1 # Supplementary Information

2

3 # Bleaching forces coral's heterotrophy on diazotrophs and *Synechococcus*

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Supplementary Information for Material and Methods

Coral collection and acclimation

Stylophora pistillata samples were collected in the New Caledonian lagoon (22°28’40.41’’S; 166°28’05.68’’E, sampling licence issued by the “Province Sud”, Government of New-Caledonia). Corals were transported from the collection site in a cooler and randomly assigned to the experimental tanks. Forty terminal branch portions (5 cm) were cut from five parent colonies, hung on nylon wires and suspended in two 20 L aquaria (twenty nubbins per aquarium) supplied with 100 µm-filtered seawater, renewed at a rate of 16.5 $L.h^{-1}$ and mixed using a submersible pump (Aquarium system, micro-jet MC 320, Mentor, OH, USA). In each tank, coral colonies were spaced apart and never touched each other. Temperature (28 ± 0.2 °C) and salinity (35.70 ± 0.02) were kept constant using heaters connected to electronic controllers and controlled by YSI MPS 556 probe (YSI, Yellow Springs, OH, USA). Corals received a constant irradiance of 120 ± 10 µmol photons $m^2.s^1$ (photoperiod 12h: 12h light: dark) using four Aquablue plus neon bulbs (blue–white, 15.000 K, Giesemann, Germany). The nubbins were acclimatized to the different conditions for three weeks before increasing the temperature. At the end of the acclimation period and in order to mimic a bleaching event, in one of the two aquaria, *S. pistillata* nubbins were exposed to a gradual increase in temperature of 0.5°C per day until reaching 31 ± 0.5 °C. These colonies appeared bleached (see *Symbiodiniaceae* densities and total chlorophyll concentrations in the **Supplementary Information for results**) and are referred to as “bleached” colonies. In the other aquarium, colonies were maintained at the initial temperature of 28 ± 0.2 °C and are referred to as “untreated” colonies. While we recognize that this can be considered as pseudoreplication, all physico-chemical parameters (temperature, salinity, nutrient concentration, pH) were rigorously controlled in each tank so that the only difference between tanks was temperature. Therefore, despite the fact that this set

38 up could be considered as pseudoreplication, results obtained are representative and the most
39 parsimonious explanation for differences observed between the two coral batches is clearly the
40 presence or absence of *Symbiodiniaceae* in corals, and not stochastic errors.
41
42 ***Experiment setup and ^{15}N assimilation from planktonic diazotrophs***
43 In March 2017 (austral summer conditions) prior the start of the experiment, plankton was
44 sampled from the lagoon by collecting and prefiltering 50 L of seawater through a 100 µm mesh
45 to exclude larger cells. The presence of planktonic diazotrophs was confirmed by observations
46 using a Zeiss Axio-Imager A2 LED (Zeiss, Jena, Germany) epifluorescence microscope
47 equipped with a green (510-560 nm) excitation filter, targeted the UCYN phycoerythrin-rich
48 cells. The sample was split into three 4.3 L polycarbonate bottles which were immediately
49 filtered on pre-combusted (450°C, 5h) GF/F filters (Whatman, St-Louis, MO, USA) for the
50 measurement of the initial (time zero) ^{15}N-enrichment of plankton. Twenty one HCl-washed
51 2.3 L polycarbonate bottles were also filled and directly spiked with 4 mL of $^{15}N_2$ (98.9 atom%
52 ^{15}N, Cambridge Isotopes Laboratories, Inc.) injected with a gas-tight syringe [1]. Each bottle
53 was shaken 20 times (to fragment the $^{15}N_2$ bubble and facilitate its dissolution) and incubated
54 for 24 h at irradiances corresponding to the sampling depth using screening, and cooled with
55 circulating surface seawater. At the end of the 24 hours of incubation and for each of the
56 experimental beakers, the 1.5 L bottles containing the labeled plankton cells were filtered and
57 concentrated onto 0.4 µm polycarbonate filters. These filters were then respectively placed in
58 50 mL of initial seawater in which plankton cells were harvested and vortexed to resuspend
59 these cells. Aliquots of 5 mL were filtered on pre-combusted (450°C, 5h) GF/F filters for the
60 measurement of the ^{15}N-enrichment of the source pool, while the remaining 45 mL, containing
61 a concentrate of planktonic cells, was added to the sixteen incubation beakers of which ten
62 contained corals. The six remaining control beakers (without coral) included four beakers to

63 analyze plankton abundances and three to determine *nifH* gene expression at the beginning and
64 the end of incubations. All beakers were immersed in a water bath to maintain the temperature
65 constant (28 ± 0.5°C) throughout the experiment. Coral branches were hung on nylon wires to
66 the top of the beakers. Subsequently, the beakers were sealed air-free using a glass slide. At the
67 end of each experiment (12 h), corals were rinsed six times with filtered seawater to remove
68 cells potentially adhered to the coral surface [2] and subsequently transferred to Ziploc bags
69 and stored at −20°C until analysis. We considered nitrogen assimilation in *Symbiodiniaceae*
70 and tissue separately as both 'compartments' use nitrogen for their own needs. *Symbiodiniaceae*
71 have the ability to take up and retain nitrogen from surrounding water [3, 4] and transfer it to
72 the host [5]. Corals can also obtain nitrogen via the ingestion of external food sources [6].
73 Hence, it was important to calculate the assimilation of nitrogen by the tissue and the
74 *Symbiodiniaceae* separately. Nitrogen assimilation rates were calculated as described in [1]
75 (Eqn 1):

76 $$N_{assimilation} = \frac{({}^{15}N_{target,Tf} - {}^{15}N_{target,0f})}{{}^{15}N_{enriched\ plankton} - {}^{15}N_{not\ enriched\ plankton}) \times t} \times [\frac{PN_{target}}{A})$$

77 Where ${}^{15}N_{target,\ tf}$ is the ^{15}N enrichment of the target pool (tissue or *Symbiodiniaceae*) at the end
78 of the incubation, ${}^{15}N_{target,\ 0f}$ is the corresponding time zero ^{15}N enrichment (natural ^{15}N
79 enrichment of the target pool at the start of the incubation). The ${}^{15}N_2$-labelled (${}^{15}N_{enriched\ plankton}$)
80 and non-labelled (${}^{15}N_{not\ enriched\ plankton}$) atom % values are considered in Eqn 1 as follows: the
81 source pool is the isotopic enrichment of the natural planktonic assemblage added to the
82 beakers. t is the incubation time in hours and PN_{target}/A is the mass (µg) of particulate nitrogen
83 of the target pool per area (cm^2) of coral skeleton surface (µg N cm^{-2} h^{-1}).

84

85 ***Experiment setup and ^{15}N assimilation from enriched seawater***

86 We also quantified the direct uptake of N_2 fixed by endosymbiotic diazotrophs. We used the
87 dissolved ${}^{15}N_2$ method [7, 8]. 20 L of seawater were previously filtered onto 0.2 µm. 4.5 L were

88 degassed and transferred to a gas-tight Tedlar® bag using silicon tubing. Subsequently, 45 mL
89 of $^{15}N_2$ (98.3 atom% ^{15}N, Cambridge Isotope Laboratories) were injected into the bag with a
90 gas-tight syringe [1]. The bubble was vigorously shaken for 20 min until its complete
91 dissolution. After 24 h of incubation at 4°C, 12 mL samples were collected from the Tedlar®
92 bag in Exetainer® vials to measure the ^{15}N-enrichment of the labeled filtered seawater. The ten
93 glass beakers were filled to 20% volume with ^{15}N-enriched seawater, 80% unenriched filtered
94 seawater and incubated for 12 h.
95

96 ***Symbiodiniaceae density and total chlorophyll concentration***

97 The tissue was removed from the skeleton using an air-pick [9] and approximately 20 mL of
98 0.45 µm filtered seawater. The slurry (20 mL) was homogenized with a Potter tissue grinder.
99 For each coral sample, the number of *Symbiodiniaceae* was counted three times by light
100 microscopy using a Neubauer's cell. A 10 mL subsample was taken from each homogenate,
101 immediately centrifuged at 6000g for 10 min (4°C) and the supernatant removed. The pellet
102 containing the *Symbiodiniaceae* was resuspended in 100% acetone for 24 h (4°C) in the dark to
103 extract chlorophyll *a* and c_2. The extracts were centrifuged at 10 000g for 15 min and
104 absorbances were read at 630, 663 and 750 nm. Chlorophyll concentrations were computed
105 according to the spectrometric equations of [10, 11]. Chlorophyll a and c_2 are given as total
106 chlorophyll. All measurements were normalized to the skeletal surface area, see below.
107

108 ***Surface normalization***

109 Symbiont densities, chlorophyll concentrations and plankton ingestion rates were standardized
110 per skeletal surface area (cm²), estimated using the paraffin wax-dipping method [12, 13].
111

112 ***Photosynthetic efficiency measurements***

113 Prior the start of the experiment, to assess the status of PSII in *Symbiodiniaceae*, photosynthetic
114 efficiency (F_v/F_m) *in hospite* was measured in aquaria using a diving-PAM fluorometer (Walz,
115 Germany) (n = 10 from each treatment). After 20 min of dark adaptation, [14] the 8 mm optical
116 fiber was maintained perpendicular to the nubbins' surface using a black-jacket at a fixed
117 distance of 5 mm guaranteeing a correct distance of the optical fiber from the coral. The initial
118 fluorescence (F_0) was measured by applying a weak pulsed red light (3 μs, LED 650 nm) on
119 dark-adapted colonies. A saturating pulse (800 ms) of bright actinic light (8,000 μmol photons
120 m^{-2} s^{-1}) was then applied to obtain the maximum fluorescence value (F_m). Variable fluorescence
121 (F_v) was calculated resulting in F_m-F_0 [15]. Fv/Fm values may be used as a quantitative measure
122 of photo-inactivation during coral bleaching [16]. Fv/Fm values of healthy corals are ranging
123 between 0.5 to 0.8. Values between 0 to 0.2 are indicative of severe bleaching [17].
124

125 ***Oxygen production***

126 Dissolved oxygen level was measured for each incubation beaker. Measurements have been
127 performed in individual small glass chambers (115 mL) supplied with seawater collected in
128 each of the beakers at the beginning (T*0*) and end (T*f*) of the experiment. Each glass chambers
129 was equipped with an O_2 sensor spot to allow an optical measure of the dissolved oxygen
130 concentration using a Fibox 4 transmitters (PreSens, Germany). The probe was calibrated
131 before each measurement against air saturated with moisture.
132

133 ***^{15}N analyses in Symbiodiniaceae, coral tissue and plankton***

134 *Symbiodiniaceae* and tissue were separated by centrifugation [2]. *Symbiodiniaceae* were dried,
135 ground and encapsulated in tin cups before isotope ratio mass spectrometry (IRMS) analyses.
136 Subsamples of each homogenate containing only coral tissue or planktonic samples were
137 filtered (15 mL) on pre-combusted GF/F filters under low pressure. All the filters were analysed

138 using an elemental analyser coupled to an IRMS (EA-IRMS, Integra CN, SerCon Ltd, Cheshire,
139 UK).
140
141 ***Prey cell count***
142 The concentration of prey cells, i.e. *Prochlorococcus*, *Synechococcus*, and picoeukaryotes,
143 were quantified at the start and end of the incubation period to check for their ingestion by the
144 corals. This abundance was determined with a FACSVerse Flow Cytometer (Becton Dickinson,
145 131 San Jose, CA, USA). Heterotrophic bacteria were detected by diluting the medium by 10
146 with filtered seawater (0.2 µm) and stained it with SYBR Green (DNA). For small
147 phytoplankton, cyanobacteria and *Prochlorococcus*, quantification was done according to red
148 fluorescence level corresponding to chlorophyll a content, and orange fluorescence linked to
149 pigment content like phycoerythrin (especially to select *Synechococcus* population) and also by
150 size sorting using 1 µm green calibration beads. *Cyanothece* cells were determined by strain-
151 specific quantitative analyses to infer their abundance from the number of *nifH* gene copies.
152
153 ***Ingestion rates***
154 Ingestion rates were assessed as clearance rate according to previous studies on corals [18, 19],
155 and calculated using the equations of [20], which take into account the natural growth and death
156 of the prey during incubations. Ingestion rates were expressed as number of prey organisms
157 ingested and normalized to the skeletal surface area.
158
159 ***Quantification of diazotrophs - DNA sampling, extraction and qPCR assays***
160 Samples for DNA analyses were filtered onto Supor filters (Pall Gelman) and stored in bead
161 beater tubes containing a mixture of 0.1-mm and 0.5-mm diameter glass beads (BioSpec
162 Products, Bartlesville, OK, USA). The tubes were stored at -80ºC until further analysis. DNA

163 was extracted using the Qiagen Plant kit with additional freeze-thaw, bead beating proteinase
164 K steps for sample preparation before the kit purification and elution to 50 µL as previously
165 described [21]. All DNA samples were quantified by Picogreen assays (Thermo Scientific). The
166 abundance of diazotrophs was determined using TaqMan qPCR assays on a StepOnePlus Real-
167 Time PCR System (Applied Biosystems), with primer-probe sets for *Trichodesmium* [22],
168 UCYN-A1 [23], UCYN-C [22], the Gammaproteobacterium γ-24774A11 [21], and Het1
169 (*Richelia-Rhizosolenia* DDAs) [22]. Each reaction (25.5 µL) consisted of 12.5 µL TaqMan
170 Universal PCR Master Mix (Applied Biosystems), 1 µL of each primer (at 10 µM, HPLC
171 purified, TAG Copenhagen, Denmark), 0.5 µL probe (TAG Copenhagen), 8 µL PCR grade
172 water, 0.25 µL bovine serum albumin (BSA), and 2 µL standard or sample (samples were pre-
173 diluted to 5 ng/µL to add 10 ng DNA to all qPCR reactions). The thermal cycling conditions
174 were 2 min at 50ºC, 10 min at 95ºC followed by 45 cycles of 15 s at 95ºC and 1 min at 60ºC.
175 Standard dilutions (10^7-10^1) were run in duplicate, samples and no-template controls (NTCs)
176 in triplicate. NTCs were undetectable. The efficiency of the primer-probe sets was 82-102%.
177 Inhibition tests were carried out on all samples and each primer-probe set by adding 2 µL of
178 10^5 standard to each sample. The efficiencies of inhibition tests ranged from 98-101%, thus we
179 consider that our samples were not inhibited. The limit of detection (LOD) and detected but not
180 quantifiable (DNQ) limits used were 1 and 8 gene copies per reaction, respectively. Samples
181 that were below LOD were designated a value of 0 in the data set, whereas gene copies higher
182 than LOD but less than DNQ were designated a conservative value of 1 *nifH* gene copy L^{-1}.
183

184 ***Statistical analyses***

185 All tests were performed using R version 3.5.1 within RStudio (Version 1.1.456, 2018). The
186 non-parametric Mann-Whitney-Wilcoxon test was first used to test for significant differences
187 in *Symbiodiniaceae* and chlorophyll concentrations, respiration and photosynthetic efficiency

188 (F_v/F_m). This test was also applied to analyze planktonic ingestion rates and nitrogen

189 assimilation rates (**Supplementary Table 1**). The *ggplot2* package (Wickham 2009) was used

190 to create the box plot figures. Throughout the manuscript, values given are expressed as mean

191 ± SD. Statistical significance was accepted at $P < 0.05$.

192 # Supplementary Information for Results

193

194 ### *Symbiodiniaceae density and chlorophyll concentration*

195 Bleached corals have 8 and 7 times less *Symbiodiniaceae* and chlorophyll concentrations than
196 untreated colonies (respectively 1.33 ± 0.33 10^6 cell cm^{-2} and 0.16 ± 0.06 10^6 cell cm^{-2}; n = 9;
197 9.91 ± 2.69 chl.cm^{-2} and 1.39 ± 1.08 chl.cm^{-2}; n = 9; Mann-Whitney-Wilcoxon test, $P < 0.01$)
198 (**Supplementary Figure S1**).

199

200 ### *Photosynthetic efficiency*

201 Prior the start of the experiment, colonies that were submitted to temperature increase, showed
202 severe visual bleaching and a complete suppression of coral photosynthetic efficiency, with
203 F_v/F_m equal to 0 (n = 10, while untreated colonies exhibited high photosynthetic efficiency with
204 $F_v/F_m = 0.721 \pm 0.017$ (n = 10).

205

206 ### *Oxygen production*

207 The oxygen level in beakers containing corals has decreased from 5.43 ± 0.01 mg O_2 L^{-1} to 4.19
208 mg ± 0.95 O_2 L^{-1} (n = 10).

209

210 ### *Picoplankton cell abundance and ingestion* rates

211 During the 12 h of incubation, concentrations of all pico- and nano-plankton groups decreased,
212 except those of bacteria. In the control beakers without coral colonies), we observe a slight
213 increase in *Prochlorococcus* and *Synechococcus* concentrations, meaning that there is a natural
214 cell growth for these two groups during the 12 hours of incubation (increase of 0.22 10^5 cell
215 mL^{-1} and 0.67 10^5 cell mL^{-1} for *Prochlorococcus* and *Synechococcus* respectively) while there
216 is decrease in picoeukaryotes cells, certainly due to predation by other taxa (- 0.62 10^3 cell mL^{-}

217 ^{1}). In terms of number of prey ingested, normalized to skeletal surface, *Prochlorococcus* was
218 quantitatively the major group ingested (1.44 ± 0.77 10^5 cell cm^{-2} h^{-1}) followed by
219 *Synechococcus* (3.08 ± 0.88 10^4 cell cm^{-2} h^{-1}) and picoeukaryotes (1.83 ± 1.61 10^3 cell cm^{-2} h^{-1}
220) regardless of the treatment (**Supplementary Table 1 and 3**). *Synechococcus* ingestion rates
221 were higher in bleached than in untreated colonies (respectively 3.79 ± 0.64 10^4 cell cm^{-2} h^{-1}
222 and 2.38 ± 0.24 10^4 cell cm^{-2} h^{-1}; Mann-Whitney-Wilcoxon test, P = 0.028).
223
224 ***Quantification of diazotrophs***
225 Analysis of the planktonic diazotroph abundances confirmed the presence of diazotrophs into
226 the incubation medium. In the control beakers, we noticed an increase in the number of *nifH*
227 gene copies L^{-1} of the tested phylotypes (UCYN-A1, UCYN-C and *Trichodesmium*) during the
228 incubation period. Conversely, in all beakers containing corals, either untreated or bleached (n
229 = 3), abundances of UCYN-A1 and UCYN-C decreased between the beginning and the end of
230 the experiment. This is not the case for *Trichodesmium*, whose abundances remain unchanged
231 (**Supplementary Table 2**). Indeed, this specific diazotroph is filamentous and often present as
232 bundles, which likely makes it harder prey to catch and ingest for corals [24].
233
234 ***Endosymbiotic diazotroph activity***
235 For coral incubations in $^{15}N_2$-enriched seawater, we measured a mean (± SD) nitrogen
236 assimilation of 3.11 ± 1.91 10^{-4} and 5.42 ± 4.95 10^{-7} respectively in *Symbiodiniaceae* and tissue
237 of bleached corals and 1.21 ± 1.45 10^{-4} and 3.14 ± 7.68 10^{-8} in *Symbiodiniaceae* and tissue of
238 untreated ones.
239
240 ***Elemental composition of pico-, nanoplankton and diazotrophs***
241 Nitrogen and carbon contents as well as nitrogen and carbon assimilation rates for each type of

242 preys are reported in **Supplementary Table 4 and 5**. Bleached corals preferentially fed on

243 *Synechococcus* that provided them 7.57 ± 1.28 10^{-4} µg N cm^{-2} h^{-1}. Specific feeding on planktonic

244 diazotrophs and *Synechococcus spp*. also represents a non-negligible source of carbon for bleached

245 corals (respectively 3.10 10^{-4} and 9.46 ± 1.60 10^{-3} µg C cm^{-2} h^{-1}).

246 **Supplementary Figure S1:** *Symbiodiniaceae* density (A) and total chlorophyll concentration
247 (B) in untreated and bleached corals before the $^{15}N_2$ isotope labelling experiment. Stars indicate
248 statistically significant differences (n = 9; mean ± SD).
249
250 **Supplementary Table 1:** Summary of statistical analyses performed with the non-parametric
251 test Mann-Whitney-Wilcoxon test.
252
253 **Supplementary Table 2:** Summary of diazotroph cell abundance (*nifH* gene copies L^{-1}) at the
254 start (*T0*) and at the end (12 h) of the incubation period into control beakers (without corals)
255 and beakers with corals (untreated and bleached) (UCYN-A1 and UCYN-C, n = 3 for each
256 taxon; mean ± SD; *Trichodesmium*, n = 9, mean ± SD).
257
258 **Supplementary Table 3:** Summary of plankton abundance (cell mL^{-1}) and ingestion rates (cell
259 cm^{-2} h^{-1}) in the incubation medium at the beginning and the end of the experiment in both
260 bleached and untreated corals and for both treatments (n = 5 for each treatments; mean ± SD).
261
262 **Supplementary Table 4:** Carbon and nitrogen cell content (µg $cell^{-1}$) and related assimilation
263 rates (µg cm^{-2} h^{-1}) calculated for *Prochlorococcus*, *Synechococcus* and picoeukaryotes using
264 literature conversion factors and the results of the present study (mean ± SD).
265
266 **Supplementary Table 5:** Carbon and nitrogen cell content (pg $cell^{-1}$) of the studied
267 diazotrophs.

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