

1 **Supplementary Information for**

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3 **Variable responses to ocean acidification among mixotrophic protists with different**
4 **lifestyles**

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21 **This PDF file contains:**

22 **Materials and Methods**

23 **Supplementary Figures S1-S7**

24 **Supplementary Tables S1-S2**

25 **Supplementary Materials and Methods**

26 *Flow cytometric cell enumeration*

27 Samples from all replicates were fixed with a mixture of glutaraldehyde (0.25% final
28 concentration) and Pluronic F68 (0.01% final concentration) [1], and were analyzed after a
29 20-minute dark incubation. For bacterial enumeration, a subsample was diluted 10 times in
30 TE buffer and stained with the nucleic acid stain SYBR Green I (Molecular Probes-
31 Invitrogen, Carlsbad, CA, USA) in a final concentration of 5×10^{-5} of the commercial stock
32 prior to enumeration. *Ochromonas* cells were detected using a red fluorescence (690/50 nm)
33 trigger and gated based on their red chlorophyll autofluorescence and forward scatter
34 properties (see Sup. Figure 1 for an example). Bacterial cells were enumerated using a green
35 fluorescence (525/40 nm) trigger and gated based on their green fluorescence and forward
36 scatter properties. In addition to routine sampling, samples of the cultures before and after
37 each transfer were fixed in the same manner, but were flash frozen in liquid nitrogen and
38 stored at -80 °C for accurate counts and growth rates over each growth period. Those samples
39 were enumerated similarly, with the addition of spiked Fluoresbrite™ Polychromatic Red 2.0
40 μm Microspheres (Polysciences, Warrington, PA, USA) as internal standards. The samples
41 from the last three transfers were used to calculate growth rates as well as relative
42 Chlorophyll fluorescence.

43 The growth rates of all replicates in the two treatments were calculated based on the last three
44 transfers. The growth rate of each replicate for a given growth period between two transfers
45 was calculated as follows:

$$46 \quad GR_{Transfer} = \frac{\ln(x_{t+\Delta t}) - \ln(x_t)}{\Delta t} \quad (\text{Eq. 1})$$

47 where $x_{t+\Delta t}$ is the *Ochromonas* count obtained by flow cytometric enumeration of the
48 sample after the growth period, x_t corresponds to the count before the growth period, and

49 Δt is the growth period in days (ranging from one to four days). The growth rate of each
50 replicate was calculated by averaging the replicate's growth rate over the last three transfers.
51 Cellular chlorophyll fluorescence was analyzed from the flow cytometry samples taken at the
52 end of the last three growth periods. To this end, the mean red fluorescence of the
53 *Ochromonas* population (gate P2 in Supp Figure 1) was divided by that of the red fluorescent
54 beads which were added as internal reference to each sample (gate P1 in Supp Figure 1). For
55 each replicate, these normalized values were then averaged over the last three transfers.

56 ***Grazing assays***

57 For preparation of Fluorescently Labeled Bacteria (FLB), a late-exponential bacterial culture
58 was harvested, concentrated by centrifugation and resuspended in PBS buffer (pH=9). The
59 dye 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) was added in a concentration of 100
60 μg per 1 ml culture. The culture was dark incubated with the dye for two hours in a 60°C
61 water bath. The excess dye was then removed by four to five rounds of centrifugation and
62 resuspension in artificial seawater. Final resuspension was done with artificial seawater and
63 aliquots were frozen at -20°C until use. When performing a grazing assay, an FLB aliquot
64 was thawed, sonicated for 10 minutes, and enumerated on the flow cytometer to determine
65 FLB concentration.

66 For detecting ingestion of FLB by *Ochromonas*, samples from T_0 and T_{30} of the grazing
67 assays were analyzed on the flow cytometer twice – once using a red fluorescence trigger for
68 detection of *Ochromonas* cells and once using a green fluorescence trigger for detecting the
69 FLB. In addition, subsamples from T_0 were stained with SYBR green to verify total bacterial
70 counts at the beginning of the assay using a green fluorescence trigger (as described above).
71 *Ochromonas* cells were identified and gated as described above. Uptake of FLB by
72 *Ochromonas* cells was then detected by following the changes in the green fluorescence

73 properties of the *Ochromonas* cells over the 30 minutes incubation. The basal green
 74 fluorescence of the population was determined based on the T₀ sample. Cells exceeding this
 75 baseline green fluorescence were termed “feeding” (see Sup. Figure 2 for an example). The
 76 percent of cells feeding at T₃₀ was corrected by subtracting the percent of cells feeding at T₀.
 77 The mean number of FLB per feeding cell was estimated based on the mean green
 78 fluorescence of the feeding cells ($\overline{GF}_{feeding}$), corrected for basal green fluorescence of
 79 *Ochromonas* ($\overline{GF}_{non\ feeding}$), and divided by the mean green fluorescence of the FLB
 80 (\overline{GF}_{FLB}):

$$81 \quad FLB \text{ per feeding cell} = \frac{\overline{GF}_{feeding} - \overline{GF}_{non\ feeding}}{\overline{GF}_{FLB}} \quad (\text{Eq. 2})$$

82 The grazing rate (G, in bacteria cell⁻¹ h⁻¹) was then calculated as:

$$83 \quad G = \frac{\text{feeding cells} \times FLB \text{ per feeding cell}}{\text{Total } Ochromonas \times \Delta t} \times \frac{\text{Total Bac}}{FLB} \quad (\text{Eq. 3})$$

84 where Δt is the incubation period in hours, *Total Bac* is the total bacterial abundance
 85 quantified with SYBR Green, and *FLB* is the FLB abundance.

86 ***Photosynthetic carbon fixation***

87 For measurements of carbon fixation, ¹⁴C labeled bicarbonate stock of 100 μCi ml⁻¹ activity
 88 was added to glass scintillation vials containing 2mL aliquots sampled from the experimental
 89 cultures (final activity of ~1 μCi ml⁻¹). We used six replicate cultures per experimental
 90 treatment, and filled three vials per replicate for ¹⁴C analysis. The vials from five of the six
 91 replicates were incubated for 1 h at three different light levels: darkness, 50, and 100 μmol
 92 photons m⁻² s⁻¹. The three vials of the remaining replicate were used for a 1 h incubation in
 93 darkness, a 1 h incubation at 100 μmol photons m⁻² s⁻¹, and one T₀ measurement with
 94 sampling immediately upon addition of the ¹⁴C – bicarbonate. This T₀ measurement was

95 used as a quality control of our ^{14}C analysis, to verify that the measured ^{14}C incorporation
96 was zero at T_0 .

97 For the ^{14}C analysis, a 100 μl sample was transferred to 4 ml Ultima Gold scintillation
98 cocktail (Revvity, Groningen, Netherlands) and total activity was immediately counted in a
99 scintillation counter ('*DPM total*' sample). Subsequently, 2 ml of 10% glacial acetic acid in
100 70% methanol were added to the remaining culture to remove inorganic carbon. Vials were
101 then dried overnight at 60°C until all liquid had evaporated. Residues were re-dissolved in 2
102 ml of MQ and 10 ml of scintillation cocktail was added for counting in the scintillation
103 counter ('*DPM fixed*' and '*DPM fixed dark*'). DPM values obtained were converted into
104 carbon fixation rates (C_{FIX} , in $\text{pg C cell}^{-1} \text{h}^{-1}$) as follows:

$$105 \quad C_{FIX} = \frac{\left(\frac{DPM_{fixed} - DPM_{fixed\ dark}}{DPM_{total}}\right) \times \left(\frac{Total\ DIC}{\Delta t}\right) \times 1.05}{Ochromonas\ abundance} \quad (\text{Eq. 4})$$

106 where 1.05 is the radioisotope discrimination factor, and Total DIC was obtained from the
107 DIC samples taken from each replicate at the day of the physiological experiments. '*DPM*
108 *fixed*' at T_0 was compared to '*DPM fixed dark*' to correct for scintillation activity in the dark
109 incubation samples.

110 ***Analysis of cell clumping***

111 Estimation of the extent of cell clumping was done through analysis of the cytograms of each
112 replicate, over the last three transfers. Each cytogram of red fluorescence vs forward scatter
113 was gated twice. One gate ('singlets') included only the main cluster of cells which
114 corresponds with single *Ochromonas* cells and contained the majority of events. This gate
115 was used to calculate the mean red fluorescence of that *Ochromonas* sample. The second gate
116 was less stringent and included also events on the diagonal, with higher red fluorescence and
117 forward scatter intensity ('all *Ochromonas*'). Using a python package, FlowCal[2], the red

118 fluorescence of each event in the ‘all *Ochromonas*’ gate was extracted, and divided by the
119 mean ‘singlets’ red fluorescence value in order to estimate the number of cells in each event.
120 The mean number of cells per event was then calculated by summing all the estimated cell
121 numbers per event across all events in a sample followed by division by the total number of
122 events in the sample. For each replicate, this value was averaged over the last three transfers.
123 While this procedure gives a quantitative estimate of cell clumping, we note that it might not
124 reflect accurate quantification of cells per event as these might not scale linearly with the
125 fluorescence signal detected by flow cytometry.

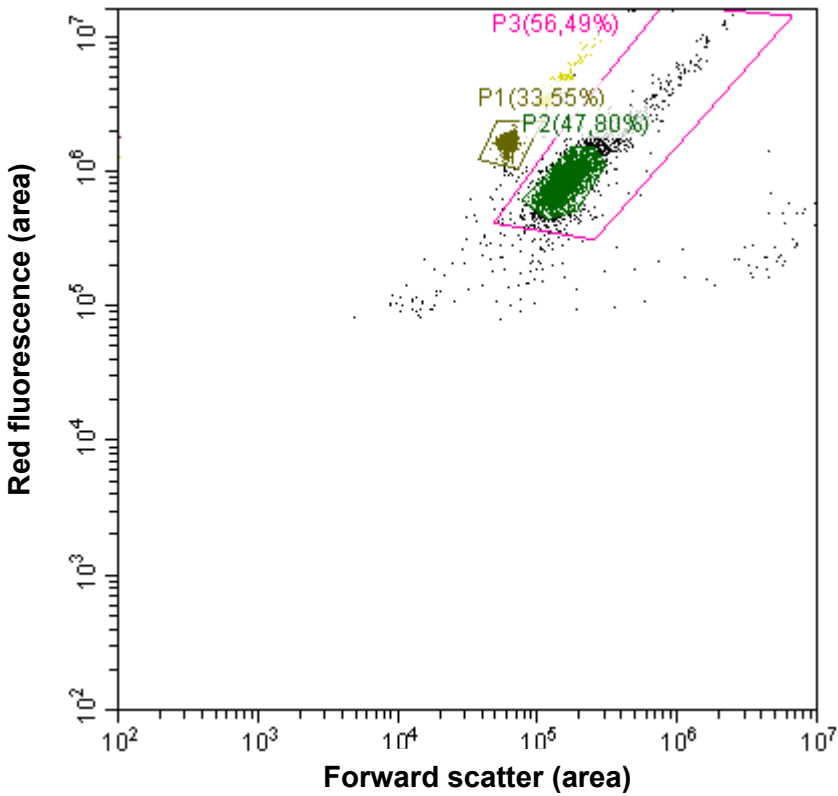
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127 References:

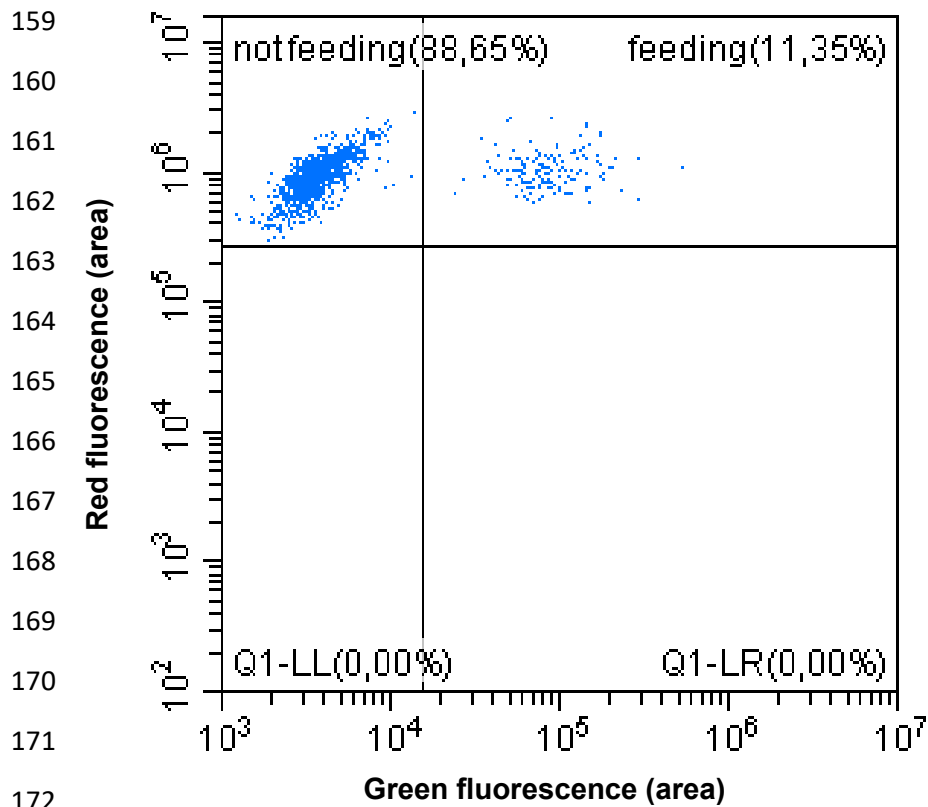
- 128 1. Marie D, Rigaut-Jalabert F, Vaultot D. An improved protocol for flow cytometry analysis
129 of phytoplankton cultures and natural samples. *Cytometry A* 2014;**85**:962–8.
130 <https://doi.org/10.1002/cyto.a.22517>
- 131 2. Castillo-Hair SM, Sexton JT, Landry BP *et al.* FlowCal: A user-friendly, open source
132 software tool for automatically converting flow cytometry data from arbitrary to calibrated
133 units. *ACS Synth Biol* 2016;**5**:774–80. <https://doi.org/10.1021/acssynbio.5b00284>

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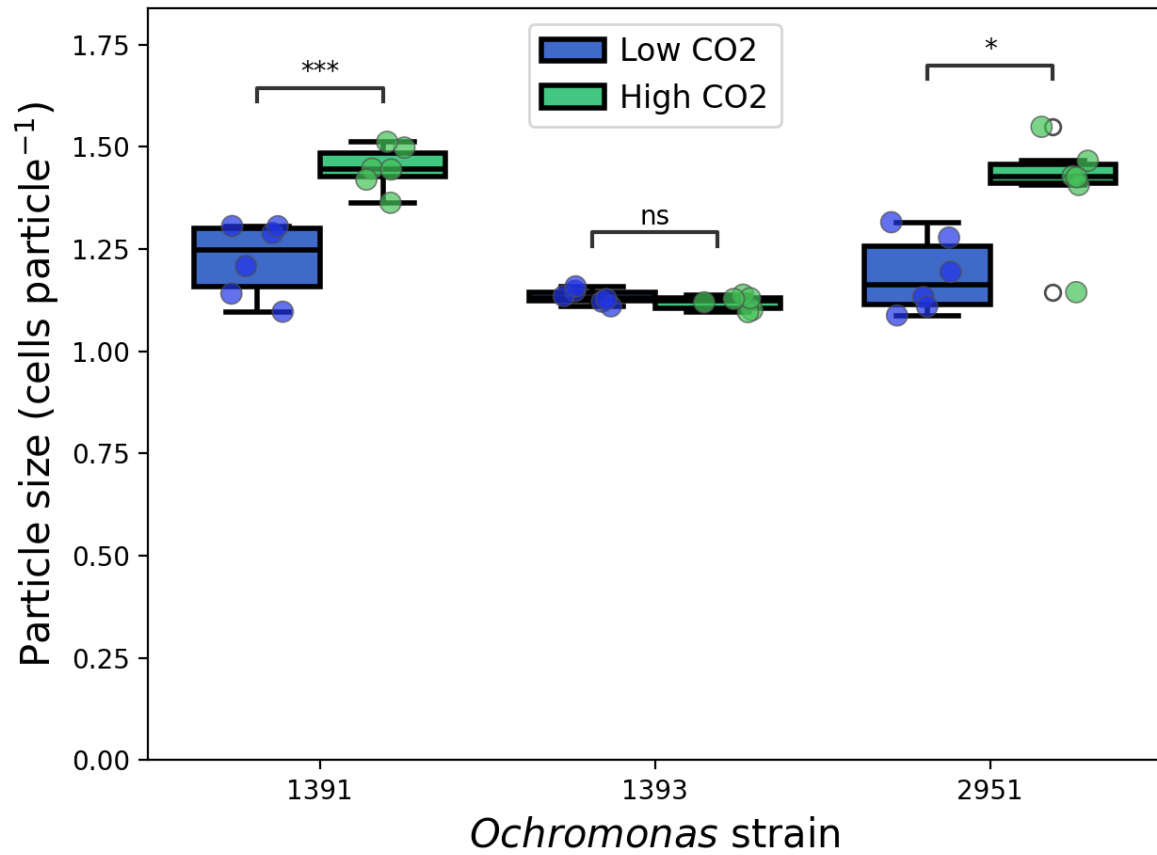


Supplementary figure S1. An example of a raw cytogram from *Ochromonas* counts on the flow cytometer. The sample, of CCMP1393 replicate from the last day of the experiment, was triggered on red fluorescence (690/50) and the following populations were gated based on their red fluorescence (y axis) and their forward scatter (x axis): gate P1 – singlets of red fluorescent beads added to the sample prior to running, gate P2 – singlets of the *Ochromonas* population, gate P3 – all *Ochromonas* cells, including events containing more than one cell.



173 Supplementary figure S2: An example of a cytogram from the grazing assay. *Ochromonas*
 174 population (gated based on Fig S1, P2 gate) is plotted based on the red and green
 175 fluorescence properties. The population is then separated based on the green fluorescence
 176 properties: The population with the low green fluorescence is termed “not feeding” while the
 177 population with higher green fluorescence is termed “feeding”

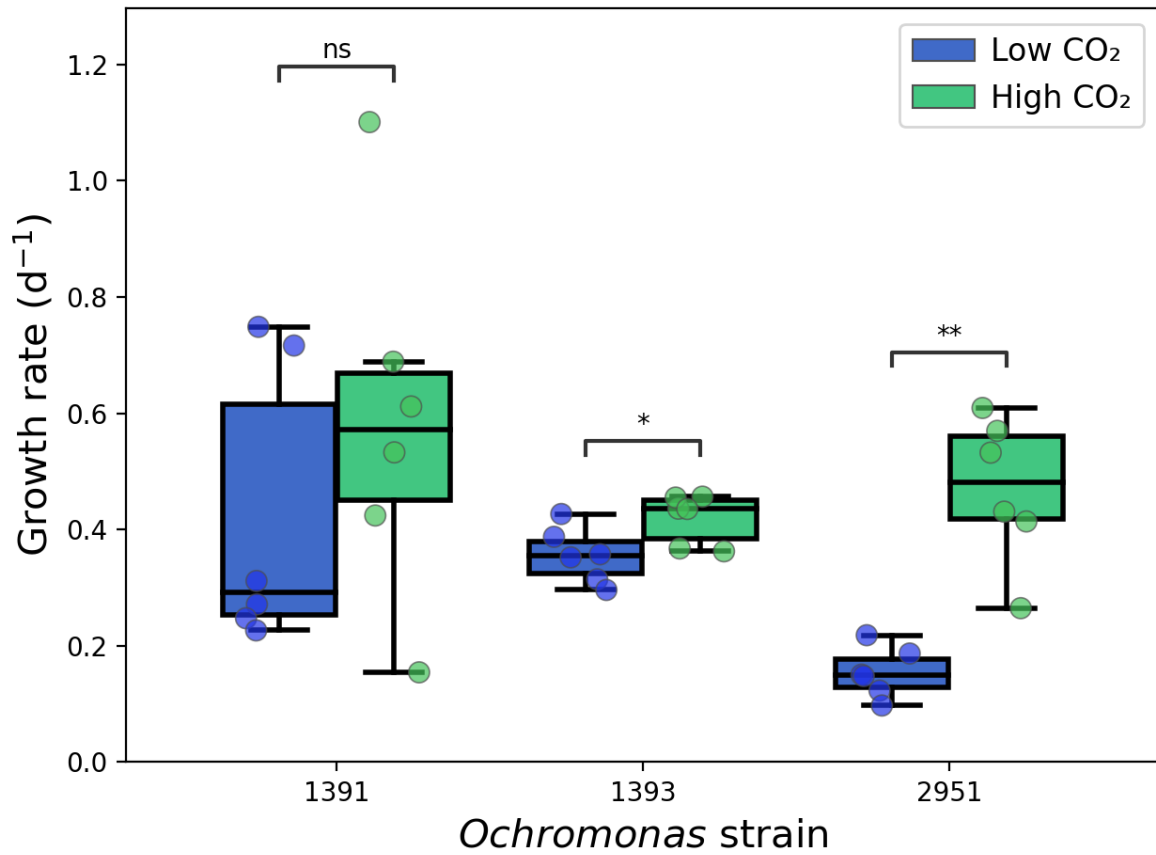
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180 Supplementary figure S3: Estimated number of cells per flow cytometric event (particle) in
 181 samples of the three *Ochromonas* strains under Low and High CO₂ treatments. Asterisks
 182 indicate significant differences between treatments (Welch's *t*-test: **p* < 0.05, ***p* < 0.01, ****p* <
 183 0.001).

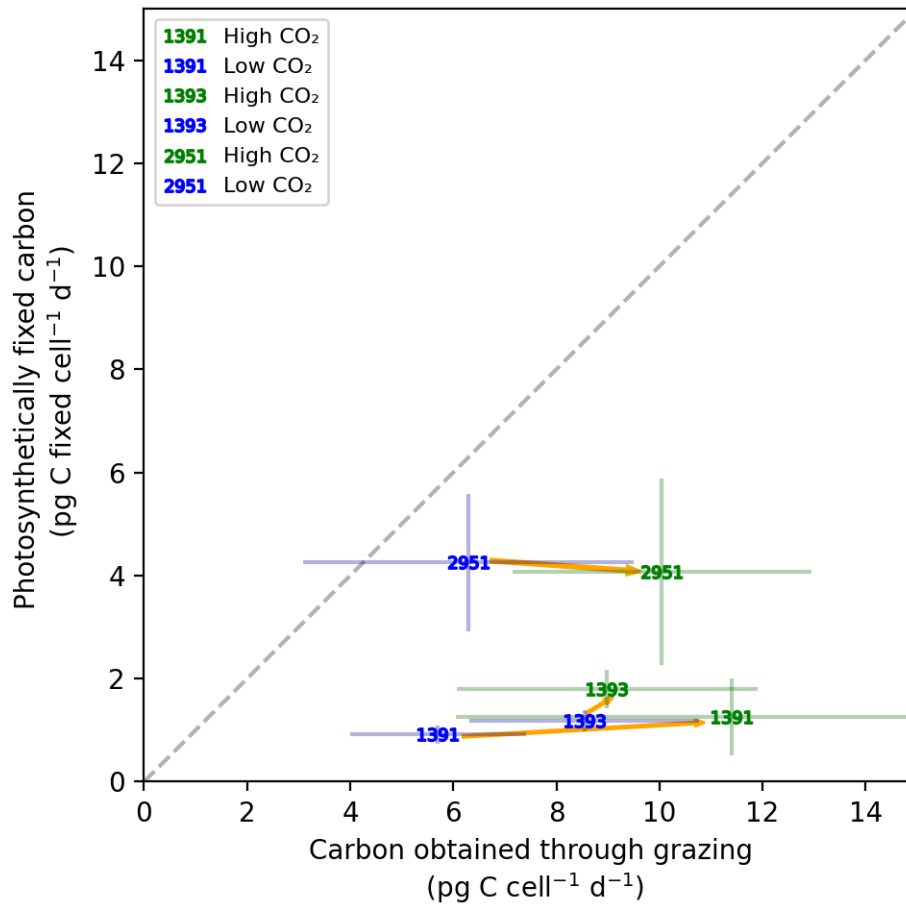
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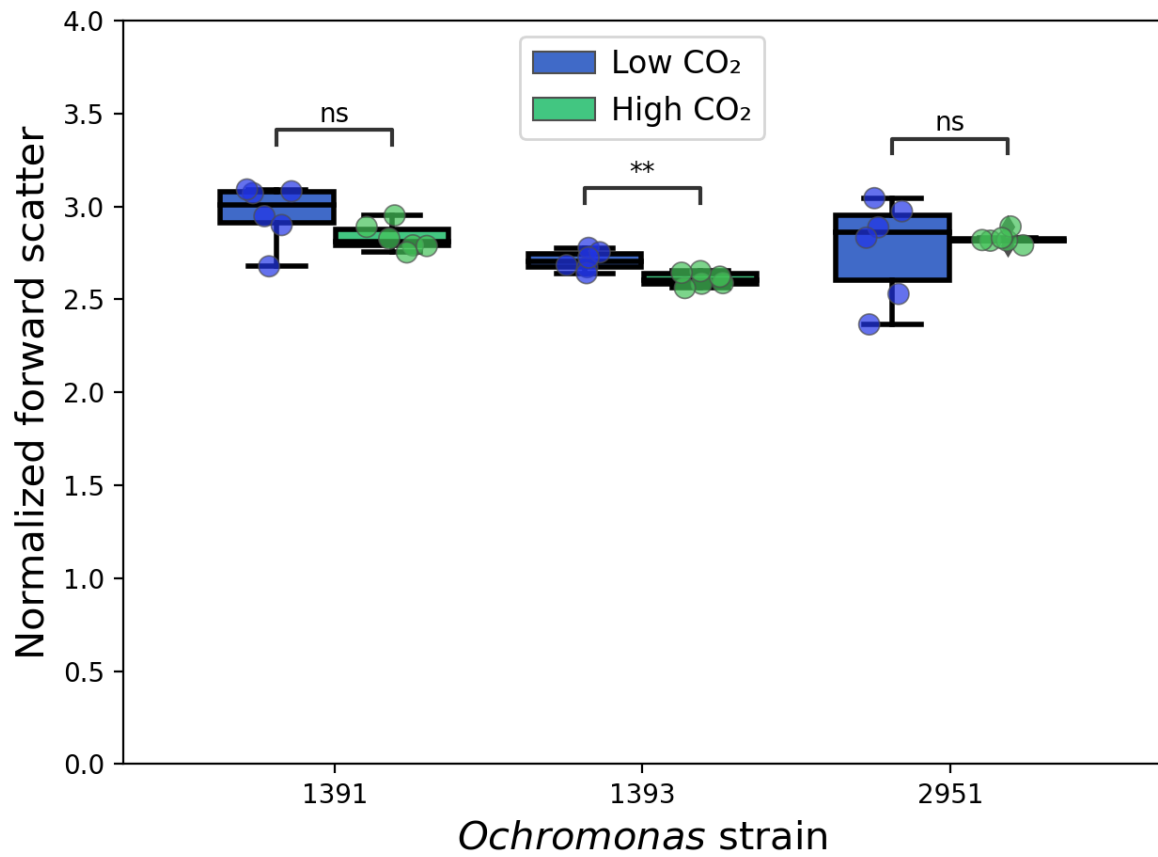
186 Supplementary figure S4: Specific growth rate of the three *Ochromonas* strains in the Low
 187 CO₂ and High CO₂ treatments, based on adjusted flowcytometric cell counts, to account for
 188 potential clumping. Asterisks indicate significant differences between treatments (Welch's *t*-
 189 test: **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

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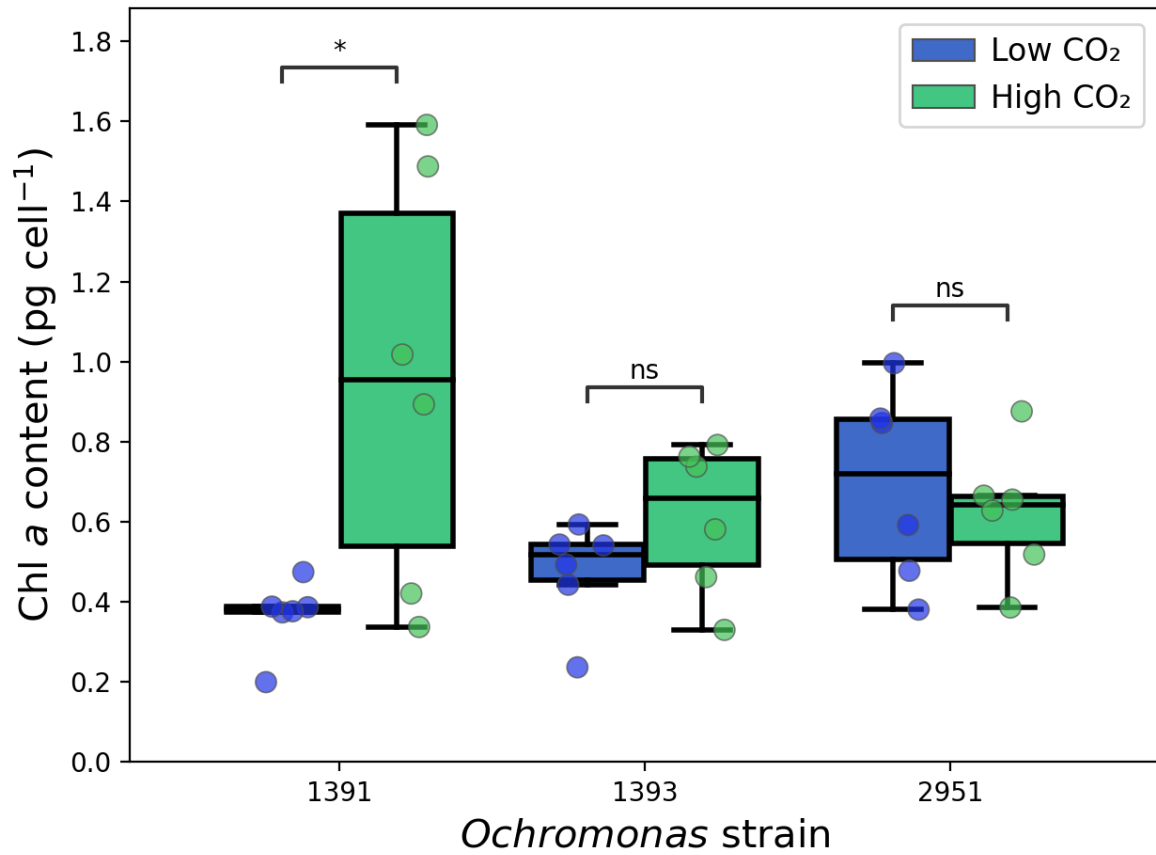
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192 Supplementary figure S5: The balance between phototrophic and heterotrophic carbon
 193 acquisition for the three *Ochromonas* strains in the low CO₂ and high CO₂ treatments. The
 194 amounts of carbon either obtained from grazing (x-axis) or from photosynthetic carbon
 195 fixation (y -axis) per day are extrapolated from measured hourly rates (Figure 3). Error bars
 196 represent standard error of the mean. The dashed grey line is the $y=x$ line, at which
 197 phototrophic and heterotrophic carbon acquisition are equal. Orange arrows are drawn from
 198 the low to the high CO₂ treatment of the same strain.



199

200 Supplementary figure S6: Relative forward scatter as a proxy for cell size of the three
 201 *Ochromonas* strains in the Low CO₂ and High CO₂ treatments, measured by flow cytometry.
 202 Asterisks indicate significant differences between treatments (Welch's *t*-test: **p* < 0.05, ***p*
 203 < 0.01, ****p* < 0.001).



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205 Supplementary figure S7: Chlorophyll *a* content of the three *Ochromonas* strains under Low
 206 and High CO₂ treatments. Asterisks indicate significant differences between treatments
 207 (Welch's *t*-test: **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

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210 **Table S1:** Carbonate chemistry of the *Ochromonas* experiments and their controls. Data show mean \pm
 211 SD (n=6), where each replicate was averaged over the last four transfers. The data are obtained from
 212 two consecutive experiments: the first experiment (e1) included *Ochromonas* CCMP2951 and
 213 CCMP1391, and the second experiment (e2) included *Ochromonas* CCMP 1393; both experiments
 214 included controls without *Ochromonas*. Asterisks in parentheses indicate significant differences of the
 215 indicated carbonate chemistry parameter between the low and high CO₂ treatments (Welch's *t*-test:
 216 **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

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Strain	Treatment	dissolved CO ₂ (ppm)	pH	DIC ($\mu\text{mol L}^{-1}$)	Alkalinity ($\mu\text{Eq L}^{-1}$)
CCMP 1391	Low CO ₂ (e1)	323 \pm 34(***)	8.31 \pm 0.01(***)	2266 \pm 8(*)	2653 \pm 42(***)
CCMP 2951	Low CO ₂ (e1)	342 \pm 74(***)	8.30 \pm 0.07(***)	2247 \pm 32(**)	2632 \pm 36(**)
CCMP 1393	Low CO ₂ (e2)	362 \pm 6(***)	8.32 \pm 0.01(***)	2585 \pm 12(**)	3013 \pm 14(***)
Control	Low CO ₂ (e1)	335 \pm 7(**)	8.30 \pm 0.01(**)	2285 \pm 72	2663 \pm 83(*)
Control	Low CO ₂ (e2)	402 \pm 12(***)	8.28 \pm 0.01(***)	2585 \pm 71	2976 \pm 82
CCMP 1391	High CO ₂ (e1)	867 \pm 46(***)	7.92 \pm 0.02(***)	2334 \pm 43(*)	2505 \pm 40(***)
CCMP 2951	High CO ₂ (e1)	890 \pm 53(***)	7.91 \pm 0.02(***)	2370 \pm 8(**)	2540 \pm 8(**)
CCMP 1393	High CO ₂ (e2)	783 \pm 34(***)	8.02 \pm 0.01(***)	2653 \pm 39(**)	2890 \pm 42(***)
control	High CO ₂ (e1)	983 \pm 135(**)	7.87 \pm 0.06(**)	2342 \pm 47	2494 \pm 63(*)
control	High CO ₂ (e2)	991 \pm 10(***)	7.92 \pm 0.01(***)	2696 \pm 67	2881 \pm 75

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219 **Table S2:** Statistical comparison of the low CO₂ treatment versus high CO₂ treatment for each of the
 220 measured experimental and physiological parameters using the Welch's *t*-test. Degrees of freedom are
 221 mentioned once per parameter as they were the same for all strains. Significant differences (*p*<0.05)
 222 are indicated in bold.

Strain	<i>1391</i>		<i>1393</i>		<i>2951</i>		df
	t statistic	p	t statistic	p	t statistic	p	
pCO₂	21.26	1.2×10⁻⁹	30.03	3.9×10⁻⁷	14.77	1.2×10⁻⁷	5
pH	-18.54	1.1×10⁻⁶	-43.80	7.9×10⁻¹⁰	-12.19	1.8×10⁻⁵	5
DIC	3.79	0.0002	4.13	0.0063	8.87	0.0002	5
alkalinity	-7.04	4.8×10⁻⁵	-6.84	0.0004	-6.17	0.0011	5
Growth Rate	0.76	0.4645	2.40	0.0375	6.26	0.0006	5
Photosynthetic C fixation	1.10	0.3175	3.46	0.0091	0.20	0.8434	5
Slope of C fixation	0.19	0.8608	2.74	0.0283	1.46	0.1938	4
Grazing rate	2.48	0.0476	0.29	0.7747	2.12	0.0598	5
Relative red fluorescence	6.62	0.0004	3.11	0.0112	2.89	0.0163	5
Fucoxanthin/Chla	1.62	0.1433	1.14	0.2842	4.01	0.0095	5
b-carotene/chla	0.18	0.8598	0.60	0.5613	4.20	0.0050	5
VAZ/chla	2.69	0.0350	0.07	0.9439	4.50	0.0046	5
Particle size (cells particle⁻¹)	5.17	0.0007	1.46	0.1741	3.21	0.0109	5
Growth rate (counts adjusted to particle size)	1.02	0.334	2.42	0.0365	5.79	0.0011	5
Forward scatter	1.78	0.1172	3.90	0.0036	0.50	0.630	5
Cellular Chlorophyll a content	2.74	0.0386	1.47	0.1752	0.59	0.5716	5

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