

# Anomalously high abundance of *Crocospaera* in the South Pacific Gyre

Mar Benavides<sup>1,\*,†</sup>, Mathieu Caffin<sup>1</sup>, Solange Duhamel<sup>2,†</sup>, Rachel Ann Foster<sup>3</sup>, Olivier Grosso<sup>1</sup>, Cécile Guieu<sup>4,5</sup>, France Van Wambeke<sup>1</sup>, Sophie Bonnet<sup>1</sup>

<sup>1</sup>Aix Marseille University, Université de Toulon, CNRS, IRD, MIO UM 110, 13288 Marseille, France

<sup>2</sup>Lamont Doherty Earth Observatory, Columbia University, Palisades, NY 10964, USA

<sup>3</sup>Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, 106 91, Sweden

<sup>4</sup>Sorbonne Universités, UPMC University Paris 06, INSU-CNRS, Laboratoire d’Océanographie de Villefranche, 181 Chemin du Lazaret, 06230 Villefranche-sur-mer, France

<sup>5</sup>Center for Prototype Climate Modeling, New York University Abu Dhabi, PO Box 129188, Abu Dhabi, United Arab Emirates

\*Corresponding author: French National Research Institute for Sustainable Development (IRD), Mediterranean Institute of Oceanography (MIO), Campus de Luminy Bâtiment Méditerranée, 13288 Marseille cedex 09, France. E-mail: [mar.benavides@ird.fr](mailto:mar.benavides@ird.fr)

**One sentence summary:** We find diazotrophically active *Crocospaera* in the clearest waters of the world's oceans.

†Present address: Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA.

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## Abstract

The unicellular diazotrophic cyanobacterium *Crocospaera* contributes significantly to fixed nitrogen inputs in the oligotrophic ocean. In the western tropical South Pacific Ocean (WTSP), these diazotrophs abound thanks to the phosphorus-rich waters provided by the South Equatorial Current, and iron provided aeolian and subsurface volcanic activity. East of the WTSP, the South Pacific Gyre (SPG) harbors the most oligotrophic and transparent waters of the world's oceans, where only heterotrophic diazotrophs have been reported before. Here, in the SPG, we detected unexpected accumulation of *Crocospaera* at 50 m with peak abundances of  $5.26 \times 10^5$  nifH gene copies l<sup>-1</sup>. The abundance of *Crocospaera* at 50 m was in the same order of magnitude as those detected westwards in the WTSP and represented 100% of volumetric N<sub>2</sub> fixation rates. This accumulation at 50 m was likely due to a deeper penetration of UV light in the clear waters of the SPG being detrimental for *Crocospaera* growth and N<sub>2</sub> fixation activity. Nutrient and trace metal addition experiments did not induce any significant changes in N<sub>2</sub> fixation or *Crocospaera* abundance, indicating that this population was not limited by the resources tested and could develop in high numbers despite the oligotrophic conditions. Our findings indicate that the distribution of *Crocospaera* can extend into subtropical gyres and further understanding of their controlling factors is needed.

**Keywords:** *Crocospaera*, oligotrophic, diazotrophs, cyanobacteria

## Introduction

The western tropical South Pacific (WTSP) is a recognized hotspot of N<sub>2</sub> fixation activity with an estimated contribution of ~21% to the global fixed nitrogen input (Bonnet et al. 2017, Tang et al. 2019). In this region, the filamentous and colony-forming *Trichodesmium* and unicellular *Crocospaera* thrive thanks to year-round surface seawater temperatures above 25°C, provision of phosphorus-rich waters transported westwards with the South Equatorial Current, and iron (Fe) inputs are predicted from island weathering and hydrothermal inputs along the Tonga-Kermadec volcanic arc (Bonnet et al. 2018, Caffin et al. 2018, Guieu et al. 2018, Messié et al. 2020).

*Crocospaera* is a unicellular diazotrophic cyanobacterium widespread in the open ocean (Moisander et al. 2010). It can be present as different phenotypes, including the small cell type (< 4 µm) and the large cell type (> 4 µm), which are known to have different extracellular polysaccharide excretion capabilities (Bench et al. 2013) and geographical distributions (Bench et al. 2016). While *Crocospaera* and *Trichodesmium* often share the same niche in the open ocean (Stenegren et al. 2018), *Crocospaera* differ from *Trichodesmium* in its N<sub>2</sub> fixation diel cycle, where *Crocospaera* fixes N<sub>2</sub> at night and *Trichodesmium* during the day (Zehr and Capone 2020). Also, these species have different strategies to

cope with iron limitation, *Crocospaera* being advantaged by its low size as well as iron reconditioning between day and night times (Saito et al. 2011). These diazotrophs also differ in their organic phosphorus uptake capabilities, with *Trichodesmium* being able of using both phosphonates (P–C bonds) and phosphoesters (P–O–C bonds; Dyhrman et al. 2006), while *Crocospaera* only has genes to process the latter (Dyhrman and Haley 2006).

At the boundary between the WTSP and the South Pacific Gyre (SPG; ~170 W longitude) *Trichodesmium* and *Crocospaera* populations decline abruptly due to the lack of sufficient Fe to sustain their growth and N<sub>2</sub> fixation activity, and are outcompeted by heterotrophic diazotrophs such as Gamma A and the unicellular non-photosynthetic cyanobacterial symbiont, UCYN-A (Bonnet et al. 2008, Halm et al. 2011, Shiozaki et al. 2018, Stenegren et al. 2018). During the OUTPACE cruise (Oligotrophic to UlTra oligotrophic PACific Experiment, doi: 10.17600/15000900), crossing the WTSP and the western flank of the SPG, we unexpectedly detected high abundances of *Crocospaera* in the ultraoligotrophic waters of the SPG. Intrigued by this accumulation, here, we inspect further the vertical distribution of *Crocospaera*, and the cell-specific N<sub>2</sub> fixation rate and abundance response to nutrient and trace metal additions in bottle incubations.

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## Materials and methods

### Sampling, hydrographic, and chemical variables

Samples were collected between March 23 and 27, 2015 at a station located in the oligotrophic SPG (18.42 S 166.06 W), during the OUTPACE cruise (Moutin et al. 2017). At this station, six consecutive casts were carried out to study the vertical distribution of the abundance, N<sub>2</sub> fixation activity and organic/inorganic nutrient limitation of *Crocospaera* (Table S1, Supporting Information).

Seawater was obtained from 11 depths in the photic layer (6, 17, 30, 40, 50, 60, 70, 90, 120, 150, and 180 m) using either a regular or trace metal clean (TMC) rosette frame (Table S1, Supporting Information) holding 24 hydrographic bottles (12 l) and sensors for temperature, salinity (SBE 911plus), chlorophyll fluorescence (Wetlabs ECO-AFL/FL, Philomath, Oregon). Inorganic nutrient concentrations (nitrate plus nitrite—NO<sub>x</sub>, and phosphate—here, referred to as dissolved inorganic phosphorus or DIP) were measured by colorimetric methods with a detection limit of 0.05  $\mu$ M (Aminot and Kérouel 2007). Dissolved organic carbon (DOC) concentrations were measured by high-temperature catalytic oxidation as described in Panagiotopoulos et al. (2019). Dissolved organic phosphorus (DOP) was measured by a wet oxidation procedure (Pujo-Pay and Raimbault 1994). Dissolved iron (dFe) concentrations were obtained by flow injection analyses as described in Bonnet and Guieu (2006), and are available from the supplementary material of Guieu et al. (2018).

### Abundance of diazotrophs

*Crocospaera* cells were enumerated and measured from 2.3 l seawater samples filtered on 2  $\mu$ m pore size 25 mm diameter polycarbonate membranes and fixed with paraformaldehyde diluted in filtered seawater (2% final concentration). The samples were stored at -80°C until enumeration and size measurement using an epifluorescence microscope (Zeiss Axioplan, Jena, Germany) fitted with a green (510–560 nm) excitation filter. They were counted on 40 fields (1.3 mm<sup>2</sup> per field; 0–2800 *Crocospaera*-like cells per field), scanned and analyzed with the ImageJ1 software. *nifH* gene copy numbers of UCYN-B (whose closest cultured relative is *Crocospaera watsonii*), UCYN-A1, UCYN-A2, *Trichodesmium*, and the *Richelia* symbionts of diatoms (diatom–diazotroph associations, DDAs): *het1*, *het2*, and *het3* were enumerated by Taq-Man quantitative PCR (qPCR) assays as described in Stenegren et al. (2018). In nutrient amendment experiments (described below), *Crocospaera* cells were counted by flow cytometry as described in Berthelot et al. (2016; Figure S1, Supporting Information).

### Bulk N<sub>2</sub> fixation rates

Bulk N<sub>2</sub> fixation rates were measured from samples collected in four consecutive casts while on station (Table S1, Supporting Information). For each profile, triplicate seawater samples were transferred to transparent polycarbonate 4.3-l bottles, closed gas-tight with silicon septa screwcaps, and spiked with 5 ml of <sup>15</sup>N<sub>2</sub> (98.9% atom%, Cambridge isotopes). The <sup>15</sup>N<sub>2</sub> gas batch used did not contain significant concentrations of other nitrogenous compounds labeled with <sup>15</sup>N, as previously confirmed (Dabundo et al. 2014, Benavides et al. 2015). The samples were incubated in situ for 24 h on a surface-tethered line at the same depths as samplings. Only at the four depths where the abundance of *Crocospaera* was maximal (i.e. 30, 40, 50, and 60 m), samples were incubated in shaded on-deck incubators at the irradiance corresponding to each of these depths using neutral density screening adapted to mimic the light intensity at each corresponding

sampling depth. Incubations were stopped by vacuum filtration of the samples onto precombusted 25 mm GF/F filters and stored at -20°C until analysis. A subsample of 1 l was filtered onto 0.2  $\mu$ m polycarbonate filters for subsequent single-cell N<sub>2</sub> fixation analyses (see below). Surface and DCM seawater samples were also filtered at time zero (not labeled or incubated) to determine the natural isotopic signature of ambient particulate nitrogen. Bulk particulate nitrogen concentrations and <sup>15</sup>N/<sup>14</sup>N ratios were determined with an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS, Integra 2, SerCon Ltd). The accuracy of the instrument was verified every ten samples using international reference materials IAEA-N-1 and IAEA-310A. The analytical precision associated with the mass determination averaged 2.8% for particulate organic nitrogen. The analytical precision associated with <sup>15</sup>N was 0.0010 atom%<sup>15</sup>N for a measured mass of 0.7  $\mu$ mol N. N<sub>2</sub> fixation rates were calculated with the equations in Montoya et al. (1996). The detection limit was 0.035 nmol N l<sup>-1</sup> d<sup>-1</sup>. To avoid an overestimation of the <sup>15</sup>N<sub>2</sub> gas equilibration time and consequent underestimation of N<sub>2</sub> fixation rates (White et al. 2020), we measured the dissolved <sup>15</sup>N enrichment in incubation bottles using membrane inlet mass spectrometry analyses as previously described (Kana et al. 1994, Bonnet et al. 2018).

### Cell-specific N<sub>2</sub> fixation rates

Single-cell rates of *Crocospaera*-like N<sub>2</sub> fixation rates were determined at 30, 40, 50, and 60 m by nanoscale secondary ion mass spectrometry (nanoSIMS 50L). To maximize the number of cells analyzed in each nanoSIMS image, *Crocospaera*-like cells were sorted with a Becton Dickinson Influx Mariner flow cytometer (BD Biosciences, Franklin Lakes, NJ) prior to nanoSIMS analyses as described in Berthelot et al. (2016). After determining the *Crocospaera*-like cluster, the cells were sorted on a 13 mm diameter, 0.2  $\mu$ m pore size polycarbonate membrane, and prepared for nanoSIMS analyses as previously described (Berthelot et al. 2016, Bonnet et al. 2016). Samples were sputtered prior to analyses with a current of ~10 pA for at least 2 min. Subsequently, a cesium primary beam of ~1.3 pA cesium (16 KeV) focused onto a ~100 nm spot diameter region was scanned across a 256 × 256 or 512 × 512 pixel raster (depending on the image size), with a counting time of 1 ms per pixel. Mass resolving power was ~8000 to resolved isobaric interferences. Negative secondary ions (<sup>12</sup>C<sup>14</sup>N<sup>-</sup>, <sup>12</sup>C<sup>15</sup>N<sup>-</sup>) were collected by electron multiplier detectors, and secondary electrons were also imaged simultaneously. A total of 10–50 serial quantitative secondary ion images were generated and were combined to create the final image. Between 20 and 100 planes were generated for each cell analyzed. The number of *Crocospaera*-like cells analyzed with the nanoSIMS ranged between 40 and 80 per sample.

The <sup>15</sup>N enrichment of target *Crocospaera*-like cells was used to calculate single-cell N<sub>2</sub> fixation rates as previously described (Foster et al. 2013, Bonnet et al. 2016). The cellular nitrogen content of *Crocospaera* cells (fmol N cell<sup>-1</sup>) was calculated on the basis of their diameter, converted to biovolume assuming a spherical shape, and converted to carbon (Verity et al. 2012). A C:N ratio of 8.5:1 was used to transform cellular carbon content to cellular nitrogen content based on sorted *Crocospaera* cells analyzed by EA-IRMS (see above). The cellular nitrogen content was multiplied by the abundance of *Crocospaera* cells (determined by microscopy counts) to assess the biomass of the whole group present in our samples.

## Whole community nutrient amendment experiment

Amendment experiments were performed inside a shipboard clean-air (Class 100) laboratory container, under strict TMC conditions according to the GEOTRACES guidelines ([www.geotraces.org/images/Cookbook.pdf](http://www.geotraces.org/images/Cookbook.pdf)). Seawater was sampled from 50 m (where the abundance of *Crocospaera* was maximal) using a TMC Teflon (PTFE) diaphragm pump connected to plastic tubing and dispensed into detergent and trace metal grade acid washed 4.3 l transparent polycarbonate bottles.

Sets of triplicate bottles received amendments of different nutrients known to limit N<sub>2</sub> fixation which included iron ('Fe', solution of ferric citrate at 10 nM final concentration), nickel ('Ni' nickel sulfate at 10 nM final concentration), DIP (suprapur sodium dihydrogen phosphate at 1  $\mu$ M final concentration), DOP (a mix of methylphosphonic acid, 2-aminoethylphosphonic acid and fructose 1,6-biphosphate at 1  $\mu$ M final concentration), and volcanic ashes from the archipelago of Vanuatu (Ash), and aerosols ('dust') collected on board during the OUTPACE cruise [as described in Guieu et al. (2018)]. Incubations were run for 48 h. The response of bulk N<sub>2</sub> fixation rates and *Crocospaera* abundance to these additions were tested by mass spectrometry and qPCR assays, respectively, as described above. Primary production rates were measured using 10% <sup>13</sup>C-bicarbonate labeling and 24 h incubations as previously described (Berthelot et al. 2017). Heterotrophic bacterial production rates were obtained from tritiated leucine incubations as detailed in Van Wambeke et al. (2018).

## Results

### Hydrological and biogeochemical environment

Hydrological conditions at our sampling stations remained fairly stable along the six consecutive casts carried out at our sampling station (Fig. 1). Temperature was  $> 29^{\circ}\text{C}$  from the surface down to 30 m at the thermocline, decreasing gradually to  $19^{\circ}\text{C}$  at 200 m (Fig. 1A). Salinity was 35.2 from the surface to 30 m and increased to  $\sim 36$  at 200 m, showing more variability among casts than temperature (Fig. 1B). The daytime cast (158) showed slightly higher oxygen and fluorescence levels than night time casts (190 and 018). Oxygen levels were  $\sim 187 \text{ ml l}^{-1}$  between the surface and 30 m, increasing to 200–210 ml l<sup>-1</sup> between 40 and 100 m, then decreasing to  $\sim 170 \text{ ml l}^{-1}$  at 200 m (Fig. 1C). Fluorescence profiles depicted a sharp deep chlorophyll maximum (DCM) at 140 m (Fig. 1D). PAR profiles reflect that the conditions were cloudy during the second cast (cast160). At the surface, PAR levels reached 250  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , decreasing exponentially with depth (Fig. 1E). The 1% PAR level was located at 175 m.

The concentrations of NO<sub>x</sub> were  $< 0.05 \mu\text{M}$  between the surface and 120 m, then increasing linearly with depth down to 3.5  $\mu\text{M}$  at 175 m (Fig. 1F). The concentrations of DIP were 0.28  $\mu\text{M}$  at the surface, stable at 0.13–0.16  $\mu\text{M}$  between 16 and 92 m, gradually increasing back to  $\sim 0.3 \mu\text{M}$  at 150 m (Fig. 1G). DOC and DOP concentrations showed stable levels at 74  $\mu\text{M}$  and 0.15  $\mu\text{M}$ , respectively, between the surface and 60 m, decreasing gradually with depth \*. A decrease in DOP concentrations between 0.15 and 0.13  $\mu\text{M}$  was observed at 60 m (Fig. 1I). The concentration of dFe was 0.4 nM at the surface and decreasing to  $\sim 0.16$ –0.2 nM between 50 and 150 m (Fig. 1J).

### Bulk and cell-specific N<sub>2</sub> fixation rates

Bulk N<sub>2</sub> fixation rates ranged between 0.2 and  $0.5 \pm 0.1 \text{ nmol N l}^{-1} \text{ d}^{-1}$  from the surface down to 30 m, increased to  $1.3 \text{ nmol N l}^{-1} \text{ d}^{-1}$

at 40 m, subsequently peaking at 50 m and reaching a maximum of  $2 \text{ nmol N l}^{-1} \text{ d}^{-1}$ , then decreasing back to  $1 \text{ nmol N l}^{-1} \text{ d}^{-1}$  at 60 m (Fig. 2A). Cell-specific *Crocospaera*-like N<sub>2</sub> fixation rates were measured at 30, 40, 50, and 60 m depth. Converting cell-specific to volumetric N<sub>2</sub> fixation rates demonstrated that *Crocospaera* was the major active diazotroph, contributing 54%–100% of bulk N<sub>2</sub> fixation rates. Cell-specific rates were  $0.08 \pm 0.02 \text{ fmol N cell}^{-1} \text{ h}^{-1}$  at the surface and peaked at  $2 \pm 0.2 \text{ fmol N cell}^{-1} \text{ h}^{-1}$  at 50 m (Fig. 2B).

### Abundance of diazotrophs

The depth-integrated (down to 120 m) abundance of *Crocospaera* in the SPG station was two to three orders of magnitude higher than observed in previous studies in the South Pacific (Figure S2, Supporting Information). At the SPG station sampled, the average volumetric abundance of UCYN-B cells was  $8.9 \pm 0.2 \times 10^4 \text{ nifH}$  gene copies l<sup>-1</sup>, their maximum abundance being measured at 50 m with  $5.3 \pm 0.2 \times 10^5 \text{ nifH}$  gene copies l<sup>-1</sup>. Epifluorescence microscopy counts showed lower abundances at the surface and 20 m ( $1.1$ – $2.5 \times 10^2 \text{ cells l}^{-1}$ ), but agreed with qPCR at finding a peak of  $2.5 \times 10^5 \text{ cells l}^{-1}$  at 50 m (Fig. 2C). The average cell diameter of the *Crocospaera*-like cells observed under the microscope and subsequently analyzed by nanoSIMS was 3.5  $\mu\text{m}$  (Figure S3, Supporting Information). Other diazotroph groups were enumerated using qPCR. *Trichodesmium*, UCYN-A1, and UCYN-A2 were only detected at 60 m with  $3.7 \times 10^2 \pm 2.2 \times 10^2$ ,  $2.9 \pm 0.2 \times 10^4$  and  $3.6 \pm 0.2 \times 10^4 \text{ nifH}$  gene copies l<sup>-1</sup>, respectively (data not shown). DDA groups Het-1, Het-2, and Het-3 were not detected.

### Microbial community response to nutrients and trace metal additions

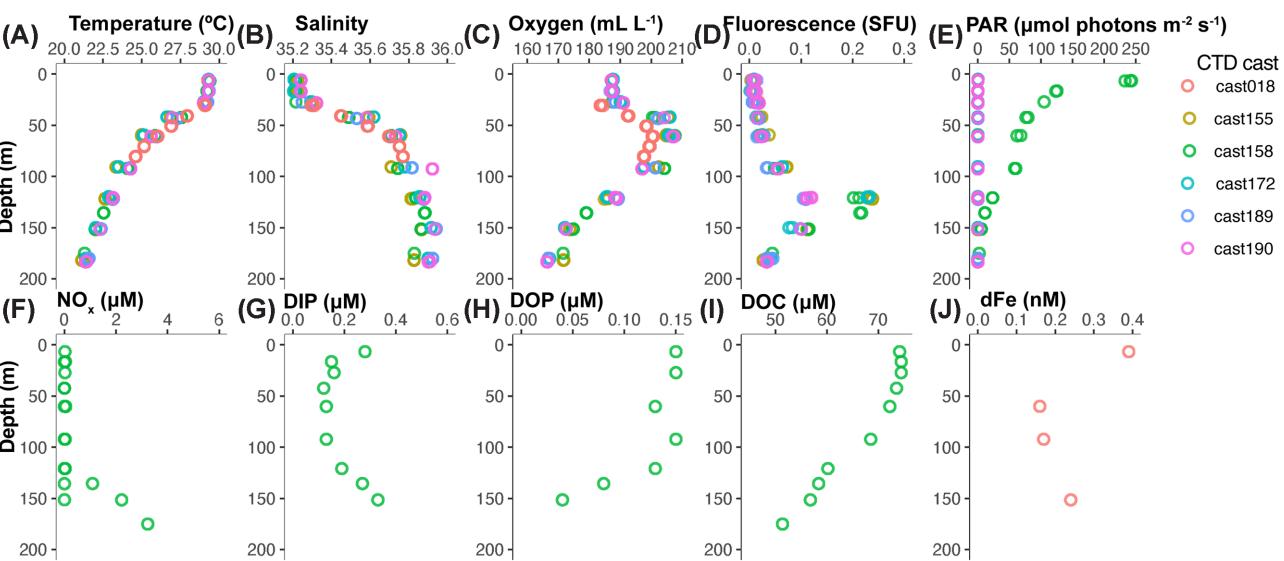
The response of the whole microbial community from 50 m to DIP, DOP, dFe, Ni, ash, or dust addition is shown in Fig. 3(A). N<sub>2</sub> fixation and primary production rates were not significantly enhanced by any of the amendments provided (Fig. 3A, one-way ANOVA  $P > .05$ ). Heterotrophic bacterial production rates were significantly enhanced by DOP additions (Fig. 3A, 0.9-fold increase, one-way ANOVA Tukey test  $P = .04$ ).

The abundance of UCYN-A1 increased significantly in response to DIP, DOP, and ash additions (one-way ANOVA Tukey test  $P = .05$ , .03, and .02, respectively), but responded negatively to additions of Ni (Fig. 3A). The abundance of *Crocospaera* did not respond to DIP, Fe, or Ni amendments, and decreased in DOP, ash, and dust amendments. However, none of these differences were statistically significant (Fig. 3A, one-way ANOVA  $P = .09$ ). Finally, *Trichodesmium* was only stimulated by DOP additions (1-fold, one-way ANOVA Tukey test  $P = .02$ ).

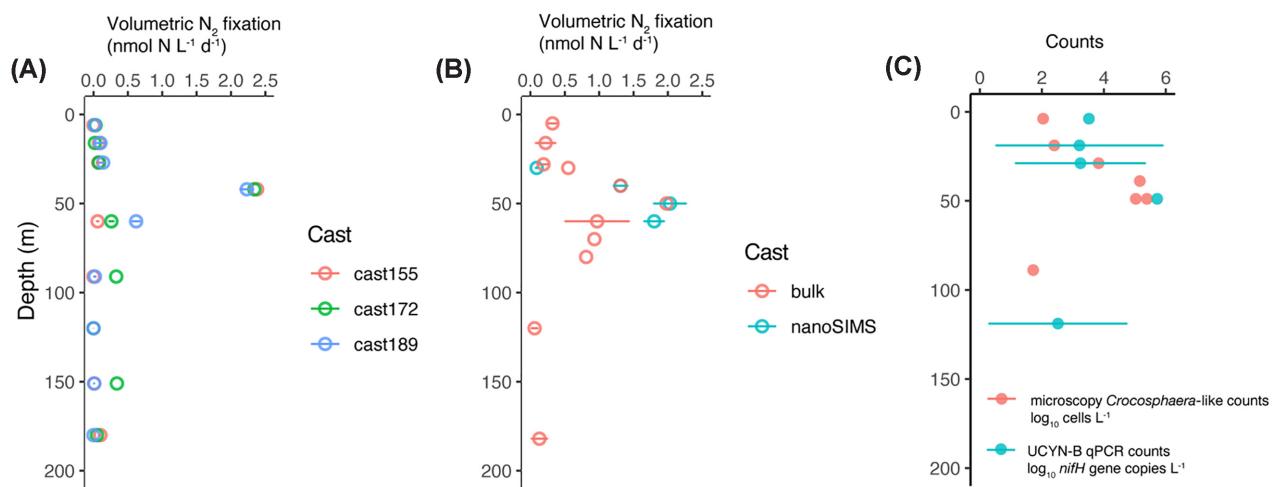
## Discussion

Previous studies have reported *Crocospaera* abundances up to  $10^7 \text{ nifH}$  gene copies l<sup>-1</sup> in the North Pacific Subtropical Gyre (Church et al. 2005, Bench et al. 2016, Wilson et al. 2017) and the WTSP (Moisander et al. 2010, Bonnet et al. 2015). However, previous studies in different parts of the SPG have reported low to undetectable *Crocospaera* (Bonnet et al. 2008, Halm et al. 2011, Shiozaki et al. 2018). Here, we report on an unexpected high abundance of *Crocospaera* peaking at 50 m in the ultraoligotrophic, waters of the SPG.

The vertical distribution of *Crocospaera* at our station in the SPG differed from that observed in the North Pacific in previous studies. In the North Pacific Subtropical Gyre, Church et al. (2005)



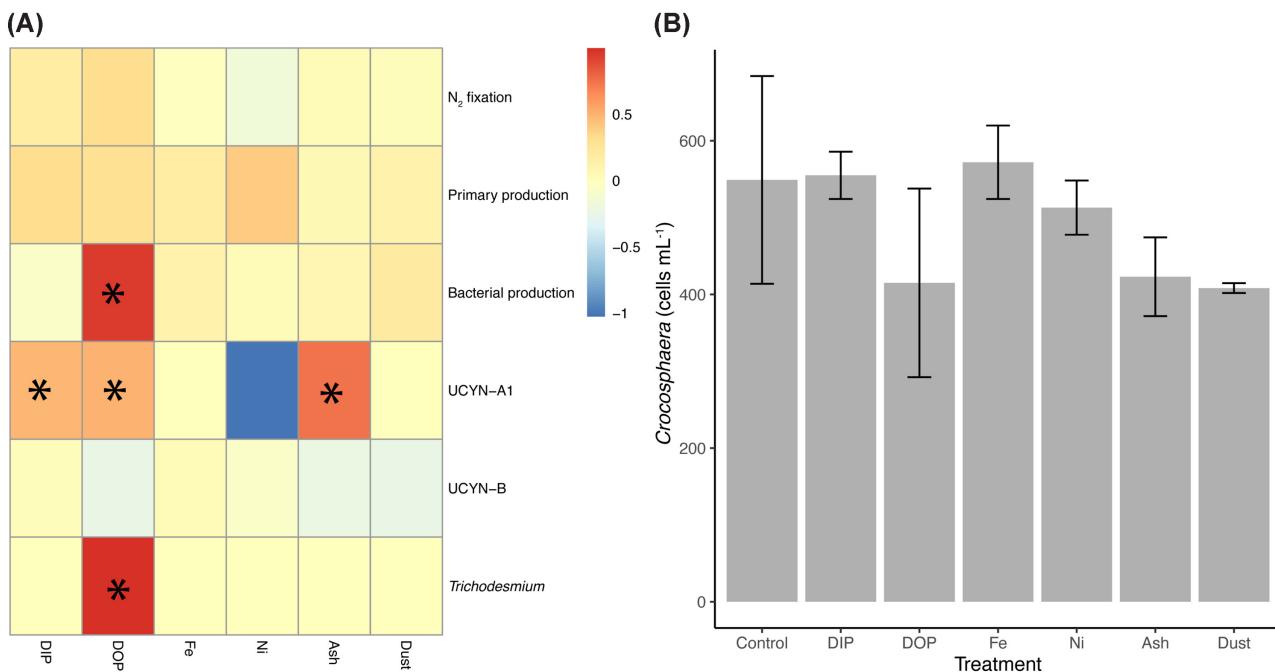
**Figure 1.** Profiles of hydrographic and chemical variables measured at the SPG station. Along four consecutive casts (cast018, cast158, cast160, and cast190) the following hydrographic variables were recorded: (A) temperature, (B) salinity, (C) dissolved oxygen, (D) fluorescence (arbitrary values), and (E) photosynthetically active radiation -PAR-. On cast018, chemical variables were recorded as follows: (F) nitrate + nitrite ( $\text{NO}_x$ ) concentrations, (G) DIP concentrations, (H) DOP concentrations, (I) DOC concentrations, and (J) dFe concentrations [the latter published in Guieu et al. (2018)].



**Figure 2.** Average bulk community and Crocosphaera-specific  $\text{N}_2$  fixation rates. (A) Volumetric  $\text{N}_2$  fixation rates from bulk seawater samples collected during consecutive casts 155, 172, and 189; (B) volumetric  $\text{N}_2$  fixation rates from cast 190 including bulk seawater and Crocosphaera-specific  $\text{N}_2$  fixation rates converted to volumetric rates considering cell abundance (blue dots); (C) vertical variability of Crocosphaera abundance quantified by epifluorescence microscopy and qPCR assays with a primer-probe set targeting UCYN-B (see Materials and methods). Error bars indicate the standard deviation of the mean. Note that replicates were not available for microscopy counts.

reported Crocosphaera abundances of  $\sim 10^3$ – $10^4$   $\text{nifH}$  gene copies  $\text{l}^{-1}$  from the surface to the mixed layer depth, which was located at  $\sim 80$  m during their study in the boreal winter. Also, at station ALOHA but during the boreal summer season, Wilson et al. (2017) found a similar vertical distribution but with abundances equal or higher than  $10^6$   $\text{nifH}$  gene copies  $\text{l}^{-1}$ . Similarly, Bench et al. (2016) and Shiozaki et al. (2018) reported abundances of  $10^5$ – $10^7$   $\text{nifH}$  gene copies  $\text{l}^{-1}$  in the upper mixed layer at various sites in the North Pacific Subtropical Gyre and the WTSP, with a sharp decrease below 50 m. This typical vertical distribution differs from that observed here, where low abundances of  $10^2$   $\text{nifH}$  gene copies  $\text{l}^{-1}$  were measured at the surface, followed by a peak of  $2.56 \times 10^5$   $\text{nifH}$  gene copies  $\text{l}^{-1}$  at 50 m and a sharp decline down to the base of the photic zone (Fig. 2A). Crocosphaera peaked at 50 m and not at the subsurface as seen in previous studies cited above. The high irra-

diance measured in the upper water column layer ( $> 200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; Fig. 1E) typical of the SPG, recognized as the clearest waters in the world's oceans (Twardowski et al. 2007) is well-above the optimum at which  $\text{N}_2$  fixation saturates in Crocosphaera (i.e.  $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; Inomura et al. 2019), explaining their accumulation at deeper levels in the water column as compared to other regions of the ocean. However, the cell-specific  $\text{N}_2$  fixation peak observed here at 50 m corresponds to  $\sim 80 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR (Fig. 1E), which would be suboptimal for Crocosphaera (Inomura et al. 2019) and does not explain their accumulation at this depth. Growth,  $\text{N}_2$  and carbon fixation are negatively affected by UV radiation in cultures of Crocosphaera (Zhu et al. 2020). While no UV data is available for our cruise, previous studies have found a deeper penetration of UV in the clear waters of the SPG as compared to other ocean regions (Morel et al. 2007). This could explain why



**Figure 3.** Microbial activity and abundance responses to nutrient and trace metal additions at 50 m in the SPG station. **(A)** Fold-change responses with respect to the control of  $\text{N}_2$  fixation, primary production, and heterotrophic bacterial production rates, and abundance of UCYN-A1, UCYN-B, and *Trichodesmium* as estimated by qPCR assays. **(B)** UCYN-B changes in abundance as a response to nutrient and trace metal additions. Error bars indicate the standard deviation of the mean. Asterisks indicate statistical significance (see Results section).

*Crocospaera* accumulated deeper in the water column during our study (Fig. 2) as compared to shallower previous observations in other open ocean regions (Church et al. 2005, Moisander et al. 2010, Wilson et al. 2017, Stenugren et al. 2018).

Previous studies have detected two subpopulations of *Crocospaera* in the South Pacific: large type ( $> 4 \mu\text{m}$ ) and small type ( $< 4 \mu\text{m}$ ; Bench et al. 2016). Genome comparisons of several *Crocospaera* strains have shown that while being very similar at the genome level, strains differ widely in their phenotype and strategies to cope with nutrient and trace metal stress (Webb et al. 2009). The large type harbors more DOP scavenging and iron stress response genes than the small type (Dyrhman and Haley 2006, Bench et al. 2013). The *Crocospaera* cells observed here belonged to the small type ( $\sim 3.5 \mu\text{m}$ ; Figure S3, Supporting Information) and peaked at 50 m, coinciding with the lowest dFe concentrations measured during our cruise (0.16 nM, Fig. 1J; Guieu et al. 2018). While these dFe concentrations are limiting for phytoplankton growth in the SPG (Behrenfeld and Kolber 1999), recent *Crocospaera* cell-specific dFe uptake rates measured in the same region ( $0.46\text{--}1.21 \text{ amol Fe cell}^{-1} \text{ d}^{-1}$ ) confirm that this diazotroph is well adapted to thrive in dFe-limited environments (Lory et al. in rev.). Alternatively, the  $\sim 2\text{--}3^\circ\text{C}$  temperature increase between 50 m and the on-deck incubators may have alleviated Fe limitation in *Crocospaera* (Yang et al. 2021).

The peak of *Crocospaera* at 50 m also coincided with a decrease in DIP concentrations at 50–60 m (Fig. 1G), while DOP concentrations did not seem to be variable (Fig. 1H) as compared to the surface. This could be expected in a small *Crocospaera* cell-type population, less genetically equipped to scavenge DOP than the large cell type (Bench et al. 2013). The locally available concentrations of dFe and DIP seemed to be sufficient to fulfill the needs of this *Crocospaera* population, since responses to either dFe or DIP additions were not observed (Fig. 3B).

West of the SPG along the OUTPACE cruise transect, *Crocospaera* abundances peaked at  $10^4\text{--}10^5 \text{ nifH}$  gene copies  $\text{l}^{-1}$  at

40 m, in the region where dFe concentrations were highest, i.e. between 175 E and  $\sim 175$  W (Guieu et al. 2018). In this region, specific *Crocospaera*-like  $\text{N}_2$  fixation rates were  $0.5\text{--}2.5 \text{ fmol N cell}^{-1} \text{ h}^{-1}$ , only contributing 6%–10% to bulk  $\text{N}_2$  fixation rates (Bonnet et al. 2018). At the SPG station, *Crocospaera* cell-specific  $\text{N}_2$  fixation ranged between 0.7 and  $1.4 \text{ fmol N cell}^{-1} \text{ h}^{-1}$ , representing 100% of bulk  $\text{N}_2$  fixation rates at all depths except at 30 m (Fig. 2C). These rates are in good agreement with previous *Crocospaera*-like cell-specific  $\text{N}_2$  fixation measurements in the North Pacific Subtropical Gyre (between  $0.1$  and  $1.1 \text{ fmol N cell}^{-1} \text{ h}^{-1}$ ; Foster et al. 2013) and the WTSP ( $2.1 \text{ fmol N cell}^{-1} \text{ h}^{-1}$ ; Berthelot et al. 2016). Similar cell-specific rates have been reported for other widespread diazotrophs such as *Trichodesmium* ( $2.3 \text{ fmol N cell}^{-1} \text{ h}^{-1}$ ; Berthelot et al. 2016) and UCYN-A1 ( $\sim 1 \text{ fmol N cell}^{-1} \text{ h}^{-1}$ ; Martínez-Pérez et al. 2016), reinforcing the good health and important diazotrophic activity of *Crocospaera* in the SPG during this study.

Overall, the high abundance,  $\text{N}_2$  fixation activity, and lack of response to any nutrient or trace metal additions of *Crocospaera* observed in the SPG station indicate that this population was healthy, active, and largely contributing to fixed nitrogen inputs in this ultraoligotrophic environment. In light of the predicted expansion of subtropical gyres (Polovina et al. 2008), our results suggest that the possibly overlooked abundance and distribution of low-nutrient adapted *Crocospaera* in the SPG deserves further study.

## Supplementary data

Supplementary data are available at [FEMSLE](https://femsle.oup.com/femsle/article/369/1/fnac039/6565802) online.

## Authors' contributions

All authors contributed to the sampling and experiments carried out onboard. M.C. performed the microscopy cell counts and nanoSIMS analyses. S.D. performed the  $^{14}\text{C}$  uptake analyses. O.G.

performed the IRMS and MIMS analyses. F.V.W. performed the heterotrophic bacterial production analyses. M.B. wrote the paper with contributions from all authors.

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**Conflicts of interests statement.** None declared.

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