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Diazotroph-derived nitrogen release and transfer under varying light intensity: insights from co-culture studies

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Biological dinitrogen (N_2) fixation is a major source of new N to surface seawater, sustaining ocean productivity. However, the fate of diazotroph-derived nitrogen (DDN), specifically its release and transfer, and the factors controlling these processes, remain poorly understood. Here, we established stable co-cultures of the major diazotrophs, filamentous *Trichodesmium erythraeum* IMS101 and unicellular *Crocosphaera watsonii* WH8501, with the pico-cyanobacterium *Synechococcus* sp. WH8102, to explore the intrinsic differences in DDN release and transfer between diazotroph strains. We found that *T. erythraeum* released similar amounts of DDN as *C. watsonii*, but had a significantly higher DDN transfer efficiency for supporting *Synechococcus* cell growth. These results implied a higher bioavailability of fixed N released by *T. erythraeum* than by *C. watsonii*. Additionally, we showed that elevated light levels significantly enhanced *T. erythraeum* DDN release and transfer. Our results provide new insights into the fate of N fixed by different diazotrophs and the environmental factors that control the process.

KEYWORDS

***Trichodesmium*, *Crocosphaera*, DDN release, DDN transfer, light**

1 Introduction

The availability of fixed nitrogen (N) is a key factor controlling phytoplankton growth throughout most of the oligotrophic oceans (Moore et al., 2013; Browning and Moore, 2023). In N-limited regions, diazotrophs convert the abundant N_2 gas into ammonia, providing significant bioavailable N that fuels primary production (Gruber and Galloway, 2008). Several studies have been conducted in recent decades to determine the biogeographical distribution and controlling factors of diazotrophs in the global oceans (Mills et al., 2004; Sohm et al., 2011; Zehr, 2011; Wen et al., 2022). However, less attention has been given to the fate of the diazotroph-derived nitrogen (DDN) in marine ecosystems, particularly its release into the dissolved pool and its potential transfer to the other pelagic

plankton. Understanding these processes and their controlling factors are essential for explaining the full impact of diazotrophs on marine N cycles, primary production, and carbon (C) export (Mulholland, 2007).

The filamentous cyanobacteria *Trichodesmium* and the free-living unicellular cyanobacterium (*Crocosphaera*) are the two main N_2-fixers throughout the (sub)tropical oligotrophic ocean (Capone et al., 1997; Masuda et al., 2024). However, studies of the release and transfer of diazotroph fixed N have predominantly focused on *Trichodesmium*, which has been reported to release 6–90% of its newly fixed N into the dissolved pool (Konno et al., 2010; Benavides et al., 2013b; Berthelot et al., 2016; Bonnet et al., 2016a; Berthelot et al., 2017; Caffin et al., 2018; Lu et al., 2018). Previous studies showed that only 15–20% of cells within a *Trichodesmium* trichome are capable of fixing N (diazocytes), while the remaining cells (vegetative cells) in a filament rely heavily on the bioavailable N supplied by the diazocytes (Berman-Frank et al., 2003; Mulholland et al., 2004). This indicates that the newly fixed N by diazocytes could be actively released into the surrounding environment and subsequently taken up by the vegetative cells and potentially other co-existing plankton (Mulholland et al., 2004). In addition, the release of DDN in natural water is not solely tied to active physiological processes but also results from dying diazotrophic cells through viral lysis, sloppy feeding, programmed cell death, etc (ONeil et al., 1996; Hewson et al., 2004; Berman-Frank et al., 2004). The released DDN can be subsequently used by other plankton in the surrounding water. For example, several studies performed in the western tropical South Pacific Ocean (WTSP) showed that 6–12% of *Trichodesmium* fixed N was transferred to non-diazotrophic plankton using nanometer scale secondary ion mass spectrometry (nanoSIMS) coupled with ^{15}N isotopic labelling and flow cytometry cell sorting (Bonnet et al., 2016c; Berthelot et al., 2016; Caffin et al., 2018).

Compared to *Trichodesmium*, less is known about the DDN release and transfer by *Crocosphaera*, which exhibits a distinctly different cell size, morphology and N_2 fixation pattern (Berman-Frank et al., 2004; Masuda et al., 2024). For example, *Crocosphaera* fixes N at night while *Trichodesmium* fixes N during the day. Moreover, *Crocosphaera* has a higher competitive capability for combining N (Masuda et al., 2022), potentially affect the utilization of its released DDN by other non-diazotrophs. Consequently, DDN fixed by *Crocosphaera* may have a distinctly different fate in the marine ecosystem to that released from *Trichodesmium*.

There have been few direct comparative studies of DDN release and transfer between *Trichodesmium* and *Crocosphaera*. A field study conducted in the WTSP found that ~20–40% of the fixed N was released to the dissolved pool when *Trichodesmium* dominated, while the DDN release was not quantifiable when *Crocosphaera* dominated (Caffin et al., 2018). In other studies, no significant difference was found in the DDN release (<10.3%) between *Trichodesmium* and *Crocosphaera* (Berthelot et al., 2015, 2016). For DDN transfer in artificially induced blooms, the efficiency of DDN transfer by *Crocosphaera* (4–5%) was only half that of *Trichodesmium* (~12%) (Berthelot et al., 2016). In contrast, field studies in similar regions reported higher transfer efficiencies for a *Crocosphaera*-dominated diazotroph community (15 ± 3%) than for a *Trichodesmium*-dominated diazotroph community (9 ± 3%) (Caffin et al., 2018). These contradictory results suggest that further comparative studies are needed to understand the intrinsic differences between the DDN release and transfer of the two types of diazotrophs.

Additionally, environmental factors such as temperature, nutrients, and light intensity regulate the N_2 fixation in *Trichodesmium* (Bell and Fu, 2005; Breitbarth et al., 2008), and thus may also impact DDN release and transfer. For example, a *Trichodesmium* culture study showed that exposure to high-light levels significantly enhanced the release of fixed N in the form of ammonium (NH_4^+) and dissolved organic N (DON) (Wannicke et al., 2009). However, field incubation experiments have shown that the percentage of DDN released into the dissolved phase increases with a decline in light intensity (Lu et al., 2018). These results indicate that changes in light intensity modulate the release of fixed N from diazotrophs. It is unclear why the results vary between culture and field studies, and further studies are therefore needed to confirm how changes in light intensity impact diazotrophic DDN release and transfer.

Here, we established a co-culture of diazotrophs (*Trichodesmium erythraeum* IMS101 and *Crocosphaera watsonii* WH8501) with a non-diazotrophic pico-cyanobacteria *Synechococcus* sp. WH8102 under various light intensities. The ecological niches of the two diazotrophs and *Synechococcus* partially overlap (Campbell et al., 2005; Flombaum et al., 2013; Shao et al., 2023). The aim was to determine the differences in DDN release and transfer between *T. erythraeum* and *C. watsonii*, and then investigate the effect of light intensity on these processes using simple co-culture systems in a laboratory setting. We found that *T. erythraeum* was more efficient in transferring DDN to *Synechococcus* than *C. watsonii*, although the overall release and transfer of DDN were not significantly different between the two stains. These results imply a higher bioavailability of released fixed N by *T. erythraeum* than *C. watsonii*. Additionally, we found that an increase in light intensity significantly enhanced DDN release and transfer by *T. erythraeum*.

2 Methods

2.1 Unialgal culture conditions

Two N_2-fixing cyanobacteria, *T. erythraeum* IMS101 and *C. watsonii* WH8501, along with one non- N_2-fixing pico-cyanobacterium, *Synechococcus* sp. WH8102, were cultured. *T. erythraeum* and *C. watsonii* were grown in Aquil-tricho medium (Hong et al., 2017) prepared with 0.22 μm-filtered and microwave-sterilized oligotrophic western North Pacific surface water. The medium was enriched with chelexed and filter-sterilized NaH_2PO_4, and supplied with filter-sterilized vitamins and trace metals, buffered with 5 μM EDTA. *Synechococcus* was also grown in Aquil-tricho medium but was enriched with 100 μM $NaNO_3$.

All algae were pre-adapted to a light intensity of 200 μE m^{-2} s^{-1} by semi-continuous culturing for more than six months. The light level was monitored using a spherical light meter (QSL-2100,

Biospherical Instruments Inc., San Diego CA, USA). The light intensity measured using the spherical light meter was approximately 2.5 times greater than that measured using a flat light meter (~80 μE m^{-2} s^{-1}). Cultures were maintained in the exponential growth stage at 27°C with a 14:10 h light–dark cycle in an algal growth chamber (AGC-850, Firstek Corp, China) before starting the co-culture. Strict sterile techniques were applied for culturing and experimental manipulations.

2.2 Co-culture setup

Exponentially growing *Synechococcus* was transferred to an inorganic-N-free Aquil-tricho medium for a 2-day N-starvation acclimation, during which cell growth ceased (Supplementary Figure S1). The N-starved *Synechococcus* was then transferred separately into exponentially growing *T. erythraeum* and *C. watsonii* cultures, and co-cultured under the same conditions as the monoculture of *T. erythraeum* and *C. watsonii*. To achieve the exponential growth of diazotrophs and *Synechococcus* in the co-culture systems, inoculation ratios were optimized in the preliminary experiments. The optimized inoculation ratios were *Synechococcus*: *T. erythraeum* = ~5–8:1 (cell number: cell number), and *Synechococcus*: *C. watsonii* = 0.003:1 (cell number: cell number), respectively, which ensured a similar growth rate of *Synechococcus* in both co-cultures (Table 1). To minimize the impact of the inoculation process, the volume of the *Synechococcus* culture transferred was kept to less than 7% of the *T. erythraeum* and *C. watsonii* culture volumes. In the co-culture system, N fixed by diazotrophs was the sole N source supporting *Synechococcus* growth. Both the diazotrophs and *Synechococcus* in the co-cultures continued exponential growth for at least 3 days (as shown in Figure 1), after which an aliquots of the co-cultures were spiked with $^{15}N_2$ gas (98.9 atom%, Cambridge Isotope Laboratories, Lot #: I-21065/AR0664758) and incubated for another 24 h to determine the total N_2 fixation rate of diazotrophs, as well as the DDN release to the dissolved phase and DDN transfer to *Synechococcus*.

To explore the impact of light intensity on DDN release and transfer, *T. erythraeum* and *Synechococcus* were adapted to a lower light intensity of 70 μE m^{-2} s^{-1}, and a higher light intensity of 750 μE m^{-2} s^{-1} for at least 3 months. Subsequently co-cultures of *T. erythraeum* and *Synechococcus* were established as described above. Additionally, to examine DDN release and transfer at an even higher light intensity, e.g., 1100 and 1800 μE m^{-2} s^{-1} (approximately equal to full noon sunlight on a cloudless day) (Lu et al., 2018), at which the stable exponential growth of *T. erythraeum* and *Synechococcus* could not be achieved due to a strong light inhibition effect, we conducted short-term (24-h) mixed incubations that were enriched with ^{15}N.

2.3 Chlorophyll a (Chl a), cell concentration, and growth rate

In the monoculture and co-cultures, *T. erythraeum* growth was monitored by daily measurements of the Chl a concentration. Briefly, *T. erythraeum* cells were collected by filtration onto 3-μm pore size polycarbonate membrane filters (PC, Millipore, Burlington, MA, USA). In the co-culture systems, because the cell size of *Synechococcus* was typically less than 1.5 μm, *Synechococcus* could not be trapped on the 3-μm PC filters. The filters were heated at 65°C for 6 min in 90% (vol/vol) methanol. After extraction, the filters were removed, and cell debris was pelleted by centrifugation. The Chl a concentration was subsequently determined by a spectrophotometric analysis following the method described by Demarsac and Houmard (1988). To count *T. erythraeum* cell numbers, photographs of *T. erythraeum* were taken using a camera (DS126281, Canon, Tokyo, Japan) connected to an inverted microscope (CKX41, Olympus, Tokyo, Japan). The total length of filaments in 1 mL culture were measured, and the cell number of ~20 filaments was counted. The average length of cells was obtained by dividing the total length of the measured filaments by their total cell number. The cell density of the culture was then calculated by dividing the total length of filaments in 1 mL culture by the average cell length. The Chl a per cell was calculated by dividing the Chl a concentration by the cell density.

The growth of *C. watsonii* and *Synechococcus* was monitored daily by measuring cell abundance using flow cytometry (Accuri™

TABLE 1 Growth rates (d^{-1}) of *Synechococcus* and two diazotrophs, *Trichodesmium erythraeum* and *Crocosphaera watsonii*, and the total N_2 fixation rates (pmol N $cell^{-1}$ d^{-1}) of the two diazotrophs in the *T. erythraeum–Synechococcus* and *C. watsonii–Synechococcus* co-culture systems under different light intensities (70, 200, and 750 μE m^{-2} s^{-1}).

Light (μE m^{-2} s^{-1})	Co-culture	n	Growth rate (d^{-1})		Total N_2 fixation rate (pmol $cell^{-1}$ d^{-1})	Initial inoculation ratio Syne: Diazotroph: (cell number: cell number)
			Diazotroph	Syne		
70	Tricho-Syne	2	0.32 ± 0.00	0.24 ± 0.07	0.18 ± 0.01	5~8: 1
200		3	0.43 ± 0.02	0.33 ± 0.03	0.31 ± 0.05	
750		2	0.34 ± 0.00	0.66 ± 0.03	0.23 ± 0.00	
200	Croco-Syne	4	0.40 ± 0.01	0.30 ± 0.02	0.02 ± 0.00	0.003: 1

To set up the co-culture systems in which diazotrophs and *Synechococcus* all sustained exponential growth, the initial inoculation ratios of *Synechococcus* versus diazotroph (cell number: cell number) were optimized in the preliminary experiments, and the optimized inoculation ratios used in this study were presented in the table. The total N_2 fixation rates are the total formation rates of diazotroph-derived nitrogen (DDN) in the co-culture systems, including both the particulate and dissolved fractions, and were normalized to diazotroph cell abundance (cell-specific, pmol N $cell^{-1}$ d^{-1}). Data are presented as the mean ± SD (n is the number of biological replicates).

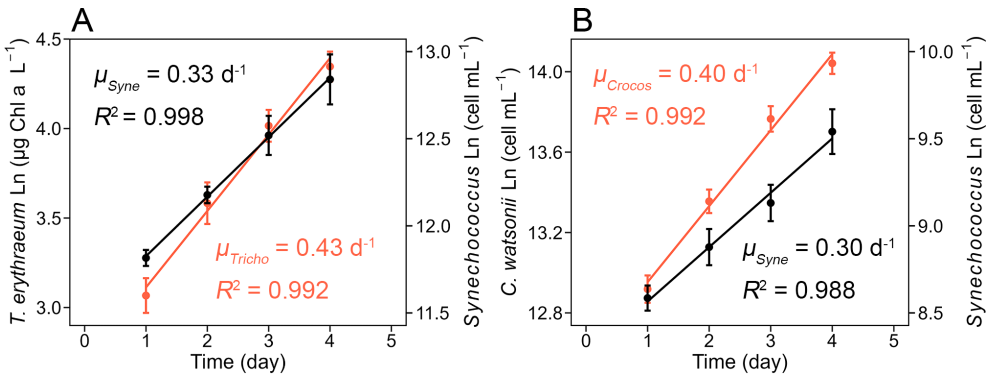

FIGURE 1
Exponential growth of *Trichodesmium erythraeum* IMS101, *Crocosphaera watsonii* WH8501, and *Synechococcus* sp. WH8102 in the *T. erythraeum*–*Synechococcus* and *C. watsonii*–*Synechococcus* co-culture systems under a light intensity of 200 μE m^{-2} s^{-1}. **(A)** Growth rates of *T. erythraeum* (μ_{Tricho}, red) and *Synechococcus* (μ_{Syne}, black) in their co-culture systems. **(B)** Growth rates of *C. watsonii* (μ_{Crocos}, red) and *Synechococcus* (μ_{Syne}, black) in their co-culture systems. The *T. erythraeum*–*Synechococcus* co-culture systems were set up at the optimized initial inoculation ratio of ~ 6:1 (*Synechococcus* cell number: *T. erythraeum* cell number), and *C. watsonii*–*Synechococcus* co-culture systems were 0.003:1 (*Synechococcus* cell number: *C. watsonii* cell number), to achieve the exponential growth of both strains. The growth of *T. erythraeum* was monitored daily by measuring the Chl a concentration, while the growth of *C. watsonii* and *Synechococcus* was monitored daily through the measurement of cell abundance. Error bars represent the standard deviation of biological replicates (n = 3 in the *T. erythraeum*–*Synechococcus* co-culture experiment, n = 4 in the *C. watsonii*–*Synechococcus* co-culture experiment).

C6, BD Biosciences, Franklin Lakes, NJ, USA). Briefly, samples were collected in 2 mL centrifuge tubes and preserved in freshly prepared 0.2-μm-filtered glutaraldehyde (0.5% vol/vol final concentration). After fixation in the dark for 15 mins, samples were frozen in liquid nitrogen and stored at -80°C until analysis. *Synechococcus* and *C. watsonii* cells were distinguished and quantified based on forward scatter and red fluorescence (670 nm).

Specific growth rates were calculated from the linear regressions of the natural logarithm of Chl a concentrations or cell densities versus time during the exponential growth phase. Each growth curve included four data points to ensure accuracy and reliability in the linear regressions.

2.4 Particulate N and C fixation rates

Particulate N_2 fixation was measured using the ^{15}N gas dissolution method (Mohr et al., 2010), while concurrently, a C fixation assay was conducted utilizing $NaH^{13}CO_3$ (99 atom% ^{13}C, Cambridge Isotope Laboratories, Cambridge, UK). The $^{15}N_2$ gas (98.9 atom%, Cambridge Isotope Laboratories, Lot #: I-21065/AR0664758) was tested and confirmed to be non-contaminated following the method of Yu et al. (2024). To prepare the ^{15}N-enriched water, 5 mL of $^{15}N_2$ gas was dissolved into 500 mL of degassed seawater. Incubations were conducted in duplicate in acid-cleaned 1-L Nalgene polycarbonate bottles. Each bottle was spiked with 30 mL of $^{15}N_2$-enriched water and $NaH^{13}CO_3$ solution to a final concentration of 200 μM, followed by incubation in an algae chamber for 24 h. The final ^{15}N enrichment [100×^{15}N/(^{15}N+^{14}N), atom%] of the N_2 pool in the incubation bottles was measured using a Membrane Inlet Mass Spectrometer (MIMS), yielding a value of 1.29 ± 0.11 atom% (n = 11). Then, cells were filtered onto 25 mm pre-combusted (450°C for 4 h) GF/75 filters (Advantec, Eden Prairie, MN, USA). Cells not enriched with ^{15}N and ^{13}C were also collected to establish the baseline enrichments for biomass ^{15}N and ^{13}C. All filters were acid fumed, dried, and then analyzed using an EA IsoLink™ IRMS system (Flash IRMS elemental analyzer coupled to a Delta V isotope ratio mass spectrometer, Thermo Fisher Scientific, Waltham, MA, USA).

The N and C contents of the GF/75 filter blanks were 0.06 ± 0.00 μmol and 1.16 ± 0.06 μmol, respectively, consistently lower than the N (>1 μmol) and C (>10 μmol) contents of the measured samples. The natural ^{15}N and ^{13}C enrichments of co-culture samples were ~0.366 atom% and ~1.081 atom%, respectively, which are significantly lower than the values in the samples spiked with $^{15}N_2$ and $NaH^{13}CO_3$ (^{15}N>0.654 atom%, ^{13}C>2.724 atom%). The average reproducibility of the ^{15}N and ^{13}C measurement of the USGS-40 standard was ± 0.0002 atom% and ± 0.0005 atom% (n = 18), respectively. The ^{15}N and ^{13}C fixation rates were calculated based on methods described by Montoya et al. (1996) and Hama et al. (1983), respectively.

2.5 Total N_2 fixation rate and DD^{15}N release into the dissolved pool

The total N_2 fixation rates were the total formation rates of DDN, including both the particulate and dissolved fractions.

To determine the DDN released to the dissolved fractions, 50 ml of the incubation waters, both with and without ^{15}N enrichment, were filtered through 0.22 μm pore size Millex-GP syringe filters (Millipore Express PLUS membrane, Millipore). The filtrates were preserved at -20°C for subsequent measurement of the concentration and ^{15}N enrichment of total dissolved nitrogen (TDN).

For measurement of TDN concentration, TDN was oxidized to NO_3^- using a purified persulfate oxidizing reagent (POR, ACS-

grade, Merck, Rathway, NJ, USA) in a 12 mL 450°C pre-combusted borosilicate glass tube (Knapp et al., 2005). The POR was recrystallized four times and prepared as alkaline POR by dissolving 6 g $K_2S_2O_8$ and 6 g NaOH (ACS-grade, Merck) in deionized water to a final volume of 100 mL. The residual NO_3^- concentration in the POR (POR blank) was determined to be less than 2 µmol L^{-1}. Following oxidation, the sample pH was adjusted to 7–8 using 6 N HCl. The concentrations of the resulting NO_3^- were measured by a chemiluminescent analysis, with a detection limit of 0.5 µmol L^{-1} (Braman and Hendrix, 1989).

Isotopic analyses of ^{15}N enrichment of TDN were conducted using the denitrifier method, which involves an isotopic analysis of the nitrous oxide produced by denitrifying *Pseudomonas aureofaciens* (Sigman et al., 2001). These analyses were performed on a Gasbench-Isotopic Ratio Mass Spectrometer (Delta V, Thermo Fisher Scientific). The rate of DDN released into the dissolved pool was calculated following Bonnet et al. (2016c):

$$DDN_{release} = \frac{^{15}N_{ex} \times TDN_{con}}{^{15}N_{sr}} \quad (1)$$

where $^{15}N_{ex}$ is the ^{15}N enrichment of the TDN fraction after 24 h of incubation relative to the time zero value, TDN_{con} is the measured TDN concentration, and $^{15}N_{sr}$ is the ^{15}N enrichment of the source N_2 pool in the incubation bottles (as mentioned above).

2.6 Cell sorting and DD^{15}N transfer to *Synechococcus*

For flow cytometry sorting (to separate the *Synechococcus* from the co-cultured diazotrophs), 0.3 to 0.7 L of co-culture algae were concentrated onto 0.22 µm pore size polycarbonate filters (47 mm) using a Nalgene polysulfone filtration unit (Item#: 300–4050, Thermo Fisher Scientific). Both the filtration unit and membrane were acid-cleaned prior to use. The cells on the filter were then resuspended into 4.5 mL of filtered seawater in a cryovial, where they were then fixed and preserved using glutaraldehyde (final concentration of 0.5% vol/vol) that had been filtered through a 0.2 µm filter. After fixation in the dark for 15 min, samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Cell sorting was conducted following the method described by Baer et al. (2017), using a BD FACSAria™ III flow cytometer equipped with 488 and 561 nm lasers and detectors for forward and side scatter at 692 and 530 nm, respectively. *Synechococcus* populations were determined based on forward scatter and orange fluorescence (530 nm). Pre-filtered 30‰ NaCl (CAS: 7647–14–5, pure-grade, Sigma-Aldrich, St. Louis, MO, USA) solution was used as sheath fluid. The sorted populations were collected in 15 mL high-clarity polypropylene conical tubes (FALCON, Corning, NY, USA) and subsequently filtered onto pre-combusted GF/75 filters. Samples were then frozen in liquid nitrogen and stored at -80°C for further analysis.

The PON and ^{15}N of the sorted cells were analyzed using the persulfate oxidation method coupled with the denitrifier method, respectively, as described above. The rate of DDN transferred to *Synechococcus* was calculated as follows:

$$DDN_{transfer} = \frac{^{15}N_{syn} \times PON_{con}}{^{15}N_{sr}} \times A \quad (2)$$

where $^{15}N_{syn}$ is the ^{15}N enrichment of *Synechococcus* after 24 h of incubation relative to the time zero value, PON_{con} is the particulate organic nitrogen content of the sorted *Synechococcus* (fmol $cell^{-1}$), A is the abundance of *Synechococcus*, and $^{15}N_{sr}$ is the ^{15}N enrichment of the source N_2 pool in the incubation bottles.

Filter and sheath blanks were measured (Supplementary Table S2) and subtracted from each analysis of mass. Based on the filter and sheath blank results, as well as the *Synechococcus* cellular N quota, a minimum of 2.5×10^7 *Synechococcus* cells were sorted to meet the quantifiable requirement.

2.7 Statistical analysis

The R software (version 4.3.0) was used to analyze data and establish the significance of differences based on a Welch two sample *t*-test or one-way ANOVA in combination with a Tukey *post hoc* test. A significance level of $p < 0.05$ was applied.

3 Results

3.1 Co-culture of diazotrophs with *Synechococcus*

The *T. erythraeum* and *C. watsonii* monocultures were grown with similar growth rates of ~0.5 d^{-1} under a light intensity of 200 µE m^{-2} s^{-1} (Table 2). The total N_2 fixation rate (including fixed N in the particulate and dissolved phases) of *T. erythraeum* was either normalized to cell number (cell specific N_2 fixation rate) or C biomass (C-specific N_2-fixation rate), and was significantly higher than that of *C. watsonii* (*t*-test, $p < 0.05$, Table 2). Additionally, *T. erythraeum* had a significantly lower C:N and C-fix: N_2-fix ratios compared to *C. watsonii* (*t*-test, $p < 0.01$, Table 2), suggesting that *T. erythraeum* was less efficient than *C. watsonii* in using the fixed N to support its own C fixation.

The monoculture of *Synechococcus* sp. grew exponentially with a rate of 0.91 d^{-1} in the medium fortified with sufficient nitrate (100 µM) and under a light intensity of 200 µE m^{-2} s^{-1} (Supplementary Figure S1). Prior to inoculation into the exponentially growing *T. erythraeum* or *C. watsonii* cultures, *Synechococcus* was acclimated to an inorganic N-free medium for 2 days until cell growth ceased (Supplementary Figure S1).

To establish co-culture systems in which diazotrophs and *Synechococcus* all sustained exponential growth, the inoculation ratios were optimized in the preliminary experiments. It was found that at inoculation ratios of *Synechococcus*: *T. erythraeum* = ~6:1 (cell number: cell number), *Synechococcus* and *T. erythraeum* grew exponentially at growth rates of 0.33 ± 0.03 and 0.43 ± 0.02 d^{-1}, respectively, under a light intensity of 200 µE m^{-2} s^{-1} (Figure 1). Therefore, the *T. erythraeum* fixed N supported the growth of

10.3389/fmars.2025.1485853

TABLE 2 Comparison of growth rates and total N_2 fixation rates between *Trichodesmium erythraeum* IMS101 and *Crocosphaera watsonii* WH8501 in monocultures.

Diazotroph	Growth rate (d^{-1})	Cell-specific N_2 fixation rate (pmol N cell $^{-1}$ d^{-1})	C-specific N_2 fixation rate (nmol N μmol C^{-1} d^{-1})	C:N	C-fix:N_2-fix
T. erythraeum	0.53 ± 0.01[a]	0.38 ± 0.03[a]	67.67 ± 1.62[a]	7.4 ± 0.1[a]	2.09 ± 0.05[a]
C. watsonii	0.51 ± 0.01[a]	0.02 ± 0.00[b]	40.49 ± 1.02[b]	10.4 ± 0.1[b]	5.59 ± 0.29[b]

Two diazotrophs were grown under a light intensity of 200 μE m^{-2} s^{-1}. The total N_2 fixation rates were the total formation rates of diazotroph-derived nitrogen (DDN), including both the particulate and dissolved fractions, and were normalized to the cell abundance (cell-specific N_2 fixation rate, pmol N $cell^{-1}$ d^{-1}) and carbon content (C-specific N_2 fixation rate, nmol N μmol C^{-1} d^{-1}) of diazotrophs, respectively. The ratios of particulate organic carbon to nitrogen (C:N) and the C fixation rate to the N_2 fixation rate (C-fix:N_2-fix) are also shown. Data are presented as the mean ± SD (n = 3). Different superscripted letters indicate significant differences ($p < 0.05$) between the two diazotroph strains (*t*-test).

Synechococcus and the growth of *Synechococcus* slightly inhibited the growth of *T. erythraeum* by about 20% (0.43 d^{-1} in co-culture vs 0.53 d^{-1} in monoculture). Additionally, the growth rate of *Synechococcus* in this co-culture system was one third of the maximum growth rate under N-replete conditions and the same light intensity (Supplementary Figure S1), suggesting a N-limitation of *Synechococcus* in the co-culture system. For the *C. watsonii*–*Synechococcus* co-culture system, the inoculation ratio of *Synechococcus*: *C. watsonii* was 0.003:1 (cell number: cell number), for both strains to sustain exponential growth at growth rates similar to those in the *T. erythraeum*-*Synechococcus* co-culture system (Figure 1). The huge difference in the inoculation ratios in the two co-culture systems was mainly due to the differences in the cell specific N_2 fixation rates of the two diazotrophs, i.e., 0.31 pmol N $cell^{-1}$ d^{-1} for *T. erythraeum* and 0.02 pmol N $cell^{-1}$ d^{-1} for *C. watsonii*, respectively (Table 1). Additionally, it is likely that the two diazotrophs released different amounts and speciation of DDN to support the growth of non-diazotrophic phytoplankton.

3.2 Comparison of DDN release and transfer in the two co-culture systems

On the 3rd day after initiating the *T. erythraeum*–*Synechococcus* and *C. watsonii*–*Synechococcus* co-culture systems (3rd day in Figure 1), ^{15}N enriched seawater was added and the culture bottles were continually incubated under the same growing conditions for 24 h. The DDN released to the dissolved phase and DDN transferred to *Synechococcus* were measured. In the two co-culture systems, the diazotrophs reached a similar biomass on the 3rd day, i.e., 516 ± 12 and 619 ± 27 μmol C L^{-1} for *T. erythraeum* and *C. watsonii*, respectively. The ^{15}N in diazotrophic (0.902 atom% and 0.738 atom% for *T. erythraeum* and *C. watsonii* respectively) and *Synechococcus* biomass (> 0.472 atom%), as well as in the dissolved pool (> 0.386 atom%) after 24 h of incubation were significantly enriched compared with the abundance of the natural isotope (~0.366 atom%, *t*-test, $p < 0.05$, Supplementary Table S1). The total volumetric N_2 fixation rate of *T. erythraeum* (34.9 ± 2.7 μmol N L^{-1} d^{-1}), including the particulate fraction collected on the filter and the fraction released into the dissolved pool, was nearly double that of *C. watsonii* (18.9 ± 3.2 μmol N L^{-1} d^{-1}). The majority of the total DDN (>95%) was contained within the diazotrophs themselves.

The rate of $DD^{15}N$ release into the dissolved pool (calculated by Equation 1) in the *T. erythraeum*–*Synechococcus* co-culture system was 507 ± 101 nmol N L^{-1} d^{-1}, with no significant difference compared to that of the *C. watsonii*–*Synechococcus* co-culture (489 ± 49 nmol N L^{-1} d^{-1}, *t*-test, Figure 2A). However, the percentage of the released $DD^{15}N$ relative to the total fixed N was slightly higher in *C. watsonii* (2.61%) compared to *T. erythraeum* (1.45%) (*t*-test, $p < 0.01$, Figure 2B). The volumetric rates of *T. erythraeum* $DD^{15}N$ transferred to *Synechococcus* (calculated by Equation 2) were significantly higher than those of the *C. watsonii* $DD^{15}N$ (*t*-test, $p < 0.01$, Figure 2A). Additionally, *T. erythraeum* had a significantly higher $DD^{15}N$ transfer efficiency (i.e., the proportion of the total fixed N, 0.36%) than *C. watsonii* (< 0.01%, *t*-test, $p < 0.01$, Figure 2B) in the co-cultures. Whereas, the total DDN release fractions (DDN released to the dissolved pool plus the DDN transferred to *Synechococcus*) did not differ significantly between the two co-culture systems.

3.3 The DDN release and transfer of *T. erythraeum* in response to changing light intensity

To explore the impact of light intensity on DDN release and transfer, we conducted additional co-cultures of *T. erythraeum* and *Synechococcus* under light intensities of 70 and 750 μE m^{-2} s^{-1}, and compared the results to the treatment under the 200 μE m^{-2} s^{-1} condition (Table 1). The initial inoculation ratios of *T. erythraeum* and *Synechococcus* were kept similar under the different light treatments (5–8:1, *Synechococcus* cell number: *T. erythraeum* cell number, Table 1). *T. erythraeum* and *Synechococcus* grew exponentially under the three light intensities. The growth rates and total N_2 fixation rates of *T. erythraeum* were significantly lower under 70 and 750 μE m^{-2} s^{-1} than under 200 μE m^{-2} s^{-1}, suggesting that 70 and 750 μE m^{-2} s^{-1} were light-limited and light-inhibited conditions, respectively, for the growth and N_2 fixation of *T. erythraeum* (Table 1). The growth rates of *Synechococcus* were lower under the low light conditions of 70 μE m^{-2} s^{-1} than 200 μE m^{-2} s^{-1}, with consistently lower total N_2 fixation rates of *T. erythraeum*

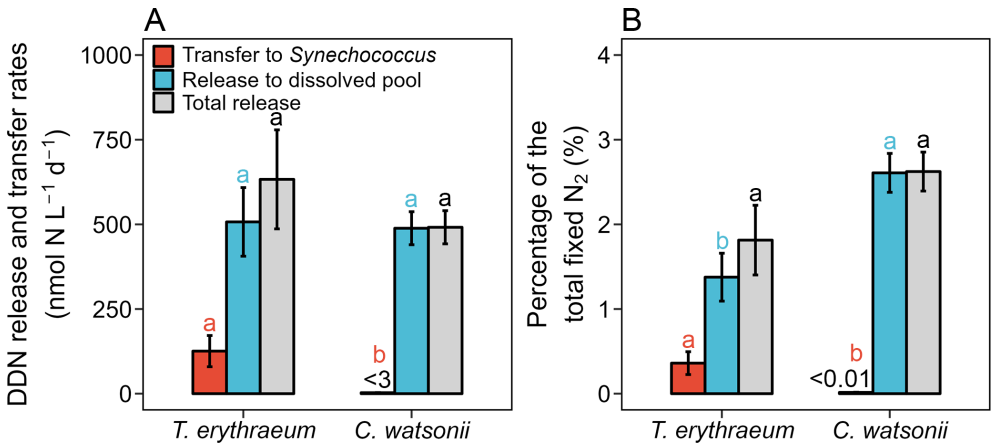

FIGURE 2
Comparison of diazotroph-derived nitrogen (DDN) release and transfer between *Trichodesmium erythraeum* IMS101 and *Crocosphaera watsonii* WH8501 in their co-culture systems under a light intensity of 200 µE m^{-2} s^{-1}. **(A)** Rates of fixed N_2 transferred to *Synechococcus* (red bars), released to the dissolved pool (blue bars), and total release (gray bars). **(B)** Percentage released and transferred DDN within the total fixed N_2. The measurement was conducted on the 3rd day after initiating the co-culture systems (3rd day in Figure 1), when the biomass of diazotrophs *T. erythraeum* and *C. watsonii* reached 515 ± 12 and 619 ± 21 µmol C L^{-1}, and the volumetric total N_2 fixation rates were 34.9 ± 2.7 and 18.9 ± 3.2 µmol N L^{-1} d^{-1}, respectively. The DDN transfer rate from *C. watsonii* to *Synechococcus* was < 3 nmol N L^{-1} d^{-1} and the percentage of total fixed N_2 was < 0.01%. The error bars represent the standard deviation of biological replicates (n = 3 in the *T. erythraeum–Synechococcus* co-culture system, n = 4 in the *C. watsonii–Synechococcus* co-culture system). Different superscripted letters indicate significant differences ($p < 0.05$) between *T. erythraeum* and *C. watsonii* (*t*-test).

under low light conditions. In contrast, the growth rates of *Synechococcus* increased when light intensity increased to 750 µE m^{-2} s^{-1}, under which N_2 fixation of *T. erythraeum* was inhibited by the high light conditions (Table 1). In line with these results, *T. erythraeum* exhibited a higher DD^{15}N transfer efficiency under 750 µE m^{-2} s^{-1} than under 70 and 200 µE m^{-2} s^{-1} (Figure 3).

Consistently, the highest percentage of DDN released into the dissolved phase was found under a light intensity of 750 µE m^{-2} s^{-1} (3.19%), although no statistically significant difference was found among the three light intensities (one-way ANOVA, $p > 0.05$, Figure 3). As a result, the total released fraction (release to the dissolved phase plus transfer to *Synechococcus*) was highest under 750 µE m^{-2} s^{-1} (Figure 3). To confirm that a high light intensity increased the DDN release, we conducted short-term (24-h) co-culture incubations under light intensities of 1100 and 1800 µE m^{-2} s^{-1}, that were approximately equivalent to full noon sunlight on a cloudless day (Lu et al., 2018). Under these two high light conditions, *T. erythraeum* and *Synechococcus* could not grow exponentially due to strong light inhibition effects; therefore, only short-term incubations were conducted. The results showed that although the total N_2 fixation rates were very low under the high light conditions of 1100 and 1800 µE m^{-2} s^{-1}, the portion of fixed N released to the dissolved pool increased with increasing light intensity and reached ~25% under 1800 µE m^{-2} s^{-1} (Figure 4).

4 Discussion

Due to the importance of DDN in supporting phytoplankton productivity and C export in oligotrophic oceans, various studies have quantified the rate or fractions of DDN release and transfer by diazotrophic cyanobacteria (Benavides et al., 2013a; Bonnet et al., 2016b, c; Berthelot et al., 2017; Caffin et al., 2018). However, these studies produced substantial variations in their results, with the reasons behind these variations rarely reported. By establishing well-controlled co-cultures of representative marine diazotrophs with the pico-cyanobacterium *Synechococcus*, we compared the intrinsic differences in DDN release and transfer between the filamentous cyanobacterial diazotroph *T. erythraeum* and the unicellular *C. watsonii*. Additionally, we discussed the importance of light intensity in modulating DDN release and transfer.

4.1 N_2 and C fixation rates of diazotrophs: *T. erythraeum* vs. *C. watsonii*

In both the monoculture and co-cultures, *T. erythraeum* exhibited higher cell-specific and C-specific N_2 fixation rates than *C. watsonii* under the same culture conditions (Tables 1; 2). These findings were aligned with previous reports in which N_2 fixation rates were measured using the conventional acetylene reduction method (Knapp et al., 2012) and the novel nanoSIMS analysis (Berthelot et al., 2016). However, *T. erythraeum* was less efficient at using the fixed N to support its own C fixation than *C. watsonii*, as indicated by the lower C-fix:N_2-fix ratios compared to *C. watsonii* (Table 2). As filamentous cyanobacterial diazotrophs, only 15–20% of its cells within a *Trichodesmium* trichome are diazocytes that are capable of N_2 fixing. It was proposed that the new N fixed by diazocytes would be actively released into the surrounding environment and subsequently taken up by the vegetative cells in the rest of the filaments or by other phytoplankton (Berman-Frank et al., 2003; Mulholland et al., 2004). However, *C. watsonii* are unicellular N_2 fixing cyanobacteria.

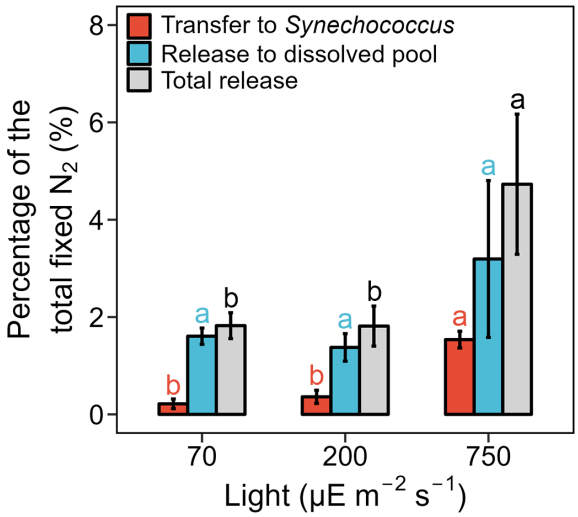

FIGURE 3
Percentage of diazotroph derived nitrogen (DDN) release and transfer in the *Trichodesmium erythraeum* IMS101 and *Synechococcus* sp. WH8102 co-culture system under different light intensities (70, 200, and 750 μE m^{-2} s^{-1}). The measurement was conducted on the 3rd day after initiating the co-culture systems, when the biomass of *T. erythraeum* reached 212 ± 10, 516 ± 12, and 149 ± 7 μmol C L^{-1}, and the volumetric total N_2 fixation rates were 9.3 ± 1.0, 34.9 ± 2.7, and 9.8 ± 0.5 μmol N L^{-1} d^{-1}, under the light intensities of 70, 200, and 750 μE m^{-2} s^{-1}, respectively. Error bars represent the standard deviation of biological replicates (n = 2 in the 70 and 750 μE m^{-2} s^{-1} treatment groups, n = 3 in the 200 μE m^{-2} s^{-1} treatment group). Different superscripted letters indicate significant differences ($p < 0.05$) among the different light intensities (one-way ANOVA followed by a Tukey *post hoc* test).

Therefore, it is likely that *T. erythraeum* may release more of its fixed DDN to the surrounding environment and be less efficient in using its own fixed DDN.

4.2 The release and transfer of DDN: *T. erythraeum* vs. *C. watsonii*

After measuring DDN release, we did not find more DDN present in the dissolved phase in the *T. erythraeum–Synechococcus* co-culture compared to the *C. watsonii–Synechococcus* co-culture. The rate of DDN release was similar between the two species (Figure 2), even though the volumetric N_2 fixation rate of *T. erythraeum* was twice as high as that of *C. watsonii*. Even when accounting for the DDN transferred to *Synechococcus*, the total amount of DDN excreted by *T. erythraeum* was similar to that of *C. watsonii* (Figure 2). However, the DDN excreted by *T. erythraeum* was more efficient in supporting *Synechococcus* cell growth in the co-cultures (Figure 2; Table 1). We propose that fixed N derived from *T. erythraeum* was likely more bioavailable for *Synechococcus*

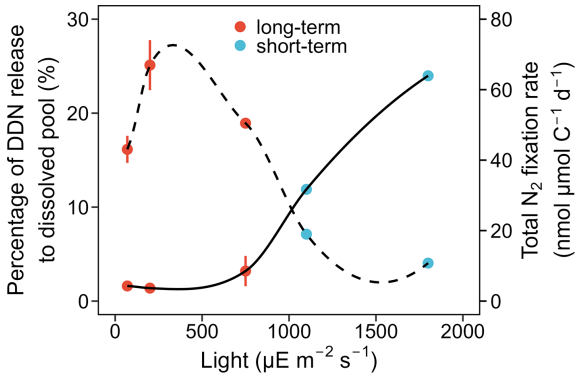

FIGURE 4
Percentage of total diazotroph-derived nitrogen (DDN) released to the dissolved pool (%) and total N_2 fixation rate (nmol μmol C^{-1} d^{-1}) of *Trichodesmium erythraeum* IMS101 in the co-cultures under different light intensities (70, 200, 750, 1100, and 1800 μE m^{-2} s^{-1}). The solid line denotes the percentage of DDN released to the dissolved pool (%), while the dashed line indicates the N_2 fixation rate. The total N_2 fixation rates are the total formation rates of DDN in the co-culture systems, including both the particulate and dissolved fractions. The red dots represent the results from the long-term (4 days) co-culture experiments presented in Figure 3 (n = 2 in the 70 and 750 μE m^{-2} s^{-1} treatment group, 3 in the 200 μE m^{-2} s^{-1} treatment group). The blue dots represent the results from short-term (24-h) co-culture experiments (n = 1 in the 1100 and 1800 μE m^{-2} s^{-1} treatment groups).

than that from *C. watsonii*. This was supported by the significantly higher DDN transfer efficiency of *T. erythraeum* (0.36%) compared to *C. watsonii* (< 0.01%) (Figure 2). It was also reported that in artificially induced blooms in the western South Pacific, a *T. erythraeum* bloom resulted in a significantly higher $^{15}NH_4^+$ enrichment in the dissolved pool than a *C. watsonii* bloom (Berthelot et al., 2016), suggesting a higher bioavailability of the fixed N released from *T. erythraeum*. Therefore, DDN released by *T. erythraeum* can contribute significantly to primary production by supporting the growth of non-diazotrophic phytoplankton (Campbell et al., 2005).

For the smaller unicellular *C. watsonii*, although the active release of its DDN played a minor role in supporting the growth of non-diazotrophic phytoplankton, it is more easily grazed by zooplankton than *T. erythraeum* and resulted in a more passive release of DDN (Caffin et al., 2018). Nevertheless, our co-culture systems provided a useful approach to compare the active DDN release and transfer by the two different diazotrophs. It should be noted that our observations of DDN release and transfer were the results obtained for 24-h of incubation after ^{15}N enrichment. The turnover time of DON could be longer than 24-h (Bronk et al., 2007), therefore, the efficiency of DDN transfer may have been underestimated in our study systems.

4.3 Impact of light intensity on the DDN release and transfer of *T. erythraeum*

Our study demonstrated the significant role of light intensity in controlling the release and transfer of DDN fixed by *T. erythraeum*. It was shown that *T. erythraeum* supported higher *Synechococcus* growth under a high light intensity of 750 μE m^{-2} s^{-1} (Table 1), which aligned well with the increased percentage of total DDN released to the dissolved pool and transferred to *Synechococcus* (Figure 3). Additionally, although even higher light intensities, e.g., 1100 and 1800 μE m^{-2} s^{-1}, inhibited the growth and N_2 fixation of *T. erythraeum*, they further increased the portion of fixed N released to the dissolved pool (Figure 4). It has been proposed that that the exudation of DDN in the form of NH_4^+ and DON could serve as a potential electron sink to protect cells from photo-damage (Wannicke et al., 2009).

However, previous field incubation experiments have found that the fraction of DDN in the dissolved phase increased when the light intensity decreased to a very low level, e.g., 15 μE m^{-2} s^{-1} (Lu et al., 2018). We found no significant difference in the fraction of DDN in the dissolved phase among the three light treatments of 70, 200, and 750 μE m^{-2} s^{-1}. The DDN in the dissolved phase was the net result of diazotroph DDN excretion and uptake by phytoplankton. Light intensity not only affects the DDN excretion rate, but also the phytoplankton N uptake rate (Wannicke et al., 2009; Han et al., 2023). Under a very low light intensity, such as 15 μE m^{-2} s^{-1}, although diazotrophs could decrease DDN excretion, phytoplankton may be short of energy for N uptake, leading to an increase in percentage of DDN in the dissolved fractions (Lu et al., 2018; Han et al., 2023). These studies highlight the important role of light intensity in controlling the diazotrophic N_2 fixation rate and DDN release rate, as well as phytoplankton N uptake rates. Our results using the simple and well-controlled co-culture system clearly showed how light intensity affects *T. erythraeum* N_2 fixation, DDN release, and transfer.

Factors other than light can also affect diazotrophic DDN release. For example, iron and phosphorus limitation can affect the DDN release of *Azotobacter vinelandii*, *T. erythraeum*, or *Nodularia* (McRose et al., 2019; Wannicke et al., 2009; Schoffelen et al., 2019). Moreover, top-down controls such as viral lysis and grazing could stimulate passive DDN release and promote C export (Bonnet et al., 2016a; Kuznecova et al., 2020). Future studies should investigate the effects of these potential factors to obtain a comprehensive understanding of the fate and role of DDN in supporting marine primary production.

5 Conclusions

By establishing co-cultures of diazotrophs with non-diazotrophic pico-cyanobacteria *Synechococcus* sp., this study provided new insights into the release and transfer of fixed N by two different strains of diazotrophs, and then investigated the impact of light intensity on these processes. First, we demonstrated that under identical culture conditions, *T. erythraeum* had a significantly higher efficiency in supporting *Synechococcus* growth than *C. watsonii*. This was evidenced by the notably higher DDN transfer efficiency of *T. erythraeum* than *C. watsonii*, although the overall exudation efficiency of fixed N (DDN release plus DDN transfer) revealed no significant differences between these two species. We therefore proposed that the higher bioavailability of the released N from *T. erythraeum* contributed to the more efficient DDN transfer of *Synechococcus*. Second, we showed that the elevated light intensity significantly increased DDN release and transfer. With the future warming of oceans, intensified seawater stratification may increase phytoplankton light exposure and could therefore alter the fate of DDN in the marine ecosystem.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

XH: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. ZW: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Investigation. TL: Methodology, Writing – original draft. HH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources,

Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2025.1485853/full#supplementary-material

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