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ORIGINAL ARTICLE

Chemical microenvironments and single-cell carbon and nitrogen uptake in field-collected colonies of *Trichodesmium* under different pCO_2

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Gradients of oxygen (O_2) and pH, as well as small-scale fluxes of carbon (C), nitrogen (N) and O_2 were investigated under different partial pressures of carbon dioxide (pCO_2) in field-collected colonies of the marine dinitrogen (N_2)-fixing cyanobacterium *Trichodesmium*. Microsensor measurements indicated that cells within colonies experienced large fluctuations in O_2, pH and CO_2 concentrations over a day–night cycle. O_2 concentrations varied with light intensity and time of day, yet colonies exposed to light were supersaturated with O_2 (up to ~200%) throughout the light period and anoxia was not detected. Alternating between light and dark conditions caused a variation in pH levels by on average 0.5 units (equivalent to 15 nmol l^{-1} proton concentration). Single-cell analyses of C and N assimilation using secondary ion mass spectrometry (SIMS; large geometry SIMS and nanoscale SIMS) revealed high variability in metabolic activity of single cells and trichomes of *Trichodesmium*, and indicated transfer of C and N to colony-associated non-photosynthetic bacteria. Neither O_2 fluxes nor C fixation by *Trichodesmium* were significantly influenced by short-term incubations under different pCO_2 levels, whereas N_2 fixation increased with increasing pCO_2. The large range of metabolic rates observed at the single-cell level may reflect a response by colony-forming microbial populations to highly variable microenvironments.

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Introduction

The biological fixation of dinitrogen (N_2) into ammonia by marine cyanobacteria has a crucial role in fueling primary production and material export in the oligotrophic open ocean (Karl *et al.*, 1997, 2008). In the North Pacific Subtropical Gyre, N_2 fixation has been estimated to support up to 50% of export production (Dore *et al.*, 2002; Böttjer *et al.*, 2017). A key contribution to the pool of fixed nitrogen (N) is provided by *Trichodesmium* sp., a filamentous, colony-forming, non-heterocystous cyanobacterium known for forming vast near-surface blooms throughout the tropical and subtropical oceans (LaRoche and Breitbarth, 2005). Observations of elevated concentrations of ammonium and dissolved organic N within *Trichodesmium* blooms (Karl *et al.*, 1992), and significant release of fixed N by *Trichodesmium* in laboratory and field studies (Mulholland, 2007) suggest that this diazotroph is an important source of new N to its associated community. Understanding the ecology and physiology of this keystone species has been complicated by its patchy distribution in the water column, occurrence as single trichomes and colonies in highly variable contributions (Letelier and Karl, 1996; Carpenter *et al.*, 2004) and its substantial epibiota (Siddiqui *et al.*, 1992; Sheridan *et al.*, 2002; Hewson *et al.*, 2009; Hmelo *et al.*, 2012; Momper *et al.*, 2015). Furthermore, the ability of *Trichodesmium* to fix N_2 during daytime, when it conducts oxygenic photosynthesis, remains enigmatic, as the N_2-fixing enzyme nitrogenase is inhibited by oxygen (O_2) both *in vitro* and *in vivo* (Gallon, 1992; Burgess and Lowe, 1996; Durner

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et al., 1996; Staal *et al.*, 2007). It was previously considered that O_2-depleted microzones in *Trichodesmium* colonies shielded nitrogenase from O_2 and thereby facilitated N_2 fixation (for example, Carpenter and Price, 1976; Paerl and Bebout, 1988). Additional O_2 protection mechanisms include the confinement of nitrogenase to specialized cells within trichomes termed diazocytes (Bergman and Carpenter, 1991; Fredriksson and Bergman, 1995), and the downregulation of photosynthesis during the period of maximum nitrogenase activity at midday (Berman-Frank *et al.*, 2001). Although these proposed mechanisms help explain how *Trichodesmium* can simultaneously fix N_2 and photosynthesize without heterocysts, many questions remain as to the short-term regulation of these processes in single trichomes and colonies (for example, Bergman *et al.*, 2013).

Application of microsensor technology to analyze small-scale chemical gradients has highlighted the influence of photosynthesis and respiration on O_2 and pH microenvironments in Baltic cyanobacterial aggregates (Ploug, 2008). Similar measurements also showed that *Trichodesmium* colonies can establish and sustain O_2 microenvironments, which are distinct from the ambient water (Paerl and Bebout, 1988). Colony microenvironments are also relevant to carbonate chemistry, as model calculations suggest that partial pressure of carbon dioxide (pCO_2) in the diffusive boundary layer of phytoplankton cells differs substantially from the ambient water (Wolf-Gladrow *et al.*, 1999; Flynn *et al.*, 2012). In a laboratory study simulating a *Trichodesmium* bloom, strong deviations in carbonate chemistry were observed, inducing precipitation of calcium carbonate (Kranz *et al.*, 2010b).

The responses of *Trichodesmium* to predicted future climate change have been investigated in a range of laboratory and field studies with varying results, including both strong positive responses (for example, Hutchins *et al.*, 2007; Levitan *et al.*, 2007; Kranz *et al.*, 2009) and no responses to elevated seawater pCO_2 (lower pH) scenarios (for example, Böttjer *et al.*, 2014; Gradoville *et al.*, 2014). Interactions of CO_2 effects on *Trichodesmium* with other environmental factors, such as light and nutrients (Kranz *et al.*, 2010a; Shi *et al.*, 2012), suggest that distinct microenvironments in *Trichodesmium* colonies may influence its CO_2 responses.

Here, we characterized O_2 and pH gradients within and around *Trichodesmium* colonies collected in the North Pacific Subtropical Gyre using microsensors. In addition, secondary ion mass spectrometry (SIMS) was used to quantify the uptake of carbon (C) and N on the single-cell level and to track its fate in these colonies. We discuss the implications of colony formation for key physiological processes in *Trichodesmium*, as well as potential consequences for its responses to ocean acidification.

Materials and methods

Trichodesmium and seawater sampling

Experiments were conducted at Station ALOHA (22° 45'N 158°00'W) in the oligotrophic North Pacific Subtropical Gyre in September 2014 on board R/V *Kilo Moana* (HOT Cruise 265). *Trichodesmium* colonies were sampled from the near-surface ocean (a depth of <10 m, unless otherwise specified, Supplementary Table S1) using a hand-held plankton net (200 μm mesh). Individual colonies (puff-shaped if not specified differently) for stable isotope incubations and microsensor measurements were picked with an inoculation loop and transferred into filtered (0.2 μm) seawater collected at Station ALOHA. Additional colonies for microsensor measurements (as specified in Supplementary Table S1) were sampled from coastal surface waters (21°15'N 157°49'W) and brought to the laboratory within 1 h of collection.

Carbonate chemistry

To manipulate carbonate chemistry, seawater was bubbled with gas mixtures of different pCO_2 levels and allowed to equilibrate at 25 °C for >12 h. For the ambient pCO_2 treatment, room air was supplied by aquarium pumps, yielding a pCO_2 of 506 ± 128 μatm and pH_{NBS} (US National Bureau of Standards (NBS) scale) of 8.1 ± 0.1 (Supplementary Table S2). For the high pCO_2 treatment, a mixture of ambient air and CO_2 (Scott Specialty Gases, Fremont, CA, USA; purity 99.995%) was prepared with a gas mixing pump (Westhoeff, Bochum, Germany), yielding a pCO_2 of 1117 ± 358 μatm and pH_{NBS} of 7.8 ± 0.1. pH levels were measured with a microelectrode (Unisense, Aarhus, Denmark) that was two-point calibrated with NBS buffers (Sigma Aldrich, Stockholm, Sweden). Samples for dissolved inorganic C (DIC) and total alkalinity (TA) were filtered (0.2 and 0.45 μm polycarbonate filters, respectively) and fixed with mercuric chloride. DIC was measured colorimetrically (QuAAtro autoanalyzer, Seal, Norderstedt, Germany). TA was determined by potentiometric titration (TitroLine alpha plus, Schott Instruments, Mainz, Germany). DIC, TA and pH_{NBS} in the experimental system were monitored on a daily basis (Supplementary Table S2).

Colony characteristics

Individual *Trichodesmium* colonies for cell counts (using microscopes Eclipse 90i, Nikon Instruments, Düsseldorf, Germany/Axiovert 135, Zeiss, Jena, Germany) were preserved in Lugol's solution (Sigma Aldrich). To quantify the number of cells per colony, the cumulative length of all trichomes in a colony was divided by the average cell length. Samples for analysis of species composition were coated with 0.1% agarose and stained with 4,6-diamidino-2-phenylindole before epi-fluorescence microscopy

(Axioplan2 imaging, Zeiss). *Trichodesmium* species were identified based on cell shape and size following Hynes *et al.* (2012). For quantification of chl *a* concentrations, 15 colonies per replicate were collected on glass fiber filters (25 mm diameter, Whatman, Maidstone, UK), and the chl *a* extracted in 5 ml of 90% acetone at − 20 °C before fluorometric analysis (Turner Designs 10-AU, Sunnyvale, CA, USA; Strickland and Parsons, 1972). For determination of particulate organic C and N (POC and PON) contents, as well as dry weight, 15–25 colonies per replicate were collected on pre-combusted glass fiber filters (25 mm, Whatman). Before mass spectrometry (ThermoFinnigan DeltaXP, Bremen, Germany), filters were acidified (HCl fume, >12 h) and dehydrated (50 °C, >12 h).

Microsensor measurements

Microsensor measurements of O_2 concentrations and pH in colonies were conducted in a custom-made flow system (Ploug and Jørgensen, 1999), where colonies were suspended in a laminar flow (0.1 mm s^{-1}) of filtered seawater with stable O_2 concentrations and pH levels. For determining gradients within and surrounding the colonies, the microelectrode was carefully advanced toward and through the colony as observed under a dissection microscope. Measurements were conducted with a Clark-type O_2 microelectrode (10 µm tip size; response time 1–3 s; Unisense) and a pH microelectrode (100 µm tip size, response time <10 s; Unisense). Seawater in the flow system was continuously bubbled with air of the respective pCO_2 and the resulting pCO_2 and pH_{NBS} values were confirmed as described above (section 'Carbonate chemistry'). Colonies were pre-incubated at the respective pCO_2 for 2–6.5 h in an on-deck incubator and subsequently acclimatized to conditions in the flow system for ~10 min. Measurements were performed at 25 °C and 1000 µmol photons m^{-2} s^{-1} unless specified otherwise (cold-white halogen lamps (5500 K), VWR International, Stockholm, Sweden).

Net community O_2 fluxes (J) in light and dark were calculated from the steady-state O_2 gradients at the colony surface (dC/dr) according to Fick's first law of diffusion, applying a diffusion coefficient (D) of 2.2593×10^{-5} cm^2 s^{-1} for O_2 (25 °C, salinity 34; Broecker and Peng, 1974):

$$J = -D\frac{dC}{dr}$$

Colony surface area and volume for normalization were calculated from measured half axes assuming ellipsoid geometry. O_2 residence time in colonies was estimated from the initial decrease in O_2 concentrations at the colony surface following a light to dark shift (Jørgensen and Revsbech, 1985).

Stable isotope incubations

To measure rates of C and N_2 fixation, single *Trichodesmium* colonies were incubated with $^{15}N_2$ gas (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and $NaH^{13}CO_3$ (Sigma Aldrich) in 5.9 ml vials (Exetainer, Labco, Lampeter, UK) at the respective pCO_2 level. Solutions of $^{15}N_2$ gas and $NaH^{13}CO_3$ in filtered seawater were prepared according to Klawonn *et al.* (2015b). The atom percent excess (AT% excess) for ^{13}C at the beginning of incubations was 4.2 ± 0.2 (ambient pCO_2) and 3.8 ± 0.1 (high pCO_2; quantified by gas chromatography isotope ratio mass spectrometry; $n = 6$). The AT % excess for ^{15}N was 4.2 ± 0.4 (ambient pCO_2) and 3.5 ± 0.3 (high pCO_2; quantified by membrane inlet mass spectrometry; $n = 7$). Day and night incubations were conducted for 11.5 h, respectively, in an on-deck incubator shaded to 50% surface irradiance (blue acrylic shielding #2069, Delvie's Plastic Inc., Salt Lake City, UT, USA) at sea-surface temperature, allowing for gentle movement of the vials to minimize diffusion limitation to the colonies. Subsequent to incubations, colonies were fixed with paraformaldehyde (2% final concentration; Electron Microscopy Sciences, Hatfield, PA, USA) for 24 h at 4 °C in darkness, filtered onto polycarbonate filters (type GTTP, 0.2 µm, Millipore, Merck, Darmstadt, Germany), washed with milliQ water and stored at room temperature.

Secondary ion mass spectrometry

After incubation with stable isotope tracers, isotopic composition of single cells was analyzed by large geometry SIMS (LG-SIMS; IMS1280, CAMECA, Gennevilliers, France) and nanoscale SIMS (nanoSIMS; nanoSIMS 50 L, CAMECA). LG-SIMS provides a higher throughput than nanoSIMS and was chosen for obtaining a large number of measurements on single *Trichodesmium* cells. The higher resolution measurements necessary for analyzing the enrichment of associated bacteria were performed by nanoSIMS.

A total of 66–135 *Trichodesmium* cells per treatment were analyzed by LG-SIMS, ensuring that mean values were stable and representative for the population (as described in Svedén *et al.* 2015). Filters with sample material were gold coated before measurements. Measurements were performed using a cesium ion (Cs^+) primary beam with a spatial resolution of 1 µm. Areas of interest (90 × 90 µm) were pre-sputtered with a beam of 3 nA (100 s) and then imaged using a 40–60 pA primary beam for 100 cycles. Secondary ion images of $^{13}C^{14}N^-$, $^{12}C^{14}N^-$ and $^{12}C^{15}N^-$ were recorded using a peak-switching routine at a mass resolution of ca. 6000 (M/ΔM). Selection of regions of interest (corresponding to single cells) and subsequent data analysis were performed using the WinImage2 software (CAMECA). Trichome morphology was additionally

analyzed by epi-fluorescence microscopy (Axioplan2 imaging, Zeiss).

Using nanoSIMS, 51–65 associated bacteria and 28–42 additional *Trichodesmium* cells were analyzed for each treatment in a subsample of one of the colonies analyzed previously by LG-SIMS. Subsequent to 4,6-diamidino-2-phenylindole staining and gold coating of the filters, associated bacteria attached to or positioned next to *Trichodesmium* cells on the filters were selected by fluorescence microscopy and marked by Laser Microdissection Microscopy (LMD6000, Leica, Wetzlar, Germany). The marked areas of interest were imaged by scanning electron microscopy (Quanta 250 FEG, FEI, Hillsboro, OR, USA). Secondary electron images were taken under high vacuum conditions using an Everhart–Thornley detector with an acceleration voltage of 2 kV for the electron beam. For nanoSIMS measurements, the areas were pre-sputtered with a Cs^+ primary ion beam of ~100 pA. During nanoSIMS analysis, the Cs^+ ion beam was focused to a nominal spot size of <100 nm in diameter and a beam current of 0.8–1.2 pA was used. Secondary ion images for $^{12}C^-$, $^{13}C^-$, $^{12}C^{14}N^-$ and $^{12}C^{15}N^-$ were simultaneously recorded. To minimize interferences, the instrument was tuned with an average mass resolution of >8000 M/ΔM. Selection of regions of interest and subsequent data analysis were performed using the Look@nanosims software (Polerecky *et al*., 2012).

C and N_2 fixation by *Trichodesmium* are presented as C- and N-specific fixation rates, respectively, which reflect cellular turnover of C through C-fixation and turnover of N through N_2 fixation. These values are equivalent to C- and N-based growth rates and are independent of cell size. Specific C and N fixation of *Trichodesmium* were calculated from AT % in the cell ($AT\%_{cell}$) and on the filter background ($AT\%_{bg}$), AT% excess of the total dissolved N_2 or DIC pool in the ambient water (measured as described above ('Stable isotope incubations'; $AT\%\,excess_{ambient}$) and the incubation time (dt) as follows (adapted from Montoya *et al*., 1996):

$$\text{specific fixation}\left[h^{-1}\right] = \frac{AT\%_{cell} - AT\%_{bg}}{AT\%\text{excess}_{ambient} \times \text{dt}}$$

Results

Trichodesmium colony characteristics

Colonies of *Trichodesmium* varied strongly in size (100–800 μm half axes), as well as in cell numbers per colony volume ($R^2 < 0.1$, ordinary least squares linear regression of cell number vs colony volume; Table 1). Although colony POC and PON contents were variable (Table 1), the ratio of POC:PON was relatively constant at 5.7 ± 0.8 (mol:mol; $n = 13$). Single colonies appeared to consist of multiple species tentatively identified as *Trichodesmium erythraeum*, *Trichodesmium thiebautii* and *Trichodesmium tenue* (Figure 1, Supplementary Table S3). Associated bacteria occurred primarily as single cells, but also included short filaments of 2–5 cells and did not show chlorophyll autofluorescence under blue light (488 nm) excitation (Figure 1).

Table 1 Key characteristics of *Trichodesmium* colonies sampled during the experiment (mean ± s.d.)

Parameter	*Unit*	*Value*
Cells	$Colony^{-1}$	5946 ± 6852 ($n = 22$)
Trichomes	$Colony^{-1}$	76 ± 63 ($n = 22$)
Volume	$mm^3\ colony^{-1}$	0.17 ± 0.19 ($n = 14$)
Half axis	μm	323 ± 106 ($n = 14$)
Dry weight	$\mu g\ colony^{-1}$	448 ± 175 ($n = 22$)
Chl *a*	$ng\ colony^{-1}$	14 ± 4 ($n = 5$)
POC	$\mu g\ colony^{-1}$	3.7 ± 1.5 ($n = 12$)
PON	$\mu g\ colony^{-1}$	0.5 ± 0.2 ($n = 12$)

Abbreviations: POC, particulate organic carbon; PON, particulate organic nitrogen.

Microenvironments and O_2 fluxes

Microsensor measurements revealed steep gradients in O_2 concentrations and pH associated with *Trichodesmium* colonies (Figures 2a and b). Repeated measurements on individual colonies in the light showed that net O_2 production of the microbial community was dependent on the time of day, with a decrease in O_2 concentrations within colonies toward noon, followed by an increase toward the afternoon and a decrease in the evening (Figure 2d). After sunset (20:00 hours), colonies were undersaturated with O_2 in both light and dark conditions, but anoxic conditions were not detected (Figure 2d). During daytime, O_2 concentrations were also dependent on light intensity (Figure 2c). Across all colonies and treatments, O_2 saturation varied between 61% and 203% (relative to air-saturated seawater, 25 °C, 34 psu). When exposed to light, O_2 saturation in the colony center was 153 ± 29% and 128 ± 41% for ambient and high pCO_2, respectively (Figure 3). In the dark, mean O_2 saturation in the center of colonies was 79 ± 10% and 75 ± 5% for ambient and high pCO_2, respectively (Figure 3). Although net O_2 production and dark respiration within colonies (Table 2) were highly correlated ($R^2 = 0.86$), weak correlation of O_2 fluxes with colony radius ($R^2 < 0.53$) reflected variability in the abundance and composition of organisms in colonies. Dark respiration was equivalent to 35 ± 12% ($n = 8$) of gross O_2 production (that is, the sum of net O_2 production and dark respiration), with O_2 residence time within colonies averaging 1.8 ± 0.9 min ($n = 9$). Differences in the pCO_2 treatments affected neither O_2 concentrations in the center of colonies (*t*-test, $P > 0.05$; Figure 3) nor O_2 fluxes (*t*-test, $P > 0.05$; Table 2).

pH_{NBS} in the center of colonies ranged from 7.2 to 8.8. In the light, mean pH_{NBS} levels in the center of

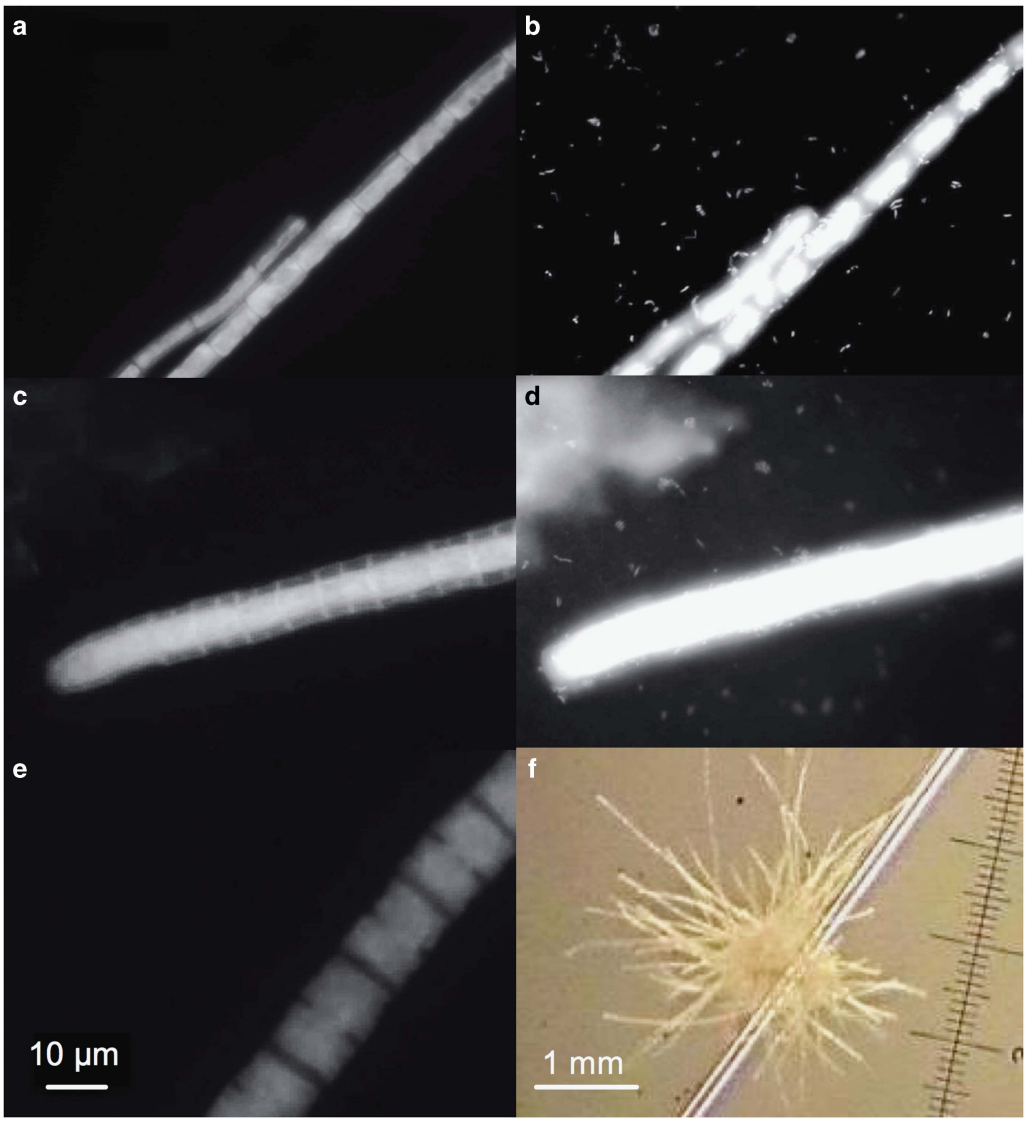

Figure 1 Morphology of *Trichodesmium* and associated bacteria. (**a**, **c**, **e**) show chlorophyll autofluorescence; (**b, d**) show 4,6-diamidino-2-phenylindole (DAPI) staining. (**a, b**) trichome type I (trichome on the left) and II (trichome on the right); (**c**, **d**) trichome type III; (**e**) trichome type IV (*cf.* Supplementary Table S3). Scale bar in **e** is valid for **a** through **e**. (**f**) Typical morphology of a puff-shaped colony.

colonies were 8.4 ± 0.2 at ambient pCO_2 (mean pH in surrounding seawater 8.1) and 8.0 ± 0.3 at high pCO_2 (mean pH in surrounding seawater 7.8; Figure 3). In the dark, pH_{NBS} levels in the center of colonies were 7.8 ± 0.2 and 7.6 ± 0.2 under ambient and high pCO_2, respectively. The respective proton concentrations were $5 \pm 3\ nmol\ l^{-1}$ (ambient pCO_2) and $12 \pm 7\ nmol\ l^{-1}$ (high pCO_2) in light, and $17 \pm 9\ nmol\ l^{-1}$ (ambient pCO_2) and $31 \pm 19\ nmol\ l^{-1}$ (high pCO_2) in dark conditions (Figure 3).

C and N_2 fixation

C and N_2 fixation were highly variable between individual *Trichodesmium* cells (Figure 4). Variation was higher between cells in different trichomes than within single trichomes. ^{15}N enrichment for cells incubated during the day ranged up to 0.75 AT% excess, equivalent to N-specific N fixation rates of up to $0.015\ h^{-1}$. C and N_2 fixation rates measured during night-time were similar to controls (that is, *Trichodesmium* colonies with no label added) in both pCO_2 treatments (Table 3). Interestingly, several trichomes showed very low ^{15}N enrichment during daytime (equivalent to N-specific N fixation $< 0.0025\ h^{-1}$) although they were highly enriched in ^{13}C relative to controls (approximately 25% of cells analyzed; for example, green squares (trichome #1), Figure 4). The reverse, that is, ^{15}N enrichment in the absence of ^{13}C enrichment occurred only in 3 out of the 320 cells analyzed. No consistent patterns in ^{13}C and ^{15}N enrichment depending on trichome width and length were observed (data not shown). C and N_2 fixation rates varied depending on cellular position within

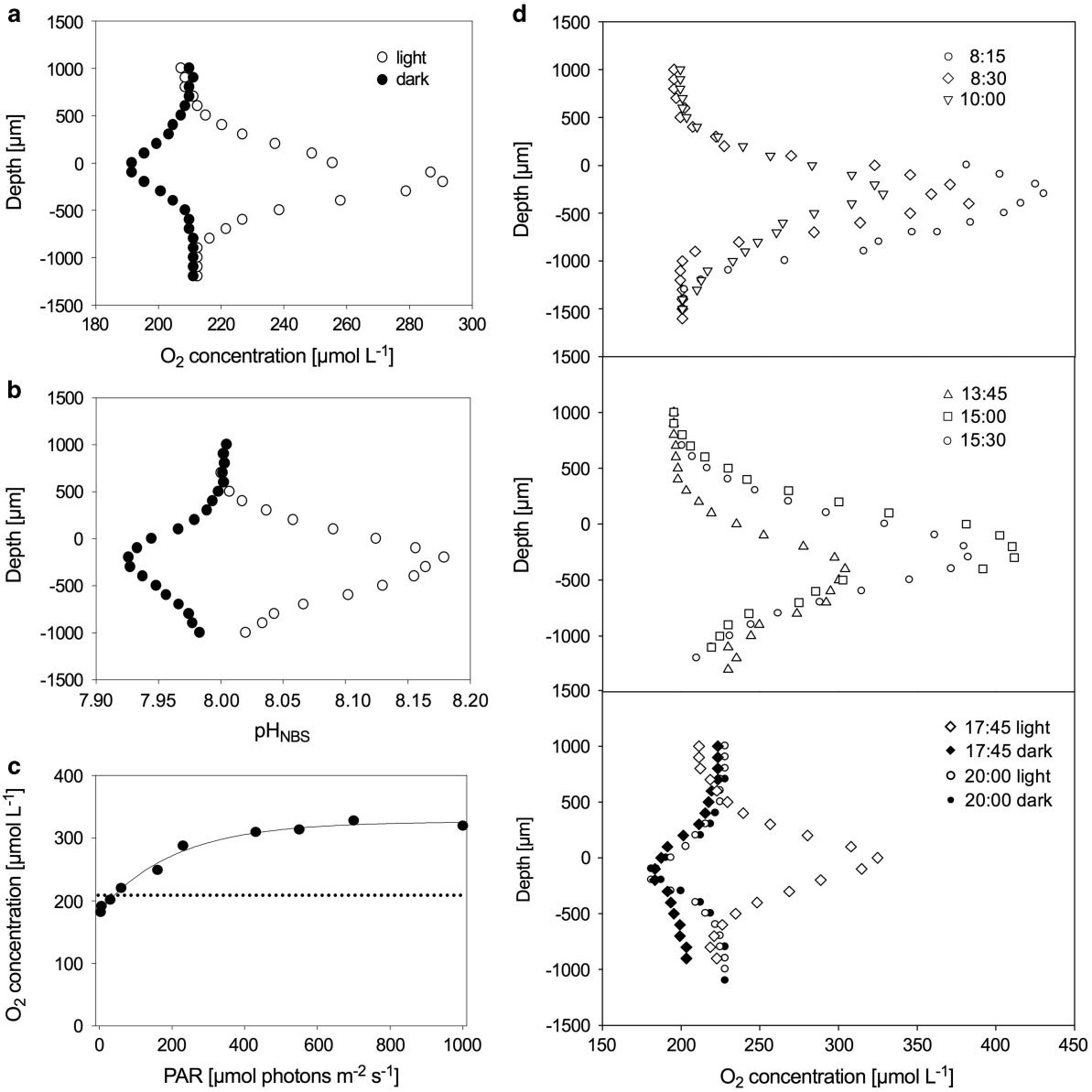

Figure 2 (**a, b**) Profiles of O_2 concentration and pH_{NBS} measured in the same *Trichodesmium* colony in light and dark (measured at 10:00 h). (**c**) Dependence of O_2 concentrations at the surface of a *Trichodesmium* colony on light intensity (measured at 16:00 h). Dotted line indicates O_2 concentration in the ambient water. (**d**) Profiles of O_2 concentrations in *Trichodesmium* colonies measured at different times of the day in light (open symbols) and dark (closed symbols); each panel shows profiles measured in one colony. The upper colony surface is at 0 µm depth.

trichomes, with decreased C fixation rates (lower by ~15%) in cells located in the central part of trichomes compared with the periphery or intermediate regions (Kruskal–Wallis test, $P<0.001$; Supplementary Figure S1). N_2 fixation rates were lower by ~50% in cells located in intermediate areas of the trichomes compared with the central and peripheral regions (Kruskal-Wallis test, $P<0.0001$).

C fixation by *Trichodesmium* during daytime was not affected by an increase in pCO_2 (Mann–Whitney test, $P>0.05$; Table 3). For samples measured by LG-SIMS, N_2 fixation during daytime was significantly elevated in the high pCO_2 treatment compared with ambient pCO_2 (Mann–Whitney test, $P<0.0001$; Table 3). However, this trend was not reflected in the smaller sample size analyzed by nanoSIMS (Table 4). The lower spatial resolution (*ca* 1 µm) of the LG-SIMS analyses allows for a greater throughput of measurements than nanoSIMS (*ca* 100 nm resolution). Hence, the 117–135 cells analyzed per treatment by LG-SIMS were assumed to be more representative for the population of *Trichodesmium* cells in these metabolically variable communities than the 28–42 cells analyzed per treatment by nanoSIMS.

Associated bacteria in colonies incubated during daytime were enriched in ^{13}C and ^{15}N, reaching on average 0.06 ± 0.07 AT% excess, independent of

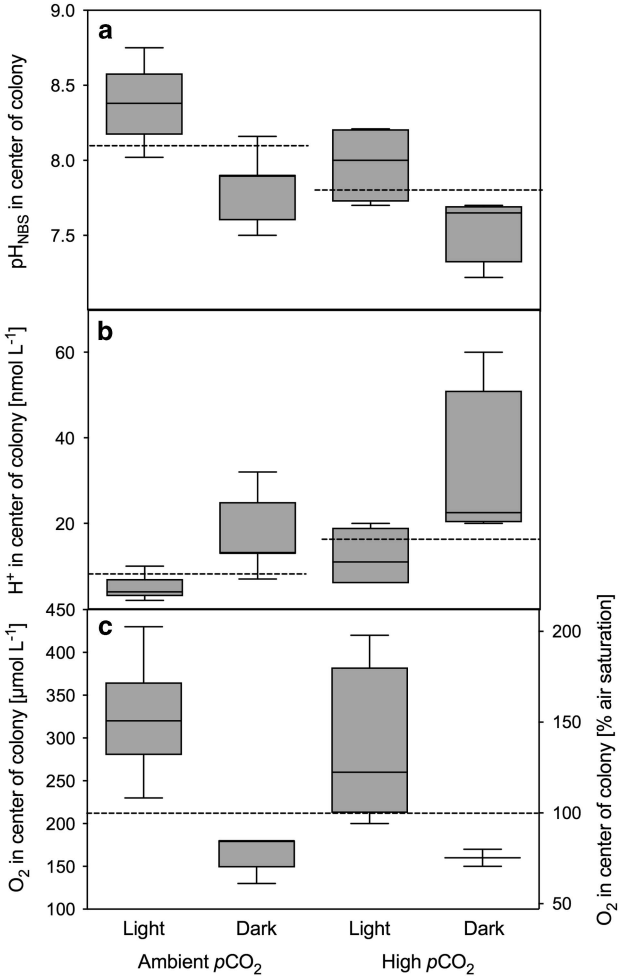

Figure 3 pH_{NBS} levels (**a**) as well as proton (H^+) concentrations (**b**) and O_2 concentrations (**c**) in the center of *Trichodesmium* colonies measured in light and dark under different pCO_2 levels in the surrounding water (ambient vs high pCO_2). Dashed lines indicate the respective conditions in the ambient water. $n \geqslant 5$, except for pH and H^+ at high pCO_2 with $n=4$ and O_2 at high pCO_2 (dark) with $n=3$. Boxes comprise the 25% and 75% quartiles; horizontal lines in the center indicate median values; whiskers indicate minimum and maximum values.

pCO_2 treatment (Mann–Whitney test, $P>0.05$; Table 4, Figure 4).

Discussion

Physiological challenges imposed by O_2 and pH microenvironments

The microsensor measurements revealed elevated O_2 concentrations and pH values within *Trichodesmium* colonies during the light period caused by photosynthesis. Such conditions in the colony center are disadvantageous for both C-acquisition and N_2 fixation. As these conditions do not occur with free-living trichomes, the benefits of colony formation, which are still unidentified, must exceed the costs incurred by these conditions. It should also be noted that the colonies analyzed in this study were relatively small compared with previous reports (for example, ~450 trichomes and 12 µg POC $colony^{-1}$ (Carpenter *et al.*, 1993); 182 trichomes and 10 µg POC $colony^{-1}$ (Letelier and Karl, 1996)), indicating that larger gradients in O_2 and pH are likely to occur.

With specific regard to O_2, concentrations measured at the colony surface as a function of light intensity (Figure 2c) indicate that *Trichodesmium* cells were acclimated to light conditions typical of the upper 10 m at Station ALOHA (http://hahana.soest.hawaii.edu/hoedylan/data/data.html) and light-saturated, but not photo-inhibited, during O_2 measurements performed at 1000 µmol photons m^{-2} s^{-1} (light saturation point (I_k) 256 µmol photons m^{-2} s^{-1}, Figure 2c). The 200% O_2 saturation observed here exceeds previous estimates of 75–100% saturation (measured at 1140 µmol photons m^{-2} s^{-1}; Paerl and Bebout, 1988), but is in agreement with estimates of ~150–200% saturation measured at <200 µmol photons m^{-2} s^{-1} by Prufert-Bebout *et al.* (1993) and Carpenter *et al.* (1990). The differences in O_2 concentrations may be due to differences in colony size, the biomass and activity of associated bacteria, or the metabolic state of *Trichodesmium*. One of the prominent temporal

Table 2 Community net O_2 production and community dark respiration in *Trichodesmium* colonies, based on microsensor measurements performed under different pCO_2 levels (ambient vs high pCO_2; mean ± s.d.)

pCO_2	*Net O_2 production*		*Dark respiration*	
	nmol O_2 $colony^{-1}$ h^{-1}	*nmol O_2 mm^{-3} h^{-1}*	*nmol O_2 $colony^{-1}$ h^{-1}*	*nmol O_2 mm^{-3} h^{-1}*
Ambient				
Mean ± s.d.	1.5 ± 0.9 ($n=10$)	20 ± 12 ($n=10$)	0.7 ± 0.4 ($n=10$)	7 ± 4 ($n=7$)
Range	0.4–3.1	3–41	0.3–1.2	2–14
High				
Mean ± s.d.	1.5 ± 1.7 ($n=4$)	11 ± 10 ($n=4$)	2.0 ± 1.9 ($n=3$)	7 ± 4 ($n=3$)
Range	0.2–3.9	4–25	0.4–4.1	3–10

Abbreviations: O_2, oxygen; pCO_2, partial pressure of carbon dioxide.
Volumetric rates are normalized to colony volume.

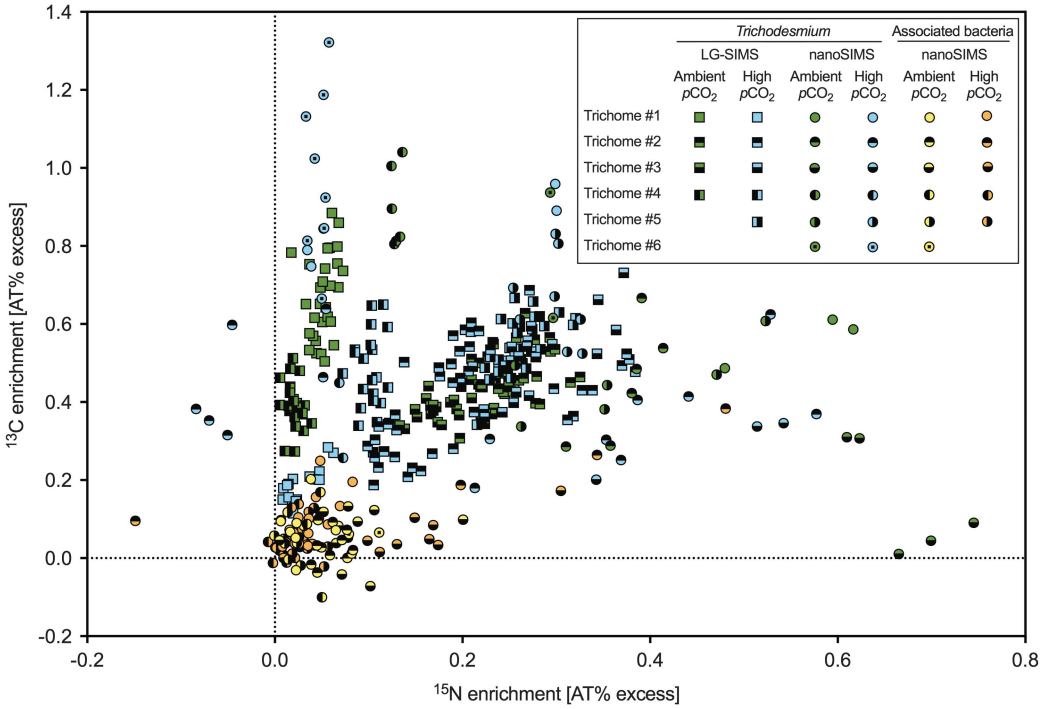

Figure 4 ^{13}C and ^{15}N enrichment in individual cells of *Trichodesmium* and associated bacteria measured by LG-SIMS (squares) and nanoSIMS (circles). Green and blue symbols represent *Trichodesmium* cells incubated during the day at ambient and high pCO_2, respectively; yellow and orange symbols represent associated bacteria incubated during the day at ambient and high pCO_2, respectively. Cells located in a single trichome or associated with the same trichome are represented by the same symbol shape and filling.

Table 3 Specific C and N fixation by *Trichodesmium* incubated at different times of day (day vs night) and under different pCO_2 levels (ambient vs high pCO_2), measured by LG-SIMS (mean ± s.d.)

	pCO_2	*C-specific C fixation* (h^{-1})	*N-specific N fixation* (h^{-1})
Day	Ambient	0.0099 ± 0.0027	0.0028 ± 0.0021 (n = 117)
	High	0.0100 ± 0.0033	0.0047 ± 0.0024 (n = 135)
Night	Ambient	0.0015 ± 0.0005	0.0004 ± 0.0001 (n = 66)
	High	0.0014 ± 0.0006	0.0004 ± 0.0002 (n = 72)
Control		0.0014 ± 0.0001	0.0004 ± 0.0001 (n = 32)

Abbreviations: C, carbon; LG-SIMS, large geometry secondary ion mass spectrometry; N, nitrogen; pCO_2, partial pressure of carbon dioxide.
Controls are measurements on *Trichodesmium* cells incubated without stable isotope tracer additions.

variances in O_2 concentrations was a midday depression in net O_2 production, with O_2 saturation decreasing from 190% to 150%, to reach $<330\ \mu mol\ O_2\ l^{-1}$ (Figure 2d). In addition, relatively high O_2 uptake compared with other colony-forming cyanobacteria (Svedén *et al.*, 2015) was observed, with dark community respiration at 35% of gross O_2 production and a light compensation point (that is, the light intensity at which colony community respiration was balanced by photosynthesis) at ~50 µmol photons $m^{-2}\ s^{-1}$ (Figure 2c), which is in line with previous findings on *Trichodesmium* (Kana, 1993). These observations suggest that the mechanisms proposed to protect nitrogenase from O_2 (Kana, 1993; Berman-Frank *et al.*, 2001) were active; however, O_2 concentrations in the colony microenvironment were still elevated substantially above ambient concentrations throughout the day (Figure 2d). Anoxia has been observed within heterotrophic, suspended microbial aggregates exceeding 1 mm in diameter (Ploug *et al.*, 1997; Klawonn *et al.*, 2015a), whereas photosynthetically active aggregates of heterocystous cyanobacteria showed elevated O_2 concentrations, in agreement with our study (Ploug, 2008). In *Trichodesmium*, which lacks the diffusion barrier provided by heterocysts, intracellular biochemical or physiological mechanisms (for example, respiration and Mehler reaction; Milligan *et al.*, 2007) are required to reduce O_2 concentrations in the vicinity of nitrogenase. The resulting intracellular O_2 gradients may differ on a single-cell level depending on cell specialization (diazocytes vs vegetative cells).

The elevated pH levels in the colony microenvironment suggest reduced CO_2 concentrations because of photosynthetic C uptake. RubisCO in cyanobacteria has an especially low affinity for CO_2 (Badger *et al.*, 1998), and *Trichodesmium* has been shown to compensate for this by highly active C concentrating mechanisms (CCM's; for example, Kranz *et al.*, 2009). Although >80% of C uptake by *Trichodesmium* was found to be met by uptake of HCO_3^- (Kranz *et al.*, 2009; Eichner *et al.*, 2015), previous studies showed notable responses in CCM activity to changes in pCO_2 (for example, twofold lower DIC half saturation concentrations in cells acclimated to 150 ppm pCO_2 (pH_{NBS} 8.56) compared with 370 ppm (pH_{NBS} 8.26); Kranz *et al.*, 2009). Also elevated O_2

Table 4 ^{13}C and ^{15}N enrichment in *Trichodesmium* and associated bacteria measured by nanoSIMS after 12-h incubations with $NaH^{13}CO_3$ (AT% excess of 4.2 ± 0.2 (ambient pCO_2) and 3.8 ± 0.1 (high pCO_2)) and $^{15}N_2$ (AT% excess of 4.2 ± 0.4 (ambient pCO_2) and 3.5 ± 0.3 (high pCO_2)) at different times (day vs night) and under different pCO_2 levels (ambient vs high pCO_2; mean ± s.d.)

	pCO_2	*Trichodesmium*		*Associated bacteria*	
		^{13}C *enrichment (AT% excess)*	^{15}N *enrichment (AT% excess)*	^{13}C *enrichment (AT% excess)*	^{15}N *enrichment (AT% excess)*
Day	Ambient	0.53 ± 0.28 (n = 28)	0.39 ± 0.19 (n = 28)	0.06 ± 0.06 (n = 51)	0.05 ± 0.04 (n = 60)
	High	0.56 ± 0.26 (n = 40)	0.21 ± 0.19 (n = 42)	0.07 ± 0.07 (n = 62)	0.06 ± 0.09 (n = 65)
Night	Ambient	ND	ND	ND	ND
	High	− 0.02 ± 0.02 (n = 4)	0.01 ± 0.00 (n = 4)	− 0.02 ± 0.06 (n = 30)	0.01 ± 0.01 (n = 40)

Abbreviations: AT% excess, atom percent excess; C, carbon; N, nitrogen; nanoSIMS, nanoscale secondary ion mass spectrometry; ND, not determined; pCO_2, partial pressure of carbon dioxide.

concentrations in the colony microenvironment may affect the CCM by increasing the risk for photorespiration: kinetic properties of RubisCO (Badger *et al.*, 1998) suggest up to 18% lower C fixation rates under O_2 concentrations measured in the colony center. Our findings thus indicate that cells have to compensate for the low CO_2 availability and high O_2 concentrations in colonies, by investing more energy into the CCM to maintain similar C fixation rates as free-living trichomes.

C and N_2 fixation and cellular interactions in Trichodesmium colonies

SIMS revealed a remarkably high variability in C and N_2 fixation between single cells, including trichomes that were photosynthetically active, but acquired no or little N by N_2 fixation (or uptake of newly fixed N released by other trichomes in the colony). A similar pattern, with only half of the photosynthetically active *Trichodesmium* cells actively fixing N_2, was recently observed in the tropical North Atlantic (Martínez-Pérez *et al.*, 2016). In contrast to previous studies demonstrating species-specific differences in N_2 fixation (Carpenter *et al.*, 1993) and substantial variability in community-level C:N fixation ratios across studies (Mulholland *et al.*, 2006), this high variability could not be explained by species identity or by differences in experimental conditions. Different N acquisition strategies, as well as differences in storage metabolism, which could cause an uncoupling of C and N fluxes during the incubation period (Finzi-Hart *et al.*, 2009), may account for some of this variation. Whereas N transfer between diazocytes and vegetative cells in *Trichodesmium* is commonly assumed to rely on extracellular diffusion (Mulholland and Capone, 2000), intercellular metabolite exchange via septal junctions was recently demonstrated in heterocystous cyanobacteria (Nürnberg *et al.*, 2015). Our observation that trichomes differed more strongly from each other than cells within the same trichome (Figure 4) indicates that N and C were transferred more efficiently along single trichomes than between different trichomes. Therefore, we hypothesize that *Trichodesmium* may have direct intercellular transport mechanisms similar to heterocystous cyanobacteria, which have not been described for this species.

The potential to separate N_2 fixation from photosynthesis in space or time has been a debated topic in *Trichodesmium* ecology. The majority of cells (~75%) in our study was enriched in both ^{13}C and ^{15}N after 12-h incubations, consistent with findings by Finzi-Hart *et al.* (2009), and supporting the perspective that fixation and intercellular transport of C and N are regulated on shorter time scales (Küpper *et al.*, 2004; Popa *et al.*, 2007). The differences in C and N_2 fixation between cells depending on their location (Supplementary Figure S1) indicate some degree of cell specialization along trichomes, consistent with the concept of diazocytes (Bergman and Carpenter, 1991; Berman-Frank *et al.*, 2001). The O_2 and pH gradients causing adverse conditions for both C and N_2 fixation in the colony center question the proposed spatial separation on a colony level (Paerl, 1994).

The ^{15}N enrichment of associated bacteria in the colonies indicates that they assimilated newly fixed ^{15}N released by *Trichodesmium*, in agreement with findings from a recent study in the South West Pacific (Bonnet *et al.*, 2016). Presumably, the ^{15}N-enriched associated bacteria did not include single-celled N_2-fixing cyanobacteria, as they showed neither chl *a* autofluorescence nor ^{15}N enrichment during night incubations. Moreover, no autotrophic host cells (as expected for non-autotrophic cyanobacteria such as UCYN-A; Thompson *et al.*, 2012) were observed. The associated bacteria are also unlikely to be heterotrophic N_2-fixers as the ^{15}N enrichment was restricted to the daytime when *Trichodesmium* fixed N_2 (Table 4), whereas heterotrophic N_2-fixers have been found to express *nifH* equally or at stronger levels at night-time compared with daytime (Church *et al.*, 2005; Moisander *et al.*, 2014). Enrichment of these non-photosynthetic bacteria in ^{13}C, in addition to ^{15}N, points toward transfer of C and N from *Trichodesmium* in the form of dissolved organic C and/or N, in line with previous reports on amino-acid release by *Trichodesmium* (Mulholland and Capone, 2000). *Trichodesmium* is also a source of transparent exopolymer particles,

which can form mucoid sheaths around trichomes (observed in our samples by scanning electron microscopy) and provide a colonization matrix for heterotrophic bacteria (Sheridan *et al.*, 2002; Berman-Frank *et al.*, 2007).

C- and N-based generation times for *Trichodesmium* were relatively long (5–28 days), in agreement with previous studies (LaRoche and Breitbarth, 2005; Mulholland *et al.*, 2006). In addition to large-scale nutrient patterns and loss processes in the water column, microenvironments may have an important role in determining *Trichodesmium* growth, with elevated pH and O_2 posing physiological constraints to C acquisition and N_2 fixation and influencing the availability of iron and phosphorus within colonies.

Implications for ocean acidification responses

The lack of CO_2-responses in O_2 evolution (observed by microsensors) and the positive effect of elevated pCO_2 on N_2 fixation (observed by LG-SIMS) are in agreement with several previous studies, including laboratory studies on *Trichodesmium* grown in single trichomes (for example, Hutchins *et al.*, 2007; Levitan *et al.*, 2007; Kranz *et al.*, 2010a) and field studies on *Trichodesmium* colonies (Hutchins *et al.*, 2009; Lomas *et al.*, 2012). Other recent field studies, in contrast, showed no or variable CO_2 effects on C and N_2 fixation (Law *et al.*, 2012; Böttjer *et al.*, 2014; Gradoville *et al.*, 2014), highlighting the variability in CO_2 responses of *Trichodesmium*.

Trichodesmium in the field is adapted to highly variable O_2 and pH levels within the colony microenvironment, with the magnitude of diurnal pH variations (~0.5 units) exceeding that of ocean acidification effects expected until the end of the century in the ambient seawater (0.3 pH units for RCP8.5; IPCC, 2013). The adaptation to high short-term pH variability may decrease the sensitivity of *Trichodesmium* colonies to the long-term trend of ocean acidification, yet the importance of the different time scales of these concurrent processes for cellular physiology is not well understood. In contrast, the energy dependence of CO_2 responses in *Trichodesmium* (for example, Kranz *et al.*, 2010a; Eichner *et al.*, 2014) suggests that the energy demands imposed by high CCM activity and O_2 protection of nitrogenase in colonies may enhance CO_2 effects, especially under low light intensities. Considering the species-specific differences in CO_2 sensitivity of *Trichodesmium* (Hutchins *et al.*, 2013), the species diversity and differences in metabolic activity between trichomes observed here seem likely to cause variable CO_2 responses. Differential CO_2 responses of *Trichodesmium* species and their associated bacteria, in turn, will affect the microbial interactions within colonies under ocean acidification.

Conclusion and outlook

In summary, *Trichodesmium* colonies were characterized by substantial gradients in O_2 concentrations and pH on a micrometer scale. In addition, the large range in O_2 and pH levels observed in light vs dark conditions suggests vast changes over day–night cycles. In an oligotrophic region often considered relatively stable, colonies thus provide variable microenvironments, requiring pronounced physiological flexibility in *Trichodesmium* and its associated organisms. The high diversity in C and N uptake rates at a single-cell level may be an adaptation of these microbial populations to thrive in fluctuating microenvironments. Future studies should address the mechanisms of C and N_2 fixation under the adverse conditions in the colony microenvironment, as well as the benefits of colony formation, the mechanisms of N transport between *Trichodesmium* cells, and the implications of different time scales in carbonate chemistry variability.

Conflict of Interest

The authors declare no conflict of interest.

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