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Better off alone? New insights in the symbiotic relationship between the flatworm *Symsagittifera roscoffensis* and the microalgae *Tetraselmis convolutae*

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1 **Better off alone? New insights in the symbiotic relationship between the**
2 **flatworm *Symsagittifera roscoffensis* and the microalgae *Tetraselmis***
3 ***convolutae***

4

5

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17

18 **Short title:** Photobiology of *Symsagittifera roscoffensis* and *Tetraselmis convolutae*

19

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21 *convolutae*

22

23 **Abstract**

24 The acoel flatworm *Symsagittifera roscoffensis* lives in obligatory symbiosis with the microalgal
25 chlorophyte *Tetraselmis convolutae*. Although this interaction has been studied for more than a century,
26 little is known on the potential reciprocal benefits of both partners, a subject that is still controversial.
27 In order to provide new insights into this question, we have compared the photophysiology of the free-
28 living microalgae to the symbiotic form in the flatworm, both acclimated at different light irradiances.
29 Photosynthesis – Irradiance curves showed that the free-living *T. convolutae* had greater photosynthetic
30 performance (i.e., oxygen production rates, ability to harvest light) than their symbiotic form, regardless
31 of the light acclimation. However, they were affected by photoinhibition under high irradiances, which
32 did not happen for the symbiotic form. The resistance of symbiotic microalgae to photoinhibition were
33 corroborated by pigment analyses, which evidenced the induction of photoprotective mechanisms such
34 as xanthophyll cycle as well as lutein and β -carotene accumulation. These processes were induced even
35 under low light acclimation and exacerbated upon high light acclimation, suggesting a global stress
36 situation for the symbiotic microalgae. We hypothesize that the internal conditions in the sub-epidermal
37 zone of the flatworm (e.g., osmotic and pH), as well as the phototaxis toward high light imposed by the
38 worm in its environment, would be major reasons for this chronic stress situation. Overall, our study
39 suggests that the relationship between *S. roscoffensis* and *T. convolutae* may be a farming strategy in
40 favor of the flatworm rather than a symbiosis with mutual benefits.

41

42

43 1. Introduction

44 Symbiosis is an interspecific interaction where two or more organisms live in close association,
45 often supported by a high level of integration of the physiology of both partners (Muscatine 1980).
46 Depending on the benefit reciprocity, symbiosis range along a continuum from parasitism, where one
47 partner benefits at the expense of the other(s), to mutualism, where all partners benefit (Tipton et al.
48 2019). Symbiosis with photosynthetic microalgae occurs in many groups of marine invertebrates such
49 as Porifera, Cnidaria, Mollusca or Chordata (Venn et al. 2008; Rumpho et al. 2011; Melo Clavijo et al.
50 2018). Among the most studied animal-algal relationships are the reef-building corals, hosting
51 photosynthetic dinoflagellates in their polyps. More discreet and less known, the flatworm
52 *Symsagittifera roscoffensis* (Graff 1891) establishes an obligatory symbiosis with the green alga
53 *Tetraselmis convolutae* (Parke and Manton 1967; Arora 2016; Arboleda et al. 2018). Their interaction
54 is set up at each generation by worm juveniles ingesting microalgae which they will keep for the lifetime
55 of the animal (Keebles 1910). The adult stage does not ingest any organic food and lives autotrophically
56 in the light, in phosphate and nitrate-rich environments, thus depending entirely on the microalgal
57 photosynthetic products (e.g., fatty acid, amino acid, organic acid) (Taylor 1974; Boyle and Smith 1975;
58 Meyer et al. 1979). During the vacuolization process, microalgae lose their cell wall, flagella and eyespot
59 and stay under the epidermis of the worm (Douglas, 1983a). Benefitting the symbiosis, *T. convolutae*
60 uses the uric acid produced by the worm as an endogenous nitrogen source (Boyle & Smith, 1975;
61 Douglas, 1983b). This association is not only biologically remarkable but also ecologically relevant, as
62 the flatworm can form large populations on sandy shores, easily noticeable as dark green patches
63 (Keebles 1910; Doonan and Gooday 1982). The exploitation of this ecological niche would generate an
64 annual primary production rates of 872.9 g C m⁻² (Doonan and Gooday 1982), a value similar to those
65 measured on coral reefs (Muscatine, 1990). Furthermore, the ability of the symbiotic algae to actively
66 uptake nitrates makes *S. roscoffensis* a possible important *in situ* nitrate interceptor in the sediment at
67 local scales (Carvalho et al. 2013).

68 In many animal-algal associations (i.e., symbiosis, keptoplasty), the fact that the photosynthetic
69 activity of the algal partner is often increased compared to the free-living form, has led to the 'host-
70 mediated enhanced photosynthetic performance' hypothesis (Johnson et al. 2006; Johnson 2011;
71 Serôdio et al. 2014; Decelle et al. 2019). By providing a suitable environment regarding light and
72 nutrient availability, the host may increase the ability of the algae to maximize photosynthetic carbon
73 fixation. In the case of *S. roscoffensis* and *T. convolutae*, the flatworm can also adjust its position along
74 the vertical light gradient of the sediment photic zone, therefore regulating light acclimation of its
75 symbiont (Serôdio et al. 2011; Worley et al. 2019). It has also been suggested that the typical circular
76 milling of the flatworm, which allow the formation of dense and stable biofilm, may promote the
77 photosynthesis of the microalgae (Sendova-Franks et al. 2018). In their study, Serôdio et al. (2011)
78 suggested that non photochemical quenching of chlorophyll fluorescence (i.e., dissipation of excess

79 energy by heat), a mechanism involved in photoprotection, could also play a role in the regulation of
80 light utilization by the symbiotic algae. This ability to maximize the photobiology of the symbiont might
81 constitute a real advantage for *T. convolutae*, in comparison to other free-living forms of non-motile
82 microalgae in intertidal areas. On the other hand, the study of Nissen et al. (2015) demonstrated that the
83 host's reaction to light stimuli was not adjusted to the symbionts' needs, which questions its behavioral
84 capacity of photosynthetic regulation (Serôdio et al. 2011). Considering the lack of evidence for a true
85 mutualistic relationship (*sensus* Mushegian and Ebert 2015) between *S. roscoffensis*-*T. convolutae*
86 (Selosse 2000; Kiers and West 2016), and the somewhat contradictory results about the behavioral
87 photosynthetic regulation of the flatworm (Serôdio et al. 2011; Nissen et al. 2015), more experiment are
88 needed to addressed the benefit of the symbiosis for the algae and a potential exploitative host control
89 by the flatworm.

90 In this context, the present study assessed the effect of the symbiosis on the photobiology of the
91 microalga *Tetraselmis convolutae* by comparing the free-living and the symbiotic forms of the alga,
92 acclimated to different light irradiance levels. To do so, we performed oxygen production measurement
93 and pigment analyses in order to understand whether the photosynthesis is increased and/or regulated
94 differently in the host compared to the free-living form.

95

96 **2. Material and methods**

97 **2.1. Biological material and culture conditions**

98 A strain of the green microalga *Tetraselmis convolutae* was isolated from one individual of the
99 acoel flatworm *Symsagittifera roscoffensis* collected on a sandy shore of the French coast of the English
100 Channel (48°44'N, 3°59'W, Roscoff, Brittany, France) on December 7th, 2007. A *T. convolutae* culture
101 was then obtained by micropipette isolation of a single cell released from the specimen. The resulting
102 monoclonal culture was maintained in filter-sterilized seawater supplemented with modified K/2
103 medium (-Tris, -Si; Keller *et al.*, 1975) at 17°C under a light irradiance of 70-80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$
104 in a 12:12 light: dark cycle. The culture was deposited in the Roscoff Culture Collection ([http://roscoff-](http://roscoff-culture-collection.org/)
105 [culture-collection.org/](http://roscoff-culture-collection.org/)) as RCC1563.

106 For the experiment, *T. convolutae* was grown in pyrex erlenmeyer flasks in autoclaved seawater
107 supplemented with 1% Provasoli solution (Provasoli et al. 1968), in a thermostat-water bath at 19-20°C.
108 The cultures were acclimated during 8 days to low (30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and high (350 μmol
109 $\text{photons m}^{-2} \text{s}^{-1}$) white light irradiance upon a 12:12 light: dark cycle, provided by an iodine metal halide
110 light source (400 W, Sylvania, Wilmington, USA). The acclimation time was chosen since it is assumed
111 that light acclimation requires one generation time, which varies between 2 hours and 6 days depending
112 on species and physiology (Reynolds 1984; Grobbelaar et al. 1992; MacIntyre et al. 2002). The
113 irradiance levels were adjusted using a quantameter (Quantum LI-190SA, LiCor, Lincoln, USA) and
114 the distance from the light source. The algal cultures were maintained in exponential growth phase by

115 diluting the cultures with fresh medium every two days and monitoring the optical density at 665 nm
116 with a spectrophotometer (Jenway 6305, JENWAY, Staffordshire, UK). *S. roscoffensis* were collected
117 on a sandy beach in Roscoff in July 2015 (48°44'N, 3°59'W, Brittany, France) and maintained in the
118 same conditions as described above for the cultures of the free-living microalgae.

119

120 2.2. Oxygen production measurements

121 We used a custom-made light chamber to perform oxygen measurements under different light
122 intensities in order to obtain Photosynthesis-Irradiance (P-I) curves. Briefly, this system consisted in a
123 marine water-bath in which the water was maintained at a constant temperature (19-20°C) with
124 continuously running water. The same white light source as described above was placed above the
125 device and a set of 5 neutral density filters (LEE Filters, Andover, UK) placed between the light source
126 and the aquarium, enabling us to set 8 different irradiance levels (from 0 to $\approx 1200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).
127 Cultures of *Tetraselmis convolutae* in their growth exponential phase and flatworms (40-50
128 individuals), along with two controls containing autoclaved seawater, were placed in 2 mL vials (3 and
129 4 replicates, respectively) and incubated at the different light irradiances. Preliminary experiments
130 allowed the determination of the proper incubation time (30-40 minutes depending on light irradiance)
131 to ensure linear oxygen production/consumption and to avoid Warburg effect (Turner and Brittain 1962).

132 Oxygen production/consumption rate, translated as net primary production (in the light) and
133 respiration rates (in the dark), were calculated by measuring the dissolved oxygen molar concentration
134 (then converted in mass concentration) at the beginning and the end of the incubation period with a non-
135 invasive optical fiber system (FIBOX 3, PreSens, Regensburg, Germany). The system was calibrated
136 prior to the experiment using sodium dithionite (0 %) and air bubbling (100 %). Net oxygen production,
137 respiration and gross oxygen production rates were corrected from controls and calculated as:

138

$$139 \quad R = \frac{\Delta O_2 \times V}{\Delta t \times \text{chl } a} \quad NP = \frac{\Delta O_2 \times V}{\Delta t \times \text{chl } a} \quad GP = NP - R$$

140

141 where NP is the net oxygen production rate ($\mu\text{g O}_2 \text{ h}^{-1} \mu\text{g chl } a^{-1}$), R the respiration rate ($\mu\text{g O}_2 \text{ h}^{-1} \mu\text{g}$
142 $\text{chl } a^{-1}$), GP the gross oxygen production rate ($\mu\text{g O}_2 \text{ h}^{-1} \mu\text{g chl } a^{-1}$), ΔO_2 the difference between initial
143 and final O_2 concentrations ($\mu\text{g O}_2$), V the volume of the chamber (mL), Δt the incubation time (h), and
144 chl *a* the chl *a* mass (μg).

145 At the end of the experiment, the cultures of *T. convolutae* free-living cells were centrifuged at
146 $4\ 500 \times g$ and the supernatant was discarded. For the symbiotic forms, the medium was carefully
147 removed with a micropipette. All samples were then stored at -20°C until further analysis. Pigments
148 were fully extracted in the dark at 4°C, during 6 h in 100 % methanol for the free-living cells and 2 h in
149 100 % ethanol for the symbiotic forms. Absorbance measurements were made using a
150 spectrophotometer, and chl *a* was quantified using the Ritchie equations (Ritchie 2008).

151

152 **2.3. HPLC pigment analysis**

153 A volume of 3 mL of exponentially growing culture of *T. convolutae* was centrifuged at 4 500
154 $\times g$ and a total of 60 *Symsagittifera* flatworms by sample were isolated in glass tubes. The cell pellets or
155 the flatworms were resuspended in 100 % acetone for an hour at -20 °C and sonicated during 5 rounds
156 of 10 sec. The extracts were then centrifuged at 20 000 $\times g$, the supernatants collected and centrifuged
157 again to ensure total removal of particles and cell debris. All sample preparations were carried out under
158 subdued light at 4°C.

159 The extracts were brought to 25 % Milli-Q water to avoid peak distortion (Zapata and Garrido
160 1991) and a volume of 100 μ L of the pigment extract was immediately injected into a High Pressure
161 Liquid Chromatography system (Hewlett-Packard HPLC 1100 Series, AGILENT, Santa Clara, USA),
162 equipped with a quaternary pump and diode array detector. Pigment separations were performed using
163 a Waters Symmetry C₈ column (150 \times 3 \times 4.6 mm, 3.5 μ m particle size) for 38 min at a flow rate of 1
164 mL min⁻¹, according to procedures previously published (Garrido et al. 2009; Lopes dos Santos et al.
165 2016). The modified protocol of Garrido *et al.*, (2009) was used in order to allow the separation of
166 neoxanthin and loroxanthin derivatives, with the solutions A = methanol:acetonitrile:aqueous pyridine
167 solution in the proportions 50:40:10 (v/v/v), and B = acetonitrile:acetone (80:20 v/v), following the
168 gradient (min, %B): 0 min, 0 %; 22 min, 40 %; 28 min, 95 %; 38 min, 95 %. In order to separate the
169 zeaxanthin from the lutein peak, the classical procedure of Zapata *et al.*, (2000) was also applied for
170 each sample, with the mobile phases A = methanol:acetonitrile:aqueous pyridine solution (0.025 M) in
171 the proportions 50:25:25 (v/v/v), and B = acetonitrile:acetone (80:20 v/v), following the same solvent
172 gradient.

173 Chlorophylls and carotenoids were detected by their absorbance at 440 nm and identified by
174 diode array spectroscopy. Pigments were identified and, when possible, the relative quantities were
175 estimated using standards derived from macroalgae and phytoplankton cultures by preparative HPLC
176 (Repeta and Bjørland 1997), using previously compiled extinction coefficients (Roy et al. 2011). The
177 deepoxydation state (DES) of the xanthophyll cycle was calculated for each sample as:

178

$$179 \quad \text{DES} = [\text{Zeaxanthin}] / ([\text{Violaxanthin}] + [\text{Antheraxanthin}] + [\text{Zeaxanthin}])$$

180

181 **2.4. Statistical analyses**

182 Oxygen production measurements were normalized to chl *a* and plotted versus irradiance. The
183 gross oxygen production was preferred to the net oxygen production to take into account the respiration
184 of the flatworm and be able to compare the rates between free-living and symbiotic microalgae forms.
185 Non-linear regression models of photosynthesis considering photoinhibition were fitted to each P-I
186 curve (Platt et al. 1980). Classical photosynthetic parameters were then calculated from the model,
187 including P_{MAX} (maximal oxygen production rate), α (initial slope of the curve), I_K (light intensity at the

188 onset of light saturation) and β (photoinhibition parameter). P_{MAX} was interpreted as the maximum,
189 potential, light-saturated, oxygen production rate under light condition without photoinhibition (Platt et
190 al. 1980).

191 Two-way factorial analyses of variance (ANOVA) were used to assess differences in model
192 parameters between *T. convolutae* forms (two levels: free-living and symbiotic) and light condition (two
193 levels: low and high light acclimation). When significant, *post hoc* multiple comparisons were carried
194 out using Tukey HSD. Normality and homocedasticity of the residuals were graphically assessed. When
195 those conditions were not fulfilled, simple factor effect was verified using the non-parametric Wilcoxon
196 test based on ranks. Changes in pigment concentrations were not statistically assessed because only
197 duplicates for free-living cells were available. Statistical analyses were performed in R version 3.3.0 (R
198 Core Team 2016).

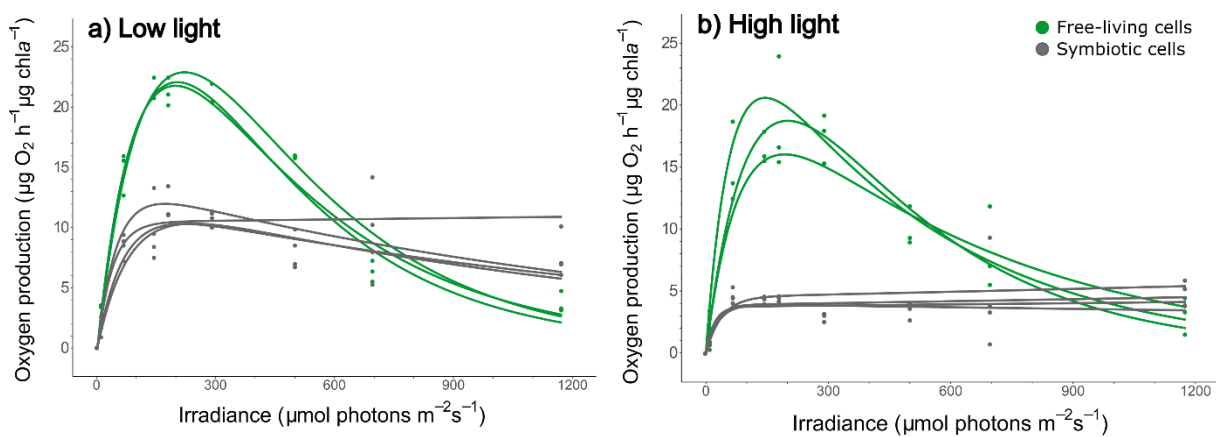
199

200 3. Results

201 3.1. Oxygen production measurements

202 P-I curves showed clear different shapes between the free-living and the symbiotic forms of
203 *Tetraselmis convolutae*, for both light irradiance acclimation (Figure 1a & b). No significant interaction
204 was found between the factors “Living-form” and “Light acclimation” (Table 2).

205



206

207 **Fig. 1** Photosynthesis-irradiance (P-I) curve of *Tetraselmis convolutae* in the free-living (green) and symbiotic
208 (grey) forms acclimated under low (a; 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and high (b; 350 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) irradiance.

209

210 Overall, the free-living cells exhibited significantly higher values than symbiotic cells for all the
211 photosynthetic parameters calculated: oxygen production rate (P_{MAX} was 3-8 fold higher in free-living
212 cells than symbiotic ones) while the initial slope of the curve (α), and the light intensity at the onset of
213 light saturation (I_K) were in average 2-fold greater in free living cells. Conversely the free-living cells
214 showed strong photoinhibition, as evidenced by the significant higher values of photoinhibition index
215 (β : 8-17 fold) (Table 1 & 2). Indeed, the oxygen production rate of the free living cells cultures strongly

216 decreased from about 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ down to values lower than those of the symbiotic algae.
 217 No significant change in respiration rate were detected.

218
 219 **Table 1** Mean (\pm SD) of maximal oxygen production rate (P_{MAX}), initial slope (α), saturation irradiance (I_K),
 220 photoinhibition index (β) and respiration rate (R), in the free-living and symbiotic forms of *Tetraselmis convolutae*,
 221 acclimated to low and high light. P_{MAX} , α , I_K and β were retrieved from the model of Platt *et al.*, (1980) whereas R
 222 was experimentally measured.

223

| | Low light | | High light | |
|--|-------------------------|-----------------------|-------------------------|-----------------------|
| | Free living cells (n=3) | Symbiotic cells (n=4) | Free living cells (n=3) | Symbiotic cells (n=4) |
| P_{MAX} ($\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1}$) | 36.1 \pm 3.3 | 12.4 \pm 1.4 | 30.9 \pm 3.9 | 4.0 \pm 0.3 |
| α ($\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1} \mu\text{mol photons m}^{-2} \text{s}^{-1}$) | 0.31 \pm 0.01 | 0.22 \pm 0.06 | 0.33 \pm 0.1 | 0.15 \pm 0.03 |
| I_K ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) | 117.1 \pm 5.3 | 59.5 \pm 19.8 | 99.3 \pm 24.2 | 27.9 \pm 7.9 |
| β ($\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1} \mu\text{mol photons m}^{-2} \text{s}^{-1}$) | 0.17 \pm 0.05 | 0.01 \pm 0 | 0.08 \pm 0.04 | 0 \pm 0 |
| R ($\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1}$) | -0.7 \pm 0.4 | -1.2 \pm 0.2 | 0.1 \pm 1.1 | -0.2 \pm 0.2 |

225
 226 In terms of light acclimated, only the I_K parameter and the respiration varied significantly,
 227 regardless of the living form (Table 2). Mean I_K was 84.2 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for low light acclimated
 228 algae compared to 58.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for high light acclimated ones. Respiration was 14-fold
 229 higher in low light acclimated cell than high-light acclimated ones (-0.96 and -0.07 $\mu\text{g O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1}$
 230 respectively).

231 **Table 2** Factorial two-way ANOVA followed by Tuckey HSD post hoc test showing the effect of light condition
 232 (low vs. high light acclimation) and living form (free-living vs. symbiotic cells) of the microalgae *Tetraselmis*
 233 *convolutae* on the initial slope (α), the saturation irradiance (I_K) and the respiration rate (R). The effect on the
 234 maximal oxygen production rate (P_{MAX}), and the photoinhibition index (β) was assessed by two distinct Wilcoxon
 235 tests. Bold numbers indicate significant level < 0.05 . LL: low light, HL: high light, FL: free-living, S: symbiotic.

236

| | P_{MAX} | | | α | | | I_K | | | β | | | R | | |
|--------------|--|----|----------------------|---|------|----------------|--|-------|----------------------|---|----|----------------------|--|------|--------------|
| | $(\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1})$ | | | $(\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1} \mu\text{mol photons m}^{-2} \text{s}^{-1})$ | | | $(\mu\text{mol photons m}^{-2} \text{s}^{-1})$ | | | $(\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1} \mu\text{mol photons m}^{-2} \text{s}^{-1})$ | | | $(\mu\text{mol O}_2 \text{h}^{-1} \mu\text{g chl a}^{-1})$ | | |
| | df | W | P | df | F | P | df | F | P | df | W | P | df | F | P |
| Light | 1 | 13 | 0.17 | 1 | 1.01 | 0.338 | 1 | 8.89 | 0.01* | 1 | 15 | 0.26 | 1 | 8.51 | 0.02* |
| Form | 1 | 48 | < 0.001*** | 1 | 15.2 | 0.002** | 1 | 54.93 | < 0.001*** | 1 | 48 | < 0.001*** | 1 | 1.48 | 0.25 |
| Light x Form | - | - | - | 1 | 1.87 | 0.2 | - | 0.63 | 0.44 | - | - | - | 1 | 0.26 | 0.62 |

Tuckey HSD post hoc test

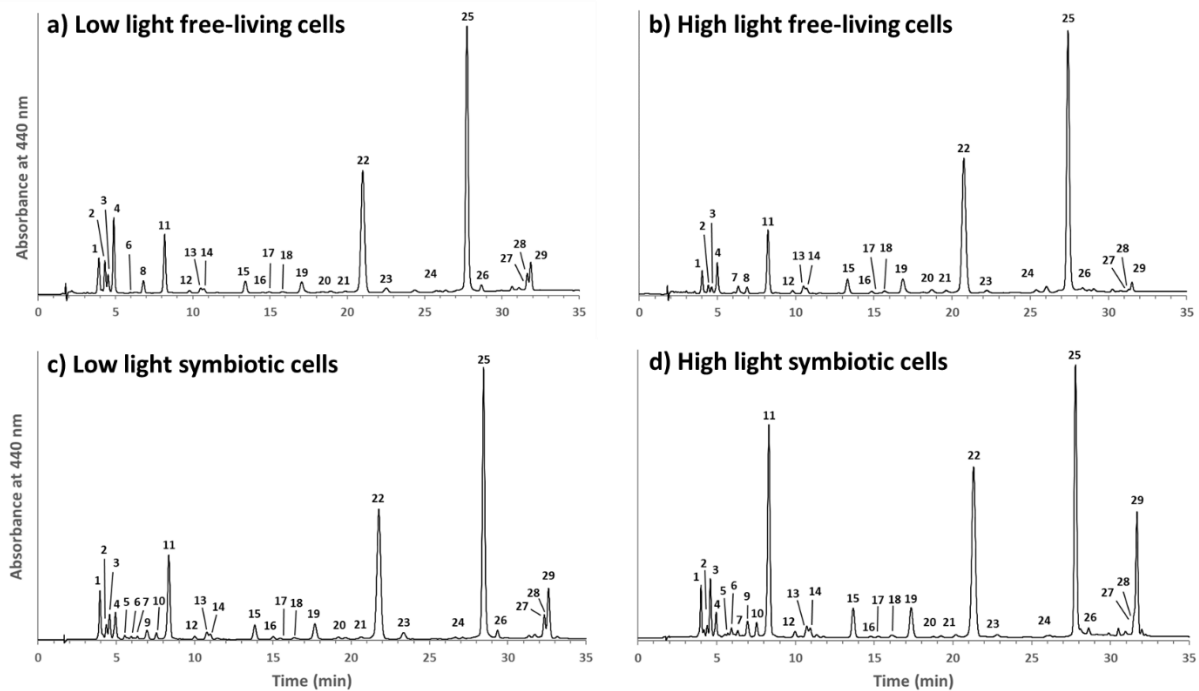
| | | | | | | | | | | | | | | | |
|-------|--------|---|---|--------|---|---|---------|---|---|--------|---|---|---|---|---------|
| Light | - | - | - | - | - | - | LL > HL | - | - | - | - | - | - | - | LL > HL |
| Form | FL > S | - | - | FL > S | - | - | FL > S | - | - | FL > S | - | - | - | - | - |

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

237
 238
 239 **3.2. HPLC pigment analysis**

240 The pigments of the free-living form of *T. convolutae*, *i.e.* in culture, were difficult to extract,
 241 even though we carried out a strong extraction procedure. It displayed a particularly complex
 242 pigmentation with the major pigments being typical of Chlorophyta microalgae (similar to the so-called
 243 PRASINO-2A group; Roy *et al.*, 2011), notably including chlorophyll *b*, lutein, violaxanthin, *cis*-
 244 neoxanthin and loroxanthin (Figure 2a & b, Table 3, Table S1). *T. convolutae* contain several acyl-
 245 loroxanthin compounds, with the two major ones probably being loroxanthin-decenoate and
 246 loroxanthin-dodecenoate as previously reported in several other *Tetraselmis* species (Garrido *et al.*

247 2009) and in *Pyramimonas parkae* (Kohata and Watanabe 1989). We also detected a high number of
 248 more or less minor pigments. Using the modified method of Garrido et al. (2009), one of them eluted
 249 early and showed absorbance properties identical to *trans*-neoxanthin (peak 1 in Table S1). Two species
 250 of carotene were detected in the cell extracts, β,β - and β,ϵ -carotene but it is probable that other forms
 251 co-eluted with them and/or with phaeophytin *a*.



252
 253 **Fig. 2** Chromatographic analysis of *Tetraselmis convolutae* pigmentation (Garrido et al. 2009) as free-living cells
 254 acclimated to low (a) and high (b) light, and as a symbiont of *Symsagittifera roscoffensis* acclimated to low (c) and
 255 high (d) light. The retention times and wavelength maxima are mentioned in the supplementary material (Table
 256 S1). 1: *trans*-neoxanthin like; 2: loroxanthin; 3: *cis*-neoxanthin; 4: violaxanthin; 5: neochrome like; 6: Auroxanthin
 257 like; 7: Antheraxanthin; 8: *cis*-neoxanthin like; 9: Auroxanthin like; 10: Dihydrolutein like; 11: Lutein (+
 258 zeaxanthin); 12: unidentified; 13: unidentified; 14: unidentified; 15: Loroxanthin decenoate; 16: Acyl Loroxanthin
 259 like; 17: Unidentified; 18: Unidentified; 19: Loroxanthin dodeceonate; 20: Unidentified; 21: Unidentified; 22:
 260 Chlorophyll *b*; 23: Chlorophyll *b* derivative; 24: Chlorophyll *a* derivatives; 25: Chlorophyll *a*; 26: Chlorophyll *a*
 261 derivative; 27: β,ϵ -carotene; 28: Phaeophytin *a*; 29: β,β -carotene.

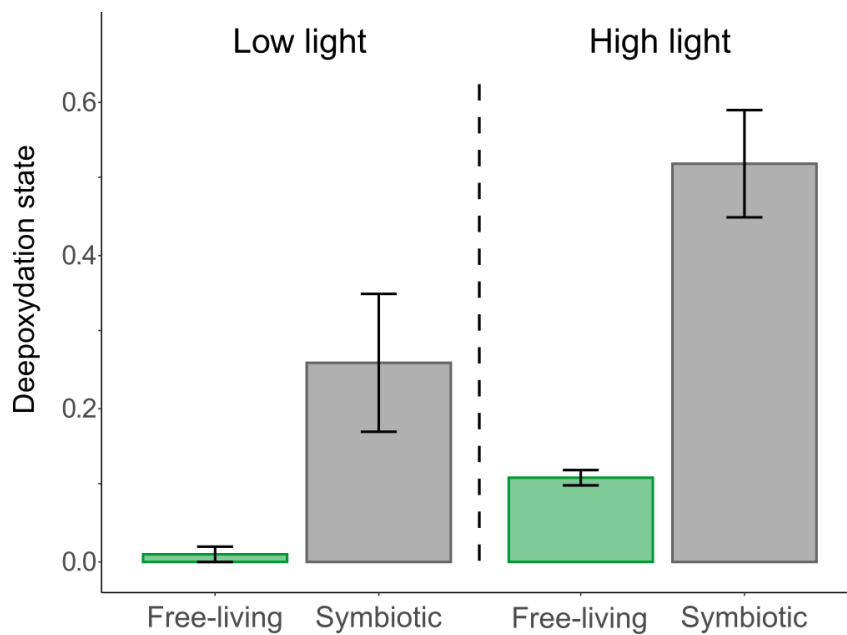
262
 263 In contrast to the cells in culture, we had no difficulty to achieve complete extraction of the pigments
 264 for the symbiotic forms. The pigmentation was globally similar to the one of the free-living forms
 265 (Figure 2c & d, Table 3). However, a higher proportion of the minor pigments neochrome and
 266 auroxanthin was measured in *hospite* microalgae, whereas these pigments were hardly detectable in the
 267 free-living cells. In addition, two other pigments showing wavelength maxima quasi identical to *cis*-
 268 neoxanthin and dihydrolutein eluted at about 4.5 and 7.5 min, respectively, only in the symbiotic form
 269 (Figure 2c & d).

270

271 **Table 3** Mean (\pm SD) of the major pigments relatively to chlorophyll *a* (mg/g) in the free-living and symbiotic
 272 forms of *Tetraselmis convolutae*, acclimated to low and high light. We were not able to quantify all the detected
 273 pigments because many of them were in too low amounts to allow purification for calibration of the HPLC system.
 274

| | Low light | | High light | |
|-------------------------|-------------------------|-----------------------|-------------------------|-----------------------|
| | Free living cells (n=2) | Symbiotic cells (n=3) | Free living cells (n=2) | Symbiotic cells (n=3) |
| Loroxanthin | 9.2 \pm 0.8 | 4.2 \pm 0.6 | 2.7 \pm 2.7 | 2.9 \pm 1.3 |
| <i>cis</i> -neoxanthin | 4.8 \pm 2.2 | 5.1 \pm 2.7 | 3.3 \pm 3.3 | 15.4 \pm 2.8 |
| Chlorophyll <i>b</i> | 479.5 \pm 18.7 | 499.3 \pm 67.2 | 549.4 \pm 32.6 | 628.5 \pm 32.7 |
| Loroxanthin decenoate | 6.9 \pm 0.1 | 7.4 \pm 1.4 | 11.1 \pm 1.0 | 16.2 \pm 0.6 |
| Loroxanthin dodeceonate | 7.8 \pm 0.2 | 9.3 \pm 1.2 | 11.6 \pm 1.1 | 18.4 \pm 0.8 |
| Lutein | 29.5 \pm 2.1 | 33.4 \pm 4.2 | 36.6 \pm 2.1 | 91.0 \pm 8.1 |
| β,β -carotene | 7.6 \pm 1.0 | 12.9 \pm 2.8 | 3.1 \pm 1.0 | 43.9 \pm 4.6 |
| Violaxanthin | 25.5 \pm 1.3 | 9.5 \pm 0.1 | 15.7 \pm 3.7 | 7.3 \pm 1.1 |
| Antheraxanthin | 0.3 \pm 0.1 | 1.8 \pm 0.4 | 5.3 \pm 1.8 | 2.7 \pm 0.2 |
| Zeaxanthin | 0.2 \pm 0.3 | 4.1 \pm 1.6 | 2.5 \pm 0.3 | 11.4 \pm 1.7 |

275
 276
 277 Acclimation to high light levels induced similar changes in the pigmentation for both free-living
 278 and symbiotic forms of *T. convolutae* (Table 3). Several pigments decreased relatively to chlorophyll *a*
 279 in response to high light, including *trans*-neoxanthin, loroxanthin, *cis*-neoxanthin (only in the free-living
 280 cells), while the chlorophyll *b*:*a* ratio remained fairly stable in all conditions. By contrast, the two major
 281 acyl-loroxanthin compounds increased in response to high light acclimation. Photoprotective
 282 mechanisms such as the xanthophyll cycle were clearly activated (Demmig-Adams and Adams 1996;
 283 Jahns and Holzwarth 2012), as shown by the successive deepoxydation of violaxanthin into
 284 antheraxanthin and zeaxanthin in both the free-living and symbiotic forms of *T. convolutae* (Figure 3).
 285 This process was accompanied by a significant lutein synthesis (Table 3).
 286



287
 288 **Fig. 3** Deepoxydation state of the xanthophyll cycle in the free-living (green) and symbiotic (grey) forms of
 289 *Tetraselmis convolutae*, acclimated to low and high light (mean \pm SD).
 290

291 Interestingly, there were clear differences in the amplitude of these acclimation processes between the
292 free-living and the symbiotic cells. Under low light, the symbiotic *T. convolutae* showed much higher
293 levels of the photoprotective pigments lutein, zeaxanthin and β -carotene. These marked differences were
294 exacerbated under high light acclimation, upon which the symbiotic cells developed high xanthophyll
295 deepoxydation state and a pronounced induction of *cis*-neoxanthin, lutein along with a strong β -carotene
296 accumulation.

297

298 **4. Discussion**

299 The symbiosis between the flatworm *S. roscoffensis* and the chlorophyte *T. convolutae* is a
300 remarkable and rare model system that allows studying the level of reciprocity of such interactions
301 (Serôdio et al. 2011; Dupont et al. 2012; Bailly et al. 2014; Nissen et al. 2015; Arboleda et al. 2018).
302 Defining the status of this symbiosis has remained nevertheless challenging. In particular, a number of
303 questions have been recently raised about egalitarian partnerships in nature, with some authors arguing
304 that there is still a lack of evidence for a mutualistic relationship for the *S. roscoffensis*-*T. convolutae*
305 couple, especially for the algae, and that the relationship might be considered as a form of imprisonment
306 of the symbiotic partner (Selosse 2000; Kiers and West 2016). In order to give new insights into these
307 questions, we studied the influence of the symbiotic lifestyle on the algal photobiology.

308

309 **4.1 The microalgal lifestyle is associated with distinct net oxygen production**

310 The oxygen production curves measured for both algal forms acclimated to two light irradiance
311 levels, unambiguously showed oxygen production was higher in algal free-living form compared to the
312 symbiotic one, at most of the tested irradiances. The only published data on the oxygen production rate
313 of *T. convolutae* (Nozawa et al. 1972), using comparable light acclimation (i.e., $73 \mu\text{mol photons m}^{-2} \text{s}^{-1}$)
314 and under similar irradiance levels (i.e., $100\text{-}200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), indicated 3-4 fold lower values
315 than in our study, for both algal living-forms. Since both light during growing and measurement period
316 were overall comparable (estimated by converting the published data, in foot-candle to units of
317 irradiance), the difference could be attributed to the measurement technique (polarographic electrode *vs.*
318 optical fiber). Interestingly, the difference between the two living forms was almost identical. The
319 maximal oxygen production rate modelled (i.e., P_{MAX}) in free-living cells, which occurred between 100
320 and $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, was 3 and 8 fold higher at low light and high light, respectively, than in
321 the symbiotic forms, while Nozawa *et al.* (1972) found a factors of 3 between the two living forms at
322 low light acclimation. To explained this difference, authors proposed that the lower net oxygen
323 production rate observed for the symbiotic form might be due to the host respiration, an increased
324 symbiont photo-respiration, shading effect thanks to the arrangement of the algae within the host, and/or
325 a reduced or host-regulated photosynthetic rate.

326 Although it is quite possible that the flatworm respired some of the oxygen produced by the
327 alga, it is unlikely that the large differences in oxygen production we observed originated only in the

328 worm respiration, especially considering the small differences in the calculated respiration rates. Our
329 data suggest that, although it was not statistically significant, the symbiotic system displays slightly
330 higher respiration rates than the free-living algae alone because of the flatworm respiration, and that the
331 microalgae respired at similar rate in any condition and lifestyle. In addition, the P-I curves of the free-
332 living and symbiotic alga showed different shapes that are typical of differences in photosynthesis
333 regulation (see below), which would thus be induced by the alga itself.

334 Our results thus suggest a different perspective to the idea that algal photosynthetic activity would
335 be enhanced in the host (Johnson 2011; Serôdio et al. 2014; Decelle et al. 2019), as the symbiosis would
336 leads here to a decrease of the global photosystem II activity of the microalgal symbiont.

337

338 **4.2 Different photoacclimation capacities in different lifestyles**

339 The different shapes of the oxygen production rate *vs.* irradiance curves strongly suggest that the
340 algae regulate the photosynthesis process differently depending on the acclimation irradiance and its
341 lifestyle. The symbiotic microalgae showed lower α and I_K values than the free-living ones, indicating
342 lower efficiency to harvest light and a saturation at lower irradiance level, respectively (Platt et al. 1980;
343 MacIntyre et al. 2002). This is often associated with a reduced functional photosynthetic antenna which,
344 in green algae, is composed of the so-called light harvesting complex proteins. Even though the chl *b:a*
345 ratio did not change, our results thus suggest a downregulation of at least some of these proteins in the
346 alga in the symbiotic form.

347 In addition, the symbiotic microalgae were much less sensitive to high light irradiances than the free-
348 living ones, as indicated by the photoinhibition phenomenon. This photoinhibition suggests a strong
349 photosystem II photoinactivation at irradiances higher than 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the free-living
350 cells. These differences between the two lifestyles were exacerbated upon high light acclimation. In
351 particular, when acclimated to 350 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the symbiotic algae were able to maintain a
352 constant photosystem II oxygen evolving up to the highest irradiance tested (i.e. $\approx 1200 \mu\text{mol photons}$
353 $\text{m}^{-2} \text{s}^{-1}$). These observations suggest that, in contrast to the free-living form, the symbiotic *T. convolutae*
354 may chronically induce photoprotection mechanisms that prevent both photoinhibition and
355 photodamages.

356 Pigment analyses showed clear differences between the two light irradiance acclimation states in
357 both lifestyles and are globally in agreement with a previous study on *Tetraselmis* photoacclimation
358 (Garrido et al. 2009). The reduction of some of the pigments in response to high light in both algal forms
359 may be related to a reduction of the functional size of the photosynthetic antenna (Melis et al. 1999).

360 The most obvious changes concerned the photoprotection mechanisms. High light acclimation
361 resulted in an increase of the photoprotective xanthophyll cycle activity (Demmig-Adams and Adams
362 1996), indicating an activation of the violaxanthin deepoxydase enzyme in the symbiont plastids. In
363 addition, lutein accumulation was observed in response to high light pre-exposure in both lifestyles.
364 Although the mechanism has not been yet extensively described, this pigment has been shown to be

365 induced in response to high light in a number of planktonic chlorophytes (see e.g. Six *et al.*, 2008;
366 Garrido *et al.*, 2009; Lopes dos Santos *et al.*, 2016) and is thought to be involved in photosystem
367 photoprotection (Niyogi *et al.* 1997; Horton and Ruban 2005; Jahns and Holzwarth 2012). Noteworthy,
368 these photoprotective mechanisms were activated much more intensively in the symbiotic lifestyle than
369 in the free-living microalgae. Indeed, the deepoxydation state of the xanthophyll cycle of the symbiotic
370 algae acclimated to 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ was twofold higher than the one of free-living algae grown
371 under 350 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Similarly, the lutein accumulation was particularly pronounced in the
372 symbiotic microalgae acclimated to high light. These photoprotection mechanisms were likely
373 associated with non-photochemical quenching of fluorescence, although we did not carry out pulse-
374 amplitude modulation (PAM) fluorimetry measurements. At last, the symbiotic microalgae accumulated
375 large amounts of β -carotene. This is a well-known phenomenon in chlorophytes, which triggers this
376 photoprotective and antioxidant mechanism in response to various types of stresses (Ahmed *et al.* 2015).

377 Our results thus suggest that the symbiotic lifestyle is associated with a stress signal in the alga. The
378 fact that such protective mechanisms are induced even under low light suggests that they can be
379 considered as a global stress, which is not necessarily directly linked to light. It is indeed well known
380 that photoprotection can be related and induced by other environmental factors than light, for instance
381 in temperature and osmotic stress situations, with the aim of decreasing the intraplastidial production of
382 reactive oxygen species originating in photosystem II activity (Foyer and Shigeoka 2011). Considering
383 that free-living cells of *T. convolutae* strain RCC1563 were grown since 2007 at a controlled temperature
384 of 17°C and that symbiotic cells naturally experienced temperature ranging from 16-22°C at the moment
385 of the collection (Doonan and Gooday 1982), the temperature of 20°C in our experiment is not
386 considered as stressful.

387

388 **4.3 The flatworm as a stressful environment for the symbiotic alga**

389 As recently observed in planktonic symbiotic Acantharians, the algal symbiont may undergo
390 profound ultrastructural and physiological changes upon inclusion in the host (Decelle *et al.* 2019). It
391 has been shown that symbiotic *Tetraselmis* have a different cell structure compare to the free-living cells
392 (Oschman 1966; Provasoli *et al.* 1968). In particular, the inclusion in the flatworm is associated with the
393 loss of a part of the cell envelope, leading to an intimate contact between the algal plasma membrane
394 and the flatworm internal medium (Douglas 1983b). The large differences in the pigment extraction
395 rates we observed are probably related to the presence of the cell envelope, since the free-living cells
396 were much more difficult to extract. The internal medium of the flatworm might thus constitute a
397 stressful environment for *T. convolutae*. As already suggested for kleptoplasts in sea slugs (Jesus *et al.*
398 2010), the direct contact between the symbiotic algae (then devoid of cell envelope) and the flatworm
399 intern media, which is very different from the seawater, can dramatically change the acido-basic balance
400 of the algal cells. Such drastic medium modification might be considered as a chronic osmotic stress
401 that might activate protective mechanisms in the alga such as, for example, the violaxanthin

402 deepoxydase gene and those involved in the lutein synthesis. The accumulation of β -carotene is also
403 very well-known to occur in osmotic stress situations, notably in *Dunaliella salina* (Oren 2005).
404 Interestingly, we detected minor amounts of the pigments neochrome and auroxanthin only in the worm
405 samples, whereas these pigments were hardly detectable in the free-living cells. In prasinophytes, these
406 two pigments are often derived from the acidic catalyse of *cis*-neoxanthin and violaxanthin, respectively
407 (Haugan and Liaaen-Jensen 1994). Even though these two carotenoids might have been synthesized
408 during the pigment extraction, their formation support the idea that the flatworm constitutes a very acidic
409 medium for the marine microalga. In addition, it is worth noting that a previous study also suggested
410 that the flatworm may be a stressful environment for the alga. Indeed, Van Bergeijk and Stal (2001)
411 showed that *in hospite* algae contain a 6-fold higher concentration of the algal molecule DMSP than in
412 the free-living cells. The induction of this protectant molecule suggests a stress situation for the algae in
413 the flatworm (Dickson and Kirst 1986; Sunda et al. 2002; Archer et al. 2010).

414

415 **4.4 From a planktonic to a benthic light niche**

416 Our results show that, in response to the inclusion in the flatworm, the algae induce permanent
417 light-dissipating photoprotection mechanisms that are typical of a pronounced stress situation. The
418 consequence is that the alga in its symbiotic lifestyle is quite resistant to light stress and is able to
419 maintain photosystem II activity up to high irradiances in spite of a lower photosynthetic efficiency than
420 free-living form. This is likely associated with a lower synthesis of photosynthetic compounds available
421 for the host (Hoogenboom et al. 2006).

422 The chronic induction of protective mechanism appears to be crucial for the survival of the alga in
423 the flatworm because the symbiosis may be associated, for the alga, to dramatic changes in the
424 osmolarity of its environment but also of its light niche. Indeed, in its planktonic form, *T. convolutae*
425 deals with light irradiances more or less attenuated in the water column, depending on the depth. By
426 contrast, in the symbiotic lifestyle, the acclimation to light is dependent of *S. roscoffensis* migrations.
427 The flatworms are often observed at the surface of the sediments at low tide and show a positive
428 phototaxis (Doonan and Gooday 1982; Serôdio et al. 2011). This behavior, associated with a shading
429 effect due to the arrangement of the algae within the host (Nozawa et al. 1972), may explain why the
430 symbiotic cells were acclimated to lower light levels than the free-living ones. However, other studies
431 showed that the flatworm could move in areas where the irradiance was higher than optimal for the
432 algae, likely harming their photosynthetic apparatus (Nissen et al. 2015). It is worth noting that during
433 our 8 days of acclimation, the flatworm did not have sediment to potentially escape from the high
434 irradiance provided. However we did observe migration pattern toward less illuminated areas, which is
435 in agreement with the photoaccumulation experiment of Serôdio et al. (2011). The microalgae must then
436 cope with “abnormally” high light irradiances and it is probable that they could not survive without
437 strong photoprotection. Thus, the symbiotic system *S. roscoffensis* – *T. convolutae* appears to use a
438 similar photophysiological strategy as the intertidal diatom-dominated microphytobenthos, which show

439 adaptation traits to high irradiances (Cartaxana et al. 2013), while using motility to select the optimal
440 light acclimation (Ezequiel et al. 2015). Our study thus highlights the physiological consequences of a
441 change from a planktonic to a benthic light niche.

442

443 **4.5 The flatworm as a parasite of the microalga?**

444 The traditional ideas behind mutualism are reciprocal benefits and partner cooperation. However,
445 this concept has been revisited for a few years toward a more parsimonious explanation known as
446 exploitative host control (Wooldridge 2010; Lesser et al. 2013; Keeling and McCutcheon 2017). For
447 instance, in *Paramecium bursaria* and its algal symbiont *Chlorella* sp., the photosynthetic efficiency of
448 the chlorophyte is higher in the free-living microalgae than in the symbiotic ones. The *Chlorella*
449 symbionts can escape their host, but only under conditions where the latter does not benefit from
450 symbiosis, and there are no conditions where this nutritional symbiosis is mutually beneficial (Lowe et
451 al. 2016). In the case of the *S. roscoffensis* - *T. convolutae* relationship, there is still no observation or
452 evidence for possibility for the microalgae to escape from the flatworm (Muscatine et al., 1974), even
453 though once free (mechanically during our isolation process), the symbiotic algae could recover cell
454 envelope, flagella and eyespot (Ian Probert, person. com.). In addition, our data suggest that the
455 microalgae undergo chronic stress in the flatworm, which induce the locking of the photosynthetic
456 apparatus in photoprotective mode, while the flatworm assimilates photosynthetic compounds from the
457 algae (Taylor 1974; Boyle and Smith 1975; Meyer et al. 1979).

458 The vertical transmission of symbionts is one important feature that can help understand whether a
459 relationship is mutualistic or parasitic (Garcia and Gerardo 2014). Vertical transmission (i.e., symbionts
460 inherited from parents) is expected to lead to a certain level of phylogenetic homogeneity of the
461 symbionts and to increase symbiont effectiveness for the host (Herre et al. 1999). In the case of *T.*
462 *convolutae*, algae are ingested as free-living cells by juveniles of *S. roscoffensis* and are therefore
463 replaced at each generation (i.e., horizontal transmission), impeding any transgenerational selection.
464 Overall, these results question the mutualistic nature of this relationship (Selosse 2000).

465 The symbiotic association between *S. roscoffensis* and *T. convoluta* could thus be a farming strategy
466 where the flatworm would use the algae to its own needs, without mutualism retribution (Kiers and West
467 2016; Sørensen et al. 2019). Further research on the molecule exchanges between both partners and on
468 the consequences of the association on the fitness of *T. convolutae* would allow a deeper understanding
469 of the nature of the relationship.

470

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480

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482

483 6. References

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662 **Supplementary material**

663

664 Table S1: Chromatogram peak identification, retention time and online spectral characteristics of the pigments
 665 detected with the modified method of Garrido et al. (2009) in the free-living and symbiotic forms of *Tetraselmis*
 666 *convolutae*.

667

| Peak # | Pigment | Retention time (min) | Wavelength maxima (nm) | | |
|--------|----------------------------------|----------------------|------------------------|-----|-----|
| 1 | <i>trans</i> -neoxanthin like | 3.85 | 418 | 442 | 471 |
| 2 | Loroxanthin | 4.21 | (423) | 447 | 474 |
| 3 | <i>cis</i> -neoxanthin | 4.48 | 414 | 438 | 466 |
| 4 | Violaxanthin | 4.86 | 414 | 441 | 470 |
| 5 | Neochrome like | 5.74 | 400 | 423 | 450 |
| 6 | Auroxanthin like | 5.94 | 381 | 402 | 427 |
| 7 | Antheraxanthin | 6.35 | (423) | 448 | 475 |
| 8 | <i>cis</i> -neoxanthin like | 4.48 | 413 | 437 | 465 |
| 9 | Auroxanthin like | 6.96 | 381 | 402 | 427 |
| 10 | Dihydrolutein like | 7.53 | 406 | 430 | 455 |
| 11 | Lutein (+ zeaxanthin) | 8.31 | (422) | 447 | 475 |
| 12 | Unidentified | 10 | 423 | 441 | 468 |
| 13 | Unidentified | 10.73 | (422) | 443 | 471 |
| 14 | Unidentified | 10.85 | (421) | 443 | 469 |
| 15 | Loroxanthin decenoate | 11.51 | (424) | 449 | 475 |
| 16 | Acyl Loroxanthin like | 12.56 | (424) | 449 | 475 |
| 17 | Unidentified | 15.49 | (423) | 444 | 472 |
| 18 | Unidentified | 16.41 | (423) | 445 | 470 |
| 19 | Loroxanthin dodecenoate | 17.81 | (424) | 449 | 476 |
| 20 | Unidentified | 19.25 | (424) | 448 | 477 |
| 21 | Unidentified | 20.59 | (423) | 444 | 472 |
| 22 | Chlorophyll <i>b</i> | 21.71 | 464 | 600 | 649 |
| 23 | Chlorophyll <i>b</i> derivative | 23.3 | 463 | 599 | 649 |
| 24 | Chlorophyll <i>a</i> derivatives | 24-27 | 430 | 617 | 663 |
| 25 | Chlorophyll <i>a</i> | 28.24 | 431 | 617 | 663 |
| 26 | Chlorophyll <i>a</i> derivative | 29.35 | 433 | 617 | 663 |
| 27 | β,ϵ -carotene | 32.33 | (424) | 448 | 476 |
| 28 | Phaeophytin <i>a</i> | 32.42 | 410 | 608 | 666 |
| 29 | β,β -carotene | 32.59 | (427) | 453 | 478 |

668

669