Chemical microenvironments and single-cell carbon and nitrogen uptake in field-collected colonies of *Trichodesmium* under different $p$CO$_2$

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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 ($p$CO$_2$) 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 $p$CO$_2$ levels, whereas N$_2$ fixation increased with increasing $p$CO$_2$. 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., 1992; 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...
et al., 1996; Staal et al., 2007). It was previously considered that O2-depleted microzones in Trichodesmium colonies shielded nitrogenase from O2 and thereby facilitated N2 fixation (for example, Carpenter and Price, 1976; Paerl and Bebout, 1988). Additional O2 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 N2 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 O2 and pH microenvironments in Baltic cyanobacterial aggregates (Ploug, 2008). Similar measurements also showed that Trichodesmium colonies can establish and sustain O2 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 (pCO2) 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 pH levels 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., 2009) and no responses to elevated seawater pCO2 (lower pH) scenarios (for example, Böttjer et al., 2014; Gradoville et al., 2014). Interactions of CO2 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 CO2 responses.

Here, we characterized O2 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 pCO2 levels and allowed to equilibrate at 25 °C for >12 h. For the ambient pCO2 treatment, room air was supplied by aquarium pumps, yielding a pCO2 of 506 ± 128 μatm and pHNBS (US National Bureau of Standards (NBS) scale) of 8.1 ± 0.1 (Supplementary Table S2). For the high pCO2 treatment, a mixture of ambient air and CO2 (Scott Specialty Gases, Freemont, CA, USA; purity 99.995%) was prepared with a gas mixing pump (Westhoff, Bochum, Germany), yielding a pCO2 of 1117 ± 358 μatm and pHNBS 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 pHNBS 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 O2 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 O2 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 O2 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 pCO2 and the resulting pCO2 and pHsys values were confirmed as described above (section ‘Carbonate chemistry’). Colonies were pre-incubated at the respective pCO2 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 O2 fluxes (J) in light and dark were calculated from the steady-state O2 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 cm2 s−1 for O2 (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. O2 residence time in colonies was estimated from the initial decrease in O2 concentrations at the colony surface following a light to dark shift (Jørgensen and Revsbech, 1985).

Stable isotope incubations
To measure rates of C and N2 fixation, single Trichodesmium colonies were incubated with 15N2 gas (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and NaH13CO3 (Sigma Aldrich) in 5.9 ml vials (Exetainer, Labco, Lampeter, UK) at the respective pCO2 level. Solutions of 15N2 gas and NaH13CO3 in filtered seawater were prepared according to Klawonn et al. (2015b). The atom percent excess (AT% excess) for 15N at the beginning of incubations was 4.2 ± 0.2 (ambient pCO2) and 3.8 ± 0.1 (high pCO2; quantified by gas chromatography isotope ratio mass spectrometry; n = 6). The AT% excess for 13C at the beginning of incubations was 4.2 ± 0.4 (ambient pCO2) and 3.5 ± 0.3 (high pCO2; 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 shading #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 13C14N−, 12C14N− and 12C13N− 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

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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 FESEM, 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 focussed 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$^{15}$N− and $^{13}$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).

CO$_2$ and NO$_2$ 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 NO$_2$ 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):

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

### 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:ol; $n=13$). 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 ± 6832 ($n=22$) |
| 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 | μg colony$^{-1}$ | 448 ± 175 ($n=22$) |
| POC       | μg colony$^{-1}$ | 3.7 ± 1.5 (n=12) |
| PON       | μg 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
colonies were $8.4 \pm 0.2$ at ambient $p$CO$_2$ (mean pH in surrounding seawater 8.1) and $8.0 \pm 0.3$ at high $p$CO$_2$ (mean pH in surrounding seawater 7.8; Figure 3). In the dark, pH$_{NBS}$ levels in the center of colonies were $7.8 \pm 0.2$ and $7.6 \pm 0.2$ under ambient and high $p$CO$_2$, respectively. The respective proton concentrations were $5 \pm 3$ nmol l$^{-1}$ (ambient $p$CO$_2$) and $12 \pm 7$ nmol l$^{-1}$ (high $p$CO$_2$) in light, and $17 \pm 9$ nmol l$^{-1}$ (ambient $p$CO$_2$) and $31 \pm 19$ nmol l$^{-1}$ (high $p$CO$_2$) 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 $p$CO$_2$ 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

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**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.
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 $p$CO$_2$ (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 $p$CO$_2$ treatment compared with ambient $p$CO$_2$ (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
CO2 treatment (Mann–Whitney test, $P > 0.05$; Table 4, Figure 4).

## Discussion

**Physiological challenges imposed by O2 and pH microenvironments**

The microsensor measurements revealed elevated O2 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 N2 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 O2 and pH are likely to occur.

With specific regard to O2, 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 O2 measurements performed at 1000 μmol photons m$^{-2}$ s$^{-1}$ (light saturation point $(I_s)$ 256 μmol photons m$^{-2}$ s$^{-1}$, Figure 2c). The 200% O2 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 O2 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

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**Table 2** Community net O2 production and community dark respiration in *Trichodesmium* colonies, based on microsensor measurements performed under different pCO2 levels (ambient vs high pCO2; mean ± s.d.)

| pCO2     | Net O2 production | Dark respiration |
|----------|------------------|------------------|
|          | nmol O2 colony$^{-1}$ h$^{-1}$ | nmol O2 mm$^{-3}$ h$^{-1}$ | nmol O2 colony$^{-1}$ h$^{-1}$ | nmol O2 mm$^{-3}$ h$^{-1}$ |
| **Ambient** | 1.5 ± 0.9 ($n=10$) | 20 ± 12 ($n=10$) | 0.7 ± 0.4 ($n=10$) | 7 ± 4 ($n=7$) |
|          | 0.4–3.1           | 3–41             | 0.3–1.2             | 2–14             |
| **High** | 1.5 ± 1.7 ($n=4$) | 11 ± 10 ($n=4$) | 2.0 ± 1.9 ($n=3$) | 7 ± 4 ($n=3$) |
|          | 0.2–3.9           | 4–25             | 0.4–4.1             | 3–10             |

Abbreviations: O2, oxygen; pCO2, partial pressure of carbon dioxide.

Volumetric rates are normalized to colony volume.
variances in O₂ concentrations was a midday depression in net O₂ production, with O₂ saturation decreasing from 190% to 150%, to reach 0.330 μmol O₂ l⁻¹ (Figure 2d). In addition, relatively high O₂ uptake compared with other colony-forming cyanobacteria (Svedén et al., 2015) was observed, with dark community respiration at 35% of gross O₂ 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 O₂ (Kana, 1993; Berman-Frank et al., 2001) were active; however, O₂ 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 O₂ 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 O₂ concentrations in the vicinity of nitrogenase. The resulting intracellular O₂ 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 CO₂ concentrations because of photosynthetic C uptake. RubisCO in cyanobacteria has an especially low affinity for CO₂ (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 HCO₃⁻ (Kranz et al., 2009; Eichner et al., 2015), previous studies showed notable responses in CCM activity to changes in pCO₂ (for example, twofold lower DIC half saturation concentrations in cells acclimated to 150 ppm pCO₂ (pHₙₕₛ 8.56) compared with 370 ppm (pHₙₕₛ 8.26); Kranz et al., 2009). Also elevated O₂

| pCO₂  | 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)   |

Table 3: Specific C and N fixation by *Trichodesmium* incubated at different times of day (day vs night) and under different pCO₂ levels (ambient vs high pCO₂), measured by LG-SIMS (mean ± s.d.)

Abbreviations: C, carbon; LG-SIMS, large geometry secondary ion mass spectrometry; N, nitrogen; pCO₂, partial pressure of carbon dioxide.

Controls are measurements on *Trichodesmium* cells incubated without stable isotope tracer additions.

Figure 4: 13C and 15N 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 pCO₂, respectively; yellow and orange symbols represent associated bacteria incubated during the day at ambient and high pCO₂, respectively. Cells located in a single trichome or associated with the same trichome are represented by the same symbol shape and filling.
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 O2 concentrations measured in the colony center. Our findings thus indicate that cells have to compensate for the low CO2 availability and high O2 concentrations in colonies, by investing more energy into the CCM to maintain similar C fixation rates as free-living trichomes.

**C and N2 fixation and cellular interactions in Trichodesmium colonies**

SIMS revealed a remarkably high variability in C and N2 fixation between single cells, including trichomes that were photosynthetically active, but acquired no or little N by N2 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 N2, was recently observed in the tropical North Atlantic (Martínez-Pérez et al., 2016). In contrast to previous studies demonstrating species-specific differences in N2 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 metabolic 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 N2 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 13C and 15N 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 N2 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 O2 and pH gradients causing adverse conditions for both C and N2 fixation in the colony center question the proposed spatial separation on a colony level (Paerl, 1994).

The 15N enrichment of associated bacteria in the colonies indicates that they assimilated newly fixed 15N released by *Trichodesmium*, in agreement with findings from a recent study in the South West Pacific (Bonnet et al., 2016). Presumably, the 15N-enriched associated bacteria did not include single-celled N2-fixing cyanobacteria, as they showed neither chl a autofluorescence nor 15N 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 N2-fixers as the 15N enrichment was restricted to the daytime when *Trichodesmium* fixed N2 (Table 4), whereas heterotrophic N2-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 13C, in addition to 15N, 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,

### Table 4 13C and 15N enrichment in *Trichodesmium* and associated bacteria measured by nanoSIMS after 12-h incubations with NaH13CO3 (AT% excess of 4.2 ± 0.2 (ambient pCO2) and 3.8 ± 0.1 (high pCO2)) and 15N2 (AT% excess of 4.2 ± 0.4 (ambient pCO2) and 3.5 ± 0.3 (high pCO2)) at different times (day vs night) and under different pCO2 levels (ambient vs high pCO2; mean ± s.d.)

| pCO2    | Trichodesmium (13C enrichment) | Associated bacteria (13C enrichment) | Trichodesmium (15N enrichment) | Associated bacteria (15N enrichment) |
|---------|-------------------------------|-------------------------------------|--------------------------------|-------------------------------------|
|         | (AT% excess)                   | (AT% excess)                        | (AT% excess)                   | (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; pCO2, partial pressure of carbon dioxide.
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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