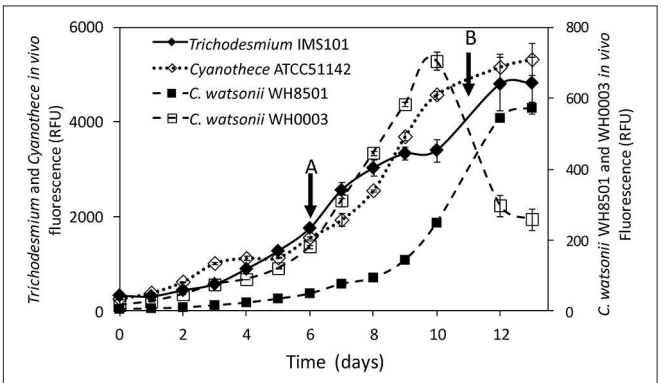

FIGURE 1 | Evolution of the *in vivo* Chl *a* fluorescence (in relative fluorescence units) in *Trichodesmium* IMS101, *Cyanothece* ATCC51142, *C. watsonii* WH8501 and WH0003 cultures. Error bars represent the standard deviation of triplicate measurements and the propagated analytical error. Black arrows represent the start of the diel cycle experiments (exp 2): on day 6 in *Cyanothece* and *C. watsonii* WH0003 cultures (A), on day 11 in *Trichodesmium* and *C. watsonii* WH8501 cultures (B).

filtered on pre-combusted (450°C, 4 h) GF/F filters, stored at −20°C until analysis by the wet-oxidation method according to Pujo-Pay and Raimbault (1994). DON concentrations were corrected from the contribution of NH_4^+ and NO_x.

N_2 Fixation and Primary Production Rates

Net N_2 fixation rates were measured using the $^{15}N_2$-enriched seawater method (Großkopf et al., 2012). The enriched seawater was prepared using the culture medium described above, which was degassed for 1 h by circulating it through a degassing membrane (mini-module®, Membrana) connected to a vacuum pump (<850 mbar) at a rate of 280 mL min^{-1}. The degassed medium was then transferred to a 2 L gas tight Tedlar® bag using silicon tubing. 10 mL of $^{15}N_2$ (98.3 atom% ^{15}N, Cambridge Isotope Laboratories) was added to the bag and the bubble was "physically broken" until its complete dissolution. $^{15}N_2$ enriched medium was then added (5% vol:vol) to the 60 mL bottles for exp 1 and to the experimental 160 mL culture flasks for exp 2. In order to measure the $^{15}N_2$ enrichment of the medium, samples were collected from Tedlar® bags in Exetainer® vials previously He-purged in order to avoid contact with atmospheric N_2. These samples were analyzed on a Membrane Inlet Mass Spectrometer (MIMS) for the determination of the $^{30}N/^{28}N$ ratio (Kana et al., 1994) using natural seawater at equilibrium with the atmosphere as a reference. The measured $^{15}N_2$ enrichment in the Tedlar® bags was 84 ± 8% resulting in a final enrichment of 4.2 ± 0.4 atom% in the incubation bottles. Net primary production (C fixation) was measured using the ^{13}C labeling method by adding a $H^{13}CO_3^-$ solution to the experimental culture bottles together with $^{15}N_2$ enriched medium, resulting in a final calculated ^{13}C enrichment of 10 atom%. For exp 1, the experimental bottles were incubated for 24 h and filtered on precombusted (450°C, 4 h) GF/F glass fiber filters (Whatman®). For exp 2, the triplicate experimental bottles were filtered every 3 h along the diel cycle and treated as described above. The PON and POC contents and the ^{15}N and ^{13}C enrichment of the cells were measured on an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS, Integra CN) calibrated using IAEA standards. The analytical precision associated with mass determination ranged between 0.2 and 2.8% of PON and between 0.8 and 4.8% of POC. The analytical precision associated with ^{15}N and ^{13}C enrichment was ± 0.0010 atom% and ± 0.0003 atom% for a measured mass of 0.7 µmol-N and 6.7 µmol-C, respectively. All the results were corrected from the blank contribution. Net N_2 fixation ($\rho N_{2,net}$) and net C fixation (ρC_{net}) rates were calculated as follows:

$$\rho N_{2,net} = \frac{\Delta R_{PON}}{R_{N_2}} \times \frac{[PON]}{\Delta t} \quad (1)$$

$$\rho C_{net} = \frac{\Delta R_{POC}}{R_C} \times \frac{[POC]}{\Delta t} \quad (2)$$

with ΔR_{PON} and ΔR_{POC} the differences in ^{15}N and ^{13}C atom% measured in the particulate matter between two time points, R_{N2} and R_C the ^{15}N and ^{13}C atom% in seawater during the incubation, [PON] and [POC] the PON and POC concentrations (µmol L^{-1}) at the given time point, and Δt the time between each sampling point. For exp 1, the cumulated net N_2 fixation was calculated as the sum of all the daily-based $\rho N_{2,net}$. For exp 2, $\rho N_{2,net}$ and ρC_{net} were divided by the number of cells in the cultures in order to express the cellular N_2 fixation rates. The final analytical precision was calculated as the analytical precision of each term accumulated according to the propagation of errors law.

^{15}N-enrichment of NH_4^+ and DON Pools

The ^{15}N-enrichment of the NH_4^+ and DON pools during exp 2 was measured using the two steps ammonium diffusion method modified from Slawyk and Raimbault (1995) and Raimbault et al. (1999). At each of the 9 time points over the diel cycle, 100 mL of the filtrate from every 160 mL flask were collected in polyethylene tubes, poisoned with $HgCl_2$ (20 µg mL^{-1} final concentration), stored at 4°C in the dark, and transferred to 500 mL borosilicate bottles just before analysis. Briefly, during the first step, all the NH_4^+ was converted into NH_3 by adding MgO (baked at 450°C for 4 h) and then trapped on an acidified GF/C glass filters (50 µL H_2SO_4 0.5 N) suspended above the sample using a stainless hook attached to the cap. 1 µmol-N of non-labeled NH_4^+ was added as a carrier to provide enough PN for mass spectrometry analyses. After 1 week of incubation at 55°C with daily agitation, filters were recovered, dried at 60°C for 24 h and stored in precombusted (450°C, 4 h) glass vials until analysis by EA-IRMS as described above for N_2 fixation. During the second step, the DON was converted to nitrate (NO_3^-) by wet oxidation. 10 mL of a digestion mixture (60 g of $K_2S_2O_8$ dissolved in 1 L of NaOH 1.5 N) was added to the borosilicate bottles, which were then autoclaved at 120°C for 30 min. The NO_3^- resulting from the oxidation was reduced in NH_4^+ by adding 200 mg of Devarda's alloy (baked at 450°C for 4 h). The NH_4^+ was then recovered by repeating the first step adding 1.5 mL of NaOH 12.5 N instead of MgO. In this second step the use of a strong base (NaOH) compensated the acidity brought by the Devarda's alloy and insured basic conditions. $NO_3^- + NO_2^-$ were also recovered during this second step, but as they are not likely released by diazotrophs (thus not ^{15}N-enriched) and considering their low concentrations (<0.2 µmol N L^{-1}) relative to DON in the culture medium, they were not discriminated from the DON pool. All the results were corrected from the blank contribution. For each incubation time of exp 2, the total amount of N_2 fixed and incorporated as PON ($\sum N_2^{PON}_{fixed}$) or released as NH_4^+ ($\sum N_2^{NH4}_{released}$) and DON ($\sum N_2^{DON}_{released}$) was calculated as:

$$\sum N_2 = \frac{\Delta R_N}{R_{N_2}} \times [N] \quad (3)$$

with ΔR_N, the ^{15}N-enrichment of the PON, NH_4^+, or DON pools and [N], the PON, DON, or NH_4^+ concentrations. The recovery of the ^{15}N in the dissolved pool allowed the calculation of the gross N_2 fixation for each incubation time as:

$$\sum N_{2,gross} = \sum N_2^{PON}_{fixed} + \sum N_2^{NH4}_{released} + \sum N_2^{DON}_{released} \quad (4)$$

In order to evaluate the accuracy of the method, two tests were conducted. First, the ability of the method to recover expected

N content and ^{15}N-enrichment of the whole cultures was tested. For this purpose, the culture medium containing *Cyanothece* and *C. watsonii* WH0003 cells was sampled after 12 h and 24 h of $^{15}N_2$-incubation. The N content of the samples was extracted as described in the second step of the diffusion method (Slawyk and Raimbault, 1995). The resulting $TN_{diffused}$ concentration was compared to the TN obtained from the sum of PON, DON, NH_4^+, and NO_x concentrations individually determined. In addition, the ^{15}N-enrichment recovered allowed the direct determination of the gross N_2 fixation as follows:

$$\sum N_2{}^{direct}_{gross} = \frac{\Delta R_{TN}}{R_{N_2}} \times [TN] \quad (5)$$

with ΔR_{TN} the ^{15}N-enrichment of the diffused TN pool and [TN] the sum of the PON, DON, NH_4^+, and NO_x concentrations. The $\sum N_2{}^{direct}_{gross}$ was compared to the $\sum N_{2,gross}$ calculated at the same times points.

The second test consisted in the evaluation of the ability of the method to isolate the NH_4^+ pool from the alkali-labile DON compounds. For this purpose several organic substrates (urea, creatine, and glutamic acid) were dissolved in 100 ml of filtered (0.2 µm) seawater (30 µmol N L^{-1} final concentrations) and processed as described in the first step of the diffusion method. The recovery of these compounds was compared to the recovery of NH_4^+ diffused in the same conditions.

An isotopic fractionation occurs during the diffusion of the NH_4^+ which tends to decrease the final ^{15}N-enrichment (Holmes et al., 1998). According to Holmes et al. (1998), the high N recovery during the NH_4^+ diffusion step (>95%) and the relatively small volumes used for the diffusion in our study limited the isotopic fractionation. The latter was thus neglected.

Results

The four strains exhibited an increase of *in vivo* chlorophyll *a* fluorescence along the 13 days of monitoring (**Figure 1**). The average growth rates calculated for each strain during the exponential growth phase were 0.30 d^{-1}, 0.26 d^{-1}, 0.37 d^{-1}, and 0.38 d^{-1} for *Trichodesmium, Cyanothece*, *C. watsonii* WH8501, and WH0003 cultures, respectively.

Fixed N_2 Released as DN Estimated from the Mass Balance Approach (Exp 1)

PON and DON concentrations continuously increased in all cultures during exp 1, while NH_4^+ concentrations remained below 0.5 µmol N L^{-1} during the 6 days of the experiment and did not show any clear pattern (**Figure 2**). The resulting increase of TN after the 6 days of the experiment was 82.7 ± 3.8 µmol N L^{-1}, 102.3 ± 2.5 µmol N L^{-1}, 37.0 ± 2.4 µmol N L^{-1}, and 88.9 ± 4.5 µmol N L^{-1} in *Trichodesmium, Cyanothece*, *C. watsonii* WH8501, and WH0003 cultures, respectively. In the meantime, net N_2 fixation rates cumulated over the 6 days of exp 1 reached 78.3 ± 9.9 µmol N L^{-1}, 84.2 ± 9.7 µmol N L^{-1}, 30.8 ± 5.2 µmol N L^{-1}, and 80.2 ± 11.0 µmol N L^{-1} in the cultures as cited above. Even though the two parameters followed the same pattern, the accumulation of TN was higher than the cumulated net N_2 fixation at the end of exp 1 in all strains

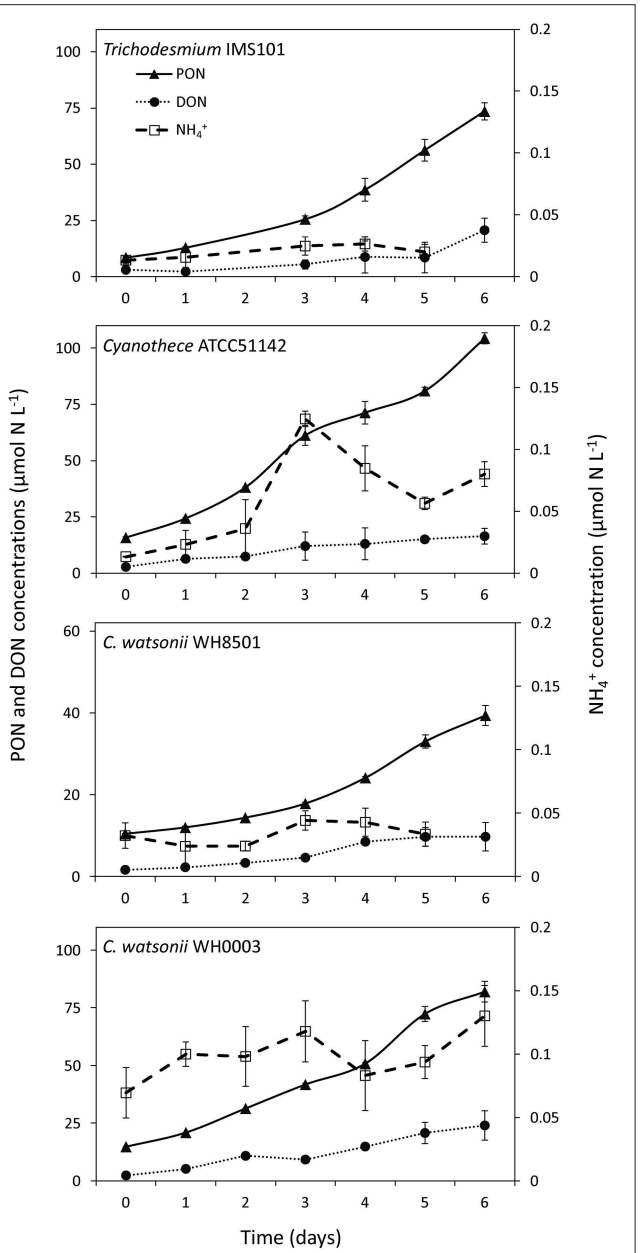

FIGURE 2 | Evolution of Particulate Organic Nitrogen (PON), Dissolved Organic Nitrogen (DON) and ammonium (NH_4^+) concentrations during exp 1. Error bars represent the standard deviation of triplicate measurements and the propagated analytical error.

(**Figure 3**). The difference accounted for the fixed N_2 released as DN during the 24 h of incubation and represented 5 ± 13, 18 ± 12, 17 ± 18, and 10 ± 15% of the gross N_2 fixation in *Trichodesmium, Cyanothece*, *C. watsonii* WH8501, and WH0003 cultures, respectively (**Table 1**). At the end of exp1, due to the large analytical uncertainties, the proportion of fixed N_2 released as DN was significantly higher than zero only in the *Cyanothece* culture (one-tailed *t*-test, $\alpha = 0.05$, $n = 3$). Furthermore, there were no statistically significant differences in fixed N_2 released as DN between the four strains (two-tailed *t*-test, $\alpha = 0.05$).

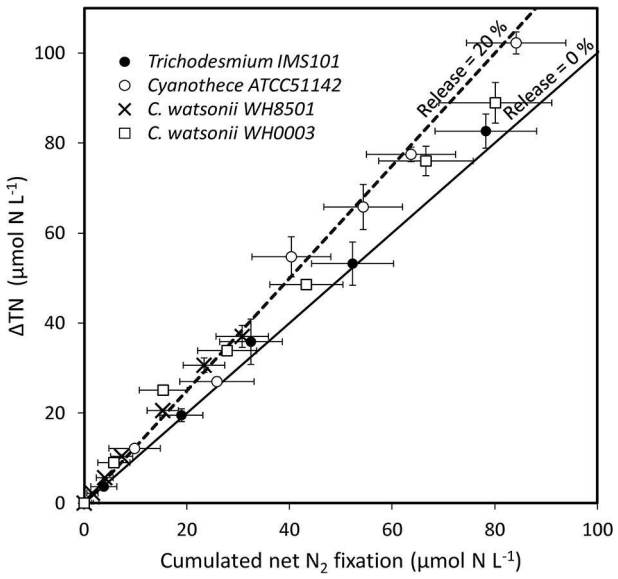

FIGURE 3 | TN accumulation (ΔTN), accounting for gross N_2 fixation, as a function of cumulated incorporation of N_2 in the PON, accounting for net N_2 fixation, in the culture media during exp 1. Error bars represent the standard deviation of triplicate measurements and the propagated analytical error. The lines show the evolution of the system assuming a proportion of fixed N_2 released as DN accounting for 0% (solid line) and 20% (dotted line) of the gross N_2 fixation.

During exp 1, the cellular C:N ratios in the studied strains were relatively stable and averaged 4.7, 7.7, 9.2, and 12.3 in *Trichodesmium*, *Cyanothece*, *C. watsonii* WH8501, and WH0003 cultures, respectively (**Table 2**). The C:N_2 fixation ratio clearly increased for all strains by a factor of up to 4, during the 6 days of incubation (**Table 2**).

Fixed N_2 Released as DN Measured from the ^{15}N-enrichment of DN (Exp 2)

During exp 2, C and N_2 fixation rates were followed over the diel cycle. N_2 fixation started at the beginning of the light period in *Trichodesmium* culture, peaked at midday at 8.5 ± 1.0 fmol N cell^{-1} h^{-1} (assuming 100 cells per trichome), and stopped at the beginning of the dark period (**Figure 4**). C fixation followed a similar pattern and peaked around midday at 223.0 ± 65.0 fmol C cell^{-1} h^{-1} (**Figure 4**). Conversely, N_2 and C fixation rates in the UCYN cultures were time-decoupled. In *Cyanothece*, *C. watsonii* WH8501 and WH0003 cultures, N_2 fixation started at the beginning of the dark period and peaked between 6 and 9 h at 1.3 ± 0.1 fmol N cell h^{-1}, 0.6 ± 0.1 fmol N cell h^{-1} and 2.5 ± 0.7 fmol N cell h^{-1}, respectively (**Figure 4**). C fixation occurred during the light period and peaked at 44.2 ± 16.1 fmol C cell h^{-1}, 14.2 ± 3.0 fmol C cell h^{-1}, and 68.6 ± 24.0 fmol C cell h^{-1} in *Cyanothece*, *C. watsonii* WH8501, and WH0003 cultures, respectively (**Figure 4**).

Although variable, DON concentrations did not show any clear pattern along the diel cycle with concentrations ranging from 6 to 19 µmol N L^{-1} (**Figure 5**). NH_4^+ concentrations ranged from 0.09 to 0.19 µmol N L^{-1}, respectively in all cultivated strains, except in the *C. watsonii* WH0003 culture, where NH_4^+ concentrations peaked after the beginning of the N_2 fixing period (dark) at 0.43 ± 0.29 µmol N L^{-1} (**Figure 5**). In the *Trichodesmium* culture, the ^{15}N-enrichments of the DON (R_{DON}) and NH_4^+ (R_{NH4}) pools during the diel cycle followed the enrichment of the PON pool (R_{PON}), i.e., it increased during the N_2 fixing period and remained stable during the non N_2 fixing period (Table S1). However, R_{DON} remained 6–20 times lower than R_{PON}, whereas R_{NH4} reached and exceeded the value of R_{PON} at the end of the N_2 fixing period. Similarly, in the UCYN cultures, the ^{15}N-enrichment of the dissolved pools also increased along the diel cycle and R_{DON} remained lower than the R_{PON}, whereas R_{NH4} equaled or exceeded R_{PON} (Table S1). For all the strains, due to the low NH_4^+ concentrations, the fixed N_2 released as NH_4^+ represented less than 0.2% of gross N_2 fixation after 24 h of incubation (**Figure 5**, Table S2). Meanwhile, the fixed N_2 released as DON did not exceed 1.6% of gross N_2 fixation (**Figure 5**, Table S2). Most of the fixed N_2 released as DN (sum of DON and NH_4^+) was in the form of DON (76–90%) in all cultures. These results indicate that while the proportion of the fixed N_2 released increased over the diel cycle in the *Trichodesmium* culture, it was maximum for UCYN strains at the beginning of the N_2 fixing period (in particular for *Cyanothece,* **Figure 5**) and tended to decrease afterwards. Nevertheless, these peaks have to be moderated by their low values and large uncertainties. No statistical significant differences were found between the strains, neither in terms of fixed N_2 released as DN (two-tailed *t*-test, $\alpha = 0.05$, $n = 6$) nor in terms of proportion of fixed N_2 released as NH_4^+ vs. DON (two-tailed *t*-test, $\alpha = 0.05$, $n = 6$) after 24 h of incubation. Our results also indicate that the proportion of fixed N_2 released as DN after 24 h of incubation for each of the strains studied was not significantly different between exp 1 and exp 2 (two-tailed *t*-test, $\alpha = 0.05$, $n = 6$).

Accuracy of the Diffusion Method

The tests conducted to evaluate the accuracy of the diffusion method indicate that more than 95% of the NH_4^+ were recovered after the first step of the diffusion (**Table 3**). The recovery of the whole N culture content after all steps (wet oxidation, conversion of NO_3^- into NH_3, and diffusion on the filter) ranged from 67 to 91% (78% on average; **Figure 6**). The method reproduces well the expected ^{15}N-enrichment as, on average, the $\sum N_{2gross}^{direct}$ gave similar results than the $\sum N_{2,gross}$ (**Figure 6**). The first step of the diffusion method had a limited impact on the hydrolysis and further diffusion of the various DON compounds tested (**Table 3**). Indeed, while the recovery of NH_4^+ was >95%, <12% of the DON compounds tested were hydrolyzed and diffused.

Discussion

Diazotrophs can fix atmospheric N_2 at high rates, and introduce new N into nutrient depleted waters of the ocean. The release of a high proportion of recently fixed N_2 to the DN pool has long been suggested based on the observed accumulation of DN during *Trichodesmium* sp. blooms (Devassy et al., 1979; Karl et al., 1997; Lenes et al., 2001). Most of the culture experiments have focused on *Trichodesmium* sp., but very few data are available for UCYN

TABLE 1 | Summary of the fixed N_2 released as DN, NH_4^+, or DON reported for diazotrophs in the literature and in this study (in percentage of the gross N_2 fixation) as a function of the methodology used.

Strain	Targeted compound	% fixed N_2 released	Method	References
CULTURES				
net $^{15}N_2$ fixation vs. gross N_2 fixation				
Trichodesmium sp.	DN	68 (46–82)[a]	bub. $^{15}N_2^b$ vs. ARA[c]	Mulholland, 2007
Trichodesmium IMS101 (continuous culture)	DN	79–90	bub. $^{15}N_2^b$ vs. ARA[c]	Mulholland and Bernhardt, 2005
Trichodesmium IMS101	DN	5 ± 13	diss. $^{15}N_2^d$ vs. ΔTN[e]	This study
Cyanothece ATCC51142	DN	18 ± 12	diss. $^{15}N_2^d$ vs. ΔTN[e]	This study
C. watsonii WH8501	DN	17 ± 18	diss. $^{15}N_2^d$ vs. ΔTN[e]	This study
C. watsonii WH0003	DN	10 ± 15	diss. $^{15}N_2^d$ vs. ΔTN[e]	This study
^{15}N recovery in dissolved pool				
Trichodesmium IMS101	DON	8.4 ± 10.4	Bronk and Glibert, 1991	Mulholland et al., 2004
Trichodesmium IMS101	NH_4^+	4.7 ± 5.5	Dudek et al., 1986	Mulholland et al., 2004
Cyanothece sp. Miami BG043511	DON	1.0 ± 0.3	Slawyk and Raimbault, 1995	Benavides et al., 2013a
Trichodesmium IMS101	DON	1.0 ± 0.5[f]	Slawyk and Raimbault, 1995	This study
Cyanothece ATCC51142	DON	1.6 ± 0.7[f]	Slawyk and Raimbault, 1995	This study
C. watsonii WH8501	DON	0.5 ± 0.1[f]	Slawyk and Raimbault, 1995	This study
C. watsonii WH0003	DON	0.5 ± 0.5[f]	Slawyk and Raimbault, 1995	This study
Trichodesmium IMS101	NH_4^+	0.1[f]	Slawyk and Raimbault, 1995	This study
Cyanothece ATCC51142	NH_4^+	0.2[f]	Slawyk and Raimbault, 1995	This study
C. watsonii WH8501	NH_4^+	0.1[f]	Slawyk and Raimbault, 1995	This study
C. watsonii WH0003	NH_4^+	0.1[f]	Slawyk and Raimbault, 1995	This study
FIELD				
net $^{15}N_2$ fixation vs. gross N_2 fixation				
Trichodesmium sp. colonies	DN	40[a]	bub. $^{15}N_2^b$ vs. ARA[c]	Mulholland, 2007
^{15}N recovery in dissolved pool				
Trichodesmium sp.	DON	50	Bronk and Glibert, 1991	Glibert and Bronk, 1994
Bulk sea water	DON	14–23	Sigman et al., 2001	Benavides et al., 2013b
Bulk sea water	DN	50 (10–84)	Tsunogai et al., 2008	Konno et al., 2010
Aphanizomenon sp. (light incubation)	NH_4^+	35	Warembourg, 1993	Ploug et al., 2010

[a] *Average and range of the studies reviewed in Mulholland (2007).*
[b] *Net N_2 fixation from the $^{15}N_2$ bubble method (Montoya et al., 1996).*
[c] *Gross N_2 fixation from acetylene reduction assay (ARA).*
[d] *Net N_2 fixation rates from the $^{15}N_2$ dissolution method (Mohr et al., 2010).*
[e] *Gross N_2 fixation from the TN accumulation in the batch cultures (ΔTN).*
[f] *After 24 h of incubation.*

(**Table 1**). Here, are discussed and compared the results of fixed N_2 released in the DN pool obtained using two independent approaches in four cultures of representative marine diazotrophs grown under identical conditions.

Methodological Considerations Regarding the Measure of the Fixed N Release

The measure of the fixed N_2 released by diazotrophs is affected by a number of methodological issues that needs to be mentioned prior to any physiological or ecological interpretation. In the literature, the values of fixed N_2 released as DN appear to be closely related to the methodological approach used (**Table 1**). The methods used can be separated into two categories: 1/ the comparison of net and gross N_2 fixation rates assessed by acetylene reduction assays (ARA) or the accumulation of TN in the batch cultures and 2/ the direct measurement of ^{15}N-enrichment in the DN pool following $^{15}N_2$ incubations (Mulholland et al., 2004; Benavides et al., 2013a, this study). Both approaches have methodological issues that are discussed below, together with recent insights taken into account in our study that tended to reconcile them.

The release estimated by the comparison of the gross and net N_2 fixation generally yield higher values as compared to the ^{15}N isotopic determination of DN (**Table 1**). Recent insights showed that the $^{15}N_2$ bubble method widely used (Montoya et al., 1996) underestimates net fixation rates by a factor of 2–6 (Mohr et al., 2010; Großkopf et al., 2012; Wilson et al., 2012) that may overestimate the release to the same extent. The large proportion of fixed N_2 released as DN obtained from previous comparisons of the gross and net N_2 fixation rates reported in **Table 1** (70–90% of the gross N_2 fixation) would be reduced to 21–27% if we consider a dissolution of 30% of the $^{15}N_2$ bubble during the 12 h of the fixing period as shown by Mohr et al. (2010). Another issue is the uncertainty of the gross rate when estimated from

TABLE 2 | Cellular C:N ratios and net C:N_2 fixation ratios along the 6 days of exp 1.

	Trichodesmium IMS101	*Cyanothece* ATCC51142	*C. watsonii* WH8501	*C. watsonii* WH0003
Day	Cellular C:N ratio			
0	5.5 (1.6)	6.3 (1.2)	8.8 (5.0)	13.0 (1.4)
1	5.3 (0.9)	7.2 (0.9)	12.9 (0.7)	12.4 (1.2)
2		7.5 (0.0)	12.2 (0.3)	12.5 (1.3)
3	4.9 (1.1)	8.4 (0.8)	7.5 (3.3)	13.0 (0.7)
4	4.5 (0.5)	9.7 (0.5)	9.8 (2.1)	13.1 (1.5)
5	3.8 (0.1)	7.0 (1.3)	5.6 (0.3)	10.1 (0.1)
6	4.3 (0.0)	11.3 (0.4)	7.3 (0.8)	13.4 (0.2)
Day	C:N_2 fixation ratio			
0	1.8 (0.9)	5.9 (0.7)	3.6 (3.0)	7.8 (1.7)
1	2.4 (0.8)	5.6 (4.9)	9.4 (9.5)	9.9 (3.6)
2	NA	9.2 (0.3)	9.2 (0.7)	10.6 (1.6)
3	2.9 (0.1)	13.2 (11.7)	10.4 (4.4)	12.3 (0.6)
4	3.8 (0.3)	NA	12.0 (1.2)	10.4 (9.0)
5	4.1 (0.4)	9.8 (0.2)	10.9 (1.4)	14.6 (1.6)
6	5.6 (0.6)	NA	13.4 (1.0)	NA

Standard deviations of triplicate measurements cumulated with analytical precision are given in parentheses.

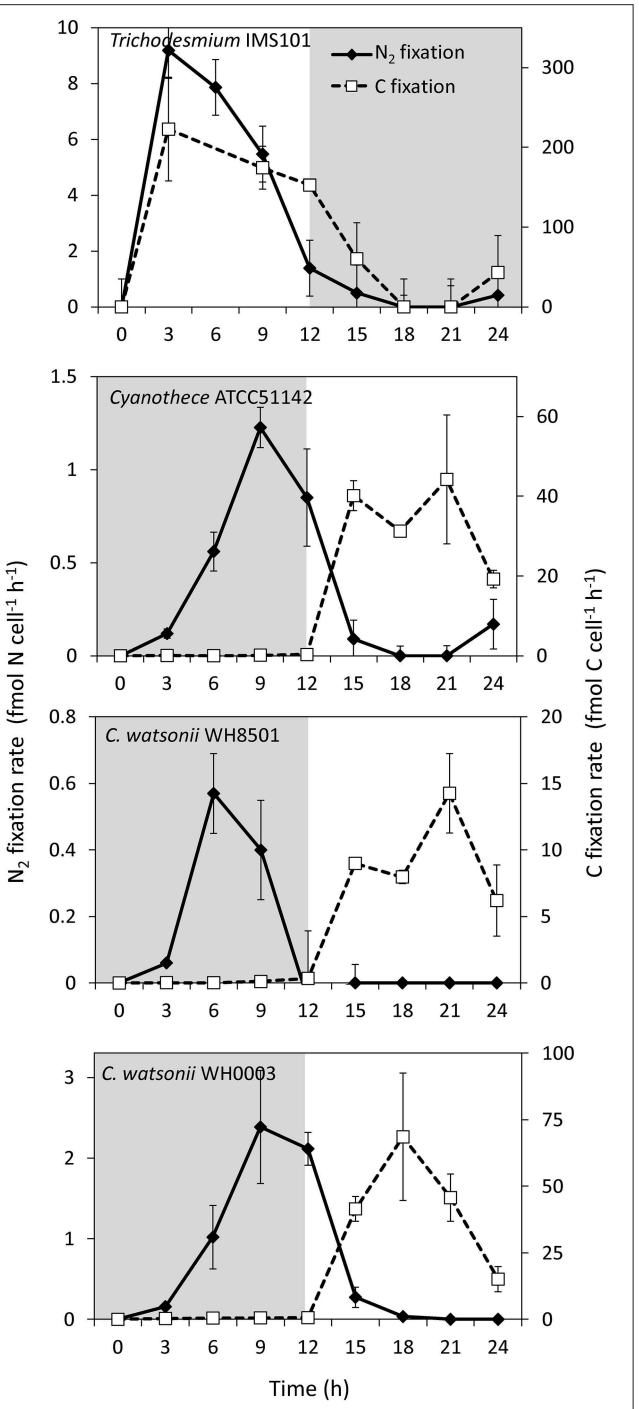

FIGURE 4 | Net N_2 and C fixation rates during exp 2 for each strain studied. Error bars represents the standard deviation of the triplicates cumulated with the analytical precision according to the propagation of errors. Gray areas represent the dark periods.

the ARA as the conversion factor between acetylene and N_2 is subjected to debate (from 3 to 4), which prevents any precise evaluation of the fixed N_2 released as DN (Mulholland, 2007). Consequently, the values of fixed N_2 released as DN estimated from the comparison of gross and net N_2 fixation rates should be considered with caution.

The recovery of the ^{15}N-enrichment in the DN pool (DON and NH_4^+) following $^{15}N_2$ incubations is the only way to directly measure the recently fixed N_2 released as DN. Two methods have been used to recover the DON: the DON separation from DIN using an ion retardation column (Bronk and Glibert, 1991), and the diffusion method (Slawyk and Raimbault, 1995, see Materials and Methods Section for details). Theoretically, both methods should be able to recover an important and representative fraction of the DON pool. However, none of the methods show 100% accuracy. Changes made in the manufacture of the retardation ion column have resulted in a variable DON isolation efficiency (McCarthy and Bronk, 2008), leading to the possibility of contaminations by DIN when using the new-build ions retardation resins. Similarly, the high temperature conditions (55°C) used for the extraction of the NH_4^+ associated with the diffusion method (Slawyk and Raimbault, 1995) hydrolyzes part of the labile DON pool leading to a biased estimate of the enrichment (McCarthy and Bronk, 2008). The tests conducted in this study showed that the hydrolysis of labile DON compounds during the NH_4^+ diffusion step occurs, albeit relatively low (<12%, **Table 1**). Further investigations should be conducted to estimate to which extent the accumulation of small fraction of hydrolyzed DON compounds may ultimately affect the measured ^{15}N-enrichment of the NH_4^+ pool. The isotopic signature recovered in both methods may thus not be equal, which hinders the direct comparison of the fixed N_2 released as DON between studies. Nevertheless, our results of fixed $^{15}N_2$ released as DON and NH_4^+ (1–8% of the gross N_2 fixation) are in the same range than the two studies that used the recovering ^{15}N in the dissolved pool for *Cyanothece* (∼1%)

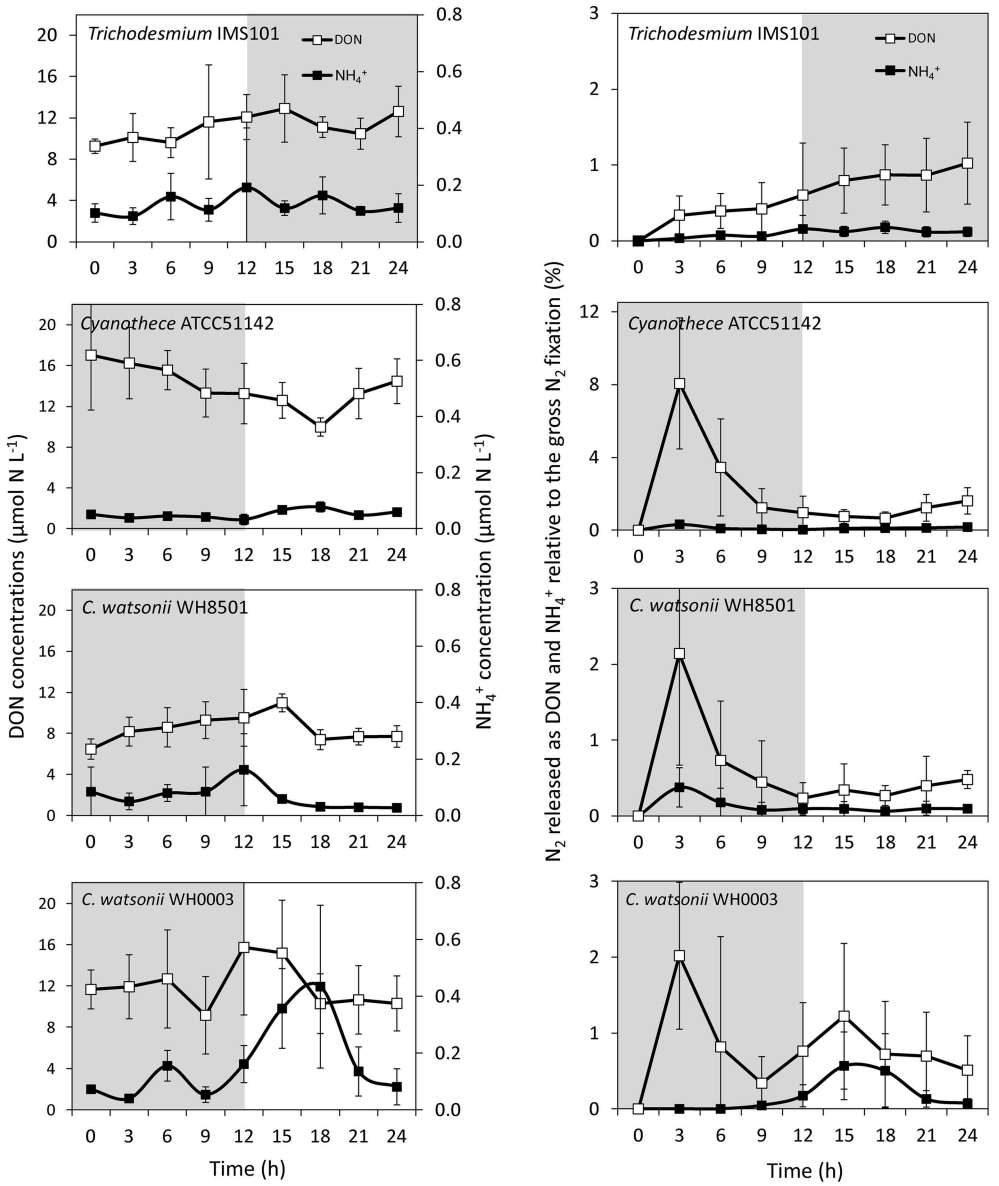

FIGURE 5 | Evolution of DON and NH_4^+ concentrations along the diel cycle (left panel) and proportion of fixed N_2 released as DON and NH_4^+ relative to gross N_2 fixation (right panel) as a function of the incubation time in each culture during exp 2. Error bars represent the standard deviation of triplicate measurements and the propagated analytical error. Gray areas represent the dark periods.

and *Trichodesmium* cultures (∼10%) (Mulholland et al., 2004; Benavides et al., 2013a).

In this study we used the $^{15}N_2$-enriched seawater method and measured the $^{15}N_2$ enrichment of the incubated medium (R_{N2}) in order to provide accurate estimates of net N_2 fixation rates. As a result, the differences between the fixed N_2 released as DN values calculated from the mass balanced approach (exp 1) used here and the ^{15}N recovery in the dissolved pool (exp 2) were reduced as compared to previous studies (**Table 1**). However, uncertainties in the determination of the R_{N2} and the propagations of the errors during the 6 days of exp 1 resulted in high uncertainties associated with the final estimates of fixed N_2 released as DN (about ±10% of the gross N_2 fixation). Alternatively, the direct recovery of the ^{15}N in the DN pool allows short incubation times and thus limits the propagation of errors. Furthermore, the final proportion of fixed N_2 released as DN compared to the gross N_2 fixation is not sensitive to the R_{N2}. Indeed, when calculating the proportion, Equation (3) is divided by Equation (4) and the R_{N2} is divided by itself. Thus, despite the recognized methodological bias, the ^{15}N recovery method appears to be the most accurate way to quantify the fixed N_2 released as DN both in culture and in field studies.

C and N Dynamics

The growth rates measured in this study are in good agreement with those reported in the literature for *Trichodesmium* sp. (LaRoche and Breitbarth, 2005), *Cyanothece* sp. (Feng et al., 2010), and *C. watsonii* sp. (Webb et al., 2009). During the diel cycle (exp 2), *Trichodesmium* net N_2 fixation rates peaked 3 h after the beginning of the light period, which is slightly earlier and in the lower end of rates (determined using ARA) previously reported (Mulholland and Bernhardt, 2005; Wannicke et al., 2009; Knapp et al., 2012). The magnitude of net N_2 fixation rates measured in the UCYN cultures are in the range of those reported in the literature determined using ARA (Dekaezemacker and Bonnet, 2011; Knapp et al., 2012; Masuda et al., 2013). The temporal uncoupling between C fixation (photosynthesis) and N_2 fixation observed over the diel cycle both in the cellular C:N ratio and fixation rates is well documented for *Cyanothece* (Sherman et al., 1998) and *C. watsonii* (Dron et al., 2012; Mohr et al., 2013). This decoupling prevents the inactivation of the nitrogenase by oxygen produced during photosynthesis (Fay, 1992; Gallon, 1992). The averaged cellular C:N ratios were different among the strains. *Trichodesmium* showed the lowest cellular C:N ratio (4.7 $\pm$ 0.6), which is at the lower end of the values reported in the literature for both culture and field experiments (LaRoche and Breitbarth, 2005; Mulholland, 2007; Holl and Montoya, 2008; Wannicke et al., 2009). In contrast, the relatively high cellular C:N ratio of the UCYN cultivated in this study (**Table 2**) is in the range of published ratios for *Cyanothece* (Benavides et al., 2013a) and in the high end for the two *C. watsonii* strains (Webb et al., 2009; Dekaezemacker and Bonnet, 2011).

Diazotrophs are known to exhibit a higher C:N_2 fixation ratio than that expected from their cellular C:N ratio or to the Redfield ratio of 6.6 (Mulholland, 2007). As an example, C:N_2 fixation ratio are on average 7 times higher than the Redfield ratio in the Sargasso Sea (Orcutt et al., 2001). Some investigators have suggested that the high C:N_2 fixation ratio in *Trichodesmium* colonies may be explained by an over C fixation to serve as carbohydrate for the ballasting effect (Villareal and Carpenter, 1990; Romans et al., 1994; White et al., 2012), or to reduce by respiration the oxygen concentration that would inhibit the nitrogenase activation. Alternatively, the discrepancy between fixation and biomass C:N ratios may be due to substantial fixed N_2 release into dissolved pool (Mulholland et al., 2004). Here, the use of the actualized $^{15}N_2$ net fixation rate method tended to bring closer the two ratios (**Table 2**) as compared to previous studies. This reduces the importance of the processes cited above, in particular the hypothesis of large fixed N_2 releases which is comforted by the low values reported here. Nevertheless, while the cellular C:N ratios of all the strains monitored here were relatively stable, the C:N_2 fixation ratios increased along exp 1, indicating a shift in the metabolism of N, C or both assimilation processes (**Table 2**). This has already been observed in batch cultures of *Trichodesmium* IMS101 (Mulholland and Capone, 2001) and *Cyanothece* sp. BG 043511 (Benavides et al., 2013a). It is possible that in response to the increase in DON availability (**Figure 2**), the organisms partly use this alternative source of N, leading to an increase in the C:N_2 fixation ratio. This is supported by studies that have measured significant uptake rates of DN compounds by *Trichodesmium* sp. (Mulholland and Capone, 1999; Mulholland et al., 1999; Orcutt

TABLE 3 | N recovered after the first step of the diffusion method for ammonium (NH_4^+) and three organic molecules solutions.

Treatment	N diffused (μmol N)	Recovery (%)
NH_4^+	2.88 (0.24)	95.9 (8.1)
Urea	0.11 (0.03)	3.7 (1.0)
Glutamic acid	0.36 (0.35)	12.1 (11.7)
Creatine	0.01 (0.02)	0.4 (0.7)

In each treatment 3 μmol N was added and let to diffuse for 1 week at 55°C (see Materials and Methods for details). Each treatment was performed in triplicates. Standard deviations are shown in parenthesis.

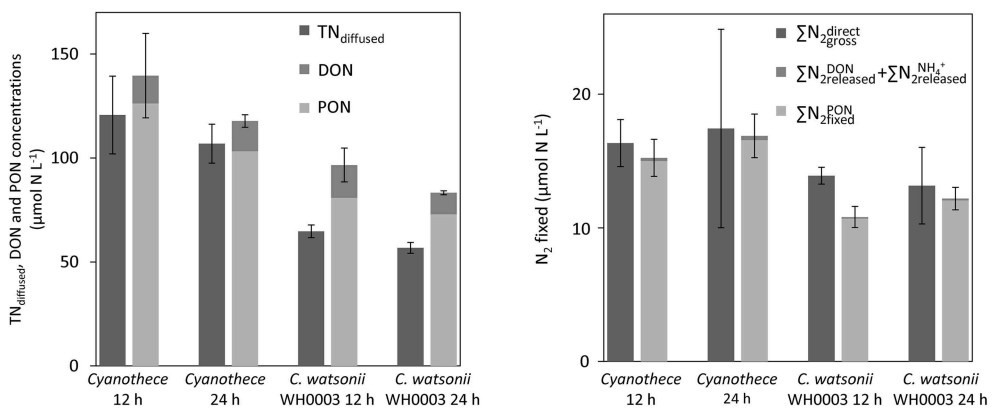

FIGURE 6 | Concentrations of TN recovered using the diffusion method ($TN_{diffused}$) and sum of PON and DON concentrations at the 12 h and 24 h time points in *Cyanothece* and *C. watsonii* WH8501 cultures (left). Gross N_2 fixation measured using the ^{15}N-enrichment recovered from the diffusion of the whole culture medium ($\sum N_2^{direct}_{gross}$) and as the sum of N_2 incorporated in PON ($\sum N_2^{PON}_{fixed}$) and released as DON ($\sum N_2^{DON}_{released}$) and NH_4^+ ($\sum N_2^{NH4}_{released}$) after 12 h and 24 h of incubation for *Cyanothece* and *C. watsonii* WH8501 (right). Error bars represent the standard deviation of triplicate measurements and the propagated analytical error.

et al., 2001; Holl et al., 2005) and *Aphanizomenon* sp. (Berman, 1999) cultures. Furthermore, changes in the ratio of N_2 fixation relative to NH_4^+ or DON uptakes with the different growth stages confirm that diazotrophs may adapt their N metabolism as a function of their environment (Mulholland and Capone, 2000, 2001).

Strong Similarities in the Release between the Filamentous and Unicellular Strains

Large differences in the N metabolism between the colony forming *Trichodesmium* and the free living UCYN strains were suspected to generate differences in the fixed N_2 release dynamics. Indeed, the slight but continuous increase of fixed N_2 release observed in the *Trichodesmium* culture contrasts with the peak observed at the beginning of the N_2 fixing period in the UCYN ones. It is possible that part of the recently fixed N_2 by UCYN during the dark period is stored in the extracellular pool before being metabolized during the photosynthesis period. However, the peaks of fixed N_2 release occurred at the very beginning of the N_2 fixation period, when the rates were still low (**Figure 4**). Thus, the absolute amount of fixed N_2 released is low compared to the total amount of N_2 fixed along the diel cycle and does not significantly differ from *Trichodesmium* after 24 h of incubation.

The low fixed N_2 release rates reported here seem to contradict the efficient transfer of fixed N_2 from diazotroph to non-diazotrophs observed in culture experiments (Agawin et al., 2007; Lee Chen et al., 2011). Nevertheless, the absence of ^{15}N accumulation in the DN pool does not neccesarly preclude intense N fluxes to transit through the dissolved pool. The measure of the fixed N_2 released as DN is the result of the recently fixed N_2 excreted out from the cells (from the intra- to the extra-cellular pools) but also of its subsequent uptake that would occur during the incubation time. Indeed, high NH_4^+ uptake rates are reported in *Trichodesmium* sp. (Mulholland and Capone, 1999, 2001; Mulholland et al., 2004) and *C. watsonii* cultures (Masuda et al., 2013). This might explain the absence of NH_4^+ accumulation in the DN pool in our cultures and the low release rates measured, but it also implies high NH_4^+ regeneration which is in good agreement with the high enrichment of the NH_4^+ pool measured in the present study (Table S2). The absence of significant differences in the release between the strains studied here suggests that the N release and regeneration occurs both for colonial/filamentous and free living diazotrophs. Thus, the release and the subsequent uptake of the recently fixed N_2 may be a way to exchange N not only for filamentous but also for unicellular and free living diazotrophs (Foster et al., 2013; Thompson and Zehr, 2013). It could also be due to the nitrogenase apparatus that is complex to modulate; the energetic cost of its flexibility could be higher than the cost of over-fixing N_2 (Dron et al., 2012). In addition, it is possible that the NH_4^+ regeneration is due to an unavoidable counter diffusion, i. e. the unionized NH_3, in equilibrium with NH_4^+, accumulated in the intracellular pool may diffuse toward the extracellular pool (Kleiner, 1985; Van Dommelen et al., 1998). High NH_4^+ uptake ability would thus prevent the diazotrophs to run out of N. Another factor that may explain efficient N_2 transfer despite low extracellular fixed N_2 releases rates is a high production of extracellular polysaccharides (EPS) as reported for *C. watsonii* (Sohm et al., 2011). Theses exudates, in which the cells are embeded, are retained on GF/F filters and may limit the diffusion of the released compounds toward the dissolved pool resulting in low fixed N_2 release as DN. However, in the field, the formation of aggregates through high EPS production may facilitate the transfer of N_2 from diazotrophs to non-diazotrophs through their spacial proximity (Paerl and Priscu, 1998; Foster et al., 2013; Klawonn et al., 2015).

Most of the fixed N_2 released as DN measured in this study was in the organic form (DON ∼80–90%). This suggests that a significant part of the fixed N_2 could be advantageous for heterotrophs through the microbial loop or phytoplankton capable of mixotrophy (Bronk et al., 2007). These results have to be taken with caution as the cultures were not axenic. Even though the bacterial contamination was limited (<0.05% of the total biomass in average), it could have played a potential role in the N turnover in the culture medium. It is possible that the released DON compounds were recycled as NH_4^+, which might prevent the accumulation of DON and thus reduce the release rates. Furthermore, part of the bacteria can pass through the GFF filter and affect the fixed N_2 released as DON. Nevertheless, despite present, no heterotrophic bacterial accumulations were observed during the course of the experiment and their low biomasses strongly suggest that the bacterial presence had a limited impact on the main conclusions of this study, namely low fixed N_2 releases in the DN.

The fixed N_2 released by diazotrophs reported in the literature is generally higher for field studies compared to culture studies (**Table 1**). Aside from the methodological bias, the physiological status of the cells is probably one of the main factors explaining this difference. Culture studies are generally performed during the exponential growth phase of cells, whereas in field studies, communities are probably composed of cells experiencing different phases of their cellular cycle that may affect the fixed N_2 released as DN (Mulholland et al., 2004). The release is also sensitive to a variety of exogenous factors occurring in the field such as intense viral lysis (Fuhrman, 1999; Hewson et al., 2004) and sloppy feeding (O'Neil and Roman, 1992; O'Neil et al., 1996), which are absent (or strongly limited) in cultures. Additionally, in the field, cells are not maintained in optimal growth conditions of temperature, nutrient and light availability, which may influence N_2 fixation rates and the quantity and quality of fixed N released. As an example, Wannicke et al. (2009) showed an increase of extracellular NH_4^+ concentrations in *Trichodesmium* IMS101 cultures exposed to drastic changes of light intensity, suggesting an increase of the fixed N_2 released as NH_4^+.

Conclusions

This study provides new insights on the recently fixed N_2 released as DN by different strains of diazotrophs and shows that, in culture, the release does not depend on the different diazotrophs strains considered. Indeed, no clear differences were

observed between filamentous and unicellular strains both in terms of quantity of fixed N_2 released as DN, and in terms of quality (DON vs. NH_4^+). The absence of significant differences suggests that release processes are shared among free living and filamentous diazotrophs. The review of previously published experiments shows that the two approaches commonly used lead to very different values of fixed N_2 released as DN. The more accurate determination of N_2 fixation rates applied here tended to reduce the gaps between both approaches and shows that the proportion of fixed N_2 released as DN is likely <10% of the gross N_2 fixation in diazotroph culture. Nevertheless, the N flux excreted out of the diazotroph cells might be higher due to probable NH_4^+ re-uptake. In natural assemblages, fixed N_2 excreted as DN may be efficiently used by the surrounding planktonic community, which is confirmed by multiple examples of cellular interactions between various diazotrophs and their close environment (Thompson and Zehr, 2013).

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Supplementary Material

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