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1 Continuous selection pressure to improve temperature acclimation of *Tisochrysis lutea*

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9

10 **Abstract.**

11 Temperature plays a key role in outdoor industrial cultivation of microalgae. Improving the thermal
12 tolerance of microalgae to both daily and seasonal temperature fluctuations can thus contribute to
13 increase their annual productivity. A long term selection experiment was carried out to increase the
14 thermal niche (temperature range for which the growth is possible) of a neutral lipid overproducing
15 strain of *Tisochrysis lutea*. The experimental protocol consisted to submit cells to daily variations of
16 temperature for 7 months. The stress intensity, defined as the amplitude of daily temperature
17 variations, was progressively increased along successive selection cycles. Only the amplitude of the
18 temperature variations were increased, the daily average temperature was kept constant along the
19 experiment. This protocol resulted in a thermal niche increase by 3°C (+16.5 %), with an
20 enhancement by 9 % of the maximal growth rate. The selection process also affected *T. lutea*
21 physiology, with a feature generally observed for 'cold-temperature' type of adaptation. The amount
22 of total and neutral lipids was significantly increased, and eventually productivity was increased by
23 34%. This seven month selection experiment, carried out in a highly dynamic environment,
24 challenges some of the hypotheses classically advanced to explain the temperature response of
25 microalgae.

26 **Keywords:** *Tisochrysis lutea*, adaptation, selectostat, temperature response, lipids, continuous
27 selection, evolution engineering.

28 **Introduction**

29 Microalgae are a promising source of resources and bulk chemicals for various applications such as
30 nutrition, aquaculture, cosmetics and, at a longer time scale, biofuels or green chemistry. However,
31 the wild type strains commonly cultivated offer poor industrial yields, limiting their profitability.
32 These wild species adapted to natural conditions do not tolerate extreme temperatures reached by
33 artificial culturing systems (photobioreactors or greenhouse cultures) with a low thermal inertia
34 [1][2]. Moreover, in temperate climates, microalgae production throughout the year is limited by the
35 low winter temperatures. This sensitivity to temperature therefore restricts production, unless
36 temperature is regulated with a high cooling/heating system involving high financial and
37 environmental costs [1]. The need to select new strains adapted to a wider range of temperature is
38 thus crucial for increasing productivity and reducing cultivation costs.

39 The steady state growth response of microalgae to temperature, i.e. the growth rate as a function of
40 temperature in acclimated and non-limiting conditions, also called the thermal reaction norm, has
41 been extensively studied [2][3], and can be accurately predicted by the model of [4]. This model is
42 parameterized by three cardinal temperatures: T_{min} and T_{max} , corresponding respectively to the
43 temperatures below and above which growth is not possible. T_{opt} , the optimum growth temperature,
44 was defined as the temperature leading to the highest growth rate (all other parameters being kept
45 constant). The temperature values ranging between T_{min} and T_{max} represent the thermal niche. The
46 selection of strains with a larger thermal niche and thus lower temperature sensitivity is a promising
47 approach to increase outdoor productivity.

48 Microalgal strains acclimate to temperature in a process similar to photoacclimation [5]. Under weak
49 thermal perturbation and on short term exposition (less than 10 generations), acclimation enhances
50 ecological fitness by modulating the flux of energy produced by photosynthesis to carbon fixation
51 rate through the Calvin cycle in a more efficient way [6]. However, when the thermal stress exceeds
52 its physiological tolerance, adaptation mechanisms are triggered. The genetic modifications triggered

53 during the adaptation process result from the selection of individuals with highest fitness in the new
54 thermal stressing conditions. Adaptation occurs by two different mechanisms: selection of the most
55 adapted individuals among the pre-existing genetic diversity of a non-monoclonal population (pre-
56 selective mutations), and *de novo* genetic mutations induced by environmental stress (adaptive
57 mutation) [7]. In this paper “adapted strain” was defined as the resulting strain supporting
58 temperature stresses that the simply “acclimated strain” do not tolerate. As it is shown later,
59 adaption also results in a different lipid profile, compared to the initial strain, when submitted to the
60 same environment. Little information exists on physiological temperature adaptation in microalgae.
61 The only available studies are for extremophile strains, resulting of a million year evolutionary
62 process of thermal adaptation. Life at low temperatures has selected individuals that could mitigate
63 the lowering of their metabolic activities and growth by synthesizing psychrophilic enzymes with
64 higher activity at cold temperatures [8][9] or stimulating enzyme production [10][11], and can
65 preserve the fluidity of their membranes by increasing the synthesis of unsaturated fatty acids
66 [12][13][3]. Conversely, under high temperature conditions that tend to denature proteins and
67 membranes [10][14], extremophile adapted microalgal species produce more Heat Shock Proteins
68 [15] or saturated fatty acids, allowing them to resist these lethal effects [14]. This study therefore
69 investigated the possibility of changing the temperature response (growth rate and physiology) of a
70 non-extremophile strain of *Tisochrysis lutea*, keeping this species for several months in continuous
71 cultures, under increasing stressful thermal conditions consisting in daily temperature shifts. This
72 species is well-used in aquaculture and mollusk hatcheries for fish larva and bivalve nutrition
73 especially because of its high content in essential polyunsaturated fatty acids. It is a fast growing
74 microalgae (μ_{\max} about 2 d^{-1}), easy to cultivate under temperate climate, requiring a pH about 7, a
75 solar irradiance of $780 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at about 30°C [16].

76 **Materials and methods**

77 **Microalgae strain.** The *Tisochrysis lutea* strain (CCAP 927/17) used in this experiment, named W2X,
78 was obtained by a selection/mutation process from the original strain CCAP 926/14. This strain is
79 characterized by a doubled triglyceride productivity [17][18]. Note that the strain used was non-
80 monoclonal and thus contained a pre-existing genetic diversity supposed to facilitate the emergence
81 of new traits.

82 **Culturing system.** The culturing system was specifically designed to maintain long-term continuous
83 cultures of microalgae in computer-controlled growing conditions. The enrichment medium was
84 prepared in several 20 L tanks (Nalgene) filled with 3 weeks-matured natural seawater, previously
85 filtered on 0.1 μm , and autoclaved at 120°C for 40 min. After cooling, f/2 medium [19] was added
86 through a 0.22 μm sterile filter. The culture vessels consisted of water jacketed, 1.9-liter plane
87 photobioreactors (thereafter named “selectiostat”) connected to a circulating programmable water
88 bath (Lauda Proline RP845). 30 to 40 min were necessary to vary the temperature from 10 to 40 °C
89 inside the selectiostats. Cultures were continuously and gently homogenized by a magnetic stirrer
90 and bubbling air. They were illuminated with LEDs (Nichia NVSL219BT 2 700°K) placed on one side of
91 the photobioreactors. The light intensity, continuously measured with a probe (SKY, SKL2620) placed
92 on the opposed side of the reactor was maintained at $I_{in} = 250 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$. pH was
93 maintained constant at 8.2 by computer-controlled microinjections of CO₂. Turbidity was measured
94 on-line using a red LED (Rodan HIRL5010) at 800 nm on one side of the reactor and a photodiode
95 (Optek OP993) on the other side. Light, pH, temperature, turbidity, and dilution rate were
96 continuously monitored by ODIN® software [20].

97 **Cleaning procedure.** As the selection experiment lasted about 260 days in stressing conditions, algae
98 biofilm was removed monthly. After saving 1 liter of culture in an autoclaved Schott bottle, the
99 different pieces of the selectiostats were dismantled and washed with milliQ water then 70 %
100 ethanol. Once dried and reassembled, the photobioreactors were sterilized with 10 % HCl and then

101 rinsed with fresh sterile medium filtered through 0.22 μm (SpiralCap, Gelman). Selectiostats were
102 then inoculated with the preserved culture, and complemented with sterile medium filtered through
103 0.22 μm with a Stepdos pump (KNF) to readjust the culture volume.

104 ***Turbidostats vs fedbatch modes.*** Two selectiostats were processed in parallel, each of them using a
105 different culturing mode. In the selectiostat named STurb (turbidostat mode), the dilution rate was
106 dynamically changed by the ODIN[®] software through a PID control algorithm to maintained turbidity
107 at a constant level corresponding approximately to a cell concentration of $9 \times 10^5 \text{ cell.mL}^{-1}$.

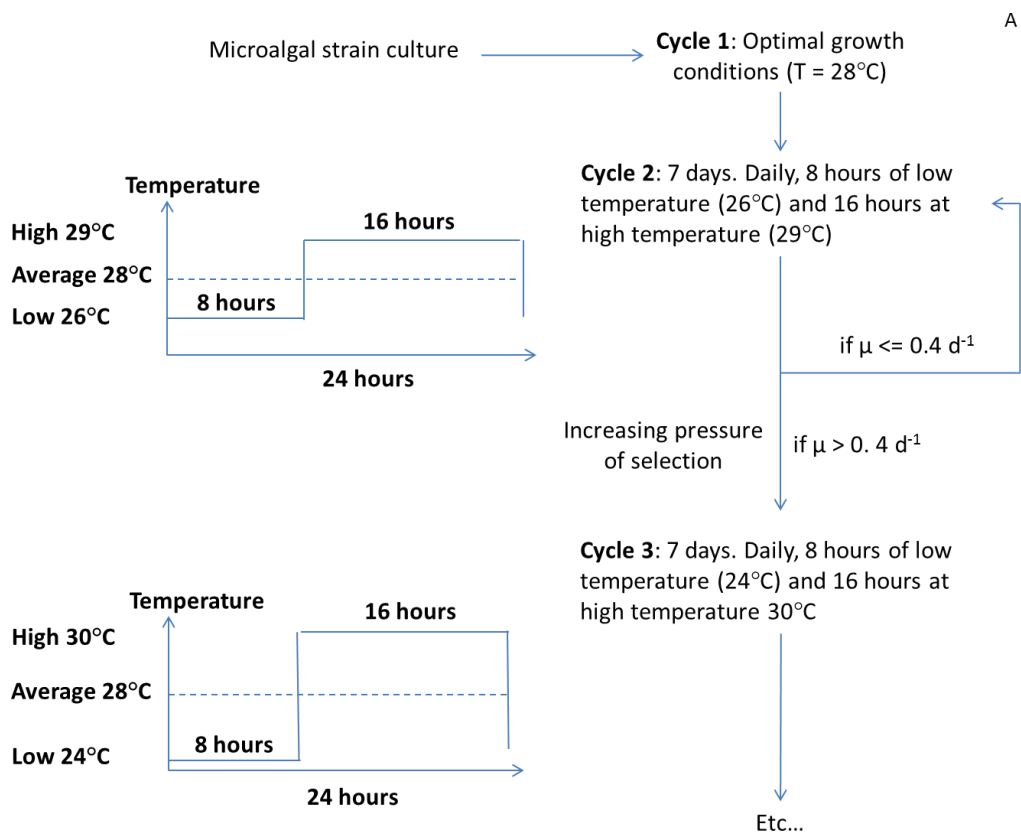
108 The biomass set point was chosen as the maximal value permitting to avoid marked light shading.
109 The nitrogen concentration in the inflowing medium permitted a biomass concentration of 3.3×10^6
110 cell.mL^{-1} . Moreover for cell concentration higher than $5 \times 10^6 \text{ cell.mL}^{-1}$, light limitation was observed in
111 the selectiostat. To be conservative, a biomass concentration below $10^6 \text{ cell.mL}^{-1}$ was chosen to fulfill
112 these conditions.

113 In the selectiostat named SFb (fed-batch mode), a fraction of the culture volume was replaced with
114 fresh sterile medium every 7 days. Only 5 to 10 % of the initial volume was kept, in order to restart
115 cultures with an initial cell density of $5 \times 10^5 \text{ cell.L}^{-1}$.

116 ***Continuous selection procedure, the "ratchet" protocol.*** Our selection method was inspired from the
117 original protocols proposed by [16] and [17], but instead of gradually increasing the culture
118 temperature, the daily average temperature was kept unchanged at 28°C along the experiment
119 (Eq.1). The stress consisted in progressively increasing the amplitude of the daily temperature
120 variation pattern. Indeed, square wave temperature variations were applied, with 8 hours at low
121 temperature (T_{low}) followed by 16 hours at high temperature (T_{high}) with an average daily
122 temperature of 28°C.

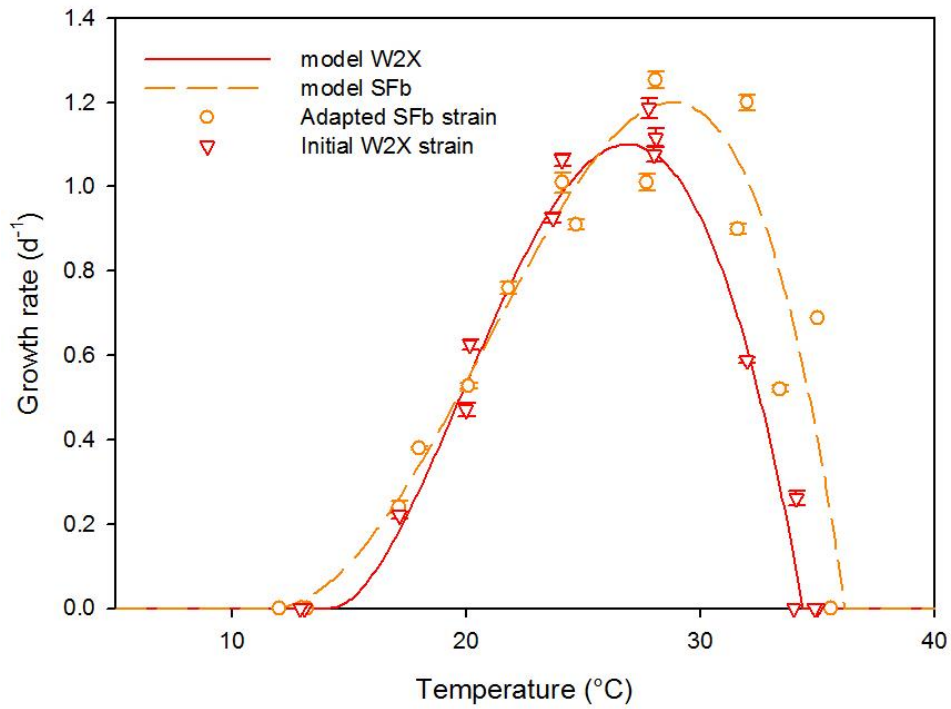
123
$$T_{\text{daily average}} = \frac{1}{24} (T_{\text{high}} \times 16 + T_{\text{low}} \times 8) \quad (1)$$

124 This daily pattern was repeated identically over 7 days (i.e. a selection cycle). This duration allowed
 125 to avoid nitrogen starvation in the fed-batch mode and to reach equilibrium in the turbidostat mode.
 126 At the end of a selection cycle, if the measured growth rate was higher than 0.4 d^{-1} (i.e. more than 50
 127 % of the maximum growth rate, meaning that cells were able to cope with temperature stress), a
 128 new cycle was implemented by reducing T_{low} by 2°C and increasing T_{high} by 1°C . Otherwise the
 129 temperature conditions were remained unchanged to provide a longer adaptation period (Fig. 1).
 130 The rationale behind this asymmetrical mode was motivated by the asymmetry of microalgal
 131 response to temperature, where the variation of the growth rate observed for temperatures higher
 132 than T_{opt} is generally steeper than below T_{opt} (Fig. 2) [4]. For the two last selection cycles 9 and 10,
 133 T_{high} was increased by only 0.5°C and T_{low} decreased by only 1°C in order to reduce the mortality
 134 resulting from the very large daily variations of temperature at the end of the experiment (Table 1).



135 Figure 1: temperature conditions applied during the 3 first cycles of the selection experiment in
136 STurb and SFb. The same protocol with increasing temperature amplitudes was used for the
137 following cycles (4 to 10).

138



139 Figure 2: growth rate measured at different temperatures in the TIP device for the initial W2X strain
140 (triangles) and the temperature selected strain SFb (open circles). Lines represent the best fit of the
141 [4] model to the data series.

# cycle	T _{Low} (°C)	T _{High} (°C)	Number of feed batches	Cycle duration (d)
1	28.0	28.0	6	42
2	26.0	29.0	1	7
3	24.0	30.0	1	7
4	22.0	31.0	2	14
5	20.0	32.0	2	14
6	18.0	33.0	2	14
7	16.0	34.0	1	7
8	14.0	35.0	2	14
9	13.0	35.5	23	161
10	12.0	36.0	3	23

142

143 Table 1: Temperature conditions applied during the selection experiment. A cycle was a set of
 144 temperature conditions (T_{min} and T_{max}) daily applied to culture during selection procedure.

145 **Cell population.** Size distribution of the cell population was monitored once or twice a day with an
 146 optical particle counter (HIAC - Royco; Pacific Scientific Instruments). Mean cell diameter and
 147 biovolume were calculated from the size distribution. The variability between triplicate samples was
 148 routinely lower than 5 %. The first and the second counting were performed at the beginning of the
 149 low and high temperature periods, respectively. Measurements were performed at least once per
 150 cycle (Fig. 3; Tab. 1).

151 The average growth rate, experimentally measured for a cycle of selection $\bar{\mu}_{Exp-SFb}$ (d⁻¹) of the SFb
 152 culture was calculated using the following equation:

$$153 \quad \bar{\mu}_{Exp-SFb} = \frac{\ln\left(\frac{BV_2}{BV_1}\right)}{t_2 - t_1} \quad (2)$$

154 where BV₁ and BV₂ were the biovolume (μm³.mL⁻¹) at time t₁ and t₂ (d), respectively. The growth rate
 155 was the average of 4 days of measurements (n > 4). The computation of the average growth rate in a
 156 continuous culture ($\bar{\mu}_{Exp-STurb}$) with permanent stress affecting growth is tricky and would have
 157 needed high frequency biomass measurements. For the routine daily monitoring of the selectiostats,
 158 we used the average dilution rate over one week. However, this proxy of average growth rate turned

159 out not to be accurate enough to support the discussion (standard deviation of measurements > 10
160 %). Therefore, the evolution of the growth rate for the turbidostat (which qualitatively follows a
161 similar trend as for the fed batch) was not used for the analyses, and only the features of the final
162 adapted strain are discussed in this paper.

163 **Growth response with respect to temperature (TIP experiment).** A special device (TIP) composed
164 with 17 photobioreactors (0.5 L) and permitting to control independently temperature, pH and light
165 intensity [16] was used to assess the temperature response on growth at constant pH and irradiance
166 [16]. Three strains were used, *T. lutea* before (W2X strain, stored at 19°C for 2 years) and after (STurb
167 and SFb, stored for one month at 21°C) the selection experiment. After one day acclimation in the
168 TIP, the exponential growth rate was estimated at 8 temperatures ranging from 12°C to 35.5°C from
169 the linear regression of the logarithm of DO₆₈₀ (n>12). Irradiance (250 μmol.m⁻².s⁻¹) and pH (7.9) were
170 maintained constant. Cell concentration was assessed by image analysis after Lugol staining on
171 Malassez slides using specific software (Samba Technologies, Meylan, France) to check the DO₆₈₀
172 measurement validity. Lipid classes and fatty acid composition were analyzed for STurb and SFb
173 strains growing at 28°C after 7.2 days of cultivation in nitrogen starvation and compared with the
174 measurements obtained during the W2X lipid comparison experiment (detail below).

175 **Characterisation of temperature response.** The three cardinal temperatures (T_{min}, T_{max} and T_{opt}) were
176 estimated for the initial W2X and for the new selected strains SFb and STurb, obtained after 10
177 months of selection, by fitting the model proposed by [4] to the TIP growth response (Fig. 2). A
178 gradient-based optimisation procedure was run and a jackknife analysis provided an estimate of
179 these parameters together with their confidence intervals [4].

180 To quantify the effect of temperature selection on the measured growth rate of SFb strain, a
181 “selection coefficient” s_{SFb} [21] was calculated as follow:

$$182 \quad s_{SFb} = \ln(\bar{\mu}_{Exp-SFb}) - \ln(\bar{\mu}_{Th-W2X}) \quad (3)$$

183 where $\bar{\mu}_{Th-W2X}$ is the theoretical growth rate of the strain W2X calculated for the same temperature
184 conditions as experienced by the culture SFb during each selection cycle, with the cardinal parameter
185 of the W2X strain:

$$186 \quad \bar{\mu}_{Th-W2X}(T_{low}, T_{high}) = \frac{7.5}{24} \mu_{W2X}(T_{low}) + \frac{15.5}{24} \mu_{W2X}(T_{high}) + \frac{1}{24} \mu_{W2X}(T_{average}) \quad (4)$$

187 where T_{low} and T_{high} are the low and high temperature values set during the selection cycles, and
188 $T_{average}$ the mean temperature experienced during the 1h transition time between low and high
189 temperatures. Selection occurs if the frequency of adapted strains in the population increases ($s > 0$)
190 [21].

191 **W2X lipid comparison experiment (benchmark experiment).** To compare the lipid content of the
192 initial W2X strain with the new adapted strains, a benchmarking experiment in similar conditions
193 than the TIP experiment was performed. Three cylindrical glass photobioreactors of 2 L were used as
194 triplicates. Temperature was controlled at 28°C and pH maintained constant at 7.9 by micro-
195 additions of CO₂ in the bubbling air. Continuous light was provided by fluorescent tubes (Dulux®1,
196 2G11, 55W/12-950, lumilux de lux, daylight, OsramSylvania) at 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and measured in the
197 center of the empty photobioreactors as in the TIP device. Cultures were gently homogenized by
198 magnetic stirring. The enrichment medium was prepared in several 20 L tanks (Nalgene) filled with 3
199 weeks-matured natural seawater, previously filtered on 0.1 μm , and autoclaved at 120°C for 40 min.
200 After cooling, f/2 medium was added through a 0.22 μm sterile filter [19]. Nitrogen concentration
201 was lowered at f/4 concentration to reach N starvation. Lipid sampling (400 mL of culture) was
202 realized at the end of the experiment during the starvation phase.

203 **Cellular content analysis.** 6.65 mL triplicates of culture were sampled weekly, 3 days after the
204 beginning of the selection cycle and just after the temperature shift from T_{high} to T_{Low} , and filtered
205 onto glass-fiber filters (Whatman GF/C, threshold 1.2 μm) precombusted at 450°C for 12h. For
206 particulate carbon and nitrogen measurement, samples were kept at 60°C until analyses were
207 performed with a CHN analyzer (2400 Series II CHNS/O, Perkin Elmer). For pigments, samples were

208 kept at -80°C, and extracted in acetone (3 ml) for 1 hour at 4°C in the dark with frequent and gentle
209 stirring. After 5 min of centrifugation (JOUAN G 412) at 2000 rpm, supernatant was analyzed with a
210 spectrophotometer (Perkin Elmer UV/Vis Spectrophotometer Lambda2). Chlorophyll *a*, *b* and total
211 carotenoid were determined using the equations of Lichtenthaler and absorbance at 470.0, 644.8
212 and 661.6 nm [22]. The variability between triplicates was lower than 7 %.

213 For adapted strains, lipid samplings were made at the end of the TIP experiment, after 7.7 days of
214 cultivation in nitrogen starved conditions at 28°C. For the W2X, lipids were sampled at the end of the
215 lipid comparison experiment (6.9 days) in N-starved conditions. Known volumes of culture were
216 centrifuged (JOUAN G 412) for 10 min at 2000 rpm, after the addition of 100 µL of Cl₃Fe (50 mg.mL⁻¹)
217 as flocculate. The lipid extraction protocol was derived from Bligh and Dyer [23]. Total lipids were
218 determined gravimetrically. Neutral lipids, glycolipids and phospholipids were separated on Extract-
219 Clean SPE Silica 500 mg/8 mL (Alltech) and eluted respectively with 6 column volumes of chloroform,
220 4 column volumes of acetone and 6 column volumes of methanol completed with 2 volumes of
221 methanol–10 % ammonia. Total lipids were placed on top of the column in chloroform. All fractions
222 were dried under vacuum and weighted to provide an estimate of each class of lipids.

223 The lipid productivity (µg_{Lipid}.mL⁻¹.d⁻¹) of the different strains during the benchmarking experiment
224 was calculated as follows:

$$225 \quad P_{lipid} = \frac{Lipid_f - Lipid_i}{t_f - t_i} \quad (5)$$

226 where *Lipid_f* and *Lipid_i* are the total lipid concentration (µg.mL⁻¹) at final and initial time respectively.

227 Note that, the initial lipid concentration was not measured (not enough matter for our lipid
228 protocol). Initial lipid content per gram carbon was thus assumed to be the same as the final content.

229 This hypothesis provides a slightly underestimated productivity, but has limited impact since initial
230 biomass is very low. Fatty acid analysis began by converting saponifiable lipids into methyl esters with
231 7 % boron trifluoride in methanol [24]. Gas chromatography (GC) of fatty acid methyl esters (FAME)
232 was carried out on a 30 m length 0.32 mm internal diameter quartz capillary column coated with

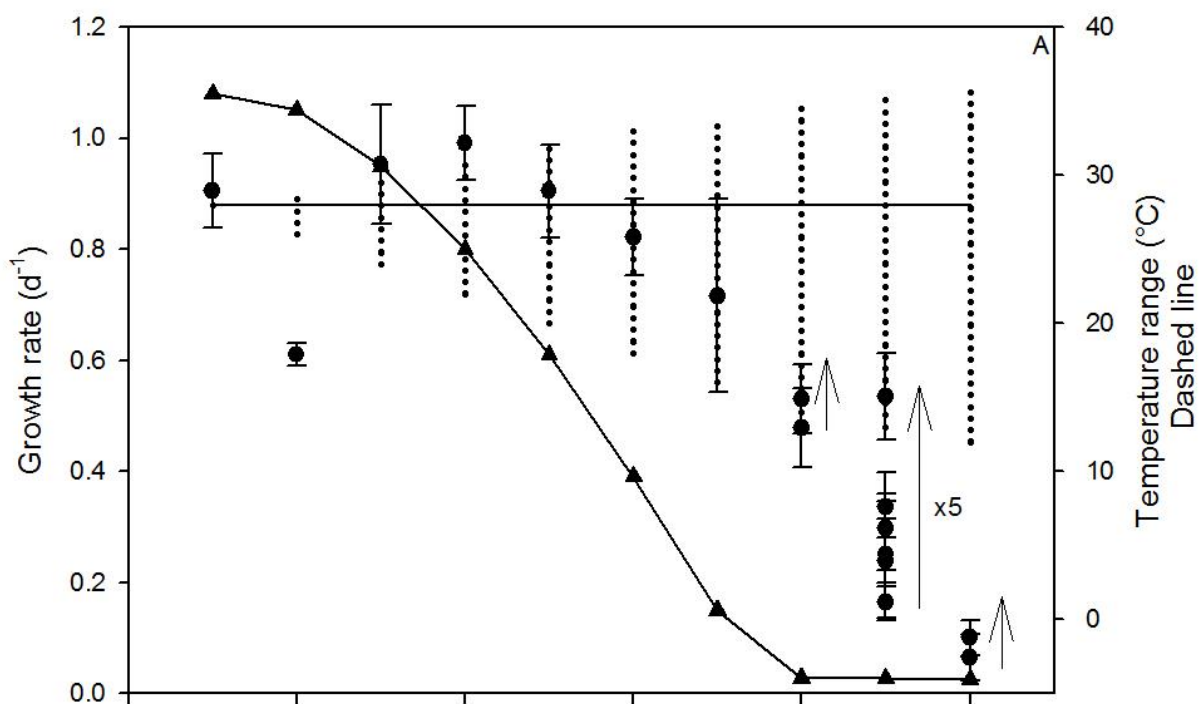
233 famewax (Restek) in a Perkin-Elmer XL autolab GC, equipped with a flame ionization detector (FID).
234 The column operated isothermally at 185°C. Helium was used as carrier gas at 7 psig. Injector and
235 detector were maintained at 250°C. The variability was routinely 3 % for major components, 1 – 9 %
236 for intermediate components and 25 % for minor components (<0.5 % of total fatty acids).
237 Measurements were relative and expressed as percentage of total fatty acid, no internal standard
238 was needed.

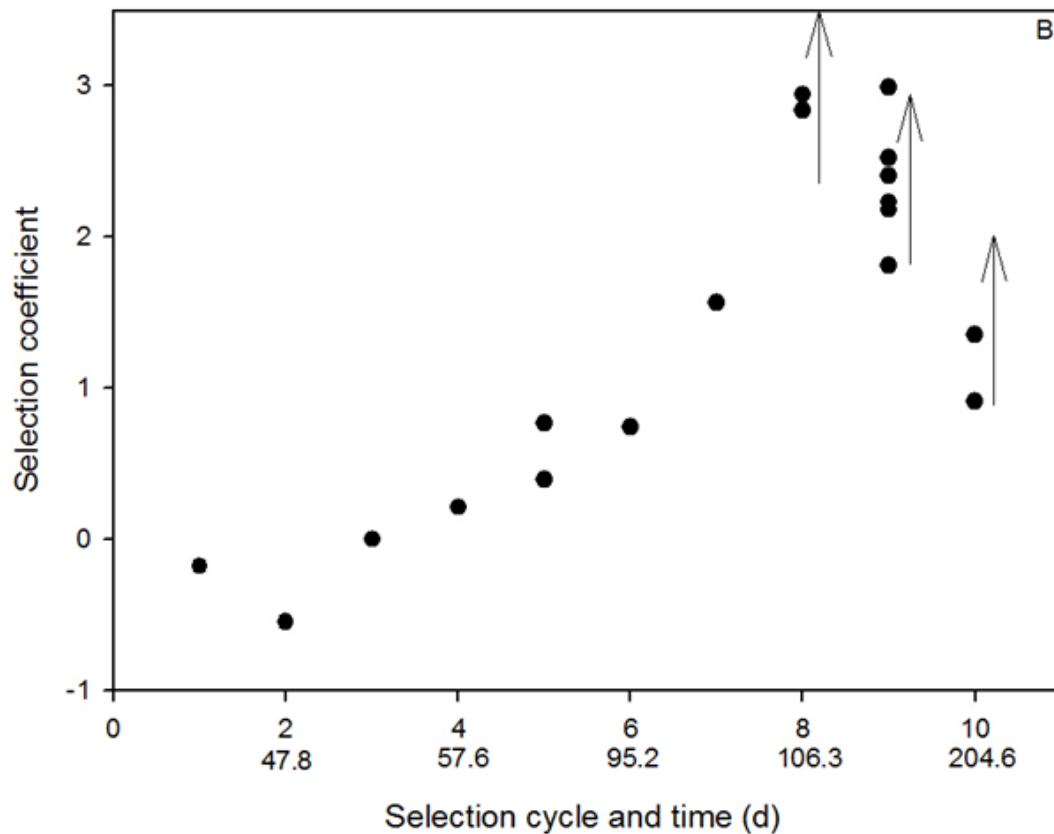
239 **Results and discussion**

240 ***From acclimation to adaptation.*** Fig. 3A shows that the theoretical growth rate of the initial strain
241 W2X calculated for the experimental temperature conditions (Eq. 4), decreased with the amplitude
242 of temperature variations. Fig. 3A also shows that, except for the very first 2 cycles, the growth rates
243 measured in the SFb selectiostat were significantly higher than the theoretical growth rate computed
244 for the parameters of the initial strain. It is also noticeable that, for the temperature conditions of
245 cycles 8, 9 and 10, the growth is still possible in the SFb selectiostat, while no growth could be
246 observed at these temperatures for the initial strain. The reason why the growth rates in the SFb
247 selectiostat measured during the two first cycles were lower than the theoretical growth rate for the
248 initial W2X strain might be due to an initial acclimation phase to the variable temperature conditions.
249 It is likely that, by maintaining the cells for a longer time in the temperature conditions of cycles 1
250 and 2, the growth rate would have progressively increased as a result of a combination of acclimation
251 and adaptation. This increase in average growth can indeed clearly be observed for the last cycles
252 which were repeated several weeks. As a matter of illustration, the average growth rate during the
253 cycle 9 was multiplied by 5 when comparing the first and the last week of the cycle (Fig. 3 A). The
254 selection coefficient s (Fig. 3 B) was positive after cycle 3; this means that the frequency of new
255 thermally adapted individuals in the population increased to the detriment of the initial strain [21].
256 This increase shows that the initial strain was not fitted to these highly changing temperature
257 conditions. From cycle 8 to 10 ($T_{\text{Low}} = 12^{\circ}\text{C}$; $T_{\text{High}} = 35.5^{\circ}\text{C}$), the transition from a cycle to the next led

258 systematically to a reduction of the selection coefficient. After the transition in the new cycle, it
 259 gradually increased with the number of generations highlighting the progressive emergence of cells
 260 better fitted to the selective pressure (Fig. 3 B; black arrows).

261 With a Luria-Delbrück fluctuation analysis [7], Costas et al. [25] studied the variance of the fitness
 262 during selection conditions and determined the origin of mutant adapted to the new thermal
 263 conditions. They increased the average growth temperature as a stressing parameter for *Isochrysis*
 264 *galbana* wild type ($T_{\max} = 28^{\circ}\text{C}$). Under a low temperature range (15 – 30°C), the increase of the strain
 265 fitness was due to acclimation or pre-selective mutations (selection from the pre-existing genetic
 266 diversity). Under a higher range (30 - 35°C), they assumed that the observed response was linked to
 267 adaptive mutations (occurrence and selection of new mutants). With our protocol, it was not
 268 possible to identify the source of fitness increase. Since the pressure of selection was dynamic, in
 269 contrast to [25], the observed selection was probably a dynamical equilibrium involving acclimation,
 270 pre-selective mutations and adaptive mutation to the emergence of a new population with a broader
 271 thermal niche and/or a higher thermal acclimation capacity.





272 Figure 3: A) Mean growth rates measured for SFb strain during each selection cycle (black circles),
 273 and theoretical growth rate calculated with the calibrated temperature model of the initial strain
 274 W2X (triangles) submitted to the same temperature conditions. Growth rate was measured at a
 275 minimum of 6 different dates on one culture (SFb, n>6), during the exponential phase to achieve a
 276 linear regression with error < 5 % and thus an accurate growth rate determination. The vertical
 277 dashed lines indicate the amplitude of temperature variations during each cycle, and the horizontal
 278 continuous line corresponds to the mean temperature maintained constant at 28°C throughout the
 279 selection experiment. B) Time change of the selection coefficient measured in SFb. When measured
 280 during consecutive weeks of the same cycle, the selection coefficient increased with time (vertical
 281 arrows).

282

283 **New strain characterization.** The new strains were obtained after 41 generations in fed-batch (SFb)
 284 and approximately 157 generations in turbidostat (STurb; value obtained from the estimate of the

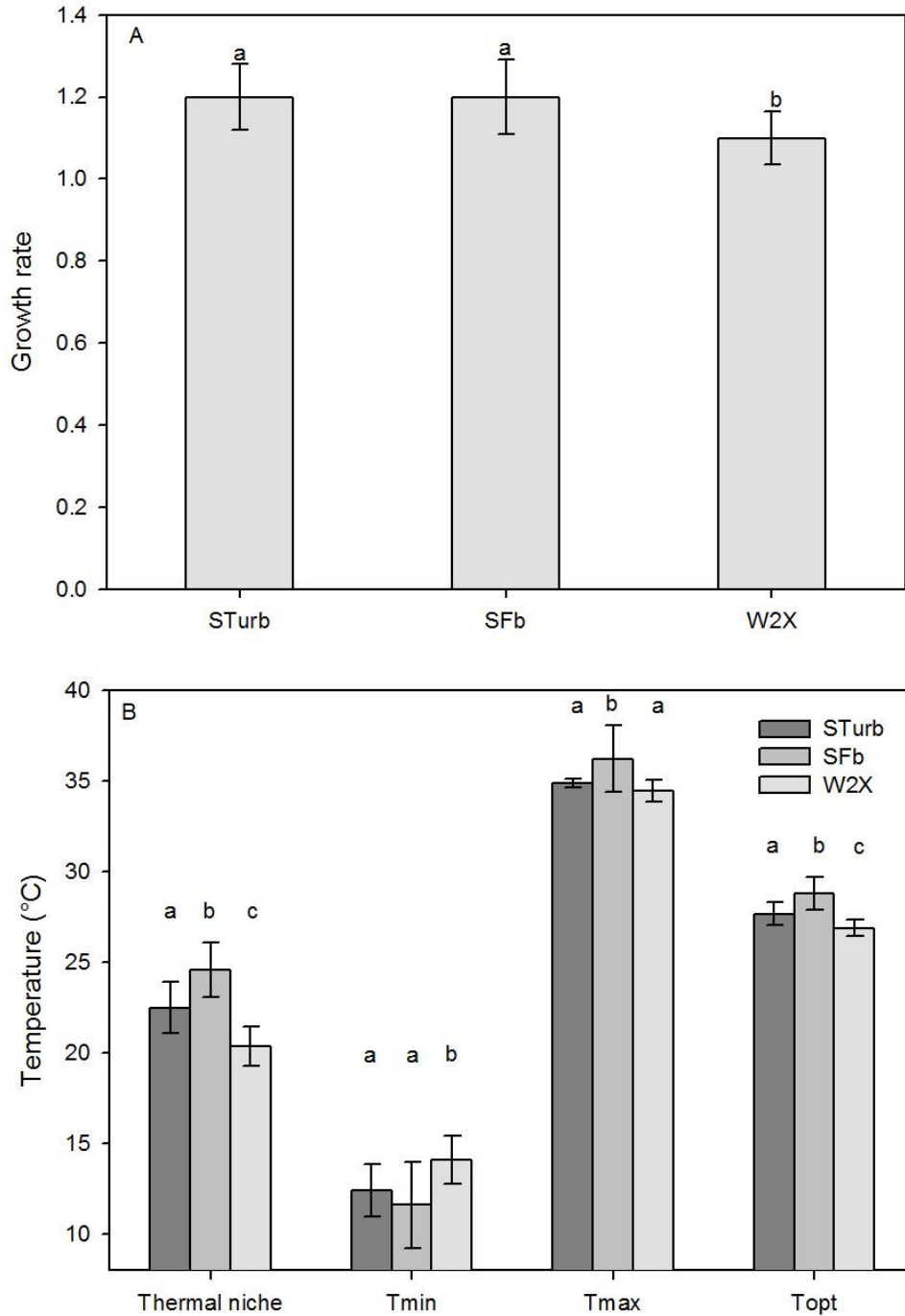
285 average growth rate of the STurb population based on the dilution rate). The thermal response of
286 these two final new strains and the initial strain W2X was characterized with the TIP [16] and
287 compared (Fig. 2). The cardinal temperatures for these new strains were assessed using the model of
288 [4]. The optimal growth rates of the two new selected strains were significantly higher than the rate
289 of the initial W2X (+9 %; statistical difference at 1 %, Student test; Fig. 4 A). This result was expected
290 because it was demonstrated mathematically and experimentally that continuous medium renewal
291 eliminates preferentially individuals that grow slower, leading to an increase of the population mean
292 growth rate [26][27]. Consistent with Eppley [28], the increase in the optimal growth rate was
293 correlated with an increase in T_{opt} (Fig. 4). However, despite the “hotter is faster” trend [29], the
294 Eppley curve normalized by the optimal growth rate of the W2X strain overestimates the evolution of
295 the optimal growth rate by 41.7 % and 51.1 % for STurb and SFb respectively.

296 The trade-off between thermal specialist and thermal generalist strains (narrow thermal niche/high
297 performances and large thermal niche/low performances respectively) is a common hypothesis in
298 literature. A microorganism with an increased thermal niche should have a lower maximal growth
299 rate. This assumption has sometimes been mathematically transcribed by a constant area beneath
300 the temperature response growth curve [29][30][31]. However, this widespread assumption in
301 evolutionary models of thermal adaptation is hardly supported by experimental data for
302 microorganisms. Bennett & Lenski [32] have refuted this assumption since they recorded, after 2000
303 generations, an increasing T_{opt} of some *E. coli* strains without modifying the thermal niche. To our
304 knowledge, our experiment with microalgae is the first to assess this assumption. The increase in
305 growth rate coincided with an increase in the thermal niche (+11 % and +22 % respectively for final
306 STurb and SFb strains; statistical difference 1 %, Student test; Fig. 4). The dynamic environment used
307 in our experiments is probably a key factor explaining the inconsistency of the trade-off theory for *T.*
308 *lutea*. The thermal niche increase was the consequence of two significant effects, an increase in T_{max}
309 and a decrease in T_{min} (Fig. 4). When comparing the initial W2X thermal niche (20°) to the thermal
310 niche of other microalgal species (30°C for *Scenedesmus sp.*, 34°C for *Dunaliella tertiolecta*, 24°C for

311 *Porphyridium cruentum* [4]), it appeared that the initial strain was probably a thermal specialist. On
312 the other hand, the new adapted strains with a thermal niche close to 25°C were closer to thermal
313 generalists.

314 Previous studies carried out with bacteria have shown that these microorganisms adapt their optimal
315 growth temperature (T_{opt}) to the average temperature of their environment [33] or slightly above as
316 hypothesized by some models [34] but very few data reinforce this hypothesis. After an adaptation
317 of three months at constant average temperatures, [11] recorded the same growth rate for
318 *Skeletonema costatum* at 3°C and 18°C. Likewise, by submitting *Isochrysis galbana* to increasing
319 constant average temperatures, [35] reported positive growth at 35°C for the adapted strain,
320 whereas the wild type did not grow at this temperature. Here, T_{opt} of the new strains increased
321 during the selection process and was at the end higher than the average temperature (28°C). This
322 evolution is consistent with [36] who compared the T_{opt} modelled for 195 species with the average
323 temperature of the species location.

324 Our experiment, carried out with intense daily temperatures variations but constant mean value,
325 highlighted that temperature adaptation is complex and depends not only on the average
326 temperature but also on the temperature range and its dynamics. It also highlighted the importance
327 to consider all the cardinal temperatures and not only the optimal growth temperature as it is too
328 often the case.



329 Figure 4: comparison between the new STurb and SFb strains and the initial W2X strain. A) Growth
 330 rate. B) Cardinal temperatures and thermal niche. Values are expressed as the mean \pm standard
 331 deviation determined by a jackknife analysis. Groups marked with the same letter (a, b or c) are not
 332 statistically different (1 % error, Student test). Different letters indicate groups statistically different.

333 **Evolution of the biochemical composition across the selection procedure.** In *T. lutea*, nitrogen
 334 starvation increases the total lipid content [37][38]. The initial W2X strain (CCAP 927/17), produces 2

335 times more neutral lipids (1.7 times more total lipids and a higher ratio of neutral to polar lipids) than
336 the wild type (CCAP 927/14; [17]). After our thermal selection experiment, it was necessary to check
337 the preservation of these highly interesting lipid properties. Lipid content of the adapted strains
338 cultivated at 28°C during the TIP experiment was compared with measurements made on the initial
339 W2X, during the benchmark experiment, in the same culture conditions. Starvation state were
340 similar (C:N ratio : 20.9, 17.7 and 18.0 ± 1.3 mol:mol respectively for SFb, STurb and W2X).

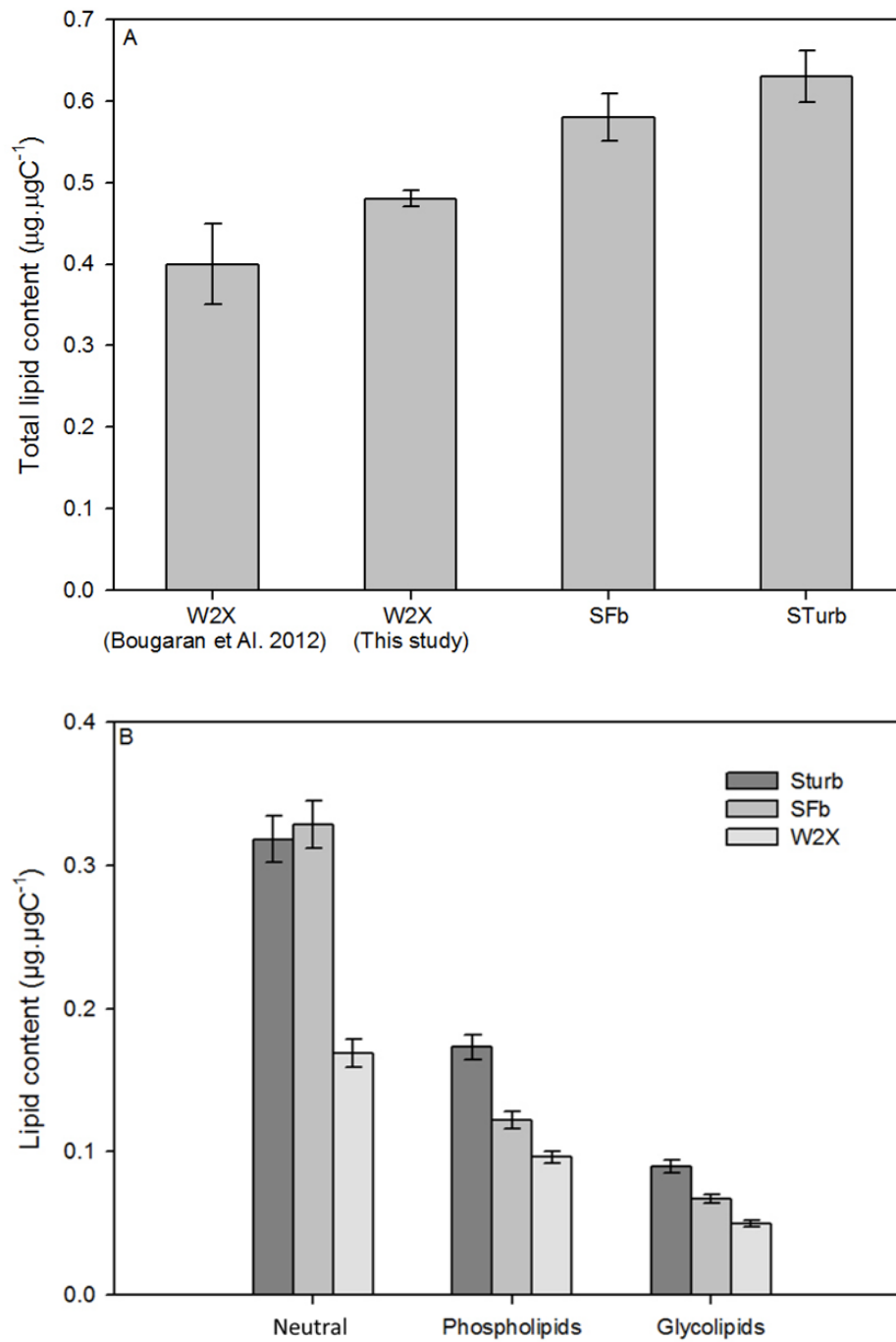
341 The adapted SFb and STurb strains had higher total lipid content than the initial W2X strain (0.58,
342 0.63 and 0.48 ± 0.01 $\mu\text{g} \cdot \mu\text{gC}^{-1}$ respectively for SFb, STurb and W2X; Fig 5A). Lipid content found for
343 the W2X was in accordance with result from [17] for the same strain (0.4 ± 0.05 $\mu\text{g} \cdot \mu\text{gC}^{-1}$). It could be
344 hypothesized that total lipids were enhanced due to the thermal stress response induced by
345 temperature variations. The acclimation response of total lipids to temperature is not clear in the
346 literature [39]. For some species, total lipids increased as temperature decreases, and reach
347 maximum levels at extreme temperatures [40][40][41][3]. Other authors found a strong link between
348 total lipid content and growth rate [42][43][14]. However, only acclimation to temperature was
349 studied without emphasis on long term evolution.

350 Moreover, the repartition in lipid classes changed strongly between the adapted and the initial
351 strains. Under nitrogen starvation, adapted strains exhibited higher neutral lipid content (+ 92 %) and
352 higher phospholipid content (+53 %) than the W2X strain (Fig. 5B). No difference was observed in the
353 relative repartition of free fatty acids between these strains (Tab. 2). In response to cold
354 temperatures, membrane cell fluidity is preserved by an increase of polyunsaturated fatty acid and a
355 decrease of saturated and monosaturated fatty acid [14]. Very few information exist on lipid class
356 response to temperature (phospholipids, glycolipids and neutral lipids). Some works suggest that an
357 increase of the temperature increases total lipids and neutral lipids [44]. Once again, these works
358 studied only acclimation. Lipid adaptation to temperature was never experimented. Lipids were
359 affected by the thermal selection procedure but the attractive lipid profile of the initial strain in term

360 of neutral lipids production was not degraded but on the contrary improved. At the end, total lipid
361 productivity of the adapted strains was increased by 34% when compared with the initial W2X (13.6,
362 14.1 and 10.3 $\mu\text{g}_{\text{Lipid}}\cdot\text{mL}^{-1}\cdot\text{d}^{-1}$ for Sturb, SFb and W2X respectively).

363 It is generally observed that warmer temperatures are more favorable to smaller cells, probably due
364 to the increase in enzymatic reaction rates [45][46]. During our selection procedure, the average cell
365 diameter was significantly decreased (ANOVA, p -value > 0.1), except during the last selection cycles
366 (9, 10) where it increased (Fig. 6 A). Moreover, the C:N ratio gradually decreased (Fig. 6 B), while the
367 Chl a:C ratio started to decrease after selection cycle 6 (Fig. 6 C). All these observations are
368 characteristic of cold adaptation pattern, as reported by [3] and [47] for *T. lutea*. This acclimation
369 features at lower temperature reflect the reallocation of cell resources (energy, carbon) necessary to
370 enhance the temperature-dependent biochemical reactions of the photosynthesis dark phase and to
371 rebalance the energy produced by the temperature-independent photochemical reactions involved
372 in the photosynthesis light phase [47].

373 These results were unexpected since the daily average temperature was constant throughout the
374 selection (28°C). Our selection protocol turned out to be more selective towards cold temperatures
375 than high temperatures, probably because the decrease in temperature at each selection cycle was
376 more severe than its increase (-2°C and +1°C respectively). Secondly, this selection protocol was
377 dynamic and necessitated a continuous thermal acclimation. This particular physiological status may
378 offer a higher plasticity and acclimation capacity.



379 Figure 5: comparison of the total lipids (A) and lipid classes (B) between the adapted Sturb and SFb
 380 strains (n = 3; measurement triplicates) and the initial W2X strain (n=3; independent culture
 381 triplicates) after nitrogen starvation.

382

383

	W2X	Standard deviation		STurb	SFb
Saturated					
ISO14:0	0.02	0.01		0.11	0.02
C14:0	23.64	0.88		27.95	23.92
ISO15:0	0.30	0.02		0.35	0.22
ANT15:0	0.14	0.02		0.13	0.15
C15:0	0.36	0.03		0.36	0.26
ISO16:0	0.04	0.01		0.14	0.08
ISO17:0	0.16	0.00		0.08	0.07
ANT17:0	0.04	0.01		0.05	0.04
C16:0	16.06	0.18		15.08	16.02
C17:0	0.08	0.00		0.05	0.05
C18:0	0.57	0.08		0.35	0.54
Total	41.86	0.89		44.64	41.37
Mono-ene					
C14:1n-5	0.71	0.08		0.78	0.62
C15:1n-8	0.03	0.01		0.03	0.03
C15:1n-6	0.01	0.00		0.02	0.02
C16:1n-7	2.98	0.23		2.93	2.26
C16:1n-5	0.32	0.01		0.34	0.46
C18:1n-9	22.27	1.14		18.03	24.37
C18:1n-7	2.94	0.24		0.96	1.43
C18:1n-5	0.45	0.05		1.32	0.53
C20:1n-9	0.10	0.01		0.07	0.04
C20:1n-7	0.03	0.00		0.00	0.04
C20:1n-5	0.08	0.01			
C22:1n-13+11	0.22	0.06		0.25	0.31
C22:1n-9	0.12	0.16		0.27	0.41
Total	30.35	0.81		24.99	30.51
Di-ene					
C16:2n-6	0.14	0.01		0.08	0.07
C16:2n-4	0.41	0.02		0.34	0.26
C18:2n-6	4.14	0.15		3.85	4.79
C20:2n-6	0.16	0.03		0.55	0.17
Total	5.32	0.13		4.82	5.29
Tri-ene					
C16:3n-4	0.05	0.01		0.02	0.01
C16:3n-3	0.23	0.01		0.20	0.17
C18:3n-6	0.20	0.02		0.11	0.11
C18:3n-3	2.25	0.08		4.05	4.75
C20:3n-6	0.13	0.03		0.10	0.09

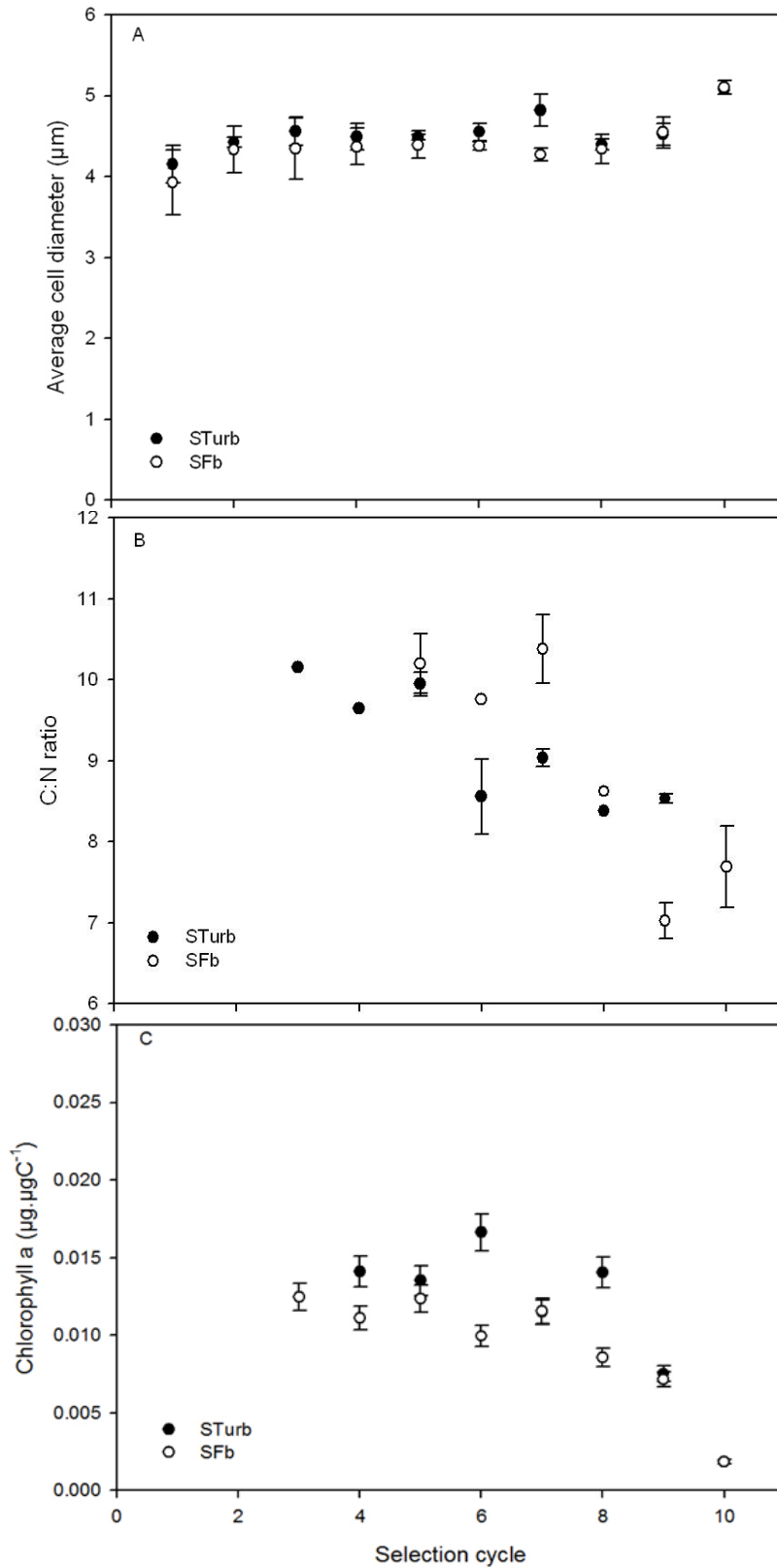
C20:3n-3	0.05	0.00		0.12	0.12
Total	2.95	0.11		4.60	5.24
Tetra-ene					
C16:4n-3	0.02	0.00		0.05	0.01
C16:4n-1	0.04	0.00		0.08	0.06
C18:4n-3	5.88	0.29		7.18	6.18
C20:4n-6	0.24	0.01		0.14	0.18
C20:4n-3	0.08	0.00		0.08	0.05
C22:4n-6	0.14	0.01		0.20	0.06
Total	6.48	0.27		7.72	6.53
Penta-ene					
C18:5n-3	1.27	0.08		0.35	0.22
C20:5n-3	0.24	0.01		0.23	0.23
C21:5n-3	0.43	0.03		0.24	0.14
C22:5n-6	1.68	0.06		1.98	1.92
C22:5n-3	0.27	0.22		0.11	0.06
Total	3.89	0.26		2.90	2.58
C22:6n-3	9.14	0.72		10.32	8.48
Total PUFA	27.78	0.28		30.36	28.11

384

385

386 Table 2: relative fatty acid composition of the two new adapted strains in comparison with the initial

387 W2X strain (n=3; independent culture triplicates) as a % of total fatty acid.



388 Figure 6: evolution of three biological markers during the selection process at the first iteration of
 389 each cycle. A) Evolution of the cell diameter. B) C: N ratio C) Chl a: Carbon ratio. Standard deviation
 390 was calculated on triplicate of independent measurements.

391 **Conclusion**

392 Dynamical selection pressure in continuous culture, for long periods of time has a strong potential
393 for domesticating strains for industrial applications. This study highlights the possibility to impact the
394 thermal niche of *T. lutea* by modifying T_{\min} and T_{\max} with fluctuating temperatures but at constant
395 daily average temperature. The increase in the thermal niche was also concomitant to an increase in
396 the maximum growth rate and associated to important physiological changes similar to a 'cold-
397 temperature' acclimation. This approach permitted to keep the initial lipid properties of the strains in
398 term of neutral lipids production. One of the most interesting features triggered by the highly
399 dynamic protocol must still be explored: the capacity of the new strains to acclimate more rapidly
400 under large temperature fluctuations.

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