PHOTOSYNTHETICA
International Journal for Photosynthesis Research

DOI 10.32615/ps.2023.007 PHOTOSYNTHETICA 61 (1): 58-72, 2023

REVIEW

Protection of nitrogenase from photosynthetic O_2 evolution in *Trichodesmium*: methodological pitfalls and advances over 30 years of research

A. HANIA[*,**], R. LÓPEZ-ADAMS[*], O. PRÁŠIL[*,**], and M. EICHNER[*,+]

Laboratory of Photosynthesis, Institute of Microbiology of the Czech Academy of Sciences, Centre Algatech, Novohradská 237 – Opatovický Mlýn, 37901 Třeboň, Czech Republic[*]
Faculty of Science, University of South Bohemia, Branišovská 1760, 37005 České Budějovice, Czech Republic[**]

Abstract

The *Trichodesmium* genus comprises some of the most abundant N_2-fixing organisms in oligotrophic marine ecosystems. Since nitrogenase, the key enzyme for N_2 fixation, is irreversibly inhibited upon O_2 exposure, these organisms have to coordinate their N_2-fixing ability with simultaneous photosynthetic O_2 production. Although being the principal object of many laboratory and field studies, the overall process of how *Trichodesmium* reconciles these two mutually exclusive processes remains unresolved. This is in part due to contradictory results that fuel the *Trichodesmium* enigma. In this review, we sift through methodological details that could potentially explain the discrepancy between findings related to *Trichodesmium*'s physiology. In doing so, we exhaustively contrast studies concerning both spatial and temporal nitrogenase protective strategies, with particular attention to more recent insights. Finally, we suggest new experimental approaches for solving the complex orchestration of N_2 fixation and photosynthesis in *Trichodesmium*.

Keywords: cyanobacteria; diazocyte; immunolabelling; method comparison; microscopy; O_2-scavenging mechanisms.

Introduction

N is central to life, as it is a fundamental component of biomass and is essential for many biological processes (Capone and Knapp 2007). In marine ecosystems, the principal sources of bioavailable inorganic N (also called fixed, reactive, or combined N) which phytoplankton can assimilate, are NH_4^+ and NO_3^- (Mulholland and

Highlights

- We analyse conflicting results on nitrogenase location and activity in *Trichodesmium*
- We highlight well-established and more novel or potential O_2-reducing mechanisms
- Finally, we discuss recent mechanistic models and propose new solving avenues

Received 2 December 2022
Accepted 6 March 2023
Published online 13 March 2023

[+]Corresponding author
e-mail: eichner@alga.cz

Abbreviations: AMCA – 7-amino-4-methylcoumarin-3-acetic acid; APC – allophycocyanin; APX – ascorbate peroxidase; CCM – CO_2-concentrating mechanism; CLSM – confocal laser scanning microscopy; cryo-SEM – cryo-scanning electron microscopy; EpiFluo-M – epifluorescence microscopy; F_0 – minimal fluorescence yield of the dark-adapted state; FKM – fluorescence kinetic microscopy; Fluo-LM – fluorescence light microscopy; FLV – flavoprotein; F_m – maximal fluorescence yield of the dark-adapted state; FRAP – ferric reducing/antioxidant power; FRRf – fast repetition rate fluorometry; F_v/F_m – maximal quantum yield of PSII photochemistry; HPLC – high-performance liquid chromatography; HRP – horseradish peroxidase; LG-SIMS – large geometry secondary ion mass spectrometry; LM – light microscopy; MnSOD – manganese superoxide dismutase; mRNA CARD-FISH – mRNA catalysed reported deposition fluorescence *in situ* hybridization; $NaH^{13}CO_3$ – ^{13}C-labelled sodium bicarbonate; NanoSIMS – nanoscale secondary ion mass spectrometry; NiSOD – nickel superoxide dismutase; PC – phycocyanin; PE – phycoerythrin; PUB – phycourobilin; ROS – reactive oxygen species; SIP – stable isotope probing; SOD – superoxide dismutase; TEM – transmission electron microscopy.

Acknowledgements: This work was supported by GACR (20-02827Y to ME, 20-17627S to OP) and OP VVV (CZ.02.2.69/0.0/0.0/20_079/0017812 to ME).
Conflict of interest: The authors declare that they have no conflict of interest.

Lomas 2008). However, both of these N forms occur in concentrations limiting primary productivity (Falkowski 1997, Gruber 2005, Bristow *et al.* 2017, Pajares and Ramos 2019). The N_2 fixation process provides the food web with biologically fixed N in the form of NH_4^+ (Postgate 1998, Mahaffey *et al.* 2005, Benavides *et al.* 2011, Zehr and Capone 2020). In the tropical and subtropical ocean gyres, the filamentous cyanobacteria of the genus *Trichodesmium* have been historically considered the most abundant among marine diazotrophs, accomplishing up to 50% of N_2 fixation, a contribution overall ranging between 60 and 80 Tg N y^{-1} (Capone *et al.* 1997, 2005; Tyrrell *et al.* 2003, Coles *et al.* 2004, Westberry and Siegel 2006, Langlois *et al.* 2008, Carpenter and Capone 2008, Benavides *et al.* 2016, Pierella Karlusich *et al.* 2021).

The breaking of the triple bond that connects the two N atoms in molecular N_2 is catalysed by nitrogenase, a highly conserved enzyme formed by two distinct proteins: dinitrogenase reductase (also called Fe protein) and dinitrogenase (also called FeMo protein; Robson and Postgate 1980, Berman-Frank *et al.* 2003). N_2 fixation is an energy-expensive process: 16 ATP molecules and 8 electrons are needed to carry out the reaction (Postgate 1998, Rascio and La Rocca 2013, Seefeldt *et al.* 2020). As part of this reaction, an equimolar amount of H_2 is produced, contributing to the high energy costs related to N_2 fixation (Tamagnini *et al.* 2002). Nitrogenase is irreversibly inhibited upon O_2 exposure (Gallon 1981, Fay 1992), thus, additional energy has to be invested for covering the indirect costs related to its protection, *i.e.*, the removal of O_2 from the cellular interior and the repair of the nitrogenase due to oxidative damage (Großkopf and LaRoche 2012). Among the 13 diazotrophic lineages, nitrogenase sensitivity to O_2 is especially hindering for cyanobacteria, since they are the only lineage that produces molecular O_2 as a by-product of their photosynthetic metabolism (Boyd and Peters 2013), and a series of strategies have been developed to ensure that both N_2 fixation and photosynthesis can be performed by the same organism (Berman-Frank *et al.* 2003).

Generally, in single-celled cyanobacteria, the separation of the two processes takes place in time, *i.e.*, N_2 fixation occurs only during the night whereas photosynthesis is carried out during the day (Schneegurt *et al.* 1994). In turn, spatial separation is typical of filamentous cyanobacteria which have evolved heterocysts, fully differentiated N_2-fixing cells within the filaments. In contrast to vegetative cells, heterocysts are characterised by a cell wall with a thick glycolipid layer and lack functional PSII which enables daytime N_2 fixation (Kumar *et al.* 2010). Heterocysts and vegetative cells are mutually dependent, with the vegetative cells supplying sugars and heterocysts delivering fixed N compounds (Golden and Yoon 2003, Mullineaux *et al.* 2008). A combination of both time and space separation has been suggested to occur uniquely within the members of the genus *Trichodesmium* (Fig. 1; Capone *et al.* 1997, Chen *et al.* 1998, Berman-Frank *et al.* 2001b).

Occurring as free filaments (also called trichomes) or as colonies (puff or tuft shaped), *Trichodesmium* is a non-heterocystous N_2 fixer (Dugdale *et al.* 1961, Bergman *et al.* 1997). Yet, several studies have reported a more subtle spatial separation, *i.e.*, confinement of nitrogenase only to a subset of cells within a filament, known as diazocytes (Fig. 1*A*; *e.g.*, Fredriksson and Bergman 1997). Contrary to heterocysts, diazocytes are not clearly distinguishable based on their morphology and do not lack PSII. Therefore, diazocytes can in principle conduct oxygenic photosynthesis (Carpenter *et al.* 1990, Siddiqui *et al.* 1992, Janson *et al.* 1994, Berman-Frank *et al.* 2001b). Additionally, no transporters for a reciprocal exchange of fixed N and C between diazocytes and adjacent vegetative cells were found until recently, implying that vegetative cells have to take up NH_4^+ released to

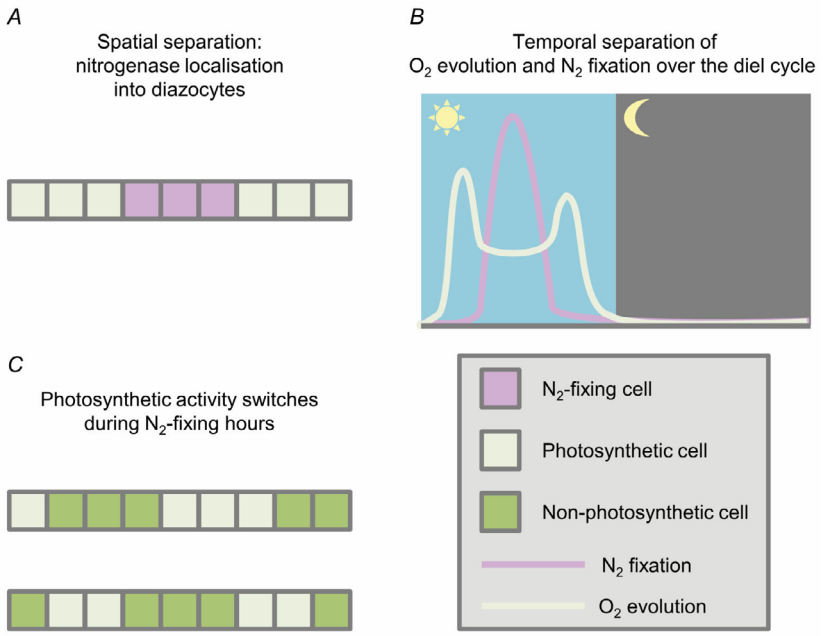

Fig. 1. Three strategies suggested to enable N_2 fixation in *Trichodesmium* that are critically discussed in this review. (*A*) Spatial separation: confinement of nitrogenase to not fully differentiated cells (diazocytes). (*B*) Temporal separation over the diel cycle: photosynthetic O_2 evolution is temporarily downregulated at noon, when N_2 fixation reaches its peak. (*C*) Photosynthetic activity switches: minute-scale switches in photosynthetic activity of single cells. The key in the box at the bottom right of the figure applies to all panels.

the environment by the diazocytes (*i.e.*, extracellular transfer; Mulholland *et al.* 2004, Mulholland and Capone 2009, Kranz *et al.* 2011). However, a recent study by Weiss *et al.* (2019) revealed septal junction architectures, *i.e.*, cell-cell joining structures (typical of heterocystous cyanobacteria) in *Trichodesmium* using cryo-scanning electron microscopy (cryo-SEM). This novel finding suggests that direct connections between diazocytes and neighbouring vegetative cells might also exist in *Trichodesmium*, favouring the model of spatial separation. In support of a mechanism for the temporal (partial) separation of N_2 fixation from O_2 production, photosynthetic O_2 evolution was reported to be temporarily downregulated at noon, when N_2 fixation reaches its peak, while photosynthetic O_2 evolution showed one peak in the morning and another one in the afternoon (Fig. 1*B*; Berman-Frank *et al.* 2001b, Kranz *et al.* 2009, Eichner *et al.* 2014, Boatman *et al.* 2019). The regulation of N_2 fixation over the diel cycle, with a peak during midday, was shown to involve nitrogenase *de novo* synthesis and reactivation of part of the enzyme pool in the morning, and degradation and inactivation in the afternoon and night (Capone *et al.* 1990, Zehr *et al.* 1993), all regulated by a circadian clock (Chen *et al.* 1996, 1998). However, in contrast to these findings, Lundgren *et al.* (2001) observed neither *de novo* synthesis nor the inactive form of nitrogenase.

Although much has been learned about *Trichodesmium* over the last century, the overall process of how this genus reconciles N_2 fixation and photosynthetic O_2 production remains unresolved. The multitude of findings on this question indeed indicate a complex regulation, and contradictory results, arguing for either spatial or temporal separation strategies, so far prevented a consensus on the mechanisms involved. For instance, early hypotheses suggested that the aggregation of *Trichodesmium* filaments in a colony is necessary for N_2 fixation because O_2 concentrations would be low in microzones within the centre of colonies (Paerl and Bebout 1988). This was ultimately shown to not be a prerequisite of N_2 fixation since free filaments were actively fixing N_2 (*e.g.*, Ohki and Fujita 1988), even at higher rates than colonies (Eichner *et al.* 2019), and O_2 concentrations within the colonies were no different (Carpenter *et al.* 1990), or even elevated (Eichner *et al.* 2017) compared to the colony surface. A number of studies detected nitrogenase in almost all cells of a filament, including photosynthetic cells (Ohki 2008, Taniuchi *et al.* 2008, Ohki and Taniuchi 2009), questioning the model of diazocytes. Moreover, it has been observed that *Trichodesmium* cells are capable of switching between different photosynthetic activity states on time scales of minutes (Fig. 1*C*; Berman-Frank *et al.* 2001b, Küpper *et al.* 2004). Most recently, analysis of *Trichodesmium*'s whole proteome dynamics showed the proteome to oscillate four times on a diel basis, with N_2 fixation and photosynthesis fluctuating simultaneously (Held *et al.* 2022). In this review, we compile studies that used different techniques, analyse methodological differences between them and systematically compare their findings, aiming to explain and reconcile apparently contradictory results. With this, we summarise the current knowledge of how N_2 fixation and photosynthesis are coordinated in *Trichodesmium* and suggest avenues to follow in solving this long-standing question.

Spatial separation strategy: Do diazocytes exist?

In the following section, we focus on *Trichodesmium* studies aimed at detecting the nitrogenase localisation and activity. These works employed a range of different species, strains, labelling methods, and visualisation techniques. The main methodological details are summarised in Table 1S (*supplement*) and results are illustrated in Fig. 2. We also group together studies that have co-localised nitrogenase with other proteins. Herein, we exclusively define diazocytes as a subset of consecutive cells within a trichome that fixes N_2.

Nitrogenase localisation along filaments by immunolabelling: Studies from 1989 to 1997 focused on field-collected *Trichodesmium* samples of various species, including *T. thiebautii*, *T. contortum*, *T. tenue*, and *T. erythraeum*, from different regions of the Atlantic Ocean. Glutaraldehyde, paraformaldehyde or liquid N_2 were used as fixative compounds, without any apparent effect on the result/conclusion. Immunolabelling employed anti-Fe protein of *Rhodospirillum rubrum*, *T. thiebautii* or universal (*i.e.*, derived from a variety of N_2-fixing bacteria, including *R. rubrum*) as primary antibodies, different sizes of gold particles conjugated to the secondary antibodies, and transmission electron microscopy (TEM) for analysis. A major difficulty in almost all these studies was the whole longitudinal sectioning of the lengthy and twisted *Trichodesmium* filaments. Although this did not allow authors to assess the localisation of nitrogenase along trichomes with certainty, different conclusions were made based on the examination of single (cross-sections) and few consecutive (oblique and longitudinal sections) cells. In the study of Paerl *et al.* (1989), nitrogenase was found in all section types and, since the absence of the enzyme was not observed, the authors concluded that 'virtually all *Trichodesmium* cells within a colony possessed nitrogenase' (Fig. 2*A*). In contrast, the cross-sectioned cells shown by Bergman and Carpenter (1991) contained nitrogenase only in 10–40% of cells, a proportion identified *via* immunogold-labelling followed by enhancement with silver deposition, and visualised *via* light microscopy (LM). Although the whole trichome sectioning never succeeded, analysis of short longitudinal sections evidenced that cells within a specific trichome were always either labelled or unlabelled. The restriction of nitrogenase into only some randomly distributed trichomes of a colony suggested that these trichomes are equivalent to heterocysts, supplying the surrounding non-N_2-fixing trichomes with NH_4^+ (Fig. 2*B*). In view of the different results with respect to Paerl *et al.* (1989), it was hypothesised that under certain conditions all *Trichodesmium* filaments may synthesise nitrogenase, or that a mixture of N_2-fixing and non-N_2-fixing *Trichodesmium* species may exist within a single colony.

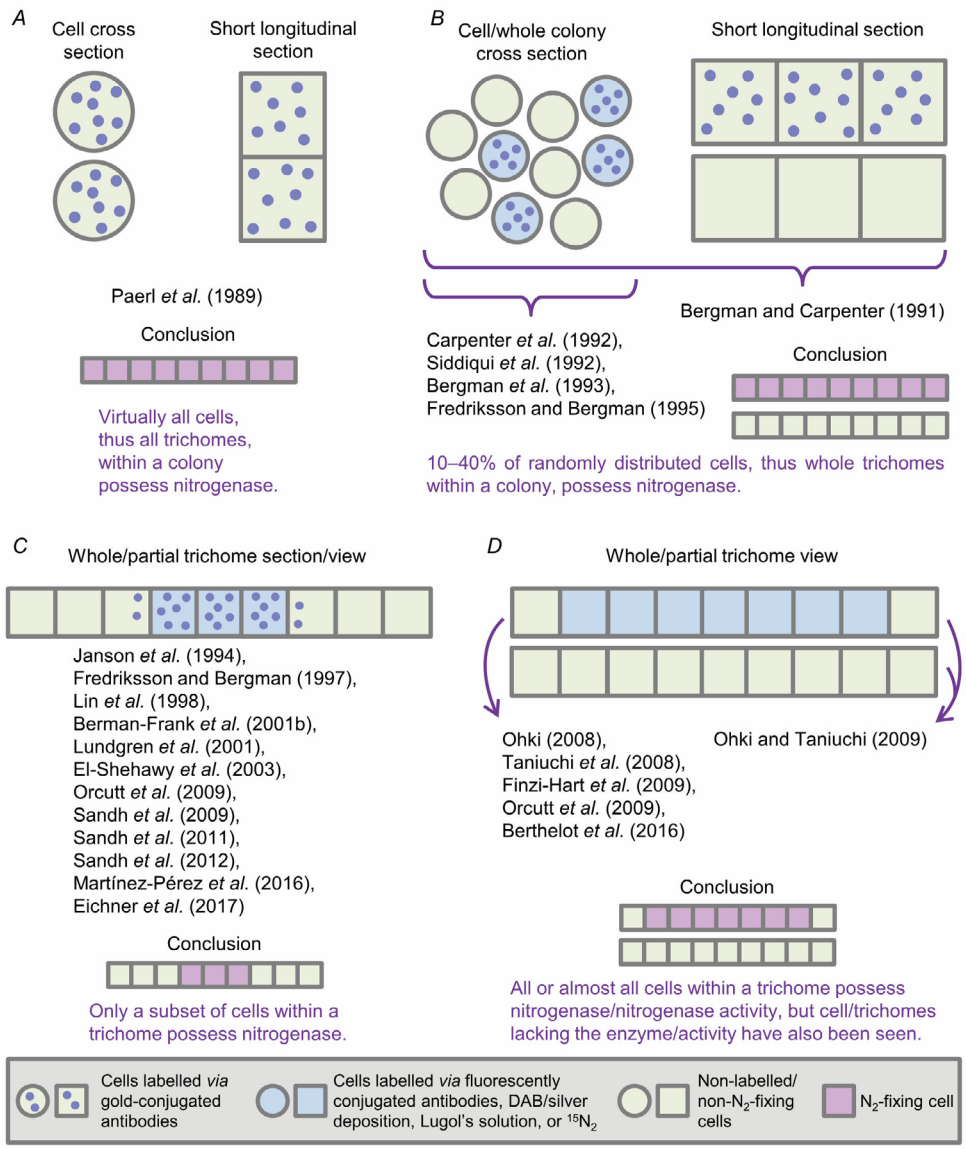

Fig. 2. Illustration of the methods, results and conclusions of studies aimed at detecting the nitrogenase localisation and activity in *Trichodesmium*. Studies are sorted into four groups according to the methods and conclusions reached. Note that electron microscopy studies generally analysed cross-sections of single cells or short longitudinal sections and extrapolated to whole trichomes, while light microscopy-based methods analysed whole trichomes. (*A*) All cells, *i.e.*, all trichomes have nitrogenase. (*B*) Only some cells, *i.e.*, trichomes, have nitrogenase. (*C*) Nitrogenase is confined into diazocytes. (*D*) Nitrogenase is present in almost all cells of a filament, but there are also filaments lacking nitrogenase. The key in the box at the bottom of the figure applies to all panels. DAB – 3,3'-diaminobenzidine.

Moreover, Carpenter *et al.* (1992), Siddiqui *et al.* (1992), and Bergman *et al.* (1993) came to similar findings as Bergman and Carpenter (1991), *i.e.*, nitrogenase being present only in certain cells representative of the whole N_2-fixing trichomes (Fig. 2*B*). Yet, the heterocyst equivalence of these N_2-fixing trichomes is questioned in the latter two works (Siddiqui *et al.* 1992, Bergman *et al.* 1993). These studies localised phycocyanin (PC), phycoerythrin (PE), Rubisco (Siddiqui *et al.* 1992), and aa_3-type cytochrome oxidase (Bergman *et al.* 1993) in all analysed cells. Such results contradicted the separation of photosynthesis and N_2 fixation into different trichomes (Fig. 2*B*; Bergman and Carpenter 1991) and indicated that respiratory mechanisms may be involved in nitrogenase protection (*see* the section 'Subcellular distribution of nitrogenase and co-localisation with other proteins').

The first evidence for a different type of nitrogenase localisation was observed by Janson *et al.* (1994). In this work, longitudinal sectioning of three whole *T. contortum* trichomes showed different concentrations of nitrogenase along the filaments. Around 15 consecutive cells in the central region, corresponding to ~10% of the total cell

number, showed intense nitrogenase labelling, whereas the enzyme labelling was drastically reduced in the 2–3 cells on the neighbouring sides of the central regions, and was almost absent in the remaining cells (Fig. 2*C*). These central regions also appeared as brighter zones in LM in filaments fixed only with glutaraldehyde. It was hypothesised that such areas were not discovered earlier due to difficulties in longitudinal sectioning or because they might be a unique feature of *T. contortum*. In the study of Fredriksson and Bergman (1995), both immunogold- and immunofluorescent-labelling were applied on central cross-sectioned regions of tuft-shaped *Trichodesmium* colonies. While gold particles once more only showed nitrogenase in some cells, the fluorescent labelling analysis of the whole cross-sectioned colonies, imaged by fluorescence light microscopy (Fluo-LM), allowed a more precise determination of the overall proportion of the randomly labelled cells, yielding an average of 14%. However, the way the analysis was conducted cannot reveal whether these nitrogenase-containing cells represent the whole trichomes (Fig. 2*B*), as suggested by Bergman and Carpenter (1991), or a limited number of cells within individual trichomes (Fig. 2*C*), as shown by Janson *et al.* (1994). Focusing on *T. tenue*, this issue was addressed in the authors' subsequent study (Fredriksson and Bergman 1997). Although trichomes were only partly sectioned, the study confirmed that consecutively arranged nitrogenase-labelled and poorly- or non-nitrogenase-labelled cells may co-exist in the same trichome, like in *T. contortum* (Janson *et al.* 1994). They formulated the hypothesis that trichomes possess central N_2-fixing cells (Fig. 2*C*), and proposed the term ‘diazocyte’ to describe these cells, which has since been a favoured model.

Studies in the following years focused on field samples of *Trichodesmium* collected from different regions of the Atlantic, Indian, and Pacific Oceans as well as the laboratory strains of *T. erythraeum*, IMS101 and NIBB1067. Additional to glutaraldehyde and paraformaldehyde, ethanol was also introduced as a fixative compound. Only in one study the preference for ethanol is expressed since glutaraldehyde and paraformaldehyde combined either with ethanol or methanol did not produce successful staining (Lin *et al.* 1998). Immunolabelling employed anti-Fe protein of *R. rubrum*, anti-Fe and/or anti-FeMo proteins (both from *Trichodesmium* sp. NIBB1067) as primary antibodies, fluorescently or horseradish peroxidase (HRP)-conjugated secondary antibodies, and results were analysed *via* Fluo-LM, EpiFluo-M (epifluorescence microscopy), confocal laser scanning microscopy (CLSM) or LM. These methods allowed the observation of intact (unsectioned) samples and facilitated the examination of a greater number of filaments. What emerges from these studies is an almost distinct separation between (*1*) those carried out on natural *Trichodesmium* populations of the Atlantic and Indian Oceans and the laboratory strain *T. erythraeum* IMS101 (isolated from North Atlantic waters by Prufert-Bebout *et al.* 1993), and (*2*) the natural populations from the Japanese waters of the Pacific Ocean and the laboratory strain *T. erythraeum* NIBB1067 (isolated from Kuroshio waters by Ohki and Fujita 1982). In the first case, nitrogenase was found to be located in a subset of cells along trichomes (Fig. 2*C*; Lin *et al.* 1998, Berman-Frank *et al.* 2001b, Lundgren *et al.* 2001, El-Shehawy *et al.* 2003, Sandh *et al.* 2009) and, apparently, this pattern was present in all examined trichomes (Lin *et al.* 1998, Lundgren *et al.* 2001, El-Shehawy *et al.* 2003). Moreover, such confinement was shown as lighter zones after treatment with both borax-buffered formalin (El-Shehawy *et al.* 2003) and Lugol's solutions, and was supported by immunogold-labelling, evidencing nitrogenase-containing cells neighboring cells lacking the enzyme (Lundgren *et al.* 2001, Sandh *et al.* 2009, 2011, 2012). The confinement of nitrogenase was not observed in the samples from the Pacific Ocean, including strain NIBB1067, where almost all cells within a filament possessed nitrogenase (Ohki 2008, Taniuchi *et al.* 2008, Ohki and Taniuchi 2009). Moreover, heterogeneity among filaments was observed, where some but not all filaments contained the enzyme (Fig. 2*D*; Ohki and Taniuchi 2009). The only geographical exception was found in the study of Lundgren *et al.* (2001), where samples collected in the Pacific Ocean also showed diazocytes. We inform the reader that the two species of the *Katagnymene* genus (*i.e.*, *K. pelagica* and *K. spiralis*) on which the study of Lundgren *et al.* (2001) was conducted are now included in the *Trichodesmium* genus and unified as one species named *T. pelagicum* (Orcutt *et al.* 2002, Lundgren *et al.* 2005, Hynes *et al.* 2012).

Aside from the sampling/isolation locations, other differences between the two groups of findings concern the antibodies used. Specifically, anti-Fe protein of *R. rubrum* was used as primary antibody in combination with fluorescently/gold-conjugated secondary antibodies in studies showing diazocytes, whereas anti-Fe and/or anti-FeMo proteins of *Trichodesmium* sp. NIBB1067 as primary antibodies and HRP-conjugated secondary antibody were used in the studies showing no diazocytes. The fluorescence approach was also tested by Ohki (2008), however, this procedure was discarded because of the masking effect of autofluorescence over the fluorescence emitted from Alexa-350, the blue fluorophore mainly used in the studies where diazocytes were observed [7-amino-4-methylcoumarin-3-acetic acid (AMCA) and Alexa-568, blue and red fluorescence, respectively, were used occasionally; Lin *et al.* 1998, Berman-Frank *et al.* 2001b]. Hence, are these opposite findings reflecting different biology (strain-specific differences) or the effect of using different antibodies and visualisation techniques? There is only one study (Orcutt *et al.* 2009) in which both types of nitrogenase labelling patterns were observed in *T. erythraeum* IMS101 (Fig. 2*C*,*D*), where a green fluorescent Qdot-525 conjugated secondary antibody was used. Authors speculated that such differences might be attributed to the different age or physiological state of the cultures, as diazocytes were observed in the 55- but not in the 35-d-old culture. However, long and intact filaments are only shown for the culture with diazocytes, while short and broken filaments are shown for the culture

with more cells containing nitrogenase but no diazocytes. Such differences in morphology and integrity of the samples could be an effect of sample handling issues, potentially leading to the misidentification of the two nitrogenase labelling patterns. Furthermore, the primary antibody used in this study was derived from Baltic Sea cyanobacteria (K. Gundersen, personal communication), hence, differences in specificity compared to antibodies used in the other studies cannot be excluded. As the above-mentioned issues with sample preparation, specificity of primary antibodies and interferences in the detection of secondary antibodies can all complicate the interpretation of immunolabelling results; studies targeting the N_2 fixation process itself rather than protein localisation can provide useful complementary insights.

Stable isotope probing (SIP) and nanoscale secondary ion mass spectrometry (NanoSIMS) to localise N_2 fixation: NanoSIMS is a surface analysis technique that allows to probe elements and their isotopes, and small molecules at high spatial resolution (~50 nm; Herrmann *et al.* 2007). NanoSIMS, in combination with SIP, can tackle the question of whether nitrogenase is fixing N_2 in a certain cell or not, linking protein localisation with enzymatic activity. In this method, images of the cellular uptake of the stable ^{15}N isotope from the incubation medium are acquired to infer N_2 fixation, whereas the uptake of ^{13}C (from ^{13}C-labelled bicarbonate, $NaH^{13}CO_3$) can be used as a proxy for photosynthetic activity.

A number of studies have successfully combined SIP and NanoSIMS to analyse metabolic activities in *Trichodesmium* (Finzi-Hart *et al.* 2009, Berthelot *et al.* 2016, Bonnet *et al.* 2016, Martínez-Pérez *et al.* 2016, Benavides *et al.* 2017, 2022; Eichner *et al.* 2017). Among them, only the study by Finzi-Hart *et al*. (2009) was specifically focused on examining N_2 fixation in *Trichodesmium* at the single-cell level. In their work, filaments of *T. erythraeum* IMS101 were incubated in isotopically-labelled medium for 8 time points (0 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h). Dozens of contiguous cells from the same trichome were imaged, and all cells showed similar ^{15}N and ^{13}C uptake from the 2-h incubations onwards (whereas shorter incubations did not result in significant enrichment), with an average positive correlation of $r^2 = 0.4$. These findings suggest the lack of spatial segregation of N_2 fixation in strain IMS101, *i.e.*, no diazocytes were identified (Fig. 2*D*). Moreover, the high spatial resolution capabilities of NanoSIMS combined with cell depth profiling analysis allowed to visualise the whole cell volume. Such examination detected intracellular inclusions rich in ^{13}C and ^{15}N identified as cyanophycin, a N-storage compound, which appeared after 4-h incubation. These inclusions were randomly distributed in all the imaged cells, in contrast to Fredriksson and Bergman's findings (Fredriksson and Bergman 1997), where they reported that the cyanophycin inclusions were present in 61% of non-nitrogenase-labelled cells, but only in 24% of nitrogenase-labelled cells. The continuous presence of cyanophycin inclusions identified by NanoSIMS could be a product of homogeneous nitrogenase activity along the trichomes, which additionally strengthens the authors' observation of no diazocytes in strain IMS101. The SIP-NanoSIMS technique was also applied to both laboratory cultures and field-collected *Trichodesmium* samples in subsequent studies, although they were not specifically aimed at investigating the presence of diazocytes. Berthelot *et al.* (2016) observed a positive correlation between the accumulation of ^{15}N and ^{13}C ($r^2 = 0.5$) in all *Trichodesmium* cells after 48-h incubation (Fig. 2*D*). Differently, Martínez-Pérez *et al.* (2016) reported that after 24-h incubation, almost all cells were fixing CO_2, but only 46% of these cells were fixing N_2. Additionally, Eichner *et al.* (2017) used SIP-NanoSIMS and large geometry SIMS (LG-SIMS) and observed that after 12-h incubation, cells located at an intermediate position in the trichome had lower N_2 fixation rates than the central or peripherally located cells, indicating heterogeneous N_2 fixation among cells within a single trichome. To our knowledge, these last two studies are the only SIP-NanoSIMS studies that potentially suggest diazocytes existence (Fig. 2*C*), although authors do not discuss this matter in detail.

It is worth noting that in the SIP-NanoSIMS method the incubation periods and initial $^{15}N_2$ enrichment could explain the diverse results. For instance, short incubations (up to 1 h) may not have been long enough for cells to show any isotopic enrichment significantly above natural abundance, and thus, evidence the presence of diazocytes, whereas long incubations (2–48 h) could have allowed the distribution of newly fixed $^{15}N_2$ among cells within a filament, masking any potential diazocytes (Finzi-Hart *et al.* 2009, Berthelot *et al.* 2016). Shorter incubation times coupled with higher $^{15}N_2$ and ^{13}C enrichments in the medium could address these issues. Besides these SIP-NanoSIMS related pitfalls, the findings in the mentioned studies show no consensus concerning the existence of diazocytes in *Trichodesmium*. Therefore, further efforts through this technique are needed to provide a wider picture of both spatial and temporal fluctuations between N_2 fixation and photosynthetic activities in *Trichodesmium*.

Subcellular distribution of nitrogenase and co-localisation with other proteins: At the single-cell level, all immunogold-labelling studies on samples collected during daytime located nitrogenase distributed throughout the cytoplasm (Fig. 2*A–C*, Table 1S; Paerl *et al.* 1989, Bergman and Carpenter 1991, Siddiqui *et al.* 1992, Carpenter *et al.* 1992, Bergman *et al.* 1993, Janson *et al.* 1994, Fredriksson and Bergman 1995, 1997; Lundgren *et al.* 2001, Sandh *et al.* 2009, 2011, 2012), whereas samples collected at night contained nitrogenase very close to the cytoplasmic membrane (Bergman and Carpenter 1991). It was observed that part of the nitrogenase pool is degraded and inactivated in the afternoon while *de novo* synthesis and reactivation take place in the morning (Capone *et al.* 1990). A recent proteomics study, in turn, showed the nitrogenase subunits NifH, NifD, and NifK to oscillate four times over a diel cycle, and indicated that, even during periods of inactive N_2 fixation,

the enzyme never fully disappears (Held *et al.* 2022). Thus, the peripherally-located nitrogenase at night may represent the inactive pool of the enzyme, while nitrogenase distributed throughout the cytoplasm may result from *de novo* synthesis during the morning (Bergman and Carpenter 1991). The absence of a specific subcellular distribution during the day suggests that N_2 fixation is not spatially separated within the cell from photosynthetic O_2 production and that other mechanisms may be involved in nitrogenase protection. Some of the above-mentioned studies have further deepened on this matter by localising other proteins related to CO_2 fixation, photosynthesis, and respiration. It is well known that in cyanobacteria CO_2 fixation is confined to polyhedral bodies called carboxysomes. Unlike eukaryotic photosynthetic organisms, cyanobacteria do not possess chloroplasts and mitochondria. The organisation of thylakoid membranes varies in cyanobacteria species from parietal (alongside the plasma membrane) to parallel, radial or fascicular arrangements (cross-passing the cytosol; Mareš *et al.* 2019). Thylakoids' organisation in *Trichodesmium* belongs to the fascicular type (Mareš *et al.* 2019) and their network was reported to depend on nitrogenase content, being tight in nitrogenase-containing cells and more loose in non-nitrogenase-containing cells (Fredriksson and Bergman 1997). Furthermore, respiration in cyanobacteria occurs on both thylakoid and plasma membranes (Falkowski and Raven 1997).

Siddiqui *et al.* (1992) individually immunolabelled PC, PE, and Rubisco, and co-immunolabelled Rubisco together with nitrogenase. Sections of single localisations displayed all proteins fairly scattered throughout the cytoplasm and mainly confined to thylakoid membranes (PC and PE) and carboxysomes (Rubisco). Following a similar pattern, the co-immunolabelling assay showed Rubisco located in all nitrogenase-containing cells. Homogeneous distribution of Rubisco was also seen in filaments of *T. erythraeum* IMS101 by fluorescence immunolocalisation (Lin *et al.* 1998). The coexistence of PC, PE, Rubisco, and nitrogenase argues against the concept that photosynthesis and N_2 fixation are spatially separated either within the cell or between non-N_2-fixing and N_2-fixing trichomes (Fig. 2*B*; Bergman and Carpenter 1991). Similarly, Janson *et al.* (1994) localised PE through single- and double-immunolabelling experiments with nitrogenase. Although the spatial confinement of nitrogenase into a subset of cells was shown in this study for the first time (Fig. 2*C*; *see* the section 'Nitrogenase localisation along filaments by immunolabelling'), no compartmentalisation was found for the PSII accessory pigment PE. Indeed, both assays again showed that PE was present in all cells and at similar levels in both nitrogenase-containing and non-nitrogenase-containing cells. Identical results of PE immunolabelling were shown also by Lundgren *et al.* (2001). The spatial pattern of oxygenic photosynthesis was further investigated by Berman-Frank *et al.* (2001b) using double-immunolabelling with fluorescent labels directed at nitrogenase and D1, a core protein of the O_2-evolving PSII. D1 was present in most cells in a trichome, including putative diazocytes. Additionally, immunolabelling performed by Bergman *et al.* (1993) addressed the respiratory protein aa_3-type cytochrome oxidase and nitrogenase. Gold markers showed aa_3-type cytochrome oxidase in all cells, including the nitrogenase-containing ones, and predominantly associated with cytoplasmic and thylakoid membranes. Moreover, the regression analysis of gold particle counts per unit of cell area of both enzymes revealed a significantly positive correlation ($r^2 = 0.9$), where cells with higher nitrogenase levels also had higher levels of aa_3-type cytochrome oxidase.

Regardless of the confinement of nitrogenase into diazocytes or not, these findings suggest that the two mutually exclusive processes of N_2 fixation and photosynthesis may take place in the same cell, and that other mechanisms must be involved in nitrogenase protection. For instance, photosynthetic proteins may be inactive in nitrogenase-containing cells, Rubisco could predominantly function as an oxygenase at mid-day (photorespiration), and/or aa_3-type cytochrome oxidase could drive O_2 consumption (dark respiration) at high rates. Such mechanisms may work together as a time separation strategy allowing N_2 fixation and photosynthetic activity to occur at different moments during the light period.

Temporal separation strategy: How are N_2 fixation and photosynthesis coordinated in time?

The downregulation of photosynthetic O_2 evolution during the peak of N_2 fixation at noon (Fig. 1*B*) is possible by a series of mechanisms, which are explored in this section, starting with bulk measurements and genome exploration, followed by single-cell measurements. The current standing on the regulation of photosynthesis in relation to N_2 fixation is illustrated in Fig. 3.

Bulk measurements and genome exploration: A number of studies indicated that, by converting O_2 into H_2O, the dark respiration process plays a significant role in lowering intracellular O_2 in *Trichodesmium* (Kana 1993, Berman-Frank *et al.* 2001b, Kranz *et al.* 2009, 2010; Eichner *et al.* 2014, 2019; Boatman *et al.* 2019). While in cyanobacteria the magnitude of dark respiration is usually <10% of the gross O_2 production, in *Trichodesmium* values from 13 to 46% with a mean value of ~20% have been observed (Kana 1993, Eichner *et al.* 2014). Boatman *et al.* (2019) have also shown dark respiration rates to be five times higher than what is required to maintain cellular metabolism. Overall, these numbers not only indicate a relevant role in decreasing O_2 (Fig. 3*A*), but also that dark respiration provides a considerable proportion of energy that fuels N_2 fixation (Boatman *et al.* 2019). Another potential mechanism to reduce O_2 in the vicinity of nitrogenase is the oxygenase activity of Rubisco. Yet, cyanobacteria possess CO_2-concentrating mechanisms (CCM) to enhance the carboxylation efficiency and reduce the risk of the wasteful process of photorespiration (Giordano *et al.* 2005, Badger *et al.* 2006). To our knowledge, only one study has demonstrated that

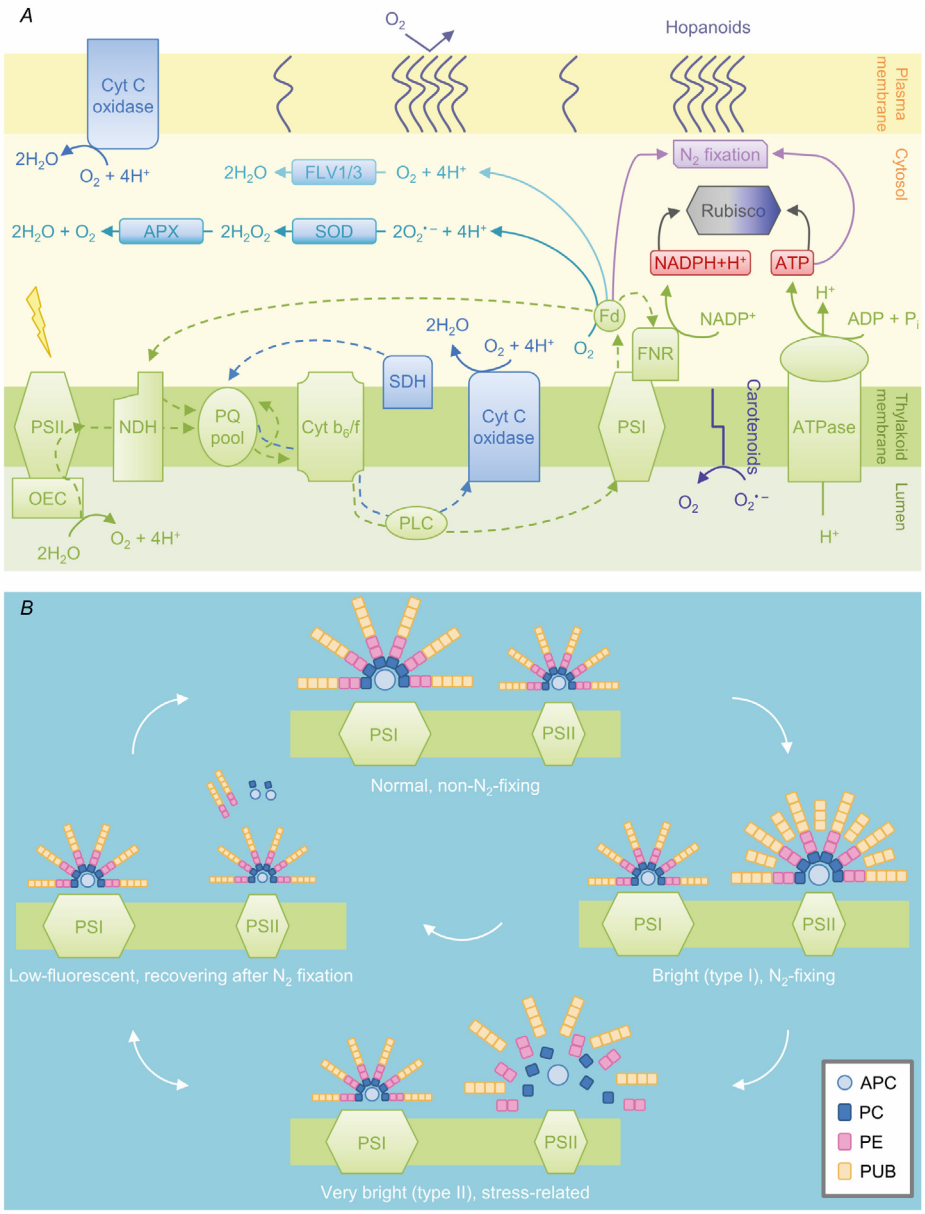

Fig. 3. Illustration of the current standing on mechanisms involved in the management of cellular O_2 concentrations in *Trichodesmium*. (*A*) Schematic representation of the major pathways of photosynthetic O_2 evolution and O_2-lowering mechanisms suggested. Complexes involved in photosynthetic light reactions and electron flow are color coded in green. Energy equivalents regenerated in photosynthesis are color coded in red and their employment in Calvin cycle and N_2 fixation in grey and purple, respectively. O_2-lowering mechanisms are color coded in different shades of blue: hopanoid lipid O_2 diffusion barrier (individually or as raft formations); photorespiration; dark respiration (complexes and electron flow); Mehler reaction; Mehler-like reaction; carotenoids. (*B*) Model of the reversible uncoupling of phycobilisomes as well as individual phycobiliproteins from the photosystems as suggested based on observations of alternating fluorescence states (modified after Küpper *et al.* 2009). The bigger size of PSI compared to PSII is representative of the high PSI:PSII ratio in *Trichodesmium*. ADP – adenosine-5'-diphosphate; APC – allophycocyanin; APX – ascorbate peroxidase; ATPase – ATP synthase; Cyt b_6/f – cytochrome b_6/f protein complex; Cyt C oxidase – cytochrome C oxidase; Fd – ferredoxin; FLV1/3 – flavoprotein1/3 heterodimer; FNR – ferredoxin NADP reductase; H_2O_2 – hydrogen peroxide; NDH – NADPH dehydrogenase; OEC – oxygen evolving complex; PC – phycocyanin; PE – phycoerythrin; P_i – inorganic phosphorus; PLC – plastocyanin; PQ pool – plastoquinone pool; PUB – phycourobilin; SDH – succinate dehydrogenase; SOD – superoxide dismutase.

photorespiration takes place at significant levels despite the existence of the CCM (Eisenhut *et al.* 2008). This study was performed on *Synechocystis* PCC6803 and the occurrence of photorespiration was evaluated by measuring glycolate concentrations using high-performance liquid chromatography (HPLC) and characterising photosynthesis with a Clark-type O_2 electrode on mutants with inactivated genes of the pathway (Eisenhut *et al.* 2008).

Unfortunately, genetic transformation of *Trichodesmium* has not been successfully performed yet. Although there is no Rubisco without photorespiration activity (Giordano *et al.* 2005), there are no studies that have specifically quantified photorespiration activity in *Trichodesmium*. Hence, its relevance for reducing O_2 during N_2 fixation is not clarified to date (Fig. 3*A*).

During photosynthesis, reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and oxygen radicals (*e.g.*, $O_2^{\bullet-}$, $^{\bullet}OH$, $HO_2^{\bullet}$), are generated. One way to neutralise the deleterious effects of ROS is through a variety of nonenzymatic antioxidants, *e.g.*, ascorbic acid, reduced glutathione, carotenoids, and flavonoids. Kelman *et al.* (2009) examined various N_2-fixing and non-N_2-fixing cyanobacteria, with particular focus on *Trichodesmium*, principally aiming at characterising the total antioxidative activity by the ferric reducing/antioxidant power (FRAP) assay, and identifing the nonenzymatic antioxidants by HPLC. It turned out that *Trichodesmium* had the highest total antioxidant activity among the studied species. Additionally, while the highest relative contribution to the total antioxidant activity was coming from the protein cell extracts (86%) in all other tested cyanobacteria, the nonpolar organic cell extract gave the highest contribution (54%) in *Trichodesmium* (Kelman *et al.* 2009). The identification of *Trichodesmium* nonpolar organic cell extract showed that this fraction contained high amounts of all-*trans*-β-carotene, and small amounts of 9-*cis*-β-carotene and retinyl palmitate (a compound originating from β-carotene). Overall, the study demonstrated that carotenoids have an important protective role against ROS in *Trichodesmium* (Fig. 3*A*).

A well-known pathway that reduces ROS in *Trichodesmium* is the Mehler reaction (Kana 1993, Berman-Frank *et al.* 2001b, Levitan *et al.* 2007, Milligan *et al.* 2007, Kranz *et al.* 2010), in which $O_2^{\bullet-}$ is converted into H_2O_2 by superoxide dismutase (SOD), and subsequently scavenged by ascorbate peroxidase (APX) into H_2O (Fig. 3*A*). *Trichodesmium* contains two SOD genes, *sodA* and *sodN*, encoding isoforms containing manganese (MnSOD) and nickel (NiSOD), respectively (Dupont *et al.* 2008). The latter has captured attention over the last years (Ho 2013, Ho *et al.* 2013, Rodriguez and Ho 2014, 2017; Held *et al.* 2021, Chen *et al.* 2022). The laboratory study by Ho (2013) showed that, under sufficient supply of P and Fe, N_2 fixation in *Trichodesmium* was limited by Ni. Increased Ni concentrations elevated both N_2 fixation rates and SOD activity, suggesting that NiSOD may be involved in nitrogenase protection from ROS (Ho 2013). The low SOD activity at low Ni concentrations also indicates that in *Trichodesmium*, there was no mutual replacement between NiSOD and MnSOD, although Mn was available in the growth medium, suggesting that MnSOD may not to be involved in $O_2^{\bullet-}$ removal (Ho 2013). Subsequent laboratory studies came to similar conclusions by varying Ni availability together with light intensity and quality (Ho *et al.* 2013, Rodriguez and Ho 2014, 2017; Chen *et al.* 2022). Notably, the highest N_2 fixation rates were observed under the condition of high Ni availability and high light intensity (Ho *et al.* 2013, Rodriguez and Ho 2014, Chen *et al.* 2022), both characteristic features of the surface oceanic waters where *Trichodesmium* blooms (Middag *et al.* 2020, Chen *et al.* 2022). Chen *et al.* (2022) also measured the concentrations of two representative photosynthetic proteins, *i.e.*, PsbA (D1) and PsaC (core subunit of PSI). These proteins also showed the highest concentrations at high Ni availability and high light intensity, providing further support that NiSOD is crucial for protecting the photosynthetic machinery of *Trichodesmium* from ROS (Chen *et al.* 2022). A study conducted on *Trichodesmium* field colonies combining mineralogical and molecular characterisation revealed that NiSOD was significantly more abundant when colonies interacted with mineral particles, where Ni availability may be enhanced (Held *et al.* 2021). While taken together these studies indicate that NiSOD may be an important player for nitrogenase protection from $O_2^{\bullet-}$ in *Trichodesmium*, further experiments are needed to shed light on the mutual replacement between NiSOD and MnSOD.

Widespread among oxygenic photosynthetic organisms, flavoproteins (FLV) carry out a mechanism that alleviates excessive reduction of the electron transfer chain (reviewed by Allahverdiyeva *et al.* 2015, Alboresi *et al.* 2019, Nikkanen *et al.* 2021). Specifically, FLVs mediate a Mehler-like reaction in which O_2 is reduced to H_2O without the concomitant production of ROS (Helman *et al.* 2003, Allahverdiyeva *et al.* 2013). Data mining of sequenced genomes has shown that up to six isoforms of FLVs are present in cyanobacteria (Flv1-4, Flv1B, 3B). While Flv2 and Flv4 are unique to β-cyanobacteria and Flv1B and Flv3B to heterocystous species, Flv1 and Flv3 are present in almost all species, including *Trichodesmium* (Zhang *et al.* 2009, Allahverdiyeva *et al.* 2015). They catalyse the Mehler-like reaction on the acceptor side of PSI, most likely by functioning as a Flv1/3 heterodimer (Helman *et al.* 2003, Allahverdiyeva *et al.* 2013, Mustila *et al.* 2016). Although direct evidence is still lacking, the presence of Flv1 and Flv3 genes in the *Trichodesmium* genome suggests that this mechanism may also be involved in lowering O_2 during N_2 fixation (Fig. 3*A*).

All the above-mentioned processes can lower O_2 concentration in the interior of the cell, but none of them can avoid O_2 diffusion from the environment across the cell membranes. Hopanoids, a class of membrane lipids, can intercalate (individually or as raft formations) into the cytoplasmic bilayer and confer this special property (Poger and Mark 2013, Belin *et al.* 2018). Cornejo-Castillo and Zehr (2019) screened publicly available genomes of marine cyanobacteria to examine the presence of hopanoid biosynthetic and modification genes. Remarkably, the authors discovered that (*1*) all non-heterocyst-forming N_2-fixing cyanobacteria had these genes (including *Trichodesmium* IMS101), whereas (*2*) none of the non-N_2-fixing and heterocyst-forming N_2-fixing cyanobacteria did. Hence, the potential occurrence of the hopanoid O_2 diffusion barrier in *Trichodesmium* (Fig. 3*A*) paves new directions for future studies.

Single-cell chlorophyll fluorescence kinetic microscopy (FKM): Chlorophyll fluorescence measurements are widely employed in photosynthesis research. The introduction of FKM has made it possible to capture and map the parameters of the chlorophyll variable fluorescence kinetics of the primary photosynthetic reactions (Küpper *et al.* 2000). In relation to N_2 fixation, FKM has been used to investigate the spatial and temporal patterns of the photosynthetic activity in *Trichodesmium* IMS101 over a diel cycle (Berman-Frank *et al.* 2001b, Küpper *et al.* 2004, 2008, 2009; Andresen *et al.* 2010).

In the earliest study of Berman-Frank *et al.* (2001b), cultures measured during non-N_2-fixing hours (early and late stages of the light phase) showed homogeneous minimal fluorescence yield of the dark-adapted state (F_0) in almost all analysed trichomes, while some zonations were observed in the maximal quantum yield of PSII photochemistry (F_v/F_m). During the N_2-fixing hours (5 to 7 h after the beginning of the light phase), some cells showed higher F_0 and lower F_v/F_m. These cells appeared as bright zones not evenly distributed along trichomes, *i.e.*, they were seen in whole filaments, central areas, and in the tips of filaments. Furthermore, individual cells along the trichomes were observed to switch between the fluorescence states within a short time range (10 to 15 min). These data collectively indicated that during the light phase, all *Trichodesmium* cells are capable of photosynthetic activity and that there is a temporally and spatially modulated photosynthetic pattern.

While providing important new insights, this first study (Berman-Frank *et al.* 2001b) could not reveal the mechanisms that regulate the on and off switching of *Trichodesmium*'s photosynthetic activity. Such analysis was conducted in subsequent studies by combining the FKM method with traditional measurements (*e.g.*, acetylene-reduction assay, pigment extraction, UV/VIS spectrometry, and protein immunoblotting), fast repetition rate fluorometry (FRRf), and 77-K fluorescence spectroscopy (Küpper *et al.* 2004, 2008, 2009; Andresen *et al.* 2010). With closer examination, it was revealed that there are four activity states: two types of bright cells [(1) & (2)], which together account for up to around 15% of cells, a low-fluorescent state (3), and a normal non-N_2-fixing state (4) (Küpper *et al.* 2009). (1) Type I (bright cells) occurred only during N_2-fixing hours and had fluorescence characteristics indicating retained PSII activity (Küpper *et al.* 2004). Because of its O_2 sensitivity, nitrogenase can presumably coexist with a functional PSII only if the latter does not lead to net O_2 production, which can be achieved, for instance, by the upregulation of O_2-scavenging processes such as the Mehler reaction. The reversible switches between fluorescence levels were again observed in a way that the authors described as analogous to a traffic lights system. Given the rapidity of the switches (minutes or even quicker), it was suggested that the changes in fluorescence intensity originated from processes that are faster than the degradation or synthesis of proteins or pigments (Küpper *et al.* 2004). Such a rapid process may be a reversible coupling/uncoupling of the phycobilisome antenna from PSII reaction centres (Fig. 3*B*; Küpper *et al.* 2004, 2009). Also, a change from the association of the phycobilisome antenna with the generally less fluorescent PSI to the association with the more fluorescent PSII may take place, leading to the so-called state II to state I transition. The high PSI:PSII ratio, characteristic of *Trichodesmium* (Berman-Frank *et al.* 2001a, 2007; Levitan *et al.* 2010a), would result in a surplus of free phycobilisomes upon this transition (Küpper *et al.* 2004). (2) Type II (very bright cells), which appeared independently of N_2-fixing hours, had fluorescence characteristics indicating hardly any PSII activity. Based on these features, in type II cells the total uncoupling of energy flow from the phycobilisome antenna to PSII is likely (Fig. 3*B*; Küpper *et al.* 2004, 2009). Overall, type I cells were assigned as N_2-fixing, whereas type II cells were associated to stress conditions (Küpper *et al.* 2004, 2008). (3) Mainly after the N_2-fixing hours, an additional type of cells with low F_0 and PSII activity was observed. These cells in low-fluorescent state may represent a fluorescence-quenching recovery period during which cells are gradually passing from the N_2-fixing to the (4) normal non-N_2-fixing state (Fig. 3*B*; Küpper *et al.* 2004).

Additionally, the contribution of individual phycobiliproteins was explored by spectrally resolved FKM in order to describe the variable composition of the *Trichodesmium* antenna (Küpper *et al.* 2009, Andresen *et al.* 2010). The analysis revealed that phycourobilins (PUB) give the highest contribution to F_0 in the type I cells, which suggests that a large proportion of PUB is coupled to PSII during the N_2-fixing hours (Küpper *et al.* 2009, Andresen *et al.* 2010). While PE contribution to F_0 was small during the N_2-fixing hours, a high contribution of PE to the maximal fluorescence yield of the dark-adapted state (F_m) was shown in cells in the low-fluorescence state (suggested to represent a recovery phase after N_2-fixation; Küpper *et al.* 2009). These results overall hint that, over a diel cycle, not only phycobilisomes can reversibly uncouple from the reaction centres, but that also individual phycobiliproteins can reversibly uncouple from the phycobilisomes (Fig. 3*B*). Hence, the extreme flexibility of the *Trichodesmium* antenna seems to play an important role in regulating photosynthesis during N_2 fixation (Küpper *et al.* 2009, Andresen *et al.* 2010).

Conclusion and outlook

Our comparison of numerous studies using immunocytochemistry revealed that nitrogenase localisation differs between *T. erythraeum* strains IMS101 (confined into diazocytes; Fig. 2*C*) and NIBB1067 (not confined; Fig. 2*D*). However, it is worth to remark that the applied immunolabelling protocols were also different (Table 1S). We suggest that a direct comparison between the two strains, by applying an identical immunolabelling procedure, could address the discrepancy between previous results. Specifically, it would reveal whether the different nitrogenase localisation is due to different biology or to previously applied protocols. In a similar manner, more SIP-NanoSIMS studies conducted at relevant time-

scales are needed to better understand the spatial patterns of N_2 fixation and photosynthesis activity. Some of the inconsistencies in the results of immunolocalisation and SIP-NanoSIMS studies could also be explained by the observation that there is not necessarily a direct correspondence between *nifH* mRNA transcript levels, Fe protein abundance, and nitrogenase enzymatic activity (Levitan *et al.* 2010b). Our understanding of the interactions of these processes will improve once protocols for addressing each regulatory level are standardised and reproducible results have been obtained for the comparison of patterns of N_2 fixation and photosynthesis across different levels, including not only protein localisation and activity but also analysis of gene expression, for example, by mRNA catalysed reported deposition fluorescence *in situ* hybridization (mRNA CARD-FISH). While in the laboratory growth conditions can be closely controlled, in nature conditions are variable and different *Trichodesmium* species may co-occur, including the species lacking all the N_2-fixation genes that were recently discovered (Delmont 2021). Hence, whether immunolabelling, SIP-NanoSIMS, and mRNA CARD-FISH laboratory results are also applicable to *Trichodesmium* in the field remains to be tested. With the aim of giving new insights into the orchestration of N_2 fixation and photosynthesis in *Trichodesmium*, such a multi-technique approach is currently underway in our research group.

On the other hand, noteworthy hints/hypotheses on *Trichodesmium*'s physiology have been recently provided by modelling studies (Eichner *et al.* 2019, Inomura *et al.* 2019, Luo *et al.* 2022, Gardner *et al.* 2023). Based on their data, Eichner *et al.* (2019) examined variation in intracellular O_2 by modelling the relation between intra- and extracellular O_2 concentrations, respiratory O_2 fluxes, and membrane permeability (ultimate model output). While direct measurements of membrane permeability in *Trichodesmium* are lacking, the model revealed that single cells can achieve anoxic conditions even under high extracellular O_2 concentrations with a cell permeability that is generally in line with literature estimates for other organisms. Similarly, results of a separate modelling approach by Inomura *et al.* (2019) suggested the existence of a diffusion barrier against O_2 providing low cell permeability. Although there is no direct experimental evidence yet, we now know that *Trichodesmium* has the genes for the biosynthesis and modification of hopanoid lipids, which could confer the low permeability property to its cell membranes (Fig. 3*A*; Cornejo-Castillo and Zehr 2019). The presence of an O_2 diffusion barrier in combination with net O_2 evolution implies that photosynthetic O_2 accumulates within the cell, imposing a high risk of oxidative stress (Eichner *et al.* 2019). However, *Trichodesmium* possesses a wide defensive arsenal against O_2 accumulation and ROS (Fig. 3*A*) consisting of carotenoids (Kelman *et al.* 2009), the Mehler reaction (*e.g.*, Levitan *et al.* 2007, Milligan *et al.* 2007, Kranz *et al.* 2010), in which NiSOD, rather than MnSOD, seems to play a crucial role (Ho 2013, Ho *et al.* 2013, Rodriguez and Ho 2014, 2017; Held *et al.* 2021, Chen *et al.* 2022), dark respiration (*e.g.*, Eichner *et al.* 2014, 2019; Boatman *et al*. 2019), and potentially the Mehler-like reaction (Zhang *et al.* 2009, Allahverdiyeva *et al.* 2015) as well as photorespiration. Cell-to-cell differences in the activity of these mechanisms may support N_2 fixation in diazocytes, as implied in a recent modelling study where the Mehler reaction was restricted to diazocytes (Gardner *et al.* 2023). The dynamics of photosynthetic activity observed by FKM suggest that intracellular O_2 concentrations and fluxes may drastically change on time-scales of minutes (Fig. 3*B*; Berman-Frank *et al.* 2001b, Küpper *et al.* 2004, 2008, 2009; Andresen *et al.* 2010).

An interesting suggestion is the potential existence of a ‘conformational protection’ of the nitrogenase protein, proposed early-on by Zehr *et al.* (1993) based on detection of two forms of nitrogenase with different molecular masses in *Trichodesmium*. More recently, a small [2Fe:2S] ferredoxin termed FeSII or the ‘Shethna protein II’ was isolated from *Azotobacter vinelandii* and a molecular mechanism, which could allow rapid and reversible switches of nitrogenase activity, was characterised (Schlesier *et al.* 2016). Considering that the residence time of O_2 calculated using the model was one sec, such a fast conformational switch could provide an efficient mechanism to protect nitrogenase in response to changes in cellular O_2 concentrations (Inomura *et al.* 2019). Directly comparing a diazocyte scenario to one without any spatial confinement of nitrogenase, model calculations by Luo *et al.* (2022) suggested that a higher growth rate can be achieved by *Trichodesmium* with spatially confined nitrogenase. However, these results were obtained assuming that C and N storage molecules can be transferred between diazocytes and photosynthetic cells. If the synthesised molecules have to be extracellularly released and subsequently taken back up, the loss to the environment would be such as to cause a growth rate much lower than that of a scenario lacking spatial confinement of nitrogenase (Luo *et al.* 2022). Importantly, septal junction architectures were recently observed in *Trichodesmium* cells (Weiss *et al.* 2019). This novel finding could support the nitrogenase spatial confinement scenario modelled by Luo *et al.* (2022). Moreover, the model showed that temporal separation between N_2 fixation and photosynthesis is maintained regardless of nitrogenase spatial confinement, although the peak times of the two processes varied (Luo *et al.* 2022).

In summary, the studies compiled in this review highlight that procedural differences may have led to misobservations and, therefore, a re-evaluation of spatial separation of N_2 fixation from photosynthesis with a consistent approach is required. Additionally, optimisation and application of techniques such as SIP-NanoSIMS and mRNA CARD-FISH may provide complementary insights, while modeling will remain to be a useful tool in predicting and understanding physiological features and responses, and providing new questions and directions for further experimental research. Hence, we postulate that solving the puzzle of N_2 fixation and photosynthesis orchestration in *Trichodesmium* requires not only the use

of consistent protocols in visualisation techniques but also the combination of multiple approaches considering different regulatory levels.

References

Alboresi A., Storti M., Cendron L., Morosinotto T.: Role and regulation of class-C flavodiiron proteins in photosynthetic organisms. – Biochem. J. **476**: 2487-2498, 2019.

Allahverdiyeva Y., Isojärvi J., Zhang P., Aro E.-M.: Cyanobacterial oxygenic photosynthesis is protected by flavodiiron proteins. – Life **5**: 716-743, 2015.

Allahverdiyeva Y., Mustila H., Ermakova M. *et al.*: Flavodiiron proteins Flv1 and Flv3 enable cyanobacterial growth and photosynthesis under fluctuating light. – P. Natl. Acad. Sci. USA **110**: 4111-4116, 2013.

Andresen E., Lohscheider J., Šetlikova E. *et al.*: Acclimation of *Trichodesmium erythraeum ISM101* to high and low irradiance analysed on the physiological, biophysical and biochemical level. – New Phytol. **185**: 173-188, 2010.

Badger M.R., Price G.D., Long B.M., Woodger F.J.: The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism. – J. Exp. Bot. **57**: 249-265, 2006.

Belin B.J., Busset N., Giraud E. *et al.*: Hopanoid lipids: from membranes to plant-bacteria interactions. – Nat. Rev. Microbiol. **16**: 304-315, 2018.

Benavides M., Agawin N.S.R., Arístegui J. *et al.*: Nitrogen fixation by *Trichodesmium* and small diazotrophs in the subtropical northeast Atlantic. – Aquat. Microb. Ecol. **65**: 43-53, 2011.

Benavides M., Berthelot H., Duhamel S. *et al.*: Dissolved organic matter uptake by *Trichodesmium* in the Southwest Pacific. – Sci. Rep.-UK **7**: 41315, 2017.

Benavides M., Bonnet S., Le Moigne F.A.C. *et al.*: Sinking *Trichodesmium* fixes nitrogen in the dark ocean. – ISME J. **16**: 2398-2405, 2022.

Benavides M., Moisander P.H., Daley M.C. *et al.*: Longitudinal variability of diazotroph abundances in the subtropical North Atlantic Ocean. – J. Plankton Res. **38**: 662-672, 2016.

Bergman B., Carpenter E.J.: Nitrogenase confined to randomly distributed trichomes in the marine cyanobacterium *Trichodesmium thiebautii*. – J. Phycol. **27**: 158-165, 1991.

Bergman B., Gallon J.R., Rai A.N., Stal L.J.: N_2 fixation by non-heterocystous cyanobacteria. – FEMS Microbiol. Rev. **19**: 139-185, 1997.

Bergman B., Siddiqui P.J.A., Carpenter E.J., Peschek G.A.: Cytochrome oxidase: Subcellular distribution and relationship to nitrogenase expression in the nonheterocystous marine cyanobacterium *Trichodesmium thiebautii*. – Appl. Environ. Microb. **59**: 3239-3244, 1993.

Berman-Frank I., Cullen J.T., Shaked Y. *et al.*: Iron availability, cellular iron quotas, and nitrogen fixation in *Trichodesmium*. – Limnol. Oceanogr. **46**: 1249-1260, 2001a.

Berman-Frank I., Lundgren P., Chen Y.B. *et al.*: Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. – Science **294**: 1534-1537, 2001b.

Berman-Frank I., Lundgren P., Falkowski P.G.: Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. – Res. Microbiol. **154**: 157-164, 2003.

Berman-Frank I., Quigg A., Finkel Z.V. *et al.*: Nitrogen-fixation strategies and Fe requirements in cyanobacteria. – Limnol. Oceanogr. **52**: 2260-2269, 2007.

Berthelot H., Bonnet S., Grosso O. *et al.*: Transfer of diazotroph-derived nitrogen towards non-diazotrophic planktonic communities: a comparative study between *Trichodesmium erythraeum*, *Crocosphaera watsonii* and *Cyanothece* sp. – Biogeosciences **13**: 4005-4021, 2016.

Boatman T.G., Davey P.A., Lawson T., Geider R.J.: CO_2 modulation of the rates of photosynthesis and light-dependent O_2 consumption in *Trichodesmium*. – J. Exp. Bot. **70**: 589-597, 2019.

Bonnet S., Berthelot H., Turk-Kubo K. *et al.*: Diazotroph derived nitrogen supports diatom growth in the South West Pacific: A quantitative study using nanoSIMS. – Limnol. Oceanogr. **61**: 1549-1562, 2016.

Boyd E.S., Peters J.W.: New insights into the evolutionary history of biological nitrogen fixation. – Front. Microbiol. **4**: 201, 2013.

Bristow L.A., Mohr W., Ahmerkamp S., Kuypers M.M.M.: Nutrients that limit growth in the ocean. – Curr. Biol. **27**: R474-R478, 2017.

Capone D.G., Burns J.A., Montoya J.P. *et al.*: Nitrogen fixation by *Trichodesmium* spp.: An important source of new nitrogen to the tropical and subtropical North Atlantic Ocean. – Global Biogeochem. Cy. **19**: GB2024, 2005.

Capone D.G., Knapp A.N.: A marine nitrogen cycle fix? – Nature **445**: 159-160, 2007.

Capone D.G., O'Neil J.M., Zehr J., Carpenter E.J.: Basis for diel variation in nitrogenase activity in the marine planktonic cyanobacterium *Trichodesmium thiebautii*. – Appl. Environ. Microb. **56**: 3532-3536, 1990.

Capone D.G., Zehr J.P., Paerl H.W. *et al.*: *Trichodesmium*, a globally significant marine cyanobacterium. – Science **276**: 1221-1229, 1997.

Carpenter E.J., Bergman B., Dawson R. *et al.*: Glutamine synthetase and nitrogen cycling in colonies of the marine diazotrophic cyanobacteria *Trichodesmium* spp. – Appl. Environ. Microb. **58**: 3122-3129, 1992.

Carpenter E.J., Capone D.G.: Nitrogen fixation in the marine environment. – In: Capone D.G., Bronk D.A., Mulholland M.R., Carpenter E.J. (ed.): Nitrogen in the Marine Environment. 2nd Edition. Pp. 141-198. Academic Press, San Diego 2008.

Carpenter E.J., Chang J., Cottrell M. *et al.*: Re-evaluation of nitrogenase oxygen-protective mechanisms in the planktonic marine cyanobacterium *Trichodesmium*. – Mar. Ecol. Prog. Ser. **65**: 151-158, 1990.

Chen C.-C., Rodriguez I.B., Chen Y.-L.L. *et al.*: Nickel superoxide dismutase protects nitrogen fixation in *Trichodesmium*. – Limnol. Oceanogr. Lett. **7**: 363-371, 2022.

Chen Y.-B., Dominic B., Mellon M.T., Zehr J.P.: Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. strain IMS101. – J. Bacteriol. **180**: 3598-3605, 1998.

Chen Y.-B., Zehr J.P., Mellon M.T.: Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. IMS 101 in defined media: evidence for a circadian rhythm. – J. Phycol. **32**: 916-923, 1996.

Coles V.J., Hood R.R., Pascual M., Capone D.G.: Modeling the impact of *Trichodesmium* and nitrogen fixation in the Atlantic Ocean. – J. Geophys. Res. **109**: C06007, 2004.

Cornejo-Castillo F.M., Zehr J.P.: Hopanoid lipids may facilitate aerobic nitrogen fixation in the ocean. – P. Natl. Acad. Sci. USA **116**: 18269-18271, 2019.

Delmont T.O.: Environmental genomics points to non-diazotrophic *Trichodesmium* species abundant and widespread in the open ocean. – P. Natl. Acad. Sci. USA **118**: e2112355118, 2021.

Dugdale R.C., Menzel D.W., Ryther J.H.: Nitrogen fixation in the

Sargasso Sea. – Deep Sea Res. **7**: 297-300, 1961.
Dupont C.L., Barbeau K., Palenik B.: Ni uptake and limitation in marine *Synechococcus* strains. – Appl. Environ. Microb. **74**: 23-31, 2008.
Eichner M., Klawonn I., Wilson S.T. *et al.*: Chemical microenvironments and single-cell carbon and nitrogen uptake in field-collected colonies of *Trichodesmium* under different pCO_2. – ISME J. **11**: 1305-1317, 2017.
Eichner M., Kranz S.A., Rost B.: Combined effects of different CO_2 levels and N sources on the diazotrophic cyanobacterium *Trichodesmium*. – Physiol. Plantarum **152**: 316-330, 2014.
Eichner M., Thoms S., Rost B. *et al.*: N_2 fixation in free-floating filaments of *Trichodesmium* is higher than in transiently suboxic colony microenvironments. – New Phytol. **222**: 852-863, 2019.
Eisenhut M., Ruth W., Haimovich M. *et al.*: The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiontically to plants. – P. Natl. Acad. Sci. USA **105**: 17199-17204, 2008.
El-Shehawy R., Lugomela C., Ernst A., Bergman B.: Diurnal expression of *hetR* and diazocyte development in the filamentous non-heterocystous cyanobacterium *Trichodesmium erythraeum*. – Microbiology **149**: 1139-1146, 2003.
Falkowski P.G.: Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. – Nature **387**: 272-275, 1997.
Falkowski P.G., Raven J.A.: Aquatic Photosynthesis. 2nd Edition. Pp. 5-10. Blackwell Science, Malden 1997.
Fay P.: Oxygen relations of nitrogen-fixation in cyanobacteria. – Microbiol. Rev. **56**: 340-373, 1992.
Finzi-Hart J.A., Pett-Ridge J., Weber P.K. *et al.*: Fixation and fate of C and N in the cyanobacterium *Trichodesmium* using nanometer-scale secondary ion mass spectrometry. – P. Natl. Acad. Sci. USA **106**: 6345-6350, 2009.
Fredriksson C., Bergman B.: Nitrogenase quantity varies diurnally in a subset of cells within colonies of the non-heterocystous cyanobacteria *Trichodesmium* spp. – Microbiology **141**: 2471-2478, 1995.
Fredriksson C., Bergman B.: Ultrastructural characterisation of cells specialised for nitrogen fixation in a non-heterocystous cyanobacterium, *Trichodesmium* spp. – Protoplasma **197**: 76-85, 1997.
Gallon J.R.: The oxygen sensitivity of nitrogenase: a problem for biochemists and micro-organisms. – Trends Biochem. Sci. **6**: 19-23, 1981.
Gardner J.J., Hodge B.-M.S., Boyle N.R.: Investigating the unique ability of *Trichodesmium* to fix carbon and nitrogen simultaneously using MiMoSA. – mSystems: e00601-20, 2023.
Giordano M., Beardall J., Raven J.A.: CO_2 concentrating mechanisms in algae: Mechanisms, environmental modulation, and evolution. – Annu. Rev. Plant. Biol. **56**: 99-131, 2005.
Golden J.W., Yoon H.-S.: Heterocyst development in *Anabaena.* – Curr. Opin. Microbiol. **6**: 557-563, 2003.
Großkopf T., LaRoche J.: Direct and indirect costs of dinitrogen fixation in *Crocosphaera watsonii* WH8501 and possible implications for the nitrogen cycle. – Front. Microbiol. **3**: 236, 2012.
Gruber N.: A bigger nitrogen fix. – Nature **436**: 786-787, 2005.
Held N.A., Sutherland K.M., Webb E.A. *et al.*: Mechanisms and heterogeneity of in situ mineral processing by the marine nitrogen fixer *Trichodesmium* revealed by single-colony metaproteomics. – ISME Commun. **1**: 35, 2021.
Held N.A., Waterbury J.B., Webb E.A. *et al.*: Dynamic diel proteome and daytime nitrogenase activity supports buoyancy in the cyanobacterium *Trichodesmium*. – Nat. Microbiol. **7**: 300-311, 2022.
Helman Y., Tchernov D., Reinhold L. *et al.*: Genes encoding A-type flavoproteins are essential for photoreduction of O_2 in cyanobacteria. – Curr. Biol. **13**: 230-235, 2003.
Herrmann A.M., Ritz K., Nunan N. *et al.*: Nano-scale secondary ion mass spectrometry – A new analytical tool in biogeochemistry and soil ecology: A review article. – Soil Biol. Biochem. **39**: 1835-1850, 2007.
Ho T.-Y.: Nickel limitation of nitrogen fixation in *Trichodesmium*. – Limnol. Oceanogr. **58**: 112-120, 2013.
Ho T.-Y., Chu T.-H., Hu C.-L.: Interrelated influence of light and Ni on *Trichodesmium* growth. – Front. Microbiol. **4**: 139, 2013.
Hynes A.M., Webb E.A., Doney S.C., Waterbury J.B.: Comparison of cultured *Trichodesmium* (Cyanophyceae) with species characterized from the field. – J. Phycol. **48**: 196-210, 2012.
Inomura K., Wilson S.T., Deutsch C.: Mechanistic model for the coexistence of nitrogen fixation and photosynthesis in marine *Trichodesmium*. – mSystems **4**: e00210-19, 2019.
Janson S., Carpenter E.J., Bergman B.: Compartmentalisation of nitrogenase in a non-heterocystous cyanobacterium: *Trichodesmium contortum*. – FEMS Microbiol. Lett. **118**: 9-14, 1994.
Kana T.M.: Rapid oxygen cycling in *Trichodesmium thiebautii*. – Limnol. Oceanogr. **38**: 18-24, 1993.
Kelman D., Ben-Amotz A., Berman-Frank I.: Carotenoids provide the major antioxidant defence in the globally significant N_2-fixing marine cyanobacterium *Trichodesmium*. – Environ. Microbiol. **11**: 1897-1908, 2009.
Kranz S.A., Dieter S., Richter K.-U., Rost B.: Carbon acquisition by *Trichodesmium*: The effect of pCO_2 and diurnal changes. – Limnol. Oceanogr. **54**: 548-559, 2009.
Kranz S.A., Eichner M., Rost B.: Interactions between CCM and N_2 fixation in *Trichodesmium*. – Photosynth. Res. **109**: 73-84, 2011.
Kranz S.A., Levitan O., Richter K.-U. *et al.*: Combined effects of CO_2 and light on the N_2-fixing cyanobacterium *Trichodesmium* IMS101: Physiological responses. – Plant Physiol. **154**: 334-345, 2010.
Kumar K., Mella-Herrera R.A., Golden J.W.: Cyanobacterial heterocysts. – Cold Spring Harb. Perspect. Biol. **2**: a000315, 2010.
Küpper H., Andresen E., Wiegert S. *et al.*: Reversible coupling of individual phycobiliprotein isoforms during state transitions in the cyanobacterium *Trichodesmium* analysed by single-cell fluorescence kinetic measurements. – BBA-Bioenergetics **1787**: 155-167, 2009.
Küpper H., Ferimazova N., Šetlík I., Berman-Frank I.: Traffic lights in *Trichodesmium*. Regulation of photosynthesis for nitrogen fixation studied by chlorophyll fluorescence kinetic microscopy. – Plant Physiol. **135**: 2120-2133, 2004.
Küpper H., Šetlík I., Seibert S. *et al.*: Iron limitation in the marine cyanobacterium *Trichodesmium* reveals new insights into regulation of photosynthesis and nitrogen fixation. – New Phytol. **179**: 784-798, 2008.
Küpper H., Šetlík I., Trílek M., Nebdal L.: A microscope for two-dimensional measurements of *in vivo* chlorophyll fluorescence kinetics using pulsed measuring light, continuous actinic light and saturating flashes. – Photosynthetica **38**: 553-570, 2000.
Langlois R.J., Hümmer D., LaRoche J.: Abundances and distributions of the dominant *nifH* phylotypes in the Northern Atlantic Ocean. – Appl. Environ. Microb. **74**: 1922-1931, 2008.

Levitan O., Brown C.M., Sudhaus S. *et al.*: Regulation of nitrogen metabolism in the marine diazotroph *Trichodesmium* IMS101 under varying temperatures and atmospheric CO_2 concentrations. – Environ. Microbiol. **12**: 1899-1912, 2010b.

Levitan O., Kranz S.A., Spungin D. *et al.*: Combined effects of CO_2 and light on the N_2-fixing cyanobacterium *Trichodesmium* IMS101: A mechanistic view. – Plant Physiol. **154**: 346-356, 2010a.

Levitan O., Rosenberg G., Setlik I. *et al.*: Elevated CO_2 enhances nitrogen fixation and growth in the marine cyanobacterium *Trichodesmium*. – Glob. Change Biol. **13**: 531-538, 2007.

Lin S., Henze S., Lundgren P. *et al.*: Whole-cell immunolocalization of nitrogenase in marine diazotrophic cyanobacteria, *Trichodesmium* spp. – Appl. Environ. Microb. **64**: 3052-3058, 1998.

Lundgren P., Janson S., Jonasson S. *et al.*: Unveiling of novel radiations within *Trichodesmium* cluster by *hetR* gene sequence analysis. – Appl. Environ. Microb. **71**: 190-196, 2005.

Lundgren P., Soderback E., Singer A. *et al.*: *Katagnymene*: Characterization of a novel marine diazotroph. – J. Phycol. **37**: 1052-1062, 2001.

Luo W., Inomura K., Zhang H., Luoa Y.-W.: N_2 fixation in *Trichodesmium* does not require spatial segregation from photosynthesis. – mSystems **7**: e00538-22, 2022.

Mahaffey C., Michaels A.F., Capone D.G.: The conundrum of marine N_2 fixation. – Am. J. Sci. **305**: 546-595, 2005.

Mareš J., Strunecký O., Bučinská L., Wiedermannová J.: Evolutionary patterns of thylakoid architecture in cyanobacteria. – Front. Microbiol. **10**: 277, 2019.

Martínez-Pérez C., Mohr W., Löscher C.R. *et al.*: The small unicellular diazotrophic symbiont, UCYN-A, is a key player in the marine nitrogen cycle. – Nat. Microbiol. **1**: 16163, 2016.

Middag R., de Baar H.J.W., Bruland K.W., van Heuven S.M.A.C.: The distribution of nickel in the West-Atlantic Ocean, its relationship with phosphate and a comparison to cadmium and zinc. – Front. Mar. Sci. **7**: 105, 2020.

Milligan A.J., Berman-Frank I., Gerchman Y. *et al.*: Light-dependent oxygen consumption in nitrogen-fixing cyanobacteria plays a key role in nitrogenase protection. – J. Phycol. **43**: 845-852, 2007.

Mulholland M.R., Bronk D.A., Capone D.G.: Dinitrogen fixation and release of ammonium and dissolved organic nitrogen by *Trichodesmium* IMS101. – Aquat. Microb. Ecol. **37**: 85-94, 2004.

Mulholland M.R., Capone D.G.: Dinitrogen fixation in the Indian Ocean. – In: Wiggert J.D., Hood R.R., Naqvi S.W.A. *et al.* (ed): Indian Ocean Biogeochemical Processes and Ecological Variability. Pp 167-186. American Geophysical Union, Washington 2009.

Mulholland M.R., Lomas M.W.: Nitrogen uptake and assimilation. – In: Capone D.G., Bronk D.A., Mulholland M.R., Carpenter E.J. (ed.): Nitrogen in the Marine Environment. 2nd Edition. Pp. 303-384. Academic Press, San Diego 2008.

Mullineaux C.W., Mariscal V., Nenninger A. *et al.*: Mechanism of intercellular molecular exchange in heterocyst-forming cyanobacteria. – EMBO J. **27**: 1299-1308, 2008.

Mustila H., Paananen P., Battchikova N. *et al.*: The flavodiiron protein Flv3 functions as a homo-oligomer during stress acclimation and is distinct from the Flv1/Flv3 hetero-oligomer specific to the O_2 photoreduction pathway. – Plant Cell Physiol. **57**: 1468-1483, 2016.

Nikkanen L., Solymosi D., Jokel M., Allahverdiyeva Y.: Regulatory electron transport pathways of photosynthesis in cyanobacteria and microalgae: Recent advances and biotechnological prospects. – Physiol. Plantarum **173**: 514-525, 2021.

Ohki K.: Intercellular localization of nitrogenase in a non-heterocystous cyanobacterium (Cyanophyte), *Trichodesmium* sp. NIBB1067. – J. Oceanogr. **64**: 211-216, 2008.

Ohki K., Fujita Y.: Laboratory culture of the pelagic blue-green alga *Trichodesmium thiebautii*: Conditions for unialgal culture. – Mar. Ecol. Prog. Ser. **7**: 185-190, 1982.

Ohki K., Fujita Y.: Aerobic nitrogenase activity measured as acetylene reduction in the marine non-heterocystous cyanobacterium *Trichodesmium* spp. grown under artificial conditions. – Mar. Biol. **98**: 111-114, 1988.

Ohki K., Taniuchi Y.: Detection of nitrogenase in individual cells of a natural population of *Trichodesmium* using immunocytochemical methods for fluorescent cells. – J. Oceanogr. **65**: 427-432, 2009.

Orcutt K.M., Rasmussen U., Webb E.A. *et al.*: Characterization of *Trichodesmium* spp. by genetic techniques. – Appl. Environ. Microb. **68**: 2236-2245, 2002.

Orcutt K.M., Ren S., Gundersen K.: Detecting proteins in highly autofluorescent cells using quantum dot antibody conjugates. – Sensors-Basel **9**: 7540-7549, 2009.

Paerl H.W., Bebout B.M.: Direct measurement of O_2-depleted microzones in marine *Oscillatoria*: relation to N_2 fixation. – Science **241**: 442-445, 1988.

Paerl H.W., Priscu J.C., Brawner D.L.: Immunochemical localization of nitrogenase in marine *Trichodesmium* aggregates: Relationship to N_2 fixation potential. – Appl. Environ. Microb. **55**: 2965-2975, 1989.

Pajares S., Ramos R.: Processes and microorganisms involved in the marine nitrogen cycle: Knowledge and gaps. – Front. Mar. Sci. **6**: 739, 2019.

Pierella Karlusich J.J., Pelletier E., Lombard F. *et al.*: Global distribution patterns of marine nitrogen-fixers by imaging and molecular methods. – Nat. Commun. **12**: 4160, 2021.

Poger D., Mark A.E.: The relative effect of sterols and hopanoids on lipid bilayers: When comparable is not identical. – J. Phys. Chem. B **117**: 16129-16140, 2013.

Postgate J.R.: Nitrogen Fixation. 3rd Edition. Cambridge University Press, Cambridge 1998.

Prufert-Bebout L., Paerl H.W., Lassen C.: Growth, nitrogen fixation, and spectral attenuation in cultivated *Trichodesmium* species. – Appl. Environ. Microb. **59**: 1367-1375, 1993.

Rascio N., La Rocca N.: Biological nitrogen fixation. – In: Elias S.A. (ed.): Reference Module in Earth Systems and Environmental Sciences. Pp. 412-419. Elsevier, Amsterdam 2013.

Robson R.L., Postgate J.R.: Oxygen and hydrogen in biological nitrogen fixation. – Annu. Rev. Microbiol. **34**: 183-207, 1980.

Rodriguez I.B., Ho T.-Y.: Diel nitrogen fixation pattern of *Trichodesmium*: the interactive control of light and Ni. – Sci. Rep.-UK **4**: 4445, 2014.

Rodriguez I.B., Ho T.-Y.: Interactive effects of spectral quality and trace metal availability on the growth of *Trichodesmium* and *Symbiodinium*. – PLoS ONE **12**: e0188777, 2017.

Sandh G., El-Shehawy R., Díez B., Bergman B.: Temporal separation of cell division and diazotrophy in the marine diazotrophic cyanobacterium *Trichodesmium erythraeum* IMS101. – FEMS Microbiol. Lett. **295**: 281-288, 2009.

Sandh G., Ran L., Xu L. *et al.*: Comparative proteomic profiles of the marine cyanobacterium *Trichodesmium erythraeum* IMS101 under different nitrogen regimes. – Proteomics **11**: 406-419, 2011.

Sandh G., Xu L., Bergman B.: Diazocyte development in the marine diazotrophic cyanobacterium *Trichodesmium*. – Microbiology **158**: 345-352, 2012.

Schlesier J., Rohde M., Gerhardt S., Einsle O.: A conformational

switch triggers nitrogenase protection from oxygen damage by Shethna protein II (FeSII). – J. Am. Chem. Soc. **138**: 239-247, 2016.

Schneegurt M.A., Sherman D.M., Nayar S., Sherman L.A.: Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece* sp. strain ATCC 51142. – J. Bacteriol. **176**: 1586-1597, 1994.

Seefeldt L.C., Yang Z.-Y., Lukoyanov D.A. *et al.*: Reduction of substrates by nitrogenases. – Chem. Rev. **120**: 5082-5106, 2020.

Siddiqui P.J.A., Carpenter E.J., Bergman B.: Ultrastructure and immunolocalization of phycobiliproteins and ribulose-1,5-bisphosphate carboxylase/oxygenase in the marine cyanobacterium *Trichodesmium thiebautii*. – J. Phycol. **28**: 320-327, 1992.

Tamagnini P., Axelsson R., Lindberg P. *et al.*: Hydrogenases and hydrogen metabolism of cyanobacteria. – Microbiol. Mol. Biol. Rev. **66**: 1-20, 2002.

Taniuchi Y., Murakami A., Ohki K.: Whole-cell immuno-cytochemical detection of nitrogenase in cyanobacteria: improved protocol for highly fluorescent cells. – Aquat. Microb. Ecol. **51**: 237-247, 2008.

Tyrrell T., Marañon E., Poulton A.J. *et al.*: Large-scale latitudinal distribution of *Trichodesmium* spp. in the Atlantic Ocean. – J. Plankton Res. **25**: 405-416, 2003.

Weiss G.L., Kieninger A.-K., Maldener I. *et al.*: Structure and function of a bacterial gap junction analog. – Cell **178**: 374-384, 2019.

Westberry T.K., Siegel D.A.: Spatial and temporal distribution of *Trichodesmium* blooms in the world's oceans. – Global Biogeochem. Cy. **20**: GB4016, 2006.

Zehr J.P., Capone D.G.: Changing perspectives in marine nitrogen fixation. – Science **368**: eaay9514, 2020.

Zehr J.P., Wyman M., Miller V. *et al.*: Modification of the Fe protein of nitrogenase in natural populations of *Trichodesmium thiebautii*. – Appl. Environ. Microb. **59**: 669-676, 1993.

Zhang P., Allahverdiyeva Y., Eisenhut M., Aro E.-M.: Flavodiiron proteins in oxygenic photosynthetic organisms: Photoprotection of photosystem II by Flv2 and Flv4 in *Synechocystis* sp. PCC 6803. – PLoS ONE **4**: e5331, 2009.

© The authors. This is an open access article distributed under the terms of the Creative Commons BY-NC-ND Licence.